Universitat Autònoma de Barcelona

Ex vivo and in vitro studies on Toll like receptors in canine Leishmania infection

Sara Montserrat i Sangrà

Ex vivo and *in vitro* studies on Toll like receptors in canine *Leishmania* infection

Tesi doctoral

Sara Montserrat i Sangrà

Directora: Laia Solano i Gallego

Departament de Medicina i Cirurgia animal

Facultat de Veterinària

Universitat Autònoma de Barcelona

Facultat de Veterinària

2018

Universitat Autònoma de Barcelona

La Dra. Laia Maria Solano Gallego, Professora Agregada del Departament de Medicina i Cirurgia Animals de la Universitat Autònoma de Barcelona,

INFORMA:

Que el treball de tesi doctoral titulat

"Ex vivo and *in vitro* studies on Toll like receptors in canine *Leishmania* infection"

del que és autora la llicenciada en biologia

Sara Montserrat i Sangrà

ha estat realitzat sota la meva direcció i compleix les condicions exigides per optar al títol de Doctora per la Universitat Autònoma de Barcelona.

I per què així consti, signo el present informe a Bellaterra, 26 de setembre de 2018.

Dra. Laia Maria Solano Gallego
Directora

Sara Montserrat i Sangrà
Doctoranda

AGRAÏMENTS

Durant tots aquests anys hi ha hagut molta gent que ha passat per la meva vida i que ha influït en més o menys grau en el meu camí per arribar fins aquí.

No m'agradaria deixar-me a ningú perquè tothom en la seva mesura conscient o inconscientment m'ha acompanyat al llarg d'aquest període tant important per a mi.

Primerament, agraïr a la meva tutora i directora de tesis, Laia Solano, que m'ha donat l'oportunitat de poder adquirir aquesta formació i perseguir el meu somni de ser doctora. Gràcies per ensenyar-me què és important i quan ho és. El rigor inculcat i la il·lusió de continuar tot i les adversitats.

A la Pamela Martínez, perquè quasibé vam començar juntes i hem seguit fent camí plegades. Per ser-hi sempre i compartir les penes i les glòries.

A totes les persones que han format part d'aquest grup durant el meu doctorat: A la Lorena Alborch, per la paciència i l'empatia, per transferir-me tots els coneixements necessaris per poder funcionar de manera autónoma, Alejandra Álvarez, Marta Baxarias i sobretot Laura Ordeix per no perdre mai la calma, per tenir sempre les paraules justes i per ajudar-me sempre que ho he necessitat. També els estudiants de grau i màster com estudiants estrangers, tots heu aportat aire fresc al grup i alguna/ algunes nocions més als meus coneixements i per ser companys i amics.

Les persones amb les quals he pogut treballar que no són figurants del nostre departament i que m'han ajudat a aconseguir aquesta fita: Anna Castelló, Anna Mercader, Lorena Serrano, Mireia Sancenon, al departament de genètica; i a la Roser Fisa i Magdalena Alcover de la Facultat de Farmàcia de la Universitat de Barcelona.

Al personal del departament de Medicina i cirurgia animals, sempre disposats a ajudar i també al personal d'anatomia patològica amb els que he compartit despatx: tot és més fácil si estàs acompanyada.

Donar les gràcies també als veterinaris involucrats en aquest projecte i per la seva amabilitat en ajudar-nos a recaudar les mostres necessàries per fer l'estudi i per la seva

dedicació. Gràcies Hospital Clínic i Veterinari de la Universitat Autònoma de Barcelona, Ars Veterinària, Mediterrani Veterinaris i a la Veterinària Montsant.

A les nenes del departamento de Microbiologia,per fer-me veure les coses des d'un altre punt de vista i per donar-me suport.

Als becaris precaris, que m'han fet aquest camí infinitament més lleuger i divertit. Un paper que en moltes ocasions, ha estat clau per continuar. Sobretot als que hem coincidit en la fase final de la tesi, compartint les punxes del roser, cadascú en la seva especialitat.

Primeiramente queria agradecer à Investigadora Doutora Carla Maia, por todo o apoio e dedicação, fazendo do meu Doutoramento mais rico. Estou grata também, pela oportunidade de ter desfrutado duma experiência enriquecedora, que foi trabalhar com toda a equipa do Instituto de Higiene e Medicina Tropical, agradecendo desde já à Sandra por todo o apoio que me prestou e à disponibilidade da Joana, que dispensou o seu tempo para não me sentir tão sozinha.

Quero agradecer também à professora Aida, pela transmissão de conhecimentos sobre o mundo das células. Ao Mestre André Pereira, pela paciência e pela sua boa disposição. À Mónica Nunes, por ser o meu anjo da guarda no Instituto. Ao Mestre José Manuel Cristóvão, pela dedicação e disponibilidade, sabendo que podia contar sempre com ele! E quero agradecer à Cláudia Cruz, por ser minha amiga e ensinar-me o que é ser português.

A chi ho conosciuto a Lisbona, soprattutto agli italiani, in particolare a Flavia Scuteri, Beatrice Tabarrini, Oriana Parrella, Nicola e Dario Aresta. Sono stati la mia famiglia per un breve tempo e li porto nel cuore. Hanno capito quanto fosse importante la mia ricerca ancor prima di sapere di cosa si trattasse e ci sono sempre stati per me. Li ringrazio per la stima che mi hanno dimostrato. Vi voglio bene.

A mis amigos de Erasmus, por seguir allí aunque pasen los años.

A les *lisensiadas*, Noemí, Maria i Mary. Ni distància ni temps. A les verdes i a les madures. Per ser el cercle on les meves preocupacions professionals s'escolten i es poden entendre fora d'horaris laborals.

Als *Farmacólogos sin parangón* per aportar-me saviesa científica de la manera més humorística i sobretot per entendre'm durant tot aquest camí: Cristina, Melania, Marc i Isidro, gràcies per l'empenta i per ser-hi.

A Biokit i a IDIBAPS Hospital Clínic. I altra vegada, sobretot a la gent que m'he endut dels dos llocs. A les Biokitas per ajudar-me sempre, abans i durant el doctorat, i als clínics per inspirar-me i convertir-se en amics meus, Cira, Núria, Manel i sobretot a la Nadia.

A les nenes noves de sempre: Meri, Sandra i Irene. Per retrobar-nos de nou i que hagi estat en un moment clau de la meva vida. Sempre donant-me suport i compartint els meus mal de caps i les meves explicacions extrallargues de la meva tesi.

Al món del bàsquet, el qual sempre m'ha aportat majoritàriament coses positives i sobretot per la bona gent que he conegut. Per compartir la meva segona passió i per ajudar-me a créixer tant a nivell personal, com professional. Els valors adquirits a través d'aquest esport no s'ensenyen enlloc; en l'àmbit professional es fa palès i han estat de gran utilitat per poder tirar la feina endavant.

Als integrants del cor de góspel de Medicina de la Universitat de Barcelona. Eternament agraïda de fer-me acabar bé el dia a cada assaig i de donar-me un toc musical en aquesta ruta.

A tots els companys de pis amb els quals he tingut el plaer de compartir tots aquests anys i que de rebot, els ha tocat conviure amb una doctoranda i tot el que comporta. Gràcies per la paciència i pel suport.

Als amics, d'arreu, que encara que jo "desaparegui", ells hi són: als de Castellserà, de Tàrrega, de Barcelona, de Girona… resumint: als que sempre hi són i als que sorprenentment també hi han estat.

Al Miquel R.

A la meva familia, tiets i cosins. Cap retrobament es salvava de comentar en quin punt em trobava del doctorat, sobretot els viatges Barcelona – Castellserà, amb cotxe es convertien en el moment "confessionari". Gràcies per ser l'orella que sempre vol escoltar.

Als meus pares, Jordi i Lourdes, perquè sóc qui sóc gràcies a ells. Mai tindré tot el que necessitaria per agraïr-los el que han fet per a mi.

I al meu germà, Marc. Em falta vocabulari per poder descriure com d'important has sigut sempre per a mi i sobretot durant aquests quatre anys. Aquest doctorat és mig teu. Gràcies.

DEDICATÒRIA

On hi ha voluntat, hi ha camí.

(George Herbert)

A la meva família.

INDICE

Listado de tablas / Tables' list

Listado de figuras / Figures' list

Abreviaciones / Abbreviations

Asint: Asintomáticos

A/G: Cociente albumina globulina

ALT: Alanina amonitransferasa

APCs: Células presentadoras de antigeno

CanL: Leishmaniosis canina

$CD4^+$: Grupo de diferenciación cuádruple

C Hep: Células hepáticas

CMI: Respuesta inmunitaria mediada por células

Con A: Concancavalina A

CO_2: Dióxido de carbon

C Ren: Células renales

CVL: Leishmaniosis canina visceral

DC: Células dentríticas

DNA: Ácido desoxiribonucleico

EDTA: Ácido Etilendiaminotetraacético

ELISA: Enzyme-linked immunosorbent assay

En: Endosomas

E I: Epitelio intestinal

GLA-SE: Adyuvante lipídico de glucopiranosilo en emulsión estable

HCV: Hospital clínico veterinario de la UAB

IFN-γ: Interferón gamma

IL-(6,12): Interleuquina-(6,12)

IMQ: Imiquimod-R837

iNos: Óxido nítrico sintasa inducible

LB: Linfocitos B

LPS: Lipofosfoglicano

LPS: Lipopolisacárido

LSA: Antigen soluble de *Leishmania*

LRV1: *Leishmania*

L. infantum: Leishmania infantum

MC: Mastocitos

µg: Microgramos

mL: Mililitros

Mo: Monocitos

Mø: Macrófagos

MPLA: Monofosforil lípido A

nm: Nanómetros

N: Neuronas

NK: Células *natural killer*

NK-κB: Factor nuclear kappa (cadena ligera) potenciador de células B activadas

NO: Óxido nítrico

RNA: Ácido ribonucleico

P: Plasmocitoides

PBS: Tampón fosfato salino

PRRs: Pattern recognition receptors

PAMPs: Pathogen associated molecular patterns

PBMCs: Células mononucleares de sangre periférica

PRRs: Receptores reconocedores de patrones

ROS: Especies reactivas de oxígeno

Rt-qPCR: Reacción quantitativa en cadena de polimerasa a tiempo real

SD: Desviación estándar

Sint: Sintomáticos

S P: Superfície celular

TH (1,2): T helper limphocytes (1,2)

TLRs: Receptores tipo Toll

TLRa: Agonista del receptor tipo Toll

TNF-α: Factor de necrosis tumoral

UAB: Universitat Autònoma de Barcelona

UPC: Ratio de orina proteína / creatinina

VL: Visceral leishmaniosis

WHO: Organización mundial de la Salud

Ø: Medio solo

↑: Sobreexpresión del gen

↓: Disminución del gen

RESUMEN / RESUM / SUMMARY

La leishmaniosis canina (CanL) es una enfermedad zoonótica causada por el protozoo *Leishmania infantum*, de distribución mundial y altamente endémica en la cuenca mediterránea. Este parásito se transmite mediante la picadura de las hembras de flebótomos, siendo el perro el hospedador y reservorio principal. Las manifestaciones clínicas de la infección por *L. infantum* en el perro son muy variables, desde una infección subclínica crónica hasta una enfermedad muy severa, que puede ser fatal. Debido a su compleja patogénesis, es importante el papel que juegan tanto la respuesta inmunitaria innata y adaptativa en esta infección. Sin embargo, existe más información en el perro sobre la respuesta inmunitaria adaptativa que sobre la innata. Los receptores tipo Toll (TLRs) son primordiales en la maquinaria del sistema inmunitario innato que facilitan la pronta detención de varias infecciones así como la activación de la cascada inflamatoria. Sin embargo, el papel de estos receptores en la infección por *L. infantum* en perros no es muy conocida y la información que hay sobre los mismos es muy limitada.

La hipótesis de esta tesis doctoral es que los TLRs tienen un papel importante en la infección por *L. infantum* debido a que estimulan la cascada inflamatoria. El objetivo general de la presente tesis doctoral fue el de investigar la expresión de TLRs en la CanL comparando con parámetros parasitológicos, clínicos, bioquímicos e inmunológicos así como evaluar el posible uso de los agonistas de TLRs en el tratamiento de esta enfermedad. Los objetivos específicos se han desarrollado en 5 estudios y se describen a continuación.

El primer objetivo específico se describe en el capítulo 3.1. y consistió en evaluar la expresión de los receptores tipo Toll 2 (TLR2) y 4 (TLR4) en sangre no estimulada de perros con leishmaniosis clínica moderada en el momento del diagnóstico y durante un año de tratamiento así como correlacionar los parámetros clínicos, bioquímicos y parasitológicos. En el capítulo 3.2., se describe el segundo experimento donde se evaluó la cuantificación relativa del TLR2 y TLR4 en perros infectados por *L. infantum* en diferentes estadíos de la enfermedad según la clasificación de LeishVet en el momento del diagnóstico. El estudio consistió en determinar además los anticuerpos y la producción de interferón gamma

(IFN-γ) específicas frente al parásito así como la parasitemia en estadio clínico I (perros con dermatitis papular) y en estadío clínicos II-III (perros con dermatitis exfoliativa o ulcerativa). El objetivo específico del tercer estudio (capítulo 3.3.) fue el de determinar la transcripción de los genes TLR2, TLR4 y el ligando 1 de muerte programada (PD-L1) en sangre estimulada con antígeno soluble de *L. infantum* (LSA) y el mitógeno Concanavalina A (Con A) en perros enfermos respondedores a la producción de IFN-γ y no respondedores y en perros sanos. El propósito del estudio *ex vivo* 3.4. fue determinar la producción de las citoquinas IFN-γ, TNF-α y IL- 6 en sangre estimulada con LSA o agonistas TLRs (TLRsa) como el TLR3a,TLR4a y TLR7a solos o con combinación de LSA y cada uno de los TLRa en perros sanos infectados naturalmente por *Leishmania.* Finalmente se realizó un experimento *in vitro* (estudio 3.5) donde se evaluó la susceptibilidad del parásito a diferentes fármacos convencionales anti-*Leishmania* (alopurinol, miltefosine o meglumine antimoniato) en ensayos de promastigotes y amastigotes en una línea celular de macrófagos caninos así como con el tratamiento de los agonistas de TLR2, 3, 4 y 7 solos o combinados con los fármacos convencionales.

Los estudios descriptivos de esta tesis han confirmado que los TLRs parecen tener un papel en la enfermedad. Los perros infectados con *Leishmania* enfermos presentan diferente expresión de los TLRs comparado con los individuos sanos, dependiendo directamente también del perfil inmunológico del animal así como del estadio clínico. Así pues, en esta tesis doctoral, se obtuvo la sobreexpresión del TLR2 en sangre no estimulada en el momento del diagnóstico en perros con enfermedad moderada (estadío II-III) al comparar con los perros sanos. Sin embargo, la expresión de TLR2 en sangre no estimulada fue igual para los perros con enfermedad leve (estadío I) y los perros sanos. Además, se produjo una disminución de la expresión del TLR2 durante un año de tratamiento y la mejoría clínica en los perros con enfermedad moderada. No obstante, no se observaron cambios en la expresión de TLR4 en el diagnóstico ni durante el tratamiento en ninguno de los perros estudiados. Es importante señalar también que la expresión de TLR2 se correlacionó con parámetros clínicos, parasitológicos e inmunológicos asociados a enfermedad de moderada a severa. Además, los perros con estadío leve I y con dermatitis

papular tenían un perfil inmunológico predominante Th1 en cambio los animales clasificados en estadíos más severos predominaba un perfil Th2. Los resultados obtenidos en sangre estimulada con LSA fueron muy interesantes y correlacionados con los hallazgos obtenidos en sangre no estimulada. Se observó una reducción en la expresión génica de los genes TLR2 y TLR4 en sangre estimulada con LSA en los perros enfermos que eran productores de IFN-γ comparado con los perros sanos, y una alta expresión de PD-L1 en todos los grupos estudiados tanto para LSA como para Con A. La sangre estimulada con TLRsa en perros sanos resultó tener una alta producción de las citoquinas IFN-γ, TNF-α y IL-6, comparadas con el medio solo. Además, se observó un efecto sinérgico proinflamatoria cuando se estimuló con TLR4a y TLR7a en combinación con LSA.

En los estudios *in vitro* se demostró la susceptibilidad del parasito a los fármacos convencionales siendo el fármaco más eficaz la miltefosina. Además, se demostró que los TLRs agonistas solos reducen la infección y se observó también sinergia en la reducción de la infección con alopurinol y los agonistas para el TLR4. No obstante, no se detectó la producción de TNF-α ni de NO en los sobrenadantes recogidos a las 72 horas. Sin embargo, sí que se detectó la transcripción de los TLR2, 4 y 7 en todas las condiciones estudiadas. En general, se demostró una disminución de la transcripción de TLR2 o sin cambios en la expresión de TLR4 y TLR7 con la infección y resultados más variables. Sin embargo, la expresión de TLRs con el tratamiento con fármacos anti-*Leishmania* convencionales solos ocon los agonistas de TLRs solos o con combinaciones de ambos fue mas variable.

En conclusión, esta tesis doctoral ha demostrado que la expresión de TLR2 en sangre no estimulada es un marcador de enfermedad de moderada a severa. Sin embargo, el TLR4 no parece ser un buen marcador para CanL en sangre no estimulada. Además, la reducción de la expresión de los TLR2 y 4 en sangre estimulada con LSA se asoció a perros enfermos respondedores a IFN-γ los cuales tienen un perfil más protector que perros no respondedores a IFN-γ. El experimento *in vitro* nos reveló que los fármacos combinados con los TLRsa o incluso los TLRsa solos pueden reducir la infección. Por este motivo, los

hallazgos encontrados en esta tesis sugieren que los TLRs podrían utilizarse como inmunoterapia, solos o combinados con fármacos convencionales.

La leishmaniosi canina (CanL) és una malaltia zoonòtica causada pel paràsit *Leishmania infantum*, de distribució mundial i altament endèmica a la conca mediterrània. Aquest paràsit es transmet mitjançant la picada de les femelles de flebòtoms, sent el gos l'hoste i reservori principal. Les manifestacions clíniques de la infecció per *L. infantum* en el gos són molt variables, des d'una infecció subclínica crònica fins a una malaltia molt severa, que pot ser fatal. Deguda a la seva complexa patogènesi, és important el paper que juguen tant la resposta immunitària innata i adaptativa en aquesta infecció. No obstant això, hi ha més informació al gos sobre la resposta immunitària adaptativa que sobre la innata. Els receptors tipus Toll (TLRs) són primordials en la maquinària del sistema immunitari innat que faciliten la ràpida detenció de diverses infeccions així com l'activació de la cascada inflamatòria. No obstant això, el paper d'aquests receptors en la infecció per *L. infantum* en gossos no és molt coneguda i la informació que hi ha sobre els mateixos és molt limitada.

La hipòtesi d'aquesta tesi doctoral és que els TLRs tenen un paper important en la infecció per *L. infantum* pel fet que estimulen la cascada inflamatòria. L'objectiu general de la present tesi doctoral va ser el d'investigar l'expressió de TLRs en la CanL comparant amb paràmetres parasitològics, clínics, bioquímics i immunològics així com avaluar el possible ús dels agonistes de TLRs en el tractament d'aquesta malaltia. Els objectius específics s'han desenvolupat en 5 estudis que es descriuen a continuació.

El primer objectiu específic es descriu en el capítol 3.1. i va consistir en avaluar l'expressió dels receptors tipus Toll 2 (TLR2) i 4 (TLR4) en sang no estimulada de gossos amb leishmaniosi clínica moderada en el moment del diagnòstic i durant un any de tractament així com correlacionar els paràmetres clínics, bioquímics i parasitològics. En el capítol 3.2., es descriu el segon experiment on es va avaluar la quantificació relativa de l'TLR2 i TLR4 en gossos infectats per *L. infantum* en diferents estadis de la malaltia segons la classificació de LeishVet en el moment del diagnòstic. L'estudi va consistir a determinar a més els anticossos i la producció d'interferó gamma (IFN-γ) específiques enfront del paràsit així com la parasitèmia en estadi clínic I (gossos amb dermatitis papular) i en estadis clínics II-III (gossos amb dermatitis exfoliativa o ulcerativa). L'objectiu específic del

tercer estudi (capítol 3.3.) Va ser el de determinar la transcripció dels gens TLR2, TLR4 i el lligant 1 de mort programada (PD-L1) en sang estimulada amb antigen soluble de *L. infantum* (LSA) i el mitogen Concanavalina a (Con A) en gossos malalts capaços de produïr IFN-γ i els no capaços i en gossos sans. El propòsit de l'estudi *ex vivo* 3.4. va ser determinar la producció de les citocines IFN-γ, TNF-α i IL-6 en sang estimulada amb LSA o agonistes TLRs (TLRsa) com el TLR3a, TLR4a i TLR7a sols o amb combinació de LSA i cadascun dels TLRa en gossos sans infectats naturalment per *Leishmània*. Finalment es va realitzar un experiment *in vitro* (estudi 3.5) on es va avaluar la susceptibilitat del paràsit a diferents fàrmacs convencionals anti-*Leishmània* (al·lopurinol, miltefosine o meglumine antimoniat) tan en promastigots com en amastigots en una línia cel·lular de macròfags canins així com amb el tractament dels agonistes de TLR2, 3, 4 i 7 sols o combinats amb els fàrmacs convencionals.

Els estudis descriptius d'aquesta tesi han confirmat que els TLRs semblen tenir un paper en la malaltia. Els gossos infectats amb *Leishmània* malalts presenten diferent expressió dels TLRs comparat amb els individus sans, depenent directament també del perfil immunològic de l'animal i de l'estadi clínic. Així doncs, en aquesta tesi doctoral, es va observar la sobreexpressió del TLR2 en sang no estimulada en el moment del diagnòstic en gossos amb malaltia moderada (estadi II-III) en comparar amb els gossos sans. No obstant això, l'expressió de TLR2 en sang no estimulada va ser igual per als gossos amb malaltia lleu (estadi I) i els gossos sans. A més, es va produir una disminució de l'expressió del TLR2 durant un any de tractament i la millora clínica en els gossos amb malaltia moderada. No obstant això, no es van observar canvis en l'expressió de TLR4 en el diagnòstic ni durant el tractament en cap dels gossos estudiats. És important assenyalar també que l'expressió de TLR2 es va correlacionar amb paràmetres clínics, parasitològics i immunològics associats a malaltia de moderada a severa. A més, els gossos amb estadi lleu I i amb dermatitis papular tenien un perfil immunològic predominant Th1, en canvi, els animals classificats en estadis més severs predominava un perfil Th2.

Els resultats obtinguts en sang estimulada amb LSA van ser molt interessants i correlacionats amb les troballes obtingudes en sang no estimulada. Es va observar una

reducció en l'expressió gènica dels gens TLR2 i TLR4 en sang estimulada amb LSA en els gossos malalts que eren productors de IFN-γ comparat amb els gossos sans i una alta expressió de PD-L1 a tots els grups estudiats tant per LSA com per amb Con A. La sang estimulada amb TLRsa en gossos sans va resultar tenir una alta producció de les citoquines TNF-α i IL-6, comparades amb el medi sol. A més, es va observar un efecte sinèrgic pro-inflamatori quan es va estimular amb TLR4a i TLR7a en combinació amb LSA.

En els estudis *in vitro* es va demostrar la susceptibilitat del paràsit als fàrmacs convencionals sent el fàrmac més eficaç la miltefosina. A més, es va demostrar que els TLRs agonistes sols redueixen la infecció i es va observar també sinergia en la reducció de la infecció amb al·lopurinol i els agonistes per al TLR4. No obstant això, no es va detectar la producció de TNF-α ni de NO en els sobrenedants recollits a les 72 hores. No obstant això, sí que es va detectar la transcripció dels TLR2, 4 i 7 en totes les condicions estudiades. En general, es va demostrar una disminució de la transcripció de TLR2 o sense canvis en l'expressió de TLR4 i TLR7 amb la infecció . No obstant això, l'expressió de TLRs després del tractament amb fàrmacs anti-*Leishmania* convencionals sols o els agonistes de TLRs sols o combinacions dels dos va ser més variable.

En conclusió, aquesta tesi doctoral ha demostrat que l'expressió de TLR2 en sang no estimulada és un marcador de malaltia de moderada a severa. No obstant això, el TLR4 no sembla ser un bon marcador per CanL en sang no estimulada. A més, la reducció de l'expressió dels TLR2 i 4 en sang estimulada amb LSA es va associar a gossos malalts productors d'IFN-γ els quals tenen un perfil més protector que gossos no productors d'IFN-γ. L'experiment *in vitro* ens va revelar que els fàrmacs combinats amb els TLRsa o fins i tot els TLRsa sols poden reduir la infecció. Per aquest motiu, els resultats trobats en aquesta tesi suggereixen que els TLRs podrien utilitzar-se com immunoteràpia, sols o combinats amb fàrmacs convencionals.

Canine leishmaniosis (CanL) is a zoonotic disease caused by the protozoan Leishmania infantum, of worldwide distribution and highly endemic in the Mediterranean basin. This parasite is transmitted by the bite of sandfly females. The dog is the main host and reservoir. The clinical manifestations of L. infantum infection in the dog are very variable and range from a chronic subclinical infection to a very severe disease, which can be fatal. Due to its wide pathogenesis, the innate and adaptive immune responses play a role in this canine infection. However, there is much more information on the adaptive immune response than on the innate one. Toll-like receptors (TLR) are essential in the machinery of the immune system that facilitates the early arrest of several infections as well as the activation of the inflammatory cascade. However, the role of these receptors in L. infantum infection in dogs is not well known and the information is very limited.

The hypothesis of this doctoral thesis is that TLRs have an important role in *L. infantum* infection in dogs because they stimulate the inflammatory cascade. The general objective of this doctoral thesis was to investigate the expression of TLRs in the CanL and compare with parasitological, clinical, biochemical and immunological parameters as well as to evaluate the possible use of TLR agonists in the treatment of this disease. The specific objectives have been developed in five studies and are described below.

The first specific objective is described in chapter 3.1. and consisted in evaluating the expression of Toll-like receptors 2 (TLR2) and 4 (TLR4) in unstimulated blood of dogs with moderate clinical leishmaniosis at the time of diagnosis and during one year of treatment as well as correlating clinical, biochemical and parasitological parameters. In chapter 3.2., we described the second experiment where the relative quantification of TLR2 and TLR4 was evaluated in dogs infected by *L. infantum* in different stages of the disease according to the LeishVet classification at the time of diagnosis. This study also investigated the antibodies and the production of interferon gamma (IFN-γ) specific to the parasite as well as the parasitemia in clinical stage I (dogs with papular dermatitis) and in clinical stages II-III (dogs with exfoliative dermatitis or ulcerative) The specific objective of the third study (Chapter 3.3.) was to determine the transcription of the TLR2, TLR4 and programmed death ligand 1 (PD-L1) genes in blood stimulated with *L. infantum* soluble

antigen (LSA) and the mitogen Concanavalin A (Con A) in sick dogs IFN-γ producers and non-IFN-γ producers and healthy dogs. The purpose of the study described in chapter 3.4. was to determine the production of the cytokines TNF-α and IL-6 in blood stimulated with LSA or TLRs agonists (TLR3, TLR4 and TLR7) alone or combined of dogs naturally infected by *Leishmania*. Finally, an *in vitro* experiment (Chapter 3.5) was carried out where the parasites' susceptibility to different conventional anti-*Leishmania* drugs (allopurinol, miltefosine or meglumine antimonate) was evaluated in promastigote and amastigote assays in a canine macrophage cell line as well as in the treatment of TLRs agonists 2, 3, 4 and 7 (TLRsa) alone or in combination with conventional drugs.

Descriptive studies of this thesis have confirmed that TLRs seem to have a role in the disease. Sick dogs infected with *Leishmania* present different expression of TLRs compared to healthy individuals, also directly depending on the immunological profile of the animal as well as the clinical stage. Thus, in this doctoral thesis, overexpression of TLR2 was obtained in unstimulated blood at the time of diagnosis in dogs with moderate disease (stage II-III) when compared with healthy dogs. However, expression of TLR2 in unstimulated blood was the same for dogs with mild disease (stage I) and healthy dogs. In addition, there was a decrease in the expression of TLR2 during one year of treatment and clinical improvement in dogs with moderate disease. However, no changes were observed in TLR4 expression at diagnosis or during treatment in any of the dogs studied. It is also important to highlight that the expression of TLR2 was correlated with clinical, parasitological and immunological parameters associated with moderate to severe disease.

In addition, dogs with mild stage I and papular dermatitis had a predominantly Th1 immunological profile whereas animals classified in more severe stages had a Th2 profile predominant. The results obtained in blood stimulated with LSA were very interesting and correlated with the findings obtained in unstimulated blood. A reduction in gene expression of the TLR2 and TLR4 genes in blood stimulated with LSA was observed in sick dogs that were IFN-γ producers compared to healthy dogs and a high expression of PD-L1 in all the groups studied for both LSA as for Con A. The blood stimulated with TLRs

agonists (TLRsa) and LSA of sick dogs turned out to have a high production of the cytokines IFN-γ, TNF-α and IL-6, compared with the medium alone. The combinations that gave the most production of cytokines of the Th1 profile are the agonists TLR4 and TLR7 each combined with LSA.

In vitro studies demonstrated the susceptibility of the parasite to conventional drugs, being miltefosine the most effective drug. In addition, it was shown that TLRsa agonists alone reduced infection, and a synergistic effect was also observed in the reduction of infection with allopurinol and agonists for TLR4. However, the production of TNF-α or NO was not detected in the supernatants collected after 72 hours. However, the transcription of TLR2, 4 and 7 was detected in all the conditions studied. In general, a decrease in the transcription of TLR2s was demonstrated or no changes in the expression of TLR4 and TLR7 with infection. However, the expression of TLRs after treatment with conventional anti-*Leishmania* drugs alone or TLR agonists alone or combination of both was more variable.

In conclusion, this doctoral thesis has shown that the expression of TLR2 in blood is not stimulated in a marker of moderate to severe disease. However, TLR4 does not appear to be a good marker for CanL in unstimulated blood. In addition, the reduced expression of TLR2 and 4 in blood stimulated with LSA was associated with sick dogs responding to IFN-γ which have a more protective profile than dogs not responding to IFN-γ. The in vitro study revealed that drugs combined with TLRsa or even TLRsa alone can reduce infection. For this reason, the findings found in this thesis are that TLRs can be used as immunotherapy or as adjuvants in future vaccines.

CAPÍTULO 1

INTRODUCCIÓN /INTRODUCTION

1. El parásito

Leishman y Donovan, dos médicos británicos, fueron los primeros en describir el parasito en el bazo de pacientes humanos en la India en el 1903 (Dedet and Pratlong, 2000; Desjeux, 1999) y de ahí deriva el nombre de una de las especies de este protozoo: *Leishmania donovani*. El género de *Leishmania* incluye más de dos docenas de especies, la mayor parte de ellas parasitan a la especie humana (Steverding, 2017). Posteriormente, Nicole y Comte identificaron *Leishmania infantum* en el perro en Túnez en 1908 (Berman, 2012).

1.1. El tripanosoma

Leishmania spp. forma parte de la familia Trypanosomatidae incluida en el género de kinetoplástidos. El nombre viene dado por la raíz en griego de Typano- (barrenillo) y soma (cuerpo). Todos los tripanosomas son parásitos de animales vertebrados. Varios tripanosomas han sido descubiertos en anfibios, reptiles, aves y mamíferos en todo el mundo (Hamilton, 2017).

La mayoría de insectos (sanguijuelas y artrópodos) transmiten muchas de las especies de tripanosoma. En este género, no hay un estadío libre de parasitismo (Hamilton, 2017).

1.1.1. El origen de la familia *Trypanosomatidae*

Los tripanosomas son kinetoplástidos, un grupo de protistas flagelados que pertenecen al fílum Euglenozoa y es caracterizado por la presencia del orgánulo con el DNA compactado llamado Kinetoplasto (Berman, 2012).

Todos los tripanosomátidos actúan como parásitos en todas las formas y estadíos de su ciclo de vida. Estudios moleculares reflejan unos cambios significativos en la taxonomía de

los tripanosomas que han revelado una gran diversidad aunque *Trypanosoma* y *Leishmania* continúan siendo los únicos parásitos de vertebrados. Parece ser que *Leishmania* ha evolucionado más que el *Trypanosoma*. Así, *T. cruzi* y otras especies de tripanosomas no comparten las mismas adaptaciones ancestrales de los parásitos de los vertebrados como el género *Leishmania* (Hamilton, 2017).

1.2. Taxonomía del protozoo *Leishmania*

Se conocen varias especies de *Leishmania*. De acuerdo con la clasificación taxonómica de las especies propuesta por Lainson y Shaw en 1987 (Lainson et al., 1987), les términos utilizados hoy en día están divididos en dos subgéneros: la *Leishmania*, presente en el Viejo mundo y el Nuevo mundo, y *Vianna*, restringida al Nuevo mundo (Novo et al., 2016; W. Peters 1987). Diversas clasificaciones se han usado para el género de *Leishmania*. Se detalla la taxonomía de *Leishmania infantum* en la figura 1.1.

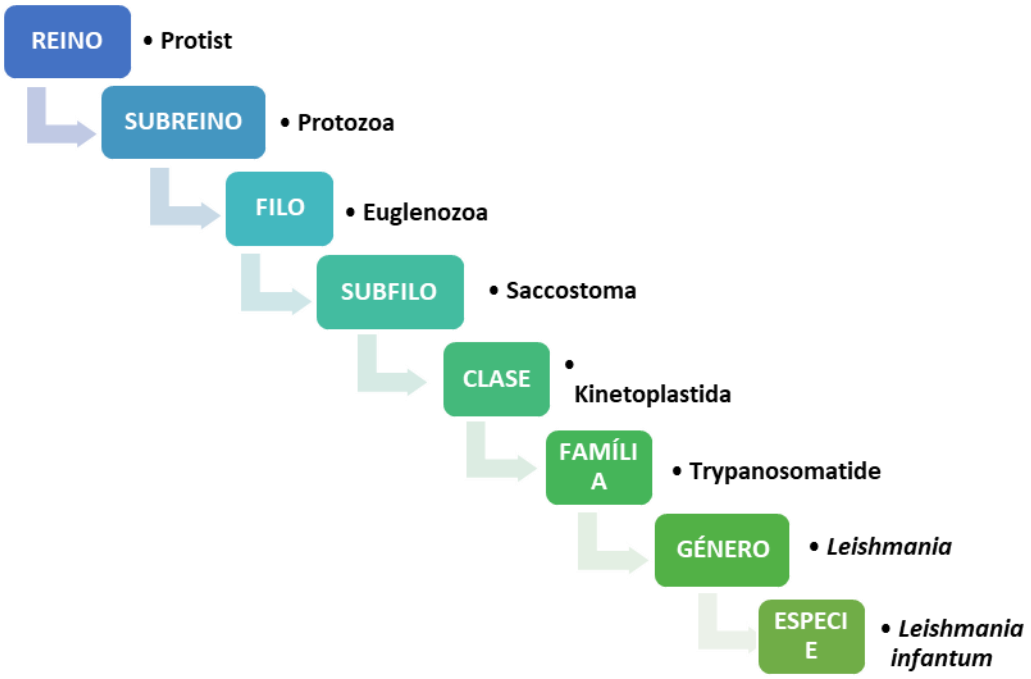

Figura 1.1. Clasificación taxonómica de la especie Leishmania infantum.

Estos dos subgéneros se diferencian por el lugar donde realiza la reproducción en el tracto digestivo del vector. La maduración de las especies del subgénero *Leishmania* está limitado a la partes alimentarias del tracto, que va des de la parte anterior hasta el pilorio, donde se encuentra la conjunción entre el intestino medio y la parte posterior de éste (crecimiento supra-pilorio), mientras que las especies del subgénero *Viannia* se puede dar tanto en el intestino medio como en el posterior (crecimiento pre-pilorio) (Novo et al., 2016).

La clasificación del género *Leishmania* es compleja. Para realizar la clasificación se emplearon los criterios como la geografía, el cuadro clínico o los nombres de quienes describieron las especies por primera vez (Killick-Kendrick, 1990). Esta situación desencadenó todo un seguido de nomenclaturas sobre las veintitrés especies que han sido descritas (figura 1.2.) (Killick-Kendrick, 1990). A pesar de las posibles confusiones, es muy importante identificar las especies que se van encontrando con el fin de tener más información sobre la epidemiología de éstas y su patogénesis para poder establecer mejor el diagnóstico y sobretodo tratamientos eficaces.

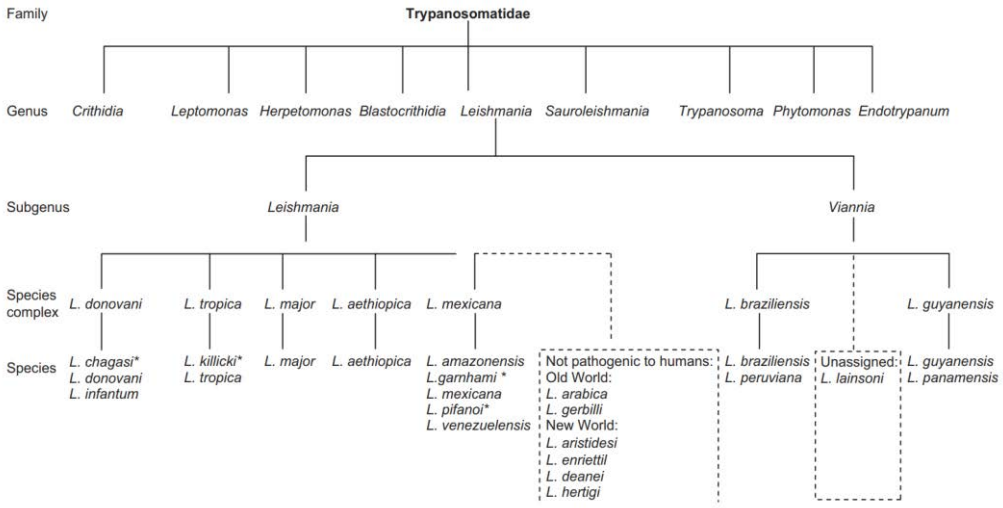

Figura 1.2. Clasificación taxonómica de Leishmania en humanos basada en parámetros intrínsecos (isoenzimas) y numeración taxonómica.

** Estatus de las especies en revisión. L. chagasi en el Nuevo mundo es la misma especie que L. infantum (WHO, 2010).*

Se han encontrado algunas especies que se han descrito solo en reptiles, de esta manera, se consideran un género separado, llamado *Sauroleishmania*, el cual se contempla como un subgénero ancestral del género de *Leishmania* (Novo et al., 2016).

La caracterización filogenética describe las relaciones parenterales entre diferentes especies de *Leishmania*, que son confirmadas por varios marcadores moleculares. Estas técnicas confirmaron la clasificación de los dos subgéneros hecha por Lainson y Shaw (Lainson et al., 1987).

El género de *Leishmania* también se puede catalogar en dos grandes categorías como las leishmaniosis zoonóticas, en las cuales el reservorio son animales domésticos o salvajes, y las leishmaniosis antropomórficas, en que los reservorios son los humanos (Grimaldi et al., 1987; Lee, 2000). En la tabla 1.1. se muestran las principales especies de *Leishmania* descritas hasta el momento.

Tabla 1.1. Principales especies de *Leishmania* que afectan a personas y animales.

ESPECIES	DISTRIBUCIÓN GEOGRÁFICA	CLASIFICACIÓN CLÍNICA EN HUMANOS	CLASIFICACIÓN SEGÚN RESERVORIO	HOSPEDADOR VERTEBRADO	REFERENCIAS
Leishmania aethiopica	Etiopía, Kenia	Cutánea, visceral	zoonótica	humanos, hyraxes	(Krayter et al., 2015; Odiwuor et al., 2011)
Leishmania amazonensis	Sud América	Cutánea, mucocutanea, visceral	zoonótica	humanos, roedores	(WHO, 2010)
Leishmania (Viannia) braziliensis	Sud América	Cutánea, mucocutánea	zoonótica	humanos, roedores	(Bustamante et al., 2012)
Leishmania donovani	África, India, Bangladesh	Visceral	antropozoonótica	humanos	*(*Poeppl et al., 2012)
Leishmania (Viannia) guyayensis	Sud américa	Cutánea	zoonótica	humanos, perros, perezosos, otros animales silvestres	*(*Poeppl et al., 2012)

Leishmania infantum (chagasi)	Cuenca Mediterránea, Sud América Oriente medio, Asia	Visceral, cutánea, mucocutánea	zoonótica	humanos, perros	*(*Alvar et al., 1990; Cantacessi et al., 2015; Freites-Martinez et al., 2015)
Leishmania major	Norte-oeste de Índia	Visceral	zoonótica	perros, animales silvestres	(Cantacessi et al., 2015)
Leishmania mexicana	México y América central	Cutánea	zoonótica	humanos, roedores	(Cantacessi et al., 2015)
L. mexicana pifanoi	Venezuela y América central	Cutánea mucocutánea	zoonótica	humanos, roedores	(Cantacessi et al., 2015)
Leishmania (Viannia) panamensis	América central	Cutánea	zoonótica	perezosos, animales silvestres	(Cantacessi et al., 2015)
Leishmania peruviana	Perú	Cutánea	zoonótica	humanos, animales silvestres	(Cantacessi et al., 2015)
Leishmania tropica	Norte Africa, Oriente medio, cuenca mediterránea	Cutánea	antropozoonótica zoonótica	humanos, roedores	(Gramiccia and Gradoni, 2005)

A lo largo de los tiempo se han utilizado diferentes métodos para clasificar el parásito, como los estudios fenotípicos basados en las isoenzimas (Hide et al., 2001) con la técnica de electroforesis, anticuerpos monoclonales, investigaciones genotípicas como la Técnica del Polimorfismo de Longitud de Fragmento (RFLPs) (Le Blancq et al., 1986) e identificación con técnicas moleculares a través de la hibridación del DNA usando la reacción en cadena de la polimerasa (PCR) para distinguir si está presente la *Leishmania* o para saber de qué especie se trata (Hide et al., 2001).

1.3. Cepas de *L. infantum*

Leishmania infantum está muy extendida geográficamente y tiene un gran rango de polimorfismos enzimáticos.

Se conocen 38 zimodemas (Gállego, 2003). La gran mayoría de cepas proceden del humano pero también de perros y de flebótomos (WHO, 2010). Las muestras estudiadas fuero criopreservadas en nitrógeno líquido en el Criobanco internacional de *Leishmania* de Montpellier, en Francia. El zimodema MON-1 es el principal responsable de la mayoría de las infecciones en el perro (Gállego, 2003; Pratlong et al., 2004). En 2004 se realizó un estudio dónde estudiando 712 izoenzimas de *Leishmania infantum* obtenidas tanto de gato, como de perro o humano. Destacaron 7 zimodemas: MON-1, MON-11, MON-24, MON-29, MON-33, MON-34, y MON-108; y descubrieron que el zimodema MON-1 era uno de los presentes en perros (Pratlong et al., 2004). Más adelante, se descubrieron más zimodemas que afectaban a *Canis familiaris* (Cardoso et al., 2002).

1.4. El vector

Los flebótomos pertenecen a la familia Diptera psychodidae.

1.4.1. Taxonomía

Se han descrito alrededor de 800 especies de flebótomos y el noventa y ocho de ellas se han confirmado como especies competentes para la transmisión de *Leishmania* (Maroli et al., 2013). El género *phlebotomus* es el más extendido en el Viejo mundo, con particularmente 42 especies reconocidas, y el género *Lutzomyia* en el Nuevo mundo, con 56 especies descritas (Killick-Kendrick,

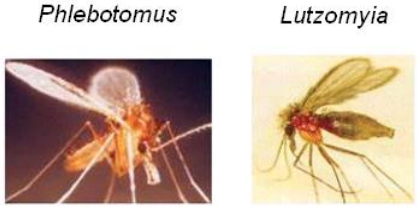

Figura 1.3. Flebótomos principales causantes de la transmisión de Leishmania *en España. Fuente: J Clin Invest. 2008 Apr; 118(4):1301-10.*

1990; WHO, 2010). En la figura 1.3. se muestran las dos clases de flebótomos. Los vectores implicados en la infección de *L. infantum* en la cuenca Mediterránea pertenecen al subgénero *Larrossius* (Absavaran et al., 2009). En España hay identificados 12 flebótomos hasta el momento: *Phlebotomus perniciosus, Phlebotomus ariasi, Phlebotomus papatasi, Phlebotomus langeroni, Phlebotomus sergenti, Phlebotomus mascitti, Phlebotomus longicuspis, Phlebotomus fortunatarum, Phlebotomus alexandri* y *Phlebotomus chabaudi Sergentomya, Minuta, S. fallax,* (Aransay et al., 2004; Saez et al., 2018; Sanchís-Marín, 1989). Los dos primeros son los principales vectores implicados en la transmisión de esta infección (Aransay et al., 2004; Martin-Sanchez et al., 1994).

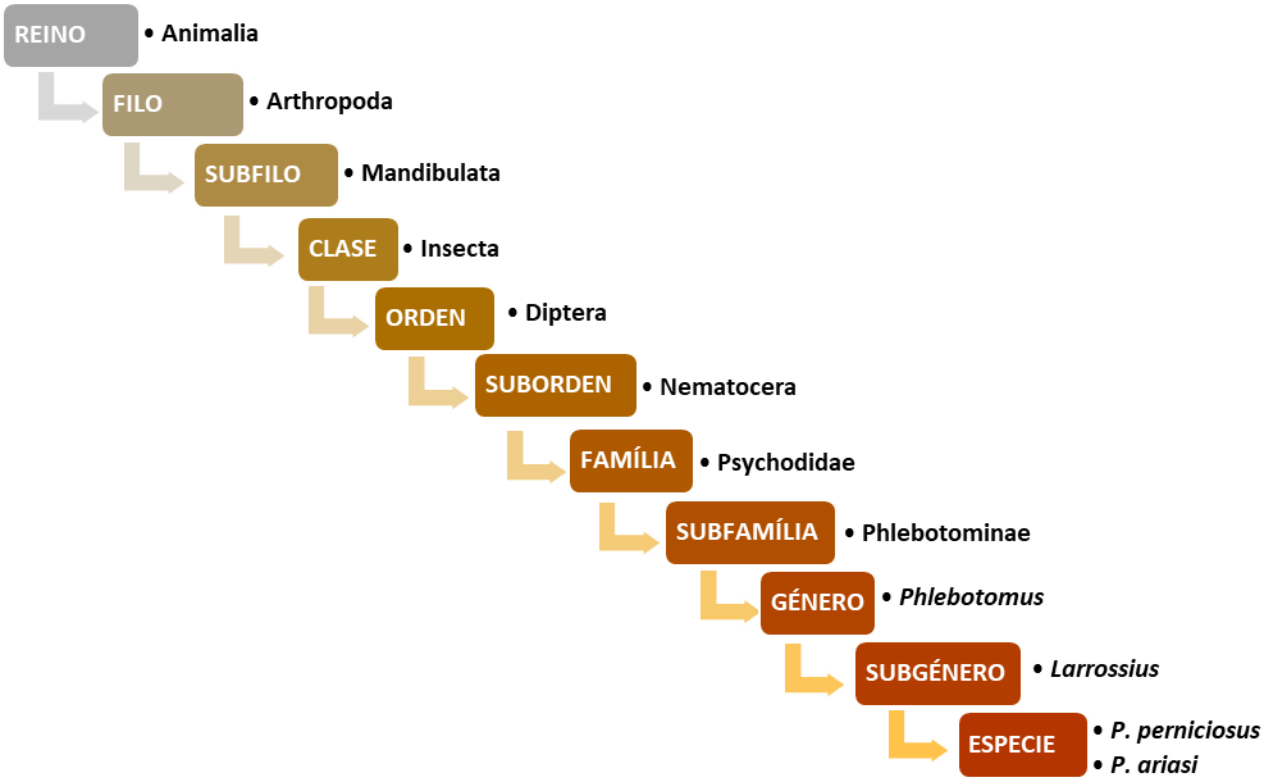

Figura 1.4. Clasificación taxonómica del flebótomo (Killick-Kendrick, 1999).

La nomenclatura del flebótomo es confusa ya que en diferentes partes del mundo otros tipos de díptera se les llaman también flebótomos (Killick-Kendrick, 1990). Por ejemplo, los *culícidos,* pertenecen a la familia *simuliidae.* A pesar de ello, las clasificaciones que están establecidas no son globalmente aceptadas, aunque comúnmente se les clasifica en la familia *Psychodidae*, y en la subfamilia *Phlebotominae*. En el diagrama 1.4. se muestra la clasificación taxonómica del flebótomo. Es importante hacer distinción entre los vectores que transmiten *Leishmania*, los cuales nombrados correctamente son los flebótomos. Éstos están agrupados en el suborden Nematocera dentro del orden Diptera (Killick-Kendrick, 1990).

1.4.2. Distribución

Los flebótomos normalmente se encuentran en partes temperadas del planeta, como en el sur de Europa, Asia, África y América central y del sur. Su dispersión se expande justo por encima de latitudes de 50º en el suroeste de Canadá y por debajo de la latitud de Francia y Mongolia. Su distribución en altitud es del nivel del mar muerto hasta 3399 metros por encima del nivel del mar en Afganistán (Killick-Kendrick, 1999).

El flebótomo *P. perniciosus* está bien distribuido en España, predominantemente en regiones áridas, y actúa como primer vector causante de la leishmaniosis. Newstead describió esta especie en 1911. *Phlebotomus ariasi* se considera un vector más esporádico que disemina la enfermedad en zonas concretas en España como en Cataluña, Aragón y Castilla y León, tal y como se muestra en la figura 1.5. (Aransay et al., 2004).

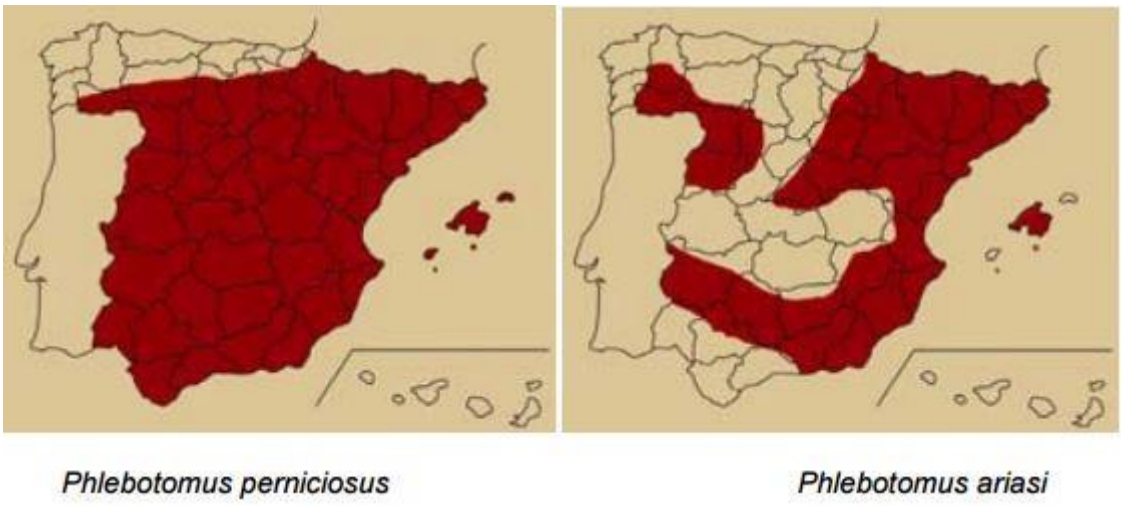

Figura 1.5.Distribución de los dos principales flebótomos en España (Aransay et al., 2004; Berta Suárez Rodríguez, 2012).

La tasa de infección por *L. infantum* en España oscila entre el 0.4 y el 4.6% en las especies de flebótomos que se muestrearon en las islas Baleares, Madrid, Tarragona, Zaragoza y Almería (Miró, 2006; Morillas Marquez et al., 1991).

1.4.3. Comportamiento y apariencia

Los flebótomos tienen una largada de 3 mm como máximo. Su color de capa abarca desde casi negra a casi blanca. Sus alas son alargadas y peludas, tal y como se muestra en la figura 1.6. (Killick-Kendrick, 1999).

Figura 1.6. Hembra de P. perniciosus alimentándose en un brazo humano.

Fuente:http://www.raywilsonbirdphotography.co.uk/Galleries/Invertebrates/vectors/sand_fly.html

Se cree que el comportamiento que tienen es de hacer saltos antes de posicionarse para picar al hospedador. Este suceso es debido a que no se dispersa mucho del lugar donde crían (Killick-Kendrick, 1999). Normalmente, pocas veces se excede de un kilómetro de su hábitat. Están condicionados por factores ambientales, pues hay una correlación positiva entre la densidad de flebótomos y la temperatura, y una correlación negativa con la humedad relativa, así se define su dinámica estacional (Tarallo et al., 2010). Un estudio nos revela que su velocidad máxima de vuelo es un poco menos de 1 m/s (Killick-Kendrick et al., 1986). La actividad del flebótomo puede ser tanto diurna como nocturna, aunque su acción se centra en la franja crepuscular, en los amaneceres y los atardeceres (Gaglio et al., 2014), y entre los 15ºC y los 28ºC, siendo favorable la ausencia de viento o lluvia (Killick-Kendrick, 1999). El momento de alimentarse ordinariamente sucede cuando es de día. Los lugares diurnos que escogen suelen ser húmedos como las bodegas, cuevas, casa con fisuras en las paredes, donde hay una vegetación densa, nidos de aves, etc (Sharma and Singh, 2008).

Normalmente las hembras son exofágicas, suelen picar en el aire libre, y exofílicas, permaneciendo en el aire libre también para la maduración de los huevos (Ferro et al., 2011; Killick-Kendrick, 1999).

1.4.4. Alimentación y reproducción

Tanto machos como hembras se alimentan de recursos naturales de azúcar como la savia de las plantas y mielada de áfidos. Las hembras, pero no los machos, también se alimentan de sangre, la cual les proporciona la nutrición para la reproducción (Schlein and Warburg, 1986).

Algunas especies son capaces de generar una primera tanda de huevos sin haberse alimentado de sangre primero, se llaman autógenas, una especie poco común neotrópica se llama autógena y partenogenética (el-Kammah, 1973).

Las especies difieren entre sí por el número de veces que se alimentan de sangre durante el periodo gonotrópico, algunas solo se alimentan una vez por cada lote de huevos y en cambio otras se alimentan varias veces de sangre por cada grupo de huevos (Killick-Kendrick et al., 1977).

1.4.5. La picadura

Cuando un flebótomo hembra pica a un vertebrado para alimentarse , inyecta su saliva dentro de la piel del vertebrado y no sólo da la oportunidad de establecer una reacción alérgica en algunos casos, sino que también se considera una parte importante para que la transmisión del parásito sea exitosa (Ribeiro et al., 1989). En estudios de laboratorio, ha sido analizado que las reacciones ante las picaduras son diferentes entre especies y eso sugiere que la composición de la saliva varía entre éstas. Muchos estudios se realizaron para confirmar esta teoría y algunos de ellos encontraron un péptido en la saliva de *Lu. longipalpis*, que era un vasodilatador (maxadilán) bastante efectivo (Qureshi et al., 1996). Se cree que este péptido ayuda al crecimiento del parásito durante la picadura. Hay estudios *in vitro* que añaden información sobre las características de la saliva de los flebótomos y se vio que de alguna forma la saliva inmunosuprimía las células del bazo y los macrófagos (Theodos and Titus, 1993). Se ha demostrado que la expresión de distintas proteínas pertenecientes a la saliva del flebótomo pueden influir a la capacidad de transmisión del parásito (Hosseini-Vasoukolaei et al., 2016; Oliveira et al., 2008).

1.5. Ciclo de vida del parásito

El ciclo de vida del parásito es asexual y heteroxeno o digenético, cuyo ciclo de vida requiere dos hospedadores: un vertebrado (mamífero) y otro invertebrado, el flebótomo, ilustrado en la figura 1.7. El parásito se disemina a través de la picadura del flebótomo hembra infectada (Killick-Kendrick, 1990).

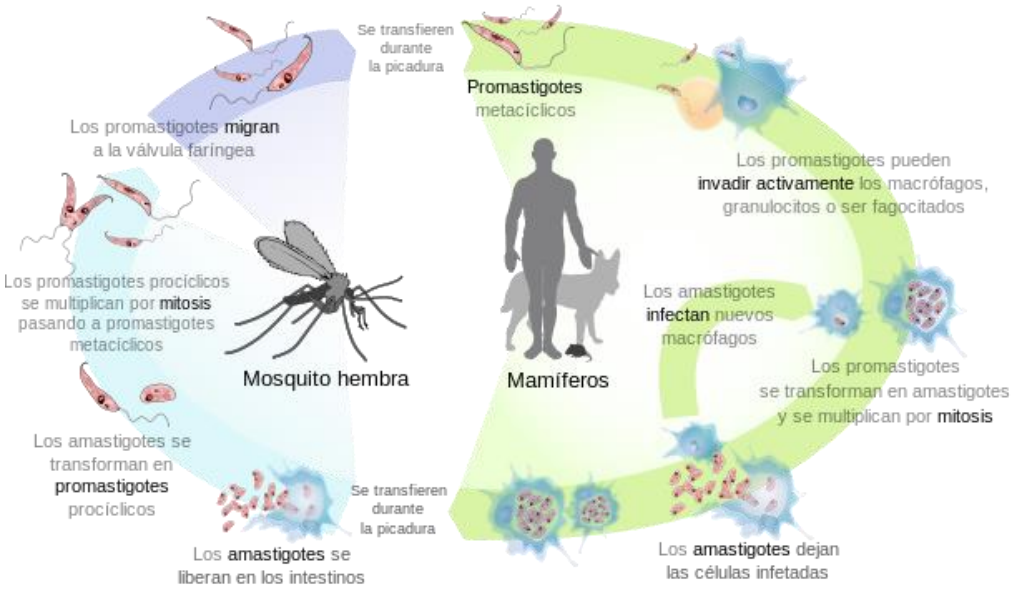

Figura 1.7. Ciclo digenético del parásito del género Leishmania *spp. Fuente: https://www.cell.com/trends/parasitology/fulltext/S1471-4922(11)00064-X?code=cell-site*

La forma promastigota infectiva es móvil y flagelada, que se encuentra en el tubo digestivo del hospedador invertebrado, y la forma amastigota se caracteriza por ser inmóvil y más redonda, sin flagelos, localizada en el hospedador vertebrado, primordialmente dentro de las células macrofágicas (Killick-Kendrick, 1990).

Los patógenos transmitidos por insectos pueden tener una mejor capacidad para expandirse rápidamente a través de la población huésped susceptible, particularmente cuando está involucrado un animal como reservorio. En la mayoría de infecciones transmitidas por sangre, los parásitos deben afrontar un ambiente hostil en el intestino de los insectos. Cuando el parásito se encuentra en el intestino medio del flebótomo, es la fase más crítica para el ciclo de vida de éste, ya que debe enfrentarse a cambios drásticos fisiológicos, bioquímicos y morfológicos (Novo et al., 2016).

Los amastigotes son cogidos de la sangre del vertebrado infectado, cuando el flebótomo hembra pica al vertebrado infectado, los amastigotes son absorbidos con la sangre y pasan al intestino del flebótomo donde allí se convertirán en metacíclicos y serán promastigotes (Kamhawi, 2006). Esta transformación se debe al cambio de temperatura y

pH del medio en que se encuentran (Bates and Rogers, 2004). Allí se multiplicarán a través de fisión binaria. Los promastigotes se mueven de manera anterior en prosboscis y son inyectados en el hospedador vertebrado cuando el flebótomo muerde a éste (Novo et al., 2016).

Los tejidos epiteliales regularmente deben hacer frente a patógenos, y el epitelio intestinal es uno de los tejidos principales entre huésped y patógeno en el tracto digestivo (Dostalova and Volf, 2012). En insectos hematófagos, los cuales actúan como vectores de enfermedades parasitarias como la tripanosomiasis o la leishmaniosis, el intestino representa el punto clave para que el parásito se desarrolle. Los promastigotes insertan el flagelo en el epitelio del tubo digestivo para quedarse anclados. Así, pueden persistir dentro del intestino cuando el resto de la sangre ingerida durante la comida es defecada (Myskova et al., 2016). El objetivo principal del parásito son los macrófagos de los hospedadores mamíferos (Bates, 2007) y el establecimiento de la infección se puede dar de tres maneras: a) por infección directa, b) por fagocitosis de los neutrófilos infectados o c) por infección silenciosa de los parásitos que se diseminan a partir de la apoptosis de los neutrófilos (Peters and Sacks, 2009).

1.6. Leishmaniosis canina

1.6.1. Epidemiología

1.6.1.1. Distribución geográfica

La CanL causada por *L. infantum* es endémica en más de 70 países en el mundo (Solano-Gallego et al., 2011) donde las dos regiones más afectadas son la cuenca mediterránea y Brasil (Solano-Gallego et al., 2009). Se desconoce la prevalencia global en perros domésticos (Gradoni, 2015) ya que la prevalencia exacta es muy difícil de cuantificar debido a que, durante el transcurso de los años, las técnicas usadas para

detectar la infección han estado varias: primero se basaban en exámenes parasitológicos mientras que los métodos más recientes son moleculares.

La organización mundial de la salud ha estimado que cerca de doce millones de personas están infectadas en todo el mundo y que cada año se reportan hasta 2 millones de casos nuevos (WHO, 2010). Los casos de leishmaniasis en humanos han documentado un aumento de infección de 42 veces más durante los años 1990 y 2010 (WHO, 2010).

Hay estudios que sugieren que hay una correlación positiva entre las prevalencias entre la leishmaniasis en humanos y la leishmaniosis canina (Cunha et al., 1995; Zoghlami et al., 2014) En el sur de Europa, se ha mostrado una seroprevalencia de un diez por ciento (Franco et al., 2011). En España, Francia, Portugal e Italia hay al menos 2 millones y medio de perros infectados de 15 millones (Moreno and Alvar, 2002).

El calentamiento global está facilitando la expansión de esta infección hacia lugares donde el flebótomo no tenía posibilidades de sobrevivir hasta ahora, por ello, se está expandiendo hacia el Norte de Europa juntamente con el hecho de que puede que haya perros importados con la enfermedad (Maia and Cardoso, 2015). Durante los últimos años, la enfermedad ha alcanzado el Norte de España y los pirineos de Francia (Chamaille et al., 2010; Miro et al., 2012). En zonas no endémicas, se han detectado casos en Bulgaria (Harizanov et al., 2013), Serbia (Savic et al., 2014), Alemania (Naucke et al., 2008), Inglaterra (Shaw et al., 2009) o Rumania (Mircean et al., 2014).

En España se muestran varios rangos de seroprevalencias des del 0% en el norte en Vizcaya y Asturias (Miro et al., 2012) hasta el 65% en la Cataluña oriental (Solano-Gallego et al., 2006). En la figura 1.8. se muestran las zonas más y menos endémicas en España en 2009. Puede ser que las diferencias encontradas entre las comunidades se deban también a las técnicas serológicas utilizadas y el tamaño muestral de cada región.

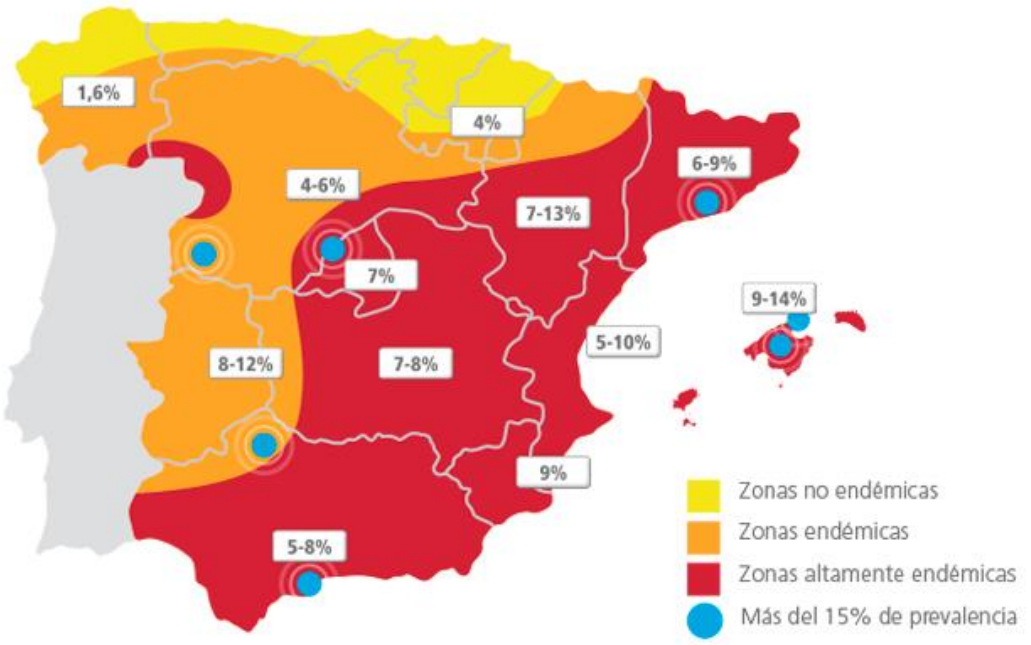

Figura 1.8. Mapa de seroprevalencia de CanL en España. Fuente: Mapa obtenido a partir de estudios y congresos europeos por P. Bourdeau: (EMOP 2004, worldLeish4 2009, ECVD 2009, RESFIZ 2009).

1.6.2. El reservorio

1.6.2.1. El reservorio principal

Los perros (*Canis familiaris*) son los principales reservorios de *L. infantum*, debido al elevado número de individuos presentes en el nicho ecológico y su estrecha relación con el vector (Alvar et al., 2004). El descubrimiento de la leishmaniosis canina en ambos lugares, los nombrados Viejo mundo y el Nuevo mundo, refleja cómo de difundido está el parásito en todo el mundo. Estos hallazgos no solo revelan una buena adaptabilidad del parásito en diferentes condiciones ambientales, la cual le hace posible ser transmitido por distintos vectores de distintas especies, géneros y subgéneros, sino que también se expanden gracias al movimiento que los animales realizan (WHO, 2014).

1.6.2.2. Otros reservorios

Los perros pueden estar en contacto con otros cánidos salvajes como el zorro o el chacal, los cuales también se infectan y son infectantes para el vector (Gramiccia and Gradoni, 2005). A pesar de ello, no se consideran fuente principal de transmisión ya que la proporción respecto a la de perros es mucho menor y la susceptibilidad en animales salvajes es también menor (Gomes et al., 2007; Luppi et al., 2008). Varias especies salvajes *canidae* como el zorro (*Vulpes* spp.), el chacal (*Canis aureus*), el lobo (*Canis lupus*) y el mapache (*Nyctereutes procyonoides*) (Millan et al., 2014) se han encontrado parasitadas por *L. infantum* tanto en el Viejo mundo como en el Nuevo mundo (WHO, 2012). En áreas endémicas se ha reportado la infección de otros animales domésticos, como los gatos (Otranto et al., 2017; Pennisi et al., 2015) o caballos. (Maia et al., 2010; Persichetti et al., 2018; Ramos-Vara et al., 1996)

En España, se han encontrado animales silvestres infectados por *Leishmania* en lobos (*Canis lupus*), zorros (*V. vulpes*), linces ibéricos (*Lynx pardinus*), ginetas (*Geneta geneta*), meloncillos (*Herpestes ichneumon*), garduñas (*Martes foina*), erizos europeos (*Erinaceus europaeus*), ratas comunes (*Rattus norvegicus*) y liebres ibéricas (*Lepus granatensis*) (Millan et al., 2016; Molina et al., 2012; Sastre et al., 2008; Sobrino et al., 2008).

1.6.2.3. Vías de transmisión de la leishmaniosis canina

El flebótomo es el único artrópodo capacitado para la transmisión del parásito de *Leishmania* spp. de manera biológicamente natural (Bates, 2007). La transmisión del parásito puede ser estacional como en la cuenca mediterránea o todo el año como en sud américa, y está condicionada tanto por la densidad de la población del vector como por la dinámica demográfica de los hospedadores vertebrados (Oliva et al., 2006). *Leishmania infantum* es un parásito obligado transmitido de huésped a huésped por diferentes vías: oral-fecal, sexual, por contacto directo, a través del agua, del aire, pero su forma principal

de transmisión es a partir de los flebótomos (Novo et al., 2016). En condiciones normales, un flebótomo pica frecuentemente al hospedador, liberando entre 10 y 500 promastigotes metacíclicos junto con los metabolitos de la digestión de la dermis del hospedador (Maia et al., 2011). Existen algunos estudios donde se ha evaluado la capacidad vectorial de otros artrópodos como las pulgas de la especie (*Ctenocephalides felis felis*) (Coutinho and Linardi, 2007), la garrapata (*Rhipicephalus sanguineus)* (Rakhshanpour et al., 2017) y también la mosca del caballo (*Trabanus impotunus*) (Coelho and Bresciani, 2013) sin demostrarse experimentalmente su rol en la transmisión del parásito (Solano-Gallego et al., 2012).

Se ha propuesto la posibilidad de que exista la transmisión directa, de perro a perro sin vectores de por medio, a través de heridas o mordeduras como por ejemplo pasa en la infección por *Babesia gibsoni* (Yeagley et al., 2009). Sin embargo, esta vía de transmisión no ha sido confirmada. La transmisión vertical (transplacentaria) se ha corroborado tanto en zonas endémicas (da Silva et al., 2009) como no endémicas (Boggiatto et al., 2011) y experimentalmente (Rosypal et al., 2005). El subministramiento de sangre de la placenta está estrechamente relacionado con la circulación sanguínea de la madre, si los macrófagos están infectados, pueden llegar al feto (Rosypal and Lindsay, 2005). Otra vía de contagio es la transmisión venérea (Silva et al., 2009). Experimentalmente, se demostró que la infección de *L. infantum* se puede transmitir de forma venérea, el 50% de las hembras sanas que copularon con machos naturalmente infectados dieron positivo mediante la técnica de PCR (Silva et al., 2009; Turchetti et al., 2014). La transfusión de sangre también es una fuente de transmisión (Owens et al., 2001).

1.6.3. Manifestaciones clínicas, inmunología y diagnóstico

Manifestaciones clínicas

No todos los perros expuestos muestran signos clínicos y las infecciones subclínicas son más frecuentes que los perros que manifiestan signos de enfermedad (Baneth et al., 2008; Solano-Gallego et al., 2001a). Así que, las manifestaciones clínicas causadas por *L. infantum* en perros pueden oscilar desde una infección crónica subclínica, una enfermedad leve que puede resolverse por sí sola hasta una enfermedad muy severa (Solano-Gallego et al., 2009).

En perros enfermos la enfermedad progresa durante un periodo de tiempo variable hacia un cuadro clínico que puede ser muy diferente en cada caso. El período de incubación de la enfermedad puede variar entre los tres meses y hasta siete años o más tras la picadura del flebótomo infectado (Koutinas and Koutinas, 2014). Por lo tanto, no se pueden atribuir unos signos clínicos específicos únicos en esta enfermedad. Como existen varios mecanismos patogénicos de la enfermedad, con diferentes órganos que pueden ser afectados a la vez, así como la particularidad de la respuesta inmunitaria de cada individuo, existe un amplio abanico de signos clínicos y alteraciones clinicopatológicas (Baneth et al., 2008). Un sistema de clasificación en estadios clínicos de la leishmaniosis canina fue desarrollado por el grupo LeishVet (www.leishvet.org/), basada en las anormalidades clínico-patológicas, signos clínicos y serología que se presentan en cada estadio clínico (Solano-Gallego et al., 2009). Con este sistema, se puede determinar un tratamiento más acotado y tener un pronóstico más acertado a la realidad. Los signos clínicos generales son la linfoadenomegalia como resultado del aumento de tamaño de los folículos linfoides, lo que resulta más fácil su palpación (Giunchetti et al., 2008b). También se ha descrito hepatomegalia en CanL pero principalmente el fallo renal es la causa principal de muerte en esta enfermedad (Solano-Gallego et al., 2009). Pérdida de peso, caquexia, letargia, palidez en las membranas mucosas, esplenomegalia, poliuria y

polidipsia, fiebre, vómitos y diarrea entre otros son signos clínicos frecuentes. Los signos cutáneos descritos en la leishmaniosis son la dermatitis exfoliativa con o sin alopecia, dermatitis ulcerativa, dermatitis nodular, dermatitis papular (ver imagen en la figura 1.9.), dermatitis pustular, hiperqueratosis nasal, hiperqueratosis plantar y onicogriposis. Los perros con leishmaniosis acostumbran a tener localizadas úlceras cutáneas y dermatitis exfoliativa alrededor de las orejas (Koutinas et al., 1999). En un área endémica, la dermatitis papular es una manifestación cutánea típica considerada de estadío leve y asociada a un buen pronóstico si no existen otras alteraciones clinicopatológicas asociadas en estos pacientes. Mientras que otras alteraciones cutáneas típicas de la leishmaniosis como la dermatitis exfoliativa o ulcerativa parece estar más asociada a estadios clínicos más severos (Esteve et al., 2015).

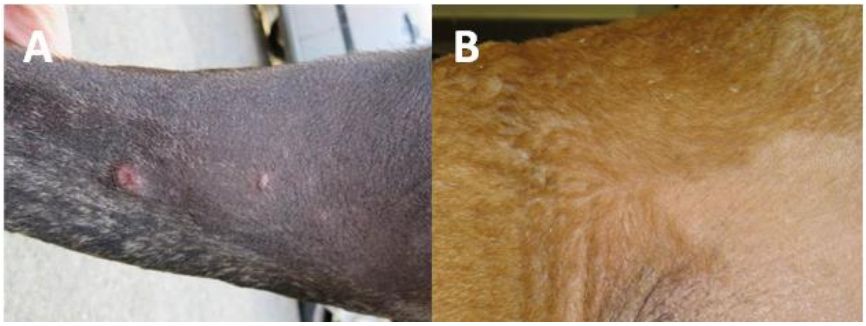

Figura 1.9. A: imagen de lesión cutánea con dermatitis papular y B: imagen de lesión cutánea con dermatitis exfoliativa. Fuente: Servicio de dermatología. HCV-UAB.

En cuanto a los signos oculares que se relacionan con la leishmaniosis son blefartitis, queratoconjuntivitis y la uveítis anterior entre otros. También pueden sufrir epistaxis, atrofia muscular, alteraciones vasculares y hasta alteraciones neurológicas (Blavier et al., 2001; Saridomichelakis and Koutinas, 2014; Solano-Gallego et al., 2011).

Los análisis de sangre son fundamentales, de ese modo se confirma el estado general del animal y se puede seguir la evolución clínica una vez empezado el tratamiento. Los hallazgos clinicopatológicos que suelen encontrarse son: anemia, leucocitosis, neutrofilia, linfopenia, hiperproteinemia con hipergammaglobulinemia, hipoalbuminemia,

reducción del ratio albuminas/ globulinas, incremento de enzimas hepáticas, proteinuria y azotemia renal (Baneth et al., 2008; Koutinas et al., 1999; Shaw et al., 2009; Solano-Gallego et al., 2001b).

Entre las enfermedades con las que se debe hacer diagnóstico diferencial son aquellas que presenten lesiones cutáneas como las dermatosis parasitarias como demodicosis, sarna sarcópica o por ejemplo dermatitis por picadura de pulga, en neoplasias y enfermedades vectoriales como ehrlichiosis, rickettsiosis o babesiosis (Baxarias et al., 2018; Bourdeau et al., 2014).

Inmunología

Los mecanismos exactos por los cuales un perro es susceptible o está protegido contra la leishmaniosis no se han descrito con claridad (Baneth et al., 2008; Miro et al., 2008). No se sabe con exactitud pero sí hay estudios que revelan que la raza podría predisponer a controlar la infección o padecer la enfermedad. Como ejemplo, los podencos ibicencos parecen tener una mayor resistencia que otras razas (Solano-Gallego et al., 2000). Sin embargo, en general, perros de pura raza parecen ser propensos (Miranda et al., 2008) a ser más susceptibles que los mestizos. Boxer, pastor alemán, rottweiler entre otras razas parecen ser más predispuestas a desarrollar la enfermedad (Penaforte et al., 2013). En la mayoría de estudios no parece haber una predisposición debido al sexo de los perros. Pero, sí que es cierto que quizá hay más estudios donde se han asociado los machos a padecer más la enfermedad que las hembras (Penaforte et al., 2013). La malnutrición produce defectos en la respuesta inmunitaria como se demostró en ratones (Perez et al., 1979) y es por este motivo que podría beneficiar la progresión de la infección a la enfermedad también en perros (Rodriguez-Cortes et al., 2017; Solano-Gallego et al., 2011). Recientemente, algunos estudios parecen sugerir que las coinfecciones permiten desarrollar la enfermedad (Angela Toepp, 2017; Baxarias et al.,

2018) y el perfil de citoquinas previo a la infección podría influir en la trayectoria de la enfermedad en el perro (Baneth et al., 2008).

La evasión del sistema inmunitario innato por parte de los parásitos está comúnmente aceptado (Sacks and Sher, 2002). Algunos de los mecanismos que se conocen hasta el momento que son empleados para ello son la remodelación fagosomal de los compartimentos y la modificación de algunas transducciones de señales (Sacks and Sher, 2002) entre otros. En los perros enfermos se ha asociado a que existe un cambio en los linfocitos T (Barbieri, 2006), los cuales hacen perder la hipersensibilidad de tipo IV o retardada frente los antígenos de *Leishmania* (Cardoso et al., 1998; Pinelli et al., 1994), la producción de IL-2 en células mononucleares de sangre periférica (PBMCs) *in vitro* (Santos-Gomes et al., 2002) así como la ausencia de la producción de IFN-γ o los linfocitos T en sangre periférica (Pinelli et al., 1994; Solano-Gallego et al., 2016b).

La gran mayoría de los perros infectados por *Leishmania*, muestran una buena respuesta inmunitaria mediada por linfocitos *in vitro* (Maia and Campino, 2008) y se ha visto que en perros subclínicos o con un perfil más leve de enfermedad, al aplicar el test de Montenegro o leishmanina, tienen una potente reacción positiva asociada a un buen pronóstico y el control de la infección (Cardoso et al., 1998; Solano-Gallego et al., 2000). La resistencia frente a este parasito se relaciona con una fuerte activación de los linfocitos CD4+ T *helper* 1 (Th1) los cuales producen IFN-γ, IL-2, TNF-α (Pinelli et al., 1994) y subsecuentemente interaccionando con los macrófagos infectados (Holzmuller et al., 2005). Por otra parte, si el parásito consigue anteponerse y modificar las señales de producción de citoquinas, las que se producirán serán del perfil Th2, como la IL-4 o IL-10, correlacionadas con la producción de anticuerpos y la progresión de la enfermedad (Hosein et al., 2017).

L. infantum parece inducir una respuesta mixta entre Th1 y Th2, de esa manera, el control de la replicación y de la progresión de la enfermedad depende del balance de estos dos tipos de respuesta inmunológica (Baneth et al., 2008; Papadogiannakis and Koutinas, 2015).

Diagnóstico

El método de diagnóstico más fiable es a través de visualización directa a través de técnicas moleculares (Moreira et al., 2007; Saridomichelakis and Koutinas, 2014; Solano-Gallego et al., 2014), o a partir de observación microscópica a través de citología o biopsias de las lesiones cutáneas u otros tejidos o fluidos biológicos.

Las técnicas inmunológicas empleadas en el diagnostico de CanL permiten valorar la respuesta inmunitaria humoral y cel·lular.

Los análisis de respuesta inmunitaria humoral se basan principalment en la detección de IgG frente al parásito. La muestra que se usa para detectar estos anticuerpos específicos anti-l*eishmania* es el suero. La sensibilidad a la prueba serológica es muy elevada debido a la respuesta inmune humoral exagerada en perros enfermos (Reis et al., 2006b; Rodriguez-Cortes et al., 2007b; Todoli et al., 2009). Un nivel elevado de anticuerpos junto con un cuadro clínico compatible suele ser suficiente para diagnosticar definitivamente la leishmaniosis clínica en los perros (Solano-Gallego et al., 2009).

Existen varias técnicas serológicas para el diagnóstico de CanL, divididas en el grupo de cuantitativas y cualitativas (Hernandez, 2015):

<u>Cuantitativas</u>

a) Inmunofluorescencia indirecta (IFI)

Ha sido considerada la técnica de referencia para el diagnóstico serológico por la Organización Mundial de Sanidad Animal (OIE) (Maia and Campino, 2008) pero su lectura es muy subjetiva al necesitar un microscopio de fluorescencia y además parece tener una menor sensibilidad en animales infectados clínicamente sanos (Mettler et al., 2005).

b) Enzimoinmunoensayo (ELISA)

Es un test especifico más sensible que la técnica IFI (Rodriguez-Cortes et al., 2013; Solano-Gallego et al., 2014). La sensibilidad puede oscilar entre el 8 y el 99.5 % (Marcondes et al., 2011). Se usan diversos antígenos clasificados en cuatro grupos según su naturaleza: extractos de promastigotes o amastigotes solubles o completos, proteínas recombinantes y proteínas purificadas (Solano-Gallego et al., 2014). La sensibilidad se ve afectada por el tipo de antígeno seleccionado (Maia and Campino, 2008; Mettler et al., 2005). Su gran ventaja es la automatización, lo que permite procesar simultáneamente un gran número de muestras.

c) Test de aglutinación directa (DAT)

Se usan promastigotes teñidos en suspensión o liofilizados para detectar respuesta inmunitaria frente a los antígenos de la superfície de *Leishmania* (Cardoso et al., 2004; Meredith et al., 1995). Tiene mucha sensibilidad pero la limitación de esta técnica es que necesita 18 horas de incubación y crear las diluciones seriadas del suero (Ferreira Ede et al., 2007; Schallig et al., 2004). Hay una variante de DAT que es también de aglutinación de cribado pero rápida (FAST). Se concentra el parásito y así el volumen de ensayo es menor, solo requiere de una dilución y la lectura es en 3 horas (Schallig et al., 2002).

d) Inmunoblotting

Con fracciones antigénicas de *Leishmania* detecta anticuerpos de la muestra. Permite valorar la fase de la infección y la respuesta al tratamiento (Lasri et al., 2003). Rutinariamente no se utiliza esta técnica ya que se necesita personal especializado y un laboratorio de investigación, pues su aplicación principal seria validar otras técnicas (Ferroglio et al., 2007).

e) Citometría de flujo

Su ventaja principal es que es rápida y precisa, pudiendo evaluar las partículas de un fluido como las IgG frente los promastigotes de *Leishmania* (Maia and Campino, 2008; Rocha et al., 2003).

Cualitativas

Principalmente se basan en el antígeno rK39, disponible en el mercado en forma de tiras de papel de nitrocelulosa impregnadas de antígeno (Mettler et al., 2005; Otranto et al., 2005) aunque también se utilizan otros tipos de antígenos. Estos kits son fáciles de usar y muy útiles para el veterinario. Presenta una especificidad media alta, su sensibilidad es de entre el 30 y 70% (Rodriguez-Cortes et al., 2013; Solano-Gallego et al., 2014), lo quepuede dar falsos negativos (Solano-Gallego et al., 2011). Sin embargo, algunos de estos kits comerciales han logrado una sensibilidad de hasta el 97% (Marcondes et al., 2011).

1.6.4. Tratamiento

A pesar de que existente varias opciones terapéuticas, hasta la fecha no existen principios activos totalmente eficaces que lleguen a una curación parasitológica completa. La mayoría de fármacos pueden permitir una curación clínica en algunos perros pero no consiguen eliminar totalmente el parásito del organismo (Hernandez, 2015; Miro et al., 2017; Noli and Auxilia, 2005).

El objetivo principal del tratamiento es alargar la esperanza y la calidad de vida, ayudar a la respuesta inmunitaria y disminuir la carga parasitaria (Maroli et al., 2010; Miro et al., 2011; Otranto et al., 2013).

Aunque el protocolo de tratamiento ha ido cambiando a lo largo de los años, en la actualidad, los fármacos más utilizados son los antimoniales o miltefosina (Baneth and Shaw, 2002) en combinación con alopurinol. Los otros fármacos más nuevos, se usan para complementar el tratamiento o para perros que no responden bien o no se puede usar el tratamiento convencional.

1) Antimoniales pentavalentes

Se han utilizado durante casi un siglo en el tratamiento para la leishmaniosis. El Glucantime® (antimoniato de n-metilglucamina) y el Pentostam® son los dos fármacos comerciales con este principio activo. Su actividad leishmanicida se debe a la inhibición selectiva de las enzimas fosfofructoquinasa y piruvato deshidrogenasa de *Leishmania* implicadas en la glicólisis y la oxidación de los ácidos grasos, bloqueando la formación del trifosfato de adenosina (ATP) y el de guanina (GTP) (Berman et al., 1985).

Actualmente la pauta marcada de administración son entre 50-100 mg/Kg, se reparte la dosis diaria total en dos veces al día durante hasta un mes y medio cuando se combina con 10 mg/Kg al día en dos veces con alopurinol (Miro et al., 2009; Solano-Gallego et al., 2011). La combinación de estos dos principios activos posee una acción sinérgica favoreciendo la tasa de curaciones y las recaídas son mucho más tardías (Baneth and Shaw, 2002; Noli and Auxilia, 2005), además que se consigue reducir el parásito en la piel, reduciendo así el poder infectante de los perros a los flebótomos (Alvar et al., 1994; Miro et al., 2011) demostrado mediante técnicas de xenodiagnóstico (Miro et al., 2011).

Hay mucha bibliografía donde se documenta que existen reacciones adversas al fármaco (Miro et al., 2017) como por ejemplo desarrollar pancreatitis (Luciani et al., 2013; Moritz et al., 1998) y que puede ser tóxico ya que los antimoniales alteran la estructura terciaria de las proteínas de muchas enzimas, dando lugar a signos de intolerancia como los vómitos, diarrea, anorexia, fiebre entre otras durante o después del tratamiento (Bourdeau et al., 2014; Slappendel and Teske, 1997).

2) Alquilfosfolípidios

Son análogos sintéticos de la lisofosfocolina. La miltefosina es una alquilfosfocolina que se desarrolló como agente antineoplásico (Hilgard et al., 1993). Se observó que actuaba con actividad parasiticida tanto *in vitro* como en *in vivo*, frente a los promastigotes y los amastigotes (Croft et al., 1996; Farca et al., 2012). Los mecanismos de acción antitumoral y leishmanicida no se conocen al detalle. Hay una teoría que propone que el principio activo incorpora a la bicapa lipídica de la membrana plasmática del parásito aumentando la permeabilidad y alterando el metabolismo de los alquil-lípidos (Lux et al., 2000). Otra teoría es que provoca alteraciones en la síntesis de fosfolípidos de la membrana flagelar dando muerte celular por apoptosis (Paris et al., 2004).

Se da este tratamiento en monoterapia en perros, es eficaz y reduce bastante la carga parasitaria (Mateo et al., 2009) pero en algún caso se ha observado un incremento de carga parasitaria a los 6 meses (Mateo et al., 2009). Actualmente la mejor estrategia es la combinación de miltefosina con alopurinol para el tratamiento CanL (Noli and Saridomichelakis, 2014). Se ha demostrado que es tan eficaz a efectos clínicos como el protocolo estándar que se usa basado en el tratamiento con alopurinol y antimoniales (Miro et al., 2009). La combinación ha demostrado ser más efectiva que la miltefosina por sí sola (Farca et al., 2012).

Los efectos adversos que pueden darse debido a la miltefosina son disorexia, vómitos y diarreas leves y autolimitantes (Miro et al., 2013). Se han descrito efectos teratogénicos, embriotóxicos y fetotóxicos en ratones (Sindermann and Engel, 2006).

La combinación del Milteforán® con el alopurinol como segunda elección para tratar CanL (Noli and Saridomichelakis, 2014).

Se han descrito efectos teratogénicos, embriotóxicos y fetotóxicos en ratones (Sindermann and Engel, 2006).

3) Alopurinol

El alopurinol es un análogo estructural de la hipoxantina, metabolizado por los parásitos de *Leishmania* para producir un análogo inactivado de inosina. Este es incorporado en el RNA del parásito causando la formación de substancias tóxicas que conllevan un error en la síntesis proteica, actuando como parasitostático (Baneth and Shaw, 2002).

Este compuesto se utiliza por toda Europa, ya que se considera seguro y económico (Torres et al., 2011). Su uso se emplea para las recidivas, porque cuando se administra por períodos más largos de cinco meses, la probabilidad es mucho menor que cuando se administra solamente antimoniato de n-metilglucanmina (Noli and Auxilia, 2005). La dosis habitual usada es de entre 10-20 mg/kg al día a largo plazo, repartida en 2 tandas, que puede durar de entre 6 a 18 meses aunque la duración del alopurinol no se ha definido del todo y dependerá de diferentes factores como el estadio clínico entre otros (Solano-Gallego et al., 2011). Perros tratados solo con alopurinol pueden mostrar mejora clínica pero se ha visto que las recidivas son frecuentes (Baneth et al., 2008; Miro et al., 2011). Aunque su toxicidad es baja, se han visto algunos episodios de fallos en la función hepática y renal cuando el uso ha sido prolongado (Torres et al., 2011), en algunos casos por haber desarrollado hipersensibilidad al tratamiento (Jung et al., 2015).

4) Anfotericina B

La anfotericina B (AmB) es un antibiótico derivado de los polienos obtenidos a partir de *Streptomyces nodosus* (Caffrey et al., 2001). Se ha demostrado su efecto contra especies de protozoos como *Leishmania* y *Naegleria* (Baneth and Shaw, 2002). Su actividad leishmanicida se basa en provocar la permeabilización de la membrana plasmática del parásito por interacción selectiva con el ergosterol presente en la misma formando poros acuosos que favorecen la pérdida de pequeños cationes como K^+, Ca^{2+} y Mg^{2+}, lo cual causa la muerte el parásito (Gray et al., 2012). La anfotericina B es el fármaco de elección en el tratamiento de la leishmaniosis visceral humana en Europa (Bern et al.,

2006). Sin embargo, la dificultad de su administración (perfusión lenta), elevado coste, resistencia del fármaco y efectos secundarios como vómitos, fiebre, temblores, anemia y nefrotoxicidad (Bern et al., 2006) en el perro han motivado el desuso de este fármaco en la práctica clínica.

5) Aminosidina

La aminosidina, también nombrada como paramomicina, es un aminoglicósido con actividad antiprotozoa y antimicrobiana. En perros también se ha combinado con antimoniales pentavalentes para mejorar su eficacia (Oliva et al., 1998). La gran limitación de este fármaco es su toxicidad renal y vestibular, estando contraindicando el uso en perros con leishmaniosis y enfermedad renal (Oliva et al., 1998). Su mecanismo de acción consiste en alterar la asociación de la subunidad ribosomal mediante el bloqueo de la síntesis proteica (Davidson et al., 2009). Se ha visto que es eficaz cuando se administra una dosis única de 15 mg/Kg durante 21 días (Athanasiou et al., 2013).

6) Pentamidina

Este principio activo es una diamidina aromática usada en el tratamiento contra la tripanosomiasis y por eso se consideró un tratamiento seguro para la leishmaniosis (Maroli et al., 2010). Su uso es limitado debido a su alto coste a parte de una serie de efectos secundarios como vómitos, salivación y diarrea entre otros (Ciaramella, 2003).

Su mecanismo de acción no está esclarecido pero se cree que altera el metabolismo de las proteínas y de los ácidos nucleicos, desorganizando el ADN (Jha et al., 1991).

La dosis recomendada es de 4 mg/kg en 3 dosis semanales durante 5 hasta 7 semanas, o 20 inyecciones en días alternos (Rhalem et al., 1999). La mayoría de perros tratados mejoran pero hay recidivas sino se utiliza con alopurinol (Baneth and Shaw, 2002).

7) Quinolonas

La Marbofloxacina es una fluoroquinolona de tercera generación disponible en medicina veterinaria, posee una actividad potente frente a numerosas bacterias gram-negativas y gram-positivas (Vouldoukis et al., 1996). Su dosis recomendada consiste en 2mg/kg al día durante 28 días (Rougier et al., 2008). Su unión a proteínas plasmáticas es baja y además presenta un gran volumen de distribución (Schneider et al., 1996). La actividad leishmanicida testada *in vitro* es a través del TNF-α y la vía iNOS, resultado de la producción de dióxido de nitrógeno de los macrófagos, así puede eliminar los amastigotes (Farca et al., 2012; Vouldoukis et al., 2006) aunque su eficacia comparado con la miltefosina es menor.

8) Derivados del imidazol

Son compuestos antifúngicos, algunos también con actividad bacteriana y protozoaria. Uno de los principios activos más empleados es el metronidazol. Es un derivado del grupo nitroimidazoles con propiedades antibacterianas, antiprotozoarias y antiinflamatorias, e *in vitro* posee actividades anti *Leishmania* (Pennisi et al., 2005). En un estudio donde se describían las interacciones del metronidazol con otros fármacos, como por ejemplo el alopurinol, se dan efectos no deseados ya que el metronidazol puede hacer aumentar la toxicidad con litio aunque grandes intoxicaciones no se hayan reportado (Miljkovic et al., 2014).

A pesar de la alta eficacia conseguida con los fármacos, éstos no han conseguido eliminar por completo el parásito del organismo, por ello, aún se deben hacer investigaciones para mejorar la eficacia de los tratamientos (Miro et al., 2017). Un camino prometedor a seguir para conseguir que el individuo afectado consiga resolver la infección, es el de combinar los fármacos con adyuvantes. Los inmunomoduladores como

son los agonistas de los receptores tipo Toll (TLR), son una estrategia emergente para poder declinar la cascada de transducción de la señal hacia un perfil inmunológico proinflamatorio tipo Th1 y así ayudar al propio sistema inmunitario a combatir contra la infección recibida. (Hosein et al., 2015). Los TLRs conectan el sistema inmunitario innato con el adaptativo. En otras enfermedades como el cáncer se usa la inmunización como terapia para mejorar las propias respuestas inmunitarias (Gnjatic et al., 2010).

1.6.5. Prevención

No hay una medida preventiva totalmente eficaz. Sin embargo, el uso tópico de repelentes o insecticidas de forma regular para evitar la picadura del flebótomo en el perro permite la reducción de la infección por *L. infantum* (Otranto and Dantas-Torres, 2013; Wylie et al., 2014). Existen otras medidas frente a los flebótomos como por ejemplo: mantener al perro dentro de la vivienda en los amaneces y atardeceres (Galvez et al., 2010; Martin-Sanchez et al., 2009), evitar la acumulación de maderas, escombros, fisuras en las paredes en la vivienda o lugares frecuentados (Sharma and Singh, 2008; Solano-Gallego et al., 2011), el rociamiento de insecticidas en las viviendas (Courtenay et al., 2002; Sharma and Singh, 2008) y telas antimosquitos protectoras y cortinas sobretodo en casos de leishmaniosis humana (Alexander and Maroli, 2003; Miró, 2006)

Los repelentes tópicos usados actualmente son:

1) Los collares

Impregnados con deltametrina. Estos collares, eficaces en varias especies de flebótomos y en varias regiones de Europa, van liberando insecticida penetrando así en el tejido subcutáneo, alcanzando la actividad máxima en dos semanas tras su aplicación. Su efecto insecticida dura hasta los seis meses y su eficacia para proteger de la picadura es del 60% (Ferroglio et al., 2008; Maroli et al., 2001). Se ha descrito alguna reacción adversa

en algún caso (Aoun et al., 2008) pero en la gran mayoría no ha habido repercusiones negativas de esta aplicación (Maroli et al., 2001).

2) Formulaciones *spot on*

Son las comúnmente llamadas pipetas que también son altamente protectoras y confieren una gran repelencia. En un día el insecticida ya se ha distribuido uniformemente por todo el estrato córneo del perro (Miro et al., 2008). Existen diferentes productos comerciales pero todos utilizan piretroides. Se administra cada 21 días con efectos repelentes en todos los casos observados experimentalmente en perros jóvenes (Otranto et al., 2007) y no se conocen reacciones adversas al producto (Miro et al., 2007; Otranto et al., 2010).

3) Pulverizadores

La base es de permetrina y piriproxifeno, con actividad repelente inmediata, alta eficacia (de un 72% aproximadamente) durante 21 días (Molina et al., 2006).

4) Vacunas

Este método sería el más eficaz para proteger contra la enfermedad de la leishmaniosis, sin embargo, aún no existe la fórmula perfecta para inmunizar ni a perros ni a humanos (Gradoni, 2001). La vacunación deberá inducir una respuesta Th1 predominante, fuerte para prevenir el establecimiento del parásito, y duradera para controlar la progresión de la enfermedad (Gradoni, 2015). Hoy en día existen vacunas comerciales para la leishmaniosis canina, pero al no proporcionar protección total, se debe combinar con repelentes tópicos (Miro et al., 2017; Oliva et al., 2014).

Para mejorar las vacunes existentes, se han realizado muchos experimentos para poder potenciar los candidatos a vacuna con adyuvantes (Palatnik-de-Sousa, 2008). Las

estrategias para diseñar una vacuna han estado varias: como vacunas a base de antígenos purificados (Moreno et al., 2014), antígenos recombinantes (Gradoni, 2015) y de ADN (Rodriguez-Cortes et al., 2007c) y a base de parásitos muertos (Giunchetti et al., 2008a).

Las vacunas se pueden clasificar en primera generación (parásito vivo), de segunda generación (antígeno purificado natural o recombinado) y de tercera generación, que son plásmidos con antígenos codificados (Rezvan and Moafi, 2015).

Las vacunas de primera generación pueden ser vivas, que consiste en inocular el parásito cultivado *in vitro* intradérmicamente (McCall et al., 2013), vivas atenuadas, que son aislados avirulentos como *L. Tarentolae* (Breton et al., 2005) parásitos atenuados experimentalmente *in vitro* (Saljoughian et al., 2013), por radiación gamma (Gorczynski, 1985), mutagénesis química (Kimsey et al., 1993).

Las vacunas de segunda generación pueden diseñarse a través de antígenos brutos de parásitos completos como la vacuna LBSap, combinación de antígenos brutos de *L. braziliensis* y saponina (Roatt et al., 2012), a partir de fracciones de la superfície del parásito purificadas de *Leishmania* tratadas en el laboratorio como por ejemplo la proteínas PSE y Q A -21 o mediante proteínas recombinantes la proteína quimérica Q obtenida mediante la combinación de cinco fragmentos altamente antigénicos (Soto et al., 1998) fusionados y clonados con *E. coli* a partir de cuatro proteínas de *L. infantum* (Fernandez Cotrina et al., 2018).

Las vacunas de tercera generación son vacunas de ADN que se insertan en un gen que codifica la proteína de interés en un plásmido bacteriano, el cual se inocula al sujeto para que exprese las proteínas *in vivo* (Wolff et al., 1990). El ADN del plásmido actúa como vehículo y adyuvante para activar los linfocitos B y T, la producción de citoquinas y la activación de la respuesta inmune innata a través del TLR9 (Krieg, 2002). Para mejorar este tipo de vacuna, se han hecho estudios donde la estrategia era la vacunación y revacunación. Consiste en vacunar con un vector que contiene el gen de interés y la revacunación contiene el mismo gen pero el vector es distinto. Se ha demostrado que este

método mejora la inmunogenicidad de este tipo de vacunas induciendo la activación de los linfocitos T (Woodland, 2004).

Otra de las estrategias emergentes para conseguir una vacuna más efectiva es mediante los adyuvantes como los agonistas de los TLRs. La activación de TLR promueve respuestas inflamatorias innatas y la inducción de inmunidad adaptativa. En los años más recientes, gracias a la evolución de las técnicas moleculares, han dado acceso al siguiente nivel en la generación de vacunas. En consecuencia, en las últimas dos décadas, cada vez más pruebas han vinculado la activación de TLR con la patogénesis de las enfermedades inmunes y el cáncer. Un buen adyuvante facilitará la coestimulación de células T y B para potenciar la producción de inmunidad adquirida específica de antígeno (Alving et al., 1986). Esta característica distintiva de un adyuvante hace que los TLR sean los candidatos principales en el desarrollo de vacunas. Aunque no se conocía en ese momento, las primeras vacunas en realidad contenían contaminantes bacterianos que activaban los TLR y servían como adyuvantes. La señalización de TLR ayuda a acelerar y mejorar la inducción de respuestas específicas de la vacuna (Alving et al., 1986). En estos aspectos, los TLR pueden considerarse un cuchillo del "ejército suizo" del sistema inmune, listos para responder en una multitud de estados infecciosos y de enfermedades (Dowling and Mansell, 2016). Algunos ligandos de TLR se han usado para crear vacunas y han ganado bastante atención debido a su capacidad para provocar una respuesta inmunitaria efectiva (Dowling and Mansell, 2016; Krishnan et al., 2009).

5) Eutanasia como medida preventiva

En Brasil es donde se ha usado este método para controlar la CanL desde la década de los años 50. Se sacrifican miles de perros al año si presentan seropositividad a *Leishmania* spp. (Dantas-Torres et al., 2012). La ineficacia de la técnica está demostrada por el creciente diagnóstico de nuevos casos de *Leishmania* visceral entre los años 1990 y 2010 en Brasil (Dantas-Torres et al., 2012). Esta opción de prevención es infructuosa porque otros animales pueden hacer de reservorio como roedores o los propios humanos

(Quinnell and Courtenay, 2009), porque basarse solamente en la serología del perro es un diagnóstico poco preciso (Silva et al., 2011) y porque el reemplazo rápido de los perros eutanasiados por perros jóvenes incrementa la proporción de animales susceptibles de la población (Nunes et al., 2008).

1.7. Inmunología

Inmunidad es el término usado para describir la habilidad del hospedador para resistir al ataque de microbios que pueden potencialmente comprometer la salud del individuo (Hoebe and Beutler, 2004). Hasta el momento, se han descrito dos tipos de respuestas inmunitarias principales que aparecen en animales vertebrados: la inmunidad innata y la adquirida. La inmunidad innata es la primera en actuar cuando existe alguna posible irrupción de algún patógeno extraño y es menos específico. En cambio, la inmunidad adaptativa colabora con la inmunidad innata pero sólo cuando ésta se ha activado primero. El papel de la inmunidad adaptativa se rige por dar una respuesta al patógeno de manera más específica (Hoebe and Beutler, 2004).

Uno de los primeros mecanismos a activarse para hacer frente a les estímulos dañinos en los tejidos es la inflamación. Las células del sistema inmunitario innato son las involucradas para mediar las respuestas inflamatorias (Kenneth Murphy, 2012). Estas células están reforzadas con un gran abanico de receptores que están preparados para detectar patrones moleculares rápidamente. La respuesta innata es la más ancestral de las dos respuestas, actuando velozmente para hacer frente a la intromisión foránea sin memorizar los aspectos de los patógenos (Kenneth Murphy, 2012).

Por el contrario, la inmunidad adaptativa, necesita de más tiempo que la inmunidad innata (puede conllevar entre horas o días) para que su respuesta se desarrolle. A pesar de ello, las infecciones son eficazmente eliminadas gracias al reconocimiento de antígenos específicos que se despliega en este tipo de respuesta (Kenneth Murphy, 2012). Además, la respuesta adaptativa permite al sistema a producir una memoria en las células

inmunológicas. Así que una vez un individuo se ha expuesto a un agente infeccioso, el cuerpo es capaz de detectarlo mucho más rápido las siguientes exposiciones que el individuo pueda experimentar frente al mismo patógeno (Kenneth Murphy, 2012).

1.7.1. Respuesta inmunitaria adquirida

La respuesta inmunitaria adaptativa se inicia cuando las células presentadores de antígeno (APCs), se encuentran con antígenos en el medio y alcanzan los órganos linfoides secundarios (Kenneth Murphy, 2012). El sistema adaptativo se comprende de linfocitos T y B, las cuales proporcionan inmunidad patógeno-específica al hospedador. Los linfocitos B producen anticuerpos específicos que neutralizan las toxinas, mientras que los linfocitos T aportan un entorno de citoquinas para poder eliminar el patógeno a partir de sus efectos citotóxicos y/o alternando con los linfocitos B (Hoebe and Beutler, 2004). La respuesta adaptativa es inducida cuando el patógeno sigue replicándose ya que ha superado las estrategias de la inmunidad innata para hacer frente cualquier invasión de un cuerpo extraño y la infección persiste (Kenneth Murphy, 2012).

Las funciones principales de las APC, son: a) activar la defensa de la inmunidad innata, b) procesar antígenos exógenos al cuerpo e iniciar la respuesta innata adaptativa y c) son capaces de regular la inmunidad adaptativa determinando si el antígeno desencadenará una respuesta mediada por anticuerpo o per el contrario será una respuesta mediada celular (Tizard, 2012). Las células dendríticas inmaduras tienen la habilidad de coger material extracelular a partir de la macropinocitosis e internalizarlo para destruir los patógenos que los PPRs fallaron en su detección. Además, las células dendríticas maduras expresan moléculas coestimulatorias proporcionando señales de proliferación y diferenciación (Guermonprez et al., 2002).

El sistema inmunitario adaptativo, los linfocitos T son las responsables de la respuesta mediada celular. La mayor acción a cargo de estas células es llevar a cabo la diferenciación del clúster 8 (CD8), la citotoxicidad. Los linfocitos T *helper* (Th), por otro lado, se encargan

de activar otras linfocitos, los CD4 (Kenneth Murphy, 2012). Los linfocitos CD4 vírgenes se pueden diferenciar en varios tipos de linfocitos T efectoras: Th1, Th2, TH17 y células T foliculares (T_{FH}) (Murphy and Reiner, 2002). Los Th1 producen citoquinas que activan a los macrófagos, cuya labor es destruir los organismos intracelulares, mientras que Th2 producen citoquinas que reclutan y activan los mastocitos, basófilos y eosinófilos. Los Th17 secretan las citoquinas de la familia IL-17, induciéndola producción de quimiocinas. Éstas, reclutan neutrófilos en el lugar de la infección. Las T_{FH} activan los linfocitos B para la producción de IFN-γ (Kenneth Murphy, 2012). A parte de estos 4 tipos de linfocitos T efectoras, existe un subconjunto llamados linfocitos T reguladores. Son una clase heterogénea y su función principal es suprimir la respuesta de los linfocitos T efectores cuando ya no son necesarios, de esta forma el organismo evita que se dé la autoinmunidad (Kenneth Murphy, 2012).

Las diferentes poblaciones de células T se han caracterizado en perro. Las células T reguladoras se encuentras bajo el nombre de FoxP3 como marcador de enfermedad (Dowling and Mansell, 2016; Pinheiro et al., 2011).

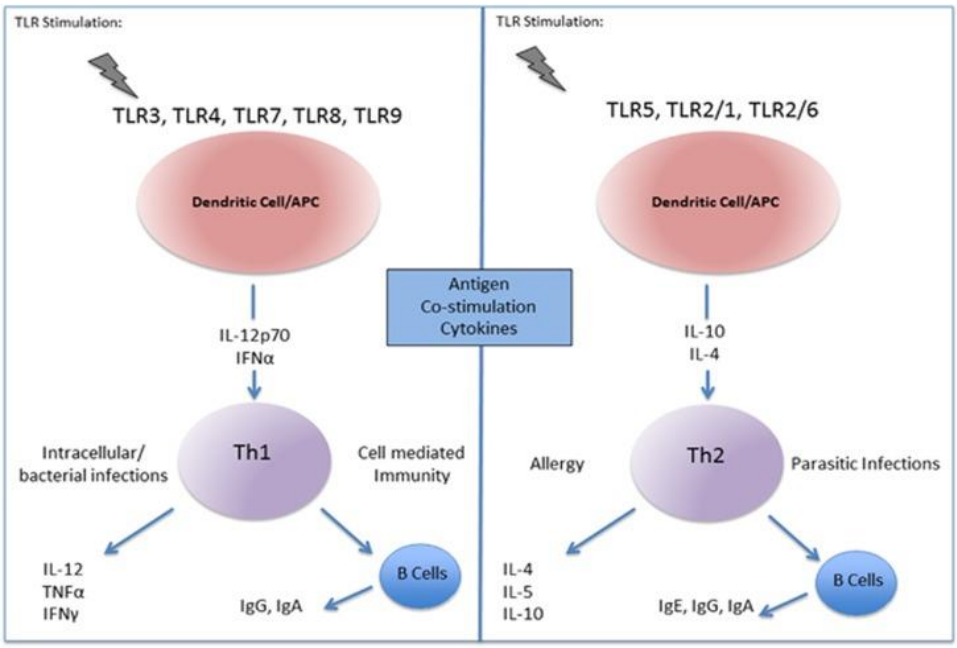

Figura 1.10. Respuestas celulares T helper 1 y los receptors tipo Toll. Fuente: (Dowling and Mansell, 2016).

1.7.2. Respuesta innata

La inmunidad innata fue descrita por Elie Metchinkoof hace más de un siglo. Los componentes de la respuesta innata son un conjunto de mecanismos que tiene el organismo que no es patógeno-específico pero pueden reconocer patrones o clases de moléculas que no pertenecen al individuo y que normalmente se encuentran en los patógenos. Las células inmunitarias como las células dendríticas y los macrófagos directamente eliminan los microbios patogénicos a través de la fagocitosis o inducen la formación de citoquinas que facilita la eliminación de éstos. La respuesta innata desencadena la siguiente respuesta inmunitaria que consistirá en el reconocimiento específico del patógeno y la memorización de éste, la respuesta inmunitaria adaptativa (Kumar et al., 2009).

Existen cuatro clases de barreras primarias que forman parte de la inmunidad innata: la anatómica, la fisiológica, la fagocítica y la inflamatoria. Las barreras anatómicas y son las primeras que actúan para combatir las infecciones que son la piel y las mucosas. Las barreras fisiológicas consisten en la temperatura, el pH; las moléculas asociadas a células y las moléculas solubles. Estas moléculas solubles incluyen las proteínas tipo lisozima, interferón y el complemento. Muchas de estas tienen las propiedades de reconocimiento de patrones, cuyas habilidades les permite reconocer una clase específica de molécula (Richard A. Goldsby, 2003).

El sistema inmunitario innato usa un gran espectro de receptores de membrana que reconocen patrones moleculares (PPRs) los cuales son expresados en la superfície de membrana, secretados en el corriente sanguíneo o también en compartimientos específicos intracelulares (Medzhitov, 2001). Este sistema inmediatamente es capaz de contener a los patógenos y no requiere de memoria celular. Existen varias funciones de las proteínas PPRs pero la mayoría de funciones consisten en hacer la fagocitosis, opsonización, activación de la coagulación y de la cascada del complemento, activación de la inflamación y la iniciación de la apoptosis. El sistema inmunitario innato responde a estructuras moleculares comunes y conocidas que se asocian a patrones moleculares de

patógenos (PAMPs), los cuales son reconocidos por los receptores de patógenos nombrados anteriormente. En la familia PPRs se incluyen los Toll like receptors (TLRs), que son una clase de PPRs. La interacción entre el receptor (PPRs) y los patrones moleculares de los patógenos (PAMPs) cataliza la respuesta inmunitaria innata (Medzhitov, 2001). Los patrones moleculares asociados a patógenos son producidos por los microbios y no por las células del hospedador, de esta forma, las células inmunitarias pueden discernir entre aquello que pertenece al cuerpo mismo de aquello que no lo es (Janssens and Beyaert, 2003). Se hallan diversas famílias de PPRs, localizadas en las superficies celulares, tanto extracelulares como intracelulares. Los receptores de membrana extracelular son los TLRs y las lectinas, mientras que los PPRs intracelulares son los receptores gen inducibles (RIG-receptors) o los que tienen un dominio donde se unen los nucleótidos oligomerizados (NOD)-like receptors, algunos de ellos se muestran en la figura 1.11. Las lectinas son proteínas solubles que se involucran principalmente en la captura de invasiones bacterianas (Shayakhmetov et al., 2010; Tizard, 2012).

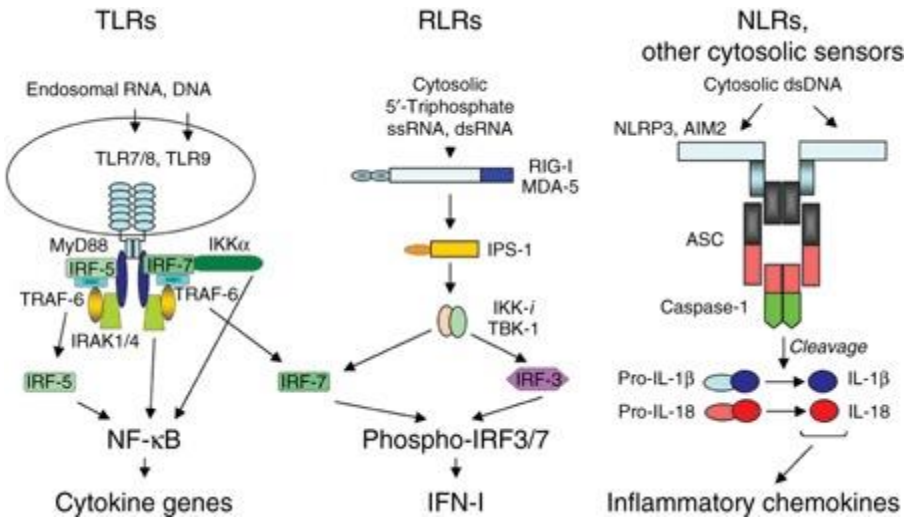

Figura 1.11.Tres clases de receptores de reconocimiento de patrones implicados en el reconocimiento de ácidos nucleicos virales en células de mamíferos. NLR, receptor similar a un dominio de oligomerización de nucleótidos; RLR, receptor de tipo I genético inducible por ácido retinoide; TLR, receptor tipo Toll. Fuente: Shayakhmetov et al., 2010.

Los macrófagos son unas de las células más conocidas del sistema inmunitario innato. Los fagocitos mononucleares se desarrollan de las células madre mieloides en la médula ósea y dan lugar a monoblastocitos y al final a los monocitos. Este proceso ocurre bajo la influencia de citoquinas conocidas como factores de estimulación. Estos monocitos, se adentran en el torrente sanguíneo circulando durante setenta y dos horas antes de transformarse en macrófagos (Tizard, 2012). Además, para una fagocitación y una buena expresión de PPRs, los macrófagos responden a la invasión de patógenos produciendo interleucinas (IL) como la IL-1, la IL-6, la IL-12, TNF-α entre otras. Esta citoquinas tiene varias funciones como la de la estimulación de las respuestas en fase aguda, catalizando así la inflamación y promoviendo la diferenciación de los linfocitos B o la secreción de IFN-γ. En la figura 1.12. se muestran qué células pertenecen a cada respuesta.

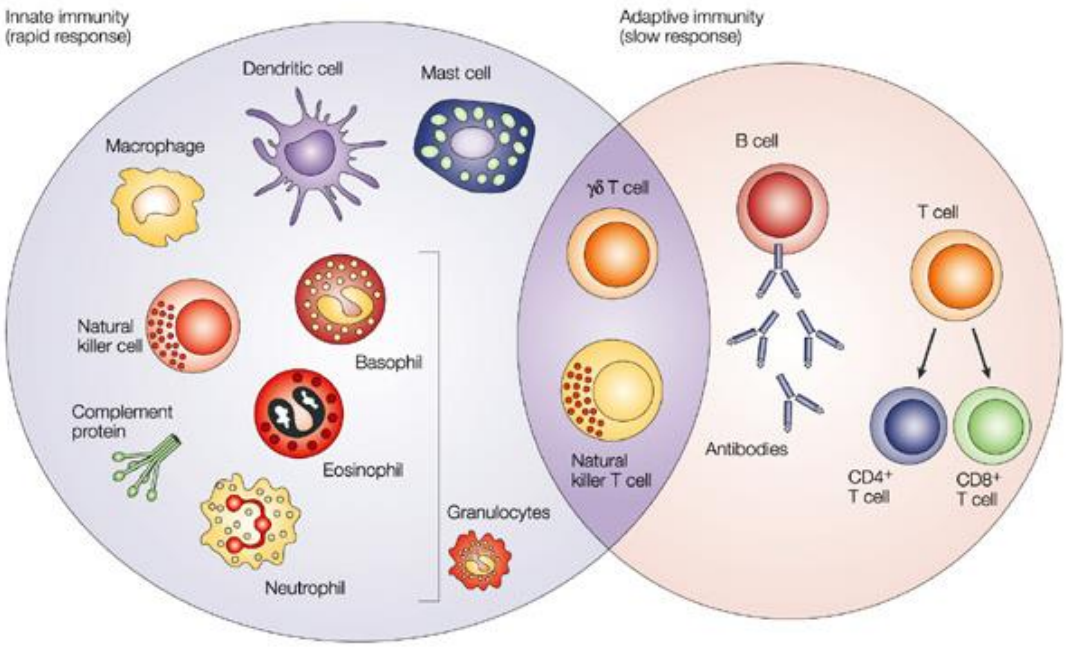

Figura 1.12. Ilustración de los diferentes tipos de células en la inmunidad innata y en la adaptativa Fuente: (Pillai, 1991).

1.7.3. Respuesta inmunitaria en la infección por *Leishmania*

Cuando la hembra del flebótomo pica a un hospedador vertebrado, se desencadena las reacciones inflamatorias en el lugar de la picadura, donde intervienen las células dendríticas, los linfocitos T y δ y los macrófagos (Teixeira et al., 2006). Estas células contienen en sus membranas los receptores tipo Toll (TLR), capaces de reconocer patrones moleculares conservados de patógenos (Medzhitov, 2001; Tuon et al., 2008). Durante este proceso se liberan quimiocinas que se encargan de reclutar los neutrófilos y las células *natural killer* (NK) y los macrófagos en la primera fase de la infección (Teixeira et al., 2006).

Cuando el flebótomo hembra que pica actúa como vector, es decir, contiene parásitos en su interior, los promastigotes después de superar las condiciones adversas del intestino, a través de la picadura pasa al cuerpo hospedador. Allí en primer lugar se enfrentará a moléculas del complemento, a anticuerpos y a células fagocíticas, que serán capaces de neutralizar la invasión hasta un 80% de los promastigotes (Lewis and Peters, 1977). Los parásitos sobreviven a la defensa del sistema inmunitario porque invaden los macrófagos, utilizándolos como "caballo de Troya" pasando desapercibidos de manera silenciosa (Laskay et al., 2003), ya que si no fuera por ellos, los parásitos serían eliminados rápidamente por las células del complemente, las NK y los neutrófilos (Almeida et al., 2013). En perros susceptibles la capacidad fagocítica de estas células es reducida (Brandonisio et al., 1990), por ello, la diseminación del parásito dependerá de la resistencia del hospedador, un animal susceptible puede tardar horas, en cambio un resistente pueden permaneces los parásitos confinados en la piel (Brandonisio et al., 1990).

La inmunidad protectora parece ser que está mediada por la acción del factor de necrosis tumoral alfa (TNF-α), la interleuquina 2 (IL-2) y el interferón gamma (IFN-γ) secretados por los linfocitos T activados, que a su vez activan también la función leishmanicida de los macrófagos a través de la producción de óxido nítrico (NO). La producción del óxido nítrico través de la sintetasa óxido nítrico inducible (iNOS) tiene un

papel importante cuando existe infección intracelular bacteriana y parasitaria (Holzmuller et al., 2018; Olekhnovitch et al., 2014). La inducción de iNOS está finamente regulada y requiere la transducción de la señal y la activación de la transcripción (STAT) para la transducción de citoquinas y factores de crecimiento y del factor nuclear potenciador de las cadenas ligeras kappa de los linfocitos B activadas (NF-κβ), ambos implicados en la respuesta celular frente a estímulos como agentes bacterianos o parasitarios para mantener la homeostasis (Olekhnovitch et al., 2014).

Los macrófagos infectados son lisados por los linfocitos T citotóxicas CD8+ en un proceso mediado por el complejo mayor de histocompatibilidad, el cual se suprime cuando la carga parasitaria es muy elevada (Pinelli et al., 1994). En perros subclínicos, prevalen los linfocitos CD8+ (Reis et al., 2006a). Los amastigotes fagocitados por los macrófagos son portados des de la piel hasta los nódulos linfáticos, exponiendo los antígenos de éstos a los linfocitos T y así activándolos. Una vez las células presentadoras de antígeno (APCs) se activan empieza la respuesta inmunitaria frente el parásito presentándose dos posibles respuestas ya nombradas: predominante Th1 asociada a proteger el animal o predominante Th2, asociada a la progresión de la enfermedad (Baneth et al., 2008) pero en los perros que presentan una diseminación descontrolada, ambas respuestas son bajas (Santos-Gomes et al., 2002).

1.7.4. Ligando del receptor de muerte programada PD-L1

Este ligando es una proteína transmembrana que tiene el papel inmunoregulador mediante la supresión de la respuesta del sistema inmunitario en situaciones donde se puede desarrollar una respuesta excesiva y crear autoinmunidad. Actúa tanto en enfermedades inmunitarias como infecciosas, enfermedades crónicas y parasitarias. La unión del ligando con su receptor PD-1 transmite la señal inhibitoria a los linfocitos T reduciendo su proliferación y así causar la apoptosis de éstas (Chemnitz et al., 2004). El ligando PD-L1 se expresa en la superfície de las células T específicas y en las células presentadoras de antigeno (APCs) (Ishida et al., 1992). Al catalizar la señal inhibitoria y se

reduce la proliferación de las células T, también se reduce la producción de citoquinas como la IFN-γ, y TNF-α (Bardhan et al., 2016; Rodrigues et al., 2014). El agotamiento de las células T mediado por el receptor PD-1 se ha demostrado en ratones con leishmaniosis visceral (Habib et al., 2018) y también en humana (Gautam et al., 2014). Además, la pérdida de la proliferación de las células $CD4^+$ T específicas para *L. infantum* en perros enfermos fue correlacionado con un aumento de la expresión de PD-1 en la superfície celular y la discapacidad de la función fagocítica (Chiku et al., 2016; Esch et al., 2013; Schaut et al., 2016).

Muy poca información está disponible en la literatura relacionada con el PD-L1 y su expresión génica en CanL (Barroso et al., 2018; Chiku et al., 2016) pero el bloqueo del ligando PD-L1 parece restaurar la función celular de las células T en modelo murino (Habib et al., 2018) y en perro (Chiku et al., 2016; Schaut et al., 2016) cuyos resultados mostraron un bajada en la carga parasitaria. Es importante remarcar que la disfunción de las células T en presencia del parásito, como la reducida apariencia o ausencia de producción de IFN-γ en sangre de perro varía según los signos clínicos que muestra el perro y su severidad (Solano-Gallego et al., 2016b).

1.8. Los receptores tipo Toll

Los receptores tipo Toll (TLRs) fueron identificados en la mosca *Drosophila* y hasta hoy se han descrito hasta 13 en mamíferos vertebrados y 10 en perros (Cusco et al., 2014; Kumar et al., 2009; Mikami et al., 2012). Los TLRs reconocen estructuras específicas en patógenos para dar la respuesta innata y finalmente la respuesta inmunitaria adaptativa antígeno-específica (Chauhan et al., 2017; Kawai and Akira, 2010). Los TLRs hasta ahora descritos con sus ligandos específicos y los tipos de células donde se expresan se encuentran descritos en la siguiente tabla (modificada de (Hosein, 2014)):

Tabla 1.2. Resumen de los receptores tipo Toll (TLRs) en mamíferos y sus ligandos principales, adaptadores y tipos de células principales donde se expresan.

Receptores	Ligando(s)	Localización del ligando	Adaptador (es)	Localización del receptor	Tipos de células	Referencias
TLR1 (heterodímer con TLR2)	Lipopeptidos triaciles	Bacteria	MyD88	Superfície celular	Mø, DC, B	(Takeuchi et al., 2002)
TLR2	Glicolípidos, lipopéptidos y lipoproteínas múltiples, HSP70	Bacterias gram positivas y células del hospedador	MyD88	Superfície celular	Mø, DC, M	(Takeuchi et al., 1999)
TLR3	ARN de doble cadena poly I:C	Virus	TRIF	Compartimento celular	DC, L B, L T	(Alexopoulou et al., 2001)
TLR4	Lipopolisacárid, fibrinógeno	Bacterias	MyD88	Superficie celular	Mø, DC, M, L B, E I	(Takeuchi et al., 1999)
TLR5	Flagelina	Bacterias	MyD88	Superficie celular	Mø, E I, DC	(Smith et al., 2003)
TLR6 (heterodimero con TLR2)	Lipopéptidos diaciles, ácido lipoteicoico	Micoplasma	MyD88, TIRAP	Compartimento celular	Mø, M, L B	(Takeuchi et al., 1999)
TLR7	ARN de cadena simple, imidazoquinolina	Compuestos antivirales y antialergénicos	MyD88	Compartimento celular	Mø DC, P, L B	(Heil et al., 2004)
TLR8	ARN de cadena simple, imidazoquinolina	Compuestos antivirales y antialergénicos	MyD88	Compartimento celular	Mø, DC, P, L B	(Heil et al., 2004)

TLR9	Oligonucleótidos ADN CpG no metilado	Bacterias	MyD88	Compartimento celular	Mø DC, P, L B, L T	(Bauer et al., 2001)
TLR10 (heterodímer con TLR1)	Péptidos diacetilados	Hongos	MyD88	Superficie celular	M, En, Mø, DC	(Govindaraj et al., 2010)
TLR11 (sólo en ratón)	Proteínas tipo porfirina, porfirinas toxoplasmaticas	*Toxoplasma gondi*	MyD88	Compartimento celular	Mø, C Hep, C Ren, eipiteli o de la vejiga	(Yarovinsky, 2014)
TLR12 (sólo en ratón)	Porfirinas toxoplasmáticas	*Toxoplasma gondi*	MyD88	Compartimento celular	N, DC, Mø, En	(Mishra et al., 2008; Yarovinsky, 2014)
TLR13 (sólo en ratón)	rRNA	Virus	MyD88, TAK1	Compartimento celular	Mø, DC, En	(Mishra et al., 2008)

Mo: monocitos, Mø: macrófagos, DC: células dentríticas, MC: mastocitos, B: células B, T: células T, En: endosomas, E I: epitelio intestinal, SP: superficie celular, C Hep: células hepáticas, C Ren: células renales, N: neuronas, P: plasmocitoides

Los TLRs son un tipo de receptores que reconocen patrones (PPR). Estos son tipo 1 que son proteínas de transmembrana expresadas por células del sistema inmunitario innato que luego da lugar a la respuesta inmunológica adaptativa (Chauhan et al., 2017). El dominio rico en leucinas (LRR) de los TLRs consiste entre 16 y 28 repeticiones en tándem de motivos LRR involucradas en el reconocimiento de los ligandos como proteínas,

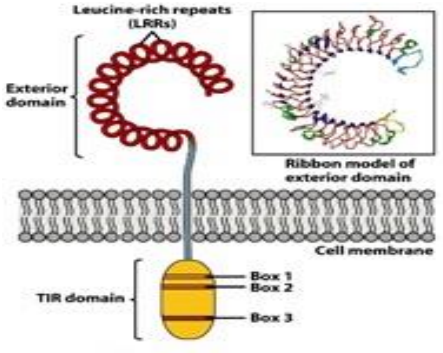

Figura 1.13. Esquema gráfico del receptor tipo Toll y sus estructuras extracelular, intracelular y tranmembrana. Kuby Immunology, Sixth edition © 2007 v.h.Freeman and Company.

azúcares, lípidos o ácidos nucleicos derivados de proteínas o péptidos. Cada LRR consiste de 20-30 aminoácidos (Kumar et al., 2009). Cuando el receptor interacciona con el ligando, las proteínas adaptadores son reclutadas al dominio interleucina toll 1 (TIR). Este dominio es una región conservada de la proteína que modula la interacción con la siguiente proteína adaptadora intracelular para la transducción de la señal (figura 1.13.).

Los TLRs inducen citoquinas interferón tipo 1, quimioquinas y moléculas coestimulatorias. La especificidad de cada TLR es dependiente del adaptador citoplasmático que se asocian al dominio TIR de los TLRs. Los TLRs tienen 4 adaptadores principales para hacer esa función como se muestra en la figura 1.14.: el factor de diferenciación mieloide 88 (MyD88), dominio TIR con adaptador a proteína (TIRAP), dominio TIR que contiene interferón inductor de proteína adaptadora (IFN)-γ (TRIF) y la molécula adaptadora relacionada con TRIF (TRAM) (Chauhan et al., 2017; Schnare et al., 2001). Los adaptadores proporcionan la activación de los factores de transcripción que regulan las respuestas inflamatorias.

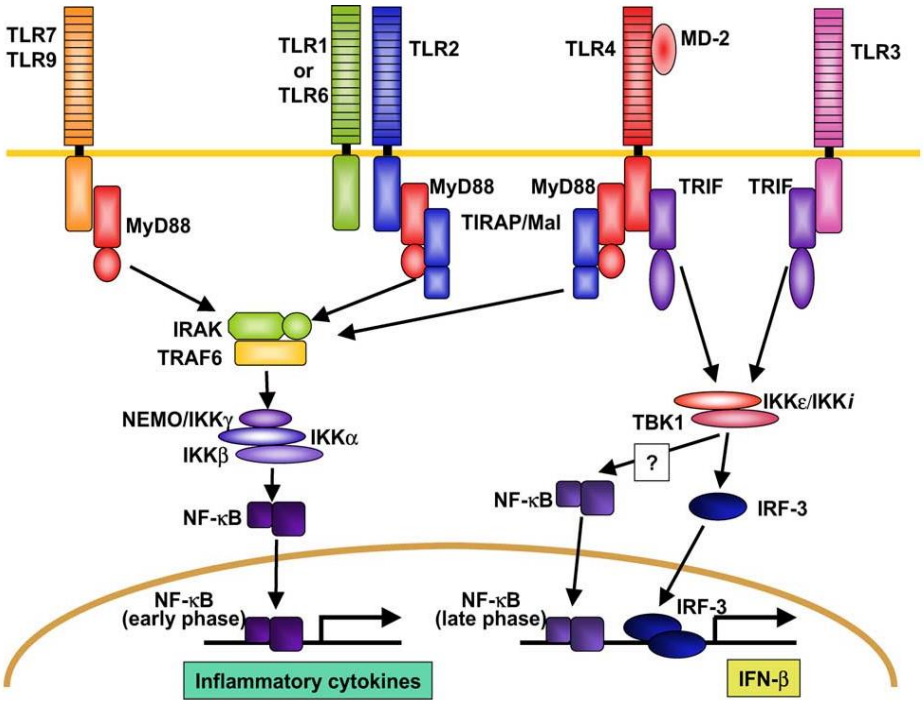

Figura 1.14. Cascada de señalización de los TLRs, Fuente: (Takeda and Akira, 2004).

MyD88 es el adaptador que se necesita para todos los TLRs exceptuando el TLR3, que necesita del adaptador TRIF (la cascada de señalización independiente de MyD88) tal como se indica en la figura 1.10 (Akira et al., 2001). Esta transducción de la señal se vio principalmente en los receptores TLR3 y TLR4, donde sus ligandos tienen la habilidad de catalizar la expresión del interferón tipo 1, lo cual requiere el reclutamiento de TRAM para activar TRIF (Wang et al., 2001) y seguir la cascada de la transducción de la señal IKKi / IKKe y TBK1, mediante la activación de IRF-3 que se transloca al núcleo y dando lugar ala expresión de interferón-β.

La vía dependiente de MyD88 se desarrolla cuando el dominio TIR se activa y recluta al adaptador IRAK-4. Éste, al unirse se fosforila y activa IRAK-1, que a su vez este último se asocia a TRAF6, todas ellas moléculas adaptadoras transductoras de la señal. TRAF6 puede terminar en 2 diferentes rutas, una conduce a la activación del factor AP-1 a través de la activación de MAP quinasa. En la otra vía se activa el complejo TAK1/ TAB que mejora la actividad del complejo IKK quinasa. Una vez activado, se induce la fosforilación y posterior degradación de IκB que finalmente conduce a la translocación nuclear del factor de transcripción NF-κB lo cual induce la expresión de citoquinas inflamatorias (Szatmary, 2012).

1.8.1. Los receptores tipo Toll y la infección por *Leishmania*

La inmunidad adaptativa es muy importante para resolver la infección. Sin embargo, hay evidencias de que la inmunidad innata juega un papel muy importante en la defensa contra la *Leishmania* (Faria et al., 2011). Se conoce que la *Leishmania* induce respuestas mediadas por TLRs. En un *estudio in vitro* observaron que *L. major* activaba regiones de IL-1α a través de la cascada se señalización de MyD88 en macrófagos (Hawn et al., 2002). En este estudio muestran que las respuestas mediadas por TLRs son importantes para la eliminar el parásito (Muraille et al., 2003). Pues, como se ha dicho antes, los receptores TLR3 es independiente de MyD88 y TLR4 puede activar las dos vías, la MyD88 y la

independiente a ésta, así que con ellos se demuestra que los TLRs tienen involucración directa con el modelo estudiado (Faria et al., 2011).

Se sabe que el TLR2 se activa con la presencia de *Leishmania*. El lipofosgoglicano (LPG) induce la estimulación y la sobre expresión del TLR2 en células *natural killer* y también mejora la producción de TNF-α y IFN-γ (Becker et al., 2003). También, se ha comprobado que la producción de NO se correlaciona con la activación de TLR2 (Kavoosi et al., 2010). En un estudio con macrófagos de ratón infectados con *L. major*, se les trató les añadió al cultivo los ligandos para TLRs solos o en combinación con CD40. Se observó que los ligandos mejoraban la sintetasa óxido nítrico inducible (iNOS) y la IL-12 generando así la producción de NO, cuya función se ha descrito que provoca un perfil protector Th1 (Chandel et al., 2014).

Estos hallazgos sugieren que la activación de MyD88 a través de los receptores tipo Toll son importantes para montar la respuesta Th1 (Osanya et al., 2011).

Existen evidencias que el TLR4 tiene un rol protector en infecciones por *L. major*. Los ratones deficientes de TLR4 disminuyeron la carga parasitaria en piel pero aumentó la supervivencia del parásito en las células hospedadoras. Este hecho fue correlacionado con una alta actividad arginasa (Kropf et al., 2004a). La activación del receptor TLR4 se ha visto relacionada también con la producción de ROS, lo cual ayuda parcialmente a la eliminación del parásito intracelular, visto en macrófagos murinos (Faria et al., 2011).

1.8.1.1. Los receptores tipo Toll y la leishmaniosis canina

Así como existen muchos estudios que determinan diversas citoquinas en la CanL, hay pocos estudios que investiguen el papel de los TLRs y la CanL (Hosein et al., 2017). A pesar de ello, existen investigaciones hechas sobre la expresión de algunos TLRs como TLR2 y TLR9 en perros en diferentes tejidos como piel o intestino (Esteve et al., 2015; Figueiredo et al., 2013). Así como, la expresión de TLR2 se ha documentado en monocitos y granulocitos de perros infectados por *Leishmania* de Brasil (Amorim et al., 2011).

CAPÍTULO 2

HIPÓTESIS Y OBJETIVOS / HYPOTHESIS AND OBJECTIVES

2.1. Hipótesis /Hypothesis

Leishmania infantum es un parásito cosmopolita que se considera endémico en más de 80 países de todo el mundo en que los perros actúan como reservorio principal, tanto para las personas como para otros animales. Un adecuado diagnóstico de la infección causada por este parásito es importante para poder contrarrestar mejor los nuevos casos que van surgiendo, sobretodo es fundamental para diferenciar perros enfermos de portadores subclínicos. Además, existe un amplio espectro clínico que se caracteriza por: Perros "menos susceptibles" que presentan una respuesta inmunitaria celular de tipo T helper 1 (Th1) que confiere protección frente la enfermedad y perros enfermos que manifiestan una respuesta inmunitaria humoral acompañada de una reducida respuesta inmunitaria celular y una alta carga parasitaria que se considera predominante cuando hay progresión de la enfermedad de tipo Th2. Se debe indagar más como esta enfermedad actúa y afecta al sistema inmunitario del hospedador, para poder saber cuáles serán las mejores estrategias para abordarlo y eliminarlo. Varios estudios sugieren que la respuesta inmunitaria innata es primordial para la resolución favorable de la leishmaniosis. Se ha demostrado que los receptores TLRs responsables de actuar en la respuesta inmunitaria innata, activan la actividad pro-inflamatoria en macrófagos infectados por la *Leishmania*, ayudando a neutralizar la infección eliminando el parásito en los modelos murinos. Aunque existen protocolos y tratamientos que han evolucionado bastante, todavía no existe ningún tratamiento que consiga erradicar la enfermedad del sujeto que la padece sin la aparición de recaídas. Se ha demostrado que los agonistas de TLRs, tanto sintéticos como naturales, son capaces de estimular los macrófagos y las células presentadoras de antígeno en roedores. También se ha sugerido en otros estudios que el uso de los agonistas de estos receptores podría ser una buena estrategia inmunoterapéutica para aumentar la respuesta específica frente el parásito. Respecto a la leishmaniosis canina o humana es muy limitada la información sobre los agonistas de TLRs así como la expresión de TLRs en diferentes tejidos en perros o personas.

Debido a la escasa información que existe sobre la respuesta innata en el perro frente la infección por *L. infantum* (Hosein et al., 2017), y el potencial efecto protector de los TLRs en esta infección (Kropf et al., 2004a), se hipotizó el posible papel protector de los TLRs en la infección por *L. infantum* en el perro. Además, los agonistas de TLRs podrían ser excelentes y útiles agentes inmunoterapéuticos en el tratamiento de la infección por *L. infantum* en el perro.

Leishmania infantum is a cosmopolitan parasite that is considered endemic in more than 80 countries around the world where dogs act as the main reservoir for both humans and other animals. An adequate diagnosis of the infection caused by this parasite is important to be able to better counteract the new cases that arise, above all it is fundamental to differentiate sick dogs from subclinical carriers. In addition, there is a broad clinical spectrum that is characterized by: "Less susceptible" dogs that have a T helper 1 (Th1) cellular immune response that confers protection against disease and sick dogs that exhibit a humoral immune response accompanied by a reduced cellular immune response and a high parasitic load that is considered predominant when there is progression of Th2 type disease. It should be investigated more as this disease acts and affects the immune system of the host, to be able to know what will be the best strategies to address it and eliminate it. Several studies suggest that the innate immune response is paramount to the favorable resolution of leishmaniasis. It has been shown that the TLR receptors responsible for acting on the innate immune response activate pro-inflammatory activity in macrophages infected by *Leishmania*, helping to neutralize the infection by eliminating the parasite in the murine models. Although there are protocols and treatments that have evolved enough, there is still no treatment that manages to eradicate the disease of the subject who suffers without the occurrence of relapse. It has been shown that TLR agonists, both synthetic and natural, are capable of stimulating macrophages and antigen-presenting cells in rodents. Also, it has been suggested in other studies that the use of the agonists of these receptors could be a good immunotherapeutic strategy to increase the specific response against the parasite. Regarding canine or human leishmaniosis, information on TLR agonists is limited, as is the expression of TLRs in different tissues in dogs or people.

Due to the scarce information that exists on the innate response in the dog against the infection by *L. infantum* (Hosein et al., 2016), and the potential protective effect of the TLRs in this infection (Kropf et al., 2004), the possible protective role of TLRs in the infection by *L. infantum* in the dog was hypothesized. In addition, TLR agonists could be

excellent and useful immunotherapeutic agents in the treatment of *L. infantum* infection in dogs.

2.2. Objetivos / Objectives

El objetivo general de esta tesis doctoral fue el de determinar el papel de los TLRs y la utilidad clínica de los agonistas de TLR como inmunoterapia en la infección por *L. infantum* en el perro. Esta tesis doctoral ha determinado el papel de los TLRs y los agonistas de TLR como agentes inmunoterapéuticos mediante los siguientes objetivos específicos que se describen a continuación:

1. Determinar la transcripción de los genes TLR2 y TLR4 en sangre no estimulada proveniente de perros sanos no infectados y perros enfermos infectados naturalmente con *L. infantum* en el momento del diagnóstico y durante un año de tratamiento. Así como relacionar los parámetros clínicos, inmunológicos y parasitológicos.
2. Determinar y comparar la transcripción de los genes TLR2 y TLR4 en sangre no estimulada proveniente de perros en diferentes estadios clínicos de leishmaniosis en el momento del diagnóstico. Así como relacionar los parámetros clínicos, inmunológicos y parasitológicos.
3. Determinar la transcripción de los genes TLR2, TLR4 y PD-L1 en sangre estimulada con LSA o CoA de perros enfermos infectados de forma natural y en perros sanos no infectados. Así como relacionar los parámetros clínicos, inmunológicos y parasitológicos.
4. Observar el efecto que causan los TLR3, TLR4 y TLR7 agonistas solos o en combinación con el antígeno de *Leishmania* en células de la sangre de perros aparentemente sanos que viven en área endémica de leishmaniosis.
5. Determinar la concentración inhibitoria máxima media (IC50) necesaria de diferentes fármacos anti-*Leishmania* convencionales para inhibir el crecimiento de promastigotes de *L. infantum* mediante técnicas de viabilidad (alamar blue) y determinar la susceptibilidad de los amastigotes de *Leishmania* con los fármacos convencionales solos o combinados con TLRs agonistas y a su vez determinar la expresión de citoquinas y TLR2, TLR4 y TLR7 así como NO.

The general objective of this doctoral thesis was to determine the role of TLRs and the clinical usefulness of TLR agonists as immunotherapy in *L. infantum* infection in dogs. This doctoral thesis has determined the role of TLRs and TLR agonists as immunotherapeutic agents through the following specific objectives that are described below:

1. To determine the transcription of the TLR2 and TLR4 genes in unstimulated blood from non-infected healthy dogs and diseased dogs naturally infected with *L. infantum* at the time of diagnosis and during one year of treatment. As well as relating the clinical, immunological and parasitological parameters.
2. To determine and compare the transcription of the TLR2 and TLR4 genes in unstimulated blood from dogs in different clinical stages of leishmaniosis at the time of diagnosis. As well as relating the clinical, immunological and parasitological parameters.
3. Determine the transcription of the TLR2, TLR4 and PD-L1 genes in blood stimulated with LSA or CoA from diseased dogs infected naturally and in healthy non-infected dogs. As well as relating the clinical, immunological and parasitological parameters.
4. Observe the effect caused by TLR3, TLR4 and TLR7 agonists alone or in combination with *Leishmania* antigen in blood cells of apparently healthy dogs that live in endemic area of leishmaniosis.
5. Determine the mean maximum inhibitory concentration (IC50) required of different conventional anti-*Leishmania* drugs to inhibit the growth of promastigotes of *L. infantum* via viability techniques (alamar blue) and determine the susceptibility of *Leishmania* amastigotes with conventional drugs alone or combined with TLRs agonists and in turn to determine the expression of cytokines and TLR2, TLR4 and TLR7 as well as NO.

CAPÍTULO 3

ESTUDIOS / STUDIES

3.1. ESTUDIO 1

TLR-2 and TLR-4 transcriptions in unstimulated blood from dogs with leishmaniosis due to *Leishmania infantum* at the time of diagnosis and during follow-up treatment

Sara Montserrat-Sangrà[1], Lorena Alborch[1], Laura Ordeix[1,2], Laia Solano-Gallego[1,*]

[1]Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain

[2]Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Bellaterra, Spain

Reprinted from Veterinary Parasitology 209 (2015) 157–163

doi: 10.1016/j.vetpar.2015.03.004. PMID: 25825146

Abstract

Innate immunity, in particular, the role of toll-like receptors (TLRs), has not been extensively studied in canine *L. infantum* infection. The main aim of this study was to determine the transcription of TLR2 and TLR4 in the blood of dogs with natural clinical leishmaniosis at the time of diagnosis and during treatment follow-up and subsequently correlate these findings with clinical, serological and parasitological data. Forty-six *Leishmania*-seropositive sick dogs with a high antibody level at the time of diagnosis were studied and compared with 34 healthy seronegative dogs. Twenty-two of these sick dogs were treated with meglumine antimoniate and allopurinol and followed-up at 30, 180 and 365 days following the start of treatment. Clinical status was defined by a thorough physical examination, complete blood count, biochemistry profile, electrophoresis of serum proteins, and urinary protein / creatinine ratio (UPC). EDTA blood was stored in RNAlater® solution before RNA extraction and cDNA production were performed. TLR2, TLR4 and three reference genes (HPRT-1, CG14980 and SDHA) were studied in each blood sample by real time PCR. The relative quantification of TLR2 was higher (mean 3.5) in sick dogs when compared with seronegative healthy dogs (mean 1.3; P=0.0001) while the relative quantification of TLR4 was similar in both groups. In addition, the relative quantification of TLR2 significantly decreased during follow-up at all time points compared with day 0 whereas no changes were observed with TLR4 transcription. A significant positive correlation was noted between TLR2 and UPC, total protein, beta and gamma globulins, specific *L. infantum* antibodies and blood parasite load while a negative correlation was observed with albumin, albumin/globulin ratio, hematocrit and hemoglobin. TLR4 transcript did not correlate with any parameter. These findings indicate an up-regulation of TLR2 transcription in unstimulated blood in naturally infected sick dogs as compared to healthy dogs suggesting active innate immune and proinflammatory responses. In addition, TLR2 transcription is reduced with clinical improvement during treatment. In contrast, TLR4 transcription appears to be similar among groups at the time

of diagnosis with no changes during treatment follow-up suggesting a less important role for this TLR in clinical canine leishmaniosis.

Keywords

TLR2; TLR4; canine leishmaniosis; blood; treatment and follow-up.

Abbreviations

ALT: Alanine aminotransferase; β-2M: Beta-2-microglobulin; CBC: Complete blood count; cDNA: Complementary Deoxyribonucleic acid; CD11b: Cluster of differentiation 11b; CG-14980: Similar to CG14980-PB; CP: Crossing point ; DAMPs: Damage-associated molecular pattern molecules; ddCt-method: Delta delta C(T) Method; EDTA: Ethylenediaminetetraacetic acid; HMBS: Hydroxymethylbilane synthase; HPRT-1: Hypoxanthine phosphoribosyltransferase 1; M value: Describes the variation of a gene compared to all other candidate genes (evaluation of gen stability); PAMPs: Pathogen-associated molecular patterns; PBMCs: Peripheral blood mononuclear cell; PCR: Polymerase chain reaction; RG: Reference gene; RGI: Reference gene index; RIN: RNA Integrity Number; RNA: Ribonucleic acid; PRRs : Pattern recognition receptors ; qPCR: Quantitative PCR; Rq: Relative quantification ;rt- PCR: Real time PCR; SDHA: Succinate dehydrogenase complex; subunit A; flavoprotein (Fp); TBP: TATA box binding protein; TG: Target gene;TLRs: Toll-like Receptors; UPC: Urinary protein / creatinine ratio ; VL: Visceral leishmaniosis; Vn/n+1 value: Pairwise variation analysis to determine optimal number of reference genes for normalization in RT-qPCR reaction.

Introduction

Canine leishmaniosis due to *Leishmania infantum* is a life threatening zoonotic disease with a wide distribution in four continents. A broad spectrum of clinical manifestation exists in this infection ranging from subclinical infection to very severe disease (Baneth et al., 2008). A clinical staging system is currently used in the clinical setting due to the fact that several degrees of disease exist ranging from mild to very severe fatal disease (Solano-Gallego et al., 2009; Solano-Gallego et al., 2011). Dogs with moderate or more severe disease are frequently treated in Europe. The common treatment employed in the clinical setting is a combination of meglumine antimoniate with allopurinol, miltefosine with allopurinol, or allopurinol alone (Noli and Saridomichelakis, 2014). Clinical cure is often associated with a reduction in parasite load and infectiousness (Miro et al., 2011) but long-term treatment is needed in the majority of dogs with moderate to severe disease (Noli and Saridomichelakis, 2014) to maintain clinical remission. The variable clinical manifestations are likely due to the differing immunogenetics within each dog leading to a variation in immune responses (Reis et al., 2010). Although adaptive immunity has been extensively investigated in canine *L. infantum* infection, limited data is available regarding innate immune responses (Hosein et al., 2017).

The innate immune system uses non-clonal sets of recognition molecules, called pattern recognition receptors (PRRs). They bind to conserved molecular structures found in large groups of pathogens termed pathogen-associated molecular pattern (PAMPs) (Akira et al., 2001). The toll-like receptors (TLR) are one of the most important pattern recognition receptor families. TLRs are important in the early host defense against pathogen and activate adapter molecules after binding to their ligand. The activated cascade then leads to induction or suppression of genes that influence the inflammatory response (Aderem and Ulevitch, 2000; Kawai and Akira, 2010).

Recent data suggests that early innate immune responses are paramount for the ultimate outcome of *Leishmania* infection (Bogdan et al., 1996; Bogdan and Rollinghoff, 1998; Liese et al., 2008). Studies have shown that TLRs activate pro-inflammatory responses (Agallou et al., 2014; Hawn et al., 2002; Oda and Kitano, 2006). However, the role of TLRs has not been studied in canine *L. infantum* infection in detail (Hosein et al., 2017). There are only few studies of TLR2 expression in different tissues in canine leishmaniosis. Previous studies have demonstrated that TLR2 is upregulated in skin, intestine and brain in canine leishmaniosis (Esteve et al., 2015; Figueiredo et al., 2013; Melo et al., 2014a). In addition, TLR2 is upregulated in other canine inflammatory diseases including inflammatory bowel disease, immune-mediated diseases and bacterial arthritis (McMahon et al., 2010; Riggio et al., 2014). TLR4 transcription was studied in several tissues such as lymph node or spleen in canine leishmaniosis (Melo et al., 2014a), however it has been scarcely investigated in experimental or natural *L. infantum* infection in dogs (Hosein et al., 2017). To the best knowledge of the authors, TLRs transcripts have not been evaluated in unstimulated whole blood from dogs with clinical leishmaniosis at the time of diagnosis or during treatment follow-up.

The correlation between TLR expression and clinical, immunological and parasitological parameters can help to better understand host-pathogen interactions in canine *L. infantum* infection and define the specific functions of certain TLRs in this disease. Based on present literature, we hypothesize that TLR2 is up regulated while TLR4 might be down-regulated or unchanged in dogs with clinical leishmaniosis at the time of diagnosis and a switch should be observed during treatment follow-up and clinical improvement.

The main objective of this study was to determine the transcription of TLR2 and TLR4 in the blood of dogs with natural clinical leishmaniosis (Leishvet stage II, moderate disease or higher (Solano-Gallego et al., 2009) at the time of diagnosis and during follow-

up treatment). Other objective was to correlate the transcription of TLR2 and TLR4 with clinical, serological and parasitological data.

Materials and methods

Dogs

Sampling and evaluation of clinical status

A thorough physical examination was performed in all healthy and sick dogs included in this study. In addition, routine laboratory testing was also performed in all dogs to further evaluate their clinical status. Thus, a full complete blood count (CBC), serum biochemical profile which included creatinine, urea, total cholesterol, total protein and alanine aminotransferase (ALT), protein serum electrophoresis, and urinalysis with urinary protein creatinine ratio (UPC) were carried out. The hematology, biochemistry and serum electrophoresis were performed using: the Siemens ADVIA120 haematology analyzer, Olympus AU400 chemistry analyzer and the Hydrasis serum electrophoresis system.

Six milliliters of blood were collected from the respective dogs by jugular or metatarsian venipuncture for routine laboratory tests above described and transferred immediately into different tubes: Ethylenediaminetetraacetic acid (EDTA) tubes and plain tubes. 0.5 ml of EDTA blood from dogs was transferred to a cryovial that contained 1.3 mL of RNAlater® solution (Ambion). Once collected, samples were left at 4ºC overnight and then frozen at minus 80ºC until further use.

All dogs enrolled in the study were privately owned pets with client informed consent; they remained under the care of their owners and were not housed for the purposes of this study or held for any period of time. Residual samples from blood EDTA

tube and serum were used in this study. Therefore, ethical approval was not required. *Leishmania infantum* antibody levels were determined by a serial dilution in house quantitative ELISA (Solano-Gallego et al., 2016a) and *Leishmania* DNA in blood was assessed by real-time PCR (Martinez et al., 2011; Solano-Gallego et al., 2016a).

Dogs with clinical leishmaniosis

A total of 46 dogs with clinical leishmaniosis attending different veterinary facilities from Catalonia (Spain) [*Fundació Hospital Clínic Veterinari*, Universitat Autònoma de Barcelona (UAB), Cerdanyola, Barcelona), *Hospital Ars Veterinària* (Barcelona), *Mediterrani Veterinaris Hospital* (Reus, Tarragona) and *Consultori Montsant* (Falset, Tarragona)] were prospectively enrolled between January 2014 and August 2015. The diagnosis was made by detection of high specific *L. infantum* antibody levels and, in some cases confirmed, by cytology of lesions, cutaneous histology and/or immunohistochemistry for *Leishmania* (Esteve et al., 2015). In addition, 38 dogs were positive by blood PCR. Both sexes were represented with 20 female and 26 male. The median age was five years with a range from six months to 10 years. Thirty-four purebred dogs belonging to about 20 breeds and 12 mixed breed dogwere included. Clinical staging was evaluated as previously reported (Solano-Gallego et al., 2009) and sick dogs were distributed as follows: stage II (moderate disease, n=39), stage III (severe disease, n=4) and stage IV (very severe disease, n=3).

After being diagnosed, twenty-two dogs classified as being in stage II (n=19) or stage III (n=3) were further followed up during treatment. All dogs were treated with meglumine antimoniate (80-100 mg/Kg/24h subcutaneous during 1 month) and allopurinol (10 mg/Kg/12h orally during at least 12 months). Dogs were monitored at days 30, 180 and 365 during treatment. In each clinical visit, a physical examination and CBC, biochemistry profile, protein serum electrophoresis, urianalysis and UPC were performed. Both genders were represented with eight female and 14 male dogs as well as purebred

and mixed-breed dogs. The remaining 24 dogs were also treated with the treatment described above and they were also followed up during treatment. However, due to economical restrictions, TLRs were not determined in these dogs.

Control healthy dogs

A total of 34 healthy dogs were enrolled between January 2014 and August 2015. Both sexes were represented with 16 females and 18 males. The median age was approximately four years, with a range from six months to 10 years. Twenty-five pure bred dogs belonging to about 15 breeds and nine mixed breed dog were included. All dogs were seronegative with a mean and standard deviation of 6.2 $\pm$ 5.5 ELISA units (EU) and blood PCR negative.

Blood DNA extraction and *Leishmania* real-time PCR

Total DNA was isolated from EDTA whole blood using DNA Gene extraction kit (Sigma Aldrich) following the manufacturer's instructions with slight modifications. Forty µl of proteinase K solution (Sigma Aldrich, St Louis, US) were added in all samples. Four hundred µl of whole blood were used for all the samples. The other steps were performed according to the manufacturer's protocol. A blood sample from a clinically healthy non-infected dog was used as a control for DNA contamination in every DNA extraction performed.

Real-time PCR (RT-PCR) was performed with an absolute quantification as previously described (Solano-Gallego et al., 2016a). Briefly, PCR mix reaction was prepared with 4µl of DNA, 10µl of master mix (TaqMan® Fast Advanced Master Mix, Life technologies, Carlsbad, US), 1µl of *Leishmania* primers and probes (Custom TaqMan® Gene Expression Assay, life technologies) or 1µl of another type of assay primers and probes (Eukaryotic 18S rRNA Endogenous Control (VIC™ / MGB Probe, Primer Limited) and

5µl of H_2O. PCR reaction was performed in duplicates for each sample and for each target gene.

In order to verify that the PCR was done successfully, a positive control for *Leishmania*, a negative control from a non-infected clinically healthy dog and a blank (well without DNA sample) were included in all the plates. PCR was carried out in a QuantStudio Flex™ 7 Real-Time PCR system (Life Technologies, Carlsbad, US). The thermal cycling profile consisted of 50ºC 2 min in order to activate the enzyme AmpErase (ThermoFisher Scientific, Waltham, US) and afterwards, a total of forty cycles were carried out. Each cycle consisted of 20 sec at 95 ºC followed by 40 cycles of 1 sec at 95 ºC and 20 sec at 60 ºC.

Absolute quantification was carried out by the interpolation of the unknown samples to the standard curve generated from a negative sample spiked with different quantities of *Leishmania* promastigotes. Depending on the value of parasitic load, the samples were classified as negative (0 parasites / ml), low positive (<10 parasites / ml), medium positive (10-100 parasites / ml), high positive (100-1000 parasites / ml) or very high positive (> 1000 parasites / ml) (Martinez et al., 2011).

RNA extraction and RNA concentration and integrity

Blood samples were thawed on ice and total RNA from 500 µL of EDTA blood was extracted using the Ribopure™ RNA blood kit (Ambion) according to the manufacturer's instructions. A DNase digestion step was included to remove contaminating genomic DNA using the Turbo™ DNase (Ambion) following the manufacturer's instructions. RNA concentration was determined by a Nanodrop device (Thermo Fisher Scientific Inc). RNA integrity and quality were assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples had a final concentration of 20-70 ng RNA/ µl per sample. All samples included in this study had a RNA Integrity Number (RIN) value greater than 7.

cDNA synthesis and PCR real time

*c*DNA was generated using the VILO Masterscript Retrotranscriptase kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was aliquoted and stored at -20°C until ready for use.

Reference gene selection

Six reference housekeeping genes (RG) [(hydroxymethylbilane synthase (HMBS), succinate dehydrogenase complex; subunit A; flavoprotein (SDHA), TATA box binding protein (TBP), similar to CG14980-PB (CG14980), hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome) (HPRT-1) and beta-2-microglobulin(β-2M)] (Table 1; Invitrogen, Thermo Fisher Scientific) were assessed by real time PCR to establish the most stable genes by the GeNorm program (Biogazelle, Belgium) with 10 representative samples. Two parameters were considered to quantify reference gene stability: M value (average expression stability) and Vn/n+1 (pairwise variation). Originally, M≤1.5 and Vn/ n+1<0.15 were regarded as acceptable levels of expression variability (Vandesompele et al., 2002). Recently, an M value below the threshold of 0.5 has been generally regarded as being typical for a stable reference gene in a relatively homogeneous sample panel (Schlotter et al., 2009). Selected reference genes were used to calculate relative quantification of target genes.

Table 3.1.1. Summary of canine reference and target genes used in this study.

Assay ID*	Gene symbol	Gene name	GenBank mRNA	GenBank referencesequence	Amplicon pairwise
Cf02622203_g1	TLR4	toll-like receptor 4	AB080363.1	NM_001002950.1	120
Cf02625049_s1	TLR2	toll-like receptor 2	EU487534.1;AJ630583.1	NM_001005264.2	69
Cf02664981_m1	SDHA	succinate dehydrogenase complex; subunit A; flavoprotein (Fp)	DQ402985.1	XM_535807.2	64
Cf02626256_m1	HPRT-1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	AY283372.1	NM_001003357.1	102
Cf02643820_m1	LOC479750	similar to CG14980-PB		XM_536878.2	78
Cf02694648_m1	HMBS	hydroxymethylbilane synthase	-	XM_546491.2	148
Cf02659077_m1	β2M	beta-2-microglobulin	-	XM_845055.1	87
Cf02637232_m1	TBP	TATA box binding protein	-	XM_849432.1	105

*All the assays are commercially available at ThermoFisher scientific.

TLR2, TLR4 and reference housekeeping genes real-time PCR

Transcription of TLR2 and TLR4 target genes (TG) as well as three reference housekeeping genes (HPRT-1, CG14980 and SDHA) were measured by qRT-PCR using the QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies). We set up the real time PCR for blood by relative standard curves by pooling 10 representative dog samples of cDNA. This pool of cDNA was diluted from 1:5 to 1:625 to make relative standard curves for the assessment of efficiency for each target gene (TLR2 and TLR4) and six reference genes described above. The efficiency (acceptable between 80 – 120%) for each assay was: TLR2= 99.5 %, TLR4= 80.4 %, CG-14980= 110.7 %, HPRT-1= 90.3 %, SDHA= 97.5 %,

TBP= 76.9 %, HMBS= 86.3 % and B-2M= 101.8 %. Primers and probes were obtained from Thermo Fisher Scientific and are listed in Table 1.

QuantStudio™ mastermix was filled in wells of standard plates (96 wells/plate) to the indicated volumes: nuclease free water (Sigma) 0.35 µL, taqman universal master mix (2x) 7.50 µL, taqman assay 20X 0.75 µL, 1/5 cDNA 6.4µl. Plates were sealed with an optic film (Applied Biosystems, Life Technologies) centrifuged and placed into a laboratory pipetting robot (Epmotion 5057 Liquid-handling robot, Eppendorf) to generate a 384 wells/plate. After that, the new plate was set into real time PCR instrument (QuantStudio™7 Flex System applied Biosystems, Thermo Fisher Scientific).

The following cycling conditions were used for all TG and reference genes: denaturation program (95ºC, 10 min), amplification and quantification program repeated 40 times (95ºC for 15 s, 60ºC for 10 s, 72ºC for 60 s with a single fluorescence measurement). The crossing point (CP) was determined for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. CP was measured at constant fluorescent level. Each reaction was carried out in triplicate. The same control case was used in triplicate as the calibrator control in each plate. All target genes (TG) per each dog were run the same day and with the same plate.

Data were analyzed by the relative quantification method with three RG (SDHA, CG14980 and HPRT1) (Livak and Schmittgen, 2001). Data were processed applying the relative quantification method comparable to the ddCt-method (2^{dd}Ct). For normalization of TG expression, the arithmetic mean of the three RG were taken for the calculation of a reference gene index (RGI) (Vandesompele et al., 2002). RT-PCR data analysis was done by the Cloudsuite software (Thermo Fisher Scientific).

Statistical analysis

The non-parametric Mann-Whitney *U* test was used to evaluate differences among groups and was calculated with the Graphpad Prism 5 program. The Spearman's rank order correlation between TLR transcripts in canine whole blood and clinical, serological and parasitological data was calculated using the Graphpad Prism 5 program. The non-parametric Wilcoxon Signed-Rank test was used to evaluate differences between different time-points during dog follow up treatments. The Wilcoxon Signed-Rank test was performed with SPSS Statistics 17.0. A significant *P* value was considered at <0.05.

Results

Reference genes

Based on the Vn/n+1 and M values, the best housekeeping genes were HPRT-1, CG14980 and SDHA. The results are shown in Figures 3.1.1. and 3.1.2.

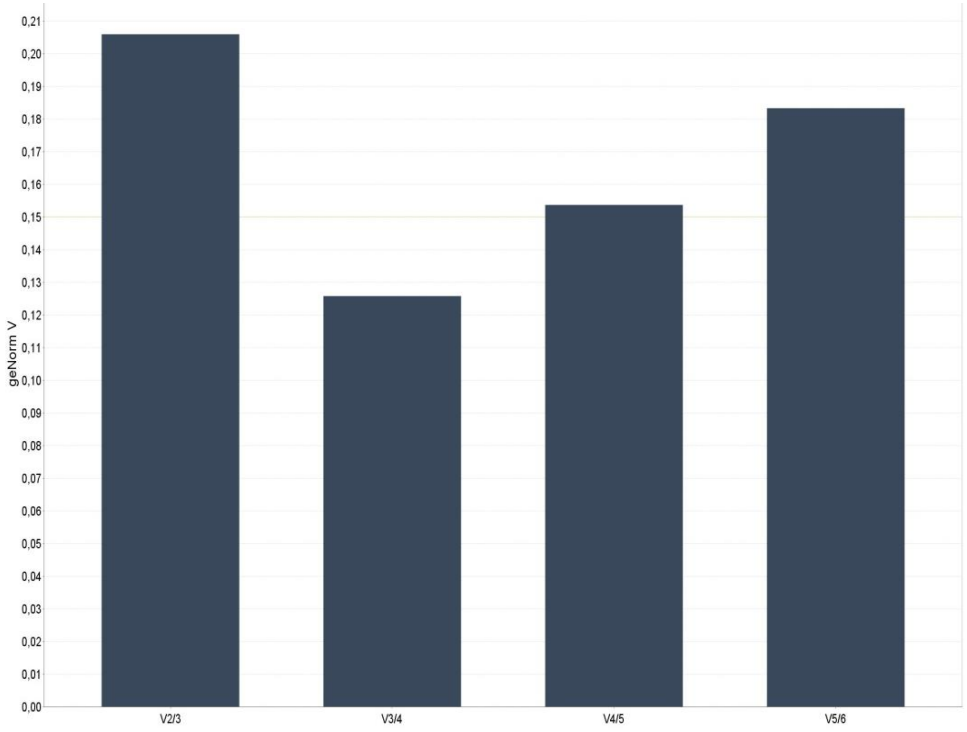

Figure 3.1.1. Determination of the optimal number of reference genes. Brown line indicates geNorm V value that needs to be below 0.15. Three references genes were selected based on results of V-value.

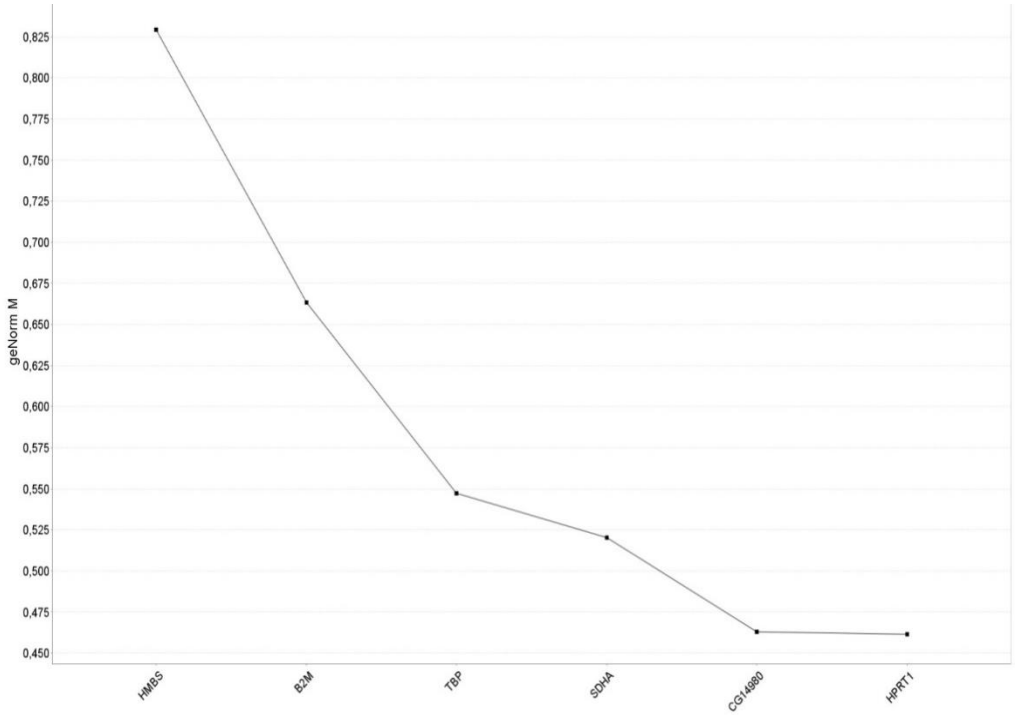

Figure 3.1.2. Average expression stability based on GeNorm programme of six references genes studied.

Diagnosis

TLRs transcripts in blood of sick dogs in comparison with healthy dogs

Relative quantification of TLR2 was significantly higher [mean 3.5 (Mann–Whitney *U* test: *Z*=-3.854, *P*<0.0001)] in sick dogs when compared with seronegative healthy dogs (mean 1.3); while relative quantification of TLR4 was similar between groups (Mann–Whitney *U* test: *Z*=-0.192, *P*=0.848) (Figure 3).

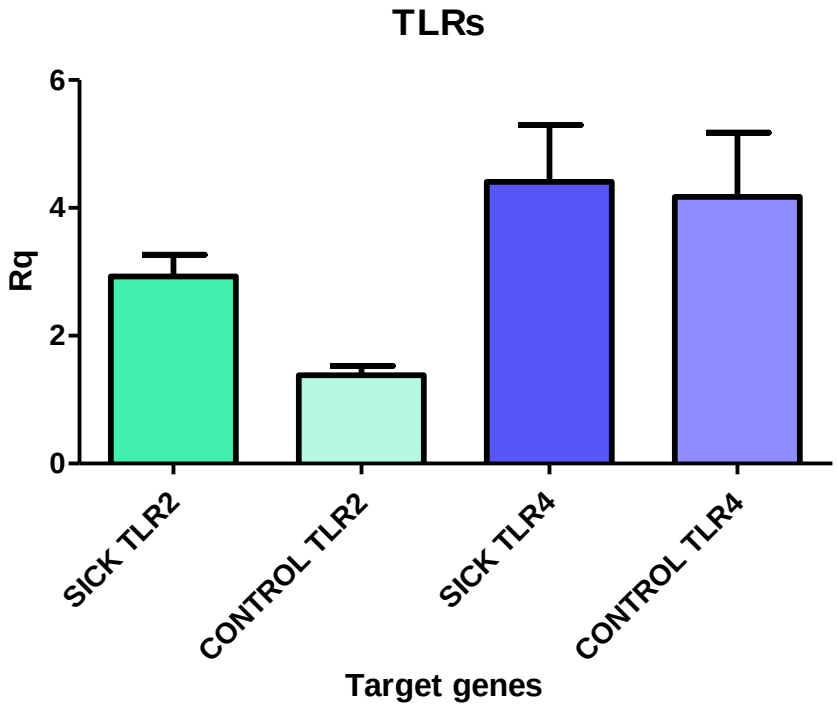

*Figure 3.1.3. Relative quantification (Rq) of TLR2 and TLR4 transcripts (mean and standard deviation) in sick and healthy dogs. *Comparison between sick dogs and seronegative healthy dogs (Mann–Whitney U test: Z=-3.854, P<0.0001).*

Correlations with TLR transcripts and clinical, serological and parasitological data

The results of Spearman's correlation between TLR transcripts and clinical, serological and parasitological parameters are listed in Table 3.1.2. A significant positive correlation was noted between TLR2 and UPC (P= 0.008), total protein (P< 0.0001), beta and gamma globulins (P<0.0001), specific *L. infantum* antibodies (P< 0.0001) and blood parasite load (P<0.0001) while a significant negative correlation was observed between TLR2 and albumin (P= 0.003), albumin/globulin ratio (A/G) (P<0.0001), hematocrit (P= 0.002) and hemoglobin (P= 0.004). TLR4 transcript did not correlate with any parameter.

Table 3.1.2. Spearman's correlation between TLR transcripts and serological, clinicopathological and parasitological parameters.

Variables (units)	P – values		Spearman correlation	
	TLR2	**TLR4**	**TLR2**	**TLR4**
TLR2		0.216		-0.137
TLR4	0.216		-0.137	
UPC	0.008[a]	0.218	0.401	0.192
Creatinine (mg/dL)	0.384	0.750	-0.100	-0.037
Urea (mg/dl)	0.108	0.590	-0.183	-0.062
Total protein (g/dL)	0.000[a]	0.036	0.437	0.233
Albumin (g/dl)	0.003[b]	0.974	-0.330	-0.004
Beta globulin (g/dL)	0.000[a]	0.644	0.397	0.054
Gamma globulin (g/dL)	0.000[a]	0.071	0.467	0.210
Ratio A/G	0.000[b]	0.284	-0.445	-0.125
Hematocrit (%)	0.002[b]	0.866	-0.389	0.022
Hemoglobin (g/dl)	0.004[b]	0.998	-0.354	0.000
L. infantum specific antibodies (ELISA units)	0.000[a]	0.694	0.462	0.044
Blood parasite load (parasite/mL)	0.000[a]	0.959	0.411	-0.006

[a]significant positive correlation; [b]significant negative correlation, P values less than 0.05

Follow up during treatment

Clinical, serological, parasitological and TLRs transcripts of sick dogs in comparison between different time points

All dogs significantly improved at days 30, 180 and 365 based on clinicopathological, serological and parasitological data. The results of clinicopathological data, serological and parasitological data during treatment follow-up are described in Table 3.1.3.

The results of TLRs transcripts in sick dogs on days 0, 30, 180 and 365 are also shown in Table 3.1.3. The relative quantification of TLR2 transcript significantly decreased during follow-up at all time points when compared with day 0 while no changes were observed with TLR4 transcript in any time point.

Table 3.1.3. Blood parasite load and antibody levels of sick dogs at diagnosis and during treatment.

	Mean ± standard deviation			
	Day 0	**Day 30**	**Day 180**	**Day 365**
Antibody levels (ELISA units)[a]	5383.17 ± 6482.49	2997.02 ± 4410.74	865.52 ± 1744.59	440.17 ± 851.96
Parasite load (parasite/ mL)[b]	27.02 ±100.70	0.28 ± 0,78	0.59 ± 2.06	0.42 ± 0.77
TLR2 transcription[c]	3.91 ± 3.64	1.75 ± 1.083	1.75 ± 1.56	1.22 ± 0.74
TLR4 transcription[d]	5.08 ± 7.60	5.05 ± 5.79	3.63 ± 3.85	5.58 ± 7.24

[a]Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test: Z = -3.847, P < 0.0001), day 180 (Wilcoxon signed-rank test: Z = -3.555, P < 0.0001), day 365 (Wilcoxon signed-rank test, Z = -4.015, P < 0.0001)]. Comparison between day 30-day 180 (Wilcoxon signed-

rank test: Z = -3.523, P < 0.0001), day 30-day 365 (Z = -3.285, P = 0.001) and day 180-day 365 (Wilcoxon signed-rank test: Z = -2.138, P = 0.033).

[b]Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test, Z = -2.669, P = 0.008), day 180 (Wilcoxon signed-rank test, Z = -2.499, P = 0.012), day 365 (Wilcoxon signed-rank test, Z = -2.417, P = 0.016)]. Comparison between day 30-day 180 (Wilcoxon signed-rank test: Z = −0.140, P = 0.889), day 30-day 365 (Z = -1.957, P = 0.050) and day 180-day 365 (Wilcoxon signed-rank test: Z = -0.0979, P = 0. 328).

[c]Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test, Z = -0.336, P = 0.005), day 180 (Wilcoxon signed-rank test, Z = -3.007, P = 0.003), day 365 (Wilcoxon signed-rank test, Z = -3.340, P = 0.001)]. Comparison between day 30-day 180 (Wilcoxon signed-rank test: Z = −0.568, P = 0.570), day 30-day 365 (Z = -2.165, P = 0.030) and day 180-day 365 (Wilcoxon signed-rank test: Z = -1.456, P = 0. 145).

[d]Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test, Z = -0.336, P = 0.737), day 180 (Wilcoxon signed-rank test, Z = -0.330, P = 0.741), day 365 (Wilcoxon signed-rank test, Z = 0.000, P = 1)]. Comparison between day 30-day 180 (Wilcoxon signed-rank test: Z = −0.434, P = 0.664), day 30-day 365 (Z = -0.644, P = 0.520) and day 180-day 365 (Wilcoxon signed-rank test: Z = -1.120, P = 0. 263).

Discussion

This study demonstrates, for the first time, an upregulation of TLR2 in unstimulated blood with clinical leishmaniosis at the time of diagnosis in dogs. Similar findings were observed in other tissues in previous studies (Esteve et al., 2015; Figueiredo et al., 2013; Hosein et al., 2015). In agreement with the present results, Esteve et al. 2015 described higher expression of TLR2 by immunohistochemistry in skin biopsies from cutaneous lesions in dogs with at least moderate disease (LeishVet stage II) when compared with dogs with mild disease due to papular dermatitis (stage I) (Esteve et al., 2015). In addition, a study from Brazil found a high parasite load along with increased frequency and expression of TLR2 in the colon of dogs with leishmaniosis (Figueiredo et al., 2013). TLR2 upregulation was also observed in brain, spleen and lymph nodes in sick dogs with natural leishmaniosis from Brazil (Melo et al., 2014a). TLR2 upregulation was also noted in skin and liver samples in a canine experimental model of infection (Hosein et al., 2015).

In contrast, a different study found that unstimulated peripheral blood mononuclear cells (PBMCs) derived from *Leishmania*-infected seropositive sick dogs from Brazil did not show differences in TLR2 expression compared with healthy dogs (Melo et al., 2014b). In addition, another study using PBMCs derived from canine blood found that CD11b + $TLR2^{+}$ cells were higher in dogs that were negative to skin *Leishmania* immunohistochemistry and xenodiagnosis than dogs with positive results for both techniques (Amorim et al., 2011). These Brazilian studies do not support our TLR2 findings however they were performed with PBMCs, the clinical status for these animals was not so well defined and the diagnosis was mainly performed by serological tests, leading to a possible cross reaction with other species of *Leishmania* in an area where they co-exist (Melo et al., 2014b). The findings of our study in conjunction with the current literature available suggest a role of TLR2 in the pathogenesis of canine leishmaniosis. TLR2 appears to be associated with moderate to very severe disease suggesting immune and proinflammatory responses due to the presence of a high *Leishmania* parasite load or cellular damage (endogenous damage-associated molecular patterns (DAMPs) also termed alarmins) as observed for other human diseases (Kang et al., 2015).

In the present study, all dogs manifested a moderate to severe clinical leishmaniosis. It is well known that clinical canine leishmaniosis is a systemic immune inflammatory disease (Baneth et al., 2008; Hosein et al., 2017; Solano-Gallego et al., 2009). Therefore, the upregulation of TLR2 transcripts in sick dogs at the time of diagnosis suggests that TLR2 appears to be a marker of inflammation in dogs as demonstrated in other canine inflammatory diseases. TLR2 upregulation has been found in inflammatory bowel diseases (McMahon et al., 2010) and immune-mediated or bacterial arthritis (Riggio et al., 2014), canine sinonasal aspergillosis and lymphoplasmacytic rhinitis (Mercier et al., 2012) as well as in female dogs with pyometra (Chotimanukul and Sirivaidyapong, 2012; Silva et al., 2010). TLR2 upregulation has been demonstrated in several inflammatory tissues including in intestinal (McMahon et al., 2010) and nasal biopsies (Mercier et al.,

2012), synovial fluid (Riggio et al., 2014) and the uterus (Chotimanukul and Sirivaidyapong, 2012). In addition, increased expression of TLR2 was also observed in PBMCs derived from dogs with steroid-responsive meningitis-arteritis (Maiolini et al., 2012).

The present results showed that TLR4 transcript in blood was unchanged among the groups studied. So far, there are few studies that have investigated TLR4 transcripts in canine leishmaniosis. Some authors found that TLR4 transcript was unchanged in the brain and spleen in agreement with the present study while TLR4 was upregulated in lymph nodes (Melo et al., 2014a) in dogs with natural leishmaniosis. Another study showed a significant downregulation of TLR4 transcription with disease progression in lymph node and in spleen samples and no change in TLR4 transcripts in skin and liver samples in an experimental canine model (Hosein et al., 2015). It is well known that there are different immune responses depending on tissues (compartmentalization) and inflammatory responses in canine leishmaniosis (Hosein et al., 2017; Melo et al., 2014a). Based on the present study and other studies, it seems that TLR4 transcription is unchanged or decreased in dogs with clinical leishmaniosis when compared with healthy dogs and might indicate a mechanism of parasite immune system evasion or a less important role for TLR4 in clinical canine leishmaniosis.

Similar to our results, differences of TLR4 transcripts have not been detected among dogs with other inflammatory diseases when compared with healthy controls (Maiolini et al., 2012) such as inflammatory bowel disease (McMahon et al., 2010), inflammatory colorectal polyps (Igarashi et al., 2015), immune-mediated arthritis and septic arthritis (Riggio et al., 2014) or lymphoplasmacytic rhinitis (Mercier et al., 2012). For example, no TLR4 transcript differences were seen in miniature dachshunds with inflammatory colorectal polyps when compared with controls (Igarashi et al., 2015). However, other studies mainly related with inflammatory diseases associated with bacterial infection showed an increase in TLR4 expression (Burgener et al., 2008;

Chotimanukul and Sirivaidyapong, 2011; Mercier et al., 2012) in agreement with the fact that TLR4 recognized bacterial lipopolysaccharides (LPS) among other molecules.

In humans, TLR expression in unstimulated whole blood has not been yet explored. However, TLRs have been investigated in *Leishmania*-specific stimulated blood in patients from Sudan with visceral leishmaniosis due to *Leishmania donovani*. TLR2 transcript was found to be highly upregulated in patients with visceral leishmaniosis (VL) when compared with healthy humans either with positive or negative leishmanin skin test results (Babiker et al., 2015) in agreement with the present study. In contrast to our results, TLR4 transcripts were also upregulated in patients with visceral leishmaniosis in *Leishmania*-specific stimulated blood (Babiker et al., 2015). Interestingly, similar findings with TLR2 and TLR4 upregulation were observed in pre-treatment splenic aspirate samples from patients with visceral leishmaniosis due to *L. donovani* from India compared to post treatment samples (Kumar et al., 2014). However, no difference in TLR2 and TLR4 transcripts was observed between unstimulated PBMCs from pre-treatment subjects, compared to post-treatment group or that of healthy endemic controls suggesting site and cell specific up regulation of mRNA expression for these TLRs during ongoing disease (Kumar et al., 2014). Other studies in humans with cutaneous leishmaniosis due to *Leishmania major* from Iran showed that the mean relative gene expression of TLR2 and TLR4 in macrophages derived from blood of patients with healing lesions were significantly higher than in patients with non-healing lesions (Tolouei et al., 2013). Another study performed in cutaneous lesions due to *Leishmania braziliensis* in humans demonstrated that TLR2 was the most common TLR expressed during active disease mainly by macrophages while TLR4 was scarcely expressed (Tuon et al., 2012).

There are no studies describing correlations with clinical, serological and parasitological parameters and TLR transcription in unstimulated blood in dogs with leishmaniosis. Therefore, there is limited knowledge related to clinical parameters and TLRs transcription in this disease. As previously mentioned, TLR2 transcription was

significantly upregulated in unstimulated blood of dogs with natural moderate to severe leishmaniosis. In addition, TLR2 was significantly correlated with laboratory abnormalities, blood parasitemia and *L. infantum* antibody level further confirming its role in dogs with moderate to very severe disease. The laboratory abnormalities that correlate with TLR2 are commonly observed in dogs with moderate to severe leishmaniosis (Solano-Gallego et al., 2011). It is also important to highlight that high antibody levels are also associated with disease severity as well as parasite load (Solano-Gallego et al., 2016b).

This study shows a significant decrease in TLR2 transcription with treatment and clinical improvement while no changes were found in TLR4 transcription. These findings reinforce the concept that TLR2 transcription in blood appears to be associated with disease severity in canine leishmaniosis and downregulation of TLR2 takes place during successful anti-*Leishmania* treatment and improvement of clinico-pathological parameters. In partial agreement with our results, unstimulated blood from Brazilian human patients with visceral leishmaniosis due to *L. infantum* presented higher percentages of CD3+ cells expressing TLR2 and TLR4, as well as cells co-expressing TLR2/TLR4 before treatment compared with post-treatment and control individuals (Gatto et al., 2015).

Conclusions

The findings presented in the current study indicate an up-regulation of TLR2 transcription in unstimulated blood of naturally infected sick dogs as compared to healthy dogs suggesting active innate immune and proinflammatory responses due to the presence of a high *Leishmania* parasite load or cellular damage as observed for other canine diseases. In addition, TLR2 transcription is reduced with clinical improvement during treatment. In contrast, TLR4 transcription appears to be unchanged at the time of diagnosis and during treatment follow-up and might indicate a mechanism of parasite immune system evasion or a less important role for this TLR in clinical canine

leishmaniosis. TLR2 expression might be used in the future as an immunological marker in research and development of vaccines and immunotherapy.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by a Spanish Ministry grant, Ministerio de Economía y competitividad (AGL2012-32498).

Dr. Laia Solano-Gallego holds a Ramón y Cajal senior researcher contract awarded by the *Ministerio de Ciencia e Innovación* (Spain) and the European Social Fund.

Acknowledgements

The authors thank all veterinarians and dog owners that contributed to this study. We are especially grateful to Dr. Marta Planellas (Hospital Clínic Veterinari of Universitat Autonoma of Barcelona), Marta Blanchart (Ars Veterinaria), Montsant Osso (Consultori Falset), Annabel Dalmau (Hospital Mediterrani), Dani Mari and Paulina Quirola (MSc students from UAB) for their clinical assistance ,sample collection and support to this study. The authors thank also Dra. Anna Castelló for her technical assistance with molecular work. The authors are also grateful to Dra. Pamela Martinez and Laura di Filippo (UAB) for their assistance with laboratory work and Dr. Shazia Hosein for English revision of the manuscript.

3.2. ESTUDIO 2

Parasite Specific Antibody Levels, Interferon-γ and TLR2 and TLR4 Transcripts in Blood from Dogs with Different Clinical Stages of Leishmaniosis.

Sara Montserrat-Sangrà[1], Laura Ordeix[1,2], Pamela Martínez-Orellana[1] and Laia Solano-Gallego[1]

[1] Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, Bellaterra, 08193, Spain; sara.montserrat@uab.cat (S.M.S.); laura.ordeix@uab.cat (L.O.); pamela.martinez@uab.cat (P.M.O.)

[2] Fundació Hospital Clínic Veterinari, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra 08193 Spain

Reprinted from Veterinary Sciences, 2018 Mar 16;5(1)

Received: 30 December 2017; Accepted: 14 March 2018; Published: date

doi: 10.3390/vetsci5010031

Abstract

Canine leishmaniosis has a wide range of disease severity from mild (stage I), to severe (stages II-III), or very severe disease (stage IV). The objective of the study was to evaluate and compare serum antibody levels, *Leishmania infantum* specific IFN-γ production and TLR2 and TLR4 transcripts in non-stimulated blood from dogs with different clinical stages at the time of diagnosis as well as blood parasitemia. Enzyme-Linked ImmunoSorbent Assay (ELISAs) were performed to determine serum antibody levels and IFN-γ production and quantitative polymerase chain reaction (*q*PCRs) in order to determine blood parasite load and TLR2 and TLR4 transcripts. Older dogs were significantly affected by more severe disease with higher antibody levels and blood parasitemia than dogs with mild disease. IFN-γ production was significantly higher in dogs with stage I disease when compared to dogs with more severe disease. Relative quantification of TLR2 in dogs with mild disease was similar to that of control dogs. On the other hand, TLR2 transcripts were significantly higher in dogs with severe disease as compared with that from control healthy dogs. No differences were found in TLR4 relative quantification between groups. This study demonstrates that dogs with different clinical stages of leishmaniosis present different levels of biological markers indicative of different immune responses.

Keywords

dog; *Leishmania infantum*; papular dermatitis; IFN-γ; TLR2; TLR4; blood parasitemia

Abbreviations

CanL: Canine leishmaniosis; CBC: counter blood cell; ELISA: Enzyme-Linked ImmunoSorbent Assay EU: ELISA units; Th: T helper cell; Toll like receptor: TLR; PCR, Polymerase chain reaction; qPCR, Quantitative PCR; RG, Reference gene; RGI, Reference gene index; RIN, RNA integrity number; RNA, Ribonucleic acid; qPCR, Quantitative PCR; Rq, Relative quantification; rt- PCR, Real time PCR

Introduction

Canine leishmaniosis (CanL) due to *Leishmania infantum* is a very pleomorphic disease. Clinically, this infection ranges from subclinical infection to very severe disease, passing through several degrees of disease (Solano-Gallego et al., 2009). According to this clinical variability, a clinical staging system that classifies the disease into four stages based on clinical signs, clinicopathological abnormalities, and measurement of anti-leishmanial antibodies, was previously described (Solano-Gallego et al., 2009).

Clinical and clinical-pathological findings observed in CanL are the consequence of complex interactions between *L. infantum* and the genetical and immunological background of the dog (Solano-Gallego et al., 2009). In fact, both innate and adaptive immune responses play a role in the outcome of *Leishmania* infection (Hosein et al., 2017; Papadogiannakis and Koutinas, 2015). However, only the adaptive immune response has been extensively investigated in dogs. The balance between the protective T-helper (Th) 1 cellular response, which was associated with the cytokine interferon-gamma (IFN-γ), and the humoral immune response mediated by Th2 lymphocytes determines the clinical manifestation of the infection. A predominantly Th1 immune response is thought to provide resistance to the development of disease. On the other hand, a predominantly Th2 immune response correlates with antibody production and disease progression (Hosein et al., 2017; Pinelli et al., 1994). Although the innate immune response has been scarcely studied in leishmaniosis, recent data suggests that it is paramount for the ultimate outcome of *Leishmania* infection (Barbosa et al., 2011; Fernandez-Bellon et al., 2005; Hosein et al., 2017). In this sense, toll like receptors (TLRs), which are one of the most important pattern recognition receptor family, are central in the early host defense against pathogen and activate adapter molecules after binding to their ligand. The activated cascade then leads to induction or suppression of genes that influence the inflammatory response (Vidya et al., 2017).

Limited information is available regarding the role of TLRs in canine *L. infantum* infection. Although, the exact role of TLRs in the pathogenesis of CanL has not been fully addressed, it would seem that there is an association between TLR2 and its pathogenesis (Hosein et al., 2017). In fact, it has been recently revealed that TLR2 upregulation in blood and skin seems to be associated with disease progression in dogs (Hosein et al., 2017) and a reduction in TLR2 transcription has been described with treatment and clinical improvement (chapter 3.1.(Montserrat-Sangra et al., 2016)). Moreover, there is a lower expression of TLR2 in skin biopsies from dogs with mild disease (papular dermatitis) when compared with dogs with moderate or severe disease (Esteve et al., 2015). TLRs other than TLR2 have been scarcely investigated in experimental or natural *L. infantum* infection in dogs (Melo et al., 2014a). Regarding TLR4, it has been described that transcription of this TLR appears to be similar among dogs with clinical leishmaniosis and healthy seronegative dogs at the time of diagnosis with no changes during treatment follow-up, indicating a less important role of this TLR in clinical leishmaniosis (chapter 3.1. (Montserrat-Sangra et al., 2016)).

Cutaneous lesions are the most common clinical signs in CanL (Ordeix L, 2013; Saridomichelakis and Koutinas, 2014) and they are clinically classified as typical (i.e., exfoliative dermatitis, ulcerative dermatitis of the bony prominences, onychogryphosis, and, in an endemic area, papular dermatitis) or atypical (i.e., muco-cutaneous nodular dermatitis, other ulcerative muco-cutaneous dermatitis than that mentioned above, sterile pustular dermatitis, or ischaemic dermatopathy) (Ordeix L, 2013). Among the cutaneous manifestations of CanL, papular dermatitis is considered to be a typical form in an endemic area and is indicative of stage I leishmaniosis (Ordeix et al., 2017; Ordeix L, 2017; Ordeix et al., 2005). In fact, dogs with papular dermatitis commonly are young dogs without any other clinical-pathological abnormalities and with low parasite load and granuloma formation in skin lesions, negative or weakly positive anti-*Leishmania* antibody levels and specific cell-mediated immune response studied by means of leishmanin skin test (Ordeix et al., 2017; Ordeix L, 2017; Ordeix et al., 2005). Furthermore, this dermatological problem is associated with the spontaneous resolution of the lesions

within 3–5 months (Bottero E, 2006). Moreover, normal-looking skin from dogs with stage I and papular dermatitis is less likely to present microscopic lesions as well as harbour the parasite when compared with dogs with moderate to severe CanL (Ordeix et al., 2017). Taken all of these findings together, papular dermatitis and stage I CanL is indicative of a protective immune response associated with a good prognosis (Bottero E, 2006; Lombardo et al., 2014; Ordeix et al., 2005). However, there are limited studies that evaluate differences in clinical staging and adaptive and innate immune responses in CanL (Martinez-Orellana et al., 2017a).

The hypothesis of this study is that dogs with stage I leishmaniosis and papular dermatitis show distinctive immunological characteristics when compared with dogs with more severe disease. Therefore, the objective of this study was to evaluate serum antibody levels, *L. infantum* specific IFN-γ production, and TLR2 and TLR4 transcripts in the blood of dogs with different clinical stages of leishmaniosis at the time of diagnosis.

Materials and Methods

Dogs

All of the dogs used in this study were privately owned pets, which were volunteered with client informed consent. They remained under the care of their owners and were not housed for the purposes of this study or held for any period of time.

Dogs with Clinical Leishmaniosis

Forty-two dogs with typical cutaneous signs due to leishmaniosis attending different veterinarian facilities from Catalonia (Spain): *Fundació Hospital Clínic Veterinari*, *Universitat Autònoma de Barcelona* (UAB), Cerdanyola, Barcelona), *Hospital Ars Veterinària* (Barcelona), *Mediterrani Veterinaris Hospital* (Reus, Tarragona), and *Consultori Montsant* (Falset, Tarragona), were prospectively enrolled. In order to evaluate its clinical status, a full complete blood count (CBC), serum biochemical profile which included

creatinine, urea, total cholesterol, total protein and alanine aminotransferase (ALT), protein serum electrophoresis, and urinalysis with urinary protein creatinine ratio (UPC) were carried out. The hematology, biochemistry, and serum electrophoresis were performed using the following laboratory equipment: Siemens ADVIA120 (Siemens Healthineers, Erlangen, Germany), chemistry analyzer Olympus AU400 (Olympus Diagnostica GmbH, Hamburg, Germany) and Hydrasis system (Sebia Hispania SA, Barcelona, Spain), respectively.

Leishmaniosis was diagnosed after visualization of amastigotes on skin cytology or biopsy with or without *Leishmania* specific immunohistochemistry, as previously described (Esteve et al., 2015), and/or the detection of specific *L. infantum* antibody levels by quantitative serology using an in-house Enzyme-Linked ImmunoSorbent Assay (ELISA) (Esteve et al., 2015). The classification of *L. infantum* antibody levels is described in table 3.2.1.

Table 3.2.1 Two-fold serial dilution ELISA classification in sera sample.

	Negative	**Very Low Positive**	**Low Positive**	**Medium Positive**	**High Positive**	**Very High Positive**
***L. infantum* antibody levels (EU)**	<35	35–100	101–500	501–9000	9001–40000	≥40000

ELISA units (EU)

Dogs were classified based on LeishVet clinical stages, as previously described (Solano-Gallego et al., 2009). In addition, blood DNA extraction and *Leishmania* quantitative polymerase chain reaction (*q*PCR) was performed with an absolute quantification of parasites/mL of blood, a standard curve was created. Ten-fold serial dilutions of known concentrations were made from a culture with 10^7 *L. infantum* promastigotes/mL. Six serial dilutions were obtained to create a standard curve. Subsequently, the value of the slope (m) and the intersection (y) of the line were

calculated. The following formula was used in order to determine the number of parasites/mL blood by PCR: (Standard deviation - y)/m. The result was multiplied by the dilution factor of DNA (x125) to achieve the number of parasites/mL (Martinez et al., 2011). The classification of parasite load, as described elsewhere (Tabar et al., 2008), is listed in table 3.2.2.

Table3.2.2. Parasite load classification in whole blood.

	Negative	Low Positive	Medium Positive	High Positive	Very High Positive
***L. infantum* Parasite load (parasites/mL)**	0	<10	10–100	101–1000	>1000

Control Healthy Dogs

A total of 34 healthy dogs were enrolled. Both sexes were represented with 16 females and 18 males. Median of age was four years, with a range from six months to 10 years. Twenty-two purebred dogs with 14 breeds were represented (golden retriever, beagle, greyhound, Labrador retriever, border collie, schnauzer, Ibizan hound, fox hound, Ariege pointer, griffon Nivernais, Bruno Jura hound, Dachshund, English setter), eight mixed-breed dogs were included and for four dogs breeds were not registered. All of the dogs were seronegative and blood *q*PCR negative.

Whole Blood Cytokine Release Assay and Determination of Canine IFN-γ

Heparinized whole blood cytokine release assay was carried out, as previously reported (Solano-Gallego et al., 2016b). Briefly, heparinized whole blood was separately mixed with three different conditions: (i) only medium (unstimulated); (ii) medium with soluble *L. infantum* antigen (LSA) at a concentration of 10 µg/mL; and, (iii) medium with the

mitogen concanavalin A (ConA, 100 mg, Medicago®, Uppsala, Sweden) at a concentration of 10 µg/mL. IFN-γ was determined in supernatants from five days after stimulation with ConA and LSA or only medium (unstimulated), as described elsewhere (Solano-Gallego et al., 2016b), by a commercial sandwich ELISA (DuoSet® ELISA by Development System R&DTM, Abingdon, UK).

Cytokine concentration from supernatants with ConA and LSA was calculated after subtracting the IFN-γ concentration obtained from supernatants with only medium (unstimulated). Thereafter, dogs were classified as IFN-γ producers when *L infantum* specific IFN-γ concentration was detectable. Dogs were classified as IFN-γ non-producers when *L. infantum* specific IFN-γ concentration was at not detectable levels (Solano-Gallego et al., 2016b).

RNA Extraction, RNA Concentration and Quality and cDNA Synthesis

Half mL of Ethylenediaminetetraacetic Acid (EDTA) blood sample was transferred to a cryovial tube that contained 1.3 mL of RNAlater (Ambion®, Thermo Fisher Scientific, Waltham, MA, USA). Then, blood samples in cryovial tubes were left at 4 °C overnight and, thereafter, frozen at −80 °C until used. Blood samples were thawed on ice and total RNA from 500 µL of EDTA blood was extracted using Ribopure RNA blood kit (Ambion®, Thermo Fisher Scientific), according to the manufacturer's instructions. A DNase digestion step was included to remove contaminating genomic DNA using TurboDNase (Ambion®, Thermo Fisher Scientific) following manufacturer's instructions. RNA concentration was determined by Nanodrop device (Thermo Fisher Scientific). RNA integrity and quality was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples had a final concentration amongst 20–70 ng RNA/µL. All samples had an RNA Integrity Number (RIN) value greater than 7. cDNA was generated using VILO masterscript retrotranscriptase (Invitrogen™, Thermo Fisher Scientific), according to the manufacturer's instructions. cDNA was stored at −20 °C until used (chapter 3.1. (Montserrat-Sangra et al., 2016)).

TLR2, TLR4 and Reference Housekeeping Genes qPCR

Transcription of TLR2 and TLR4 target genes (TG), as well as three reference housekeeping genes (RG: HPRT-1, CG14980 and SDHA), was measured by *q*PCR using QuantStudio™ 7 Flex Real-Time PCR System, array card, desktop (Life Technologies, Carlsbad, CA, USA) as previously described (chapter 3.1. (Montserrat-Sangra et al., 2016)). Briefly, we calculated the efficiency of target and reference amplification genes and determined optimal efficiencies amplifications of 100% and acceptable in the range (±20%). The baseline and threshold were established for each gene and all of the samples were processed in triplicate. The same control sample was used as a calibrator in every plate to determine relative quantification of all the dogs studied. Data were processed while applying the relative quantification method comparable to the delta-delta-cycle threshold value (ddCt) method (2^{dd}Ct) (chapter 3.1. (Montserrat-Sangra et al., 2016)). Quantitative PCR data analysed was done by the Cloudsuite software (Life technologies™, Thermo Fisher Scientific). For normalization of TG, expression the arithmetic mean of the three RG was taken for the calculation of a reference gene index (RGI) (chapter 3.1. (Montserrat-Sangra et al., 2016)).

Statistical Analysis

Statistical analyses were performed using the IBM SPSS Statistics Base 22.0 program for Windows software (IBM Corporation, Armonk, NY, USA).

Standard descriptive statistics [median and range (minimum and maximum values)] were calculated for quantitative variables. The non-parametric Mann–Whitney test was used to evaluate differences among groups. Categorical data were expressed as percentage and statistical analysis was performed while using the Fisher's exact test to compare results among independent variables. Differences were considered significant at a 5% significance level ($P < 0.05$).

Results

Dogs with Clinical Leishmaniosis

Based on clinicopathological(Solano-Gallego et al., 2009) findings and *L. infantum*-specific antibody levels, dogs were clinically classified, as previously reported (Solano-Gallego et al., 2009), and were distributed as follows: group A (stage I and papular dermatitis, n = 20, Figure 3.2.1) and group B (stage II–III and ulcerative or exfoliative dermatitis, n = 22, Figure 3.2.2).

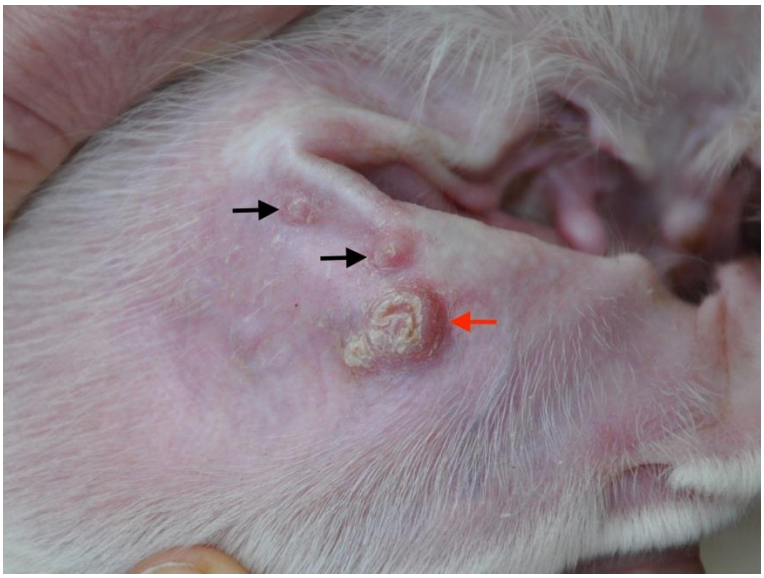

Figure3.2.1. Two raised solid lesions smaller than 0.5 cm in diameter (papules, black arrows) and one raised solid lesion wider than higher (plaque, red arrow) in the inner aspect of a pinna of an Ibizan Hound dog with papular dermatitis and stage I leishmaniosis (Group A).

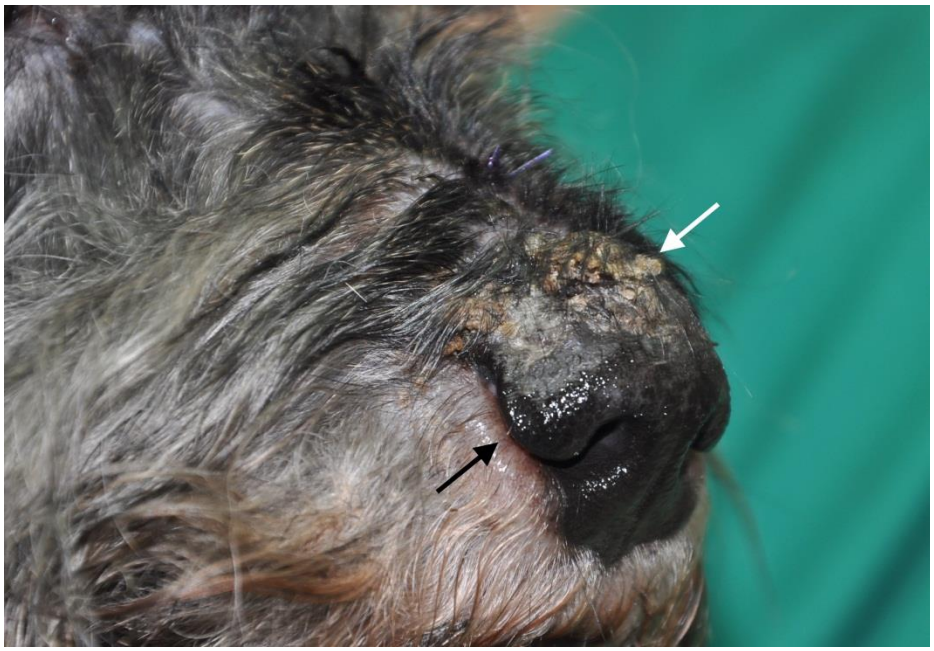

Figure3.2.2. Crusting and scaling in the dorsal part of the muzzle (white arrow) and perinasal depigmentation (black arrow) in a schnauzer dog with stage II leishmaniosis (Group B).

Ten females and ten males composed group A. Median of age was 10 months (5–98). Twelve pure bred dogs with three breeds represented and eight mixed breed dogs were included. Ten out 20 dogs were Ibizan hound dogs, one was a German pointer, and one was a Dachshund. Ten females and 12 males constituted group B. Median of age was 52.5 months (8–153). Fifteen pure bred dogs with 12 breeds represented (two French bull dogs, two Labrador retrievers, and one of each doberman, pinscher, American bull dog, schnauzer, boxer, Akita inu, greyhound, Brittany) and seven mixed breed dogs were included. The difference of age between groups was statistically significant (Mann-Whitney U-test, Z = 3.66999, P = 0.00024). Dogs from group A did not present laboratory abnormalities, while dogs from group B presented typical laboratory abnormalities of clinical leishmaniosis.

Blood *Leishmania* qPCR

Based on *Leishmania q*PCR dogs from group A were classified as negative (n = 12), low positive (n = 3), or medium positive (n = 1). The parasite load evaluation was not possible

in four dogs from this group due to lack of sample. On the other hand, dogs from group B were classified as negative (n = 7), low (n = 9), medium (n = 4) and high positive (n = 2). A higher percentage of positive *Leishmania* *q*PCR in blood were found in group B when compared with group A (Fisher´s exact test, P = 0.0201).

Group A dogs had lower parasite load (median and range (R): 0 parasite/mL (0–33.49)) than those from group B (median and R: 2.54 (0–475.07); Mann-Whitney U-test, Z = −2.664, P = 0.012).

Leishmania infantum Specific Antibody Levels

Dogs from group A were serologically classified as follows: negative (n = 11), very low positive (n = 3), low positive (n = 4), and medium positive (n = 2). On the other hand, dogs from group B were classified as very low positive (n = 1), low positive (n = 4), medium positive (n = 12), high positive (n = 3), and very high positive (n = 2). A higher percentage of seroreactive dogs was found in group B when compared with group A (Fisher´s exact test, P = 0.0001).

Group A dogs had lower levels of antibodies (median and R: 25 (5.7–529.5) EU) than those from group B (median and R: 1609 (86.8–90730.2) EU); Mann-Whitney U-test, Z = 3.53323, P = 0.00042).

Leishmania infantum specific IFN-γ production after blood stimulation

Cytokine analysis was performed in 19 out of 20 dogs from group A and in 15 out of 22 dogs from group B due to lack of samples. Fifteen of 19 dogs (79%) from group A and six out 15 (40%) from group B were classified as IFN-γ producers (Fisher exact test, P = 0.0337).

The median and R of IFN-γ production from groups A and B were 710 (0–20300) pg/mL and 0 (0-2413) pg/mL, respectively. There was a higher IFN-γ production in group A dogs when compared to those of group B (Mann-Whitney U-test, Z = 2.45047, P = 0.0143).

TLRs Transcripts

TLRS transcripts are presented in Figure 3. Relative quantification of TLR2 in group A (median and R: 1.2 (0.6–4.2) folds) was similar than that of control dogs (median and R: 1.2 (0.1–3.2) folds, Mann-Whitney U-test, Z = −0.24897, P = 0.8). On the other hand, relative quantification of TLR2 in group B (median and R: 1.9 (0.9–12.4)) was significantly higher when compared with that from control healthy dogs (Mann-Whitney U-test, Z = −2.41119, P = 0.016). Although no statistically significant, the expression of TLR2 in the group A was lower than that of the group B (Mann-Whitney U-test, Z = 1.6225, P = 0.1).

The expression of TLR4 mRNA in control dogs was of a median and R of 2.6 (0.5–13) folds and was similar to that of dogs from group A (median and R: 4.4 (0.7–19.8) folds; Mann-Whitney U-test, Z = −0.92475, P = 0.36) and to that from group B (median: 3.7 (1.1–32.8) folds); Mann-Whitney U-test, Z = −1.88358, P = 0.06). Although not statistically significant, TLR4 mRNA expression in dogs with leishmaniosis was higher than that of control group.

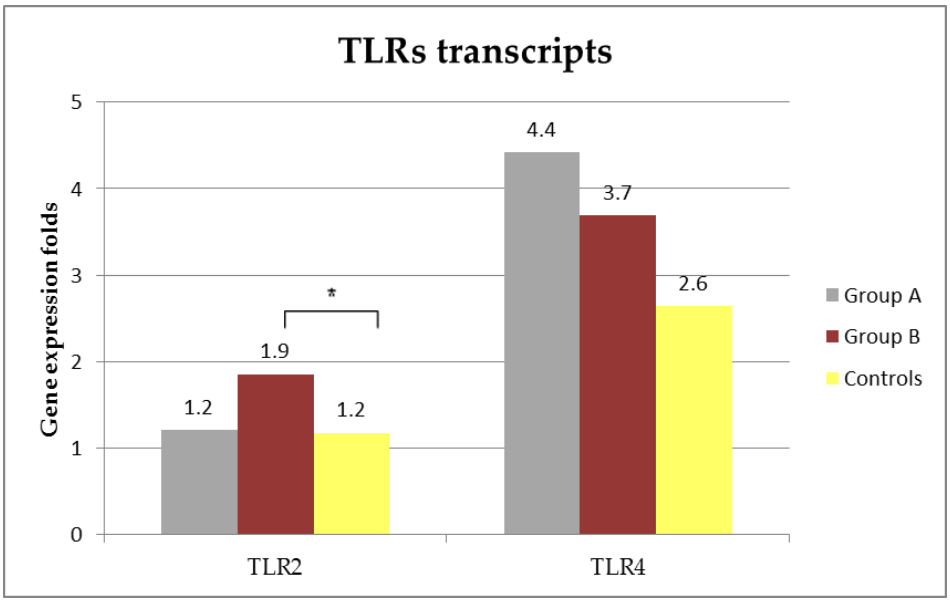

*Figure3.2.3. Median of Toll like receptors (TLR) 2 and TLR4 transcripts in non-stimulated blood from diseased and control dogs (*Mann-Whitney U-test, Z = −2.41119, P = 0.016).*

Discussion

The results of the present study support our hypothesis that dogs with stage I leishmaniosis and papular dermatitis show distinctive immunological characteristics when compared with dogs with more severe disease at the time of diagnosis.

Dogs with stage I and papular dermatitis reported herein were more frequently serologically negative than the dogs with stage II–III. Moreover, in agreement with a previous study (Ordeix et al., 2017), we demonstrated that dogs with stage I and papular dermatitis had significantly lower levels of *Leishmania* antibodies than dogs with more severe disease. Clinical CanL is often associated with a humoral response (Th2 driven), which is non-protective and denotes failure to control the infection. Serologically negative and/or low positive dogs studied herein were further diagnosed by means of skin cytology, histopathology, and/or PCR. It is well established that dogs with mild clinical signs, such as solitary lymphadenomegaly or papular dermatitis, may present with negative to low positive antibody levels (Solano-Gallego et al., 2017b), as described in the present study. Therefore, lack or low humoral immune response elicited in dogs with stage I and papular dermatitis, may be indicative of a polarized specific cellular immune response (Th1 driven), which might confer protection against disease progression.

In the present study, dogs with stage I and papular dermatitis were more commonly IFN-γ producers than dogs with more severe disease. In addition, *Leishmania*-specific IFN-γ concentrations were superior in dogs with papular dermatitis than in dogs with clinically more severe cutaneous forms. However, it is noteworthy those four dogs with mild disease were IFN-γ non–producers and that six dogs with more severe disease were IFN-γ producers. The results of the present study are in agreement with previous studies, which have shown that IFN-γ non-producers are usually classified in a more severe clinical staging than IFN-γ producers (Martinez-Orellana et al., 2017a), and that IFN-γ concentration increase with long-term anti-*Leishmania* treatment, together with clinical improvement in dogs that do not produce IFN-γ at diagnosis (Solano-Gallego et al., 2016b). Although, limited information regarding *L. infantum* specific IFN-γ production in stimulated blood in sick dogs is available, it seems that this assay is a reliable method of

measurement of T cell-mediated immunity in CanL (Carson et al., 2009) and human leishmaniosis (Singh and Sundar, 2014). It is well established that Th1 lymphocytes mainly drive IFN-γ production in stimulated blood. However, natural killer (NK) cells, which play important roles in innate immune responses, may secrete this cytokine in response to IL-12. NK cells constitute 5% to 15% of the mononuclear cells in the blood. Therefore, the proportion of IFN-γ detected in blood from this origin is likely to be low. Identification of circulating cellular subsets expressing this immune marker was not carried out in the present study; therefore IFN-γ production cannot be conclusively attributed to an adaptive immune response (Pillai, 1991). A presumed T cellular immune response in dogs with stage I and papular dermatitis, is, therefore, thought to provide resistance to the progression of disease in those patients. In fact, previous published data have demonstrated a good clinical outcome with spontaneous resolution and an apparent lack of disease progression in dogs affected of papular dermatitis and stage I (Bottero E, 2006; Lombardo et al., 2014). Moreover, a recent study have revealed a lack of disease progression in a cohort of untreated dogs with papular dermatitis and stage I leishmaniosis, followed during one year after the diagnosis (Ordeix L, 2017). However, it is interesting to highlight that those four IFN-γ non–producer dogs with papular dermatitis and stage I leishmaniosis would deserve a close and strict follow-up.

Another limitation of this study is that cytokine immune profile related to Th2 immune response (i.e., TGF-β, IL10 or IL-4) was not evaluated in these dogs. Analysis of these cytokines in stimulated blood from dogs with leishmaniosis has been scarcely described, although an association was found between parasite specific IL-10 production and disease progression (Boggiatto et al., 2010; Killick-Kendrick et al.). Using the same method that as employed in the present study, *L. infantum* specific IL-10 production after blood stimulation did not seem to be a disease severity marker since it was not detected in 64% of the patients and in those that was detected there was no correlation with the severity of the disease (Solano-Gallego et al., 2016b). Therefore, although polarized Th1 immune response is strongly suggested in dogs with stage I and papular dermatitis, a Th2 like cytokine immune profile has not been ruled out in the present study.

There are limited data available on expression of TLRs in CanL. Therefore, the role of TLRs in the pathogenesis of this disease has not been fully addressed (Amorim et al., 2011; Figueiredo et al., 2013; Hosein et al., 2015; Melo et al., 2014a; Melo et al., 2014b). This study demonstrates that dogs with stage I and papular dermatitis presented a TLR2 transcription in the non-stimulated blood that was similar to healthy control dogs. On the other hand, there was a significantly higher up regulation of TLR2 in non-stimulated blood from dogs with severe clinical leishmaniosis at the time of diagnosis when compared with healthy control dogs. Although not being statistically significant, TLR2 transcripts were lower in stage I and papular dermatitis than in dogs with clinically more severe CanL. Although the cell source of increase in the expression of TLR2 was not determined in the present study, it is likely that the main source of the upregulation of TLR2 was firstly neutrophils and secondly monocytes and NK cells. Neutrophils are the predominant white cells in whole blood in canines. In addition, it has been also demonstrated that TLR2 protein is easily detectable by flow cytometry on the canine peripheral blood granulocyte and monocyte cell surface, and is rarely present on lymphocytes (Bazzocchi et al., 2005). This is similar to what is found in humans where lymphocytes do not express TLR2 in unstimulated blood (Flo et al., 2001). Dogs with moderate clinical leishmaniosis have a high parasite load in several tissues, including the bone marrow (Becker et al., 2003; Reis et al., 2006b), and this might be the reason for a major up regulation of TLR2 in unstimulated blood, as *Leishmania* lipophosphoglycans are recognized by TLR2 (Becker et al., 2003) and it is likely that other parasite ligands are also involved in TLR2 recognition of *Leishmania* (Halliday et al., 2016). In addition, myeloid hyperplasia is a common finding in bone marrow of sick dogs with leishmaniosis (Paltrinieri et al., 2016). Neutrophils and monocytes are released from the bone marrow to the peripheral blood, and thereafter they migrate to tissues where they die. Neutrophils or monocytes do not recirculate in peripheral blood. So, we hypothesize that it is likely that an upregulation of TLR2 happens in the bone marrow of dogs with more severe disease due to high parasite loads (Reis et al., 2009; Reis et al., 2006b). This high expression of TLR2 remains in neutrophils when they circulate in the peripheral blood, while it does not occur in dogs with mild disease.

Our results in unstimulated blood are in agreement with a previous study that described higher expression of TLR2 by immunohistochemistry in skin biopsies from cutaneous lesions in dogs with at least moderate disease (LeishVet stage II) when compared with dogs with mild disease due to papular dermatitis (Esteve et al., 2015). When considering these facts, it would seem that the transcription of TLR2 is not compartmentalized between the dermal tissue and the blood in diseased dogs. TLR2 appears to be a marker of inflammation in dogs, as demonstrated in the present study and in other canine inflammatory diseases, such as inflammatory bowel disease, immunomediated or bacterial arthritis, canine sino-nasal aspergillosis, and lymphoplasmacytic rhinitis or pyometra (Chotimanukul and Sirivaidyapong, 2012; McMahon et al., 2010; Mercier et al., 2012; Riggio et al., 2014). Based on published information, TLR2 up regulation appears to be associated with moderate to severe disease, suggesting an active innate immune and proinflammatory responses due to the presence of a high *Leishmania* parasite load or cellular damage (endogenous damage-associated molecular patterns (DAMPs), also called alarmins) (Kang et al., 2015), as mentioned above. In a study from Brazil, a high parasite load was found along with increased frequency and expression of TLR2 in cells from the colon of sick dogs (Figueiredo et al., 2013). In addition, the up regulation of TLR-2 genes has been positively associated with parasite load in the skin of naturally infected dogs (Pereira-Fonseca et al., 2017). According to this, parasite load in papular dermatitis has been demonstrated to be lower than in more severe cutaneous CanL states (Ordeix et al., 2017). Moreover, dogs with papular dermatitis and stage I leishmaniosis show less microscopic skin lesions and parasite load in clinically healthy skin than dogs with more severe disease (Ordeix et al., 2017). Therefore, a lower up regulation of TLR2 in this profile of dogs may be associated with this lower *Leishmania* parasite load. In addition, dogs with papular dermatitis and stage I leishmaniosis described in this study presented a significantly lower blood parasitemia when compared with dogs with at least moderate disease.

The present results showed that TLR4 transcript in non-stimulated blood is unchanged among the groups studied. This finding is in agreement with previous studies performed on CanL (chapter 3.1.(Montserrat-Sangra et al., 2016)), in which TLR4 transcription was unchanged when compared with healthy dogs, and might indicate a mechanism of parasite immune system evasion or a less important role of this TLR in clinical CanL. This is in agreement with studies that were performed in cutaneous lesions due to *Leishmania braziliensis* in humans, which demonstrated that TLR2 is the most common TLR expressed during active disease mainly by macrophages while TLR4 is scarcely expressed (Tuon et al., 2012).

Although TLRs represent an important component of the innate immunity, there are many other inflammatory factors involved in this initial immune response, such as immunity cells, cell-associated molecules other than TLRs, and soluble molecules. Therefore, further studies regarding the analysis of these other factors is necessary in order to elucidate how innate immune responses prime the adaptive immune responses in *L. infantum* infection in dogs.

Conclusions

In conclusion, this study demonstrates that dogs with stage I and papular dermatitis present lower specific antibody levels and blood parasitemia and higher specific IFN-γ concentrations after blood stimulation than dogs with more severe disease. Moreover, TLR2 transcript in dogs with mild disease was similar to that of healthy control dogs. On the other hand, TLR2 transcript in the blood of dogs with at least stage II was significantly higher than in healthy control dogs. The results from this study show distinctive immune responses in dogs with CanL. Moreover, immunological characteristics that were observed in dogs with stage I and papular dermatitis, together with low bood parasitemia, are indicative of the control of *Leishmania* infection.

Acknowledgements

The authors thank all veterinarians and dog owners that contributed to this study. Specially, we are grateful to Dr. Marta Planellas (*Fundació Hospital Clínic Veterinari de la Universitat Autònoma de Barcelona*), Marta Blanchart (*Ars Veterinaria*), Montsant Osso (*Consultori Falset*), Annabel Dalmau (*Hospital Mediterrani*), Dani Mari and Paulina Quirola (master students from UAB) for their clinical assistance and for collecting samples and their support to this study. The authors thank also Dra. Anna Castelló and Dr. Lorena Alborch for their technical assistance to set up molecular work. The authors are also grateful to Laura di Filippo (UAB) for the assistance with laboratory work. This work was supported by Spanish ministry grants, *Ministerio de Economía y competitividad* and FEDER, EU (AGL2012-32498) and (AGL2015-68477). Dr. Laia Solano-Gallego held a Ramón y Cajal senior researcher contract awarded by the *Ministerio de Ciencia e Innovación* (Spain) and the European Social Fund.

Author contributions

Laia Solano Gallego and Laura Ordeix designed the research study. Laura Ordeix, Laia Solano-Gallego and Pamela Martínez-Orellana coordinated the veterinary clinics enrolled. Laura Ordeix and Laia Solano-Gallego examined clinically the majority of patients. Sara Montserrat-Sangrà set up molecular work. Sara Montserrat Sangrà performed all molecular work of this study. Pamela Martínez Orellana performed the majority of serological testing, whole cytokine release assay and canine IFN-γ ELISA. All authors contributed with data analysis and interpretation. Laura Ordeix and Sara Montserrat-Sangrà contributed equally in the first draft and revised the manuscript. Laia Solano Gallego revised thoroughly the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

3.3. ESTUDIO 3

TLR2, TLR4 and PD-L1 transcripts in canine whole blood after stimulation with *Leishmania infantum* in health and disease

Sara Montserrat-Sangrà[1], Pamela Martínez-Orellana, Laura Ordeix[1,2], Laia Solano-Gallego[1,*]

[1]Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain

[2]Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Bellaterra, Spain

Original research study

Abstract

The roles of toll-like receptors (TLRs) and programmed death-ligand 1 (PD-L1) in canine *Leishmania infantum* infection has not been extensively investigated. The aim of this study was to evaluate TLR2, TLR4 and PD-L1 transcripts in whole blood after stimulation with *L. infantum* antigen in healthy (n=10) and sick (n=23) dogs with leishmaniosis at the time of diagnosis and to correlate these transcripts with clinical, immunological and parasitological data. Blood from all dogs was stimulated with *L. infantum* soluble antigen (LSA) and Concanavalin A (CoA), and compared with un-stimulated medium as control. Clinical status was established by a thorough physical examination, complete blood count, biochemical profile, serum electrophoresis, and urinary protein/creatinine ratio. Sick dogs were further classified as IFN-γ producers (n=13) and IFN-γ non-producers (n=10). RNA extraction and cDNA production were performed on all blood samples in order to measure TLR2, TLR4 and PD-L1 gene expression as well as the expression of three reference genes (HPRT-1, CG14980 and SDHA) by real time PCR. In sick IFN-γ producer dogs, a downregulation of TLR4 (P=0.009) and TLR2 (P=0.087) transcripts after blood stimulation with LSA was found when compared with medium. A significant upregulation of PD-L1 transcripts after blood stimulation with LSA (healthy P=0.036; sick dogs IFN-γ producers P=0.023 and IFN-γ non-producer P=0.017) and CoA (healthy P=0.028; sick IFN-γ producers P=0.028 and IFN-γ non-producer P=0.005) was noted in all groups studied. TLR2 transcript after blood stimulation with LSA was significantly lower (mean 0.662) in sick IFN-γ producers when compared with healthy dogs (mean 1.719, P = 0.042). In contrast, no differences were found between the three groups studied for TLR4 and PD-L1 transcripts after blood stimulation with LSA or CoA. Significant correlations were noted between TLR2, TLR4 and PD-L1 gene expression after blood stimulation and clinical, serological and parasitological parameters. In conclusion, this study demonstrated for the first time that there was a downregulation of TLR2 and TLR4 transcripts in LSA stimulated blood from sick IFN-γ producer dogs compared with healthy controls while no differences in these transcript productions were

observed in IFN-γ non-producer dogs. In contrast, a pronounced upregulation of PD-L1 transcript after LSA and CoA stimulation was noted in all groups studied. The results of the present study provide with further insights into the role of these receptors and ligand in clinical canine leishmaniosis.

Abbreviations

ALT, Alanine aminotransferase; β-2M, Beta-2-microglobulin; CanL, canine leishmaniosis; CoA, concanavalin A; CBC, Complete blood count; cDNA, Complementary Deoxyribonucleic acid; CD11b, Cluster of differentiation 11b; CG-14980, LOC647357 similar to CG14980-PB; CP, Crossing point; ddCt-method, Delta delta C(T) Method; EDTA, Ethylenediaminetetraacetic acid; HMBS, Hydroxymethylbilane synthase; HPRT-1, Hypoxanthine phosphoribosyltransferase 1; IFN-γ, Interferon gamma; iNOS, inductible nitric oxide synthase; M value, Average expression stability; NO, nitric oxide; PD 1, Programmed death 1 (receptor); PD-L1; Programmed death ligand 1, PAMPs, Pathogen-associated molecular patterns; PBMC, Peripheral blood mononuclear cell; PCR, Polymerase chain reaction; qPCR, Quantitative PCR; RG, Reference gene; RGI, Reference gene index; RIN, RNA integrity number; RNA, Ribonucleic acid; PRRs, Pattern recognition receptors; qPCR, Quantitative PCR; Rq, Relative quantification; rt- PCR, Real time PCR; ROS, Reactive oxygen species; SDHA, Succinate dehydrogenase complex, subunit A; flavoprotein (Fp); TBP, TATA box binding protein; TG, Target gene; TLRs, Toll-like receptors; UPC, Urinary protein/creatinine ratio; VL, Visceral leishmaniosis; Vn/n + 1 value, Pairwise variation analysis to determine optimal number of reference genes for normalization in RT-qPCR reaction.

Keywords

Toll-like receptors (TLRs), Programmed dead ligand 1 (PD-L1), *Leishmania infantum*, gene expression, blood, dog

Introduction

Canine leishmaniosis (CanL) is a zoonotic infectious disease caused by the protozoan *Leishmania infantum* and transmitted by the bites of female phlebotomine sand-flies. This neglected disease concerns primarily countries from Asia, South Africa, Latin America and the Mediterranean basin in Europe (Desjeux, 2004).

The outcome of CanL is variable. Infected dogs can develop clinical self-limiting illness or severe disease while others remain as chronic and subclinical carriers (Baneth et al., 2008; Hosein et al., 2017). Due to the wide spectrum of disease manifestations and severity, dogs are classified in several clinical stages of leishmaniosis. Four clinical stages have been established ranging from stage I-mild disease to stage IV-very severe disease (Solano-Gallego et al., 2017b; Solano-Gallego et al., 2009). The clinical diagnosis of CanL is complex, due to the existence of subclinical infections and a wide spectrum of disease manifestations. For these reasons, several diagnostic techniques are available in the clinical setting such as cytology/histology + immunohistochemistry, serology and molecular techniques (Solano-Gallego et al., 2017b).

Susceptibility or resistance to *L. infantum* infection in canines appears to be due to the polarization of T helper 1 (Th1) and Th2-like immune responses. Classically, protection from canine *L. infantum* infection is associated with activation of Th1 cells producing interferon gamma (IFN-γ), interleukin 2 (IL-2) and tumor necrosis factor alpha (TNF-α) which induce the expression of Inducible nitric oxide synthase (iNOS) driving L-arginine metabolism toward nitric oxide (NO) production (Holzmuller et al., 2006a; Pinelli et al., 1995; Wanasen and Soong, 2008) or reactive oxygen substances (ROS) (Roma et al., 2016) and thus capable of controlling infection. However, the role of T helper (Th) type cytokines in CanL has not been as well defined as in rodent models (Grinnage-Pulley et al., 2017; Kauffmann et al., 2018). There is evidence that *Leishmania* infection induces a Th1/Th2 mixed response in dogs (Hosein et al., 2017). The production of parasite specific IFN-γ in healthy but infected dogs after blood stimulation is well known (Barbieri, 2006; Martinez-Orellana et al., 2017b) but it is encountered also in sick dogs with mild to moderate

disease (Chapter 3.2. (Montserrat-Sangra et al., 2018)) and in dogs with clinical improvement after treatment (Martinez-Orellana et al., 2017a). A predominant Th2-like response has been associated with disease progression and severity of illness. Sick dogs with moderate to very severe disease presented a decrease or absence of IFN-γ production (Solano-Gallego et al., 2016b) in blood and an increase of anti-Leishmania antibody levels and parasite load, which are detrimental for the animal (Rodriguez-Cortes et al., 2017; Solano-Gallego et al., 2016b). Production of interleukin 10 (IL-10) has been documented in peripheral blood mononuclear cells (PBMCs) after *Leishmania* antigen stimulation from sick dogs (Boggiatto et al., 2010) in agreement with human cases (Rogers and Titus, 2004). However, IL-10 does not appear to be a marker of disease and degree of severity based on recent studies performed in *ex vivo* whole blood assays after *Leishmania* antigen stimulation in canines (Rodriguez-Cortes et al., 2017; Solano-Gallego et al., 2016b).

The cellular basis and mechanisms for the development of T-cell unresponsiveness and dysfunction in CanL are not fully understood. Programmed death-1 (PD1) and its ligand (PD-L1) are accessory molecules expressed on T cells and antigen-presenting cells (APCs), respectively (Bardhan et al., 2016). Their ligation triggers inhibitory signals that diminish T cell proliferation and cytokine production such as IL-2, IFN-γ, and TNF-α from T lymphocytes in chronic infections and parasitic diseases (Bardhan et al., 2016; Rodrigues et al., 2014). PD-1 mediated T-cell exhaustion has been demonstrated in several models of murine visceral leishmaniosis (Habib et al., 2018) as well as in human visceral leishmaniosis (Gautam et al., 2014). Furthermore, the loss of *L. infantum*-specific $CD4^+$ T cell proliferation in sick dogs was correlated with increased surface expression of PD-1 and impairment of phagocyte function (Chiku et al., 2016; Esch et al., 2013; Schaut et al., 2016). In contrast, very limited information is available regarding PD-L1 ligand expression in *Leishmania* infection (Barroso et al., 2018; Chiku et al., 2016) but blockage of PDL-1 ligands appears to restore T-cell function in murine models (Habib et al., 2018) and dogs (Chiku et al., 2016; Schaut et al., 2016) which resulted in a significant decrease in the parasite burden. It is important to highlight that parasite specific T-cell dysfunction such as

reduced or absent IFN-γ production in the blood of sick dogs varies depending on disease severity and clinical staging (Solano-Gallego et al., 2016b). However, to the best knowledge of the authors, studies evaluating markers of cell exhaustion in CanL have not been able to distinguish between sick IFN-γ producer *versus* non-producer dogs.

Toll-like receptors are non-clonal sets of pathogen recognition receptors (PRRs) which are usually the first receptors of the immune cells that have to fight off an infection (Werling and Jungi, 2003). The innate immune response starts recognizing the pathogen by immune receptors and is afterwards involved in the opsonization of foreign substances (Dunkelberger and Song, 2010). The location of these receptors is on the immune surface of antigen presenting cells such as macrophages and dendritic cells (DCs) and they can skew the differentiation of activated T cells to different effector T cell subsets. Thus, TLRs play an important role in the early immune response catalyzing which signal transduction has to be produced to confront the infection (Chauhan et al., 2017). The transcription of some TLRs in dogs with CanL has been studied in several tissues including spleen, brain and PBMCs cells (Galdino et al., 2016; Grano et al., 2018), skin (Hosein et al., 2015); (Pereira-Fonseca et al., 2017) and canine un-stimulated whole blood (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018). However, to the best knowledge of the authors, there are no studies in dogs evaluating TLR transcription in whole blood after stimulation with *Leishmania* antigen.

Therefore, the aim of the present study was to evaluate the gene expression of TLR2, TLR4 and PD-L1 in whole blood after stimulation with *L. infantum* antigen in healthy non-infected and sick dogs with leishmaniosis (IFN-γ producers *versus* non-producers) at the time of diagnosis. In addition, another goal was to correlate these transcripts with clinical, immunological and parasitological data.

Material and methods

Dogs with clinical leishmaniosis

Twenty-three dogs with clinical leishmaniosis attending different veterinary facilities in Catalonia (Spain) [Fundació *Hospital Clínic Veterinari Universitat Autònoma de Barcelona* (Cerdanyola, Barcelona), *Hospital Ars Veterinària* (Barcelona), *Hospital Mediterrani Veterinaris* (Reus, Tarragona) and *Consultori Montsant* (Falset, Tarragona)] were prospectively enrolled between January 2014 and August 2015. The diagnosis was performed by detection of specific *L. infantum* antibody levels by an endpoint in-house enzyme linked immunosorbent assay (ELISA) as previously described (Solano-Gallego et al., 2016a) and, in some cases confirmed, by cytology of lesions, cutaneous histology and/or immunohistochemistry for *Leishmania* (Esteve et al., 2015). Blood *Leishmania* real time polymerase chain reaction (RT-PCR) (Solano-Gallego et al., 2016a) was also performed. Furthermore, routine laboratory tests were also carried out for all dogs to appraise their clinical status. Hence, a complete blood count (CBC) with blood smear examination, serum biochemical profile that included creatinine, urea, total protein, protein serum electrophoresis, and urinalysis with urinary protein creatinine ratio (UPC) were performed. The hematology, biochemistry and serum electrophoresis were done using the Siemens ADVIA120 hematology analyzer, Olympus AU400 chemistry analyzer and the Hydrasis serum electrophoresis system. Both sexes were represented with nine females and fourteen males. The median age was 4.2 years with a range from five months to eight years. Nineteen purebred dogs belonging to fourteen breeds (Dobermann, Beagle, Irish setter, Akita inu, Greyhound, Dachshund, Golden retriever, German shepherd, Boxer, Schnauzer, American Staffordshire terrier, Gascon Saintongeois, Ibizan hound) and four-mixed breed dogs were included. Clinical staging was evaluated as previously reported (Solano-Gallego et al., 2009) in sick dogs. Furthermore, sick dogs were classified based on parasite specific IFN-γ production as IFN-γ producers and non-

producers after blood stimulation with *L. infantum* soluble antigen (Solano-Gallego et al., 2016b). All dogs chosen belonged to stage II with the exception of two dogs that were classified in stage III due to proteinuria with a urinary protein creatinine ratio with a mean value of 1.8. Dogs were classified prior to the experiment in order to have a homogeneous sampling. All dogs enrolled in the study remained under the care of their owners and were not housed for the purposes of this study or held for any period. Additionally, an informed consent was signed for each case.

Control dogs

Ten healthy dogs were included in the study as a control group. Both sexes were represented with seven females and three males. The median age was 4.2 years, with a range from twelve months to 10 years. Six pure breed dogs belonging to about four different breeds (Ibizan hound, Labrador retriever, Bruno jura hound, Ariegeois pointer) and four mixed breed dogs were included in the control group. All dogs were ELISA seronegative and blood RT-PCR negative for *L. infantum*.

IFN-γ release whole blood cultured assay

Five hundred µL heparinized whole blood were cultured as previously described (Solano-Gallego et al., 2016b) with some modifications. Briefly, three different conditions were established: (1) medium alone (Ø); (2) medium with soluble *L. infantum* antigen (LSA) at a concentration of 10 µg/mL provided by Dr. Cristina Riera (*L. infantum* antigen 5 mg/mL, Facultat de Farmàcia, Universitat de Barcelona); (3) medium with mitogen Concanavalin A (Con A, 100 mg Medicago® Uppsala, Sweden) at a concentration of 10 µg/mL.

The plates were incubated at 37 °C in 5% CO_2. Samples were collected at 48 hours and 5 days and centrifuged at 300 rpm for 10 min. The supernatant was collected and stored at -80ºC to perform sandwich ELISA for canine IFN-γ (DuoSet ® ELISA by Development System R & D™, Abingdon, UK). Then, the pellet was resuspended in 1.25 mL of RNAlater (Waltham, Massachussetts, US) and stored at −80 °C until used. The pellet containing blood cells was used to carry out gene expression. The two collected tubes (48 hrs and 5 days) were mixed and RNA extracted together in order to obtain an optimal amount of RNA to perform the PCR technique.

RNA extraction and RNA quality

Blood in RNAlater was extracted using the Ribopure™ RNA blood kit (Ambion, Carlsbad, California, US) according to the manufacturer's instructions with slight modifications. In the lysis step, an additional 50% of the total volume per reagent was added to 1000 µl of heparinized blood pellet in order to achieve a greater lysis cell disruption. It was incubated for 20 minutes to separate RNA and DNA phases. RNA concentration was determined by a Nanodrop device (Thermo Fisher Scientific Inc, Carlsbad, California, US). RNA quality was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, US). A final concentration of 20–70 ng RNA/ µL per sample was obtained. All samples included in this study had a RNA Integrity Number (RIN) value greater than 7.

cDNA synthesis and PCR real time

VILO Masterscript Retrotranscriptase kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, US) was used to generate cDNA according to the manufacturer's instructions. The reaction mixture was incubated at 25ºC for 10 minutes followed by 60

minutes at 42ºC and the reaction was stopped after 5 minutes at 85ºC. The cDNA was aliquoted and stored at −20ºC until use.

qPCR gene expression

To analyze the expression of TLR2, TLR4 and PD-L1 (target genes) in stimulated blood, suitable reference genes (Succinate dehydrogenase complex; subunit A; Flavoprotein (SDHA), Hypoxanthine phos-phoribosyltransferase 1 (Lesch-Nyhan syndrome) (HPRT-1), TATA Box binding protein (TBP), similar to CG14980-PB (CG14980) were used as previously described (chapter 3.1. (Montserrat-Sangra et al., 2016)). The primers used in these assays are listed in table 3.3.1.

Table3.3.1. Summary of canine reference and target genes used in this study.

Assay ID[a]	Gene symbol	Gene name	Genebank mRNA	Genebanck Reference Sequence	Amplicon Pairwise
Cf02625049_s1	TLR2	Toll-like receptor 2	AB080363.1	NM_001005264.2	69
Cf02622203 g1	TLR4	Toll-like receptor 4	AB080363.1	NM 001002950.1	120
APG2FND	PD-L1	Programmed dead ligand 1	NM_001291972.1	NM_001291972.1	164
Cf02626256_m1	HPRT1	Hypoxanthine phosphoribosyltransferase 1	AY283372.1	NM_001003357.1	102
Cf02664981_m1	SDHA	succinate dehydrogenase complex; subunit A; flavoprotein	XM_535807.2	DQ402985.1	64
Cf02643820_m1	LOC479750	similar to CG14980-PB	XM_536878.2	-	78

[a]All the assays are commercially available from Thermo Fisher scientific.

Amplification was performed in the QuantStudio™ 12K Flex System Real-Time PCR (Thermo Fisher Scientific, Carlsbad, California, US) using TaqMan® Universal Master Mix II with UNG (Applied Biosystems, Foster City, California, US). Plates (96 wells/plate) were filled with 0.35 µL nuclease free water (Sigma, San Luis, Missouri, US), 7.50 µL TaqMan Universal master mix (2×), 0.75 µL TaqMan assay 20, 6.4 µL 1/5 cDNA. Plates were closed with an optical film (Applied Biosystems) centrifuged in order to mix the samples and were placed into a laboratory pipetting robot (Epmotion 5057 Liquid-handlingrobot, Eppendorf, Hamburg, Germany) to generate a 384 wells/plate. Then, the generated 384

wells plates were transferred into a real time PCR device. The PCR components and the PCR cycler conditions were identical for the all transcripts. Denaturation program (95ºC, 10 min), amplification and quantification program repeated 40 times (95ºC for 15 s, 60ºC for 10 s, 72ºC for 60 s) with a single fluorescence measurement. The baseline and threshold was automatically defined for the program in each run. Each sample was performed in triplicate for all the target and reference genes and a calibrator sample was employed as control in each plate.

Statistical analysis

The Mann-Whitney U test was used for evaluation of unpaired continuous variables among different dog groups. The Wilcoxon Signed-Rank test was used for paired continuous variables. Spearman's correlation was performed in order to observe relationships between gene transcription and clinical, serological and parasitological findings. The SPSS 17.0 statistics software (Chicago, Illinois, US) was employed for all statistical analysis. Significant differences were considered when P value was <0.05.

Results

Immunological, parasitological, biochemical and hematological findings

The most relevant parameters of the immunological and hematobiochemical findings from sick dogs are listed in table 3.3.2**.**

Table3.3.2. Immunological, parasitological and hematobiochemical findings in IFN-γ producers and IFN-γ non-producers.

Parameters	IFN-γ producers (mean ± SD)	IFN- γ non-producers (mean ± SD)	P value
Creatinine (mg/dL)	0.9 ± 0.2	0.8 ± 0.2	0.127
Urea (mg/dl)	32.3 ± 16.0	30.0 ± 8.7	0.975
Urinary protein creatinine ratio	0.4 ± 0.6	0.7 ± 0.6	0.089
Total protein (g/dL)	6.9 ± 1.3	10.2 ± 1.0	<0.0001
Albumin (g/dl)	2.9 ± 0.5	2.2 ± 0.5	0.001
Beta globulin (g/dL)	1.4 ± 0.9	2.0 ± 0.3	0.088
Gamma globulin (g/dL)	1.1 ± 1.0	4.5 ± 1.6	<0.0001
Ratio albumin/globulin	1.0 ± 0.4	0.3 ± 0.1	0.001
Hematocrit (%)	42.7 ± 8.7	28.2 ± 7.0	0.651
Hemoglobin (g/dL)	14.6 ± 2.4	9.4 ± 2.7	0.588
Leucocytes (cell/µL)	8790.7 ± 3060.0	9101.0 ± 3983.5	0.794
Lymphocytes (cell/µL)	2047.6 ± 1053.9	1943.7 ± 1353.6	0.794
Neutrophils (cell/µL)	5606.0 ± 1931.7	6267.2 ± 2932.2	0.556
Platelets (10^3 / µL)	255.1 ± 87.0	211.3 ± 83.0	0.184
LSA IFN- γ (pg/mL)	3256.6 ± 5022.6	8.1 ± 20.1	<0.0001
CoA IFN- γ (pg/mL)	6542.2 ± 5865.4	6376.8 ± 5141.4	0.605
***L. infantum* specific antibodies (ELISA units)**	666.5 ± 803.3[e]	23990.4 ± 26124.4[e]	<0.0001

Parasite load (parasite/mL)	4.1 ± 7.0	93.0 ± 251.7	0.128

LSA: *L. infantum* soluble antigen, CoA: concanavalin A

Differences among TLRs and PD-L1 transcripts in blood stimulated with medium as control, LSA and CoA

The TLRs and PD-L1 transcript results of stimulated blood from healthy control dogs and IFN-γ producer and IFN-γ non-producer sick dogs are summarized in table 3.3.3.

Table 3.3.3. Table 0.1. Relative quantification of TLR2, TLR4 and PD-L1 transcripts blood stimulation with medium alone, LSA or CoA from different dog groups.

Group of dogs (number of dogs)	**TLR2 (mean ± SD)**			**TLR4 (mean ± SD)**			**PD-L1 (mean ± SD)**		
	Medium	**LSA**	**Con A**	**Medium**	**LSA**	**Con A**	**Medium**	**LSA**	**Con A**
Healthy controls (n=10)	1.1 ±1.4	0.7 ±0.3	0.3 ±0.3	2.5 ±1.6	1.8 ±1	0.6 ±0.5	74.1 ±54.8	136 ±86.2	192.7 ±164.5
IFN-γ producer sick dogs (n=13)	0.7 ±0.4	0.5 ±1.0	0.4 ±0.5	4 ±4.1	1 ±1.1	0.4 ±0.6	135.1 ±231.8	290 ±283.3	248.2 ±268.9
IFN-γ non-producer sick dogs (n=10)	1 ±0.8	0.7 ±0.7	0.5 ±0.8	3 ±2	3.1 ±1.5	1.1 ±1.3	65.3 ±39.2	116.3 ±92.3	299.4 ±131.6

LSA: *L. infantum* soluble antigen, CoA: concanavalin A

Results of TLR2:

Controls: medium > Con A (Wilcoxon signed rank test: $P = 0.038$); LSA > Con A (Wilcoxon signed rank test: $P = 0.028$)

IFN-γ producers: medium > LSA (Wilcoxon signed rank test: *P* = 0.087), medium > Con A (Wilcoxon signed rank test: *P* = 0.013)

IFN-γ non-producers: medium > Con A (Wilcoxon signed rank test: *P* = 0.007)

Results of TLR4:

Controls: medium > Con A (Wilcoxon signed rank test: *P* = 0.028); LSA > Con A (Wilcoxon signed rank test: *P* = 0.017)

IFN-γ producers: medium > LSA (Wilcoxon signed rank test: *P* = 0.009), medium > Con A (Wilcoxon signed rank test: *P* = 0.084)

IFN-γ non-producers: medium > Con A (Wilcoxon signed rank test: *P* = 0.005)

Results of PD-L1:

Controls: medium < LSA (Wilcoxon signed rank test: *P* = 0.036), medium < Con A (Wilcoxon signed rank test: *P* = 0.028)

IFN-γ producers: medium < LSA (Wilcoxon signed rank test: *P* =0.023), medium < Con A (Wilcoxon signed rank test: *P* = 0.028)

IFN-γ non-producers: medium < Con A (Wilcoxon signed rank test: *P* = 0.005), LSA <Con A (Wilcoxon signed rank test: *P* =0.007), medium < LSA (Wilcoxon signed rank test: *P* = 0.017)

TLR4 expression in LSA stimulated blood from sick IFN-γ producer dogs (mean= 1.304) was lower than medium as control (mean= 4.809, Wilcoxon signed rank test: *P* < 0.009). TLR2 expression in LSA stimulated blood from IFN-γ producer dogs (mean= 0.518) had a non-significant tendency to be lower when compared with medium (mean= 0.735, Wilcoxon signed rank test: P < 0.087). No differences were found in TLRs transcripts in healthy control and sick IFN-γ non-producer dogs when blood was stimulated with LSA.

TLR2 and TLR4 gene expression was statistically significant downregulated after Con A stimulation when compared with medium alone in all group studied (table 3.3.3.).

A significant upregulation of PD-L1 transcripts after blood stimulation with LSA (Wilcoxon signed rank test: healthy P=0.036, IFN-γ producers P=0.023 and IFN-γ non-producer P=0.017) and CoA (Wilcoxon signed rank test: healthy P=0.028, IFN-γ producers P=0.028 and IFN-γ non-producer P=0.005) was noted in all groups studied when compared with medium alone.

Differences between TLRs and PD-L1 transcripts when compared dog groups

The results are shown in table 4. Relative quantification of TLR2 in LSA stimulated blood was significantly higher (mean= 1.79, Mann–Whitney U test: Z = -2,047, P < 0.0042) in control dogs when compared with IFN-γ producer sick dogs (mean= 0.662) while relative quantification of TLR4 LSA stimulated blood was not significantly different between control and IFN-γ producer sick dogs (Mann–Whitney U test: Z = -1,488, P = 0.148). Nonetheless, no differences between the three groups studied were found for PD-L1 transcripts after blood stimulation with LSA or CoA.

Table 3.3.4. Relative quantification of TLR2, TLR4 and PD-L1 transcripts from different groups of dogs after blood stimulation (values are expressed as ratio LSA or CoA stimulus/medium as control).

	TLR2 (mean ± SD)		**TLR4 (mean ± SD)**		**PD-L1 (mean ± SD)**	
Group of dogs (number of dogs)	**Ratio LSA/medium**	**Ratio Con A/ medium**	**Ratio LSA / medium**	**Ratio Con A / medium**	**Ratio LSA / medium**	**Ratio Con A / medium**
Healthy controls (n=10)	1.7 ±2.8 [a]	1.1 ±2.3	1.3 ±1.8	0.6 ±1.0	2.7 ±2.6	3.7 ±3.8
IFN-γ producer sick dogs (n=13)	0.6 ±0.9 [a]	0.4 ±0.4	0.5 ±0.6	0.4 ±0.5	3.8 ±2.7	4.3 ±3.7
IFN-γ non producer sick dogs (n=10)	0.9 ±0.6	0.4 ± 0.4	1.0 ±1.1	0.3 ±0.2	2.0 ±1.1	6.9 ±7.2

LSA: *L. infantum* soluble antigen, Con A: concanavalin A

[a]Controls LSA TLR2 > IFN-γ producers LSA TLR2 (Mann-Whitney U-test: $P = 0.042$)

Correlations with TLRs and PD-L1 transcripts and clinical, serological and parasitological data

The results of Spearman's correlation between TLRs transcripts and clinical, serological and parasitological parameters are listed in supplementary table S-3.3.1.

Significant correlations were noted between stimulated blood gene expression and clinical, serological and parasitological parameters in all dogs. Interestingly, TLR2 expression from LSA stimulated blood was positively correlated with total protein (P <

0.001), gamma globulin (P < 0.007) and *L. infantum* specific antibody levels (P < 0.011) whereas it was negatively correlated with hematocrit (P< 0.028), hemoglobin (P < 0.010) and LSA IFN-γ (P < 0.001). TLR4 expression from LSA stimulated blood was only negatively correlated with the parasite load (P < 0.007) and LSA IFN-γ (P < 0.007). PD-L1 gene expression from LSA stimulated blood was correlated positively with PD-L1 gene expression CoA stimulated blood (P < 0.001) and LSA IFN-γ (P < 0.027) and negatively with total protein (P < 0.042), gamma globulin (P < 0.044) and *L. infantum* specific antibodies (P < 0.032).

Discussion

The present study evaluated TLR2, TLR4 and PD-L1 transcripts in healthy controls and in dogs with clinical leishmaniosis. Sick dogs were further classified as IFN-γ producers and IFN-γ non-producers. Sick IFN-γ producer dogs presented lower *L. infantum* specific antibodies, parasitemia as well as less clinicopathological abnormalities when compared with sick IFN-γ non-producers as previously documented (Montserrat-Sangra et al., 2018; Solano-Gallego et al., 2016b). Therefore, generally speaking, sick IFN-γ producer dogs were in a less severe disease status than IFN-γ non-producers (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018).

To our best knowledge, this study shows, for the first time, a downregulation of TLR2 transcripts in blood from sick IFN-γ producer dogs naturally infected with *L. infantum* after LSA stimulation when compared with medium alone. In addition, a decrease in TLR2 expression after LSA blood stimulation was only found in sick IFN-γ producers when compared to controls and sick IFN-γ non-producers. Moreover, TLR2 transcripts were negatively correlated with LSA IFN-γ. A previous study performed in non-stimulated blood demonstrated higher TLR2 expression in sick dogs classified at least in stage II when compared to healthy controls (Montserrat-Sangra et al., 2016). Furthermore, TLR2 transcription was reduced with clinical improvement during treatment (Montserrat-

Sangra et al., 2016). It is important to remark that dogs with stage I-papular dermatitis (mild disease) presented a TLR2 transcription in the non-stimulated blood that was similar to healthy control dogs (Montserrat-Sangra et al., 2018). It is also important to highlight that high expression of TLR2 has also been documented in other tissues such as skin (Esteve et al., 2015; Pereira-Fonseca et al., 2017) and colon (Figueiredo et al., 2013) in dogs with moderate to severe disease. Therefore, TLR2 expression appears to be associated with moderate to very severe disease suggesting immune and proinflammatory responses due to the presence of a high *Leishmania* parasite load or cellular damage (endogenous damage-associated molecular patterns (DAMPs) also termed alarmins) as observed in other infection diseases in humans (Kang et al., 2015).

There is very limited knowledge related to clinical, serological and parasitological parameters and TLRs transcription in this disease. In this study, significant correlations were found between *L. infantum* stimulated blood gene expression and clinical, serological and parasitological parameters. Interestingly, TLR2 expression after LSA blood stimulation was positively correlated with total protein, gamma globulin and *L. infantum* specific antibody levels whereas it was negatively correlated with hematocrit and hemoglobin. These findings are similar and in agreement with the results obtained in a previous study where TLR2 transcripts were measured in non-stimulated blood in sick dogs infected with *L. infantum* (Chapter 3.1.(Montserrat-Sangra et al., 2016)). In this previous study, a significant positive correlation was noted between TLR2 transcripts and UPC, total protein, beta and gamma globulins, specific *L. infantum* antibodies and blood parasite load while a negative correlation was observed with albumin, albumin/globulin ratio, hematocrit and hemoglobin (Montserrat-Sangra et al., 2016). Therefore, upregulation of TLR2 appears to be positively associated with clinicopathological findings indicative of disease severity (Chapter 3.1. and 3.2. (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018)).

TLR4 expression in several tissues has been more rarely investigated in CanL. This study show, for the first time, a significant downregulation of TLR4 transcripts in LSA stimulated blood from sick IFN-γ producer dogs naturally infected with *L. infantum* when compared with medium alone. Furthermore, TLR4 transcripts from LSA stimulated blood were negatively correlated with parasite load and LSA IFN-γ concentration. However, no significant differences in TLR4 transcripts were observed in sick IFN-γ non-producer dogs in agreement with other studies (Chapter 3.1. and 3.2. (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018)). Other studies demonstrated that TLR4 transcript in non-stimulated blood was unchanged among healthy and dogs with clinical leishmaniosis (Chapter 3.1. and 3.2. (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018)) even with different clinical stages. In human patients with visceral leishmaniosis, TLR4 transcripts were upregulated in *Leishmania*-specific stimulated blood (Babiker et al., 2015). Some other authors have also found no changes in TLR4 transcripts in the brain and spleen while TLR4 was upregulated in lymph nodes in dogs with natural leishmaniosis (Melo et al., 2014a). Another study, in an experimental canine model, showed a significant downregulation of TLR4 transcription with disease progression in lymph node and in spleen samples and no change in TLR4 transcripts in skin and liver samples (Hosein et al., 2015). Other studies mainly of inflammatory responses associated with bacterial infection showed an increase in TLR4 expression (Burgener et al., 2008; Chotimanukul and Sirivaidyapong, 2011; Mercier et al., 2012).

It is interesting to highlight that in the present study, a significant reduction of both TLR2 and TLR4 was observed after LSA blood stimulation in sick dogs with a predominant IFN-γ production. In addition, a negative correlation was noted between TLR2 and TLR4 and LSA IFN-γ concentration while a negative correlation was also seen with parasite load but only with TLR4. Furthermore, similar results were observed with a decrease of TLR2 and TLR4 transcripts after CoA blood stimulation with a potent production of IFN-γ by all groups of dogs studied. These findings suggest a reduction of TLRs expression in the presence of IFN-γ. The cross-talk between cytokines and TLRs is well known (Chandel et al., 2014; Chauhan et al., 2017) as well as between TLRs with

themselves. In addition, the redundant and regulatory roles of cytokines and TLRs are also well known (Chauhan et al., 2017). Therefore, it is likely that a negative feedback is produced when there is an activation of effector T cells in conjunction with the production of high levels of IFN-γ as observed in the present study. In agreement with our results, silencing TLR2 decreases intracellular parasite load in *Leishmania* infected macrophages (Majumdar et al., 2014). In addition, macrophages derived from peripheral blood from *Leishmania* infected dogs showed a decrease in TLR2 transcripts compared with healthy non-infected dogs (Melo et al., 2014b). Furthermore, the present findings are in agreement with the results from studies in non-stimulated blood from humans with visceral leishmaniosis where a high expression of TLR2 and TLR4 was associated with decreased production of IFN-γ, IL-17 and nitric oxide (Gatto et al., 2015).

In the present study, we obtained similar results in all groups studied regarding PD-L1 expression. Blood stimulated with LSA and CoA raised PD-L1 gene expression in all groups when compared with non-stimulated blood samples. We expected to observe a difference in PD-L1 transcripts between IFN-γ producers and non-producers as a marker of cell exhaustion (Esch et al., 2013; Rodrigues et al., 2014) but this was not the case in the present study. Furthermore, PD-L1 gene expression from LSA stimulated blood was correlated positively with PD-L1 gene expression CoA stimulated blood and LSA IFN-γ and negatively with total protein, gamma globulin, and *L. infantum* specific antibodies. Therefore, our findings are in disagreement with previous literature (Barroso et al., 2018; Habib et al., 2018). It is likely that the long *ex-vivo* canine blood culture massively activated apoptotic markers such as PD-L1 after LSA and CoA blood stimulation in the dog samples studied. For this reason, due to the limited number of dogs studied, differences were not observed. Further studies need to be done with other markers, different assays and cell populations to discriminate between sick dogs with milder disease, IFN-γ producers, and dogs with more severe disease and absence of IFN-γ production.

Conclusions

This study demonstrated, for the first time, a downregulation of TLR2 and TLR4 transcripts in LSA stimulated blood from sick IFN-γ producer dogs compared with healthy controls, while no differences were noted in IFN-γ non-producer dogs. In contrast, a pronounced upregulation of PD-L1 transcript after LSA and CoA stimulation was noted in all groups studied. Altogether, the results of the present study provide further insights into the role of these receptors and ligand in clinical canine leishmaniosis.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by a Spanish Ministry grant, Ministerio de Economía y competitividad (AGL2012-32498 and AGL2015-68477). Dr. Laia Solano-Gallego held a Ramón y Cajal senior researcher contract awarded by the Ministerio de Ciencia e Innovación (Spain) and the European Social Fund.

Acknowledgements

The authors thank all veterinarians and dog owners that contributed to this study. Specially, we are grateful to Marta Planellas (Fundació Hospital Clínic Veterinari de la Universitat Autònoma de Barcelona), Marta Blanchart (Ars Veterinaria), Montsant Osso (Consultori Falset), Dani Mari and Paulina Quirola (master students from UAB) for their clinical assistance and for collecting samples and their support to this study.

Supplementary table S-3.3.5. Spearman's correlation in all dogs TLR transcripts and serological, clinicopathological and parasitological parameters.

PARAMETERS	Ratio LSA TLR2		Ratio Con A TLR2		Ratio LSA TLR4		Ratio Con A TLR4		Ratio LSA PD-L1		Ratio Con A PD-L1	
	Correlation Coefficient	P-value	Correlation Coefficient	P-value	Correlation Coefficient	P-value	Correlation Coefficient	P-value	Correlation Coefficient	P-value	Correlation Coefficient	P-value
Ratio LSA TLR2	-	-	-0.017	0.917	0.013	0.944	0.096	0.608	-0.097	0.592	0.218	0.223
Ratio Con A TLR2	-0.017	0.927	-	-	0.250	0.168	-0.018	0.923	0.037	0.841	0.114	0.533
Ratio LSA TLR4	0.013	0.944	0.250	0.168	-	-	0.066	0.725	0.036	0.843	0.127	0.480
Ratio Con A TLR4	0.096	0.608	-0.018	0.9323	0.066	0.725	-	-	0.465	0.008	0.351	0.053
Ratio LSA PD-L1	-0.097	0.592	0.037	0.841	0.036	0.843	0.465	0.008	-	-	0.538	0.001
Ratio Con A PD-L1	0.218	0.223	0.114	0.533	0.127	0.480	0.351	0.053	0.528	0.001	-	-
UPC	0.152	0.674	-0.311	0.382	-0.128	0.724	0.616	0.058	-0.049	0.894	0.043	0.907
Creatinine (mg/dL)	0.192	0.418	0.309	0.197	0.051	0.830	0.096	0.695	-0.046	0.847	-0.101	0.671
Urea (mg/dL)	-0.153	0.507	-0.411	0.072	0.094	0.687	0.302	0.195	0.310	0.171	0.390	0.081
Total protein (g/dL)	0.623	0.001	0.373	0.080	0.082	0.704	-0.093	0.682	-0.418	0.042	-0.011	0.958
Albumin (g/dl)	0.039	0.870	0.087	0.724	-0.235	0.319	-0.165	0.500	0.257	0.274	-0.272	0.274
Beta globulin (g/dL)	0.128	0.591	0.418	0.075	0.337	0.146	0.004	0.989	-0.432	0.057	0.134	0.574

Gamma globulin (g/dL)	0.581	0.007	0.097	0.692	-0.084	0.724	0.045	0.856	-0.455	0.044	-0.074	0.757
Ratio albumin/globulin	-0.332	0.166	-0.183	0.438	-0.372	0.117	0.061	0.810	0.426	0.069	-0.188	0.442
Hematocrit (%)	-0.491	0.028	0.006	0.980	0.044	0.855	-0.410	0.091	-0.026	0.912	0.084	0.724
Hemoglobin (g/dl)	-0.561	0.010	-0.089	0.718	0.011	0.962	-0.290	0.243	0.024	0.920	0.072	0.764
***L. infantum* specific antibodies (ELISA units)**	0.489	0.011	0.231	0.267	0.045	0.825	0.072	0.738	-0.422	0.032	0.121	0.557
Parasite load (parasites/mL)	0.199	0.341	-0.091	0.674	-0.522	0.007	0.159	0.467	0.088	0.674	-0.063	0.766
LSA IFN-γ (pg/mL)	-0.545	0.001	-0.178	0.329	-0.462	0.007	0.-087	0.643	0.385	0.027	0.095	0.599
Con A IFN-γ (pg/mL)	-0.234	0.89	0.017	0.927	-0.266	0.135	-0.004	0.961	0.117	0.519	0.181	0.314

In blue color are highlighted significant findings. LSA: *L. infantum* soluble antigen, Con A: concanavalin A, UPC: urinary protein/creatinine ratio.

3.4 ESTUDIO 4

Cytokine effect of TLR3, TLR4 and TLR7 agonists alone or associated with LSA on blood from dogs living in an endemic area of leishmaniosis

Pamela Martínez-Orellana[1], Sara Montserrat-Sangrà[1], Paulina Quirola-Amores[1], Noemí González[1], Laia Solano-Gallego[1*]

[1]Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain

In revision (Journal of Parasitology Research)

Abstract

Activation of Toll like receptors (TLRs) has been shown to play an important role in leishmaniosis by enhancing the parasite specific immune responses to control infection. However, the role of TLR agonists has not been studied in detail in dogs. The aim of this study was to determine the effect of TLR3, TLR4 and TLR7 receptor agonists (TLR3a, TLR4a and TLR7a) alone or in combination with *Leishmania infantum* antigen (LSA) on TNF-α and IL-6 production in blood from dogs living in endemic areas of canine leishmaniosis (CanL). Twenty-four healthy dogs from Catalonia (n=14) and Ibizan hound dogs from the island of Mallorca (n=10) were enrolled. Whole blood with TLR3a, TLR4a and TLR7a alone or combined with LSA was cultured separately, and IFN-γ, TNF-α and IL-6 were measured by ELISA. A significant increase of TNF-α was found for all conditions studied compared to medium. Stimulation with TLR4a *(p=0.0001)* and TLR7a *(p=0.005)* presented a significantly marked increase in TNF-α and IL-6 production compared to TLR3a. Importantly, significantly higher TNF-α production was found in LSA+TLR4a *(p=0.0001)* stimulated blood and LSA+TLR7a *(p=0.005)* compared to LSA. All dogs showed higher TNF-α production after LSA+TLR7a compared to TLR7a *(p=0.047)* and LSA+TLR3a compared to TLR3a *(p=0.052)*. These findings indicate a marked inflammatory cytokine effect of TLR4a and TLR7a on blood from healthy dogs living in endemic areas of CanL. Additionally, LSA+TLR7a promoted a synergistic pro-inflammatory effect with TNF-α in all dogs. Those findings suggest an active role of TLRa in pro-inflammatory responses, which might be strongly involved in the process of disease resolution.

Keywords

Leishmania infantum, dog, TLR3, TLR4 and TLR7 agonists, whole blood assay, cytokines

Abbreviations

CanL: Canine leishmaniosis; CO_2: Carbon dioxide; CD4: Cluster of cuadruple differentiation; CMI: Cell-Mediated Immune; ConA: Concavalin A; DC: Dentritic cells; EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; GLA-SE: Glucopyranosyl lipid adjuvant in stable emulsion; IFN-γ: Interferon gamma; IL-12: Interleukin 12; IL-6: Interleukin 6; IMQ: Imiquimod-R837; iNos: Nitric oxide synthase; LPG: Lipophosphoglycan; LPS : Lipopolysaccharide; LRV1: *Leishmania* RNA virus-1; LSA: *Leishmania* soluble antigen; mL: milliliters; MPLA: Monophosphoryl lipid A; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NK: Natural killers; nm: Nanometers; NO: Nitric oxide; PBMCs: Peripheral blood mononuclear cells; PAMPs: Pathogen-associated molecular patterns; PRR: Patter Recognition Receptors; ROS: Reactive oxygen species; SD: standard deviation; Th1: T helper 1; Th2: T helper 2; TLRs: Toll-like receptors; TLRa: Toll-like receptors agonist; TNF-α: Tumor necrosis factor alpha; VL: Visceral leishmaniosis; VLP: Virus-like particles; Ø: Medium alone.

Introduction

Leishmaniosis are a group of protozoan diseases caused by several species of *Leishmania* (class Kinetoplastea, family Trypanosomatidae). More than 70 countries are endemic for *Leishmania* infection, including countries in the Mediterranean basin, the Middle East, Asia, Africa and South America (Baneth et al., 2008). *Leishmania infantum* is the specie most commonly associated with canine infections (Solano-Gallego et al., 2009). In endemic Mediterranean areas, dogs are infected through the bite of sand flies of the genus *Phlebotomus* in the warmer months, from April to November (Alten et al., 2016; Rossi et al., 2008; Solano-Gallego et al., 2011).

Canine leishmaniosis (CanL) can be manifested with a range of clinical signs and laboratory abnormalities that include non-pruritic skin lesions such as exfoliative dermatitis and ulcerations, local or generalized lymphadenomegaly, weight loss, poor appetite, ocular lesions, epistaxis, lameness, renal failure and diarrhea among others (Solano-Gallego et al., 2017a). In addition, different degrees of disease severity exist (Solano-Gallego et al., 2017a). Moreover, persistent subclinical infections in dogs are common in endemic areas such as the Mediterranean basin (Shokri et al., 2017; Sideris et al., 1999).

Dogs of any breed are susceptible to *L. infantum* infection, but breeds such as Bulldogs, Dobermanns (Foglia Manzillo et al., 2013), Boxers, German shepherds and Rottweilers seem to be more susceptible to develop CanL (Solano-Gallego et al., 2009). However, Ibizan hounds are especially resistant to develop the disease living in endemic areas such as Mallorca island (Solano-Gallego et al., 2000). A mixed T helper 1 and T helper 2 (Th1/Th2) responses with a dominant Th1 profile is required by humans and dogs for protection against viscerotropic infection by *L. donovani* and *L. infantum* (Carrillo and Moreno, 2009; Khadem and Uzonna, 2014). Classically, Th1 responses are associated with resistance and Th2 responses are associated with susceptibility in dogs (Barbieri, 2006; Gradoni, 2015), humans (Kammoun-Rebai et al., 2017) and in rodent models (Sacks and Noben-Trauth, 2002). Moreover, it is demonstrated in both canine and murine models that Th1 cytokines as IFN-γ and TNF-α activate macrophages to kill *L. infantum* via increasing the nitric oxide (NO) pathway (Green et al., 1990; Holzmuller et al., 2006b; Zafra et al., 2008).

The cell-mediated immune (CMI) response is the major defence against CanL (Hosein et al., 2017; Tripathi et al., 2007) and indispensable for the resolution of this parasitic infection (Carrera et al., 1996; Quinnell et al., 2003). One clear example is observed in Th1 responses developed by resistance Ibizan hound (Solano-Gallego et al.,

2000). In contrast, disease development and severity in CanL is often correlated with a marked humoral response and the abrogation of Th1 cytokines production (Martinez-Orellana., 2017; Solano-Gallego et al., 2016b), which cannot control the infection.

Toll-Like Receptors (TLR) are type 1 membrane proteins that belong to the group of patter recognition receptors (PRRs) (Medzhitov, 2001). Ten TLR are found in dogs (Cusco et al., 2014) and they bind conserved molecular structures found in large groups of pathogen-associated molecular patterns (PAMPs). TLRs are located either in plasma membrane or internal membranes mainly of macrophages, dendritic cells (DC), natural killer (NK) cells and lymphocytes (T and B) and induce pro-inflammatory cytokines, type-1 IFN, chemokines and co-stimulatory molecules (Medzhitov, 2001; Takeda and Akira, 2004) as well as shaping adaptive immunity (Kumar et al., 2009). TLRs have been shown to play an important role in leishmaniosis (Tuon et al., 2008) because they are one of the most important non-clonal sets of PRR's families (Takeda and Akira, 2004).

There are several studies that have demonstrated the importance of TLR3 (Flandin et al., 2006; Ganguli et al., 2015), TLR4 (Kropf et al., 2004a) and TLR7 (Craft et al., 2014) in recognition, control and protection against *Leishmania* infection. Those previous works are based mainly on investigations of *Leishmania major* in a murine model (Tuon et al., 2008). However, limited information is available about the function of TLRs in canine *L. infantum* infections(Hosein et al., 2017).

TLR agonists are natural and synthetic molecular structures (PAMPs) (Gnjatic et al., 2010) that bind to TLRs with powerful immunostimulants potential. The ability of TLR agonists it is to activate signalling pathways to manage innate and acquired immunity (Steinhagen et al., 2011), therefore they amplifies parasite immune responses by stimulating the production of pro-inflammatory cytokines, which might play an important role in controlling *Leishmania* infection (Ribeiro-Gomes et al., 2007). The current treatment of CanL is not sufficiently effective and the use TLR agonists as adjuvants for

vaccine or as immunotherapy could be an interesting tool against CanL. Previously, TLR2 agonist (Pam3CSK4, a synthetic derivative of triacylated lipoproteins) alone enhances the production of the inflammatory cytokines TNF-α and IL-6 in dogs with clinical leishmaniosis, infected "resistant" and healthy non-infected. Furthermore, a combination of *L. infantum* soluble antigen (LSA) +TLR2a promoted a synergistic pro-inflammatory effect with TNF-α in Ibizan hounds (Martinez-Orellana et al., 2017b). The hypothesis of this study was that TLR3, TLR4 and TLR7 agonists (TLR3a, TLR4a and TLR7a) alone will enhance the production of inflammatory cytokines in canine *ex-vivo* whole blood. In addition, we hypothesized that the combination of TLR agonists with LSA promotes a synergistic release of inflammatory cytokines when compared with LSA or TLRs ligands alone. Therefore, the main objective of the present study was to investigate and expand the knowledge on the effect of other TLRs receptor agonist different than TLR2a as TLR3a [Poly (I:C)], TLR4a [Monophosphoryl Lipid A (MPLA)] and TLR7a [Imiquimod (IMQ)] alone or in combination with *L. infantum* antigen on *ex-vivo* whole blood from apparently healthy dogs living in an endemic area of leishmaniosis (Catalonia and the Island of Mallorca, Spain).

Materials and methods

Dogs and sampling

The study was conducted in Camprodon (Catalonia, Spain) in July 2015 (n=14) and Mallorca (Balearic Islands, Spain) in February 2017 (n=10), both endemic areas of CanL. Twenty-four apparently clinically healthy outdoors dogs of both sexes (eleven females and thirteen males) were enrolled. Age ranged between 8 month and 9 years and the median was 3.5 years old. Veterinarians subjected all dogs to physical examination and found that they did not present any clinical signs and were apparently healthy with the exception of three dogs that presented only papular dermatitis due to *L. infantum*, a mild form of clinical leishmaniosis with commonly good prognosis in dogs (Ordeix et al., 2005).

Collection of blood samples for this study was performed in accordance with veterinary protocols under aseptic conditions. Approximately 7-10 mL of blood from jugular or cephalic venipuncture was collected and immediately transferred into sterile tubes divided into: 1-2 tubes with sodium heparin (6 mL volume each) for whole blood assay, 1 tube with ethylenediaminetetraacetic acid (EDTA) (2 mL volume) and 1 tube for serum with clotting accelerator (2-4 mL volume each) for *L. infantum* specific antibodies and hematology profile. Blood sampling was obtained as screening for *L. infantum* infection and for checking general health status.

The dog owners gave their consent for blood sampling. Detection of antibodies against *L. infantum* using an in-house quantitative enzyme linked immunosorbent assay (ELISA) was performed on sera of all dogs as previously described (Solano-Gallego et al., 2016a).

Whole blood cytokine release assay

Heparinized whole blood cytokine release assay was performed as previously described (Martinez-Orellana et al., 2017b). Briefly, blood from Mallorca dogs (n=10) was stimulated as follows: 1) medium alone (ϕ), 2) medium with *L. infantum* soluble antigen (LSA) (provided by Dr. Cristina Riera, *Facultat de Farmacia*, *Universitat de Barcelona*) at a working concentration of 10 μg/mL (ϕ+LSA), 3) medium with concanavalin A (ConA) a strong lymphocyte mitogen (Medicago® Uppsala, Sweden) at a working concentration of 10 μg/mL (ϕ+ConA),4) medium with TLR3a [Poly(I:C) Invivogen® San Diego, USA] at a working concentration of 10 μg/mL (ϕTLR3a), or TLR4a [Monophosphoryl Lipid A (MPLA), Invivogen® San Diego, USA] at a working concentration of 1 μg/mL (ϕTLR4a), or TLR7a [Imiquimod-R837 (IMQ) Invivogen®, San Diego, USA] at a working concentration of 5 μg/mL receptor agonist (ϕTLR7a) and 5) medium with TLR3a, TLR4a or TLR7a at concentrations described above and LSA at a concentration described above (LSA+TLR3a or TLR4a or TLR7a). In the case of Catalonian dogs (n=14), blood was stimulated with all

TLRs agonists with the exception of TLR7a. All conditions were run in duplicates and incubated for 48 hours and 5 days at 37ºC in 5% CO_2 enriched environment. After 48h and 5 days the samples were collected and centrifuged individually at 300 g for 10 minutes and the supernatant was collected and stored at -80ºC until used. Cytokine concentrations were measured in supernatants collected at 48h for IL-6 and TNF-α and at 5 days for IFN-γ.

Sandwich ELISA for canine cytokines

Cytokine analysis of IFN-γ, TNF-α and IL-6 was performed according to manufacturer's instructions (DuoSet® ELISA by Development System R&D TM, Abingdon, UK) using 96 well plate flat bottom (Costar® Corning, New York, USA). Slight modifications were done for IFN-γ ELISA (Martinez-Orellana., 2017; Solano-Gallego et al., 2016b). Standard concentrations of IFN-γ started with 2000 pg/mL followed by two-fold dilutions in reagent diluent (R&D systems, Minneapolis, USA) until 31.25 pg/mL was reached. Standard curve for TNF-α started with 1000 pg/mL and two-fold dilutions were made until 15.6 pg/mL concentration. Standard curve for IL-6 started with 4000 pg/mL and two-fold dilutions were made until 62.5 pg/mL concentration. All values under the last standard concentration were considered undetectable for each cytokine (Martinez-Orellana., 2017). Duplicates of all supernatants studied were performed in all ELISAs. Optical density was measured with an ELISA reader (Anthos Reader 2020, Cambridge, UK) at wavelength of 450 nm. The standard curve for each cytokine was calculated using a computer generated four parameter logistic curve-fit with program myassays (http://www.myassays.com/). Plate was repeated when the R2-value of standard curve was below 0.98. Dogs were classified as IFN-γ producers when *L. infantum* specific IFN-γ concentration was detectable after subtracting medium alone. Dogs were classified as IFN-γ non-producers when *L. infantum* specific IFN-γ concentration subtracting medium alone was at non-detectable levels (Solano-Gallego et al., 2016b).

Statistical analysis

A non-parametric Wilcoxon signed-rank test was used to compare among related several treatment. Differences were considered significant with a 5% significance level *($p<0.05$).* The statistical analysis was performed using the SPSS 17.0 for Windows software (SPSS Inc., USA). Graphs were performed using excel GraphPad Prism 7 (GraphPad Software, La Jolla, USA).

Results

Clinical data

All 14 dogs from Catalonia, were clinically healthy based on physical examination and hematological parameters; additionally all dogs were seronegative. There were nine males and five females with a median of age of 50.5 months and an age range from 12 moths to 9 years old. Four mixed Fox hounds, two Dachshund, two Brussels griffon, one English pointer, two Ariegeois pointer, one Ibizan hound, one English setter and one Bruno Jura hound were studied.

Seven out of 10 dogs from Mallorca were seronegative for *L. infantum* antibodies and three were low positive, two of them with papular dermatitis and one without any lesion. There were six females and four males. All ten dogs were Ibizan hounds. The median of age was 30 months with a range from 8 month to 6 years.

Specific *L. infantum* antibody levels and IFN-γ production

Clinically healthy dogs (n=24) presented a mean ±standard deviation (mean ± SD): 16.6 ± 21.6 EU of specific *L. infantum* antibody levels.

Levels of IFN-γ of all animals under study (n=24) after LSA stimulation (mean ± SD of 584.5 ± 1131.4 EU) and Con A stimulation (mean ± SD of 8433.2 ± 7457.1 EU) were significantly higher than medium alone (mean ± SD of 12.0 ± 29.3 EU). The results from IFN-γ production at 5 days after stimulation with LSA and ConA from all dogs studied are shown in Figure 3.4.1. Additionally, fourteen (58.3%) out of twenty-four dogs were classified as parasite-specific IFN-γ producers.

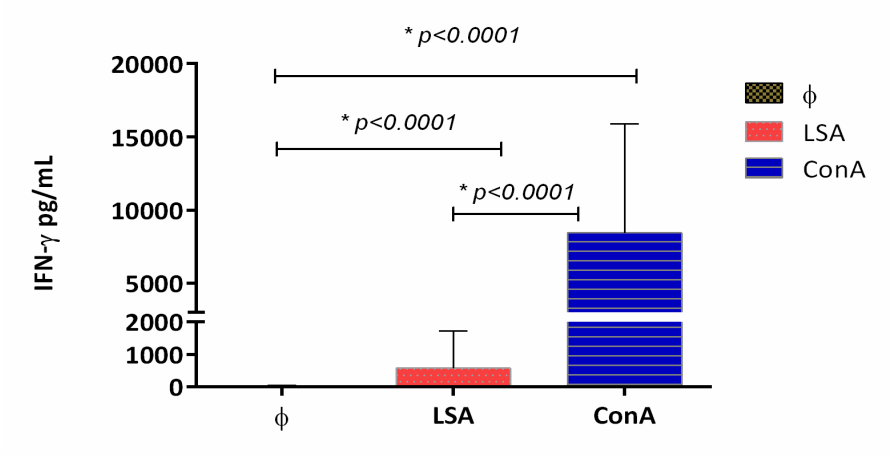

Figure 3.4.1. LSA specific IFN-γ production in blood ex-vivo after LSA and ConA stimulation in all dogs studied. Medium alone (Ø); Leishmania *soluble antigen (LSA); concanavalin A (ConA).*

TNF-α concentration

The results of TNF-α concentration for each treatment condition in all dogs studied (n=24) are summarized in Table 1. There was a significant increase of TNF-α production in samples supplemented with TLR4a *(p=0.0001)* and TLR7a (P= 0.005) when compared with TLR3a in all dogs studied and also on TLR4a (P= 0.0001) and TLR7a (P= 0.005) in combination with LSA comparing with LSA+TLR3a. Levels of TNF-α of all animals studied were significantly higher for all treatments [ϕ+LSA (P= 0.005), ϕ+ConA (P= 0.0001), ϕ+TLR3a (P= 0.044), ϕ+TLR4a (P= 0.0001), ϕ+TLR7a (P= 0.005), LSA+TLR3a (P= 0.025), LSA+TLR4a (P= 0.0001) and LSA+TLR7a (P= 0.005)] when compared to medium alone.

Moreover, higher production of TNF-α was observed after stimulation with LSA+TLR4a (P=0.0001) and LSA+TLR7a (P= 0.005) compared to LSA alone. Also, LSA+TLR3a was higher than ϕ+TLR3a (P= 0.052).

Table 3.4.1. Results of TNF-α and IL-6 concentrations after stimulation at each condition in all dogs studied (n=24).

Cytokines (pg/mL)	**Treatment conditions (mean ± SD)***								
	Φ	**ϕ+LSA**	**ϕ+ConA**	**ϕ+TLR3a**	**ϕ+TLR4a**	**ϕ+TLR7a**	**LSA+TLR3a**	**LSA+TLR4a**	**LSA+TLR7a**
TNF-α	9.6±17.1	27.1±32.7	107.7±107.7	17.2±28.0	116.3±10.9	92.0±56.1	18.8±17.2	116.1±73.8	157.1±78.1
IL-6	15.1±19.5	12.6±19.0	24.3±22.7	17.0±26.8	76.7±39.9	55.1±32.2	16.9±20.4	80.6±39.3	75.4±39.7

*mean ±standard deviation (mean ± SD), medium alone (Ø); *Leishmania* soluble antigen (LSA); concanavalin A (ConA); TLR3 (Poly (I:C) receptor agonist (Ø+TLR3a) and TLR3a and LSA (LSA+ TLR3a); TLR4 (MPLA) receptor agonist (Ø+TLR4a) and TLR4a and LSA (LSA+ TLR4a); TLR7 (IMQ) receptor agonist (Ø+TLR7a) and TLR7a and LSA (LSA+ TLR7a)

IL-6 concentration

The results of IL-6 concentration for each condition in all dogs studied (n=24) are summarized in Table 1. As observed with TNF-α, a significant increase of IL-6 production in TLR4a *(p=0.001)* and TLR7a *(p=0.005)* stimulated blood was found when compared with TLR3a in all dogs studied and also in TLR4a *(p=0.001)* and TLR7a *(p=0.005)* stimulated blood in combination with LSA compared with LSA+TLR3a. Levels of IL-6 of all animals studied were significantly higher after ϕ+ConA *(p=0.019)*, ϕ+TLR4a *(p=0.0001)*, ϕ+TLR7a *(p=0.008*), LSA+TLR4a *(p=0.0001*) and LSA+TLR7a *(p=0.005)* treatments when compared to medium alone. Moreover, higher production of IL-6 was observed after LSA+TLR4a *(p=0.0001*) and LSA+TLR7a *(p=0.005*) compared to LSA alone. No statistically significant differences were found when the different TLRs agonists were compared with the LSA treatment.

Discussion

The present results indicated for the first time a pronounced effect of TLR4a and TLR7a on cytokine production in blood from apparently healthy dogs living in endemic areas of CanL suggesting an active role of the innate and pro-inflammatory immune responses when compared with other TLR agonist or treatment conditions. In contrast, stimulation of TLR3 by seems to have a less marked effect on cytokine production, supported by a statistically significant smaller production of pro-inflammatory cytokines when compared with TLR4 and TLR7 agonists.

Improvement of vaccines and treatment strategies is imperative against leishmaniosis. In this context, new adjuvants are required to elicit an intense immune response needed for protection (Eskandari et al., 2014). In particular, the vaccine adjuvant properties of TLR7a and resiquimod (TLR7/8a) have been previously studied (Johnston and Bystryn, 2006; Weeratna et al., 2005). Interestingly, very promising results were obtained from

dogs stimulated blood with a synergic pro-inflammatory effect of TLR7a when combined with LSA, reflected in a higher production of TNF-α. A similar synergic effect of TLR2a in Ibizan hounds was previously observed but it was slightly less intense that the one observed here for TLR7a (Martinez-Orellana et al., 2017b).

In agreement with the effect of TLR7a associated with LSA in this study, a previous *L. major* mouse model study described TLR7a and/or TLR7/8a as adjuvants (Zhang and Matlashewski, 2008). It was found that *Leishmania* antigen alone was not protective to subsequent challenge unless administrated with TLR7/8a (Zhang and Matlashewski, 2008). According to the synergic effect observed in the present study, Emami et al. observed an inferior Th1 response induced in Balb/c mice by TLR7a nanoliposomal immunization alone when compared to LSA together with TLR7a formulations suggesting that TLR7a did not function well in the absence of antigen (Emami et al., 2018). Contrarily, another study in a murine model showed that TLR7/8a seems to decreases/inhibit cytokine production induced by LSA (Rostamian and Niknam, 2017). Therefore, TLR7a appear to be good candidates for use as adjuvants for vaccines and immunotherapy in dogs. On the other hand, several studies performed in murine models and in humans showed promising results for the use of TLR7a as treatment in cutaneous (Khalili et al., 2011) and visceral leishmaniasis (Craft et al., 2014). Further studies are needed in order to characterize the therapeutic role of TLR7 in CanL.

Ligands of TLR4 have been broadly used in human vaccines as adjuvants and are the only TLR receptor agonist approved due to their safety and the potent enhancement of the immune response (Carter et al., 2016). In the present study, a marked cytokine production was observed after blood stimulation with a TLR4 Medium alone (Ø); *Leishmania* soluble antigen (LSA); concanavalin A (ConA).

Agonist alone and also when associated with LSA, supporting the role of TLR4 ligands as adjuvant. Recently, several studies have evaluated the use of TLR4a as adjuvant during *Leishmania* infection. Particularly, a vaccine antigen formulated with the TLR4a glucopyranosyl lipid adjuvant in stable emulsion (GLA-SE) proved to induce antigen-

specific responses that protected mice against *L. donovani* infection and also demonstrated that prophylactic mouse immunization resulted in a marked reduction of the parasite spleen burden and the induction of memory CD4 T lymphocytes enhancing IFN-γ, TNF-α and IL-2 production (Duthie et al., 2017). Moreover, an innovative virus-like particles (VLP) vaccine loaded with sand fly saliva antigen together with *Leishmania* antigens and GLA-SE as an adjuvant improved both cellular and humoral immune responses in a murine model and in human peripheral blood mononuclear cells (PBMCs) (Cecilio et al., 2017).

In agreement with the results presented here, a previous study revealed a significant down-regulation of several TLRs including TLR4 during disease progression in lymph nodes of experimentally infected dogs (Hosein et al., 2015) suggesting a role of TLR4 in controlling infection as shown in murine models of *L. major* infection where TLR4 appeared to have a protective role (Antoniazi et al., 2004; Kropf et al., 2004a). In contrast, so far, TLR4 transcription in unstimulated blood appears to be similar in healthy dogs and dogs with different clinical stages of CanL at the time of diagnosis as well as during treatment follow-up (Chapter 3.1. and 3.2. (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018)). Further studies need to elucidate the role of TLR4 in dogs in different clinical conditions and tissues.

The higher production of TNF-α and IL-6 after TLR4a blood stimulation when compared to un-treated blood are in agreement with several studies carried out in murine and human leishmaniasis where TLR4 regulated the initial pro-inflammatory response (Kropf et al., 2004a; Srivastava et al., 2012). Additionally, and as observed by others (Galdino et al., 2016), stimulation with LSA + TLR4a showed an augmentation in TNF-α production when compared with LSA stimulus alone. Particularly, a similar study to the one presented here revealed that activation of TLR4 by lipopolysaccharide (LPS) augments TNF-α production by human PBMCs previously exposed to *L. braziliensis* amastigotes, suggesting a crucial role for TLR4 in TNF-α induction and subsequently in controlling leishmanial infection (Galdino et al., 2016). Similar results were observed in a murine

model of *L. major* immunization where LSA ability to reduce parasite load was significant statistically increased when was associated to TLR4a (Emami et al., 2018; Rostamian and Niknam, 2017). In view of the previous information together with the results presented here, TLR4 ligands should be also considerate in future studies as adjuvant for vaccines and immunotherapy in dogs.

In the present study, the effect of cytokine production of TLR3a was significantly less pronounced when compared with TLR4 and TLR7 agonists. Interestingly, all dogsshowed higher production of TNF-α after stimulation with TLR3a associated with LSA when compared with medium aloneand with TLR3a. Recently, the essential role of TLR3a in inducing a Th1 response as adjuvant was demonstrated in a murine model of a *L. (L.) amazonensis* vaccine based on total antigenic extract derived from promastigotes (Sanchez et al., 2017). Therefore, Poly (I:C) demonstrated its safety and ability to induce protection and its potential for being a promising candidate for *Leishmania* vaccines development. In contrast, other studies in which *Leishmania* RNA virus-1 (LRV1) was used to bind and stimulate endosomal TLR3 pathways in a murine model of *L. guayanensis* infection confirmed that TLR3 is the dominant route of inflammatory pathology in this particular model (Eren et al., 2016; Hartley et al., 2018). Further research conducted on *L. infantum* infection in dogs is necessary to elucidate the potential use of TLR3a as vaccine adjuvant or immunotherapy in sick dogs.

TNF-α is a powerful pro-inflammatory cytokine that promotes together with IFN-γ macrophage activation which is crucial for *Leishmania* parasite control both in dogs (Holzmuller et al., 2005) and humans (Green et al., 1990; Roach et al., 1991). However, little had been investigated on the function of TNF-α and IL-6 in dogs with clinical leishmaniosis. Moreover, less information is available related to dogs living in endemic areas of CanL and how these responses could affect the outcome of the disease in infected/exposed dogs. In the present study, a higher TNF-α response to LSA was observed when compared to medium alone in all dogs studied while this was not noted for IL-6, suggesting a more important role of TNF-α in inducing a pro-inflammatory

response to *Leishmania* parasite. Accordingly, in previous studies, the use of TNF-α antagonists in subclinically-infected people promoted the reactivation of infection by *Leishmania* spp. and the emergence of clinical signs of human leishmaniasis (Campos et al., 2018; Marcoval and Penin, 2017; Neumayr et al., 2013).

Conclusion

These findings indicate that TLR4a and TLR7a have a marked effect on cytokines production in *ex-vivo* blood from apparently healthy dogs living in endemic areas of CanL. These findings suggested an active role of TLRs on pro-inflammatory immune responses, which might be strongly involved in the process of disease resolution in dogs. In contrast, TLR3a seems to have a less pronounced effect on cytokine production in the present *ex-vivo* model. Results presented here encourage further studies in order to evaluate the potential use of TLR receptor agonist as vaccine adjuvant or as immunomodulatory therapy in *Leishmania* infection in dogs.

Conflicts of interests

The authors declare that they have no competing interests.

Funding

This study was supported by Spanish ministry grants, *Ministerio de Economia y competitividad* and FEDER, EU (AGL2012-32498 and AGL2015-68477). Dr. Laia Solano-Gallego held a Ramón y Cajal senior researcher contract awarded by the *Ministerio de Ciencia e Innovación* (Spain) and the European Social Fund.

Acknowledgements

The authors want to thank all veterinarians and dog owner that contributed to this study. We are especially grateful to Oscar Cabezón (UAB) and Cati Lladó (Hospital Mon Veterinari, Mallorca) for collecting samples. Cèlia Cerezuela and Irina Casado Nuñez (UAB) who helped to laboratory work performance.

Author's contributions

LSG designed the research study. LSG and PMO set up laboratory work. LSG coordinated the veterinary clinics enrolled. LSG, CL and PQ were involved in field studies. PMO and SM performed whole blood assay and collection of supernatants. PMO, PQ, NG performed all serological and cytokine testing. LSG and PMO contributed with data analysis and interpretation. LSG and PMO wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

3.5. ESTUDIO 5

In vitro susceptibility of a *Leishmania infantum* strain isolated from a dog and treated with conventional anti-*Leishmania* drugs alone or in combination with Toll-like receptor agonists

Montserrat-Sangrà, S.[1], Maia, C.[2], Campino, L.[2], Solano-Gallego, L[1*].

[1]Dep. Medicina i Cirurgia Animals, Facultat de veterinària, Universitat Autònoma de Barcelona (Spain)

[2]Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, UNL, Lisboa (Portugal)

Original research study

Abstract

Domestic dogs are the main hosts and reservoir hosts for *Leishmania infantum* infection in Mediterranean basin. Currently, allopurinol, miltefosine and meglumine antimoniate are the most frequently used drugs for treating dogs with clinical leishmaniosis. Toll-like receptors agonists (TLRa) appear to be immune modulators and might be used for immunotherapy in dogs. The main objective of this study was to test the in vitro susceptibility of *L. infantum* promastigotes and intracellular amastigotes to common anti-*Leishmania* drugs alone or in combination with TLR2a, TLR4a, TLR3a or TLR7a. The production of nitric oxide, cytokine TNF-α and the expression of the TLRs by macrophages was also evaluated. Percentage of infection was significantly lower ($P < 0.05$), in cells treated alone with the different TLRs agonists and with the three antileishmanial drugs when compared to untreated infected cells. No synergistic effect was observed when a combination of one drug and one TLRa was used. Intensity of infection was also significantly lower ($P < 0.05$) in cells treated alone with the different antileishmanial drugs or TLRs when compared to untreated infected cells as well as with combinations of allopurinol and TLR4a and allopurinol and TLR7a. TNF-α and NO production was not detected in any condition studied. A downregulation of TLR2 transcript and no changes of TLR4 and TLR7 expression were found in infected macrophages in comparison with non-infected ones. Interestingly, TLR7 uregulation was noted when infected cells were treated with miltefosine alone as well as a TLR4 uregulation in non infected cells treated with allopurinol. In contrast, TLR2 downregulation was found after treatment with allopurinol and miltefosine in non-infected cells. Generally, TLRs upregulation was observed when infected macrophages were treated with TLRs agonists alone. However, when drugs were added in combination with TLRs agonists, the TLRs gene expression was variable. These findings demonstrated that all TLRa and conventional drugs studied alone reduced *L. infantum* infection in an *in vitro* canine model. In addition, allopurinol combined with TR4a synergistically decrease *L. infantum* infection. These results suggest that the use of this combination may be helpful as alternative treatments in clinical canine leishmaniosis.

Keywords

Canine leishmaniosis, *Leishmania infantum*, toll like receptors, gene expression, treatment, *in vitro* susceptibility, rt-PCR.

Abbreviations

CanL: Canine leishmaniosis; FDA: Food and Drug administration; FCy: Fragment crystallizable region y, *L. infantum*: *Leishmania infantum*; MTD: Maximum tolerated dose; Pattern recognition receptors: PRRs; SPSS: Statistical Package for Social Sciences, TLRs: Toll like receptors.

Introduction

Canine leishmaniosis is a zoonotic parasitic disease caused by *Leishmania infantum* in Mediterranean basin and is transmitted by the bite of phlebotomine sand flies. In Europe, millions of dogs are affected by this severe disease which is mainly distributed in other continents such as Asia, North Africa and South America (Duprey et al., 2006). Domestic dogs are considered the major hosts and reservoir hosts for *L. infantum*. Despite the lack of pathognomonic manifestations, the commonest clinical signs in dogs are cutaneous alterations, local or generalized lymphoadenomegaly, loss of body weight, liver and spleen enlargement, ocular lesions, epistaxis, onycogryphosis and lameness (Blavier et al., 2001).

Epidemiological diversity of this disease makes it impossible to implement a unique prevention protocol (Croft and Coombs, 2003; Hadighi et al., 2006; Khatami et al., 2007). The current vaccines are no hundred per cent effective and the only successful way to treat canine leishmaniosis is by the institution of anti-*Leishmania* drugs (Palatnik-de-Sousa, 2012). Pentavalent antimonials were the first medicines employed for human (Frezard et al., 2009; Giavedoni et al., 2015) and canine leishmaniosis treatment (Noli and Auxilia, 2005). Drug resistance is documented for this drug in both humans (Faraut-Gambarelli et al., 1997; Laurent et al., 2007) and canines (Carrió, 2002; Gomez Perez et al.,

2016; Gramiccia et al., 1992) as well as side effects (Laguna et al., 1999; Pagliano et al., 2003). Miltefosine, an antitumoral oral compound, has recently emerged as an effective antileishmanial drug (Dorlo et al., 2012; Sundar and Olliaro, 2007). Another relevant drug designed to reduce *Leishmania* infection is allopurinol. However, the long-term use of allopurinol is limited due to the evidence of urinary side effects (Planellas, 2018; Torres et al., 2011) and drug resistance (Yasur-Landau et al., 2016; Yasur-Landau et al., 2017). Although animal models are well established for drug testing, they are not suitable for large-scale primary drug screenings (Fumarola et al., 2004). Therefore, several assays to test *Leishmania* susceptibility to new compounds *in vitro* have been developed using promastigotes and amastigotes (De Muylder et al., 2011; Ginouves et al., 2017).

The prime immune responses in resistance to *Leishmania* infection is mediated by antigen presenting cells such as dendritic cells or macrophages. In addition, macrophages are the foremost host cells of *Leishmania* spp., whose have a strong evasion strategy (Liu and Uzonna, 2012). Macrophages microbicidal functions are suppressed by the parasite through changing signaling pathways once the macrophages recognize *Leishmania* flagellum proteins. These contacts allow the parasite to alter the phagocytic activity of these immune cells by inducing specific mammalian phosphatases that are able to avoid signaling, thus allowing the establishment of the infection (Soulat and Bogdan, 2017). Thus, different surface receptors, such as complement receptors or mannose receptors, take part in the course of infection and each one have a different effect. While complement receptors encourage favorable conditions for parasite survival, fragment crystallizable region y (FCy) receptors mediate inflammatory pathways that catalyzes the activation of enzymes and nicotinamide adenine dinucleotide phosphate oxidase along with the generation of an oxidative burst thus enhancing parasite clearance (Dos-Santos et al., 2016).

The innate immunity system is composed for various non-clonal sets of recognition molecules called pattern recognition receptors (PRRs) promoting anti-microbial defense

mechanisms. Among PRRs, Toll-like receptors (TLRs) are the main studied innate sensors and ten are recognized in dogs so far (Cusco et al., 2014; Mikami et al., 2012; Roach et al., 2005). These receptors are able to recognize different pathogens, through binding conserved molecular regions termed pathogen-associated molecular patterns (Akira et al., 2001). TLRs are mainly expressed in phagocytes and antigen presenting cells, such as neutrophils, macrophages and dendritic cells, which are the major innate cells that respond to *Leishmania* infection (Liu and Uzonna, 2012; Sacramento et al., 2017).

Because TLR provide a link among innate and adaptive immunity, TLR agonists are being tested as an adjuvant to enhance immune responses (Mifsud et al., 2014). The outcomes of signaling TLR pathway provokes the production of pro-inflammatory cytokines, adhesion molecules and co-stimulatory molecules that might control *Leishmania* infection (Gazzinelli and Denkers, 2006). Some TLR agonists are approved for human use in several clinical conditions by food and drugs administration (FDA) while others have reached clinical studies in phases I, II and III (Hussein et al., 2014). Interestingly, there are some evidence that TLR agonists can be a good tool to improve the effectiveness of treatments; for instance, TLR4 and TLR9 agonists were observed to confer protection against *Leishmania* infection in rodent models (Daifalla et al., 2012; Raman et al., 2010). Therefore, the main objective of this study was to test the *in vitro* susceptibility of *L. infantum* promastigotes and amastigotes to common anti-*Leishmania* drugs alone or in combination with several TLR agonists (TLRa), to determine the production of nitric oxide and of the pro-inflammatory cytokine TNF-α as well as TLRs expression.

Materials and methods

Parasites

Leishmania infantum MCAN/PT/05/IMT 373 promastigotes isolated from a dog with leishmaniosis were used in this study. Promastigotes were cultured at +26ºC in Schneider's insect medium (Sigma Aldrich, Saint Louis, US), pH 6.0, containing 20% heat-inactivated fetal calf serum (FCS) (ThermoFisher, Waltham, US), 50 mg/ml gentamicin solution (Sigma) and 1% human urine (Maia et al., 2007).

Drugs and TLR agonists

Meglumine antimoniate, [Antimony-N-methyl-glucamine (Glucantime®, Merial)], allopurinol (Sigma Aldrich St Louis, MO) and miltefosine (Sigma Aldrich St Louis) were used. Stock final solutions were prepared in ultrapure water for allopurinol (5 mg/mL) and miltefosine (100 µg/mL). Glucantime® (300 mg/mL) was used directly. TLR-2a (Pam3CSK4, Invivogen, San Diego, US), TLR-3a (Poly (I:C) Invivogen, San Diego, US), TLR-4a (MPLA-SM, Invivogen, San Diego, US) and TLR7a (Imiquimod-R837, Invivogen, San Diego, US) were tested alone and in combination with one drug. All drugs and TLR agonists solutions were stored at 4ºC until used.

The half inhibitory concentration (IC50) promastigote susceptibility

Promastigote drug susceptibility was determined by using Alamar blue (Sigma Aldrich, San Louis, US) resazurin reaction. Promastigotes (1 x 10^6 parasites/mL) in the stationary phase of *in vitro* growth were plated (100 µl/well) in 96-well flat-bottomed microfilter plates (Thermofisher, Waltham, US) for 2 days at 24ºC, in the presence of 2-

fold serially diluted concentrations of the studied drugs (100 µl/well). Starting concentrations of meglumine antimoniate, allopurinol and miltefosine were 300 mg/mL, 5 mg/mL and 100 µg/mL, respectively. After 48h of incubation, 10% alamar blue of the total volume was added to each well and incubated for 24h at 24ºC. Optical density (OD) was measured spectrophotometrically by fluorescence at 535nm excitation, 595 nm emission (Triad multimode detector, Dynex Technologies, Chantilly, US). Relative viability was calculated from the ratio of the OD readings in parasites exposed to compounds *versus* those not exposed. For each drug four independent experiments were performed with three replicates each to calculate the inhibitory concentrations that kill 50% of *Leishmania* promastigotes (IC50).

Drug cytotoxicity assay

Macrophages (1 x 10^6 cells/ mL) derived from canine monocyte-macrophage malignant histiocytosis DH82 (ATCC® CRL-10389™) cell line in the logarithmic phase of growth were incubated in 16 chamber Lab Tek culture slides (ThermoFisher, Waltham US) in 200 µL of RPMI 1640 (Sigma Aldrich, St Louis, US) plus 10% FCS medium (complete RPMI). After 24 hours of cells settlement on the wells surface drugs were added. Starting concentration of meglumine antimoniate, allopurinol and miltefosine were 300 mg/mL, 5 mg/mL and 100 µg/mL, respectively. Drugs were serial diluted 2 fold eleven times in order to establish the Maximum tolerated dose (MTD), i.e. the drug concentration producing 25% decrease in cell number (Maia et al., 2007). After 72 h of incubation cells at 37ºC, 5% CO_2, 10% alamar blue of the total volume was added to each well and incubated for 24h at 37ºC and cytotoxicity was measured by spectrophotometrically by fluorescence at 535nm excitation, 595 nm emission (Triad multimode detector, Dynex Technologies, Chantilly, US). Maximum tolerated dose for DH82 cells to each drug was 1.9 mg/mL for meglumine antimoniate, 0.6 mg/mL for allopurinol and 2.2 µg/mL for miltefosine.

Intracellular amastigote susceptibility assay

DH82 macrophages (1 x 10^6 cells/ mL) in the logarithmic phase of growth were incubated in 16 chamber Lab Tek culture slides in 200 µL of complete RPMI. After 24 hours of cells settlement on the wells surface, the medium was removed and further infected with 200 µL of complete RPMI containing promastigotes (stationary phase) at 5:1 parasite:host cell ratio for 24 h. After incubation the cells were washed with PBS (pH 7.2) twice to remove non-internalized parasites prior to the addition of the drugs. Cells were incubated at 37ºC for 72h with the different drugs alone or in combination with the TLRa. Maximum tolerated dose for each anti-*Leishmania* drug described above (allopurinol 0.6 mg/ mL, miltefosine 2.2 µg/ µL and meglumine antimoniate 1.9 mg/ mL) was used in combination with the different TLR agonists (TLR2a (0.3 µg/mL), TLR3a (1000µg/mL),TLR4a (1µg/mL), and TLR7a (1000µg/mL). Macrophages non-infected with no drugs or TLR agonists and macrophages infected with no drugs or TLR agonists were used as controls. Three independent assays were performed. In each assay, four replicates were made in order to use the cells of two replicates to calculate the percentage and intensity of infection and the other two to evaluate the expression of the different TLRs. After 72 h of incubation, the supernatants of the four replicates were collected to calculate NO and TNFa production and cells were stained with giemsa or kept in RNA later. The percentage of infected cells (i.e. number of infected cells per 100 macrophages) and the intensity of infection (i.e. number of parasites per cell) were determined by light microscopy under oil (×1000) by counting at least 100 cells per well (Maia et al., 2007). Three independently experiments were carried out in triplicate to determine the IC_{50} of each drug compared to untreated infected control cultures.

TNF-α and nitric oxide concentrations

The supernatant of each treatment was collected after 72 h of incubation and used to measure TNF-α concentrations by ELISA technique following manufacturer's

instructions (DuoSet ® ELISA by Development System R & D™, Abingdon, UK). Briefly, using 96 well plate flat bottom (Costar® Corning, New York, USA). TNF-α started with 1000 pg/mL and two-fold dilutions were made until 15.6 pg/mL. All values under the last standard concentration were considered undetectable. Optical density was measured with an ELISA reader (Anthos Reader 2020, Cambridge, UK) at wavelength of 450 nm. The standard curve was calculated using a computer generated four parameter logistic curve-fit with program myassays (http://www.myassays.com/).

The accumulation of NO produced by cultured macrophages was determined using Griess reagent (Loria-Cervera et al., 2013). The NO concentration was calculated from a standard curve generated with sodium nitrite standard from 12.5 to 100 µM. Supernatants of cultured cells were mixed with 60 mM sulfanilamide in 2.5 % of phosphoric acid and incubated at room temperature (RT) in the dark during five minutes. Afterwards, N-1-naphthylethylenediamine dihidrochloride in 2.5 % of phosphoric acid was added and incubated in the dark during 5 minutes at RT. Optical density was measured spectrophotometrically on an ELISA plate reader reader (ELISA Reader Anthos 2020) at 550 nm. The NO2 concentration was calculated from a standard curve generated with sodium nitrite standard from 12.5 to 100 µM (Tsikas, 2007). Duplicates were performed in the two techniques.

Evaluation of kinetics of TNF-α concentration at 24, 48 and 72 h

An additional study was performed with non-infected DH82 cells (see Figure 3.5.3) stimulated with all TLRa studied to assess the kinetics of TNF-α at 24, 48 and 72 hours. DH-82 macrophages (1 x 10^6 cells/ mL) in logarithmic phase growth were incubated in 16 chamber Lab Tek culture slides in 200 µL of complete RPMI. After 24 hours of cells settlement on the wells surface, the medium was removed and resuspended with 200 µL of complete RPMI + TLR agonists: TLR-2a, TLR-3a, TLR-4a (and TLR-7a). The macrophages were incubated during three time points (24, 48 and 72 h) in order to evaluate TNF-α

kinetics over time. The supernatant was collected in every time point, after 24, 48 and 72 h of incubation. Two replicates were cultured for each time point. ELISA technique was performed as described above.

TLRs and reference gene transcripts by real time PCR

Two replicates of each experiment were designated for evaluating TLR gene expression. Gene expression of TLR2, TLR4 and TLR7 was carried out in the QuantStudioTM12k Flex Real-Time PCR System (Applied biosystems, Thermo Fisher Scientific). RNA extraction from DH82 cells was performed by NZYol (NZYTech - Genes & Enzymes) following manufacturer's instructions. RNA concentration was determined by Nanodrop® 1000 device (ThermoFisher Scientific, Waltham, US). cDNA was produced by VILO Masterscript Retrotranscriptase kit (ThermoFisher, Waltham, US) according to the manufacturer's instructions. The cDNA was aliquoted and stored at −80ºC until use.

Six reference housekeeping genes (RG) (Table 3.5.1.; ThermoFisher, Waltham, US) ((hydroxymethyl-bilane synthase (HMBS), succinate dehydrogenase complex; subunit A; flavoprotein (SDHA), TATA box binding protein (TBP), similar to CG14980-PB (CG14980), hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome) (HPRT-1) and beta-2-microglobulin(β–2 M)) were assessed by real time PCR with 11 representative samples to establish the most stable genes by the GeNorm program (Biogazelle, Belgium).

Table3.5.1 .Summary of canine reference and target genes used in this study.

ASSAY ID[a]	GENE SYMBOL	GENE NAME	GENEBANK mRNA	GENEBANK REFERENCE SEQUENCE	AMPLICON PAIRWISE
Cf02625049_s1	TLR2	Toll-like receptor 2	AB080363.1	NM_001005264.2	69
Cf02622203 g1	TLR4	Toll-like receptor 4	AB080363.1	NM 001002950.1	120
Cf02710573_s1	TLR7	Toll-like receptor 7	DQ333224.1;AB248956.1	NM_001048124.1	124
Cf02626256_m1	HPRT1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	AY283372.1	NM_001003357.1	102
Cf02659077_m1	B2M	beta-2-microglobulin	-	XM_845055.1;XM_535458.2	87

[a]All the assays are commercially available from Thermo Fisher scientific.

Two parameters were considered to quantify reference gene stability: M value (average expression stability) and Vn/n + 1 (pairwise variation). The lowest M values have the most stable expression genes and therefore the best candidates to act as housekeeping genes were β-2M and HPRT-1 (Figures 3.5.1. and 3.5.2.). The two selected reference genes were used to calculate relative quantification of target genes (TLR2, TLR4 and TLR7). The gene expression of each TLR transcript in macrophages was determined by PCR relative quantification calculated by 2ΔΔCt method. Each transcript is expressed as the mean of three independent experiments with three replicates ± SD. Rt-PCR procedures were performed as previously described in chapter 3.1. (Montserrat-Sangra et al., 2016).

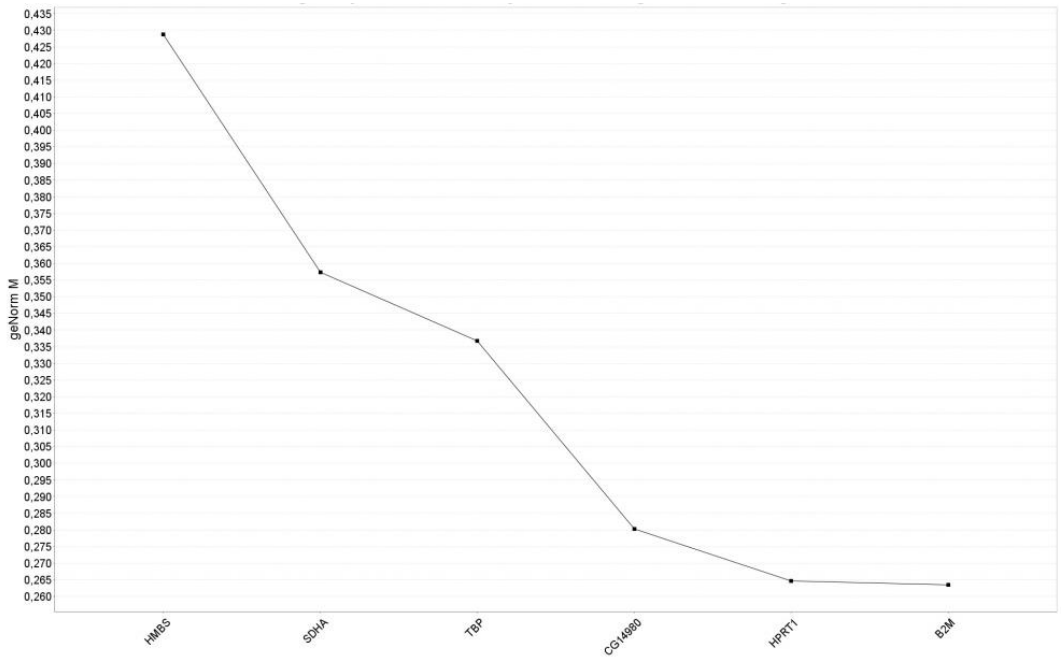

Figure 3.5.1. Average expression stability (M value) based on GeNorm program of six references genes studied in DH82 cells.

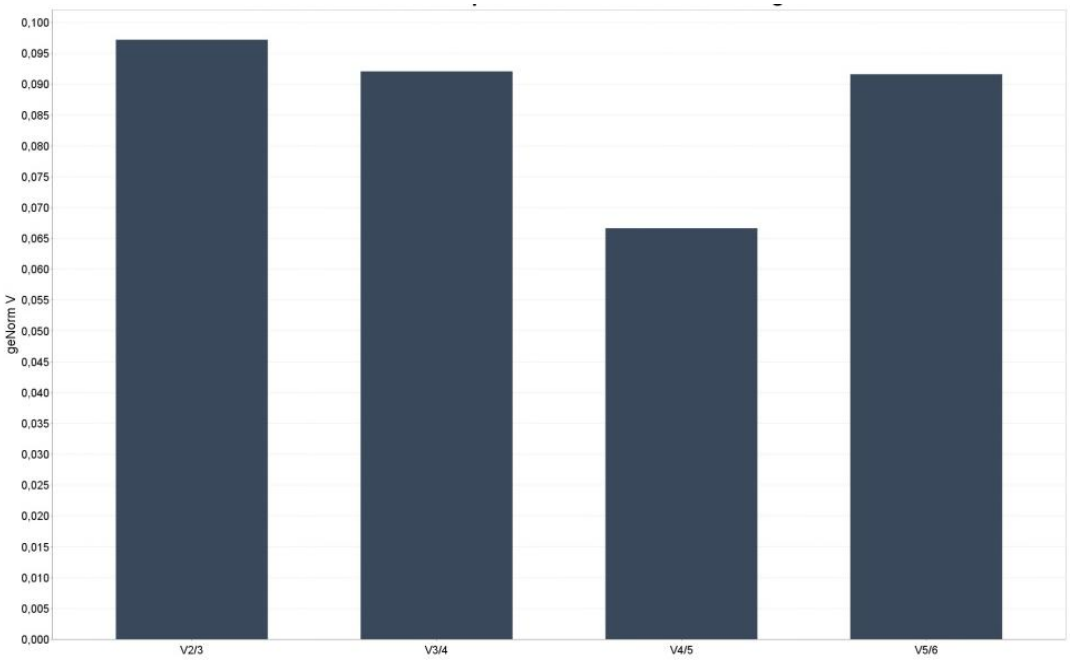

Figure 3.5.2. Determination of the optimal number of reference genes in DH82 cells. Brown line indicates Vn/n+1 value that needs to be below 0.15. Two references genes were selected based on results of Vn/n+1 value.

Statistical analysis

The half Inhibitory Concentration (IC50) was calculated through GraphPad Prism 5.0 (GraphPad Software, San Diego, USA), and "Statistical Package for Social Sciences" (SPSS®) 17.0 software (SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.).

The non-parametric Wilcoxon Signed-Rank test was used to evaluate differences between several conditions. The Wilcoxon Signed-Rank test was performed with SPSS Statistics 17.0. A significant P value was considered at ≤ 0.05.

Results

The half inhibitory concentration (IC50) promastigote susceptibility

The IC50 value for allopurinol, miltefosine and meglumine antimoniate was 0.50 mg/mL, 0.53 µL/ mL and 79.61 mg/ mL, respectively.

Percentage of infected cells

The percentage of infection is shown in table 3.5.2. Percentage of infection was significantly lower in cells treated with TLR2a (P=0.001), TLR3a (P=0.000), TLR4a (P=0.001), TLR7a (P=0.001), allopurinol (P=0.043), miltefosine (P=0.028) and meglumine antimoniate (P=0.043) alone when compared with untreated infected cells. Significant differences were found between infected macrophages treated with miltefosine and infected macrophages treated with TLR2a (P=0.028), TLR3a (P=0.043) and TLR4a (*P*=0.043). No other differences were found when comparing infected macrophages alone and infected macrophages treated with drugs in combination with other TLR agonists.

Table3.5.2. Percentage of infected macrophagic cells with L. infantum 72h after treatment with drugs and TLRs agonists.

Conditions with TLRs agonist	mean ± SD[a]	Conditions with allopurinol	mean ± SD[b]	Conditions with miltefosine	mean ± SD[c]	Conditions with meglumine antimoniate	mean ± SD[d]
M+Inf*	88.6 ± 5.2	**M+Inf+ALLOP**	78 ± 6.5	**M+Inf+MILT**	65.8 ± 4.1	**M+Inf+MEG**	77.3 ± 9.4
M+Inf+TLR2	77.8 ± 7.2	**M+Inf+ALLOP+TLR2**	83.2 ± 2.9	**M+Inf+ MILT+TLR2**	78.8 ± 5.5	**M+Inf+ MEG+TLR2**	78.8 ± 4.7
M+Inf+TLR3	80.4 ± 5.9	**M+Inf+ALLOP+TLR3**	85.7 ± 6.6	**M+Inf+ MILT+TLR3**	83.2 ± 8.2	**M+Inf+ MEG+TLR3**	77.3 ± 5
M+Inf+TLR4	78.8 ± 8.6	**M+Inf+ALLOP+TLR4**	81.3 ± 10.3	**M+Inf+ MILT+TLR4**	72.8 ± 5.7	**M+Inf+ MEG+TLR4**	66.8 ± 6.7
M+Inf+TLR7	73.2 ± 8	**M+Inf+ALLOP+TLR7**	73.7 ± 3.6	**M+Inf+ MILT+TLR7**	77 ± 9.4	**M+Inf+ MEG+TLR7**	73.3 ± 11.1

*Abbreviations: M: Macrophages; Inf: Infection; TLR: Toll-like receptor agonist; ALLOP: Allopurinol; MILT: Miltefosine; MEG: Meglumine antimoniate.

[a]M+Inf > M+Inf+TLR2, M+Inf > M+Inf+TLR3, M+Inf > M+Inf+TLR4, M+Inf > M+Inf+TLR7 ($P < 0.0001$)

[b]M+Inf > M+Inf+ALLOP and M+Inf+ALLOP < M+Inf+ALLOP+TLR2 ($P = 0.043$)

[C]M+Inf > M+Inf+MILT and M+Inf+MILT < M+Inf+TLR2 ($P = 0.028$)

M+Inf+MILT < M+Inf+TLR3, M+Inf+MILT < M+Inf+TLR4 ($P = 0.043$)

[d]M+Inf > M+Inf+MEG ($P = 0.04$)

Intensity of infection

The results of intensity of infection per each condition are summarized in table 3.5.3. A significant lower intensity of infection was noted when infected cells were treated with TLR2a (P=0.001), TLR3a (P<0.0001), TLR4a (P<0.0001), TLR7a (P<0.0001), allopurinol (P=0.028), miltefosine (P=0.028) and meglumine antimoniate (P=0.043) alone and with combinations of allopurinol + TLR4a (P=0.043) and allopurinol + TLR7a (P=0.027) when compared with untreated infected cells.

Table3.5.3. Intensity of L. infantum macrophagic infection in vitro during 24h of L. infantum promastigotes infection and 72h of drugs and TLRs agonists incubation after infection.*

Conditions with TLRs agonists	Amastigotes mean/cell ± SD[a]	Conditions with allopurinol	Amastigotes mean/cell ± SD[b]	Conditions with miltefosine	Amastigotes mean/cell ± SD[c]	Conditions with meglumine antimoniate	Amastigotes mean/cell ± SD[d]
M+Inf	4.2 ± 1.0	**M+Inf+ALLOP**	3.1 ± 1.1	**M+Inf+MILT**	2.2 ± 0.2	**M+Inf+MEG**	2.2 ± 0.5
M+Inf+ TLR2	3.3 ± 1.1	**M+Inf+ALLOP+TLR2**	2.9 ± 1.4	**M+Inf+MILT+ TLR2**	2.2 ± 0.5	**M+Inf+MEG+ TLR2**	2.7 ± 0.3
M+Inf+ TLR3	3.1 ± 0.8	**M+Inf+ALLOP+TLR3**	3 ± 1.3	**M+Inf+MILT+ TLR3**	2.2 ± 0.4	**M+Inf+MEG+ TLR3**	2.6 ± 0.3
M+Inf+ TLR4	3.5 ± 1.0	**M+Inf+ALLOP+TLR4**	1.7 ± 0.3	**M+Inf+MILT+ TLR4**	2.2 ± 0.1	**M+Inf+MEG+ TLR4**	2.2 ± 0.3
M+Inf+ TLR7	3.0 ± 0.8	**M+Inf+ALLOP+TLR7**	2 ± 1.0	**M+Inf+MILT+ TLR7**	2.1 ± 0.2	**M+Inf+MEG+ TLR7**	2.3 ± 0.7

Abbreviations: M: Macrophages; Inf: Infection; TLR: Toll-like receptor agonists; ALLOP: Allopurinol; MILT: Miltefosine; MEG: Meglumine antimoniate.

[a]M+Inf > M+Inf+TLR2, M+Inf > M+Inf+TLR3, M+Inf > M+Inf+TLR4, M+Inf > M+Inf+TLR7 (P < 0.0001)

[b]M+Inf+ALLOP > M+Inf+ALLOP+TLR4 (P = 0.043)

M+Inf+TLR4 > M+Inf+ALLOP+TLR4 (P = 0.043)

M+Inf+ALLOP > M+Inf+ALLOP+TLR7 (P = 0.028)

[c]M+Inf+MILT < M+Inf (P = 0.028)

M+Inf+MILT < M+Inf+TLR2 (P = 0.028)

M+Inf+MILT > M+Inf+TLR3 (P = 0.046)

[d]M+Inf+MEG < M+Inf (*P* = 0.043)

TNF-α and NO concentrations

TNF-α concentration was not detected in any condition studied at 72h. In the additional study performed with non-infected DH82 cells stimulated with the different TLRa at 24, 48 and 72 hours, TNF-α concentration was maximum at 24 h and after that a decrease was noted with no detectable levels at 72 h in the majority of TLRa (figure 3.5.3). NO concentration was also undetectable in all conditions studied at 72 h.

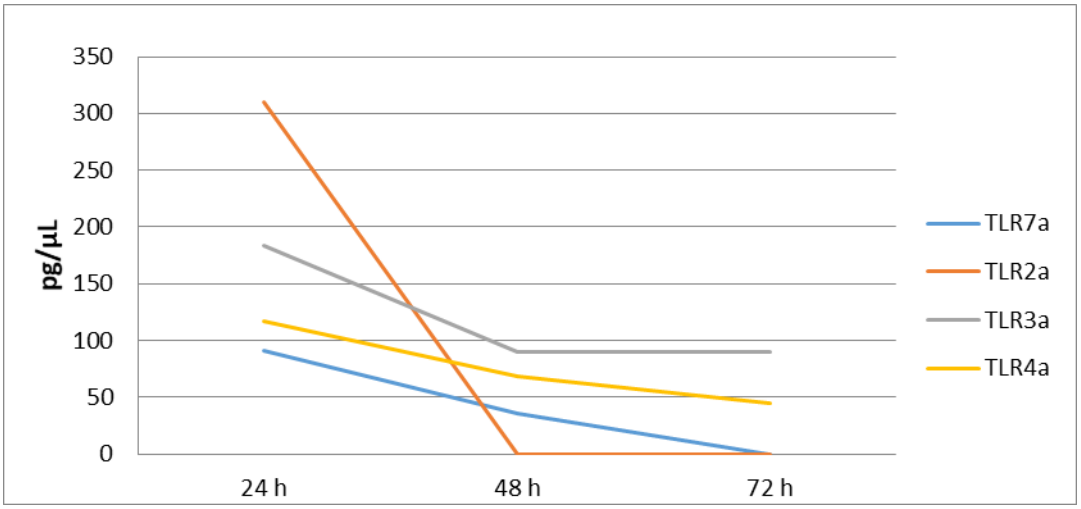

Figure 3.5.3. Mean TNF-α concentration in cultured macrophages stimulated with four TLRs agonists at three different time points

TLR2, TLR4 and TLR7 transcripts

The results of TLR2, TLR4 and TLR7 transcripts are summarized in table 3.5.4. A downregulation of TLR2 transcript and no changes of TLR4 and TLR7 expression were found in infected macrophages in comparison with non-infected ones. Interestingly, TLR7 uregulation was noted when infected cells were treated with miltefosine alone as well as a TLR4 uregulation in non infected cells treated with allopurinol. In contrast, TLR2

downregulation was found after treatment with allopurinol and miltefosine in non-infected cells. Generally, TLRs upregulation was observed when infected macrophages were treated with TLRs agonists alone. However, when drugs were added in combination with TLRs agonists, the TLRs gene expression was variable.

TLR2 gene expression

Differences were observed on TLR2 transcripts between non-infected macrophages (mean 1.2 ± 0.4) and infected macrophages (mean 1.1 ± 0.6, P = 0.05) and also between TLR2a + miltefosine non infected macrophages (mean 4.1 ± 3.5) and TLR2a + miltefosine infected macrophages (mean 0.6 ± 0.3, P = 0.012) (table 3.5.4). Moreover, a strong trend of high TLR2 gene expression in infected macrophages (mean 1.3 ± 0.9) compared with non-infected after stimulation with TLR2a was found (mean 1.0 ± 0.7, P = 0.062).

When non-infected DH82 cells were compared with those not infected but treated with the different compounds, the expression of TLR2 gene had significant differences when stimulation with TLR2a (mean 0.9 ± 0.7), TLR3a (mean 0.6 ± 0.5, P = 0.001), TLR7a (mean 0.6 ± 0.5, P = 0.008) and allopurinol (0.8 ± 0.4, P=0.025) and miltefosine (0.6 ± 0.6, P= 0.018). The combination of TLR2a + allopurinol (mean 0.5 ± 0.3) had higher TLR2 expression compared with TLR3a + allopurinol (mean 0.3 ± 0.2, P = 0.036). TLR2a + miltefosine (4.1 ± 3.5) had a significant higher TLR2 expression when compared with TLR4a + miltefosine (mean 1.1 ± 1.1, P = 0.046) and TLR7a + miltefosine (mean 2.3 ± 2.6, P = 0.028).

When infected DH82 cells were compared with those infected and treated with the studied drugs and TLRs, the upregulation of TLR2 transcripts was significantly marked in macrophages stimulated with TLR2a (mean 1.3 ± 0.9) when compared with TLR3a (0.8 ± 0.6, P < 0.0001), TLR4a (mean 0.6 ± 0.4, P = 0.002) and TLR7a (mean 0.7 ± 0.6, P = 0.002).

TLR2 transcription was significantly different (P =0.008) between TLR2a+ allopurinol (mean 0.7 ± 0.5) and TLR3a +allopurinol (mean 0.4 ± 0.2). Significant differences on TLR2 transcript between TLR2a + miltefosine (0.6 ± 0.3) TLR3a + miltefosine (1.5 ± 1.4, P = 0.043) and TLR4a + miltefosine (mean 1.9 ± 0.9, P = 0.018) were also observed. Significant difference on TLR2 transcripts was noted between TLR2a + meglumine (mean 0.5 ± 0.4) and TLR7a + meglumine (mean 0.6 ± 0.3, P = 0.028).

Table 3.5.4. TLR2 gene expression in canine macrophages treated with TLRs agonists or conventional anti-Leishmania drugs alone

Conditions*	TLR2 (mean±SD)	Conditions	TLR2 (mean+SD)
M	1.2 ± 0.4[a,w,y]	M+Inf	1.1 ± 0.5 [a]
M+TLR2	1.0 ± 0.7[b,c]	M+ Inf +TLR2	1.3 ± 0.9[d,e,f,k,]
M+TLR3	0.6 ± 0.5[b,]	M+ Inf +TLR3	0.8 ± 0.6 [d]
M+TLR4	0.8 ± 0.7	M+ Inf +TLR4	0.6 ± 0.4[e]
M+TLR7	0.6 ± 0.5[c,]	M+ Inf +TLR7	0.7 ± 0.6[f]
M+ ALLOP	0.8 ± 0.4[w]	M+Inf+ALLOP	0.7 ± 0.6
M+ ALLOP+TLR2	0.5 ± 0.3[g,h]	M+Inf+ALLOP+TLR2	0.7 ± 0.5 [k]
M+ ALLOP+TLR3	0.3 ± 0.2[g,i]	M+Inf+ALLOP+TLR3	0.4 ± 0.2 [k,l]
M+ ALLOP+TLR4	0.5 ± 0.4[j]	M+Inf+ALLOP+TLR4	0.3 ± 0.2[m]
M+ ALLOP+TLR7	0.8 ± 0.7 [h,j]	M+Inf+ALLOP+TLR7	0.7 ± 0.6 [c,l,m]
M+ MILT	0.6 ± 0.6[y]	M+Inf+MILT	1.13 ± 0.6
M+ MILT+TLR2	4.1 ± 3.5[n,o,p]	M+Inf+ MILT+TLR2	0.6 ± 0.3[n,r,s]
M+ MILT+TLR3	1.0 ± 0.7	M+Inf+ MILT+TLR3	1.5 ± 1.4[r,t]
M+ MILT+TLR4	1.1 ± 1.1 [o,q]	M+Inf+ MILT+TLR4	1.9 ± 0.9[s]
M+ MILT+TLR7	2.3 ± 2.56[p,q]	M+Inf+ MILT+TLR7	0.8 ± 0.4[t]
M+ MEG	0.8 ± 0.4	M+Inf+MEG	0.8 ± 0.6
M+ MEG+TLR2	0.3 ± 0.2[u,]	M+Inf+ MEG+TLR2	0.5 ± 0.4[v]
M+ MEG+TLR3	0.4 ± 0.2 [u]	M+Inf+ MEG+TLR3	0.4 ± 0.3
M+ MEG+TLR4	0.5 ± 0.2[g]	M+Inf+ MEG+TLR4	0.9 ± 0.9
M+ MEG+TLR7	0.9 ± 0.3	M+Inf+ MEG+TLR7	0.6 ± 0.3 [v]

*Abbreviations: M: Macrophages; TLR: Toll like receptor; Inf: infection; ALLOP: Allopurinol; MILT: Miltefosine; MEG: Meglumine antimoniate; SD: Standard deviation.

[a]P = 0.050, [b]P = 0.001, [c]P = 0.008, [d]P < 0.0001, [e]P = 0.002, [f]P = 0.002,[g]P = 0.036, [h]P = 0.012, [i]P = 0.012, [j]P = 0.063, [k]P = 0.008, [l]P = 0.008, [m]P = 0.008, [n]P = 0.012, [o]P = 0.046, [p]P = 0.028, [q]P = 0.043, [r], [s]P = 0.018, [t]P = 0.043, [u]P = 0.063, [v]P = 0.028, [w]P = 0.025, [y]P = 0.018

TLR4 gene expression

TLR4 transcription was significantly different between non infected (mean 1.1 ± 0.3) and infected cells stimulated with TLR2a (mean 1.8 ± 1.0, P = 0.043) and between non-infected cells and infected cells stimulated with TLR4a (mean 2.0 ± 0.4, P = 0.012) as well as between non-infected cells and infected cells treated either with TLR3a + allopurinol (P = 0.025) or with TLR2a + meglumine (P = 0.012) (Table 3.5.5).

When non-infected DH82 cells were compared with different treatments, TLR4 transcription was significantly different between TLR2a (mean 0.9 ± 0.4), TLR3a (mean 0.5 ± 0.3, P = 0.007), TLR7a (mean 0.5 ± 0.4, P = 0.010) and in those stimulated with allopurinol (1.6 ± 0.4, P= 0.012). TLR2a + allopurinol group (mean 1.9 ± 1.6) had different TLR4 gene expression compared with TLR4a + allopurinol (mean 4.1 ± 0.4, P=0.008) and TLR7a+ allopurinol (mean 4.1 ± 4.3, P = 0.015). TLR4 expression was also noted different between TLR2a + miltefosine (mean 19.3 ± 10.7) and TLR4a + miltefosine (mean 5.8 ± 2.3, P = 0.043). Differences on TLR4 transcripts between TLR2a + meglumine (mean 1.9 ± 1.3) and TLR4a + meglumine (mean 0.9 ± 0.5, P = 0.012) and TLR7a + meglumine (mean 1.4 ± 0.9, P = 0.036) were also observed.

When infected DH82 cells were compared with different treatments, TLR2a alone (mean 1.7 ± 0.9) generated a higher expression of TLR4 gene when compared with TLR3a alone (1.0 ± 0.9, P = 0.019). TLR4 transcripts were also different in TLR2a + allopurinol (mean 3.4 ±3.6) compared with TLR3a + allopurinol (mean 1.0 ± 0.9, P = 0.051) and TLR4a + allopurinol (mean 2.0 ± 1.8, P = 0.046). TLR2a + miltefosine (mean 9.4 ± 6.5) provided a higher expression of TLR4 transcript compared with TLR4a + miltefosine (mean 6.2 ± 5.6, P = 0.028).

Table 3.5.5. TLR4 gene expression in canine macrophages treated with TLRs agonists or

Conditions*	TLR4 (mean±SD)	Conditions	TLR4 (mean+SD)
M	1.1 ± 0.3[v]	M+Inf	1.8 ± 1.0
M+TLR2	0.9 ± 0.4[a,c,d]	M+ Inf +TLR2	1.8 ± 0.9[a,e]
M+TLR3	0.5 ± 0.3[c]	M+ Inf +TLR3	1.1 ± 1.0[e,f]
M+TLR4	0.5 ± 0.3[b]	M+ Inf +TLR4	2.0 ± 1.8[b,f,g]
M+TLR7	0.5 ± 0.4[d]	M+ Inf +TLR7	1.0 ± 1.3 [p,g]
M+ ALLOP	1.6 ± 0.4[v]	M+Inf+ALLOP	4.0±4.5[t]
M+ ALLOP+TLR2	1.9 ± 1.6[i,j]	M+Inf+ALLOP+TLR2	3.4±3.6[l,m]
M+ ALLOP+TLR3	2.1 ± 2.1[h,k]	M+Inf+ALLOP+TLR3	3.1±2.9[h,l,n]
M+ ALLOP+TLR4	4.1 ± 0.4[i]	M+Inf+ALLOP+TLR4	1.2±0.4[m,n,o]
M+ ALLOP+TLR7	4.1 ± 4.3[j,k]	M+Inf+ALLOP+TLR7	1.7±0.6[j,o]
M+ MILT	2.5 ± 4.3	M+Inf+MILT	2.0 ± 0.3
M+ MILT+TLR2	19.3 ± 10.7	M+Inf+ MILT+TLR2	9.4 ± 6.5[p]
M+ MILT+TLR3	6.3 ± 1.2	M+Inf+ MILT+TLR3	15.4 ± 12.7[q]
M+ MILT+TLR4	5.8 ± 2.3	M+Inf+ MILT+TLR4	6.2 ± 5.6[p,q,r]
M+ MILT+TLR7	6.5 ± 0.9	M+Inf+ MILT+TLR7	11.4 ± 8.3[r]
M+ MEG	1.2 ± 0.6	M+Inf+MEG	5.9 ± 7.8
M+ MEG+TLR2	1.9 ± 1.3[s,t,u]	M+Inf+ MEG+TLR2	0.6 ± 0.2[s]
M+ MEG+TLR3	1.2 ± 0.3	M+Inf+ MEG+TLR3	0.4 ± 0.2
M+ MEG+TLR4	0.9 ± 0.5 [t]	M+Inf+ MEG+TLR4	34.9 ± 68.2
M+ MEG+TLR7	1.4 ± 0.9[u]	M+Inf+ MEG+TLR7	0.9 ± 0.5

conventional anti-Leishmania drugs alone

*Abbreviations: M: Macrophages; TLR: Toll like receptor; Inf: infection; ALLOP: Allopurinol; MILT: Miltefosine; MEG: Meglumine antimoniate; SD: Standard deviation.

[a]P = 0.043, [b]P = 0.012, [c]P = 0.007, [d]P = 0.010, [e]P = 0.019, [f]P = 0.001, [g]P = 0.034, [h]P = 0.025, [i]P = 0.008, [j]P = 0.015, [k]P = 0.017, [l]P = 0.051, [m]P = 0.046, [n]P = 0.028, [o]P = 0.028, [p]P = 0.028, [q]P = 0.046, [r]P = 0.043, [s]P = 0.025, [t]P = 0.012, [u]P = 0.036, [v]P = 0.012.

TLR7 gene expression

Differences were noted on TLR7 transcription between non-infected cells and infected cell treated with TLR2a (mean 1.3 ± 1.4, P = 0.028) (Table 3.5.6). TLR7 expression was also different when cells were treated with several combinations with miltefosine as listed below: 1) TLR2a + miltefosine non-infected cells (mean 18.3 ± 15.2) *versus* TLR2a + miltefosine infected cells (mean 11.3 ± 7.4, P = 0.043); 2) TLR3a + miltefosine non infected

cells (mean 22.3 ± 15.9) *versus* TLR3a + miltefosine infected cells (mean 11.2 ± 15.0, P = 0.012) and 3) TLR7a + miltefosine non infected cells (mean 34.3 ± 21.8) *versus* TLR7a + miltefosine infected cells (mean 5.1 ± 3.9, P = 0.028). Furthermore, differences were noted on TLR7 transcription as follows: 1) TLR2a + meglumine non infected cells (mean 2.2 ± 2.4) *versus* TLR2a + meglumine infected cells (mean 1.0 ± 0.2, P = 0.018); 2) TLR7a + meglumine non-infected cells (mean 0.8 ± 1.1) *versus* TLR7a + meglumine infected cells (mean 0.7 ± 0.6, P = 0.008).

Higher TLR7 gene expression was noted in non-infected cells after stimulation of TLR2a when compared with TLR7a (P = 0.006) as well as in those stimulated withTLR2a+ miltefosine and TLR3a+ miltefosine (P =0.028).

In infected macrophages, differences on TLR7 transcripts were observed between infected macrophages (0.9 ± 1.3) and infected macrophages treated with allopurinol (0.2 ± 0.2, P= 0.036) and also when infected macrophages where treated with miltefosine (6.1 ± 7.3) compared to infected non treated macrophages (P = 0.012). Further significant differences are described below: 1) TLR2a alone (mean 1.4 ± 1.2) *versus* TLR3a alone (mean 1.8 ± 4.0, P = 0.004), 2) TLR2a+ miltefosine (mean 11.3 ± 7.4) *versus* TLR3a + miltefosine (mean 11.4 ± 15.0, P = 0.028); 3) TLR2a + meglumine (mean 1.0 ± 0.6) *versus* TLR3a + meglumine (mean 0.4 ± 0.2, P = 0.018) and 4) TLR4a + meglumine (mean 0.2 ± 0.2, P = 0.028) and TLR7a + meglumine (mean 0.8 ± 0.6, P = 0.021).

Table 3.5.6. TLR7 gene expression in canine macrophages treated with TLRs agonists or conventional anti-Leishmania drugs alone

Conditions*	TLR7 (mean±SD)	Conditions	TLR7 (mean+SD)
M	0.9 ± 0.4	M+Inf	0.9 ± 1.3[u,v]
M+TLR2	1.3 ± 1.4[a]	M+ Inf +TLR2	1.4 ± 1.2[a,c]
M+TLR3	1.2 ± 1.1	M+ Inf +TLR3	1.8 ± 3.4[c,d,e]
M+TLR4	1.2 ± 0.7[b]	M+ Inf +TLR4	3.5 ± 5.3[b,d]
M+TLR7	0.6 ± 0.4	M+ Inf +TLR7	0.9 ± 0.3[e]
M+ ALLOP	0.3 ± 0.2	M+Inf+ALLOP	0.2 ± 0.2[u]
M+ ALLOP+TLR2	1.2 ± 0.9	M+Inf+ALLOP+TLR2	1.8 ± 1.5[g]
M+ ALLOP+TLR3	0.7 ± 0.5[f]	M+Inf+ALLOP+TLR3	0.5 ± 0.5 [g,h]
M+ ALLOP+TLR4	1.9 ± 1.8[f]	M+Inf+ALLOP+TLR4	1.5 ± 07[h]
M+ ALLOP+TLR7	1.5 ± 1.2	M+Inf+ALLOP+TLR7	2.2 ± 2.4
M+ MILT	21.9 ± 27.7	M+Inf+MILT	6.1 ± 7.3[v]
M+ MILT+TLR2	18.3 ± 15.2[i,l]	M+Inf+ MILT+TLR2	11.4 ± 7.4[i,n]
M+ MILT+TLR3	22.3 ± 15.9[j,l,m]	M+Inf+ MILT+TLR3	11.4 ± 15.0[j,n]
M+ MILT+TLR4	41.9 ± 20.7	M+Inf+ MILT+TLR4	4.0 ± 3.4
M+ MILT+TLR7	34.3± 21.8[k,m]	M+Inf+ MILT+TLR7	5.1 ± 3.9[k]
M+ MEG	0.8 ± 0.3	M+Inf+MEG	2.0 ± 2.6
M+ MEG+TLR2	2.2 ± 2.4[o]	M+Inf+ MEG+TLR2	1.0 ± 0.6[o,q,r]
M+ MEG+TLR3	1.7 ± 1.9	M+Inf+ MEG+TLR3	0.4 ± 0.2[q,s]
M+ MEG+TLR4	1.1 ± 0.8[p]	M+Inf+ MEG+TLR4	0.2 ± 0.2[p,s]
M+ MEG+TLR7	0.8 ± 1.1	M+Inf+ MEG+TLR7	0.8 ± 0.6[j,r]

*Abbreviations: M: Macrophages; TLR: Toll like receptor; Inf: infection; ALLOP: Allopurinol; MILT: Miltefosine; MEG: Meglumine antimoniate; SD: Standard deviation.

[a]P = 0.028, [b]P = 0.012, [c]P = 0.004, [d]P = 0.002, [e]P = 0.028, [f]P = 0.008, [g]P = 0.063, [h]P = 0.008, [i]P = 0.043, [j]P = 0.012, [k]P = 0.028, [l]P = 0.028, [m]P = 0.018, [n]P = 0.028, [o]P = 0.018, [p]P = 0.008, [q]P = 0.018, [r]P = 0.028, [s]P = 0.021, [u]P = 0.036, [v]P = 0.012.

Discussion

Monitoring *Leishmania* susceptibility to conventional drugs and to new compounds *in vitro* is a requirement to evaluate drug efficacy and to discover an adequate system to optimize the therapy in *Leishmania* infections (Maia et al., 2013) as well as to determine drug resistance as proven in humans (Croft et al., 2006; Hendrickx et al., 2018) and dogs (Gramiccia et al., 1992; Yasur-Landau et al., 2016; Yasur-Landau et al., 2017) for different

conventional anti-*Leishmania* drugs. In the present study, the most potent drug to reduce by half the number of promastigotes was miltefosine (0.53 µg/ mL) and the less potent was meglumine antimoniate (79.61 mg/ mL), which is in agreement with previous results obtained using a *L.* infantum (IMT373) strain isolated from a human (Maia et al., 2013). The IC susceptibility found in the present study for miltefosine was 10 folds lower than the study described above (Maia et al., 2013) but the experiment conditions were slightly different. It is already described that medium cultures can influence the adaptavility and the parasite growth (Oliveira, 2011) and also IC50 is different depending on drugs used and the parasite drug resistance (Carrió, 2002).

In vitro macrophagic assays evaluating the susceptibility of *L. infantum* strains to miltefosine are scarce in the literature (Maia et al., 2013). In another study using another *Leishmania* species, the miltefosine susceptibility of *Leishmania donovani* promastigotes was also confirmed (Kulshrestha et al., 2013). In a *in vivo* murine model study demonstrated that miltefosine is active against *L. donovani* and *L. infantum* (Kuhlencord et al., 1992) and Escobar and colleages demonstrated that *L. donovani* was the most susceptible specie to miltefosine when compared with other species such as *L.aethiopica, L. tropica, L. mexicana, L. panamensis* and *L. major* (Escobar et al., 2002). In addition, the IC50 of miltefosine in other studies are similar to the ones obtained in the present study. The findings of this study and previous studies (Kuhlencord et al., 1992) suggest that the strains of *L. donovani* complex (*L. donovani* and *L. infantum*) appear to present a similar susceptibility to miltefosine (Escobar et al., 2002).

Allopurinol is also a common drug used in CanL treatment alone or in combination with miltefosine (Gradoni et al., 2008; Solano-Gallego et al., 2009). However, allopurinol treatment alone appear to lead to relapse in dogs (Saridomichelakis et al., 2005; Yasur-Landau et al., 2016). Furthermore, there is new evidence on literature that parasite seems to adapt easily to this compound (Yasur-Landau et al., 2018; Yasur-Landau et al., 2017) and develop drug resistance (Yasur-Landau et al., 2016). The findings of these studies question the use of allopurinol as long-term monotherapy treatment due to the risk of

relapse (Yasur-Landau et al., 2016). In the same study (Yasur-Landau et al., 2016), the *in vitro* susceptibility of promastigotes isolated from dogs with clinical signs after three or more months of treatment, was 0.9 mg/mL but this was the highest dose compared with dogs treated for the first time (0.2 mg/mL) or asymptomatic dogs (0.2 mg/mL). In the present study, we found an allopurinol concentration of 0.5 mg/mL and therefore, in between, of the highest and lowest concentration from the previous studied mentioned above (Yasur-Landau et al., 2016). However, it is important to highlight that in another study, IC50 susceptibility for allopurinol was 3.7 mg/mL with the same strain (Maia et al., 2013) employed in the present study, seven fold higher approximately compared with the present results. Again, the different conditions and parasite strains might be the reasons of the different IC50 values between studies.

Regarding to meglumine antimoniate, Faraut-Gambarelli et al. (1997) observed a primary and secondary unresponsiveness to this drug in human leishmaniosis (Faraut-Gambarelli et al., 1997). In the present study, meglumine antimoniate was the less powerful drug for killing promastigotes *in vitro*. Interestingly, in a previous study where the *in vitro* susceptibility of this strain was also tested (Maia et al., 2013), the IC50 (7.3 mg/mL) was ten folds less than in the present study which might be due to the different batch of the drug or to the selection of less sensitive clones to this drug among the cultured parasites. In a Brazilian study different sensitivity to meglumine antimoniate depending on the *Leishmania* species was observed. The IC50 of *Leishmania braziliensis* to this drug ranged between 0.8 to 9.5 mg/mL, whereas other *Leishmania* species such as *L. tropica, L. mexicana* or *L. amazonensis* had values ranging from 10-60 mg/mL (Rilza, 2007). Therefore, *Leishmania* species is an influence factor to determine the IC50 susceptibility to this drug.

In the present study, the percentage and intensity of infection was significantly lower in macrophagic cells treated with all conventional drugs studied alone when compared with untreated infected cells. These findings are not surprising due to the fact that it is

well known that these three drugs tested have leishmanicidal and leishmaniostatic effects in *L. infantum* infection in murine models (Borborema et al., 2018; Farca et al., 2012). *Leishmania infantum in vitro* susceptibility assays to the common drugs used in human and canine leishmaniosis treatment are scarce (Gomez Perez et al., 2016; Maia et al., 2013). Besides, there are only few studies that approached similar experiments *in vitro* macrophagic assays (Maia et al., 2013). In primary mouse peritoneal macrophages, a slightly drug potency with miltefosine against intracellular *Leishmania donovani* amastigotes with IC50 ranged from 0.16 to 1.5 µg/mL was found (Vermeersch et al., 2009). The drugs tested are used currently to treat leishmaniosis in dogs (Miró, 2006; Miro et al., 2009). Therefore, our results of the diminution of the percentage of infection and the intensity of infection are in concordance with update research published (Manna et al., 2008).

Despite there is a long way to go, some encouraging results were found using TLRs agonists in macrophages cultures in the present study. The percentage and intensity of infection was significantly lower in cells treated with all TLRs agonists alone when compared with untreated infected cells. These results are in agreement with the ones found in a similar canine experimental study using DH82 cells infected with *L. infantum* after stimulation with TLR2 agonist (Hosein, 2014). There are limited studies about immunotherapies applied to CanL using TLRs agonist with promising results (Miro et al., 2017; Roatt et al., 2017; Toepp et al., 2018). In an *in vivo* mouse model using TLR2a protection against *L. major* infection was achieved (Huang et al., 2015). TLR7 agonists such as imiquimod or resquimod were studied as topically or systematically administration and demonstrated anti-leishmanial activity alone in *in vivo* murine models against *L. infantum* (Craft et al., 2014) and *L. donovani* infections (Buates and Matlashewski, 1999; Peine et al., 2014).

Interestingly, the combinations of allopurinol with TLR4a or with TLR7a reduced the number of amastigotes per cell when compared with untreated infected cells. Allopurinol

is the most common drug used for CanL and is well known that is an effective drug for canines. However, long-term treatment is frequently needed in dogs with moderate to severe disease (Miro et al., 2009). Moreover, it is commonly used in combination with meglumine antimoniate or miltefosine (Miro et al., 2009; Travi et al., 2018) to improve clinical disease in dogs with moderate to severe disease. In fact, many evidences have shown that allopurinol should be used in combination with another compound to prevent the relapse of canine patients (Maia et al., 2013; Yasur-Landau et al., 2016). Moreover, long-term treatment with allopurinol can induce urinary adverse side effects in dogs (Torres et al., 2016). Hence, we might found a great combination to test clinically in dogs to reduce shortcomings of current allopurinol treatment in canines. The combination of allopurinol with TLR agonists might permit shorten the length of treatment and decrease the dose currently used. It is important to highlight that combination of TLR agonist such as imiquimod and conventional anti-*Leishmania* drugs such as meglumine antimoniate has been proved to be efficacious in human cutaneous leishmaniosis (Arevalo et al., 2001; Shamsi Meymandi et al., 2011). In an experimental *in vivo* murine visceral leishmaniosis model an enhancement in the therapeutic efficacy of miltefosine in combination with a synthetic TLR2 agonist (Pam3Cys) was demonstrated (Shakya et al., 2012). However, so far, these types of treatments have not been employed clinically in sick dogs infected with *L. infantum*.

Even though TNF-α were not detected at 72 h for any treatment condition, it is properly known that TLRs can modulate Th1 pathway responses causing the secretion of inflammatory cytokines in dogs (Martinez-Orellana., 2017) and immunoprotective response components (Melo et al., 2014b). It is very likely that the absence of production of this cytokine was due to the kinetics of its production in this model as explained in result section. Moreover, a previous study demonstrated that TLR2, TLR4 and TLR3 agonists were able to induce the secretion TNF-α and IL-6 by non-infected and *L. infantum* infected DH82 cells at 48 h (Hosein, 2014; Martinez-Orellana., 2017). Moreover, another experiment showed that TLR-2 and TLR-4 agonist enhances the production of inflammatory cytokines in a canine *ex-vivo* whole blood assay model (Martinez-Orellana et

al., 2017b). In addition, a combination of TLR-2 and TLR-4 agonist with *L. infantum* antigen promoted a synergistic pro-inflammatory effect with TNF-α (Martinez-Orellana., 2017).

Furthermore, to the best knowledge of the authors, this study was the first to investigate cytokines and TLRs in this amastigotes-canine DH82 cells assay with conventional antileishmanial drugs as previously studied in an *ex vivo* model with humans PBMC (Gonzalez-Fajardo et al., 2015). Unfortunately, this study fails to detect cytokine production after *in vitro* treatment of canine cells with conventional drugs. It is important to remark that in other similar *in vitro* assays with different *Leishmania* species treatment with miltefosine elicited cytokine and TLRs expression (Das et al., 2012; Mukherjee et al., 2012). Nitric oxide was also not detectable in the present study as previously reported in a similar model performed in dogs (Hosein, 2014) as well as in canine PBMCs (Esch et al., 2013). It is important to highlight that canines appear to produce more reactive oxygen (ROS) substances than NO (Esch et al., 2013; Panaro et al., 2008) probably due to the fact that they appear to have a reduced activity of enzyme inducible nitric oxide syntase (iNOS) (Nathan, 2006) as humans. Unfortunately, in the present study was not possible to measure ROS instead of NO. In agreement with the other immunological findings of the present study, minor differences were observed on TLRs transcripts when conventional antileishmanial drugs were tested alone. For example, an upregulation of TLR4 was obtained in non infected cells when treated with allopurinol opposite of what was found previously in a Western diet induced obesity murine model (Aroor et al., 2017) in kidney cells treated with allopurinol. Furthermore, a TLR7 upregulation was observed when cells were treated with miltefosine. It is interesting to highlight that miltefosine links to TLR4 (Das et al., 2012) but we only observed upregulation of TLR7 and not TLR4. The present findings are difficult to interpret due to the fact that similar studies are not available. Further studies need to investigate the immunological effect of conventional anti-*Leishmania* drugs in canines.

In vitro studies about TLRs expression are very scarce in the literature (Hosein, 2014). However, several studies describe the beneficial role of TLR involvement in the clinical resolution of parasitic infections, including in TLR pathways and *Leishmania* infection (Becker et al., 2003; Hosein, 2014). Interestingly, in the present study, no differences were found on TLR4 and TLR7 transcripts when infected and non-infected macrophages were compared and only a slightly downregulation on TLR2 expression was observed with infection which is in agreement with the well-known silent effect of the parasite on the host cell (Hosein, 2014). This process lets the parasite establish the infection while the immune system does not recognize the parasite and therefore, the parasite evades the immune system (Hosein et al., 2017). The delay on cytokines production was related to the silent establishment of the infection as previous observed in a murine model (Belkaid et al., 2001) and in dogs (Santos-Gomes et al., 2002).

In the present study, a higher expression of TLR2 in infected macrophages compared with non-infected after stimulation with TLR2a (Pam3CK4) was noted. These findings are in agreement with a previous study performed in similar conditions (Hosein, 2014). It has been reported that TLR2 agonist can restore the functional capacity of CD8 lymphocytosis when patients show characteristics of cellular exhaustion such is described in chronic infections (Hernandez-Ruiz et al., 2010). This ligand has been shown to have also a protective role in other diseases (Chen et al., 2009; Hosein, 2014). In addition, an upregulation of TLR2 was observed when cells were treated with miltefosine in combination with several TLRs agonists studied when compared with allopurinol or meglumine antimoniate.

A similar pattern was observed with TLR4 gene expression. Interestingly, a higher expression of TLR4 was found when infected cells were compared with non-infected cells after stimulation with TLR4a. TLR4 participate actively in the immune response against parasitic infections. Despite TLR4 is not as well described as TLR2 in *Leishmania* infection, it has been reported that through TLR4 pathway there is the production of proinflammatory cytokines such as TNF-α and IL-1 for the proper control of infection in

mice (Kropf et al., 2004a; Kropf et al., 2004b; Tolouei et al., 2013; Whitaker et al., 2008). Regarding TLR7 gene expression, no differences were found between control macrophages and infected macrophages after stimulation with TLR7a. TLR7 is intracellularly expressed on endosomes membranes where it recognizes nucleotides from intracellular pathogens (Mikami et al., 2012). It is well known that activating TLR7 induces an increase of IFN-α and IL-12, TNF-α and Th1 immune responses (Aderem and Ulevitch, 2000). In fact, the use of topical resiquimod (a TLR7) provided protection against *L. infantum* infection mice (Craft et al., 2014).

Conclusions

The present study gives new insights about the direct effect of the studied TLR agonists in reducing *L. infantum* infection in an *in vitro* canine model. In addition, allopurinol combined with TR4a appears to synergistically decrease the number of parasites in *L. infantum* infected cells. These results suggest that the use of these combinations may be helpful as alternative treatments in clinical canine leishmaniosis.

Funding

This study was supported by a Spanish Ministry grant, Ministerio de Economía y competitividad (AGL2015-68477) and by Fundação para a Ciência e a Tecnologia for funds to GHTM –UID/Multi/04413/2013. Dr. Laia Solano-Gallego held a Ramón y Cajal senior researcher contract awarded by the Ministerio de Ciencia e Innovación (Spain) and the European Social Fund. Dr. Carla Maia has the support of the Portuguese Ministry of Education and Science (via Fundação para a Ciência e a Tecnologia), through an Investigator Starting Grant (IF/01302/2015).

Acknowledgements

The authors thank *Instituto de Medicina e Higiene Tropical (Universidade NOVA de* Lisboa) of Lisboa its hospitality. We are especially grateful to Professor Aïda Esteves, MSc. José Manuel Cristóvão, Dr. Mónica Nunes and MSc André Pereira from IHMT for their technical assistance with cellular and molecular work and Dr. Pamela Martínez from UAB for her support and work.

CAPÍTULO 4

DISCUSIÓN / DISCUSSION

La leishmaniosis canina es una enfermedad con una inmunopatogénesis muy compleja así como el diagnóstico, el tratamiento y su prevención (Pennisi, 2015).

Se conoce que el parásito tiene varias estrategias de evasión del sistema inmunitario del hospedador, cuyos mecanismos le hace persistir y sobrevivir en el hospedador vertebrado (Gupta et al., 2013). La primera barrera que encuentra el parásito es el sistema inmunitario innato, el cual es poco específico para poder hacer frente con total éxito a la invasión de éste.

Los TLRs junto con otros receptores celulares juegan un papel muy importante en la respuesta inmunitaria y sobre todo en cómo se va a desarrollar la respuesta adaptativa (Kumar et al., 2009) frente a la infección por *L. infantum*. En los últimos años, la investigación sobre los TLRs ha ganado importancia en el terreno de la leishmaniosis murina y humana (Tuon et al., 2008) tanto en el estudio de la inmunopatogénesis como su uso en inmunoterapia e inmunomodulación (Faria et al., 2012). Así, los TLRs se han estudiado como herramienta adyuvante para los fármacos ya establecidos o también como adyuvantes para vacunas (Steinhagen et al., 2011; Zhang and Matlashewski, 2008).

Los experimentos llevados a cabo en este proyecto describen el papel que tienen losTLRs en la infección por *L. infantum* en el perro ya sea en condiciones naturales como en condiciones de cultivo *in vitro* o *ex vivo*.

Aunque la inmunidad innata se haya estudiado poco en leishmaniosis canina causada por *L. infantum* y por tanto también los TLRs, existe un estudio donde se demuestra que *L. infantum* aumenta la expresión de los TLR2 y TLR4 en el bazo durante la infección temprana en un modelo murino (Cezario et al., 2011). Sin embargo, aunque los modelos de ratón o hámster (Nieto et al., 2011) son buenos modelos de aproximación

para el perro, pueden existir diferencias entre los modelos de ratón o hámster y el modelo canino (Baneth et al., 2008; Hosein, 2014). Los estudios realizados en esta tesis doctoral demuestran por primera vez que la expresión de los receptores TLRs en sangre estimulada y no estimulada de perros se ve afectada por la infección por *Leishmania*.

Estudios con perros naturalmente infectados

Sangre no estimulada

En el estudio que se describe en capítulo 3.1., se encuentra una sobre expresión en la sangre no estimulada del gen TLR2 en el momento del diagnóstico en perros enfermos con leishmaniosis moderada. La transcripción de TLR2 se reduce con la instauración del tratamiento y mejoría clínica. Sin embargo, no se obtuvieron diferencias entre la transcripción del TLR4 entre perros enfermos y sanos en el momento del diagnóstico o durante el tratamiento. Existen resultados similares que dan robustez a nuestros hallazgos, donde en diferentes tejidos, otros autores encuentran alterada la expresión de TLR2 cuando existe enfermedad asociada a la infección por *L. infantum* (Figueiredo et al., 2014; Hosein et al., 2015). Estos estudios se resumen en la tabla 4.1. Todos ellos encontraron una sobreexpresión del TLR2 en biopsias de piel (Esteve et al., 2015) o en biopsias de piel de test intradérmicos de leishmanina positivos (Ordeix et al., 2018), en colon (Figueiredo et al., 2013) hasta en cerebro, bazo (Melo et al., 2014a) e hígado (Hosein et al., 2015). Los resultados obtenidos en el estudio de la presente tesis están en consonancia con la mayor parte de los estudios publicados en la literatura científica. Sin embargo, existe un estudio realizado en Brasil donde se relata resultados que no soportan los demostrados en esta tesis doctoral (Amorim et al., 2011). Es importante señalar que este estudio no es completamente equiparable debido a las diferencias en los métodos empleados, las condiciones clínicas y geográficas (Amorim et al., 2011). En la literatura se

han encontrado algunas discrepancias de resultados dependiendo del tejido: en sangre se ha encontrado tanto una sobreexpresión como una disminución del TLR2, y en bazo, tanto el TLR2 y TLR4 se encuentran diferencias en los resultados en los diferentes estudios consultados (Ver tabla 4.2).

Tabla 4.1. Resumen de los resultados encontrados en los estudios publicados sobre la expresión de TLR2 y TLR4 en la leishmaniosis canina.

Expresión de TLR2 (referencias)*	**Expresión de TLR4 (referencias)**
Sangre: ↑ (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018) (capítulos 3.1 y 3.2) ↓ (Amorim et al., 2011)	PBMCs:↓ (Melo et al., 2014b)
Colon: ↑ (Figueiredo et al., 2013)	Bazo: ↑ (Melo et al., 2014a) ↓ (Grano et al., 2018
Cerebro: ↑ (Melo et al., 2014a)	Linfonodo: ↑ (Melo et al., 2014a) ↓ (Hosein et al., 2015)
Bazo: ↑ (Melo et al., 2014a) ↓ (Grano et al., 2018)	
Plexo coroide: ↑ (Melo et al., 2014a)	
Linfonodo: ↑ (Hosein et al., 2015; Melo et al., 2014a)	
Piel: ↑ (Esteve et al., 2015; Hosein et al., 2015; Ordeix et al., 2018)	
Hígado: ↑ (Hosein et al., 2015)	

*Abreviaciones: ↑: sobreexpresión del gen; ↓: disminución del g

Tabla 4.2. Resumen de publicaciones relacionadas con la expresión de los TLRS en la infección por L. infantum en perros. Modificada de (Hosein et al., 2017)

TLRs estudiados	Tejido evaluado	Técnica utilizada	Número y tipos de sujetos en estudio	Características	Ubicación geográfica	Resultados	Referencias
TLR2	Sangre	Citometría de flujo	Total en estudio n= 48 Xeno+/ IHQ+ n=17 Xeno-/ IHQ- n=16	Naturalmente infectados	Brasil	Sangre: ↑ TLR2 en Xeno-/ IHQ-	(Amorim et al., 2011)
TLR2, TLR9	Yeyuno, colon	Citometría de flujo	Sanos n=6 Asint n=12 Sint n=12	Naturalmente infectados	Brasil	Yeyuno: ↑ TLR9 comparado con colon Colon: ↑ TLR2 y la carga parasitaria comparado con yeyuno	(Figueiredo et al., 2013)
TLR2, TLR4	PBMCs	Citometría de flujo	Sanos n=60	Naturalmente infectados	Brasil	PBMCs: ↓TLR4 No diferencias en TLR2	(Melo et al., 2014b)
TLR2, TLR4, TLR9	Cerebro, bazo plexo coroide, linfonodo	Rt-PCR	Sanos n=4 Sint n=15	Naturalmente infectados	Brasil	Cerebro: ↑ TLR2 Bazo: ↑ TLR2,TLR4 Plexo coroide: ↑ TLR2,TLR9 Linfonodo: ↑ TLR2,TLR4	(Melo et al., 2014a)
TLR2, TLR3, TLR4, TLR9	Linfonodo, bazo, piel, hígado	Rt-PCR	Sanos n=10 Asint n= 24 Sint n=7	Experimentalmente infectados	Reino unido y España	Linfonodo: ↓TLR3,TLR4,TLR9 / ↑ TLR2 (15 m.p.i) Bazo: ↓TLR4 (6 y 15 m.p.i.) Piel:↑ TLR2, TLR9 (6 m.p.i.) Hígado: ↑ TLR2 (15 m.p.i.)	(Hosein et al., 2015)
TLR1-7,TLR9	Sangre (macrófagos)	Rt-PCR	Total n=12	Experimentalmente infectados *in vitro* macrófagos	Brasil	↑ TLR2-6 macrófagos infectados ↓ TLR1, TLR7 macrófagos infectados Ø TLR9	(Turchetti et al., 2015)
TLR2	Piel	IHQ	Sanos n=6 Sint S-I n=11	Naturalmente infectados	Reino unido y	Piel: ↓ TLR2 en Sint S-I comparado con Sint S-II y S-III	(Esteve et al., 2015)

			Sint S-II y S-III n=10		España		
TLR2, TLR2, TLR5, TLR9	PBMCs	Citometría de flujo	Sanos y vacunados n= 45	Vacunados con Leishmune®	Brasil	Monocitos: ↑ TLR2,TLR4,TLR5,TLR9	(Moreira et al., 2016)
TLR1-TLR10	Cerebro, bazo	Rt- PCR	Sanos n=4 Sint n= 21	Naturalmente infectados	Brasil y Francia	Bazo:↑ TLR5,TLR9 ↓ TLR2-4, TLR10 Cerebro: no diferencias	(Grano et al., 2018)
TLR2,TLR4	Hígado (células de kupffer)	Rt- PCR	Sanos n=12	Experimentalmente infectados y tratados con MgA)	Brasil y Portugal	↑ TLR2, TLR4	(Rodrigues et al., 2017)
TLR2	Biopsias de piel	qPCR	Sanos n=8 Enfermos n=29	Naturalmente infectados	Brasil	↑ TLR2	(Pereira-Fonseca et al., 2017)

TLR: Receptor tipo Toll; Xeno: Xenodiagnóstico; PBMCs: células mononucleares de sangre periférica; IHQ: Inmunohistoquímica; Rt-qPCR- reacción quantitativa en cadena de la polimerasa a tiempo real; Asint: Asintomáticos, Sint: sintomáticos; ↑: sobreexpresión del gen; ↓: disminución del gen; S-I, II, III: estadío clínico LeishVet I,II,II; MgA: Antimoniato de meglumine; Asint: asintomáticos; Sint: sintomáticos; m.p.i.: meses post infección.

Los resultados sobre la expresión del TLR4 en sangre no estimulada, nos revelaron que la expresión de este gen no participa tanto como puede participar el TLR2 ya que su expresión puede variar al largo del tratamiento pero no de forma significativa. Hay estudios donde también han encontrado el mismo hallazgo que los descritos en esta tesis doctoral en diferentes tejidos, como en bazo y cerebro, pero encontraron una sobreexpresión en linfonodos (Melo et al., 2014a). Parece ser que la expresión de este gen es más variable, en otro estudio observaron que el gen del TLR4 disminuía drásticamente conforme avanzaba la enfermedad en tejidos de linfonodo y bazo mientras que en otros tejidos como el hígado o la piel no se denotaron cambios (Hosein et al., 2015). Estos resultados están en acorde con lo que se conoce sobre la compartimentalización de las respuestas inmunitarias que dependen de cada tejido (Hosein et al., 2015; Melo et al., 2014a; Rodriguez-Cortes et al., 2016). Hay que tener en cuenta que existe un menor número de estudios realizados sobre el TLR4 comparado con el TLR2. Los hallazgos encontrados en estos estudios demuestran que la transcripción de los TLRs se ve afectada dependiendo del tejido donde se observa y que cada gen de TLR puede expresarse o no correlativamente a los otros receptores de su familia.

La importancia de categorizar la severidad de la enfermedad debida a la infección por *L. infantum* en estadios clínicos, es relevante a la hora de diagnosticar además los tratamientos serán más acertados y con mejores pronósticos (Solano-Gallego et al., 2009). Partimos de la hipótesis que los perros clasificados en estadío I (leve) presentaban un perfil inmunológico distinto a los perros clasificados en estadíos más severos en el momento del diagnóstico (Solano-Gallego et al., 2016a). Los resultados obtenidos en esta tesis doctoral fueron que los niveles de anticuerpos específicos de los perros sanos eran significativamente más bajos que los niveles que se mostraban en los perros con leishmaniosis clínica, como ha sido ampliamente confirmado por otros estudios (Rodriguez-Cortes et al., 2007a; Rodriguez-Cortes et al., 2017). Sin embargo, en la presente tesis doctoral, se demuestra en un estudio comparativo descriptivo como los perros con estadios leves (Leishvet estadio I) presentaron niveles de anticuerpos más bajos así como una menor frecuencia de

positividad al comparar con perros en estadios clínicos más severos (ver capítulo 3.2.). Además, los perros estudiados en el capítulo 3.2. con dermatitis papular y clasificados como estadío I presentaban mayores niveles de IFN-γ específicos así como una porcentual mayor de perros respondedores a esta citoquina que los perros con estadíos más severos. La leishmaniosis canina suele estar asociada a la respuesta humoral Th2, la cual resulta ser un perfil no protector frente la intrusión del parásito. Se ha demostrado ya con anterioridad que los perfiles de los perros que resuelven las pápulas o tienen signos clínicos que se adecuan al estadío I, tienen pocos niveles de anticuerpos específicos así como una buena respuesta celular como es una alta producción de IFN-γ (Martinez-Orellana et al., 2017a). Además, se ha demostrado en los perros enfermos con leishmaniosis moderada que mejoraban, un incremento de la producción de IFN-γ (Martinez-Orellana et al., 2017a; Solano-Gallego et al., 2016b).

En cuanto a la expresión de los TLRs, la transcripción del TLR2 de los perros con dermatitis papular y estadío I fue similar a la de los perros sanos, y se detectó una sobre expresión de este gen en la sangre de los perros con estadíos más avanzados de la enfermedad como previamente descrito en el capítulo 3.1. Se ha observado que los perros con enfermedad de estadio moderado, tienen una alta carga parasitaria en diferentes tejidos incluyendo la médula (Reis et al., 2009) esto podría ser un motivo por el cual el TLR2 reconoce el ligando lipofosfoglicano de la *Leishmania* (Becker et al., 2003). Es importante también señalar que el TLR2 se correlacionó con otros parámetros inmunológicos, parasitológicos y clinicopatológicos asociados a una enfermedad de moderada a severa (ver capítulos 3.1 y 3.2.) (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018). El TLR2 parece ser un marcador de la inflamación en perro, tal como se demuestra en otros estudios con enfermedades inflamatorias caninas como por ejemplo la artritis bacteriana (Riggio et al., 2014) o la enfermedad inflamatoria del intestino (McMahon et al., 2010) así como la demodicosis canina, que muestra una sobre expresión de este gen cuando la enfermedad empeora, como se muestra en la tabla 4.3 (Kumari et al., 2018).

Así pues, se ha comprobado que el perfil inmunológico de los estadios leves es diferente al de los estadios más severos, donde la expresión del TLR2 junto con el

título de anticuerpos específicos y la carga parasitaria son más elevados juntamente con una reducida producción de IFN-γ en el grupo de perros infectados con un estadio más severo.

Tabla 4.3. Resumen de los estudios publicados relacionados con la expresión de TLRs en el perro en distintas enfermedades diferentes a la leishmaniosis.

TLRs estudiados	Tejido evaluado	Técnica utilizada	Enfermedad	Número y tipo de perros estudiados	Resultados	Referencias
TLR2,TLR4, TLR9	Sangre	rt-qPCR	Furunclurosis anal	Sanos n=12 Enfermos n=8	Macrófagos: ↑TLR2	(House et al., 2008)
TLR2,TLR4 TLR9	Biopsia de intestino	rt- qPCR	Enteropatías crónicas	Sanos n= 16 Enfermos n=35	Mucosa del duodeno y colon: ↑TLR2,TLR4, TLR9	(Burgener et al., 2008)
TLR2,TLR4	Biopsia intestinal	rt-PCR	Enteropatías crónicas	Sanos n=17 Enfermos n=20	↑TLR2	(McMahon et al., 2010)
TLR2, TLR4, TLR5 TLR9	Biopsia intestinal	rt-qPCR	Enteropatías crónicas	Sanos n=10 Enfermos n=13	Todas las secciones:↑TLR4 ↓TLR5	(Allenspach et al., 2010)
TLR2-5, TLR9	Sangre periférica	IHQ	Meningitis	Sanos n=6 Enfermos n=74	TLR4, TLR9 se asocian a sustentadores de la inflamación. Sangre (excepto en leucocitos): ↑TLR2,TLR4,TLR5, TLR9	(Maiolini et al., 2012)
TLR2, TLR4 TLR9	Sangre y biopsia nasal	PCR convencional	Aspergilosis sino-nasal	Enfermo n = 9	No diferencias significativas	(Mercier et al., 2014)
TLR2-4,TLR5, TLR7,TLR9	Líquido sinovial	qPCR	Artritis canina	Sanos n=6 Enfermos n= 34	Líquido sinovial:↑TLR2,TLR7	(Riggio et al., 2014)
TLR2,TLR4, TLR5,TLR9	Sangre	qPCR	Enteropatías crónicas	Sanos n=11 Enfermos n=17	Sangre: ↑TLR2,TLR4,TLR9	(Schmitz et al., 2014)
TLR1, TLR2, TLR4, TLR6-TLR10	Biopsia colorectal	qPCR	Pólipos colorectales	Sanos n=21 Enfermos n=24	Pólipo y mucosa del colon: ↑TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10,	(Igarashi et al., 2014)
TLR2, TLR4	Biopsia de hígado	qPCR	*Shunt* Portosistémico congénito	Perros con cirugía n=49 Controles n= -	↑TLR4 con atenuación completa, parcial y en buena circulación portal Ø TLR2	(Tivers et al., 2015)
TLR1,TLR2, TLR4,TLR9	Sangre	PCR y genotipado SNP	CDV, HC, Microfilarias, NC	Total n=189	TLR4 se asocia a más de una infección: sugiere un papel importante en enfermedades infecciosas	(Necesankova et al., 2016)
TLR2, TLR4	Biopsia colorectal	Hibridación *in situ*	Pólipos colorectales	Total n=10	Mucosa colorectal inflamada: ↑TLR2,TLR4 Epitelio colon :↑TLR2,TLR4	(Yokoyama et al., 2017)
TLR2,TLR4, TLR6	Sangre	Rt-qPCR	Demodicosis	E.Generalizado n=8 E. Localizado n=8 Sanos n=8	↑TLR2 E. Generalizado comparado E. Localizado ↓TLR4, TLR6 E. Localizado y generalizado comparado con sanos	(Kumari et al., 2018)

TLR: Receptor tipo Toll; E.: enfermos; Rt-qPCR: reacción quantitativa en cadena de la polimerasa a tiempo real; CDV: virus del moquillo canino; HC: Hepatozoonosis canina; NC: infección por *Neospora caninum*.

Sangre estimulada con LSA y Con A

En el capítulo 3.3. dónde se estimula la sangre de perro enfermo infectado y sano no infectado para ver cuál es el patrón de expresión génica del TLR2, TLR4 y el PD-L1, los perros enfermos fueron subclasificados en productores de IFN-γ y no productores de IFN-γ. Los productores de esta citoquina, mostraron bajos niveles de anticuerpos y también una baja parasitemia. Además las alteraciones clinicopatológicas eran menos severas que la de los perros no productores de IFN-γ, y por tanto se considera que tienen una enfermedad más leve. Después de la estimulación con LSA, se observó una baja expresión de TLR2 de los perros productores de IFN-γ en comparación con el medio y con los perros no productores de IFN-γ. Este estudio descrito en el capítulo 3.3., confirma los resultados descritos en sangre no estimulada donde la expresión de TLR2 se asocia a la gravedad de la enfermedad (capítulos 3.1 y 3.2) (Montserrat-Sangra et al., 2016; Montserrat-Sangra et al., 2018). Otros estudios dan peso a nuestros resultados (Esteve et al., 2015; Pereira-Fonseca et al., 2017) ya que demuestran lo mismo en otros tejidos. La asociación entre la expresión del TLR2 y la enfermedad moderada o severa, concuerda con que el TLR actúe en las respuestas inmunitarias pro-inflamatorias (Kang et al., 2015). Se observaron correlaciones positivas entre la expresión del TLR2 después de la estimulación con LSA y las proteínas totales, las gamma globulinas y anticuerpos específicos mientras que la correlación negativa se dio entre la expresión de TLR2 después de la estimulación por LSA y los valores de hematocrito y hemoglobina. Estos resultados ya fueron obtenidos en el primer estudio (capítulo 3.1.) siendo observados en sangre no estimulada, lo que confirma los resultados recabados.

En cuanto a la expresión del TLR4, se vio una baja expresión de éste después de la estimulación por LSA en los perros infectados productores de IFN-γ comparado con el medio sin estimular. El tránscrito TLR4 estimulado con LSA se correlacionó negativamente con las concentraciones de IFN-γ. A todo esto, no se llegó a observar diferencias estadísticas entre la expresión génica del TLR4 en perros enfermos que no

producían IFN-γ ni con el grupo control. En pacientes humanos sí se ha detectado diferencias en la expresión del TLR4 donde éste se sobre expresaba (Babiker et al., 2015) aunque otros autores también han encontrado resultados similares a los nuestros dependiendo del tejido (Hosein et al., 2015; Melo et al., 2014a).

En el estudio descrito en el capítulo 3.3., se vio una baja expresión de los receptores TLR2 y TLR4 cuando la sangre se estimuló con Con A en todos los grupos estudiados. Se sabe que la comunicación entre citoquinas y TLRs existe (Chandel et al., 2014). Estos hallazgos sugieren un *feedback* negativo con los linfocitos T, es decir que cuando existen alta producción de IFN- γ se observa una disminución del TLR2 (Gatto et al., 2015).

Sobre la expresión del tránscrito del PD-L1, obtuvimos resultados similares en todos los grupos. Se observó en sangre estimulada tanto con LSA con Con A una alta expresión de este gen, comparado con el medio solo. Esperábamos encontrar diferencias entre los productores de IFN-γ y los que no la producen, como un marcador de agotamiento celular (Esch et al., 2013), pero no fue así. Los tránscritos del PD-L1 estimulados con LSA se correlacionaron positivamente con los estimulados con Con A y con LSA IFN-γ y negativamente con las proteínas totales, las gamma globulinas y los niveles de anticuerpos específicos. Estos resultados no están soportados por la literatura encontrada (Barroso et al., 2018; Habib et al., 2018). Quizá en el presente estudio se activó una fuerte muerte celular y por eso se obtuvo una marcada expresión de PD-L1 en todos los grupos, ya que éste ligando actúa como marcador apoptótico (Hofmeyer et al., 2011). Se cree que no se consiguieron diferencias entre grupos debido al tiempo de recolección de las muestras. Debido a estas circunstancias, diferencias entre los grupos de estudio no se pudieron observar. Se deben realizar más estudios para describir mejor el papel del PD-L1 en la leishmaniosis canina. Es importante comentar que este es el primer estudio donde se describe la expresión de PD-L1 en la leishmaniosis canina. Además, desafortunadamente, no estudiamos el PD-1 que se expresa en los linfocitos y sí que parece aumentar en perros con una

disminución de la respuesta celular como descrito previamente(Chiku et al., 2016; Esch et al., 2013). Es probable que con este marcador sí que hubiéramos obtenido diferencias entre perros productores de IFN-γ y no productores.

Sangre estimulada con agonistas de TLRs

En el estudio en área endémica con perros clínicamente sanos y seronegativos, se recolectó sangre para describir el perfil de citoquinas que presentaban cuando la sangre se estimulaba con LSA, con el mitógeno Con A y con los agonistas de los receptores TLR3, TLR4 y TLR7. La estimulación con LSA y Con A de la sangre de los 24 perros resultó producir unos niveles altos de IFN-γ comparado con el medio. Se ha descrito con anterioridad en nuestro grupo que los perros sanos tienen un alta producción de IFN-γ (Martinez-Orellana et al., 2017a; Solano-Gallego et al., 2016b). En cuanto al a producción del TNF-α, se asocia a perfil Th1, más "resistente" frente a la infección, pues en nuestros resultados se vio un incremento de estas citoquinas cuando la sangre estuvo estimulada con los agonistas de TLRs solos y Con A comparado con el medio. Se encontraron diferencias en TLR4a y TLR7a cuando lo comparamos con la estimulación de la sangre con TLR3a y comparado con LSA y también los tratamientos combinados de TLR4a + LSA y TLR7a + LSA comparándolos con la combinación TLR3a + LSA. Se demostró que los TLR4a y TLR7a presentaban una potente producción de citoquinas al comparar con el TLR3a. Así pues, estos resultados sugieren que los agonistas de TLR4 y TLR7 podrían tener un papel más importante en la infección por *Leishmania* que otros agonistas como el TLR3, ya que éstos son capaces de generar TNF-α y IL-6, citoquinas conocidas en la leishmaniosis que ayudan al control de la infección (Pereira-Fonseca et al., 2017). Un estudio realizado en ratones con el agonista imiquimod aumentó la eficacia del fármaco usado en terapia durante la infección por *L. major* (Khalili et al., 2011). La diferencia entre las citoquinas TNF- α y IL-6 en este estudio fue que el TNF-α mostró una producción estadísticamente más alta cuando se comparó con el LSA y el medio solos, en cambio no se observaron diferencias en la producción de IL-6. Estos ligandos se están empezando a

experimentar en ratones para crear nuevas vacunas como adyuvantes para humanos y en un futuro para perros (Carter et al., 2016; Weeratna et al., 2005). Aunque en nuestro estudio el TLR3a no haya destacado, hay un estudio en ratón que el TLR3a es capaz de inducir respuesta Th1 (Sanchez et al., 2017). Estos hallazgos nos indican que los agonistas de los TLR pueden ayudar al sistema inmunitario a crear los productos necesarios para hacer frente a la infección por *Leishmania*, aunque se deben realizar más estudios para probar la combinación perfecta y su seguridad para hacer los tratamientos y/ o las vacunas mucho más eficientes.

Estudio in vitro

Macrófagos

En el capítulo 3.5., se describen los estudios *in vitro* realizados con ensayos de promastigotes y amastigotes. En macrófagos caninos se estableció la susceptibilidad IC50 de los amastigotes de *L. infantum* para los fármacos convencionales anti-*Leishmania*. En este caso los resultados obtenidos fueron: en alopurinol 0.50 mg/mL, en la miltefosina 0.53 µL/ mL y el meglumine antimoniato 79.61 mg/ mL. El fármaco más potente para eliminar las formas amastigotas del parásito fue el de la miltefosina y el que menos el de meglumine antimoniato. Existen varios estudios en los cuales demuestran que la miltefosina es un fármaco potente (Kulshrestha et al., 2013). Hoy en día, este fármaco combinado con el alopurinol, es uno de los protocolos terapéuticos utilizados para apaliar la infección por *Leishmania* en perros y controlar la enfermedad (Miró, 2006; Miro et al., 2009).

De acuerdo con los resultados obtenidos con amastigotes en esta tesis doctoral, el porcentaje de infección y la intensidad de infección fue menor en las células tratadas con los diferentes fármacos solos comparado con las células infectadas no tratadas y también se observó una disminución cuando las células habían sido tratadas con TLRs agonistas solos en comparación con las células infectadas sin tratamiento. Aunque se esperaban estos resultados, hay pocos estudios hechos en *in vitro* para poder comparar. Uno de ellos es el estudio realizado por Hosein et al. en

2014 que observan también reducción de amastigotes en las células DH82 (Hosein, 2014) con el tratamiento de TLR2a. Existen algunos estudios realizados en modelo murino que utilizando el TLR2a confería protección al ratón (Huang et al., 2015). Además, en la presente tesis doctoral, la combinación del alopurinol con TLR4a o con el TLR7a también reducían el número de amastigotes por célula comparado con las células no tratadas.

A pesar de que la literatura describe la participación del NO y de varias citoquinas como por ejemplo el TNF-α (Hosein, 2014) para hacer frente la enfermedad, en este estudio *in vitro* no se consiguió obtener resultados de estos dos compuestos. Sin embargo, en el estudio 3.4 (*ex vivo*) realizado con sangre de perro infectado estimulada con agonistas, sí que la producción de las citoquinas observadas fueron cuantificables y relevantes para describir el papel de los receptores tipo Toll. Relacionado con la expresión de los TLR2, TLR4 y TLR7, en este estudio hemos encontrado varios resultados dependiendo de la combinación entre fármaco y agonista. Los resultados a destacar son que no se obtuvieron cambios en el patrón de transcripción del TLR4 y TLR7 y si una reducción del TLR2 frente a la infección por *L. infantum* al igual que no hubo producción de TNF-α. Estos resultados están de acuerdo con la consolidación de la infección del parásito que se conoce que se produce de manera silenciosa para evadir el sistema inmunitario (Hosein et al., 2017). El parásito tiene mecanismos para pasar desapercibido en el sistema, de esta manera, retarda la respuesta inmunitaria y tiene tiempo para establecer la infección (Hosein et al., 2017). Similares resultados se encontraron al tratar los macrófagos tanto infectados como no infectados con fármacos convencionales solos, ya que tampoco se observaron cambios en el TNF-α y pocos cambios en la transcripción de TLRs.

En el presente estudio, sí que se obtuvo una sobre expresión del TLR2 en macrófagos infectados respecto al control después de la estimulación con el TLR2a. Un estudio similar hecho en Londres sustenta nuestros resultados: 48h post infección, la expresión del TLR2 aumentaba después de la estimulación con TLR2a (Hosein, 2014), cuyos resultados se corresponden con los de este estudio, aunque en éste se observara la expresión del gen a las 72h. También, en la presente tesis, se encontró

significancia estadística en la expresión del TLR4 cuando fue estimulado con su agonista, TLR4a. Los macrófagos infectados mostraron una mayor expresión de TLR4 comparado con aquellos no infectados. Tanto el TLR2a como el TLR4a es conocida su actividad protectora frente a la infección por *Leishmania* (Majumdar et al., 2014).

A pesar de no haber encontrado muchas diferencias en este estudio en la expresión del TLR7, hay estudios publicados donde se describe que tiene una función también protectora induciendo citoquinas proinflamatorias beneficiosas para contrarrestar la infección (Aderem and Ulevitch, 2000).

Este estudio aporta información sobre las posibles combinaciones de fármacos con adyuvantes en forma de TLRa para estimular la respuesta temprana del organismo y hacer frente a la infección de manera más rápida, logrando así un tratamiento más efectivo.

CAPÍTULO 5

CONCLUSIONES / CONCLUSIONS / CONCLUSIONS

- ✓ Los perros con enfermedad leve presentaron diferentes características inmunológicas clínicas, parasitológicas e innatas y adaptativas.
- ✓ La regulación positiva de TLR2 en sangre parece estar asociada con leishmaniosis clínica moderada a grave.
- ✓ La regulación negativa de TLR2 en sangre no estimulada se observó durante el seguimiento del tratamiento y la mejoría clínica en perros con leishmaniosis moderada a grave.
- ✓ La transcripción de TLR2 en sangre no estimulada parece ser un buen marcador de la gravedad de la enfermedad útil también para la monitorización del tratamiento.
- ✓ La transcripción de TLR4 en sangre no estimulada no discrimina entre el estado de salud y la leishmaniosis canina clínica
- ✓ La transcripción de TLR4 en sangre no estimulada no parece ser un marcador de la gravedad de la enfermedad y no es útil para la monitorización del tratamiento.
- ✓ Se observó una regulación a la baja de las transcripciones de TLR2 y TLR4 después de la estimulación sanguínea con antígeno de Leishmania solo en perros productores de IFN-γ enfermos.
- ✓ TLR4a y TLR7a tienen un efecto más pronunciado sobre la producción de citoquinas ex vivo en sangre de perros aparentemente sanos que viven en áreas endémicas de CanL en comparación con TLR3a.
- ✓ La combinación de LSA + TLR7a promovió el efecto sinérgico proinflamatorio en sangre de perros aparentemente sanos que viven en áreas endémicas de CanL.
- ✓ Los agonistas de TLR estudiados solos reducen la infección por *Leishmania* en un modelo canino *in vitro*.
- ✓ La combinación de TLR4a con alopurinol mostró una disminución sinérgica en la infección por *L. infantum* en un modelo canino *in vitro*.

- ✓ Els gossos amb malaltia lleu van presentar diferents característiques parasitològiques e immunològiques de la resposta adaptativa al comparar amb gossos amb malatia mes severa.
- ✓ La regulació positiva de TLR2 en sang no estimulada sembla estar associada amb leishmaniosi clínica de moderada a greu.
- ✓ La regulació negativa de TLR2 en sang no estimulada es va observar durant el seguiment del tractament i la millora clínica en gossos amb leishmaniosi moderada i greu.
- ✓ La transcripció de TLR2 en sang no estimulada sembla ser un bon marcador de la gravetat de la malaltia útil també per al monitoratge del tractament.
- ✓ La transcripció de TLR4 a la sang no estimulada no discrimina entre l'estat de salut i la leishmaniosi canina clínica
- ✓ La transcripció de TLR4 a la sang no estimulada no sembla ser un marcador de la gravetat de la malaltia i no és útil per al monitoratge del tractament.
- ✓ Es va observar una regulació a la baixa de les transcripcions del TLR2 i TLR4 després de l'estimulació sanguínia amb antigen de *Leishmania* només en gossos malalts productors de IFN-γ.
- ✓ TLR4a i TLR7a tenen un efecte més pronunciat sobre la producció de citoquines *ex vivo* en sang de gossos aparentment sans que viuen en àrees endèmiques de CanL en comparació amb TLR3a.
- ✓ La combinació de LSA + TLR7a va promoure l'efecte sinèrgic proinflamatori en sang de gossos aparentment sans que viuen en àrees endèmiques de CanL.
- ✓ Els agonistes de TLR estudiats sols redueixen la infecció per *Leishmania* en un model caní *in vitro*.
- ✓ La combinació de TLR4a amb al·lopurinol va mostrar una disminució sinèrgica en la infecció per *L. infantum* en un model caní *in vitro.*

- ✓ Dogs with mild disease presented distinctive parasitological and adaptive immunological features when compared with dogs with more severe disease.
- ✓ TLR2 transcription in unstimulated blood appears to be a good marker of disease severity useful also for treatment monitoring.
- ✓ TLR2 upregulation in unstimulated blood appears to be associated with moderate to severe clinical leishmaniosis.
- ✓ TLR2 downregulation in unstimulated blood was noted during treatment follow-up and clinical improvement in dogs with moderate to severe leishmaniosis.
- ✓ TLR4 transcription in unstimulated blood does not discriminate between health status and clinical canine leishmaniosis and for this reason, it does not appear to be a marker of disease severity.
- ✓ TLR4 transcription in unstimulated blood is not useful for the monitoring of treatment.
- ✓ A downregulation of TLR2 and TLR4 transcriptions after blood stimulation with *Leishmania* antigen was noted only in sick IFN-γ producer dogs.
- ✓ TLR4a and TLR7a have a more pronounced effect on cytokines production *ex-vivo* in blood from apparently healthy dogs living in endemic areas of CanL compared to TLR3a.
- ✓ The combination of LSA+TLR7a promoted a synergistic pro-inflammatory effect in blood from apparently healthy dogs living in endemic areas of CanL.
- ✓ TLR agonists studied alone reduced *L. infantum* infection in an *in vitro* canine model.
- ✓ The combination of TLR4a with allopurinol showed a synergistic decrease in *L. infantum* infection in an *in vitro* canine model.

CAPÍTULO 6

REFERENCIAS / REFERENCES

Absavaran, A., Rassi, Y., Parvizi, P., Oshaghi, M., Abaie, M., Rafizadeh, S., Mohebali, M., Zarea, Z., Javadian, E., 2009. Identification of Sand flies of the Subgenus *Larroussius* based on Molecular and Morphological Characters in North Western Iran. Iranian journal of arthropod-borne diseases 3, 22-35.

Aderem, A., Ulevitch, R.J., 2000. Toll-like receptors in the induction of the innate immune response. Nature 406, 782-787.

Agallou, M., Dotsika, E., Frydas, S., Karagouni, E., 2014. Toll-like receptor 4 promotes control of *Leishmania infantum* infection through inducement of leishmanicidal activity in host macrophages: role of mitogen activated kinases. Journal of biological regulators and homeostatic agents 28, 41-52.

Akira, S., Takeda, K., Kaisho, T., 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nature immunology 2, 675-680.

Alexander, B., Maroli, M., 2003. Control of phlebotomine sandflies. Medical and veterinary entomology 17, 1-18.

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732-738.

Almeida, A.B., Sousa, V.R., Gasparetto, N.D., da Silva, G.F., Figueiredo, F.B., Dutra, V., Nakazato, L., Madeira, M.F., 2013. Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples. Diagnostic microbiology and infectious disease 76, 321-324.

Alten, B., Maia, C., Afonso, M.O., Campino, L., Jimenez, M., Gonzalez, E., Molina, R., Banuls, A.L., Prudhomme, J., Vergnes, B., Toty, C., Cassan, C., Rahola, N., Thierry, M., Sereno, D., Bongiorno, G., Bianchi, R., Khoury, C., Tsirigotakis, N., Dokianakis, E., Antoniou, M., Christodoulou, V., Mazeris, A., Karakus, M., Ozbel, Y., Arserim, S.K., Erisoz Kasap, O., Gunay, F., Oguz, G., Kaynas, S., Tsertsvadze, N., Tskhvaradze, L., Giorgobiani, E., Gramiccia, M., Volf, P., Gradoni, L., 2016. Seasonal Dynamics of Phlebotomine Sand Fly Species Proven Vectors of Mediterranean Leishmaniasis Caused by *Leishmania infantum*. PLoS neglected tropical diseases 10, e0004458.

Alvar, J., Ballesteros, J.A., Soler, R., Benito, A., van Eys, G.J., Schoone, G.J., Cabrer, B., 1990. Mucocutaneous leishmaniasis due to *Leishmania* (*Leishmania*) *infantum*: biochemical characterization. The American journal of tropical medicine and hygiene 43, 614-618.

Alvar, J., Canavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. Advances in parasitology 57, 1-88.

Alvar, J., Molina, R., San Andres, M., Tesouro, M., Nieto, J., Vitutia, M., Gonzalez, F., San Andres, M.D., Boggio, J., Rodriguez, F., et al., 1994. Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. Annals of tropical medicine and parasitology 88, 371-378.

Alving, C.R., Richards, R.L., Moss, J., Alving, L.I., Clements, J.D., Shiba, T., Kotani, S., Wirtz, R.A., Hockmeyer, W.T., 1986. Effectiveness of liposomes as potential carriers of vaccines: applications to cholera toxin and human malaria sporozoite antigen. Vaccine 4, 166-172.

Allenspach, K., House, A., Smith, K., McNeill, F.M., Hendricks, A., Elson-Riggins, J., Riddle, A., Steiner, J.M., Werling, D., Garden, O.A., Catchpole, B., Suchodolski, J.S., 2010. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. Veterinary microbiology 146, 326-335.

Amorim, I.F., Silva, S.M., Figueiredo, M.M., Moura, E.P., Castro, R.S., Lima, T.K., Gontijo Nde, F., Michalick, M.S., Gollob, K.J., Tafuri, W.L., 2011. Toll receptors type-2 and CR3 expression of

canine monocytes and its correlation with immunohistochemistry and xenodiagnosis in visceral leishmaniasis. PloS one 6, e27679.

Angela Toepp, M.L., Tara Grinnage-Pulley, Adam Lima, Carolyne Bennett, Geneva Wilson, Michael Anderson, Hailie Fowler, Jill Saucier, Jesse Buch, Phyllis Tyrrell, G. Clark, Ramaswamy Chandrashekar, Christine Petersen 2017. (Abstract) Co-infection with tick-borne disease causes clinical progression of Leishmaniosis in dogs. (The University of Iowa, Iowa City, USA, College of Public Health Center for Emerging Infectious Diseases).

Antoniazi, S., Price, H.P., Kropf, P., Freudenberg, M.A., Galanos, C., Smith, D.F., Muller, I., 2004. Chemokine gene expression in toll-like receptor-competent and -deficient mice infected with *Leishmania major*. Infection and immunity 72, 5168-5174.

Aoun, K., Chouihi, E., Boufaden, I., Mahmoud, R., Bouratbine, A., Bedoui, K., 2008. [Efficacy of Deltamethrine-impregnated collars Scalibor in the prevention of canine leishmaniasis in the area of Tunis]. Archives de l'Institut Pasteur de Tunis 85, 63-68.

Aransay, A.M., Testa, J.M., Morillas-Marquez, F., Lucientes, J., Ready, P.D., 2004. Distribution of sandfly species in relation to canine leishmaniasis from the Ebro Valley to Valencia, northeastern Spain. Parasitology research 94, 416-420.

Arevalo, I., Ward, B., Miller, R., Meng, T.C., Najar, E., Alvarez, E., Matlashewski, G., Llanos-Cuentas, A., 2001. Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod, an immunomodulator. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 33, 1847-1851.

Aroor, A.R., Jia, G., Habibi, J., Sun, Z., Ramirez-Perez, F.I., Brady, B., Chen, D., Martinez-Lemus, L.A., Manrique, C., Nistala, R., Whaley-Connell, A.T., Demarco, V.G., Meininger, G.A., Sowers, J.R., 2017. Uric acid promotes vascular stiffness, maladaptive inflammatory responses and proteinuria in western diet fed mice. Metabolism: clinical and experimental 74, 32-40.

Athanasiou, L.V., Saridomichelakis, M.N., Kontos, V.I., Spanakos, G., Rallis, T.S., 2013. Treatment of canine leishmaniosis with aminosidine at an optimized dosage regimen: a pilot open clinical trial. Veterinary parasitology 192, 91-97.

Babiker, D.T., Bakhiet, S.M., Mukhtar, M.M., 2015. *Leishmania donovani* influenced cytokines and Toll-like receptors expression among Sudanese visceral leishmaniasis patients. Parasite immunology 37, 417-425.

Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends in parasitology 24, 324-330.

Baneth, G., Shaw, S.E., 2002. Chemotherapy of canine leishmaniosis. Veterinary parasitology 106, 315-324.

Barbieri, C.L., 2006. Immunology of canine leishmaniasis. Parasite immunology 28, 329-337.

Barbosa, M.A., Alexandre-Pires, G., Soares-Clemente, M., Marques, C., Rodrigues, O.R., De Brito, T.V., Da Fonseca, I.P., Alves, L.C., Santos-Gomes, G.M., 2011. Cytokine gene expression in the tissues of dogs infected by *Leishmania infantum*. Journal of comparative pathology 145, 336-344.

Bardhan, K., Anagnostou, T., Boussiotis, V.A., 2016. The PD1:PD-L1/2 Pathway from Discovery to Clinical Implementation. Frontiers in immunology 7, 550.

Barroso, D.H., Falcao, S.A.C., da Motta, J.O.C., Sevilha Dos Santos, L., Takano, G.H.S., Gomes, C.M., Favali, C.B.F., de Lima, B.D., Sampaio, R.N.R., 2018. PD-L1 May Mediate T-Cell Exhaustion in a Case of Early Diffuse Leishmaniasis Caused by *Leishmania (L.) amazonensis*. Frontiers in immunology 9, 1021.

Bates, P.A., 2007. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. International journal for parasitology 37, 1097-1106.

Bates, P.A., Rogers, M.E., 2004. New insights into the developmental biology and transmission mechanisms of *Leishmania*. Current molecular medicine 4, 601-609.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G.B., 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proceedings of the National Academy of Sciences of the United States of America 98, 9237-9242.

Baxarias, M., Alvarez-Fernandez, A., Martinez-Orellana, P., Montserrat-Sangra, S., Ordeix, L., Rojas, A., Nachum-Biala, Y., Baneth, G., Solano-Gallego, L., 2018. Does co-infection with vector-borne pathogens play a role in clinical canine leishmaniosis? Parasites & vectors 11, 135.

Bazzocchi, C., Mortarino, M., Comazzi, S., Bandi, C., Franceschi, A., Genchi, C., 2005. Expression and function of Toll-like receptor 2 in canine blood phagocytes. Veterinary immunology and immunopathology 104, 15-19.

Becker, I., Salaiza, N., Aguirre, M., Delgado, J., Carrillo-Carrasco, N., Kobeh, L.G., Ruiz, A., Cervantes, R., Torres, A.P., Cabrera, N., Gonzalez, A., Maldonado, C., Isibasi, A., 2003. *Leishmania* lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. Molecular and biochemical parasitology 130, 65-74.

Belkaid, Y., Hoffmann, K.F., Mendez, S., Kamhawi, S., Udey, M.C., Wynn, T.A., Sacks, D.L., 2001. The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. The Journal of experimental medicine 194, 1497-1506.

Berman, J.D., Waddell, D., Hanson, B.D., 1985. Biochemical mechanisms of the antileishmanial activity of sodium stibogluconate. Antimicrobial agents and chemotherapy 27, 916-920.

Berman, J.J., 2012. Taxonomic Guide to Infectious Diseases, 374 p.

Bern, C., Adler-Moore, J., Berenguer, J., Boelaert, M., den Boer, M., Davidson, R.N., Figueras, C., Gradoni, L., Kafetzis, D.A., Ritmeijer, K., Rosenthal, E., Royce, C., Russo, R., Sundar, S., Alvar, J., 2006. Liposomal amphotericin B for the treatment of visceral leishmaniasis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 43, 917-924.

Berta Suárez Rodríguez, B.I.F., Sara Santos Sanz,María José Sierra Moros, Ricardo Molina Moreno, Jenaro Astray Mochales, Carmen Amela Heras, 2012. Situación epidemiológica y delos factores de riesgo de transmisión de *Leishmania infantum* en España. Revista Española de Salud Pública 86, 555-564.

Blavier, A., Keroack, S., Denerolle, P., Goy-Thollot, I., Chabanne, L., Cadore, J.L., Bourdoiseau, G., 2001. Atypical forms of canine leishmaniosis. Veterinary journal 162, 108-120.

Bogdan, C., Gessner, A., Solbach, W., Rollinghoff, M., 1996. Invasion, control and persistence of *Leishmania* parasites. Current opinion in immunology 8, 517-525.

Bogdan, C., Rollinghoff, M., 1998. The immune response to *Leishmania*: mechanisms of parasite control and evasion. International journal for parasitology 28, 121-134.

Boggiatto, P.M., Gibson-Corley, K.N., Metz, K., Gallup, J.M., Hostetter, J.M., Mullin, K., Petersen, C.A., 2011. Transplacental transmission of *Leishmania infantum* as a means for continued disease incidence in North America. PLoS neglected tropical diseases 5, e1019.

Boggiatto, P.M., Ramer-Tait, A.E., Metz, K., Kramer, E.E., Gibson-Corley, K., Mullin, K., Hostetter, J.M., Gallup, J.M., Jones, D.E., Petersen, C.A., 2010. Immunologic indicators of clinical progression during canine *Leishmania infantum* infection. Clinical and vaccine immunology : CVI 17, 267-273.

Borborema, S.E.T., Osso Junior, J.A., Tempone, A.G., de Andrade Junior, H.F., do Nascimento, N., 2018. Pharmacokinetic of meglumine antimoniate encapsulated in phosphatidylserine-

liposomes in mice model: A candidate formulation for visceral leishmaniasis. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 103, 1609-1616.

Bottero E, P.M., Viglione M., 2006. Lesioni papulari indotte da *Leishmania spp*. in 8 cani giovani. Veterinaria 1.

Bourdeau, P., Saridomichelakis, M.N., Oliveira, A., Oliva, G., Kotnik, T., Galvez, R., Foglia Manzillo, V., Koutinas, A.F., Pereira da Fonseca, I., Miro, G., 2014. Management of canine leishmaniosis in endemic SW European regions: a questionnaire-based multinational survey. Parasites & vectors 7, 110.

Brandonisio, O., Carelli, G., Altamura, M., Varvara, B., Ceci, L., 1990. Circulating immune complexes and autoantibodies in canine leishmaniasis. Parassitologia 32, 275-281.

Breton, M., Tremblay, M.J., Ouellette, M., Papadopoulou, B., 2005. Live nonpathogenic parasitic vector as a candidate vaccine against visceral leishmaniasis. Infection and immunity 73, 6372-6382.

Buates, S., Matlashewski, G., 1999. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. The Journal of infectious diseases 179, 1485-1494.

Burgener, I.A., Konig, A., Allenspach, K., Sauter, S.N., Boisclair, J., Doherr, M.G., Jungi, T.W., 2008. Upregulation of toll-like receptors in chronic enteropathies in dogs. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine 22, 553-560.

Bustamante, M., Diaz, M., Espinoza, J., Parrado, R., Reithinger, R., Garcia, A.L., 2012. Sand fly fauna in Chapare, Bolivia: an endemic focus of *Leishmania* (Viannia) *braziliensis*. Journal of medical entomology 49, 1159-1162.

Caffrey, P., Lynch, S., Flood, E., Finnan, S., Oliynyk, M., 2001. Amphotericin biosynthesis in Streptomyces nodosus: deductions from analysis of polyketide synthase and late genes. Chemistry & biology 8, 713-723.

Campos, M.B., Lima, L., de Lima, A.C.S., Vasconcelos Dos Santos, T., Ramos, P.K.S., Gomes, C.M.C., Silveira, F.T., 2018. Toll-like receptors 2, 4, and 9 expressions over the entire clinical and immunopathological spectrum of American cutaneous leishmaniasis due to *Leishmania (V.) braziliensis* and *Leishmania (L.) amazonensis*. PloS one 13, e0194383.

Cantacessi, C., Dantas-Torres, F., Nolan, M.J., Otranto, D., 2015. The past, present, and future of Leishmania genomics and transcriptomics. Trends in parasitology 31, 100-108.

Cardoso, L., Neto, F., Sousa, J.C., Rodrigues, M., Cabral, M., 1998. Use of a leishmanin skin test in the detection of canine *Leishmania*-specific cellular immunity. Veterinary parasitology 79, 213-220.

Cardoso, L., Rodrigues, M., Santos, H., Schoone, G.J., Carreta, P., Varejao, E., van Benthem, B., Afonso, M.O., Alves-Pires, C., Semiao-Santos, S.J., Rodrigues, J., Schallig, H.D., 2004. Sero-epidemiological study of canine *Leishmania spp.* infection in the municipality of Alijo (Alto Douro, Portugal). Veterinary parasitology 121, 21-32.

Cardoso, L., Santos, H., Cordeiro-da-Silva, A., Pratlong, F., Dedet, J.P., Rodrigues, M., 2002. *Leishmania infantum* MON-98: infection in a dog from Alto Douro, Portugal. Acta tropica 83, 83-85.

Carrera, L., Gazzinelli, R.T., Badolato, R., Hieny, S., Muller, W., Kuhn, R., Sacks, D.L., 1996. *Leishmania* promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. The Journal of experimental medicine 183, 515-526.

Carrillo, E., Moreno, J., 2009. Cytokine profiles in canine visceral leishmaniasis. Veterinary immunology and immunopathology 128, 67-70.

Carrió, P.M., 2002. In vitro susceptibility to pentavalent antimony in *Leishmania infantum* strains is not modified during *in vitro* or *in vivo* passages but is modified after host treatment with meglumine antimoniate. MBMC Pharmacology 2, 6.

Carson, C., Antoniou, M., Ruiz-Arguello, M.B., Alcami, A., Christodoulou, V., Messaritakis, I., Blackwell, J.M., Courtenay, O., 2009. A prime/boost DNA/Modified vaccinia virus Ankara vaccine expressing recombinant *Leishmania* DNA encoding TRYP is safe and immunogenic in outbred dogs, the reservoir of zoonotic visceral leishmaniasis. Vaccine 27, 1080-1086.

Carter, D., Fox, C.B., Day, T.A., Guderian, J.A., Liang, H., Rolf, T., Vergara, J., Sagawa, Z.K., Ireton, G., Orr, M.T., Desbien, A., Duthie, M.S., Coler, R.N., Reed, S.G., 2016. A structure-function approach to optimizing TLR4 ligands for human vaccines. Clinical & translational immunology 5, e108.

Cecilio, P., Perez-Cabezas, B., Fernandez, L., Moreno, J., Carrillo, E., Requena, J.M., Fichera, E., Reed, S.G., Coler, R.N., Kamhawi, S., Oliveira, F., Valenzuela, J.G., Gradoni, L., Glueck, R., Gupta, G., Cordeiro-da-Silva, A., 2017. Pre-clinical antigenicity studies of an innovative multivalent vaccine for human visceral leishmaniasis. PLoS neglected tropical diseases 11, e0005951.

Cezario, G.A., de Oliveira, L.R., Peresi, E., Nicolete, V.C., Polettini, J., de Lima, C.R., Gatto, M., Calvi, S.A., 2011. Analysis of the expression of toll-like receptors 2 and 4 and cytokine production during experimental *Leishmania chagasi* infection. Memorias do Instituto Oswaldo Cruz 106, 573-583.

Ciaramella, P.C.M., 2003. Canine Leishmaniasis: Clinical and Diagnostic aspects. veterenary Learn 25, 358-369.

Coelho, W.M., Bresciani, K.D., 2013. Molecular and parasitological detection of *Leishmania spp*. in a dipteran of the species *Tabanus importunus*. Revista brasileira de parasitologia veterinaria = Brazilian journal of veterinary parasitology : Orgao Oficial do Colegio Brasileiro de Parasitologia Veterinaria 22, 605-607.

Courtenay, O., Quinnell, R.J., Garcez, L.M., Shaw, J.J., Dye, C., 2002. Infectiousness in a cohort of brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. The Journal of infectious diseases 186, 1314-1320.

Coutinho, M.T., Linardi, P.M., 2007. Can fleas from dogs infected with canine visceral leishmaniasis transfer the infection to other mammals? Veterinary parasitology 147, 320-325.

Craft, N., Birnbaum, R., Quanquin, N., Erfe, M.C., Quant, C., Haskell, J., Bruhn, K.W., 2014. Topical resiquimod protects against visceral infection with *Leishmania infantum chagasi* in mice. Clinical and vaccine immunology : CVI 21, 1314-1322.

Croft, S.L., Coombs, G.H., 2003. Leishmaniasis--current chemotherapy and recent advances in the search for novel drugs. Trends in parasitology 19, 502-508.

Croft, S.L., Snowdon, D., Yardley, V., 1996. The activities of four anticancer alkyllysophospholipids against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei*. The Journal of antimicrobial chemotherapy 38, 1041-1047.

Croft, S.L., Sundar, S., Fairlamb, A.H., 2006. Drug resistance in leishmaniasis. Clinical microbiology reviews 19, 111-126.

Cunha, S., Freire, M., Eulalio, C., Critosvao, J., Netto, E., Johnson, W.D., Jr., Reed, S.G., Badaro, R., 1995. Visceral leishmaniasis in a new ecological niche near a major metropolitan area of Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 89, 155-158.

Cusco, A., Sanchez, A., Altet, L., Ferrer, L., Francino, O., 2014. Non-synonymous genetic variation in exonic regions of canine Toll-like receptors. Canine genetics and epidemiology 1, 11.

Chamaille, L., Tran, A., Meunier, A., Bourdoiseau, G., Ready, P., Dedet, J.P., 2010. Environmental risk mapping of canine leishmaniasis in France. Parasites & vectors 3, 31.

Chandel, H.S., Pandey, S.P., Shukla, D., Lalsare, K., Selvaraj, S.K., Jha, M.K., Saha, B., 2014. Toll-like receptors and CD40 modulate each other's expression affecting *Leishmania major* infection. Clinical and experimental immunology 176, 283-290.

Chauhan, P., Shukla, D., Chattopadhyay, D., Saha, B., 2017. Redundant and regulatory roles for Toll-like receptors in *Leishmania* infection. Clinical and experimental immunology 190, 167-186.

Chemnitz, J.M., Parry, R.V., Nichols, K.E., June, C.H., Riley, J.L., 2004. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. Journal of immunology 173, 945-954.

Chen, J., Xu, W., Zhou, T., Ding, Y., Duan, J., Huang, F., 2009. Inhibitory role of toll-like receptors agonists in *Plasmodium yoelii* liver stage development. Parasite immunology 31, 466-473.

Chiku, V.M., Silva, K.L., de Almeida, B.F., Venturin, G.L., Leal, A.A., de Martini, C.C., de Rezende Eugenio, F., Dos Santos, P.S., de Lima, V.M., 2016. PD-1 function in apoptosis of T lymphocytes in canine visceral leishmaniasis. Immunobiology 221, 879-888.

Chotimanukul, S., Sirivaidyapong, S., 2011. Differential expression of Toll-like receptor 4 (TLR4) in healthy and infected canine endometrium. Theriogenology 76, 1152-1161.

Chotimanukul, S., Sirivaidyapong, S., 2012. The localization of Toll-like receptor 2 (TLR2) in the endometrium and the cervix of dogs at different stages of the oestrous cycle and with pyometra. Reproduction in domestic animals = Zuchthygiene 47 Suppl 6, 351-355.

da Silva, S.M., Ribeiro, V.M., Ribeiro, R.R., Tafuri, W.L., Melo, M.N., Michalick, M.S., 2009. First report of vertical transmission of *Leishmania* (Leishmania) *infantum* in a naturally infected bitch from Brazil. Veterinary parasitology 166, 159-162.

Daifalla, N.S., Bayih, A.G., Gedamu, L., 2012. *Leishmania donovani* recombinant iron superoxide dismutase B1 protein in the presence of TLR-based adjuvants induces partial protection of BALB/c mice against Leishmania major infection. Experimental parasitology 131, 317-324.

Dantas-Torres, F., Solano-Gallego, L., Baneth, G., Ribeiro, V.M., de Paiva-Cavalcanti, M., Otranto, D., 2012. Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. Trends in parasitology 28, 531-538.

Das, S., Rani, M., Pandey, K., Sahoo, G.C., Rabidas, V.N., Singh, D., Das, P., 2012. Combination of paromomycin and miltefosine promotes TLR4-dependent induction of antileishmanial immune response *in vitro*. The Journal of antimicrobial chemotherapy 67, 2373-2378.

Davidson, R.N., den Boer, M., Ritmeijer, K., 2009. Paromomycin. Transactions of the Royal Society of Tropical Medicine and Hygiene 103, 653-660.

De Muylder, G., Ang, K.K., Chen, S., Arkin, M.R., Engel, J.C., McKerrow, J.H., 2011. A screen against *Leishmania* intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit. PLoS neglected tropical diseases 5, e1253.

Dedet, J., Pratlong, F., 2000. [Taxonomy of *Leishmania* and geographical distribution of leishmaniasis]. Annales de dermatologie et de venereologie 127, 421-424.

Desjeux, P., 1999. Global control and *Leishmania* HIV co-infection. Clinics in dermatology 17, 317-325.

Desjeux, P., 2004. Leishmaniasis: current situation and new perspectives. Comparative immunology, microbiology and infectious diseases 27, 305-318.

Dorlo, T.P., Balasegaram, M., Beijnen, J.H., de Vries, P.J., 2012. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. The Journal of antimicrobial chemotherapy 67, 2576-2597.

Dos-Santos, A.L., Carvalho-Kelly, L.F., Dick, C.F., Meyer-Fernandes, J.R., 2016. Innate immunomodulation to trypanosomatid parasite infections. Experimental parasitology 167, 67-75.

Dostalova, A., Volf, P., 2012. *Leishmania* development in sand flies: parasite-vector interactions overview. Parasites & vectors 5, 276.

Dowling, J.K., Mansell, A., 2016. Toll-like receptors: the swiss army knife of immunity and vaccine development. Clinical & translational immunology 5, e85.

Dunkelberger, J.R., Song, W.C., 2010. Complement and its role in innate and adaptive immune responses. Cell research 20, 34-50.

Duprey, Z.H., Steurer, F.J., Rooney, J.A., Kirchhoff, L.V., Jackson, J.E., Rowton, E.D., Schantz, P.M., 2006. Canine visceral leishmaniasis, United States and Canada, 2000-2003. Emerging infectious diseases 12, 440-446.

Duthie, M.S., Pereira, L., Favila, M., Hofmeyer, K.A., Reed, S.J., Metangmo, S., Townsend, S., Laurance, J.D., Picone, A., Misquith, A., Hossain, F., Ghosh, P., Khan, M.A.A., Guderian, J., Bailor, H.R., Liang, H., Vergara, J., Oliveira, F., Howard, R.F., Kamhawi, S., Mondal, D., Coler, R.N., Valenzuela, J.G., Reed, S.G., 2017. A defined subunit vaccine that protects against vector-borne visceral leishmaniasis. NPJ vaccines 2, 23.

el-Kammah, K.M., 1973. Studies of autogeny in *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae). Journal of medical entomology 10, 261-263.

Emami, T., Rezayat, S.M., Khamesipour, A., Madani, R., Habibi, G., Hojatizade, M., Jaafari, M.R., 2018. The role of MPL and imiquimod adjuvants in enhancement of immune response and protection in BALB/c mice immunized with soluble *Leishmania* antigen (SLA) encapsulated in nanoliposome. Artificial cells, nanomedicine, and biotechnology, 1-10.

Eren, R.O., Reverte, M., Rossi, M., Hartley, M.A., Castiglioni, P., Prevel, F., Martin, R., Desponds, C., Lye, L.F., Drexler, S.K., Reith, W., Beverley, S.M., Ronet, C., Fasel, N., 2016. Mammalian Innate Immune Response to a *Leishmania*-Resident RNA Virus Increases Macrophage Survival to Promote Parasite Persistence. Cell host & microbe 20, 318-328.

Escobar, P., Matu, S., Marques, C., Croft, S.L., 2002. Sensitivities of *Leishmania* species to hexadecylphosphocholine (miltefosine), ET-18-OCH(3) (edelfosine) and amphotericin B. Acta tropica 81, 151-157.

Esch, K.J., Juelsgaard, R., Martinez, P.A., Jones, D.E., Petersen, C.A., 2013. Programmed death 1-mediated T cell exhaustion during visceral leishmaniasis impairs phagocyte function. Journal of immunology 191, 5542-5550.

Eskandari, F., Talesh, G.A., Parooie, M., Jaafari, M.R., Khamesipour, A., Saberi, Z., Abbasi, A., Badiee, A., 2014. Immunoliposomes containing Soluble *Leishmania* Antigens (SLA) as a novel antigen delivery system in murine model of leishmaniasis. Experimental parasitology 146, 78-86.

Esteve, L.O., Saz, S.V., Hosein, S., Solano-Gallego, L., 2015. Histopathological findings and detection of Toll-like receptor 2 in cutaneous lesions of canine leishmaniosis. Veterinary parasitology 209, 157-163.

Faraut-Gambarelli, F., Piarroux, R., Deniau, M., Giusiano, B., Marty, P., Michel, G., Faugere, B., Dumon, H., 1997. *In vitro* and *in vivo* resistance of *Leishmania infantum* to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. Antimicrobial agents and chemotherapy 41, 827-830.

Farca, A.M., Miniscalco, B., Badino, P., Odore, R., Monticelli, P., Trisciuoglio, A., Ferroglio, E., 2012. Canine leishmaniosis: *in vitro* efficacy of miltefosine and marbofloxacin alone or in combination with allopurinol against clinical strains of *Leishmania infantum*. Parasitology research 110, 2509-2513.

Faria, M.S., Reis, F.C., Azevedo-Pereira, R.L., Morrison, L.S., Mottram, J.C., Lima, A.P., 2011. *Leishmania* inhibitor of serine peptidase 2 prevents TLR4 activation by neutrophil elastase promoting parasite survival in murine macrophages. Journal of immunology 186, 411-422.

Faria, M.S., Reis, F.C., Lima, A.P., 2012. Toll-like receptors in *Leishmania* infections: guardians or promoters? Journal of parasitology research 2012, 930257.

Fernandez-Bellon, H., Solano-Gallego, L., Rodriguez, A., Rutten, V.P., Hoek, A., Ramis, A., Alberola, J., Ferrer, L., 2005. Comparison of three assays for the evaluation of specific cellular immunity to *Leishmania infantum* in dogs. Veterinary immunology and immunopathology 107, 163-169.

Fernandez Cotrina, J., Iniesta, V., Monroy, I., Baz, V., Hugnet, C., Maranon, F., Fabra, M., Gomez-Nieto, L.C., Alonso, C., 2018. A large-scale field randomized trial demonstrates safety and efficacy of the vaccine LetiFend(R) against canine leishmaniosis. Vaccine 36, 1972-1982.

Ferreira Ede, C., de Lana, M., Carneiro, M., Reis, A.B., Paes, D.V., da Silva, E.S., Schallig, H., Gontijo, C.M., 2007. Comparison of serological assays for the diagnosis of canine visceral leishmaniasis in animals presenting different clinical manifestations. Veterinary parasitology 146, 235-241.

Ferro, C., Marin, D., Gongora, R., Carrasquilla, M.C., Trujillo, J.E., Rueda, N.K., Marin, J., Valderrama-Ardila, C., Alexander, N., Perez, M., Munstermann, L.E., Ocampo, C.B., 2011. Phlebotomine vector ecology in the domestic transmission of American cutaneous leishmaniasis in Chaparral, Colombia. The American journal of tropical medicine and hygiene 85, 847-856.

Ferroglio, E., Centaro, E., Mignone, W., Trisciuoglio, A., 2007. Evaluation of an ELISA rapid device for the serological diagnosis of *Leishmania infantum* infection in dog as compared with immunofluorescence assay and Western blot. Veterinary parasitology 144, 162-166.

Ferroglio, E., Poggi, M., Trisciuoglio, A., 2008. Evaluation of 65% permethrin spot-on and deltamethrin-impregnated collars for canine *Leishmania infantum* infection prevention. Zoonoses and public health 55, 145-148.

Figueiredo, M.M., Amorim, I.F., Pinto, A.J., Barbosa, V.S., Pinheiro Lde, J., Deoti, B., Faria, A.M., Tafuri, W.L., 2013. Expression of Toll-like receptors 2 and 9 in cells of dog jejunum and colon naturally infected with *Leishmania infantum*. BMC immunology 14, 22.

Figueiredo, M.M., Deoti, B., Amorim, I.F., Pinto, A.J., Moraes, A., Carvalho, C.S., da Silva, S.M., de Assis, A.C., de Faria, A.M., Tafuri, W.L., 2014. Expression of regulatory T cells in jejunum, colon, and cervical and mesenteric lymph nodes of dogs naturally infected with *Leishmania infantum*. Infection and immunity 82, 3704-3712.

Flandin, J.F., Chano, F., Descoteaux, A., 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. European journal of immunology 36, 411-420.

Flo, T.H., Halaas, O., Torp, S., Ryan, L., Lien, E., Dybdahl, B., Sundan, A., Espevik, T., 2001. Differential expression of Toll-like receptor 2 in human cells. Journal of leukocyte biology 69, 474-481.

Foglia Manzillo, V., Di Muccio, T., Cappiello, S., Scalone, A., Paparcone, R., Fiorentino, E., Gizzarelli, M., Gramiccia, M., Gradoni, L., Oliva, G., 2013. Prospective study on the incidence and progression of clinical signs in naive dogs naturally infected by *Leishmania infantum*. PLoS neglected tropical diseases 7, e2225.

Franco, A.O., Davies, C.R., Mylne, A., Dedet, J.P., Gallego, M., Ballart, C., Gramiccia, M., Gradoni, L., Molina, R., Galvez, R., Morillas-Marquez, F., Baron-Lopez, S., Pires, C.A., Afonso, M.O., Ready, P.D., Cox, J., 2011. Predicting the distribution of canine leishmaniasis in western Europe based on environmental variables. Parasitology 138, 1878-1891.

Freites-Martinez, A., Cordoba, S., Bermejo, A., Borbujo, J., 2015. Mucocutaneous leishmaniasis caused by *Leishmania infantum* var Lombardi in an immunocompetent patient, Spain. Enfermedades infecciosas y microbiologia clinica 33, 499-500.

Frezard, F., Demicheli, C., Ribeiro, R.R., 2009. Pentavalent antimonials: new perspectives for old drugs. Molecules 14, 2317-2336.

Fumarola, L., Spinelli, R., Brandonisio, O., 2004. *In vitro* assays for evaluation of drug activity against *Leishmania spp.* Research in microbiology 155, 224-230.

Gaglio, G., Brianti, E., Napoli, E., Falsone, L., Dantas-Torres, F., Tarallo, V.D., Otranto, D., Giannetto, S., 2014. Effect of night time-intervals, height of traps and lunar phases on sand fly collection in a highly endemic area for canine leishmaniasis. Acta tropica 133, 73-77.

Galdino, H., Jr., Saar Gomes, R., Dos Santos, J.C., Pessoni, L.L., Maldaner, A.E., Marques, S.M., Gomes, C.M., Dorta, M.L., de Oliveira, M.A., Joosten, L.A., Ribeiro-Dias, F., 2016. *Leishmania* (Viannia) *braziliensis* amastigotes induces the expression of TNF-alpha and IL-10 by human peripheral blood mononuclear cells *in vitro* in a TLR4-dependent manner. Cytokine 88, 184-192.

Galvez, R., Descalzo, M.A., Miro, G., Jimenez, M.I., Martin, O., Dos Santos-Brandao, F., Guerrero, I., Cubero, E., Molina, R., 2010. Seasonal trends and spatial relations between environmental/meteorological factors and leishmaniosis sand fly vector abundances in Central Spain. Acta tropica 115, 95-102.

Gállego, M.C., J.; Castillejo, S.; Fisa, R.; Iniesta L.; López, P; Montoya, L; Riera, C; Tebar, S. & Portús, M. , 2003. Banco de cepas de *Leishmania* de la Universidad de Barcelona Revista Ibérica de Parasitología 63, 43-45.

Ganguli, P., Chowdhury, S., Chowdhury, S., Sarkar, R.R., 2015. Identification of Th1/Th2 regulatory switch to promote healing response during leishmaniasis: a computational approach. EURASIP journal on bioinformatics & systems biology 2015, 13.

Gatto, M., de Abreu, M.M., Tasca, K.I., de Assis Golim, M., da Silva, L.D., Simao, J.C., Fortaleza, C.M., de Campos Soares, A.M., Calvi, S.A., 2015. The involvement of TLR2 and TLR4 in cytokine and nitric oxide production in visceral leishmaniasis patients before and after treatment with anti-leishmanial drugs. PloS one 10, e0117977.

Gautam, S., Kumar, R., Singh, N., Singh, A.K., Rai, M., Sacks, D., Sundar, S., Nylen, S., 2014. CD8 T cell exhaustion in human visceral leishmaniasis. The Journal of infectious diseases 209, 290-299.

Gazzinelli, R.T., Denkers, E.Y., 2006. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nature reviews. Immunology 6, 895-906.

Giavedoni, P., Iranzo, P., Fuertes, I., Estrach, T., Alsina Gibert, M., 2015. Cutaneous leishmaniasis: 20 years' experience in a Spanish tertiary care hospital. Actas dermo-sifiliograficas 106, 310-316.

Ginouves, M., Simon, S., Nacher, M., Demar, M., Carme, B., Couppie, P., Prevot, G., 2017. *In vitro* sensitivity of cutaneous *Leishmania* promastigote isolates circulating in French Guiana to a set of drugs. The American journal of tropical medicine and hygiene 96, 1143-1150.

Giunchetti, R.C., Correa-Oliveira, R., Martins-Filho, O.A., Teixeira-Carvalho, A., Roatt, B.M., de Oliveira Aguiar-Soares, R.D., Coura-Vital, W., de Abreu, R.T., Malaquias, L.C., Gontijo, N.F., Brodskyn, C., de Oliveira, C.I., Costa, D.J., de Lana, M., Reis, A.B., 2008a. A killed *Leishmania* vaccine with sand fly saliva extract and saponin adjuvant displays immunogenicity in dogs. Vaccine 26, 623-638.

Giunchetti, R.C., Martins-Filho, O.A., Carneiro, C.M., Mayrink, W., Marques, M.J., Tafuri, W.L., Correa-Oliveira, R., Reis, A.B., 2008b. Histopathology, parasite density and cell phenotypes

of the popliteal lymph node in canine visceral leishmaniasis. Veterinary immunology and immunopathology 121, 23-33.

Gnjatic, S., Sawhney, N.B., Bhardwaj, N., 2010. Toll-like receptor agonists: are they good adjuvants? Cancer journal 16, 382-391.

Gomes, R.B., Mendonca, I.L., Silva, V.C., Ruas, J., Silva, M.B., Cruz, M.S., Barral, A., Costa, C.H., 2007. Antibodies against *Lutzomyia longipalpis* saliva in the fox *Cerdocyon thous* and the sylvatic cycle of *Leishmania chagasi*. Transactions of the Royal Society of Tropical Medicine and Hygiene 101, 127-133.

Gomez Perez, V., Garcia-Hernandez, R., Corpas-Lopez, V., Tomas, A.M., Martin-Sanchez, J., Castanys, S., Gamarro, F., 2016. Decreased antimony uptake and overexpression of genes of thiol metabolism are associated with drug resistance in a canine isolate of *Leishmania infantum*. International journal for parasitology. Drugs and drug resistance 6, 133-139.

Gonzalez-Fajardo, L., Fernandez, O.L., McMahon-Pratt, D., Saravia, N.G., 2015. *Ex vivo* host and parasite response to antileishmanial drugs and immunomodulators. PLoS neglected tropical diseases 9, e0003820.

Gorczynski, R., 1985. Immunization of susceptible BALB/c mice against *Leishmania braziliensis*. I. Resistance induced using as immunogen adherent or nonadherent cells from infected mice. Cell immunology 94, 1-10.

Govindaraj, R.G., Manavalan, B., Lee, G., Choi, S., 2010. Molecular modeling-based evaluation of TLR10 and identification of potential ligands in Toll-like receptor signaling. PloS one 5, e12713.

Gradoni, L., 2001. An update on antileishmanial vaccine candidates and prospects for a canine *Leishmania* vaccine. Veterinary parasitology 100, 87-103.

Gradoni, L., 2015. Canine *Leishmania* vaccines: still a long way to go. Veterinary parasitology 208, 94-100.

Gradoni, L., Soteriadou, K., Louzir, H., Dakkak, A., Toz, S.O., Jaffe, C., Dedet, J.P., Campino, L., Canavate, C., Dujardin, J.C., 2008. Drug regimens for visceral leishmaniasis in Mediterranean countries. Tropical medicine & international health : TM & IH 13, 1272-1276.

Gramiccia, M., Gradoni, L., 2005. The current status of zoonotic leishmaniases and approaches to disease control. International journal for parasitology 35, 1169-1180.

Gramiccia, M., Gradoni, L., Orsini, S., 1992. Decreased sensitivity to meglumine antimoniate (Glucantime) of *Leishmania infantum* isolated from dogs after several courses of drug treatment. Annals of tropical medicine and parasitology 86, 613-620.

Grano, F.G., Dos, S.S.J.E., Melo, G.D., de Souza, M.S., Lima, V.M.F., Machado, G.F., 2018. Toll-like receptors and cytokines in the brain and in spleen of dogs with visceral leishmaniosis. Veterinary parasitology 253, 30-38.

Gray, K.C., Palacios, D.S., Dailey, I., Endo, M.M., Uno, B.E., Wilcock, B.C., Burke, M.D., 2012. Amphotericin primarily kills yeast by simply binding ergosterol. Proceedings of the National Academy of Sciences of the United States of America 109, 2234-2239.

Green, S.J., Crawford, R.M., Hockmeyer, J.T., Meltzer, M.S., Nacy, C.A., 1990. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. Journal of immunology 145, 4290-4297.

Grimaldi, G., Jr., David, J.R., McMahon-Pratt, D., 1987. Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. The American journal of tropical medicine and hygiene 36, 270-287.

Grinnage-Pulley, T.L., Kabotso, D.E.K., Rintelmann, C.L., Roychoudhury, R., Schaut, R.G., Toepp, A.J., Gibson-Corley, K.N., Parrish, M., Pohl, N.L.B., Petersen, C.A., 2017. *Leishmania*-derived trimannose modulates inflammatory response to significantly reduce *Leishmania (L.) major*-induced lesions. Infection and immunity.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C., Amigorena, S., 2002. Antigen presentation and T cell stimulation by dendritic cells. Annual review of immunology 20, 621-667.

Gupta, G., Oghumu, S., Satoskar, A.R., 2013. Mechanisms of immune evasion in leishmaniasis. Advances in applied microbiology 82, 155-184.

Habib, S., El Andaloussi, A., Elmasry, K., Handoussa, A., Azab, M., Elsawey, A., Al-Hendy, A., Ismail, N., 2018. PDL-1 Blockade Prevents T Cell Exhaustion, Inhibits Autophagy, and Promotes Clearance of *Leishmania donovani*. Infection and immunity 86.

Hadighi, R., Mohebali, M., Boucher, P., Hajjaran, H., Khamesipour, A., Ouellette, M., 2006. Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS medicine 3, e162.

Halliday, A., Bates, P.A., Chance, M.L., Taylor, M.J., 2016. Toll-like receptor 2 (TLR2) plays a role in controlling cutaneous leishmaniasis *in vivo*, but does not require activation by parasite lipophosphoglycan. Parasites & vectors 9, 532.

Hamilton, P.B., Stevens, J.R., 2017. American Trypanosomiasis Chagas Disease, In: University of Exeter, Exeter, United Kingdom, pp. 321-344.

Harizanov, R., Rainova, I., Tzvetkova, N., Kaftandjiev, I., Bikov, I., Mikov, O., 2013. Geographical distribution and epidemiological characteristics of visceral leishmaniasis in Bulgaria, 1988 to 2012. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin 18, 20531.

Hartley, M.A., Eren, R.O., Rossi, M., Prevel, F., Castiglioni, P., Isorce, N., Desponds, C., Lye, L.F., Beverley, S.M., Drexler, S.K., Fasel, N., 2018. *Leishmania guyanensis* parasites block the activation of the inflammasome by inhibiting maturation of IL-1beta. Microbial cell 5, 137-149.

Hawn, T.R., Ozinsky, A., Underhill, D.M., Buckner, F.S., Akira, S., Aderem, A., 2002. *Leishmania major* activates IL-1 alpha expression in macrophages through a MyD88-dependent pathway. Microbes and infection / Institut Pasteur 4, 763-771.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-1529.

Hendrickx, S., Guerin, P.J., Caljon, G., Croft, S.L., Maes, L., 2018. Evaluating drug resistance in visceral leishmaniasis: the challenges. Parasitology 145, 453-463.

Hernandez-Ruiz, J., Salaiza-Suazo, N., Carrada, G., Escoto, S., Ruiz-Remigio, A., Rosenstein, Y., Zentella, A., Becker, I., 2010. CD8 cells of patients with diffuse cutaneous leishmaniasis display functional exhaustion: the latter is reversed, in vitro, by TLR2 agonists. PLoS neglected tropical diseases 4, e871.

Hernandez, L., 2015. Estudio de la infección por *Leishmania infantum* en perro: utilidad de las técnicas diagnósticas no invasivas y nuevas alternativas terapéuticas. Thesis. Iniversidad complutense de Madrid, Madrid.

Hide, M., Banuls, A.L., Tibayrenc, M., 2001. Genetic heterogeneity and phylogenetic status of *Leishmania* (Leishmania) *infantum* zymodeme MON-1: epidemiological implications. Parasitology 123, 425-432.

Hilgard, P., Klenner, T., Stekar, J., Unger, C., 1993. Alkylphosphocholines: a new class of membrane-active anticancer agents. Cancer chemotherapy and pharmacology 32, 90-95.

Hoebe, K., Beutler, B., 2004. LPS, dsRNA and the interferon bridge to adaptive immune responses: Trif, Tram, and other TIR adaptor proteins. Journal of endotoxin research 10, 130-136.

Hofmeyer, K.A., Jeon, H., Zang, X., 2011. The PD-1/PD-L1 (B7-H1) pathway in chronic infection-induced cytotoxic T lymphocyte exhaustion. Journal of biomedicine & biotechnology 2011, 451694.

Holzmuller, P., Bras-Goncalves, R., Lemesre, J.L., 2006a. Phenotypical characteristics, biochemical pathways, molecular targets and putative role of nitric oxide-mediated programmed cell death in *Leishmania*. Parasitology 132 Suppl, S19-32.

Holzmuller, P., Cavaleyra, M., Moreaux, J., Kovacic, R., Vincendeau, P., Papierok, G., Lemesre, J.L., 2005. Lymphocytes of dogs immunised with purified excreted-secreted antigens of *Leishmania infantum* co-incubated with *Leishmania* infected macrophages produce IFN gamma resulting in nitric oxide-mediated amastigote apoptosis. Veterinary immunology and immunopathology 106, 247-257.

Holzmuller, P., Geiger, A., Nzoumbou-Boko, R., Pissarra, J., Hamrouni, S., Rodrigues, V., Dauchy, F.A., Lemesre, J.L., Vincendeau, P., Bras-Goncalves, R., 2018. Trypanosomatid Infections: How Do Parasites and Their Excreted-Secreted Factors Modulate the Inducible Metabolism of l-Arginine in Macrophages? Frontiers in immunology 9, 778.

Holzmuller, P., Hide, M., Sereno, D., Lemesre, J.L., 2006b. *Leishmania infantum* amastigotes resistant to nitric oxide cytotoxicity: Impact on *in vitro* parasite developmental cycle and metabolic enzyme activities. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 6, 187-197.

Hosein, S., 2014. Innate Immunity in canine *Leishmania infantum* infection; what role do toll like receptros play? Classic. University of London, Royal Veterinary College.

Hosein, S., Blake, D.P., Solano-Gallego, L., 2017. Insights on adaptive and innate immunity in canine leishmaniosis. Parasitology 144, 95-115.

Hosein, S., Rodriguez-Cortes, A., Blake, D.P., Allenspach, K., Alberola, J., Solano-Gallego, L., 2015. Transcription of Toll-Like Receptors 2, 3, 4 and 9, FoxP3 and Th17 Cytokines in a Susceptible Experimental Model of Canine *Leishmania infantum* Infection. PloS one 10, e0140325.

Hosseini-Vasoukolaei, N., Idali, F., Khamesipour, A., Yaghoobi-Ershadi, M.R., Kamhawi, S., Valenzuela, J.G., Edalatkhah, H., Arandian, M.H., Mirhendi, H., Emami, S., Jafari, R., Saeidi, Z., Jeddi-Tehrani, M., Akhavan, A.A., 2016. Differential expression profiles of the salivary proteins SP15 and SP44 from Phlebotomus papatasi. Parasites & vectors 9, 357.

House, A.K., Gregory, S.P., Catchpole, B., 2008. Pattern-recognition receptor mRNA expression and function in canine monocyte/macrophages and relevance to canine anal furunuclosis. Veterinary immunology and immunopathology 124, 230-240.

Huang, L., Hinchman, M., Mendez, S., 2015. Coinjection with TLR2 agonist Pam3CSK4 reduces the pathology of leishmanization in mice. PLoS neglected tropical diseases 9, e0003546.

Hussein, W.M., Liu, T.Y., Skwarczynski, M., Toth, I., 2014. Toll-like receptor agonists: a patent review (2011 - 2013). Expert opinion on therapeutic patents 24, 453-470.

Igarashi, H., Ohno, K., Fujiwara-Igarashi, A., Kanemoto, H., Fukushima, K., Goto-Koshino, Y., Uchida, K., Tsujimoto, H., 2015. Functional analysis of pattern recognition receptors in miniature dachshunds with inflammatory colorectal polyps. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 77, 439-447.

Igarashi, H., Ohno, K., Maeda, S., Kanemoto, H., Fukushima, K., Uchida, K., Tsujimoto, H., 2014. Expression profiling of pattern recognition receptors and selected cytokines in miniature dachshunds with inflammatory colorectal polyps. Veterinary immunology and immunopathology 159, 1-10.

Ishida, Y., Agata, Y., Shibahara, K., Honjo, T., 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. The EMBO journal 11, 3887-3895.

Janssens, S., Beyaert, R., 2003. Role of Toll-like receptors in pathogen recognition. Clinical microbiology reviews 16, 637-646.

Jha, S.N., Singh, N.K., Jha, T.K., 1991. Changing response to diamidine compounds in cases of kala-azar unresponsive to antimonial. The Journal of the Association of Physicians of India 39, 314-316.

Johnston, D., Bystryn, J.C., 2006. Topical imiquimod is a potent adjuvant to a weakly-immunogenic protein prototype vaccine. Vaccine 24, 1958-1965.

Jung, J.W., Kim, D.K., Park, H.W., Oh, K.H., Joo, K.W., Kim, Y.S., Ahn, C., Lee, K.W., Cho, S.H., Min, K.U., Kang, H.R., 2015. An effective strategy to prevent allopurinol-induced hypersensitivity by HLA typing. Genetics in medicine : official journal of the American College of Medical Genetics 17, 807-814.

Kamhawi, S., 2006. Phlebotomine sand flies and *Leishmania* parasites: friends or foes? Trends in parasitology 22, 439-445.

Kammoun-Rebai, W., Bahi-Jaber, N., Naouar, I., Toumi, A., Ben Salah, A., Louzir, H., Meddeb-Garnaoui, A., 2017. Human cellular and humoral immune responses to *Phlebotomus papatasi* salivary gland antigens in endemic areas differing in prevalence of *Leishmania major* infection. PLoS neglected tropical diseases 11.

Kang, J.W., Kim, S.J., Cho, H.I., Lee, S.M., 2015. DAMPs activating innate immune responses in sepsis. Ageing research reviews 24, 54-65.

Kauffmann, F., Meert, E., de Jonge, K., Elkrim, Y., Hanot Mambres, D., Denis, O., Muraille, E., Magez, S., De Trez, C., 2018. STAT6 Mediates Footpad Immunopathology in the Absence of IL-12p40 Following Infection of Susceptible BALB/c Mice With *Leishmania major*. Frontiers in immunology 9, 503.

Kavoosi, G., Ardestani, S.K., Kariminia, A., Alimohammadian, M.H., 2010. *Leishmania major* lipophosphoglycan: discrepancy in Toll-like receptor signaling. Experimental parasitology 124, 214-218.

Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology 11, 373-384.

Kenneth Murphy, P.T., 2012. Janeway's Immunobiology. Garland Science, London.

Khadem, F., Uzonna, J.E., 2014. Immunity to visceral leishmaniasis: implications for immunotherapy. Future microbiology 9, 901-915.

Khalili, G., Dobakhti, F., Mahmoudzadeh-Niknam, H., Khaze, V., Partovi, F., 2011. Immunotherapy with Imiquimod increases the efficacy of Glucantime therapy of *Leishmania major* infection. Iranian journal of immunology : IJI 8, 45-51.

Khatami, A., Firooz, A., Gorouhi, F., Dowlati, Y., 2007. Treatment of acute Old World cutaneous leishmaniasis: a systematic review of the randomized controlled trials. Journal of the American Academy of Dermatology 57, 335 e331-329.

Killick-Kendrick, R., 1990. Phlebotomine vectors of the leishmaniases: a review. Medical and veterinary entomology 4, 1-24.

Killick-Kendrick, R., 1999. The biology and control of phlebotomine sand flies. Clinics in dermatology 17, 279-289.

Killick-Kendrick, R., Leaney, A.J., Ready, P.D., Molyneux, D.H., 1977. *Leishmania* in phlebotomid sandflies. IV. The transmission of Leishmania mexicana amazonensis to hamsters by the bite of experimentally infected *Lutzomyia longipalpis*. Proceedings of the Royal Society of London. Series B, Biological sciences 196, 105-115.

Killick-Kendrick, R., Wilkes, T.J., Bailly, M., Bailly, I., Righton, L.A., 1986. Preliminary field observations on the flight speed of a phlebotomine sandfly. Transactions of the Royal Society of Tropical Medicine and Hygiene 80, 138-142.

Kimsey, P.B., Theodos, C.M., Mitchen, T.K., Turco, S.J., Titus, R.G., 1993. An avirulent lipophosphoglycan-deficient *Leishmania major* clone induces CD4+ T cells which protect susceptible BALB/c mice against infection with virulent *L. major.* Infection and immunity 61, 5205-5213.

Koutinas, A.F., Koutinas, C.K., 2014. Pathologic mechanisms underlying the clinical findings in canine leishmaniasis due to *Leishmania infantum/chagasi*. Veterinary pathology 51, 527-538.

Koutinas, A.F., Polizopoulou, Z.S., Saridomichelakis, M.N., Argyriadis, D., Fytianou, A., Plevraki, K.G., 1999. Clinical considerations on canine visceral leishmaniasis in Greece: a retrospective study of 158 cases (1989-1996). Journal of the American Animal Hospital Association 35, 376-383.

Krayter, L., Schnur, L.F., Schonian, G., 2015. The genetic relationship between *Leishmania aethiopica* and *Leishmania tropica* revealed by comparing microsatellite profiles. PloS one 10, e0131227.

Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. Annual review of immunology 20, 709-760.

Krishnan, J., Lee, G., Choi, S., 2009. Drugs targeting Toll-like receptors. Archives of pharmacal research 32, 1485-1502.

Kropf, P., Freudenberg, M.A., Modolell, M., Price, H.P., Herath, S., Antoniazi, S., Galanos, C., Smith, D.F., Muller, I., 2004a. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. Infection and immunity 72, 1920-1928.

Kropf, P., Freudenberg, N., Kalis, C., Modolell, M., Herath, S., Galanos, C., Freudenberg, M., Muller, I., 2004b. Infection of C57BL/10ScCr and C57BL/10ScNCr mice with *Leishmania major* reveals a role for Toll-like receptor 4 in the control of parasite replication. Journal of leukocyte biology 76, 48-57.

Kuhlencord, A., Maniera, T., Eibl, H., Unger, C., 1992. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. Antimicrobial agents and chemotherapy 36, 1630-1634.

Kulshrestha, A., Bhandari, V., Mukhopadhyay, R., Ramesh, V., Sundar, S., Maes, L., Dujardin, J.C., Roy, S., Salotra, P., 2013. Validation of a simple resazurin-based promastigote assay for the routine monitoring of miltefosine susceptibility in clinical isolates of *Leishmania donovani*. Parasitology research 112, 825-828.

Kumar, H., Kawai, T., Akira, S., 2009. Toll-like receptors and innate immunity. Biochemical and biophysical research communications 388, 621-625.

Kumar, R., Singh, O.P., Gautam, S., Nylen, S., Sundar, S., 2014. Enhanced expression of Toll-like receptors 2 and 4, but not 9, in spleen tissue from patients with visceral leishmaniasis. Parasite immunology 36, 721-725.

Kumari, P., Nigam, R., Choudhury, S., Singh, S.K., Yadav, B., Kumar, D., Garg, S.K., 2018. *Demodex canis* targets TLRs to evade host immunity and induce canine demodicosis. Parasite immunology 40.

Laguna, F., Lopez-Velez, R., Pulido, F., Salas, A., Torre-Cisneros, J., Torres, E., Medrano, F.J., Sanz, J., Pico, G., Gomez-Rodrigo, J., Pasquau, J., Alvar, J., 1999. Treatment of visceral leishmaniasis in HIV-infected patients: a randomized trial comparing meglumine antimoniate with amphotericin B. Spanish HIV-*Leishmania* Study Group. Aids 13, 1063-1069.

Lainson, R., Ryan, L., Shaw, J.J., 1987. Infective stages of *Leishmania* in the sandfly vector and some observations on the mechanism of transmission. Memorias do Instituto Oswaldo Cruz 82, 421-424.

Laskay, T., van Zandbergen, G., Solbach, W., 2003. Neutrophil granulocytes--Trojan horses for *Leishmania major* and other intracellular microbes? Trends in microbiology 11, 210-214.

Lasri, S., Sahibi, H., Natami, A., Rhalem, A., 2003. Western blot analysis of *Leishmania infantum* antigens using sera from pentamidine-treated dogs. Veterinary immunology and immunopathology 91, 13-18.

Laurent, T., Rijal, S., Yardley, V., Croft, S., De Doncker, S., Decuypere, S., Khanal, B., Singh, R., Schonian, G., Kuhls, K., Chappuis, F., Dujardin, J.C., 2007. Epidemiological dynamics of antimonial resistance in *Leishmania donovani*: genotyping reveals a polyclonal population structure among naturally-resistant clinical isolates from Nepal. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 7, 206-212.

Le Blancq, S.M., Schnur, L.F., Peters, W., 1986. *Leishmania* in the Old World: 1. The geographical and hostal distribution of *L. major* zymodemes. Transactions of the Royal Society of Tropical Medicine and Hygiene 80, 99-112.

Lee, J.J. 2000. Illustrated Guide To The Protozoa (Allen press), p. 689.

Lewis, D.H., Peters, W., 1977. The resistance of intracellular *Leishmania* parasites to digestion by lysosomal enzymes. Annals of tropical medicine and parasitology 71, 295-312.

Liese, J., Schleicher, U., Bogdan, C., 2008. The innate immune response against *Leishmania* parasites. Immunobiology 213, 377-387.

Liu, D., Uzonna, J.E., 2012. The early interaction of *Leishmania* with macrophages and dendritic cells and its influence on the host immune response. Frontiers in cellular and infection microbiology 2, 83.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Lombardo, G., Pennisi, M.G., Lupo, T., Chicharro, C., Solano-Gallego, L., 2014. Papular dermatitis due to *Leishmania infantum* infection in seventeen dogs: diagnostic features, extent of the infection and treatment outcome. Parasites & vectors 7, 120.

Loria-Cervera, E.N., Sosa-Bibiano, E.I., Villanueva-Lizama, L.E., Van Wynsberghe, N.R., Canto-Lara, S.B., Batun-Cutz, J.L., Andrade-Narvaez, F.J., 2013. Nitric oxide production by *Peromyscus yucatanicus* (Rodentia) infected with *Leishmania* (Leishmania) *mexicana*. Memorias do Instituto Oswaldo Cruz 108, 172-177.

Luciani, A., Sconza, S., Civitella, C., Guglielmini, C., 2013. Evaluation of the cardiac toxicity of N-methyl-glucamine antimoniate in dogs with naturally occurring leishmaniasis. Veterinary journal 196, 119-121.

Luppi, M.M., Malta, M.C., Silva, T.M., Silva, F.L., Motta, R.O., Miranda, I., Ecco, R., Santos, R.L., 2008. Visceral leishmaniasis in captive wild canids in Brazil. Veterinary parasitology 155, 146-151.

Lux, H., Heise, N., Klenner, T., Hart, D., Opperdoes, F.R., 2000. Ether--lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether--lipid analogues in *Leishmania*. Molecular and biochemical parasitology 111, 1-14.

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Veterinary parasitology 158, 274-287.

Maia, C., Cardoso, L., 2015. Spread of *Leishmania infantum* in Europe with dog travelling. Veterinary parasitology 213, 2-11.

Maia, C., Gomes, J., Cristovao, J., Nunes, M., Martins, A., Rebelo, E., Campino, L., 2010. Feline *Leishmania* infection in a canine leishmaniasis endemic region, Portugal. Veterinary parasitology 174, 336-340.

Maia, C., Nunes, M., Marques, M., Henriques, S., Rolao, N., Campino, L., 2013. *In vitro* drug susceptibility of *Leishmania infantum* isolated from humans and dogs. Experimental parasitology 135, 36-41.

Maia, C., Rolao, N., Nunes, M., Goncalves, L., Campino, L., 2007. Infectivity of five different types of macrophages by *Leishmania infantum*. Acta tropica 103, 150-155.

Maia, C., Seblova, V., Sadlova, J., Votypka, J., Volf, P., 2011. Experimental transmission of *Leishmania infantum* by two major vectors: a comparison between a viscerotropic and a dermotropic strain. PLoS neglected tropical diseases 5, e1181.

Maiolini, A., Carlson, R., Tipold, A., 2012. Toll-like receptors 4 and 9 are responsible for the maintenance of the inflammatory reaction in canine steroid-responsive meningitis-arteritis, a large animal model for neutrophilic meningitis. Journal of neuroinflammation 9, 226.

Majumdar, S.B., Bhattacharya, P., Bhattacharjee, S., Majumder, S., Banerjee, S., Majumdar, S., 2014. Toll like receptor 2 and CC chemokine receptor 5 cluster in the lipid raft enhances the susceptibility of *Leishmania donovani* infection in macrophages. Indian journal of experimental biology 52, 17-29.

Manna, L., Reale, S., Vitale, F., Picillo, E., Pavone, L.M., Gravino, A.E., 2008. Real-time PCR assay in *Leishmania*-infected dogs treated with meglumine antimoniate and allopurinol. Veterinary journal 177, 279-282.

Marcondes, M., Biondo, A.W., Gomes, A.A., Silva, A.R., Vieira, R.F., Camacho, A.A., Quinn, J., Chandrashekar, R., 2011. Validation of a *Leishmania infantum* ELISA rapid test for serological diagnosis of *Leishmania chagasi* in dogs. Veterinary parasitology 175, 15-19.

Marcoval, J., Penin, R.M., 2017. Evolution of cutaneous leishmaniasis in the last 30 years in a tertiary hospital of the European Mediterranean coast. International journal of dermatology 56, 750-753.

Maroli, M., Feliciangeli, M.D., Bichaud, L., Charrel, R.N., Gradoni, L., 2013. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Medical and veterinary entomology 27, 123-147.

Maroli, M., Gradoni, L., Oliva, G., Castagnaro, M., Crotti, A., Lubas, G., Paltrinieri, S., Roura, X., Zini, E., Zatelli, A., 2010. Guidelines for prevention of leishmaniasis in dogs. Journal of the American Veterinary Medical Association 236, 1200-1206.

Maroli, M., Mizzon, V., Siragusa, C., D'Oorazi, A., Gradoni, L., 2001. Evidence for an impact on the incidence of canine leishmaniasis by the mass use of deltamethrin-impregnated dog collars in southern Italy. Medical and veterinary entomology 15, 358-363.

Martin-Sanchez, J., Guilvard, E., Acedo-Sanchez, C., Wolf-Echeverri, M., Sanchiz-Marin, M.C., Morillas-Marquez, F., 1994. *Phlebotomus perniciosus* Newstead, 1911, infection by various zymodemes of the *Leishmania infantum* complex in the Granada province (southern Spain). International journal for parasitology 24, 405-408.

Martin-Sanchez, J., Morales-Yuste, M., Acedo-Sanchez, C., Baron, S., Diaz, V., Morillas-Marquez, F., 2009. Canine leishmaniasis in southeastern Spain. Emerging infectious diseases 15, 795-798.

Martinez-Orellana, P., Mari-Martorell, D., Montserrat-Sangra, S., Ordeix, L., Baneth, G., Solano-Gallego, L., 2017a. *Leishmania infantum-specific* IFN-gamma production in stimulated blood from dogs with clinical leishmaniosis at diagnosis and during treatment. Veterinary parasitology 248, 39-47.

Martinez-Orellana, P., Quirola-Amores, P., Montserrat-Sangra, S., Ordeix, L., Llull, J., Alvarez-Fernandez, A., Solano-Gallego, L., 2017b. The inflammatory cytokine effect of Pam3CSK4 TLR2 agonist alone or in combination with *Leishmania infantum* antigen on *ex-vivo* whole blood from sick and resistant dogs. Parasites & vectors 10, 123.

Martinez-Orellana., M.-S.S., Solano-Gallego L. 2017. (Abstract) Characterization of inflammatory cytokine induction in different TLRs agonists treated canine macrophage *in vitro* model before or after *Leishmania infantum* infection (Plos Collections, Drugs for Neglected Diseases initiative).

Martinez, V., Quilez, J., Sanchez, A., Roura, X., Francino, O., Altet, L., 2011. Canine leishmaniasis: the key points for qPCR result interpretation. Parasites & vectors 4, 57.

Mateo, M., Maynard, L., Vischer, C., Bianciardi, P., Miro, G., 2009. Comparative study on the short term efficacy and adverse effects of miltefosine and meglumine antimoniate in dogs with natural leishmaniosis. Parasitology research 105, 155-162.

McCall, L.I., Zhang, W.W., Ranasinghe, S., Matlashewski, G., 2013. Leishmanization revisited: immunization with a naturally attenuated cutaneous *Leishmania donovani* isolate from Sri Lanka protects against visceral leishmaniasis. Vaccine 31, 1420-1425.

McMahon, L.A., House, A.K., Catchpole, B., Elson-Riggins, J., Riddle, A., Smith, K., Werling, D., Burgener, I.A., Allenspach, K., 2010. Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease. Veterinary immunology and immunopathology 135, 158-163.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. Nature reviews. Immunology 1, 135-145.

Melo, G.D., Silva, J.E., Grano, F.G., Homem, C.G., Machado, G.F., 2014a. Compartmentalized gene expression of toll-like receptors 2, 4 and 9 in the brain and peripheral lymphoid organs during canine visceral leishmaniasis. Parasite immunology 36, 726-731.

Melo, L.M., Perosso, J., Almeida, B.F., Silva, K.L., Somenzari, M.A., de Lima, V.M., 2014b. Effects of P-MAPA immunomodulator on Toll-like receptor 2, ROS, nitric oxide, MAPKp38 and IKK in PBMC and macrophages from dogs with visceral leishmaniasis. International immunopharmacology 18, 373-378.

Mercier, E., Peters, I.R., Day, M.J., Clercx, C., Peeters, D., 2012. Toll- and NOD-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. Veterinary immunology and immunopathology 145, 618-624.

Mercier, E., Peters, I.R., Farnir, F., Lavoue, R., Day, M., Clercx, C., Peeters, D., 2014. Assessment of Toll-like receptor 2, 4 and 9 SNP genotypes in canine sino-nasal aspergillosis. BMC veterinary research 10, 187.

Meredith, S.E., Kroon, N.C., Sondorp, E., Seaman, J., Goris, M.G., van Ingen, C.W., Oosting, H., Schoone, G.J., Terpstra, W.J., Oskam, L., 1995. Leish-KIT, a stable direct agglutination test based on freeze-dried antigen for serodiagnosis of visceral leishmaniasis. Journal of clinical microbiology 33, 1742-1745.

Mettler, M., Grimm, F., Capelli, G., Camp, H., Deplazes, P., 2005. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. Journal of clinical microbiology 43, 5515-5519.

Mifsud, E.J., Tan, A.C., Jackson, D.C., 2014. TLR Agonists as Modulators of the Innate Immune Response and Their Potential as Agents Against Infectious Disease. Frontiers in immunology 5, 79.

Mikami, T., Miyashita, H., Takatsuka, S., Kuroki, Y., Matsushima, N., 2012. Molecular evolution of vertebrate Toll-like receptors: evolutionary rate difference between their leucine-rich repeats and their TIR domains. Gene 503, 235-243.

Miljkovic, V., Arsic, B., Bojanic, Z., Nikolic, G., Nikolic, L., Kalicanin, B., Savic, V., 2014. Interactions of metronidazole with other medicines: a brief review. Die Pharmazie 69, 571-577.

Millan, J., Ferroglio, E., Solano-Gallego, L., 2014. Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. Parasitology research 113, 2005-2014.

Millan, J., Travaini, A., Zanet, S., Lopez-Bao, J.V., Trisciuoglio, A., Ferroglio, E., Rodriguez, A., 2016. Detection of Leishmania DNA in wild foxes and associated ticks in Patagonia, Argentina, 2000 km south of its known distribution area. Parasites & vectors 9, 241.

Miranda, S., Roura, X., Picado, A., Ferrer, L., Ramis, A., 2008. Characterization of sex, age, and breed for a population of canine leishmaniosis diseased dogs. Research in veterinary science 85, 35-38.

Mircean, V., Dumitrache, M.O., Mircean, M., Bolfa, P., Gyorke, A., Mihalca, A.D., 2014. Autochthonous canine leishmaniasis in Romania: neglected or (re)emerging? Parasites & vectors 7, 135.

Miro, G., Cardoso, L., Pennisi, M.G., Oliva, G., Baneth, G., 2008. Canine leishmaniosis--new concepts and insights on an expanding zoonosis: part two. Trends in parasitology 24, 371-377.

Miro, G., Checa, R., Montoya, A., Hernandez, L., Dado, D., Galvez, R., 2012. Current situation of *Leishmania infantum* infection in shelter dogs in northern Spain. Parasites & vectors 5, 60.

Miro, G., Galvez, R., Fraile, C., Descalzo, M.A., Molina, R., 2011. Infectivity to *Phlebotomus perniciosus* of dogs naturally parasitized with *Leishmania infantum* after different treatments. Parasites & vectors 4, 52.

Miro, G., Galvez, R., Mateo, M., Montoya, A., Descalzo, M.A., Molina, R., 2007. Evaluation of the efficacy of a topically administered combination of imidacloprid and permethrin against *Phlebotomus perniciosus* in dog. Veterinary parasitology 143, 375-379.

Miró, G., Molina, R., , 2006. Leishmaniosis canina: Manejo clínico y situación actual en EspañaBarcelona, 114 p.

Miro, G., Montoya, A., Roura, X., Galvez, R., Sainz, A., 2013. Seropositivity rates for agents of canine vector-borne diseases in Spain: a multicentre study. Parasites & vectors 6, 117.

Miro, G., Oliva, G., Cruz, I., Canavate, C., Mortarino, M., Vischer, C., Bianciardi, P., 2009. Multicentric, controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. Veterinary dermatology 20, 397-404.

Miro, G., Petersen, C., Cardoso, L., Bourdeau, P., Baneth, G., Solano-Gallego, L., Pennisi, M.G., Ferrer, L., Oliva, G., 2017. Novel Areas for Prevention and Control of Canine Leishmaniosis. Trends in parasitology 33, 718-730.

Mishra, B.B., Gundra, U.M., Teale, J.M., 2008. Expression and distribution of Toll-like receptors 11-13 in the brain during murine neurocysticercosis. Journal of neuroinflammation 5, 53.

Molina, R., Jimenez, M.I., Cruz, I., Iriso, A., Martin-Martin, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (Lepus granatensis) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Veterinary parasitology 190, 268-271.

Molina, R., Miro, G., Galvez, R., Nieto, J., Descalzo, M.A., 2006. Evaluation of a spray of permethrin and pyriproxyfen for the protection of dogs against *Phlebotomus perniciosus*. The Veterinary record 159, 206-209.

Montserrat-Sangra, S., Alborch, L., Ordeix, L., Solano-Gallego, L., 2016. TLR-2 and TLR-4 transcriptions in unstimulated blood from dogs with leishmaniosis due to *Leishmania*

infantum at the time of diagnosis and during follow-up treatment. Veterinary parasitology 228, 172-179.

Montserrat-Sangra, S., Ordeix, L., Martinez-Orellana, P., Solano-Gallego, L., 2018. Parasite Specific Antibody Levels, Interferon-gamma and TLR2 and TLR4 Transcripts in Blood from Dogs with Different Clinical Stages of Leishmaniosis. Veterinary sciences 5.

Moreira, M.A., Luvizotto, M.C., Garcia, J.F., Corbett, C.E., Laurenti, M.D., 2007. Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. Veterinary parasitology 145, 245-252.

Moreira, M.L., Costa-Pereira, C., Alves, M.L., Marteleto, B.H., Ribeiro, V.M., Peruhype-Magalhaes, V., Giunchetti, R.C., Martins-Filho, O.A., Araujo, M.S., 2016. Vaccination against canine leishmaniosis increases the phagocytic activity, nitric oxide production and expression of cell activation/migration molecules in neutrophils and monocytes. Veterinary parasitology 220, 33-45.

Moreno, J., Alvar, J., 2002. Canine leishmaniasis: epidemiological risk and the experimental model. Trends in parasitology 18, 399-405.

Moreno, J., Vouldoukis, I., Schreiber, P., Martin, V., McGahie, D., Gueguen, S., Cuisinier, A.M., 2014. Primary vaccination with the LiESP/QA-21 vaccine (CaniLeish) produces a cell-mediated immune response which is still present 1 year later. Veterinary immunology and immunopathology 158, 199-207.

Morillas Marquez, F., Sanchis Marin, M.C., Martin Sanchez, J., Acedo Sanchez, C., 1991. On *Phlebotomus perniciosus* Newstead, 1911 (Diptera, Phlebotomidae) in the Province of Almeria in southeastern Spain. Parassitologia 33 Suppl, 437-444.

Moritz, A., Steuber, S., Greiner, M., 1998. Clinical follow-up examination after treatment of canine leishmaniasis. The Tokai journal of experimental and clinical medicine 23, 279-283.

Mukherjee, A.K., Gupta, G., Adhikari, A., Majumder, S., Kar Mahapatra, S., Bhattacharyya Majumdar, S., Majumdar, S., 2012. Miltefosine triggers a strong proinflammatory cytokine response during visceral leishmaniasis: role of TLR4 and TLR9. International immunopharmacology 12, 565-572.

Muraille, E., De Trez, C., Brait, M., De Baetselier, P., Leo, O., Carlier, Y., 2003. Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to *Leishmania major* infection associated with a polarized Th2 response. Journal of immunology 170, 4237-4241.

Murphy, K.M., Reiner, S.L., 2002. The lineage decisions of helper T cells. Nature reviews. Immunology 2, 933-944.

Myskova, J., Dostalova, A., Penickova, L., Halada, P., Bates, P.A., Volf, P., 2016. Characterization of a midgut mucin-like glycoconjugate of *Lutzomyia longipalpis* with a potential role in *Leishmania* attachment. Parasites & vectors 9, 413.

Nathan, C., 2006. Role of iNOS in human host defense. Science 312, 1874-1875; author reply 1874-1875.

Naucke, T.J., Menn, B., Massberg, D., Lorentz, S., 2008. Sandflies and leishmaniasis in Germany. Parasitology research 103 Suppl 1, S65-68.

Necesankova, M., Vychodilova, L., Albrechtova, K., Kennedy, L.J., Hlavac, J., Sedlak, K., Modry, D., Janova, E., Vyskocil, M., Horin, P., 2016. MYD88 and functionally related genes are associated with multiple infections in a model population of Kenyan village dogs. Molecular biology reports 43, 1451-1463.

Neumayr, A.L., Morizot, G., Visser, L.G., Lockwood, D.N., Beck, B.R., Schneider, S., Bellaud, G., Cordoliani, F., Foulet, F., Laffitte, E.A., Buffet, P., Blum, J.A., 2013. Clinical aspects and

management of cutaneous leishmaniasis in rheumatoid patients treated with TNF-alpha antagonists. Travel medicine and infectious disease 11, 412-420.

Nieto, A., Dominguez-Bernal, G., Orden, J.A., De La Fuente, R., Madrid-Elena, N., Carrion, J., 2011. Mechanisms of resistance and susceptibility to experimental visceral leishmaniosis: BALB/c mouse versus Syrian hamster model. Veterinary research 42, 39.

Noli, C., Auxilia, S.T., 2005. Treatment of canine Old World visceral leishmaniasis: a systematic review. Veterinary dermatology 16, 213-232.

Noli, C., Saridomichelakis, M.N., 2014. An update on the diagnosis and treatment of canine leishmaniosis caused by *Leishmania infantum* (syn. *L. chagasi*). Veterinary journal 202, 425-435.

Novo, S.P., Leles, D., Bianucci, R., Araujo, A., 2016. The process of *Leishmania* infection - disease and new perspectives of paleoparasitology. Revista do Instituto de Medicina Tropical de Sao Paulo 58, 45.

Nunes, C.M., Lima, V.M., Paula, H.B., Perri, S.H., Andrade, A.M., Dias, F.E., Burattini, M.N., 2008. Dog culling and replacement in an area endemic for visceral leishmaniasis in Brazil. Veterinary parasitology 153, 19-23.

Oda, K., Kitano, H., 2006. A comprehensive map of the toll-like receptor signaling network. Molecular systems biology 2, 2006 0015.

Odiwuor, S., De Doncker, S., Maes, I., Dujardin, J.C., Van der Auwera, G., 2011. Natural *Leishmania donovani/Leishmania aethiopica* hybrids identified from Ethiopia. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases 11, 2113-2118.

Olekhnovitch, R., Ryffel, B., Muller, A.J., Bousso, P., 2014. Collective nitric oxide production provides tissue-wide immunity during *Leishmania* infection. The Journal of clinical investigation 124, 1711-1722.

Oliva, G., Gradoni, L., Cortese, L., Orsini, S., Ciaramella, P., Scalone, A., de Luna, R., Persechino, A., 1998. Comparative efficacy of meglumine antimoniate and aminosidine sulphate, alone or in combination, in canine leishmaniasis. Annals of tropical medicine and parasitology 92, 165-171.

Oliva, G., Nieto, J., Foglia Manzillo, V., Cappiello, S., Fiorentino, E., Di Muccio, T., Scalone, A., Moreno, J., Chicharro, C., Carrillo, E., Butaud, T., Guegand, L., Martin, V., Cuisinier, A.M., McGahie, D., Gueguen, S., Canavate, C., Gradoni, L., 2014. A randomised, double-blind, controlled efficacy trial of the LiESP/QA-21 vaccine in naive dogs exposed to two *Leishmania infantum* transmission seasons. PLoS neglected tropical diseases 8, e3213.

Oliva, G., Scalone, A., Foglia Manzillo, V., Gramiccia, M., Pagano, A., Di Muccio, T., Gradoni, L., 2006. Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. Journal of clinical microbiology 44, 1318-1322.

Oliveira, A.C., 2011. Metaciclogénese de *Leishmania spp in vitro* – Análise cinética e morfológica, Dissertação de Mestrado em Parasitologia Médica. Universidade Nova de Lisboa, IHMT.

Oliveira, F., Lawyer, P.G., Kamhawi, S., Valenzuela, J.G., 2008. Immunity to distinct sand fly salivary proteins primes the anti-*Leishmania* immune response towards protection or exacerbation of disease. PLoS neglected tropical diseases 2, e226.

Ordeix, L., Dalmau, A., Osso, M., Llull, J., Montserrat-Sangra, S., Solano-Gallego, L., 2017. Histological and parasitological distinctive findings in clinically-lesioned and normal-looking skin of dogs with different clinical stages of leishmaniosis. Parasites & vectors 10, 121.

Ordeix L, F.A., 2013. Manifestaciones clínicas cutáneas. In: Solano-Gallego L, editor. Leishmaniosis canina. Una revisión actualizada. . Servet, 69-95.

Ordeix L, R.A., Martínez-Orellana P, Montserrat-Sangrà S, Solano-Gallego L., 2017. Clinical follow-up of a series of dogs with papular dermatitis due to *Leishmania infantum*. Veterinary dermatology 28, abstract.

Ordeix, L., Silva, J., Llull, J., Quirola, P., Montserrat-Sangra, S., Martinez-Orellana, P., Solano-Gallego, L., 2018. Histological and Immunological Description of the Leishmanin Skin Test in Ibizan Hounds. Journal of comparative pathology 158, 56-65.

Ordeix, L., Solano-Gallego, L., Fondevila, D., Ferrer, L., Fondati, A., 2005. Papular dermatitis due to *Leishmania spp*. infection in dogs with parasite-specific cellular immune responses. Veterinary dermatology 16, 187-191.

Osanya, A., Song, E.H., Metz, K., Shimak, R.M., Boggiatto, P.M., Huffman, E., Johnson, C., Hostetter, J.M., Pohl, N.L., Petersen, C.A., 2011. Pathogen-derived oligosaccharides improve innate immune response to intracellular parasite infection. The American journal of pathology 179, 1329-1337.

Otranto, D., Dantas-Torres, F., 2013. The prevention of canine leishmaniasis and its impact on public health. Trends in parasitology 29, 339-345.

Otranto, D., Dantas-Torres, F., de Caprariis, D., Di Paola, G., Tarallo, V.D., Latrofa, M.S., Lia, R.P., Annoscia, G., Breitshwerdt, E.B., Cantacessi, C., Capelli, G., Stanneck, D., 2013. Prevention of canine leishmaniosis in a hyper-endemic area using a combination of 10% imidacloprid/4.5% flumethrin. PloS one 8, e56374.

Otranto, D., de Caprariis, D., Lia, R.P., Tarallo, V., Lorusso, V., Testini, G., Dantas-Torres, F., Latrofa, S., Diniz, P.P., Mencke, N., Maggi, R.G., Breitschwerdt, E., Capelli, G., Stanneck, D., 2010. Prevention of endemic canine vector-borne diseases using imidacloprid 10% and permethrin 50% in young dogs: a longitudinal field study. Veterinary parasitology 172, 323-332.

Otranto, D., Napoli, E., Latrofa, M.S., Annoscia, G., Tarallo, V.D., Greco, G., Lorusso, E., Gulotta, L., Falsone, L., Basano, F.S., Pennisi, M.G., Deuster, K., Capelli, G., Dantas-Torres, F., Brianti, E., 2017. Feline and canine leishmaniosis and other vector-borne diseases in the Aeolian Islands: Pathogen and vector circulation in a confined environment. Veterinary parasitology 236, 144-151.

Otranto, D., Paradies, P., Lia, R.P., Latrofa, M.S., Testini, G., Cantacessi, C., Mencke, N., Galli, G., Capelli, G., Stanneck, D., 2007. Efficacy of a combination of 10% imidacloprid/50% permethrin for the prevention of leishmaniasis in kennelled dogs in an endemic area. Veterinary parasitology 144, 270-278.

Otranto, D., Paradies, P., Sasanelli, M., Leone, N., de Caprariis, D., Chirico, J., Spinelli, R., Capelli, G., Brandonisio, O., 2005. Recombinant K39 dipstick immunochromatographic test: a new tool for the serodiagnosis of canine leishmaniasis. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 17, 32-37.

Owens, S.D., Oakley, D.A., Marryott, K., Hatchett, W., Walton, R., Nolan, T.J., Newton, A., Steurer, F., Schantz, P., Giger, U., 2001. Transmission of visceral leishmaniasis through blood transfusions from infected English foxhounds to anemic dogs. Journal of the American Veterinary Medical Association 219, 1076-1083.

Pagliano, P., Rossi, M., Rescigno, C., Altieri, S., Coppola, M.G., Gramiccia, M., Scalone, A., Gradoni, L., Faella, F., 2003. Mediterranean visceral leishmaniasis in HIV-negative adults: a retrospective analysis of 64 consecutive cases (1995-2001). The Journal of antimicrobial chemotherapy 52, 264-268.

Palatnik-de-Sousa, C.B., 2008. Vaccines for leishmaniasis in the fore coming 25 years. Vaccine 26, 1709-1724.

Palatnik-de-Sousa, C.B., 2012. Vaccines for canine leishmaniasis. Frontiers in immunology 3, 69.

Paltrinieri, S., Gradoni, L., Roura, X., Zatelli, A., Zini, E., 2016. Laboratory tests for diagnosing and monitoring canine leishmaniasis. Veterinary clinical pathology 45, 552-578.

Panaro, M.A., Brandonisio, O., de Caprariis, D., Cavallo, P., Cianciulli, A., Mitolo, V., Otranto, D., 2008. Canine leishmaniasis in Southern Italy: a role for nitric oxide released from activated macrophages in asymptomatic infection? Parasites & vectors 1, 10.

Papadogiannakis, E.I., Koutinas, A.F., 2015. Cutaneous immune mechanisms in canine leishmaniosis due to *Leishmania infantum*. Veterinary immunology and immunopathology 163, 94-102.

Paris, C., Loiseau, P.M., Bories, C., Breard, J., 2004. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. Antimicrobial agents and chemotherapy 48, 852-859.

Peine, K.J., Gupta, G., Brackman, D.J., Papenfuss, T.L., Ainslie, K.M., Satoskar, A.R., Bachelder, E.M., 2014. Liposomal resiquimod for the treatment of *Leishmania donovani* infection. The Journal of antimicrobial chemotherapy 69, 168-175.

Penaforte, K.M., Belo, V.S., Teixeira-Neto, R.G., Ribeiro, R.A., de Oliveira, R.B., Schettini, D.A., da Silva, E.S., 2013. *Leishmania infection* in a population of dogs: an epidemiological investigation relating to visceral leishmaniasis control. Revista brasileira de parasitologia veterinaria = Brazilian journal of veterinary parasitology : Orgao Oficial do Colegio Brasileiro de Parasitologia Veterinaria 22, 592-596.

Pennisi, M.G., 2015. Leishmaniosis of companion animals in Europe: an update. Veterinary parasitology 208, 35-47.

Pennisi, M.G., De Majo, M., Masucci, M., Britti, D., Vitale, F., Del Maso, R., 2005. Efficacy of the treatment of dogs with leishmaniosis with a combination of metronidazole and spiramycin. The Veterinary record 156, 346-349.

Pennisi, M.G., Persichetti, M.F., Serrano, L., Altet, L., Reale, S., Gulotta, L., Solano-Gallego, L., 2015. Ticks and associated pathogens collected from cats in Sicily and Calabria (Italy). Parasites & vectors 8, 512.

Pereira-Fonseca, D.C.M., Oliveira-Rovai, F.M., Rodas, L.A.C., Beloti, C.A.C., Torrecilha, R.B.P., Ito, P., Avanco, S.V., Cipriano, R.S., Utsunomiya, Y.T., Hiramoto, R.M., Calvo-Bado, L., Courtenay, O., Machado, G.F., Lima, V.M.F., Nunes, C.M., 2017. Dog skin parasite load, TLR-2, IL-10 and TNF-alpha expression and infectiousness. Parasite immunology 39.

Perez, H., Malave, I., Arredondo, B., 1979. The effects of protein malnutrition on the course of *Leishmania mexicana* infection in C57Bl/6 mice: nutrition and susceptibility to leishmaniasis. Clinical and experimental immunology 38, 453-460.

Persichetti, M.F., Pennisi, M.G., Vullo, A., Masucci, M., Migliazzo, A., Solano-Gallego, L., 2018. Clinical evaluation of outdoor cats exposed to ectoparasites and associated risk for vector-borne infections in southern Italy. Parasites & vectors 11, 136.

Peters , R.K.-K., 1987. The Leishmaniases in Biology and Medicine. Volume I: Biology and Epidemiology. Academic press, London.

Peters, N.C., Sacks, D.L., 2009. The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. Cellular microbiology 11, 1290-1296.

Pillai, A.A.A.H.L.S., 1991. Cellular and Molecular Immunology, Vol 9th Edition.

Pinelli, E., Gonzalo, R.M., Boog, C.J., Rutten, V.P., Gebhard, D., del Real, G., Ruitenberg, E.J., 1995. *Leishmania infantum*-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. European journal of immunology 25, 1594-1600.

Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., del Real, G., Ruitenberg, J., 1994. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. Infection and immunity 62, 229-235.

Pinheiro, D., Singh, Y., Grant, C.R., Appleton, R.C., Sacchini, F., Walker, K.R., Chadbourne, A.H., Palmer, C.A., Armitage-Chan, E., Thompson, I., Williamson, L., Cunningham, F., Garden, O.A., 2011. Phenotypic and functional characterization of a CD4(+) CD25(high) FOXP3(high) regulatory T-cell population in the dog. Immunology 132, 111-122.

Planellas, X.R., Y. Espada1, R. Novellas, C. Anselmi, L. Solano-Gallego, 2018. A prospective study of urinary adverse effects of allopurinol treatment for canine Leishmaniosis. Journal Veterinary International Medicine 32.

Poeppl, W., Burgmann, H., Auer, H., Mooseder, G., Walochnik, J., 2012. *Leishmania* (Viannia) *guyanensis* infection, Austria. Emerging infectious diseases 18, 1534-1535.

Pratlong, F., Rioux, J.A., Marty, P., Faraut-Gambarelli, F., Dereure, J., Lanotte, G., Dedet, J.P., 2004. Isoenzymatic analysis of 712 strains of *Leishmania infantum* in the south of France and relationship of enzymatic polymorphism to clinical and epidemiological features. Journal of clinical microbiology 42, 4077-4082.

Quinnell, R.J., Courtenay, O., 2009. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology 136, 1915-1934.

Quinnell, R.J., Kennedy, L.J., Barnes, A., Courtenay, O., Dye, C., Garcez, L.M., Shaw, M.A., Carter, S.D., Thomson, W., Ollier, W.E., 2003. Susceptibility to visceral leishmaniasis in the domestic dog is associated with MHC class II polymorphism. Immunogenetics 55, 23-28.

Qureshi, A.A., Asahina, A., Ohnuma, M., Tajima, M., Granstein, R.D., Lerner, E.A., 1996. Immunomodulatory properties of maxadilan, the vasodilator peptide from sand fly salivary gland extracts. The American journal of tropical medicine and hygiene 54, 665-671.

Rakhshanpour, A., Malmasi, A., Mohebali, M., Nabian, S., Mirhendi, H., Zarei, Z., Dalimi, A., Mohammadiha, A., Akhoundi, B., Azarm, A., 2017. Transmission of *Leishmania infantum* by *Rhipicephalus sanguineus* (Acari: Ixodidae) in Dogs. Iranian journal of parasitology 12, 482-489.

Raman, V.S., Bhatia, A., Picone, A., Whittle, J., Bailor, H.R., O'Donnell, J., Pattabhi, S., Guderian, J.A., Mohamath, R., Duthie, M.S., Reed, S.G., 2010. Applying TLR synergy in immunotherapy: implications in cutaneous leishmaniasis. Journal of immunology 185, 1701-1710.

Ramos-Vara, J.A., Ortiz-Santiago, B., Segales, J., Dunstan, R.W., 1996. Cutaneous leishmaniasis in two horses. Veterinary pathology 33, 731-734.

Reis, A.B., Giunchetti, R.C., Carrillo, E., Martins-Filho, O.A., Moreno, J., 2010. Immunity to *Leishmania* and the rational search for vaccines against canine leishmaniasis. Trends in parasitology 26, 341-349.

Reis, A.B., Martins-Filho, O.A., Teixeira-Carvalho, A., Giunchetti, R.C., Carneiro, C.M., Mayrink, W., Tafuri, W.L., Correa-Oliveira, R., 2009. Systemic and compartmentalized immune response in canine visceral leishmaniasis. Veterinary immunology and immunopathology 128, 87-95.

Reis, A.B., Teixeira-Carvalho, A., Giunchetti, R.C., Guerra, L.L., Carvalho, M.G., Mayrink, W., Genaro, O., Correa-Oliveira, R., Martins-Filho, O.A., 2006a. Phenotypic features of circulating leucocytes as immunological markers for clinical status and bone marrow parasite density in dogs naturally infected by *Leishmania chagasi*. Clinical and experimental immunology 146, 303-311.

Reis, A.B., Teixeira-Carvalho, A., Vale, A.M., Marques, M.J., Giunchetti, R.C., Mayrink, W., Guerra, L.L., Andrade, R.A., Correa-Oliveira, R., Martins-Filho, O.A., 2006b. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs

naturally infected by *Leishmania* (Leishmania) *chagasi*. Veterinary immunology and immunopathology 112, 102-116.

Rezvan, H., Moafi, M., 2015. An overview on *Leishmania* vaccines: A narrative review article. Veterinary research forum : an international quarterly journal 6, 1-7.

Rhalem, A., Sahibi, H., Lasri, S., Jaffe, C.L., 1999. Analysis of immune responses in dogs with canine visceral leishmaniasis before, and after, drug treatment. Veterinary immunology and immunopathology 71, 69-76.

Ribeiro-Gomes, F.L., Moniz-de-Souza, M.C., Alexandre-Moreira, M.S., Dias, W.B., Lopes, M.F., Nunes, M.P., Lungarella, G., DosReis, G.A., 2007. Neutrophils activate macrophages for intracellular killing of *Leishmania major* through recruitment of TLR4 by neutrophil elastase. Journal of immunology 179, 3988-3994.

Ribeiro, J.M., Vachereau, A., Modi, G.B., Tesh, R.B., 1989. A novel vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. Science 243, 212-214.

Richard A. Goldsby, T.J.K., Barbara A. Osborne, Janis Kuby, 2003. Immunology. Freeman, New York.

Riggio, M.P., Lappin, D.F., Bennett, D., 2014. Bacteria and Toll-like receptor and cytokine mRNA expression profiles associated with canine arthritis. Veterinary immunology and immunopathology 160, 158-166.

Rilza, G.A.-C., Sergio C., F. Mendonça, Heather Callahan, Andreia C. Portal, Max Grögl, 2007. Sensitivity of Leishmania braziliensis promastigotes to meglumine antimoniate (Glucantime) is higher than that of other *Leishmania* species and correlates with response to therapy in american tegumentary Leishmaniasis. Journal Parasitology 93, 688-693.

Roach, J.C., Glusman, G., Rowen, L., Kaur, A., Purcell, M.K., Smith, K.D., Hood, L.E., Aderem, A., 2005. The evolution of vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences of the United States of America 102, 9577-9582.

Roach, T.I., Kiderlen, A.F., Blackwell, J.M., 1991. Role of inorganic nitrogen oxides and tumor necrosis factor alpha in killing *Leishmania donovani* amastigotes in gamma interferon-lipopolysaccharide-activated macrophages from Lshs and Lshr congenic mouse strains. Infection and immunity 59, 3935-3944.

Roatt, B.M., Aguiar-Soares, R.D., Reis, L.E., Cardoso, J.M., Mathias, F.A., de Brito, R.C., da Silva, S.M., Gontijo, N.F., Ferreira, S.A., Valenzuela, J.G., Correa-Oliveira, R., Giunchetti, R.C., Reis, A.B., 2017. A Vaccine Therapy for Canine Visceral Leishmaniasis Promoted Significant Improvement of Clinical and Immune Status with Reduction in Parasite Burden. Frontiers in immunology 8, 217.

Roatt, B.M., Aguiar-Soares, R.D., Vitoriano-Souza, J., Coura-Vital, W., Braga, S.L., Correa-Oliveira, R., Martins-Filho, O.A., Teixeira-Carvalho, A., de Lana, M., Figueiredo Gontijo, N., Marques, M.J., Giunchetti, R.C., Reis, A.B., 2012. Performance of LBSap vaccine after intradermal challenge with *L. infantum* and saliva of *Lu. longipalpis*: immunogenicity and parasitological evaluation. PloS one 7, e49780.

Rocha, R.D., Gontijo, C.M., Santos, S.M., Carvalho, A.T., Ferrari, T.C., Oliveira, R.C., Marques, M.J., Mayrink, W., Martins-Filho, O.A., 2003. [Performance analysis of the research of IgG antibodies alive promastigote anti-forms of *Leishmania* (V.) *braziliensis* (AAPV-IgG), for flow cytometry in the diagnosis of the leishmaniasis american tegumentar]. Revista da Sociedade Brasileira de Medicina Tropical 36 Suppl 2, 15-18.

Rodrigues, A., Santos-Mateus, D., Alexandre-Pires, G., Valerio-Bolas, A., Rafael-Fernandes, M., Pereira, M.A., Ligeiro, D., de Jesus, J., Alves-Azevedo, R., Lopes-Ventura, S., Santos, M., Tomas, A.M., Pereira da Fonseca, I., Santos-Gomes, G., 2017. *Leishmania infantum* exerts immunomodulation in canine Kupffer cells reverted by meglumine antimoniate. Comparative immunology, microbiology and infectious diseases 55, 42-52.

Rodrigues, V., Cordeiro-da-Silva, A., Laforge, M., Ouaissi, A., Akharid, K., Silvestre, R., Estaquier, J., 2014. Impairment of T cell function in parasitic infections. PLoS neglected tropical diseases 8, e2567.

Rodriguez-Cortes, A., Carrillo, E., Martorell, S., Todoli, F., Ojeda, A., Martinez-Florez, A., Urniza, A., Moreno, J., Alberola, J., 2016. Compartmentalized Immune Response in Leishmaniasis: Changing Patterns throughout the Disease. PloS one 11, e0155224.

Rodriguez-Cortes, A., Fernandez-Bellon, H., Ramis, A., Ferrer, L., Alberola, J., Solano-Gallego, L., 2007a. Leishmania-specific isotype levels and their relationship with specific cell-mediated immunity parameters in canine leishmaniasis. Veterinary immunology and immunopathology 116, 190-198.

Rodriguez-Cortes, A., Martori, C., Martinez-Florez, A., Clop, A., Amills, M., Kubejko, J., Llull, J., Nadal, J.M., Alberola, J., 2017. Canine Leishmaniasis Progression is Associated with Vitamin D Deficiency. Scientific reports 7, 3346.

Rodriguez-Cortes, A., Ojeda, A., Lopez-Fuertes, L., Timon, M., Altet, L., Solano-Gallego, L., Sanchez-Robert, E., Francino, O., Alberola, J., 2007b. A long term experimental study of canine visceral leishmaniasis. International journal for parasitology 37, 683-693.

Rodriguez-Cortes, A., Ojeda, A., Lopez-Fuertes, L., Timon, M., Altet, L., Solano-Gallego, L., Sanchez-Robert, E., Francino, O., Alberola, J., 2007c. Vaccination with plasmid DNA encoding KMPII, TRYP, LACK and GP63 does not protect dogs against *Leishmania infantum* experimental challenge. Vaccine 25, 7962-7971.

Rodriguez-Cortes, A., Ojeda, A., Todoli, F., Alberola, J., 2013. Performance of commercially available serological diagnostic tests to detect *Leishmania infantum* infection on experimentally infected dogs. Veterinary parasitology 191, 363-366.

Rogers, K.A., Titus, R.G., 2004. The human cytokine response to *Leishmania major* early after exposure to the parasite *in vitro*. The Journal of parasitology 90, 557-563.

Roma, E.H., Macedo, J.P., Goes, G.R., Goncalves, J.L., Castro, W., Cisalpino, D., Vieira, L.Q., 2016. Impact of reactive oxygen species (ROS) on the control of parasite loads and inflammation in *Leishmania amazonensis* infection. Parasites & vectors 9, 193.

Rossi, E., Bongiorno, G., Ciolli, E., Di Muccio, T., Scalone, A., Gramiccia, M., Gradoni, L., Maroli, M., 2008. Seasonal phenology, host-blood feeding preferences and natural *Leishmania* infection of *Phlebotomus perniciosus* (Diptera, Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome province, Italy. Acta tropica 105, 158-165.

Rostamian, M., Niknam, H.M., 2017. Evaluation of the adjuvant effect of agonists of toll-like receptor 4 and 7/8 in a vaccine against leishmaniasis in BALB/c mice. Molecular immunology 91, 202-208.

Rosypal, A.C., Lindsay, D.S., 2005. Non-sand fly transmission of a North American isolate of *Leishmania infantum* in experimentally infected BALB/c mice. The Journal of parasitology 91, 1113-1115.

Rosypal, A.C., Troy, G.C., Zajac, A.M., Frank, G., Lindsay, D.S., 2005. Transplacental transmission of a North American isolate of *Leishmania infantum* in an experimentally infected beagle. The Journal of parasitology 91, 970-972.

Rougier, S., Vouldoukis, I., Fournel, S., Peres, S., Woehrle, F., 2008. Efficacy of different treatment regimens of marbofloxacin in canine visceral leishmaniosis: a pilot study. Veterinary parasitology 153, 244-254.

Sacks, D., Noben-Trauth, N., 2002. The immunology of susceptibility and resistance to *Leishmania major* in mice. Nature reviews. Immunology 2, 845-858.

Sacks, D., Sher, A., 2002. Evasion of innate immunity by parasitic protozoa. Nature immunology 3, 1041-1047.

Sacramento, L.A., da Costa, J.L., de Lima, M.H., Sampaio, P.A., Almeida, R.P., Cunha, F.Q., Silva, J.S., Carregaro, V., 2017. Toll-Like Receptor 2 Is Required for Inflammatory Process Development during *Leishmania infantum* Infection. Frontiers in microbiology 8, 262.

Saez, V.D., Morillas-Marquez, F., Merino-Espinosa, G., Corpas-Lopez, V., Morales-Yuste, M., Pesson, B., Baron-Lopez, S., Lucientes-Curdi, J., Martin-Sanchez, J., 2018. *Phlebotomus langeroni Nitzulescu* (Diptera, Psychodidae) a new vector for *Leishmania infantum* in Europe. Parasitology research 117, 1105-1113.

Saljoughian, N., Taheri, T., Zahedifard, F., Taslimi, Y., Doustdari, F., Bolhassani, A., Doroud, D., Azizi, H., Heidari, K., Vasei, M., Namvar Asl, N., Papadopoulou, B., Rafati, S., 2013. Development of novel prime-boost strategies based on a tri-gene fusion recombinant *L. tarentolae* vaccine against experimental murine visceral leishmaniasis. PLoS neglected tropical diseases 7, e2174.

Sanchez, M.V., Elicabe, R.J., Di Genaro, M.S., Germano, M.J., Gea, S., Garcia Bustos, M.F., Salomon, M.C., Scodeller, E.A., Cargnelutti, D.E., 2017. Total *Leishmania* antigens with Poly(I:C) induce Th1 protective response. Parasite immunology 39.

Sanchís-Marín, C.G.J.M.-M.F.M., 1989. Los flebótomos en España. Revista de sanidad e higiene pública 63, 15-34.

Santos-Gomes, G.M., Rosa, R., Leandro, C., Cortes, S., Romao, P., Silveira, H., 2002. Cytokine expression during the outcome of canine experimental infection by *Leishmania infantum*. Veterinary immunology and immunopathology 88, 21-30.

Saridomichelakis, M.N., Koutinas, A.F., 2014. Cutaneous involvement in canine leishmaniosis due to *Leishmania infantum* (syn. *L. chagasi*). Veterinary dermatology 25, 61-71, e22.

Saridomichelakis, M.N., Mylonakis, M.E., Leontides, L.S., Billinis, C., Koutinas, A.F., Galatos, A.D., Gouletsou, P., Diakou, A., Kontos, V.I., 2005. Periodic administration of allopurinol is not effective for the prevention of canine leishmaniosis (*Leishmania infantum*) in the endemic areas. Veterinary parasitology 130, 199-205.

Sastre, N., Francino, O., Ramirez, O., Ensenat, C., Sanchez, A., Altet, L., 2008. Detection of *Leishmania infantum* in captive wolves from Southwestern Europe. Veterinary parasitology 158, 117-120.

Savic, S., Vidic, B., Grgic, Z., Potkonjak, A., Spasojevic, L., 2014. Emerging Vector-Borne Diseases - Incidence through Vectors. Frontiers in public health 2, 267.

Schallig, H.D., Cardoso, L., Hommers, M., Kroon, N., Belling, G., Rodrigues, M., Semiao-Santos, S.J., Vetter, H., 2004. Development of a dipstick assay for detection of *Leishmania*-specific canine antibodies. Journal of clinical microbiology 42, 193-197.

Schallig, H.D., Schoone, G.J., Beijer, E.G., Kroon, C.C., Hommers, M., Ozbel, Y., Ozensoy, S., da Silva, E.S., Cardoso, L.M., da Silva, E.D., 2002. Development of a fast agglutination screening test (FAST) for the detection of anti-*Leishmania* antibodies in dogs. Veterinary parasitology 109, 1-8.

Schaut, R.G., Lamb, I.M., Toepp, A.J., Scott, B., Mendes-Aguiar, C.O., Coutinho, J.F., Jeronimo, S.M., Wilson, M.E., Harty, J.T., Waldschmidt, T.J., Petersen, C.A., 2016. Regulatory IgDhi B Cells Suppress T Cell Function via IL-10 and PD-L1 during Progressive Visceral Leishmaniasis. Journal of immunology 196, 4100-4109.

Schlein, Y., Warburg, A., 1986. Phytophagy and the feeding cycle of *Phlebotomus papatasi* (Diptera: Psychodidae) under experimental conditions. Journal of medical entomology 23, 11-15.

Schlotter, Y.M., Veenhof, E.Z., Brinkhof, B., Rutten, V.P., Spee, B., Willemse, T., Penning, L.C., 2009. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in

skin biopsies of healthy dogs and dogs with atopic dermatitis. Veterinary immunology and immunopathology 129, 115-118.

Schmitz, S., Henrich, M., Neiger, R., Werling, D., Allenspach, K., 2014. Stimulation of duodenal biopsies and whole blood from dogs with food-responsive chronic enteropathy and healthy dogs with Toll-like receptor ligands and probiotic Enterococcus faecium. Scandinavian journal of immunology 80, 85-94.

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S., Medzhitov, R., 2001. Toll-like receptors control activation of adaptive immune responses. Nature immunology 2, 947-950.

Schneider, M., Thomas, V., Boisrame, B., Deleforge, J., 1996. Pharmacokinetics of marbofloxacin in dogs after oral and parenteral administration. Journal of veterinary pharmacology and therapeutics 19, 56-61.

Shakya, N., Sane, S.A., Vishwakarma, P., Gupta, S., 2012. Enhancement in therapeutic efficacy of miltefosine in combination with synthetic bacterial lipopeptide, Pam3Cys against experimental Visceral Leishmaniasis. Experimental parasitology 131, 377-382.

Shamsi Meymandi, S., Javadi, A., Dabiri, S., Shamsi Meymandi, M., Nadji, M., 2011. Comparative histological and immunohistochemical changes of dry type cutaneous leishmaniasis after administration of meglumine antimoniate, imiquimod or combination therapy. Archives of Iranian medicine 14, 238-243.

Sharma, U., Singh, S., 2008. Insect vectors of *Leishmania*: distribution, physiology and their control. Journal of vector borne diseases 45, 255-272.

Shaw, S.E., Langton, D.A., Hillman, T.J., 2009. Canine leishmaniosis in the United Kingdom: a zoonotic disease waiting for a vector? Veterinary parasitology 163, 281-285.

Shayakhmetov, D.M., Di Paolo, N.C., Mossman, K.L., 2010. Recognition of virus infection and innate host responses to viral gene therapy vectors. Molecular therapy : the journal of the American Society of Gene Therapy 18, 1422-1429.

Shokri, A., Fakhar, M., Teshnizi, S.H., 2017. Canine visceral leishmaniasis in Iran: A systematic review and meta-analysis. Acta tropica 165, 76-89.

Sideris, V., Papadopoulou, G., Dotsika, E., Karagouni, E., 1999. Asymptomatic canine leishmaniasis in Greater Athens area, Greece. European journal of epidemiology 15, 271-276.

Silva, D.A., Madeira, M.F., Teixeira, A.C., de Souza, C.M., Figueiredo, F.B., 2011. Laboratory tests performed on *Leishmania* seroreactive dogs euthanized by the leishmaniasis control program. Veterinary parasitology 179, 257-261.

Silva, E., Leitao, S., Henriques, S., Kowalewski, M.P., Hoffmann, B., Ferreira-Dias, G., da Costa, L.L., Mateus, L., 2010. Gene transcription of TLR2, TLR4, LPS ligands and prostaglandin synthesis enzymes are up-regulated in canine uteri with cystic endometrial hyperplasia-pyometra complex. Journal of reproductive immunology 84, 66-74.

Silva, F.L., Oliveira, R.G., Silva, T.M., Xavier, M.N., Nascimento, E.F., Santos, R.L., 2009. Venereal transmission of canine visceral leishmaniasis. Veterinary parasitology 160, 55-59.

Sindermann, H., Engel, J., 2006. Development of miltefosine as an oral treatment for leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 100 Suppl 1, S17-20.

Singh, O.P., Sundar, S., 2014. Whole blood assay and visceral leishmaniasis: Challenges and promises. Immunobiology 219, 323-328.

Slappendel, R.J., Teske, E., 1997. The effect of intravenous or subcutaneous administration of meglumine antimonate (Glucantime) in dogs with leishmaniasis. A randomized clinical trial. The Veterinary quarterly 19, 10-13.

Smith, K.D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M.A., Barrett, S.L., Cookson, B.T., Aderem, A., 2003. Toll-like receptor 5 recognizes a conserved site on flagellin

required for protofilament formation and bacterial motility. Nature immunology 4, 1247-1253.

Sobrino, R., Ferroglio, E., Oleaga, A., Romano, A., Millan, J., Revilla, M., Arnal, M.C., Trisciuoglio, A., Gortazar, C., 2008. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. Veterinary parasitology 155, 198-203.

Solano-Gallego, L., Cardoso, L., Pennisi, M.G., Petersen, C., Bourdeau, P., Oliva, G., Miro, G., Ferrer, L., Baneth, G., 2017a. Diagnostic Challenges in the Era of Canine *Leishmania infantum* Vaccines. Trends in parasitology. 33, 706-717.

Solano-Gallego, L., Di Filippo, L., Ordeix, L., Planellas, M., Roura, X., Altet, L., Martinez-Orellana, P., Montserrat, S., 2016a. Early reduction of *Leishmania infantum*-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. Parasites & vectors 9, 235.

Solano-Gallego, L., Koutinas, A., Miro, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Veterinary parasitology 165, 1-18.

Solano-Gallego, L., Llull, J., Arboix, M., Ferrer, L., Alberola, J., 2001a. Evaluation of the efficacy of two leishmanins in asymptomatic dogs. Veterinary parasitology 102, 163-166.

Solano-Gallego, L., Llull, J., Osso, M., Hegarty, B., Breitschwerdt, E., 2006. A serological study of exposure to arthropod-borne pathogens in dogs from northeastern Spain. Veterinary research 37, 231-244.

Solano-Gallego, L., Llull, J., Ramos, G., Riera, C., Arboix, M., Alberola, J., Ferrer, L., 2000. The Ibizian hound presents a predominantly cellular immune response against natural *Leishmania* infection. Veterinary parasitology 90, 37-45.

Solano-Gallego, L., Miro, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., The LeishVet, G., 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasites & vectors 4, 86.

Solano-Gallego, L., Montserrrat-Sangra, S., Ordeix, L., Martinez-Orellana, P., 2016b. *Leishmania infantum*-specific production of IFN-gamma and IL-10 in stimulated blood from dogs with clinical leishmaniosis. Parasites & vectors 9, 317.

Solano-Gallego, L., Morell, P., Arboix, M., Alberola, J., Ferrer, L., 2001b. Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. Journal of clinical microbiology 39, 560-563.

Solano-Gallego, L., Rossi, L., Scroccaro, A.M., Montarsi, F., Caldin, M., Furlanello, T., Trotta, M., 2012. Detection of *Leishmania infantum* DNA mainly in *Rhipicephalus sanguineus* male ticks removed from dogs living in endemic areas of canine leishmaniosis. Parasites & vectors 5, 98.

Solano-Gallego, L., Villanueva-Saz, S., Carbonell, M., Trotta, M., Furlanello, T., Natale, A., 2014. Serological diagnosis of canine leishmaniosis: comparison of three commercial ELISA tests (Leiscan, ID Screen and Leishmania 96), a rapid test (Speed Leish K) and an in-house IFAT. Parasites & vectors 7, 111.

Soto, M., Requena, J.M., Quijada, L., Alonso, C., 1998. Multicomponent chimeric antigen for serodiagnosis of canine visceral leishmaniasis. Journal of clinical microbiology 36, 58-63.

Soulat, D., Bogdan, C., 2017. Function of Macrophage and Parasite Phosphatases in Leishmaniasis. Frontiers in immunology 8, 1838.

Srivastava, A., Singh, N., Mishra, M., Kumar, V., Gour, J.K., Bajpai, S., Singh, S., Pandey, H.P., Singh, R.K., 2012. Identification of TLR inducing Th1-responsive *Leishmania donovani* amastigote-specific antigens. Molecular and cellular biochemistry 359, 359-368.

Steinhagen, F., Kinjo, T., Bode, C., Klinman, D.M., 2011. TLR-based immune adjuvants. Vaccine 29, 3341-3355.

Steverding, D., 2017. The history of leishmaniasis. Parasites & vectors 10, 82.

Sundar, S., Olliaro, P.L., 2007. Miltefosine in the treatment of leishmaniasis: Clinical evidence for informed clinical risk management. Therapeutics and clinical risk management 3, 733-740.

Szatmary, Z., 2012. Molecular biology of toll-like receptors. General physiology and biophysics 31, 357-366.

Tabar, M.D., Roura, X., Francino, O., Altet, L., Ruiz de Gopegui, R., 2008. Detection of *Leishmania infantum* by real-time PCR in a canine blood bank. The Journal of small animal practice 49, 325-328.

Takeda, K., Akira, S., 2004. TLR signaling pathways. Seminars in immunology 16, 3-9.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., Akira, S., 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11, 443-451.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R.L., Akira, S., 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. Journal of immunology 169, 10-14.

Tarallo, V.D., Dantas-Torres, F., Lia, R.P., Otranto, D., 2010. Phlebotomine sand fly population dynamics in a leishmaniasis endemic peri-urban area in southern Italy. Acta tropica 116, 227-234.

Teixeira, M.J., Teixeira, C.R., Andrade, B.B., Barral-Netto, M., Barral, A., 2006. Chemokines in host-parasite interactions in leishmaniasis. Trends in parasitology 22, 32-40.

Theodos, C.M., Titus, R.G., 1993. Salivary gland material from the sand fly *Lutzomyia longipalpis* has an inhibitory effect on macrophage function *in vitro*. Parasite immunology 15, 481-487.

Tivers, M.S., Lipscomb, V.J., Smith, K.C., Wheeler-Jones, C.P., House, A.K., 2015. Lipopolysaccharide and toll-like receptor 4 in dogs with congenital portosystemic shunts. Veterinary journal 206, 404-413.

Tizard, I., 2012. Veterinary Immunology. Elsevier and Saunders, St Louis, 552 p.

Todoli, F., Solano-Gallego, L., Ojeda, A., Quintana, J., Lloret, A., Roura, X., Alberola, J., Rodriguez-Cortes, A., 2009. Anti-*Leishmania* IgA in urine samples from dogs with clinical leishmaniasis. Veterinary parasitology 159, 17-23.

Toepp, A., Larson, M., Wilson, G., Grinnage-Pulley, T., Bennett, C., Leal-Lima, A., Anderson, B., Parrish, M., Anderson, M., Fowler, H., Hinman, J., Kontowicz, E., Jefferies, J., Beeman, M., Buch, J., Saucier, J., Tyrrell, P., Gharpure, R., Cotter, C., Petersen, C., 2018. Randomized, controlled, double-blinded field trial to assess *Leishmania* vaccine effectiveness as immunotherapy for canine leishmaniosis. Vaccine.

Tolouei, S., Hejazi, S.H., Ghaedi, K., Khamesipour, A., Hasheminia, S.J., 2013. TLR2 and TLR4 in cutaneous leishmaniasis caused by *Leishmania major*. Scandinavian journal of immunology 78, 478-484.

Torres, M., Bardagi, M., Roura, X., Zanna, G., Ravera, I., Ferrer, L., 2011. Long term follow-up of dogs diagnosed with leishmaniosis (clinical stage II) and treated with meglumine antimoniate and allopurinol. Veterinary journal 188, 346-351.

Torres, M., Pastor, J., Roura, X., Tabar, M.D., Espada, Y., Font, A., Balasch, J., Planellas, M., 2016. Adverse urinary effects of allopurinol in dogs with leishmaniasis. The Journal of small animal practice 57, 299-304.

Travi, B.L., Cordeiro-da-Silva, A., Dantas-Torres, F., Miro, G., 2018. Canine visceral leishmaniasis: Diagnosis and management of the reservoir living among us. PLoS neglected tropical diseases 12, e0006082.

Tripathi, P., Singh, V., Naik, S., 2007. Immune response to *Leishmania*: paradox rather than paradigm. FEMS immunology and medical microbiology 51, 229-242.

Tsikas, D., 2007. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 851, 51-70.

Tuon, F.F., Amato, V.S., Bacha, H.A., Almusawi, T., Duarte, M.I., Amato Neto, V., 2008. Toll-like receptors and leishmaniasis. Infection and immunity 76, 866-872.

Tuon, F.F., Fernandes, E.R., Duarte, M.I., Amato, V.S., 2012. Expression of TLR2 and TLR4 in lesions of patients with tegumentary American leishmaniasis. Revista do Instituto de Medicina Tropical de Sao Paulo 54, 159-163.

Turchetti, A.P., da Costa, L.F., Romao Ede, L., Fujiwara, R.T., da Paixao, T.A., Santos, R.L., 2015. Transcription of innate immunity genes and cytokine secretion by canine macrophages resistant or susceptible to intracellular survival of *Leishmania infantum*. Veterinary immunology and immunopathology 163, 67-76.

Turchetti, A.P., Souza, T.D., Paixao, T.A., Santos, R.L., 2014. Sexual and vertical transmission of visceral leishmaniasis. Journal of infection in developing countries 8, 403-407.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology 3, RESEARCH0034.

Vermeersch, M., da Luz, R.I., Tote, K., Timmermans, J.P., Cos, P., Maes, L., 2009. *In vitro* susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. Antimicrobial agents and chemotherapy 53, 3855-3859.

Vidya, M.K., Kumar, V.G., Sejian, V., Bagath, M., Krishnan, G., Bhatta, R., 2017. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. International reviews of immunology, 1-17.

Vouldoukis, I., Drapier, J.C., Nussler, A.K., Tselentis, Y., Da Silva, O.A., Gentilini, M., Mossalayi, D.M., Monjour, L., Dugas, B., 1996. Canine visceral leishmaniasis: successful chemotherapy induces macrophage antileishmanial activity via the L-arginine nitric oxide pathway. Antimicrobial agents and chemotherapy 40, 253-256.

Vouldoukis, I., Rougier, S., Dugas, B., Pino, P., Mazier, D., Woehrle, F., 2006. Canine visceral leishmaniasis: comparison of *in vitro* leishmanicidal activity of marbofloxacin, meglumine antimoniate and sodium stibogluconate. Veterinary parasitology 135, 137-146.

Wanasen, N., Soong, L., 2008. L-arginine metabolism and its impact on host immunity against *Leishmania* infection. Immunologic research 41, 15-25.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., Chen, Z.J., 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412, 346-351.

Weeratna, R.D., Makinen, S.R., McCluskie, M.J., Davis, H.L., 2005. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine 23, 5263-5270.

Werling, D., Jungi, T.W., 2003. TOLL-like receptors linking innate and adaptive immune response. Veterinary immunology and immunopathology 91, 1-12.

Whitaker, S.M., Colmenares, M., Pestana, K.G., McMahon-Pratt, D., 2008. *Leishmania pifanoi* proteoglycolipid complex P8 induces macrophage cytokine production through Toll-like receptor 4. Infection and immunity 76, 2149-2156.

WHO 2010. world Health Statistics, p. 177.

WHO, 2012. Leishmaniasis control. 216.

WHO 2014. World Health Statistics, p. 180.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P.L., 1990. Direct gene transfer into mouse muscle *in vivo*. Science 247, 1465-1468.

Woodland, D.L., 2004. Jump-starting the immune system: prime-boosting comes of age. Trends in immunology 25, 98-104.

Wylie, C.E., Carbonell-Antonanzas, M., Aiassa, E., Dhollander, S., Zagmutt, F.J., Brodbelt, D.C., Solano-Gallego, L., 2014. A systematic review of the efficacy of prophylactic control measures for naturally occurring canine leishmaniosis. Part II: topically applied insecticide treatments and prophylactic medications. Preventive veterinary medicine 117, 19-27.

Yarovinsky, F., 2014. Innate immunity to *Toxoplasma gondii* infection. Nature reviews. Immunology 14, 109-121.

Yasur-Landau, D., Jaffe, C.L., David, L., Baneth, G., 2016. Allopurinol Resistance in *Leishmania infantum* from Dogs with Disease Relapse. PLoS neglected tropical diseases 10, e0004341.

Yasur-Landau, D., Jaffe, C.L., David, L., Doron-Faigenboim, A., Baneth, G., 2018. Resistance of *Leishmania infantum* to allopurinol is associated with chromosome and gene copy number variations including decrease in the S-adenosylmethionine synthetase (METK) gene copy number. International journal for parasitology. Drugs and drug resistance 8, 403-410.

Yasur-Landau, D., Jaffe, C.L., Doron-Faigenboim, A., David, L., Baneth, G., 2017. Induction of allopurinol resistance in *Leishmania infantum* isolated from dogs. PLoS neglected tropical diseases 11, e0005910.

Yeagley, T.J., Reichard, M.V., Hempstead, J.E., Allen, K.E., Parsons, L.M., White, M.A., Little, S.E., Meinkoth, J.H., 2009. Detection of *Babesia gibsoni* and the canine small *Babesia* 'Spanish isolate' in blood samples obtained from dogs confiscated from dogfighting operations. Journal of the American Veterinary Medical Association 235, 535-539.

Yokoyama, N., Ohta, H., Yamazaki, J., Kagawa, Y., Ichii, O., Khoirun, N., Morita, T., Osuga, T., Lim, S.Y., Sasaki, N., Morishita, K., Nakamura, K., Takiguchi, M., 2017. Localization of Toll-like Receptor (TLR) 2 and TLR4 mRNA in the Colorectal Mucosa of Miniature Dachshunds with Inflammatory Colorectal Polyps. Journal of comparative pathology 156, 183-190.

Zafra, R., Jaber, J.R., Perez-Ecija, R.A., Barragan, A., Martinez-Moreno, A., Perez, J., 2008. High iNOS expression in macrophages in canine leishmaniasis is associated with low intracellular parasite burden. Veterinary immunology and immunopathology 123, 353-359.

Zhang, W.W., Matlashewski, G., 2008. Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against *Leishmania major* in BALB/c mice. Infection and immunity 76, 3777-3783.

Zoghlami, Z., Chouihi, E., Barhoumi, W., Dachraoui, K., Massoudi, N., Helel, K.B., Habboul, Z., Hadhri, M.H., Limam, S., Mhadhbi, M., Gharbi, M., Zhioua, E., 2014. Interaction between canine and human visceral leishmaniases in a holoendemic focus of Central Tunisia. Acta tropica 139, 32-38.

ANNEX

Veterinary Parasitology 228 (2016) 172–179

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

TLR-2 and TLR-4 transcriptions in unstimulated blood from dogs with leishmaniosis due to *Leishmania infantum* at the time of diagnosis and during follow-up treatment

Sara Montserrat-Sangrà[a], Lorena Alborch[a], Laura Ordeix[a,b], Laia Solano-Gallego[a,*]

[a] Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Bellaterra, Spain
[b] Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Bellaterra, Spain

ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form 6 September 2016
Accepted 8 September 2016

Keywords:
TLR2
TLR4
Canine leishmaniosis
Blood
Treatment and Follow-up

ABSTRACT

Innate immunity, in particular, the role of toll-like receptors (TLRs), has not been extensively studied in canine *L. infantum* infection. The main aim of this study was to determine the transcription of TLR2 and TLR4 in the blood of dogs with natural clinical leishmaniosis at the time of diagnosis and during treatment follow-up and subsequently correlate these findings with clinical, serological and parasitological data. Forty-six *Leishmania*-seropositive sick dogs with a high antibody level at the time of diagnosis were studied and compared with 34 healthy seronegative dogs. Twenty-two of these sick dogs were treated with meglumine antimoniate and allopurinol and followed-up at 30, 180 and 365 days following the start of treatment. Clinical status was defined by a thorough physical examination, complete blood count, biochemistry profile, electrophoresis of serum proteins, and urinary protein/creatinine ratio (UPC). EDTA blood was stored in RNAlater® solution before RNA extraction and cDNA production were performed. TLR2, TLR4 and three reference genes (HPRT-1, CG14980 and SDHA) were studied in each blood sample by real time PCR. The relative quantification of TLR2 was higher (mean 3.5) in sick dogs when compared with seronegative healthy dogs (mean 1.3; $P = 0.0001$) while the relative quantification of TLR4 was similar in both groups. In addition, the relative quantification of TLR2 significantly decreased during follow-up at all time points compared with day 0 whereas no changes were observed with TLR4 transcription. A significant positive correlation was noted between TLR2 and UPC, total protein, beta and gamma globulins, specific *L. infantum* antibodies and blood parasite load while a negative correlation was observed with albumin, albumin/globulin ratio, hematocrit and hemoglobin. TLR4 transcript did not correlate with any parameter. These findings indicate an up-regulation of TLR2 transcription in unstimulated blood in naturally infected sick dogs as compared to healthy dogs suggesting active innate immune and proinflammatory responses. In addition, TLR2 transcription is reduced with clinical improvement during treatment. In contrast, TLR4 transcription appears to be similar among groups at the time of diagnosis with no changes during treatment follow-up suggesting a less important role for this TLR in clinical canine leishmaniosis.

Abbreviations: ALT, Alanine aminotransferase; β-2M, Beta-2-microglobulin; CBC, Complete blood count; cDNA, Complementary Deoxyribonucleic acid; CD11b, Cluster of differentiation 11b; CG-14980, Similar to CG14980-PB; CP, Crossing point; DAMPs, Damage-associated molecular pattern molecules; ddCt-method, Delta delta C(T) Method; EDTA, Ethylenediaminetetraacetic acid; HMBS, Hydroxymethylbilane synthase; HPRT-1, Hypoxanthine phosphoribosyltransferase 1; M value, Average expression stability; PAMPs, Pathogen-associated molecular patterns; PBMC, Peripheral blood mononuclear cell; PCR, Polymerase chain reaction; RG, Reference gene; RGI, Reference gene index; RIN, RNA integrity number; RNA, Ribonucleic acid; PRRs, Pattern recognition receptors; qPCR, Quantitative PCR; Rq, Relative quantification; rt- PCR, Real time PCR; SDHA, Succinate dehydrogenase complex, subunit A, flavoprotein (Fp); TBP, TATA box binding protein; TG, Target gene; TLRs, Toll-like receptors; UPC, Urinary protein/creatinine ratio; VL, Visceral leishmaniosis; Vn/n + 1 value, Pairwise variation analysis to determine optimal number of reference genes for normalization in RT-qPCR reaction.

* Corresponding author.
E-mail addresses: sara.montserrat@uab.cat (S. Montserrat-Sangrà), lorena.alborch@uab.cat (L. Alborch), laura.ordeix@uab.cat (L. Ordeix), laia.solano@uab.cat (L. Solano-Gallego).

http://dx.doi.org/10.1016/j.vetpar.2016.09.005
0304-4017/

1. Introduction

Canine leishmaniosis due to *Leishmania infantum* is a life threatening zoonotic disease with a wide distribution in four continents. A broad spectrum of clinical manifestation exists in this infection ranging from subclinical infection to very severe disease (Baneth et al., 2008). A clinical staging system is currently used in the clinical setting due to the fact that several degrees of disease exist ranging from mild to very severe fatal disease (Solano-Gallego et al., 2009, 2011). Dogs with moderate or more severe disease are frequently treated in Europe. The common treatment employed in the clinical setting is a combination of meglumine antimoniate with allopurinol, miltefosine with allopurinol, or allopurinol alone (Noli and Saridomichelakis, 2014). Clinical cure is often associated with a reduction in parasite load and infectiousness (Miró et al., 2011) but long-term treatment is needed in the majority of dogs with moderate to severe disease (Noli and Saridomichelakis, 2014) to maintain clinical remission. The variable clinical manifestations are likely due to the differing immunogenetics within each dog leading to a variation in immune responses (Reis et al., 2010). Although adaptive immunity has been extensively investigated in canine *L. infantum* infection, limited data is available regarding innate immune responses (Hosein et al., 2016).

The innate immune system uses non-clonal sets of recognition molecules, called pattern recognition receptors (PRRs). They bind to conserved molecular structures found in large groups of pathogens termed pathogen-associated molecular patterns (PAMPs) (Akira et al., 2001). The toll-like receptors (TLRs) are one of the most important pattern recognition receptor families. TLRs are important in the early host defense against pathogen and activate adapter molecules after binding to their ligand. The activated cascade then leads to induction or suppression of genes that influence the inflammatory response (Aderem and Ulevitch, 2000; Kawai and Akira, 2010).

Recent data suggests that early innate immune responses are paramount for the ultimate outcome of *Leishmania* infection (Bogdan et al., 1996; Bogdan and Rollinghoff, 1998; Liese et al., 2008). Studies have shown that TLRs activate pro-inflammatory responses (Agallou et al., 2014; Hawn et al., 2002; Oda and Kitano, 2006). However, the role of TLRs has not been studied in canine *L. infantum* infection in detail (Hosein et al., 2016). There are only few studies of TLR2 expression in different tissues in canine leishmaniosis. Previous studies have demonstrated that TLR2 is upregulated in skin, intestine and brain in canine leishmaniosis (Esteve et al., 2015; Figueiredo et al., 2013; Melo et al., 2014a). In addition, TLR2 is upregulated in other canine inflammatory diseases including inflammatory bowel disease, immune-mediated diseases and bacterial arthritis (McMahon et al., 2010; Riggio et al., 2014). TLR4 transcription was studied in several tissues such as lymph node or spleen in canine leishmaniosis (Melo et al., 2014a), however it has been scarcely investigated in experimental or natural *L. infantum* infection in dogs (Hosein et al., 2016). To the best knowledge of the authors, TLRs transcripts have not been evaluated in unstimulated whole blood from dogs with clinical leishmaniosis at the time of diagnosis or during treatment follow-up.

The correlation between TLR expression and clinical, immunological and parasitological parameters can help to better understand host-pathogen interactions in canine *L. infantum* infection and define the specific functions of certain TLRs in this disease. Based on present literature, we hypothesize that TLR2 is up regulated while TLR4 might be down-regulated or unchanged in dogs with clinical leishmaniosis at the time of diagnosis and a switch should be observed during treatment follow-up and clinical improvement.

The main objective of this study was to determine the transcription of TLR2 and TLR4 in the blood of dogs with natural clinical leishmaniosis (Leishvet stage II, moderate disease or higher (Solano-Gallego et al., 2009) at the time of diagnosis and during follow-up treatment). Other objective was to correlate the transcription of TLR2 and TLR4 with clinical, serological and parasitological data.

2. Materials and methods

2.1. Dogs

2.1.1. Sampling and evaluation of clinical status

A thorough physical examination was performed in all healthy and sick dogs included in this study. In addition, routine laboratory testing was also performed in all dogs to further evaluate their clinical status. Thus, a full complete blood count (CBC), serum biochemical profile which included creatinine, urea, total cholesterol, total protein and alanine aminotransferase (ALT), protein serum electrophoresis, and urinalysis with urinary protein creatinine ratio (UPC) were carried out. The haematology, biochemistry and serum electrophoresis were performed using: the Siemens ADVIA120 haematology analyzer, Olympus AU400 chemistry analyzer and the Hydrasis serum electrophoresis system.

Six milliliters of blood were collected from the respective dogs by jugular or metatarsian venipuncture for routine laboratory tests above described and transferred immediately into different tubes: ethylenediaminetetraacetic acid (EDTA) tubes and plain tubes. Half ml of EDTA blood from dogs was transferred to a cryovial that contained 1.3 ml of RNAlater® solution (Ambion). Once collected, samples were left at 4 °C overnight and then frozen at minus 80 °C until further use.

All dogs enrolled in the study were privately owned pets with client informed consent; they remained under the care of their owners and were not housed for the purposes of this study or held for any period of time. Residual samples from blood EDTA tube and serum were used in this study. Therefore, ethical approval was not required. *Leishmania infantum* antibody levels were determined by a serial dilution in house quantitative ELISA (Solano-Gallego et al., 2016a) and *Leishmania* DNA in blood was assessed by real-time PCR (Martinez et al., 2011; Solano-Gallego et al., 2016a).

2.1.2. Dogs with clinical leishmaniosis

A total of 46 dogs with clinical leishmaniosis attending different veterinary facilities from Catalonia (Spain) [*Fundació Hospital Clínic Veterinari*, Universitat Autònoma de Barcelona (UAB), Cerdanyola, Barcelona), *Hospital Ars Veterinària* (Barcelona), *Mediterrani Veterinaris Hospital* (Reus, Tarragona) and *Consultori Montsant* (Falset, Tarragona)] were prospectively enrolled between January 2014 and August 2015. The diagnosis was made by detection of high specific *L. infantum* antibody levels and, in some cases confirmed, by cytology of lesions, cutaneous histology and/or immunohistochemistry for *Leishmania* (Esteve et al., 2015). In addition, 38 dogs were positive by blood PCR. Both sexes were represented with 20 female and 26 male. The median age was five years with a range from six months to 10 years. Thirty-four purebred dogs belonging to about 20 breeds and 12 mixed breed dog were included. Clinical staging was evaluated as previously reported (Solano-Gallego et al., 2009) and sick dogs were distributed as follows: stage II (moderate disease, n = 39), stage III (severe disease, n = 4) and stage IV (very severe disease, n = 3).

After being diagnosed, twenty-two dogs classified as being in stage II (n = 19) or stage III (n = 3) were further followed up during treatment. All dogs were treated with meglumine antimoniate (80–100 mg/Kg/24 h subcutaneously during 1 month) and allopurinol (10 mg/Kg/12 h orally during at least 12 months). Dogs were monitored at days 30, 180 and 365 during treatment. In each clinical

visit, a physical examination and CBC, biochemistry profile, protein serum electrophoresis, urianalysis and UPC were performed. Both genders were represented with eight female and 14 male dogs as well as purebred and mixed-breed dogs. The remaining 24 dogs were also treated with the treatment described above and they were also followed up during treatment. However, due to economical restrictions, TLRs were not determined in these dogs.

2.1.3. Control healthy dogs

A total of 34 healthy dogs were enrolled between January 2014 and August 2015. Both sexes were represented with 16 females and 18 males. The median age was approximately four years, with a range from six months to 10 years. Twenty-five pure bred dogs belonging to about 15 breeds and nine mixed breed dog were included. All dogs were seronegative with a mean and standard deviation of 6.2 ± 5.5 ELISA units (EU) and blood PCR negative.

2.2. Blood DNA extraction and *Leishmania* real-time PCR

Total DNA was isolated from EDTA whole blood using DNA Gene extraction kit (Sigma Aldrich) following the manufacturer's instructions with slight modifications. Forty μl of proteinase K solution (Sigma Aldrich) were added in all samples. Four hundred μl of whole blood were used for all the samples. The other steps were performed according to the manufacturer's protocol. A blood sample from a clinically healthy non-infected dog was used as a control for DNA contamination in every DNA extraction performed.

Real-time PCR (RT-PCR) was performed with an absolute quantification as previously described (Solano-Gallego et al., 2016a). Briefly, PCR mix reaction was prepared with 4 μl of DNA, 10 μl of master mix (TaqMan® Fast Advanced Master Mix, life technologies), 1 μl of *Leishmania* primers and probes (Custom TaqMan® Gene Expression Assay, life technologies) or 1 μl of another type of assay primers and probes (Eukaryotic 18S rRNA Endogenous Control (VIC™ *M* GB Probe, Primer Limited)) and 5 μl of H_2O. PCR reaction was performed in duplicates for each sample and for each target gene.

In order to verify that the PCR was done successfully, a positive control for *Leishmania*, a negative control from a non-infected clinically healthy dog and a blank (well without DNA sample) were included in all the plates. PCR was carried out in a QuantStudio Flex™ 7 Real-Time PCR system (Life Technologies). The thermal cycling profile consisted of 50 °C 2 min in order to activate the enzyme AmpErase (Thermo Fisher Scientific) and afterwards, a total of forty cycles were carried out. Each cycle consisted of 20 s at 95 °C followed by 40 cycles of 1 s at 95 °C and 20 s at 60 °C.

Absolute quantification was carried out by the interpolation of the unknown samples to the standard curve generated from a negative sample spiked with different quantities of *Leishmania* promastigotes. Depending on the value of parasitic load, the samples were classified as negative (0 parasites/ml), low positive (<10 parasites/ml), medium positive (10–100 parasites/ml), high positive (100–1000 parasites/ml) or very high positive (> 1000 parasites/ml) (Martinez et al., 2011).

2.3. RNA extraction and RNA concentration and integrity

Blood samples were thawed on ice and total RNA from 500 μl of EDTA blood was extracted using the Ribopure™ RNA blood kit (Ambion) according to the manufacturer's instructions. A DNase digestion step was included to remove contaminating genomic DNA using the Turbo™ DNase (Ambion) following the manufacturer's instructions. RNA concentration was determined by a Nanodrop device (Thermo Fisher Scientific Inc). RNA integrity and quality were assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples had a final concentration of 20–70 ng RNA/μl per sample. All samples included in this study had a RNA Integrity Number (RIN) value greater than 7.

2.4. cDNA synthesis and PCR real time

*c*DNA was generated using the VILO Masterscript Retrotranscriptase kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was aliquoted and stored at −20 °C until ready for use.

2.5. Reference gene selection

Six reference housekeeping genes (RG) [(hydroxymethylbilane synthase (HMBS), succinate dehydrogenase complex; subunit A; flavoprotein (SDHA), TATA box binding protein (TBP), similar to CG14980-PB (CG14980), hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome) (HPRT-1) and beta-2-microglobulin(β–2 M)] (Table 1; Invitrogen, Thermo Fisher Scientific) were assessed by real time PCR to establish the most stable genes by the GeNorm program (Biogazelle, Belgium) with 10 representative samples. Two parameters were considered to quantify reference gene stability: M value (average expression stability) and Vn/n + 1 (pairwise variation). Originally, $M \leq 1.5$ and $Vn/n + 1 < 0.15$ were regarded as acceptable levels of expression variability (Vandesompele et al., 2002). Recently, an M value below the threshold of 0.5 has been generally regarded as being typical for a stable reference gene in a relatively homogeneous sample panel (Schlotter et al., 2009). Selected reference genes were used to calculate relative quantification of target genes.

2.6. TLR2, TLR4 and reference housekeeping genes real-time PCR

Transcription of TLR2 and TLR4 target genes (TG) as well as three reference housekeeping genes (HPRT-1, CG14980 and SDHA) were measured by qRT-PCR using the QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies). We set up the real time PCR for blood by relative standard curves by pooling 10 representative dog samples of cDNA. This pool of cDNA was diluted from 1:5 to 1:625 to make relative standard curves for the assessment of efficiency for each target gene (TLR2 and TLR4) and six reference genes described above. The efficiency (acceptable between 80 and 120%) for each assay was: TLR2 = 99.5%, TLR4 = 80.4%, CG-14980 = 110.7%, HPRT-1 = 90.3%, SDHA = 97.5%, TBP = 76.9%, HMBS = 86.3% and B-2M = 101.8%. Primers and probes were obtained from Thermo Fisher Scientific and are listed in Table 1.

QuantStudio™ mastermix was filled in wells of standard plates (96 wells/plate) to the indicated volumes: 0.35 μl nuclease free water (Sigma), 7.50 μl taqman universal master mix (2×), 0.75 μl taqman assay 20, 6.4 μl 1/5 cDNA. Plates were sealed with an optic film (Applied Biosystems, Life Technologies) centrifuged and placed into a laboratory pipetting robot (Epmotion 5057 Liquid-handling robot, Eppendorf) to generate a 384 wells/plate. After that, the new plate was set into real time PCR instrument (QuantStudio™7 Flex System applied Biosystems, Thermo Fisher Scientific).

The following cycling conditions were used for all TG and reference genes: denaturation program (95 °C, 10 min), amplification and quantification program repeated 40 times (95 °C for 15 s, 60 °C for 10 s, 72 °C for 60 s with a single fluorescence measurement). The crossing point (CP) was determined for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. CP was measured at constant fluorescent level. Each reaction was carried out in triplicate. The same control case was used in triplicate as the calibrator control in each plate. All target genes (TG) per each dog were run the same day and with the same plate.

Table 1
Summary of canine reference and target genes used in this study.

Assay ID[a]	Gene symbol	Gene name	GenBank mRNA	GenBank reference sequence	Amplicon pairwise
Cf02622203_g1	TLR4	toll-like receptor 4	AB080363.1	NM_001002950.1	120
Cf02625049_s1	TLR2	toll-like receptor 2	EU487534.1;AJ630583.1	NM_001005264.2	69
Cf02664981_m1	SDHA	succinate dehydrogenase complex; subunit A; flavoprotein (Fp)	DQ402985.1	XM_535807.2	64
Cf02626256_m1	HPRT-1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	AY283372.1	NM_001003357.1	102
Cf02643820_m1	LOC479750	similar to CG14980-PB		XM_536878.2	78
Cf02694648_m1	HMBS	hydroxymethylbilane synthase	–	XM_546491.2	148
Cf02659077_m1	β2M	beta-2-microglobulin	–	XM_845055.1	87
Cf02637232_m1	TBP	TATA box binding protein	–	XM_849432.1	105

[a] All the assays are commercially available at Thermo Fisher scientific.

Data were analyzed by the relative quantification method with three RG (SDHA, CG14980 and HPRT1) (Livak and Schmittgen, 2001). Data were processed applying the relative quantification method comparable to the ddCt-method (2^{dd}Ct). For normalization of TG expression, the arithmetic mean of the three RG were taken for the calculation of a reference gene index (RGI) (Vandesompele et al., 2002). RT-PCR data analysis was done by the Cloudsuite software (Thermo Fisher Scientific).

2.7. Statistical analysis

The non-parametric Mann-Whitney *U* test was used to evaluate differences among groups and was calculated with the Graphpad Prism 5 program. The Spearman's rank order correlation between TLR transcripts in canine whole blood and clinical, serological and parasitological data was calculated using the Graphpad Prism 5 program. The non-parametric Wilcoxon Signed-Rank test was used to evaluate differences between different time-points during dog follow up treatments. The Wilcoxon Signed-Rank test was performed with SPSS Statistics 17.0. A significant *P* value was considered at <0.05.

3. Results

3.1. Reference genes

Based on the Vn/n+1 and M values, the best housekeeping genes were HPRT-1, CG14980 and SDHA. The results are shown in Figs. 1 and 2.

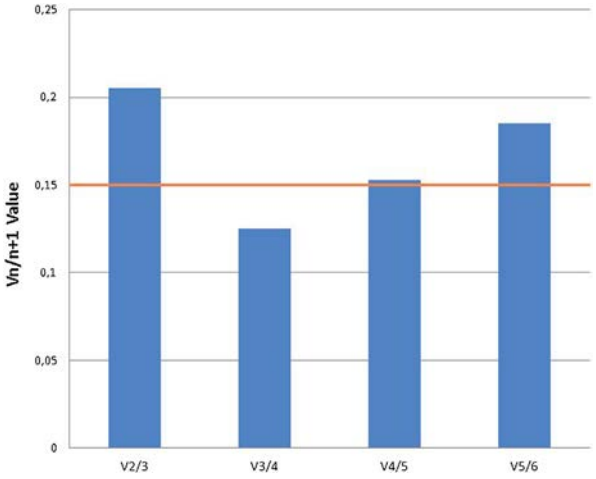

Fig. 1. Determination of the optimal number of reference genes. Brown line indicates Vn/n+1 value that needs to be below 0.15. Three references genes were selected based on results of Vn/n+1 value.

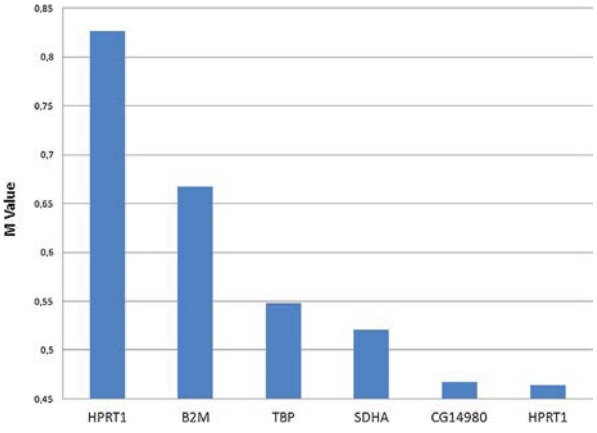

Fig. 2. Average expression stability (M value) based on GeNorm program of six references genes studied.

3.2. Diagnosis

3.2.1. TLRs transcripts in blood of sick dogs in comparison with healthy dogs

Relative quantification of TLR2 was significantly higher [mean 3.5 (Mann–Whitney *U* test: $Z = -3.854$, $P < 0.0001$)] in sick dogs when compared with seronegative healthy dogs (mean 1.3); while relative quantification of TLR4 was similar between groups (Mann–Whitney *U* test: $Z = -0.192$, $P = 0.848$) (Fig. 3).

3.2.2. Correlations with TLR transcripts and clinical, serological and parasitological data

The results of Spearman's correlation between TLR transcripts and clinical, serological and parasitological parameters are listed in Table 2. A significant positive correlation was noted between TLR2 and UPC ($P = 0.008$), total protein ($P < 0.0001$), beta and gamma globulins ($P < 0.0001$), specific *L. infantum* antibodies ($P < 0.0001$) and blood parasite load ($P < 0.0001$) while a significant negative correlation was observed between TLR2 and albumin ($P = 0.003$), albumin/globulin ratio (A/G) ($P < 0.0001$), hematocrit ($P = 0.002$) and hemoglobin ($P = 0.004$). TLR4 transcript did not correlate with any parameter.

3.3. Follow up during treatment

3.3.1. Clinical, serological, parasitological and TLRs transcripts of sick dogs in comparison between different time points

All dogs significantly improved at days 30, 180 and 365 based on clinicopathological, serological and parasitological data. The results

Table 2
Spearman's correlation between TLR transcripts and serological, clinicopathological and parasitological parameters.

Variables (units)	P – values		Spearman correlation	
	TLR2	TLR4	TLR2	TLR4
TLR2		0.216		−0.137
TLR4	0.216		−0.137	
UPC	0.008[a]	0.218	0.401	0.192
Creatinine (mg/dL)	0.384	0.750	−0.100	−0.037
Urea (mg/dl)	0.108	0.590	−0.183	−0.062
Total protein (g/dL)	0.000[a]	0.036	0.437	0.233
Albumin (g/dl)	0.003[b]	0.974	−0.330	−0.004
Beta globulin (g/dL)	0.000[a]	0.644	0.397	0.054
Gamma globulin (g/dL)	0.000[a]	0.071	0.467	0.210
Ratio albium/globulin	0.000[b]	0.284	−0.445	−0.125
Hematocrit (%)	0.002[b]	0.866	−0.389	0.022
Hemoglobin (g/dl)	0.004[b]	0.998	−0.354	0.000
Leishmania infantum specific antibodies (ELISA units)	0.000[a]	0.694	0.462	0.044
Blood parasite load (parasite/mL)	0.000[a]	0.959	0.411	−0.006

[a] A significant positive correlation. *P* values less than 0.05.
[b] A significant negative correlation *P* values less than 0.05.

Table 3
TLRs transcripts, blood parasite load and antibody levels of sick dogs at diagnosis and during treatment.

	Mean ± standard deviation			
	Day 0	Day 30	Day 180	Day 365
Antibody levels (ELISA units)[a]	5383.17 ± 6482.49	2997.02 ± 4410.74	865.52 ± 1744.59	440.17 ± 851.96
Parasite load (parasite/ mL)[b]	27.02 ±100.70	0.28 ± 0,78	0.59 ± 2.06	0.42 ± 0.77
TLR2 transcription[c]	3.91 ± 3.64	1.75 ± 1.083	1.75 ± 1.56	1.22 ± 0.74
TLR4 transcription[d]	5.08 ± 7.60	5.05 ± 5.79	3.63 ± 3.85	5.58 ± 7.24

[a] Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test: $Z = -3.847$, $P < 0.0001$), day 180 (Wilcoxon signed-rank test: $Z = -3.555$, $P < 0.0001$), day 365 (Wilcoxon signed-rank test, $Z = -4.015$, $P < 0.0001$)]. Comparison between day 30-day 180 (Wilcoxon signed-rank test: $Z = -3.523$, $P < 0.0001$), day 30-day 365 ($Z = -3.285$, $P = 0.001$) and day 180-day 365 (Wilcoxon signed-rank test: $Z = -2.138$, $P = 0.033$).
[b] Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test, $Z = -2.669$, $P = 0.008$), day 180 (Wilcoxon signed-rank test, $Z = -2.499$, $P = 0.012$), day 365 (Wilcoxon signed-rank test, $Z = -2.417$, $P = 0.016$)]. Comparison between day 30-day 180 (Wilcoxon signed-rank test: $Z = -0.140$, $P = 0.889$), day 30-day 365 ($Z = -1.957$, $P = 0.050$) and day 180-day 365 (Wilcoxon signed-rank test: $Z = -0.0979$, $P = 0.328$).
[c] Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test, $Z = -0.336$, $P = 0.005$), day 180 (Wilcoxon signed-rank test, $Z = -3.007$, $P = 0.003$), day 365 (Wilcoxon signed-rank test, $Z = -3.340$, $P = 0.001$)]. Comparison between day 30-day 180 (Wilcoxon signed-rank test: $Z = -0.568$, $P = 0.570$), day 30-day 365 ($Z = -2.165$, $P = 0.030$) and day 180-day 365 (Wilcoxon signed-rank test: $Z = -1.456$, $P = 0.145$).
[d] Comparison between day 0 and the other time points [day 30 (Wilcoxon signed-rank test, $Z = -0.336$, $P = 0.737$), day 180 (Wilcoxon signed-rank test, $Z = -0.330$, $P = 0.741$), day 365 (Wilcoxon signed-rank test, $Z = 0.000$, $P = 1$)]. Comparison between day 30-day 180 (Wilcoxon signed-rank test: $Z = -0.434$, $P = 0.664$), day 30-day 365 ($Z = -0.644$, $P = 0.520$) and day 180-day 365 (Wilcoxon signed-rank test: $Z = -1.120$, $P = 0.263$).

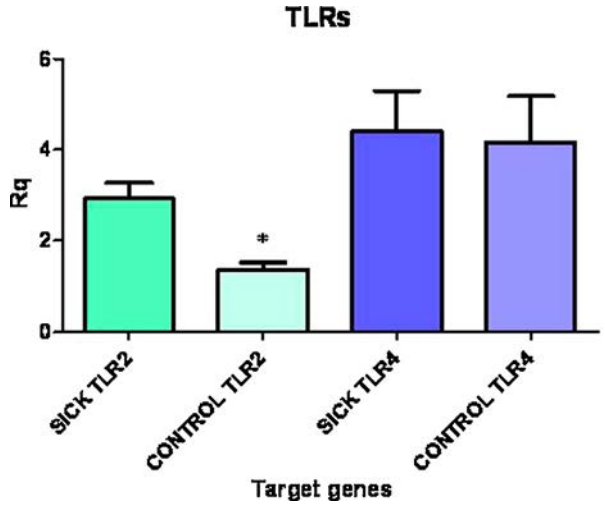

Fig. 3. Relative quantification (Rq) of TLR2 and TLR4 transcripts (mean and standard deviation) in sick and healthy dogs. *Comparison between sick dogs and seronegative healthy dogs (Mann–Whitney *U* test: $Z = -3.854$, $P < 0.0001$).

of clinicopathological data, serological and parasitological data during treatment follow-up are described in Table 3.

The results of TLRs transcripts in sick dogs on days 0, 30, 180 and 365 are also shown in Table 3. The relative quantification of TLR2 transcript significantly decreased during follow-up at all time points when compared with day 0 while no changes were observed with TLR4 transcript in any time point.

4. Discussion

This study demonstrates, for the first time, an upregulation of TLR2 in unstimulated blood of dogs with clinical leishmaniosis at the time of diagnosis. Similar findings were observed in other tissues in previous studies (Esteve et al., 2015; Figueiredo et al., 2013; Hosein et al., 2015). In agreement with the present results, Esteve et al., 2015 described higher expression of TLR2 by immunohistochemistry in skin biopsies from cutaneous lesions in dogs with at least moderate disease (LeishVet stage II) when compared with dogs with mild disease due to papular dermatitis (stage I) (Esteve et al., 2015). In addition, a study from Brazil found a high parasite load along with increased frequency and expression of TLR2 in the colon of dogs with leishmaniosis (Figueiredo et al., 2013). TLR2 upregulation was also observed in brain, spleen and lymph nodes in sick dogs with natural leishmaniosis from Brazil (Melo et al., 2014a). TLR2 upregulation was also noted in skin and liver samples in a canine experimental model of infection (Hosein et al., 2015).

In contrast, a different study found that unstimulated peripheral blood mononuclear cells (PBMCs) derived from *Leishmania*-infected seropositive sick dogs from Brazil did not show differences

in TLR2 expression compared with healthy dogs (Melo et al., 2014b). In addition, another study using PBMCs derived from canine blood found that CD11b+TLR2+ cells were higher in dogs that were negative to skin *Leishmania* immunohistochemistry and xenodiagnosis than dogs with positive results for both techniques (Amorim et al., 2011). These Brazilian studies do not support our TLR2 findings however they were performed with PBMCs, the clinical status for these animals was not so well defined and the diagnosis was mainly performed by serological tests, leading to a possible cross reaction with other species of *Leishmania* in an area where they co-exist (Melo et al., 2014b). The findings of our study in conjunction with the current literature available suggest a role of TLR2 in the pathogenesis of canine leishmaniosis. TLR2 appears to be associated with moderate to very severe disease suggesting immune and proinflammatory responses due to the presence of a high *Leishmania* parasite load or cellular damage (endogenous damage-associated molecular patterns (DAMPs) also termed alarmins) as observed for other human diseases (Kang et al., 2015).

In the present study, all dogs manifested a moderate to severe clinical leishmaniosis. It is well known that clinical canine leishmaniosis is a systemic immune inflammatory disease (Baneth et al., 2008; Hosein et al., 2016; Solano-Gallego et al., 2009). Therefore, the upregulation of TLR2 transcripts in sick dogs at the time of diagnosis suggests that TLR2 appears to be a marker of inflammation in dogs as demonstrated in other canine inflammatory diseases. TLR2 upregulation has been found in inflammatory bowel diseases (McMahon et al., 2010) and immune-mediated or bacterial arthritis (Riggio et al., 2014), canine sinonasal aspergillosis and lymphoplasmacytic rhinitis (Mercier et al., 2012) as well as in female dogs with pyometra (Chotimanukul and Sirivaidyapong, 2012; Silva et al., 2010). TLR2 upregulation has been demonstrated in several inflammatory tissues including in intestinal (McMahon et al., 2010) and nasal biopsies (Mercier et al., 2012), synovial fluid (Riggio et al., 2014) and the uterus (Chotimanukul and Sirivaidyapong, 2012). In addition, increased expression of TLR2 was also observed in PBMCs derived from dogs with steroid-responsive meningitis-arteritis (Maiolini et al., 2012).

The present results showed that TLR4 transcript in blood was unchanged among the groups studied. So far, there are few studies that have investigated TLR4 transcripts in canine leishmaniosis. Some authors found that TLR4 transcript was unchanged in the brain and spleen in agreement with the present study while TLR4 was upregulated in lymph nodes (Melo et al., 2014a) in dogs with natural leishmaniosis. Another study showed a significant downregulation of TLR4 transcription with disease progression in lymph node and in spleen samples and no change in TLR4 transcripts in skin and liver samples in an experimental canine model (Hosein et al., 2015). It is well known that there are different immune responses depending on tissues (compartmentalization) and inflammatory responses in canine leishmaniosis (Hosein et al., 2016; Melo et al., 2014a). Based on the present study and other studies, it seems that TLR4 transcription is unchanged or decreased in dogs with clinical leishmaniosis when compared with healthy dogs and might indicate a mechanism of parasite immune system evasion or a less important role for TLR4 in clinical canine leishmaniosis.

Similar to our results, differences of TLR4 transcripts have not been detected among dogs with other inflammatory diseases when compared with healthy controls (Maiolini et al., 2012) such as inflammatory bowel disease (McMahon et al., 2010), inflammatory colorectal polyps (Igarashi et al., 2015), immune-mediated arthritis and septic arthritis (Riggio et al., 2014) or lymphoplasmacytic rhinitis (Mercier et al., 2012). For example, no TLR4 transcript differences were seen in miniature dachshunds with inflammatory colorectal polyps when compared with controls (Igarashi et al., 2015). However, other studies mainly related with inflammatory diseases associated with bacterial infection showed an increase in TLR4 expression (Burgener et al., 2008; Chotimanukul and Sirivaidyapong, 2011; Mercier et al., 2012) in agreement with the fact that TLR4 recognized bacterial lipopolysaccharides (LPS) among other molecules.

In humans, TLR expression in unstimulated whole blood has not been yet explored. However, TLRs have been investigated in *Leishmania*-specific stimulated blood in patients from Sudan with visceral leishmaniosis due to *Leishmania donovani*. TLR2 transcript was found to be highly upregulated in patients with visceral leishmaniosis (VL) when compared with healthy humans either with positive or negative leishmanin skin test results (Babiker et al., 2015) in agreement with the present study. In contrast to our results, TLR4 transcripts were also upregulated in patients with visceral leishmaniosis in *Leishmania*-specific stimulated blood (Babiker et al., 2015). Interestingly, similar findings with TLR2 and TLR4 upregulation were observed in pre-treatment splenic aspirate samples from patients with visceral leishmaniosis due to *L. donovani* from India compared to post treatment samples (Kumar et al., 2014). However, no difference in TLR2 and TLR4 transcripts was observed between unstimulated PBMCs from pre-treatment subjects, compared to post-treatment group or that of healthy endemic controls suggesting site and cell specific up regulation of mRNA expression for these TLRs during ongoing disease (Kumar et al., 2014). Other studies in humans with cutaneous leishmaniosis due to *Leishmania major* from Iran showed that the mean relative gene expression of TLR2 and TLR4 in macrophages derived from blood of patients with healing lesions were significantly higher than in patients with non-healing lesions (Tolouei et al., 2013). Another study performed in cutaneous lesions due to *Leishmania braziliensis* in humans demonstrated that TLR2 was the most common TLR expressed during active disease mainly by macrophages while TLR4 was scarcely expressed (Tuon et al., 2012).

There are no studies describing correlations with clinical, serological and parasitological parameters and TLR transcription in unstimulated blood in dogs with leishmaniosis. Therefore, there is limited knowledge related to clinical parameters and TLRs transcription in this disease. As previously mentioned, TLR2 transcription was significantly upregulated in unstimulated blood of dogs with natural moderate to severe leishmaniosis. In addition, TLR2 was significantly correlated with laboratory abnormalities, blood parasitemia and *L. infantum* antibody level further confirming its role in dogs with moderate to very severe disease. The laboratory abnormalities that correlate with TLR2 are commonly observed in dogs with moderate to severe leishmaniosis (Solano-Gallego et al., 2011). It is also important to highlight that high antibody levels are also associated with disease severity as well as parasite load (Solano-Gallego et al., 2016b).

This study shows a significant decrease in TLR2 transcription with treatment and clinical improvement while no changes were found in TLR4 transcription. These findings reinforce the concept that TLR2 transcription in blood appears to be associated with disease severity in canine leishmaniosis and downregulation of TLR2 takes place during successful anti-*Leishmania* treatment and improvement of clinico-pathological parameters. In partial agreement with our results, unstimulated blood from Brazilian human patients with visceral leishmaniosis due to *L. infantum* presented higher percentages of CD3+ cells expressing TLR2 and TLR4, as well as cells co-expressing TLR2/TLR4 before treatment compared with post-treatment and control individuals (Gatto et al., 2015).

5. Conclusions

The findings presented in the current study indicate an upregulation of TLR2 transcription in unstimulated blood of naturally

infected sick dogs as compared to healthy dogs suggesting active innate immune and proinflammatory responses due to the presence of a high *Leishmania* parasite load or cellular damage as observed for other canine diseases. In addition, TLR2 transcription is reduced with clinical improvement during treatment. In contrast, TLR4 transcription appears to be unchanged at the time of diagnosis and during treatment follow-up and might indicate a mechanism of parasite immune system evasion or a less important role for this TLR in clinical canine leishmaniosis. TLR2 expression might be used in the future as an immunological marker in research and development of vaccines and immunotherapy.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by a Spanish Ministry grant, Ministerio de Economía y competitividad (AGL2012-32498).

Dr. Laia Solano-Gallego holds a Ramón y Cajal senior researcher contract awarded by the *Ministerio de Ciencia e Innovación* (Spain) and the European Social Fund.

Acknowledgements

The authors thank all veterinarians and dog owners that contributed to this study. We are especially grateful to Dr. Marta Planellas (Hospital Clínic Veterinari of Universitat Autònoma of Barcelona), Marta Blanchart (Ars Veterinaria), Montsant Osso (Consultori Falset), Annabel Dalmau (Hospital Mediterrani), Dani Mari and Paulina Quirola (MSc students from UAB) for their clinical assistance,sample collection and support to this study. The authors thank also Dra. Anna Castelló for her technical assistance with molecular work. The authors are also grateful to Dra. Pamela Martinez and Laura di Filippo (UAB) for their assistance with laboratory work and Dr. Shazia Hosein for English revision of the manuscript.

References

Aderem, A., Ulevitch, R.J., 2000. Toll-like receptors in the induction of the innate immune response. Nature 406, 782–787.

Agallou, M., Dotsika, E., Frydas, S., Karagouni, E., 2014. Toll-like receptor 4 promotes control of *Leishmania infantum* infection through inducement of leishmanicidal activity in host macrophages: role of mitogen activated kinases. J. Biol. Regul. Homeost. Agents 28, 41–52.

Akira, S., Takeda, K., Kaisho, T., 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2, 675–680.

Amorim, I.F., Silva, S.M., Figueiredo, M.M., Moura, E.P., Castro, R.S., Lima, T.K., Gontijo Nde, F., Michalick, M.S., Gollob, K.J., Tafuri, W.L., 2011. Toll receptors type-2 and CR3 expression of canine monocytes and its correlation with immunohistochemistry and xenodiagnosis in visceral leishmaniasis. PLoS One 6, e27679.

Babiker, D.T., Bakhiet, S.M., Mukhtar, M.M., 2015. *Leishmania donovani* influenced cytokines and toll-like receptors expression among Sudanese visceral leishmaniasis patients. Parasite Immunol. 37, 417–425.

Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008. Canine leishmaniosis – new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 24, 324–330.

Bogdan, C., Rollinghoff, M., 1998. The immune response to *Leishmania*: mechanisms of parasite control and evasion. Int. J. Parasitol. 28, 121–134.

Bogdan, C., Gessner, A., Solbach, W., Rollinghoff, M., 1996. Invasion, control and persistence of *Leishmania* parasites. Curr. Opin. Immunol. 8, 517–525.

Burgener, I.A., Konig, A., Allenspach, K., Sauter, S.N., Boisclair, J., Doherr, M.G., Jungi, T.W., 2008. Upregulation of toll-like receptors in chronic enteropathies in dogs. J. Vet. Int. Med. 22, 553–560.

Chotimanukul, S., Sirivaidyapong, S., 2011. Differential expression of Toll-like receptor 4 (TLR4) in healthy and infected canine endometrium. Theriogenol 76, 1152–1161.

Chotimanukul, S., Sirivaidyapong, S., 2012. The localization of Toll-like receptor 2 (TLR2) in the endometrium and the cervix of dogs at different stages of the oestrous cycle and with pyometra. Reprod. Domest. Anim. 47 (Suppl. (6)), 351–355.

Esteve, L.O., Saz, S.V., Hosein, S., Solano-Gallego, L., 2015. Histopathological findings and detection of Toll-like receptor 2 in cutaneous lesions of canine leishmaniosis. Vet. Parasitol. 209, 157–163.

Figueiredo, M.M., Amorim, I.F., Pinto, A.J., Barbosa, V.S., Pinheiro Lde, J., Deoti, B., Faria, A.M., Tafuri, W.L., 2013. Expression of Toll-like receptors 2 and 9 in cells of dog jejunum and colon naturally infected with *Leishmania infantum*. BMC Immunol. 14, 22.

Gatto, M., de Abreu, M.M., Tasca, K.I., de Assis Golim, M., da Silva, L.D., Simao, J.C., Fortaleza, C.M., de Campos Soares, A.M., Calvi, S.A., 2015. The involvement of TLR2 and TLR4 in cytokine and nitric oxide production in visceral leishmaniasis patients before and after treatment with anti-leishmanial drugs. PLoS One 10, e0117977.

Hawn, T.R., Ozinsky, A., Underhill, D.M., Buckner, F.S., Akira, S., Aderem, A., 2002. *Leishmania major* activates IL-1 alpha expression in macrophages through a MyD88-dependent pathway. Microbes Infect. 4, 763–771.

Hosein, S., Rodriguez-Cortes, A., Blake, D.P., Allenspach, K., Alberola, J., Solano-Gallego, L., 2015. Transcription of Toll-Like Receptors 2, 3, 4 and 9, FoxP3 and Th17 cytokines in a susceptible experimental model of canine *Leishmania infantum* infection. PLoS One 10, e0140325.

Hosein, S., Blake, D.P., Solano-Gallego, L., 2016. Insights on adaptive and innate immunity in canine leishmaniosis. Parasitol, 1–21.

Igarashi, H., Ohno, K., Fujiwara-Igarashi, A., Kanemoto, H., Fukushima, K., Goto-Koshino, Y., Uchida, K., Tsujimoto, H., 2015. Functional analysis of pattern recognition receptors in miniature dachshunds with inflammatory colorectal polyps. J. Vet. Med. Sci. 77, 439–447.

Kang, J.W., Kim, S.J., Cho, H.I., Lee, S.M., 2015. DAMPs activating innate immune responses in sepsis. Ageing Res. Rev. 24, 54–65.

Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384.

Kumar, R., Singh, O.P., Gautam, S., Nylen, S., Sundar, S., 2014. Enhanced expression of toll-like receptors 2 and 4 but not 9, in spleen tissue from patients with visceral leishmaniasis. Parasite Immunol. 36, 721–725.

Liese, J., Schleicher, U., Bogdan, C., 2008. The innate immune response against *Leishmania* parasites. Immunobiol 213, 377–387.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. Methods 25, 402–408.

Maiolini, A., Carlson, R., Tipold, A., 2012. Toll-like receptors 4 and 9 are responsible for the maintenance of the inflammatory reaction in canine steroid-responsive meningitis-arteritis, a large animal model for neutrophilic meningitis. J. Neuroinflammation 9, 226.

Martinez, V., Quilez, J., Sanchez, A., Roura, X., Francino, O., Altet, L., 2011. Canine leishmaniasis: the key points for qPCR result interpretationt. Parasite Vectors 4, 57.

McMahon, L.A., House, A.K., Catchpole, B., Elson-Riggins, J., Riddle, A., Smith, K., Werling, D., Burgener, I.A., Allenspach, K., 2010. Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease. Vet. Immunol. Immunopathol. 135, 158–163.

Melo, G.D., Silva, J.E., Grano, F.G., Homem, C.G., Machado, G.F., 2014a. Compartmentalized gene expression of toll-like receptors 2, 4 and 9 in the brain and peripheral lymphoid organs during canine visceral leishmaniasis. Parasite Immunol. 36, 726–731.

Melo, L.M., Perosso, J., Almeida, B.F., Silva, K.L., Somenzari, M.A., de Lima, V.M., 2014b. Effects of P-MAPA immunomodulator on Toll-like receptor 2, ROS, nitric oxide, MAPKp38 and IKK in PBMC and macrophages from dogs with visceral leishmaniasis. Int. Immunopharmacol. 18, 373–378.

Mercier, E., Peters, I.R., Day, M.J., Clercx, C., Peeters, D., 2012. Toll- and NOD-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. Vet. Immunol. Immunopathol. 145, 618–624.

Miró, G., Galvez, R., Fraile, C., Descalzo, M.A., Molina, R., 2011. Infectivity to *Phlebotomus perniciosus* of dogs naturally parasitized with *Leishmania infantum* after different treatments. Parasite Vectors 4, 52.

Noli, C., Saridomichelakis, M.N., 2014. An update on the diagnosis and treatment of canine leishmaniosis caused by *Leishmania infantum* (syn. *L. chagasi*). Vet. J. 202, 425–435.

Oda, K., Kitano, H., 2006. A comprehensive map of the toll-like receptor signaling network. Mol. Syst. Biol. 2 (2006), 0015.

Reis, A.B., Giunchetti, R.C., Carrillo, E., Martins-Filho, O.A., Moreno, J., 2010. Immunity to *Leishmania* and the rational search for vaccines against canine leishmaniasis. Trends Parasitol. 26, 341–349.

Riggio, M.P., Lappin, D.F., Bennett, D., 2014. Bacteria and Toll-like receptor and cytokine mRNA expression profiles associated with canine arthritis. Vet. Immunol. Immunopathol. 160, 158–166.

Schlotter, Y.M., Veenhof, E.Z., Brinkhof, B., Rutten, V.P., Spee, B., Willemse, T., Penning, L.C., 2009. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis. Vet. Immunol. Immunopathol. 129, 115–118.

Silva, E., Leitao, S., Henriques, S., Kowalewski, M.P., Hoffmann, B., Ferreira-Dias, G., da Costa, L.L., Mateus, L., 2010. Gene transcription of TLR2, TLR4, LPS ligands and prostaglandin synthesis enzymes are up-regulated in canine uteri with cystic endometrial hyperplasia-pyometra complex. J. Reprod. Immunol. 84, 66–74.

Solano-Gallego, L., Koutinas, A., Miro, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical

staging, treatment and prevention of canine leishmaniosis. Vet. Parasitol. 165, 1–18.
Solano-Gallego, L., Miro, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., The LeishVet, G., 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasite Vectors 4, 86.
Solano-Gallego, L., Di Filippo, L., Ordeix, L., Planellas, M., Roura, X., Altet, L., Martinez-Orellana, P., Montserrat, S., 2016a. Early reduction of *Leishmania infantum*-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. Parasite Vectors 9, 235.
Solano-Gallego, L., Montserrrat-Sangra, S., Ordeix, L., Martinez-Orellana, P., 2016b. *Leishmania infantum*-specific production of IFN-gamma and IL-10 in stimulated blood from dogs with clinical leishmaniosis. Parasite Vectors 9, 317.
Tolouei, S., Hejazi, S.H., Ghaedi, K., Khamesipour, A., Hasheminia, S.J., 2013. TLR2 and TLR4 in cutaneous leishmaniasis caused by *Leishmania major*. Scand. J. Immunol. 78, 478–484.
Tuon, F.F., Fernandes, E.R., Duarte, M.I., Amato, V.S., 2012. Expression of TLR2 and TLR4 in lesions of patients with tegumentary American leishmaniasis. Rev. Inst. Med. Trop. Sao Paulo 54, 159–163.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (RESEARCH0034).

veterinary sciences

MDPI

Article

Parasite Specific Antibody Levels, Interferon-γ and TLR2 and TLR4 Transcripts in Blood from Dogs with Different Clinical Stages of Leishmaniosis

Sara Montserrat-Sangrà [1], Laura Ordeix [1,2], Pamela Martínez-Orellana [1] and Laia Solano-Gallego [1,*]

[1] Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; sara.montserrat@uab.cat (S.M.-S.); laura.ordeix@uab.cat (L.O.); pamela.martinez@uab.cat (P.M.-O.)

[2] Fundació Hospital Clínic Veterinari, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

* Correspondence: laia.solano@uab.cat; Tel.: +34-93-581-8533

Received: 30 December 2017; Accepted: 14 March 2018; Published: 16 March 2018

Abstract: Canine leishmaniosis has a wide range of disease severity from mild (stage I), to severe (stages II–III), or very severe disease (stage IV). The objective of the study was to evaluate and compare serum antibody levels, *Leishmania infantum* specific IFN-γ production and TLR2 and TLR4 transcripts in non-stimulated blood from dogs with different clinical stages at the time of diagnosis as well as blood parasitemia. Enzyme-Linked ImmunoSorbent Assay (ELISAs) were performed to determine serum antibody levels and IFN-γ production and quantitative polymerase chain reaction (*q*PCRs) in order to determine blood parasite load and TLR2 and TLR4 transcripts. Older dogs were significantly affected by more severe disease with higher antibody levels and blood parasitemia than dogs with mild disease. IFN-γ production was significantly higher in dogs with stage I disease when compared to dogs with more severe disease. Relative quantification of TLR2 in dogs with mild disease was similar to that of control dogs. On the other hand, TLR2 transcripts were significantly higher in dogs with severe disease as compared with that from control healthy dogs. No differences were found in TLR4 relative quantification between groups. This study demonstrates that dogs with different clinical stages of leishmaniosis present different levels of biological markers indicative of different immune responses.

Keywords: dog; *Leishmania infantum*; papular dermatitis; IFN-γ; TLR2; TLR4; blood parasitemia

1. Introduction

Canine leishmaniosis (CanL) due to *Leishmania infantum* is a very pleomorphic disease. Clinically, this infection ranges from subclinical infection to very severe disease, passing through several degrees of disease [1]. According to this clinical variability, a clinical staging system that classifies the disease into four stages based on clinical signs, clinicopathological abnormalities, and measurement of anti-leishmanial antibodies, was previously described [1].

Clinical and clinical-pathological findings observed in CanL are the consequence of complex interactions between *L. infantum* and the genetical and immunological background of the dog [1]. In fact, both innate and adaptive immune responses play a role in the outcome of *Leishmania* infection [2,3]. However, only the adaptive immune response has been extensively investigated in dogs. The balance between the protective T-helper (Th) 1 cellular response, which was associated with the cytokine interferon-gamma (IFN-γ), and the humoral immune response mediated by Th2 lymphocytes determines the clinical manifestation of the infection. A predominantly Th1 immune response is thought to provide resistance to the development of disease. On the other

hand, a predominantly Th2 immune response correlates with antibody production and disease progression [2,4]. Although the innate immune response has been scarcely studied in leishmaniosis, recent data suggests that it is paramount for the ultimate outcome of *Leishmania* infection [2,5,6]. In this sense, toll like receptors (TLRs), which are one of the most important pattern recognition receptor family, are central in the early host defense against pathogen and activate adapter molecules after binding to their ligand. The activated cascade then leads to induction or suppression of genes that influence the inflammatory response [7].

Limited information is available regarding the role of TLRs in canine *L. infantum* infection. Although, the exact role of TLRs in the pathogenesis of CanL has not been fully addressed, it would seem that there is an association between TLR2 and its pathogenesis [2]. In fact, it has been recently revealed that TLR2 upregulation in blood and skin seems to be associated with disease progression in dogs [2,8], and a reduction in TLR2 transcription has been described with treatment and clinical improvement [8]. Moreover, there is a lower expression of TLR2 in skin biopsies from dogs with mild disease (papular dermatitis) when compared with dogs with moderate or severe disease [9]. TLRs other than TLR2 have been scarcely investigated in experimental or natural *L. infantum* infection in dogs [10]. Regarding TLR4, it has been described that transcription of this TLR appears to be similar among dogs with clinical leishmaniosis and healthy seronegative dogs at the time of diagnosis with no changes during treatment follow-up, indicating a less important role of this TLR in clinical leishmaniosis [8].

Cutaneous lesions are the most common clinical signs in CanL [11,12] and they are clinically classified as typical (i.e., exfoliative dermatitis, ulcerative dermatitis of the bony prominences, onychogryphosis, and, in an endemic area, papular dermatitis) or atypical (i.e., muco-cutaneous nodular dermatitis, other ulcerative muco-cutaneous dermatitis than that mentioned above, sterile pustular dermatitis, or ischaemic dermatopathy) [12]. Among the cutaneous manifestations of CanL, papular dermatitis is considered to be a typical form in an endemic area and is indicative of stage I leishmaniosis [13–15]. In fact, dogs with papular dermatitis commonly are young dogs without any other clinical-pathological abnormalities and with low parasite load and granuloma formation in skin lesions, negative or weakly positive anti-*Leishmania* antibody levels and specific cell-mediated immune response studied by means of leishmanin skin test [13–15]. Furthermore, this dermatological problem is associated with the spontaneous resolution of the lesions within 3–5 months [16]. Moreover, normal-looking skin from dogs with stage I and papular dermatitis is less likely to present microscopic lesions as well as harbour the parasite when compared with dogs with moderate to severe CanL [14]. Taken all of these findings together, papular dermatitis and stage I CanL is indicative of a protective immune response associated with a good prognosis [13,16,17]. However, there are limited studies that evaluate differences in clinical staging and adaptive and innate immune responses in CanL [18].

The hypothesis of this study is that dogs with stage I leishmaniosis and papular dermatitis show distinctive immunological characteristics when compared with dogs with more severe disease. Therefore, the objective of this study was to evaluate serum antibody levels, *L. infantum* specific IFN-γ production, and TLR2 and TLR4 transcripts in the blood of dogs with different clinical stages of leishmaniosis at the time of diagnosis.

2. Materials and Methods

2.1. Dogs

All of the dogs used in this study were privately owned pets, which were volunteered with client informed consent. They remained under the care of their owners and were not housed for the purposes of this study or held for any period of time.

2.2. Dogs with Clinical Leishmaniosis

Forty-two dogs with typical cutaneous signs due to leishmaniosis attending different veterinarian facilities from Catalonia (Spain): Fundació Hospital Clínic Veterinari, Universitat Autònoma de Barcelona (UAB), Cerdanyola, Barcelona), Hospital Ars Veterinària (Barcelona), Mediterrani Veterinaris Hospital (Reus, Tarragona), and Consultori Montsant (Falset, Tarragona), were prospectively enrolled. In order to evaluate its clinical status, a full complete blood count (CBC), serum biochemical profile which included creatinine, urea, total cholesterol, total protein and alanine aminotransferase (ALT), protein serum electrophoresis, and urinalysis with urinary protein creatinine ratio (UPC) were carried out. The hematology, biochemistry, and serum electrophoresis were performed using the following laboratory equipment: Siemens ADVIA120 (Siemens Healthineers, Erlangen, Germany), chemistry analyzer Olympus AU400 (Olympus Diagnostica GmbH, Hamburg, Germany) and Hydrasis system (Sebia Hispania SA, Barcelona, Spain), respectively.

Leishmaniosis was diagnosed after visualization of amastigotes on skin cytology or biopsy with or without *Leishmania* specific immunohistochemistry, as previously described [9], and/or the detection of specific *L. infantum* antibody levels by quantitative serology using an in-house Enzyme-Linked ImmunoSorbent Assay (ELISA) [19]. The classification of *L. infantum* antibody levels is described in Supplementary Table S1. Dogs were classified based on LeishVet clinical stages, as previously described [1]. In addition, blood DNA extraction and *Leishmania* quantitative polymerase chain reaction (*q*PCR) was performed with an absolute quantification of parasites/mL of blood, a standard curve was created. Ten-fold serial dilutions of known concentrations were made from a culture with 10^7 *L. infantum* promastigotes/mL. Six serial dilutions were obtained to create a standard curve. Subsequently, the value of the slope (m) and the intersection (y) of the line were calculated. The following formula was used in order to determine the number of parasites/mL blood by PCR: (Standard deviation − y)/m. The result was multiplied by the dilution factor of DNA (x125) to achieve the number of parasites/mL [20]. The classification of parasite load, as described elsewhere [21], is listed in Table S2.

2.3. Control Healthy Dogs

A total of 34 healthy dogs were enrolled. Both sexes were represented with 16 females and 18 males. Median of age was four years, with a range from six months to 10 years. Twenty-two purebred dogs with 14 breeds were represented (golden retriever, beagle, greyhound, Labrador retriever, border collie, schnauzer, Ibizan hound, fox hound, Ariege pointer, griffon Nivernais, Bruno Jura hound, Dachshund, English setter), eight mixed-breed dogs were included and for four dogs breeds were not registered. All of the dogs were seronegative and blood *q*PCR negative.

2.4. Whole Blood Cytokine Release Assay and Determination of Canine IFN-γ

Heparinized whole blood cytokine release assay was carried out, as previously reported [22]. Briefly, heparinized whole blood was separately mixed with three different conditions: (i) only medium (unstimulated); (ii) medium with soluble *L. infantum* antigen (LSA) at a concentration of 10 μg/mL; and, (iii) medium with the mitogen concanavalin A (ConA, 100 mg, Medicago®, Uppsala, Sweden) at a concentration of 10 μg/mL. IFN-γ was determined in supernatants from five days after stimulation with ConA and LSA or only medium (unstimulated), as described elsewhere [22], by a commercial sandwich ELISA (DuoSet® ELISA by Development System R&DTM, Abingdon, UK).

Cytokine concentration from supernatants with ConA and LSA was calculated after subtracting the IFN-γ concentration obtained from supernatants with only medium (unstimulated). Thereafter, dogs were classified as IFN-γ producers when *L infantum* specific IFN-γ concentration was detectable. Dogs were classified as IFN-γ non-producers when *L. infantum* specific IFN-γ concentration was at not detectable levels [22].

2.5. RNA Extraction, RNA Concentration and Quality and cDNA Synthesis

Half mL of Ethylenediaminetetraacetic Acid (EDTA) blood sample was transferred to a cryovial tube that contained 1.3 mL of RNAlater (Ambion®, Thermo Fisher Scientific, Waltham, MA, USA). Then, blood samples in cryovial tubes were left at 4 °C overnight and, thereafter, frozen at −80 °C until used. Blood samples were thawed on ice and total RNA from 500 µL of EDTA blood was extracted using Ribopure RNA blood kit (Ambion®, Thermo Fisher Scientific), according to the manufacturer's instructions. A DNase digestion step was included to remove contaminating genomic DNA using TurboDNase (Ambion®, Thermo Fisher Scientific) following manufacturer's instructions. RNA concentration was determined by Nanodrop device (Thermo Fisher Scientific). RNA integrity and quality was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples had a final concentration amongst 20–70 ng RNA/µL. All samples had an RNA Integrity Number (RIN) value greater than 7. cDNA was generated using VILO masterscript retrotranscriptase (Invitrogen™, Thermo Fisher Scientific), according to the manufacturer's instructions. cDNA was stored at −20 °C until used [8].

2.6. TLR2, TLR4 and Reference Housekeeping Genes qPCR

Transcription of TLR2 and TLR4 target genes (TG), as well as three reference housekeeping genes (RG: HPRT-1, CG14980 and SDHA), was measured by *q*PCR using QuantStudio™ 7 Flex Real-Time PCR System, array card, desktop (Life Technologies, Carlsbad, CA, USA) as previously described [8]. Briefly, we calculated the efficiency of target and reference amplification genes and determined optimal efficiencies amplifications of 100% and acceptable in the range (±20%). The baseline and threshold were established for each gene and all of the samples were processed in triplicate. The same control sample was used as a calibrator in every plate to determine relative quantification of all the dogs studied. Data were processed while applying the relative quantification method comparable to the delta-delta-cycle threshold value (ddCt)-method (2^{dd}Ct) [8]. Quantitative PCR data analysed was done by the Cloudsuite software (Life technologies™, Thermo Fisher Scientific). For normalization of TG, expression the arithmetic mean of the three RG was taken for the calculation of a reference gene index (RGI) [8].

2.7. Statistical Analysis

Statistical analyses were performed using the IBM SPSS Statistics Base 22.0 program for Windows software (IBM Corporation, Armonk, NY, USA).

Standard descriptive statistics [median and range (minimum and maximum values)] were calculated for quantitative variables. The non-parametric Mann-Whitney test was used to evaluate differences among groups. Categorical data were expressed as percentage and statistical analysis was performed while using the Fisher's exact test to compare results among independent variables. Differences were considered significant at a 5% significance level ($p < 0.05$).

3. Results

3.1. Dogs with Clinical Leishmaniosis

Based on clinicopathological findings and *L. infantum*-specific antibody levels, dogs were clinically classified, as previously reported [1], and were distributed as follows: group A (stage I and papular dermatitis, $n = 20$, Figure 1) and group B (stage II–III and ulcerative or exfoliative dermatitis, $n = 22$, Figure 2).

Ten females and ten males composed group A. Median of age was 10 months (5–98). Twelve pure bred dogs with three breeds represented and eight mixed breed dogs were included. Ten out 20 dogs were Ibizan hound dogs, one was a German pointer, and one was a Dachshund. Ten females and 12 males constituted group B. Median of age was 52.5 months (8–153). Fifteen pure bred dogs with 12 breeds represented (two French bull dogs, two Labrador retrievers, and one of each doberman,

pinscher, American bull dog, schnauzer, boxer, Akita inu, greyhound, Brittany) and seven mixed breed dogs were included. The difference of age between groups was statistically significant (Mann-Whitney U-test, Z = 3.66999, p = 0.00024). Dogs from group A did not present laboratory abnormalities, while dogs from group B presented typical laboratory abnormalities of clinical leishmaniosis.

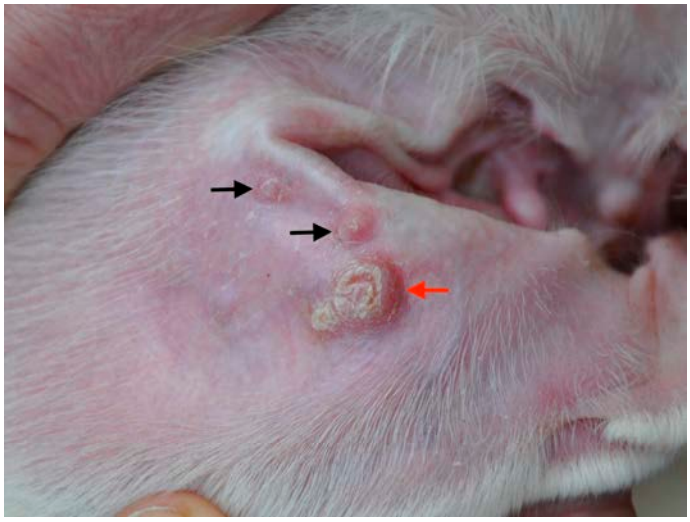

Figure 1. Two raised solid lesions smaller than 0.5 cm in diameter (papules, black arrows) and one raised solid lesion wider than higher (plaque, red arrow) in the inner aspect of a pinna of an Ibizan Hound dog with papular dermatitis and stage I leishmaniosis (Group A).

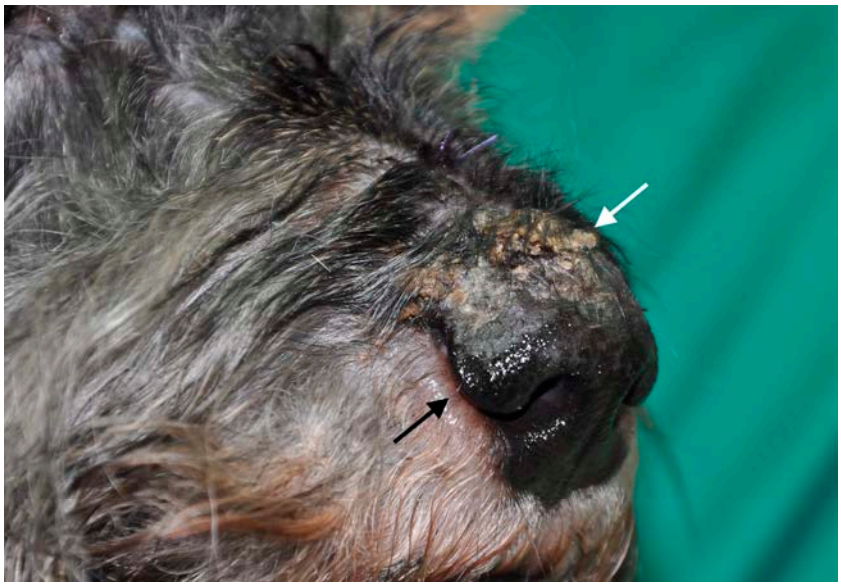

Figure 2. Crusting and scaling in the dorsal part of the muzzle (white arrow) and perinasal depigmentation (black arrow) in a schnauzer dog with stage II leishmaniosis (Group B).

3.2. Blood Leishmania qPCR

Based on *Leishmania* *q*PCR dogs from group A were classified as negative (n = 12), low positive (n = 3), or medium positive (n = 1). The parasite load evaluation was not possible in four dogs from this group due to lack of sample. On the other hand, dogs from group B were classified as negative (n = 7), low (n = 9), medium (n = 4) and high positive (n = 2). A higher percentage of positive *Leishmania* *q*PCR in blood were found in group B when compared with group A (Fisher´s exact test, p = 0.0201).

Group A dogs had lower parasite load (median and range (R): 0 parasite/mL (0–33.49)) than those from group B (median and R: 2.54 (0–475.07); Mann-Whitney U-test, Z = −2.664, p = 0.012).

3.3. *Leishmania infantum* Specific Antibody Levels

Dogs from group A were serologically classified as follows: negative (n = 11), very low positive (n = 3), low positive (n = 4), and medium positive (n = 2). On the other hand, dogs from group B were classified as very low positive (n = 1), low positive (n = 4), medium positive (n = 12), high positive (n = 3), and very high positive (n = 2). A higher percentage of seroreactive dogs was found in group B when compared with group A (Fisher´s exact test, p = 0.0001).

Group A dogs had lower levels of antibodies (median and R: 25 (5.7–529.5) EU) than those from group B (median and R: 1609 (86.8–90730.2) EU); Mann-Whitney U-test, Z = 3.53323, p = 0.00042).

3.4. *Leishmania infantum* Specific IFN-γ Production after Blood Stimulation

Cytokine analysis was performed in 19 out of 20 dogs from group A and in 15 out of 22 dogs from group B due to lack of samples. Fifteen of 19 dogs (79%) from group A and six out 15 (40%) from group B were classified as IFN-γ producers (Fisher exact test, p = 0.0337).

The median and R of IFN-γ production from groups A and B were 710 (0–20300) pg/mL and 0 (0–2413) pg/mL, respectively. There was a higher IFN-γ production in group A dogs when compared to those of group B (Mann-Whitney U-test, Z = 2.45047, p = 0.0143).

3.5. TLRs Transcripts

TLRS transcripts are presented in Figure 3. Relative quantification of TLR2 in group A (median and R: 1.2 (0.6–4.2) folds) was similar than that of control dogs (median and R: 1.2 (0.1–3.2) folds, Mann-Whitney U-test, Z = −0.24897, p = 0.8). On the other hand, relative quantification of TLR2 in group B (median and R: 1.9 (0.9–12.4)) was significantly higher when compared with that from control healthy dogs (Mann-Whitney U-test, Z = −2.41119, p = 0.016). Although no statistically significant, the expression of TLR2 in the group A was lower than that of the group B (Mann-Whitney U-test, Z = 1.6225, p = 0.1).

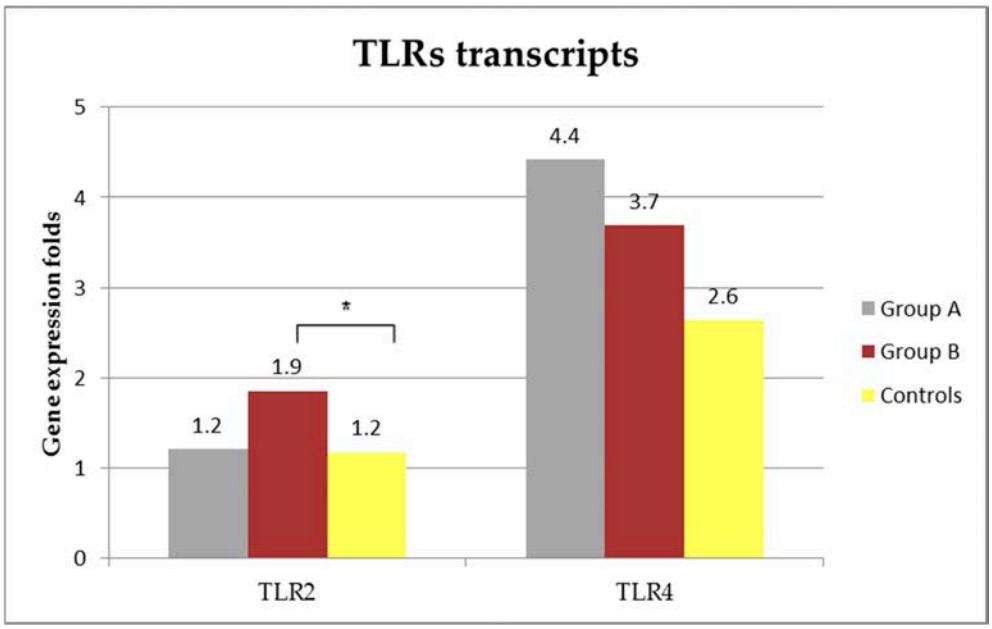

Figure 3. Median of Toll like receptors (TLR)2 and TLR4 transcripts in non-stimulated blood from diseased and control dogs (* Mann-Whitney U-test, Z = −2.41119, p = 0.016).

The expression of TLR4 mRNA in control dogs was of a median and R of 2.6 (0.5–13) folds and was similar to that of dogs from group A (median and R: 4.4 (0.7–19.8) folds; Mann-Whitney U-test, $Z = -0.92475$, $p = 0.36$) and to that from group B (median: 3.7 (1.1–32.8) folds); Mann-Whitney U-test, $Z = -1.88358$, $p = 0.06$). Although not statistically significant, TLR4 mRNA expression in dogs with leishmaniosis was higher than that of control group.

4. Discussion

The results of the present study support our hypothesis that dogs with stage I leishmaniosis and papular dermatitis show distinctive immunological characteristics when compared with dogs with more severe disease at the time of diagnosis.

Dogs with stage I and papular dermatitis reported herein were more frequently serologically negative than the dogs with stage II–III. Moreover, in agreement with a previous study [14], we demonstrated that dogs with stage I and papular dermatitis had significantly lower levels of *Leishmania* antibodies than dogs with more severe disease. Clinical CanL is often associated with a humoral response (Th2 driven), which is non-protective and denotes failure to control the infection. Serologically negative and/or low positive dogs studied herein were further diagnosed by means of skin cytology, histopathology, and/or PCR. It is well established that dogs with mild clinical signs, such as solitary lymphadenomegaly or papular dermatitis, may present with negative to low positive antibody levels [23], as described in the present study. Therefore, lack or low humoral immune response elicited in dogs with stage I and papular dermatitis, may be indicative of a polarized specific cellular immune response (Th1 driven), which might confer protection against disease progression.

In the present study, dogs with stage I and papular dermatitis were more commonly IFN-γ producers than dogs with more severe disease. In addition, *Leishmania*-specific IFN-γ concentrations were superior in dogs with papular dermatitis than in dogs with clinically more severe cutaneous forms. However, it is noteworthy that four dogs with mild disease were IFN-γ non–producers and that six dogs with more severe disease were IFN-γ producers. The results of the present study are in agreement with previous studies, which have shown that IFN-γ non-producers are usually classified in a more severe clinical staging than IFN-γ producers [18], and that IFN-γ concentration increase with long-term anti-*Leishmania* treatment, together with clinical improvement in dogs that do not produce IFN-γ at diagnosis [22]. Although, limited information regarding *L. infantum* specific IFN-γ production in stimulated blood in sick dogs is available, it seems that this assay is a reliable method of measurement of T cell-mediated immunity in CanL [24] and human leishmaniosis [25]. It is well established that Th1 lymphocytes mainly drive IFN-γ production in stimulated blood. However, natural killer (NK) cells, which play important roles in innate immune responses, may secrete this cytokine in response to IL-12. NK cells constitute 5% to 15% of the mononuclear cells in the blood. Therefore, the proportion of IFN-γ detected in blood from this origin is likely to be low. Identification of circulating cellular subsets expressing this immune marker was not carried out in the present study; therefore IFN-γ production cannot be conclusively attributed to an adaptive immune response [26]. A presumed T cellular immune response in dogs with stage I and papular dermatitis, is, therefore, thought to provide resistance to the progression of disease in those patients. In fact, previous published data have demonstrated a good clinical outcome with spontaneous resolution and an apparent lack of disease progression in dogs affected of papular dermatitis and stage I [16,17]. Moreover, a recent study have revealed a lack of disease progression in a cohort of untreated dogs with papular dermatitis and stage I leishmaniosis, followed during one year after the diagnosis [15]. However, it is interesting to highlight that those four IFN-γ non-producer dogs with papular dermatitis and stage I leishmaniosis would deserve a close and strict follow-up.

Another limitation of this study is that cytokine immune profile related to Th2 immune response (i.e., TGF-β, IL10 or IL-4) was not evaluated in these dogs. Analysis of these cytokines in stimulated blood from dogs with leishmaniosis has been scarcely described, although an association was found between parasite specific IL-10 production and disease progression [27]. Using the same method that

as employed in the present study, *L. infantum* specific IL-10 production after blood stimulation did not seem to be a disease severity marker since it was not detected in 64% of the patients and in those that was detected there was no correlation with the severity of the disease [22]. Therefore, although polarized Th1 immune response is strongly suggested in dogs with stage I and papular dermatitis, a Th2 like cytokine immune profile has not been ruled out in the present study.

There are limited data available on expression of TLRs in CanL. Therefore, the role of TLRs in the pathogenesis of this disease has not been fully addressed [10,28–31]. This study demonstrates that dogs with stage I and papular dermatitis presented a TLR2 transcription in the non-stimulated blood that was similar to healthy control dogs. On the other hand, there was a significantly higher up regulation of TLR2 in non-stimulated blood from dogs with severe clinical leishmaniosis at the time of diagnosis when compared with healthy control dogs. Although not being statistically significant, TLR2 transcripts were lower in stage I and papular dermatitis than in dogs with clinically more severe CanL. Although the cell source of increase in the expression of TLR2 was not determined in the present study, it is likely that the main source of the upregulation of TLR2 was firstly neutrophils and secondly monocytes and NK cells. Neutrophils are the predominant white cells in whole blood in canines. In addition, it has been also demonstrated that TLR2 protein is easily detectable by flow cytometry on the canine peripheral blood granulocyte and monocyte cell surface, and is rarely present on lymphocytes [32]. This is similar to what is found in humans where lymphocytes do not express TLR2 in unstimulated blood [33]. Dogs with moderate clinical leishmaniosis have a high parasite load in several tissues, including the bone marrow [34,35], and this might be the reason for a major up regulation of TLR2 in unstimulated blood, as *Leishmania* lipophosphoglycans are recognized by TLR2 [36] and it is likely that other parasite ligands are also involved in TLR2 recognition of *Leishmania* [37]. In addition, myeloid hyperplasia is a common finding in bone marrow of sick dogs with leishmaniosis [38]. Neutrophils and monocytes are released from the bone marrow to the peripheral blood, and thereafter they migrate to tissues where they die. Neutrophils or monocytes do not recirculate in peripheral blood. So, we hypothesize that it is likely that an upregulation of TLR2 happens in the bone marrow of dogs with more severe disease due to high parasite loads [34,35]. This high expression of TLR2 remains in neutrophils when they circulate in the peripheral blood, while it does not occur in dogs with mild disease.

Our results in unstimulated blood are in agreement with a previous study that described higher expression of TLR2 by immunohistochemistry in skin biopsies from cutaneous lesions in dogs with at least moderate disease (LeishVet stage II) when compared with dogs with mild disease due to papular dermatitis [9]. When considering these facts, it would seem that the transcription of TLR2 is not compartmentalized between the dermal tissue and the blood in diseased dogs. TLR2 appears to be a marker of inflammation in dogs, as demonstrated in the present study and in other canine inflammatory diseases, such as inflammatory bowel disease, immunomediated or bacterial arthritis, canine sino-nasal aspergillosis, and lymphoplasmacytic rhinitis or pyometra [39–42]. Based on published information, TLR2 up regulation appears to be associated with moderate to severe disease, suggesting an active innate immune and proinflammatory responses due to the presence of a high *Leishmania* parasite load or cellular damage (endogenous damage-associated molecular patterns (DAMPs), also called alarmins) [43], as mentioned above. In a study from Brazil, a high parasite load was found along with increased frequency and expression of TLR2 in cells from the colon of sick dogs [29]. In addition, the up regulation of TLR-2 genes has been positively associated with parasite load in the skin of naturally infected dogs [44]. According to this, parasite load in papular dermatitis has been demonstrated to be lower than in more severe cutaneous CanL states [14]. Moreover, dogs with papular dermatitis and stage I leishmaniosis show less microscopic skin lesions and parasite load in clinically healthy skin than dogs with more severe disease [14]. Therefore, a lower up regulation of TLR2 in this profile of dogs may be associated with this lower *Leishmania* parasite load. In addition, dogs with papular dermatitis and stage I leishmaniosis described in this study presented a significantly lower blood parasitemia when compared with dogs with at least moderate disease.

The present results showed that TLR4 transcript in non-stimulated blood is unchanged among the groups studied. This finding is in agreement with previous studies performed on CanL [8], in which TLR4 transcription was unchanged when compared with healthy dogs, and might indicate a mechanism of parasite immune system evasion or a less important role of this TLR in clinical CanL. This is in agreement with studies that were performed in cutaneous lesions due to *Leishmania braziliensis* in humans, which demonstrated that TLR2 is the most common TLR expressed during active disease mainly by macrophages while TLR4 is scarcely expressed [45].

Although TLRs represent an important component of the innate immunity, there are many other inflammatory factors involved in this initial immune response, such as immunity cells, cell-associated molecules other than TLRs, and soluble molecules. Therefore, further studies regarding the analysis of these other factors is necessary in order to elucidate how innate immune responses prime the adaptive immune responses in *L. infantum* infection in dogs.

5. Conclusions

In conclusion, this study demonstrates that dogs with stage I and papular dermatitis present lower specific antibody levels and blood parasitemia and higher specific IFN-γ concentrations after blood stimulation than dogs with more severe disease. Moreover, TLR2 transcript in dogs with mild disease was similar to that of healthy control dogs. On the other hand, TLR2 transcript in the blood of dogs with at least stage II was significantly higher than in healthy control dogs. The results from this study show distinctive immune responses in dogs with CanL. Moreover, immunological characteristics that were observed in dogs with stage I and papular dermatitis, together with low bood parasitemia, are indicative of the control of *Leishmania* infection.

Supplementary Materials: The following are available online at http://www.mdpi.com/2306-7381/5/1/31/s1, Table S1: Two-fold serial dilution ELISA classification in sera sample, Table S2: Parasite load classification in whole blood.

Acknowledgments: The authors thank all veterinarians and dog owners that contributed to this study. Specially, we are grateful to Marta Planellas (Fundació Hospital Clínic Veterinari de la Universitat Autònoma de Barcelona), Marta Blanchart (Ars Veterinaria), Montsant Osso (Consultori Falset), Annabel Dalmau (Hospital Mediterrani), Dani Mari and Paulina Quirola (master students from UAB) for their clinical assistance and for collecting samples and their support to this study. The authors thank also Dra. Anna Castelló and Lorena Alborch for their technical assistance to set up molecular work. The authors are also grateful to Laura di Filippo (UAB) for the assistance with laboratory work. This work was supported by Spanish ministry grants, Ministerio de Economía y competitividad and FEDER, EU (AGL2012-32498) and (AGL2015-68477). Laia Solano-Gallego held a Ramón y Cajal senior researcher contract awarded by the Ministerio de Ciencia e Innovación (Spain) and the European Social Fund.

Author Contributions: Laia Solano-Gallego and Laura Ordeix designed the research study. Laura Ordeix, Laia Solano-Gallego and Pamela Martínez-Orellana coordinated the veterinary clinics enrolled. Laura Ordeix and Laia Solano-Gallego examined clinically the majority of patients. Sara Montserrat-Sangrà set up molecular work. Sara Montserrat Sangrà performed all molecular work of this study. Pamela Martínez-Orellana performed the majority of serological testing, whole cytokine release assay and canine IFN-γ ELISA. All authors contributed with data analysis and interpretation. Laura Ordeix and Sara Montserrat-Sangrà contributed equally in the first draft and revised the manuscript. Laia Solano-Gallego revised thoroughly the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Solano-Gallego, L.; Koutinas, A.; Miro, G.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet. Parasitol.* **2009**, *165*, 1–18. [CrossRef] [PubMed]
2. Hosein, S.; Blake, D.P.; Solano-Gallego, L. Insights on adaptive and innate immunity in canine leishmaniosis. *Parasitology* **2017**, *144*, 95–115. [CrossRef] [PubMed]
3. Papadogiannakis, E.I.; Koutinas, A.F. Cutaneous immune mechanisms in canine leishmaniosis due to *Leishmania infantum*. *Vet. Immunol. Immunopathol.* **2015**, *163*, 94–102. [CrossRef] [PubMed]

4. Pinelli, E.; Killick-Kendrick, R.; Wagenaar, J.; Bernadina, W.; del Real, G.; Ruitenberg, J. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania infantum*. *Infect. Immun.* **1994**, *62*, 229–235. [PubMed]
5. Barbosa, M.A.; Alexandre-Pires, G.; Soares-Clemente, M.; Marques, C.; Rodrigues, O.R.; De Brito, T.V.; Da Fonseca, I.P.; Alves, L.C.; Santos-Gomes, G.M. Cytokine gene expression in the tissues of dogs infected by *Leishmania infantum*. *J. Comp. Pathol.* **2011**, *145*, 336–344. [CrossRef] [PubMed]
6. Fernandez-Bellon, H.; Solano-Gallego, L.; Rodriguez, A.; Rutten, V.P.; Hoek, A.; Ramis, A.; Alberola, J.; Ferrer, L. Comparison of three assays for the evaluation of specific cellular immunity to *Leishmania infantum* in dogs. *Vet. Immunol. Immunopathol.* **2005**, *107*, 163–169. [CrossRef] [PubMed]
7. Vidya, M.K.; Kumar, V.G.; Sejian, V.; Bagath, M.; Krishnan, G.; Bhatta, R. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. *Int. Rev. Immunol.* **2017**, 1–17. [CrossRef] [PubMed]
8. Montserrat-Sangrà, S.; Alborch, L.; Ordeix, L.; Solano-Gallego, L. TLR-2 and TLR-4 transcriptions in unstimulated blood from dogs with leishmaniosis due to *Leishmania infantum* at the time of diagnosis and during follow-up treatment. *Vet. Parasitol.* **2016**, *228*, 172–179. [CrossRef] [PubMed]
9. Esteve, L.O.; Saz, S.V.; Hosein, S.; Solano-Gallego, L. Histopathological findings and detection of Toll-like receptor 2 in cutaneous lesions of canine leishmaniosis. *Vet. Parasitol.* **2015**, *209*, 157–163. [CrossRef] [PubMed]
10. Melo, G.D.; Silva, J.E.; Grano, F.G.; Homem, C.G.; Machado, G.F. Compartmentalized gene expression of Toll-like receptors 2, 4 and 9 in the brain and peripheral lymphoid organs during canine visceral leishmaniasis. *Parasite. Immunol.* **2014**, *36*, 726–731. [CrossRef] [PubMed]
11. Saridomichelakis, M.N.; Koutinas, A.F. Cutaneous involvement in canine leishmaniosis due to *Leishmania infantum* (syn. *L. Chagasi*). *Vet. Dermatol.* **2014**, *25*, 61–71, e22. [CrossRef] [PubMed]
12. Ordeix, L.; Fondati, A. *Manifestaciones clínicas cutáneas en Leishmaniosis Canina. Una Revisión Actualizada*; Solano-Gallego, L., Ed.; Servet: Zaragoza, Spain, 2013; pp. 69–95.
13. Ordeix, L.; Solano-Gallego, L.; Fondevila, D.; Ferrer, L.; Fondati, A. Papular dermatitis due to *Leishmania* spp. infection in dogs with parasite-specific cellular immune responses. *Vet. Dermatol.* **2005**, *16*, 187–191. [CrossRef] [PubMed]
14. Ordeix, L.; Dalmau, A.; Osso, M.; Llull, J.; Montserrat-Sangrà, S.; Solano-Gallego, L. Histological and parasitological distinctive findings in clinically-lesioned and normal-looking skin of dogs with different clinical stages of leishmaniosis. *Parasit.Vect.* **2017**, *10*, 121. [CrossRef] [PubMed]
15. Ordeix, L.; Rodríguez, A.; Martínez-Orellana, P.; Montserrat-Sangrà, S.; Solano-Gallego, L. Clinical follow-up of a series of dogs with papular dermatitis due to *Leishmania infantum*. *Vet. Dermatol.* **2017**, *28*, 549.
16. Bottero, E.; Poggi, M.; Viglione, M. Lesioni papulari indotte da *Leishmania* spp. in 8 cani giovani. *Veterinaria* **2006**, *1*, 33–36.
17. Lombardo, G.; Pennisi, M.G.; Lupo, T.; Chicharro, C.; Solano-Gallego, L. Papular dermatitis due to *Leishmania infantum* infection in seventeen dogs: Diagnostic features, extent of the infection and treatment outcome. *Parasites Vectors* **2014**, *7*, 120. [CrossRef] [PubMed]
18. Martínez-Orellana, P.; Montserrat-Sangrà, S.; Ordeix, L.; Solano-Gallego, L. *Leishmania infantum*-specific IFN-γ production in stimulated blood from dogs with clinical leishmaniosis at diagnosis and during treatment. *Vet. Parasitol.* **2017**, *248*, 39–47. [CrossRef] [PubMed]
19. Solano-Gallego, L.; Di Filippo, L.; Ordeix, L.; Planellas, M.; Roura, X.; Altet, L.; Martínez-Orellana, P.; Montserrat, S. Early reduction of *Leishmania infantum*-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. *Parasites Vectors* **2016**, *9*, 235. [CrossRef] [PubMed]
20. Martinez, V.; Quilez, J.; Sanchez, A.; Roura, X.; Francino, O.; Altet, L. Canine leishmaniasis: The key points for qpcr result interpretation. *Parasites Vectors* **2011**, *4*, 57. [CrossRef] [PubMed]
21. Tabar, M.D.; Roura, X.; Francino, O.; Altet, L.; Ruiz de Gopegui, R. Detection of *Leishmania infantum* by real-time PCR in a canine blood bank. *J. Small Anim. Pract.* **2008**, *49*, 325–328. [CrossRef] [PubMed]
22. Solano-Gallego, L.; Montserrrat-Sangrà, S.; Ordeix, L.; Martínez-Orellana, P. *Leishmania infantum*-specific production of IFN-gamma and IL-10 in stimulated blood from dogs with clinical leishmaniosis. *Parasites Vectors* **2016**, *9*, 317. [CrossRef] [PubMed]
23. Solano-Gallego, L.; Cardoso, L.; Pennisi, M.G.; Petersen, C.; Bourdeau, P.; Oliva, G.; Miró, G.; Ferrer, L.; Baneth, G. Diagnostic challenges in the era of canine *Leishmania infantum* vaccines. *Trends. Parasitol.* **2017**, *33*, 706–717. [CrossRef] [PubMed]

24. Carson, C.; Antoniou, M.; Ruiz-Arguello, M.B.; Alcami, A.; Christodoulou, V.; Messaritakis, I.; Blackwell, J.M.; Courtenay, O. A prime/boost DNA/modified vaccinia virus ankara vaccine expressing recombinant *Leishmania* DNA encoding tryp is safe and immunogenic in outbred dogs, the reservoir of zoonotic visceral leishmaniasis. *Vaccine* **2009**, *27*, 1080–1086. [CrossRef] [PubMed]
25. Singh, O.P.; Sundar, S. Whole blood assay and visceral leishmaniasis: Challenges and promises. *Immunobiology* **2014**, *219*, 323–328. [CrossRef] [PubMed]
26. Abbas, A.; Lichtman, A.H.; Pillai, S. *Cellular and Molecular Immunology*, 7th ed.; Elsevier: Philadelphia, PA, USA, 2012.
27. Boggiatto, P.M.; Ramer-Tait, A.E.; Metz, K.; Kramer, E.E.; Gibson-Corley, K.; Mullin, K.; Hostetter, J.M.; Gallup, J.M.; Jones, D.E.; Petersen, C.A. Immunologic indicators of clinical progression during canine *Leishmania infantum* infection. *Clin. Vaccine Immunol.* **2010**, *17*, 267–273. [CrossRef] [PubMed]
28. Hosein, S.; Rodriguez-Cortes, A.; Blake, D.P.; Allenspach, K.; Alberola, J.; Solano-Gallego, L. Transcription of Toll-like receptors 2, 3, 4 and 9, Foxp3 and Th17 cytokines in a susceptible experimental model of canine *Leishmania infantum* infection. *PLoS ONE* **2015**, *10*, e0140325. [CrossRef] [PubMed]
29. Figueiredo, M.M.; Amorim, I.F.; Pinto, A.J.; Barbosa, V.S.; Pinheiro Lde, J.; Deoti, B.; Faria, A.M.; Tafuri, W.L. Expression of Toll-like receptors 2 and 9 in cells of dog jejunum and colon naturally infected with *Leishmania infantum*. *BMC Immunol.* **2013**, *14*, 22. [CrossRef] [PubMed]
30. Melo, L.M.; Perosso, J.; Almeida, B.F.; Silva, K.L.; Somenzari, M.A.; de Lima, V.M. Effects of p-MAPA immunomodulator on Toll-like receptor 2, ROS, Nitric oxide, MAPKp38 and IKK in PBMC and macrophages from dogs with visceral leishmaniasis. *Int. Immunopharmacol.* **2014**, *18*, 373–378. [CrossRef] [PubMed]
31. Amorim, I.F.; Silva, S.M.; Figueiredo, M.M.; Moura, E.P.; Castro, R.S.; Lima, T.K.; Gontijo Nde, F.; Michalick, M.S.; Gollob, K.J.; Tafuri, W.L. Toll receptors type-2 and Cr3 expression of canine monocytes and its correlation with immunohistochemistry and xenodiagnosis in visceral leishmaniasis. *PLoS ONE* **2011**, *6*, e27679. [CrossRef] [PubMed]
32. Bazzocchi, C.; Mortarino, M.; Comazzi, S.; Bandi, C.; Franceschi, A.; Genchi, C. Expression and function of Toll-like receptor 2 in canine blood phagocytes. *Vet. Immunol. Immunopathol.* **2005**, *104*, 15–19. [CrossRef] [PubMed]
33. Flo, T.H.; Halaas, O.; Torp, S.; Ryan, L.; Lien, E.; Dybdahl, B.; Sundan, A.; Espevik, T. Differential expression of Toll-like receptor 2 in human cells. *J. Leukoc. Biol.* **2001**, *69*, 474–481. [PubMed]
34. Reis, A.B.; Teixeira-Carvalho, A.; Vale, A.M.; Marques, M.J.; Giunchetti, R.C.; Mayrink, W.; Guerra, L.L.; Andrade, R.A.; Correa-Oliveira, R.; Martins-Filho, O.A. Isotype patterns of immunoglobulins: Hallmarks for clinical status and tissue parasite density in brazilian dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. *Vet. Immunol. Immunopathol.* **2006**, *112*, 102–116. [CrossRef] [PubMed]
35. Reis, A.B.; Martins-Filho, O.A.; Teixeira-Carvalho, A.; Giunchetti, R.C.; Carneiro, C.M.; Mayrink, W.; Tafuri, W.L.; Correa-Oliveira, R. Systemic and compartmentalized immune response in canine visceral leishmaniasis. *Vet. Immunol. Immunopathol.* **2009**, *128*, 87–95. [CrossRef] [PubMed]
36. Becker, I.; Salaiza, N.; Aguirre, M.; Delgado, J.; Carrillo-Carrasco, N.; Kobeh, L.G.; Ruiz, A.; Cervantes, R.; Torres, A.P.; Cabrera, N.; et al. *Leishmania* Lipophosphoglycan (LPG) activates NK cells through Toll-like receptor-2. *Mol. Biochem. Parasitol.* **2003**, *130*, 65–74. [CrossRef]
37. Halliday, A.; Bates, P.A.; Chance, M.L.; Taylor, M.J. Toll-like receptor 2 (TLR2) plays a role in controlling cutaneous leishmaniasis in vivo, but does not require activation by parasite Lipophosphoglycan. *Parasites Vectors* **2016**, *9*, 532. [CrossRef] [PubMed]
38. Paltrinieri, S.; Gradoni, L.; Roura, X.; Zatelli, A.; Zini, E. Laboratory tests for diagnosing and monitoring canine leishmaniasis. *Vet. Clin. Pathol.* **2016**, *45*, 552–578. [CrossRef] [PubMed]
39. McMahon, L.A.; House, A.K.; Catchpole, B.; Elson-Riggins, J.; Riddle, A.; Smith, K.; Werling, D.; Burgener, I.A.; Allenspach, K. Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease. *Vet. Immunol. Immunopathol.* **2010**, *135*, 158–163. [CrossRef] [PubMed]
40. Riggio, M.P.; Lappin, D.F.; Bennett, D. Bacteria and Toll-like receptor and cytokine mRNA expression profiles associated with canine arthritis. *Vet. Immunol. Immunopathol.* **2014**, *160*, 158–166. [CrossRef] [PubMed]
41. Chotimanukul, S.; Sirivaidyapong, S. The localization of Toll-like receptor 2 (TLR2) in the endometrium and the cervix of dogs at different stages of the oestrous cycle and with pyometra. *Reprod. Domest. Anim.* **2012**, *47*, 351–355. [CrossRef] [PubMed]

42. Mercier, E.; Peters, I.R.; Day, M.J.; Clercx, C.; Peeters, D. Toll- and Nod-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. *Vet. Immunol. Immunopathol.* **2012**, *145*, 618–624. [CrossRef] [PubMed]
43. Kang, J.W.; Kim, S.J.; Cho, H.I.; Lee, S.M. Damps activating innate immune responses in sepsis. *Ageing Res. Rev.* **2015**, *24*, 54–65. [CrossRef] [PubMed]
44. Pereira-Fonseca, D.C.M.; Oliveira-Rovai, F.M.; Rodas, L.A.C.; Beloti, C.A.C.; Torrecilha, R.B.P.; Ito, P.; Avanco, S.V.; Cipriano, R.S.; Utsunomiya, Y.T.; Hiramoto, R.M.; et al. Dog skin parasite load, TLR-2, IL-10 and TNF-alpha expression and infectiousness. *Parasites Immunol.* **2017**, *39*, e12493. [CrossRef] [PubMed]
45. Tuon, F.F.; Fernandes, E.R.; Duarte, M.I.; Amato, V.S. Expression of TLR2 and TLR4 in lesions of patients with tegumentary american leishmaniasis. *Rev. Inst. Med. Trop. Sao Paulo* **2012**, *54*, 159–163. [CrossRef] [PubMed]