ANALYSIS OF HOST RESPONSES AND FITNESS IN DIFFERENT PANDEMIC H1N1 (2009) INFLUENZA VIRUS IN MICE AND FERRETS

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SUMMARY/RESUMEN

1. SUMMARY

Influenza is a worldwide public health concern, being one of the most common infectious diseases and a highly contagious airborne pathology. From April 2009, a new influenza A H1N1 virus with swine origin gave rise to the emergence of worldwide outbreaks which was subsequently declared as a pandemic situation. Nowadays pdmH1N1 2009 virus continues on circulation and generally triggers mild and self-limiting infections. Nevertheless, a small percentage of the patients require hospitalization and specialized attention in Intensive Care Units (ICUs). Noteworthy, in ICU patients an increased proinflammatory cytokine production has been identified. This observation would suggest the hypothesis that the heterogeneity in the outcome of pdmH1N1 2009 influenza virus infection could be due not only to differential fitness/virulence of the diverse circulating pandemic virus strains but also to the host immune environment that may contribute to severe respiratory pathogenesis, probably by an exacerbated immune response associated to hypercytokinemia.

To test such hypothesis the work was divided in four studies describing the experiments performed in mice and ferrets with pdmH1N1 2009 viruses isolated during the 2009 outbreak from patients that showed mild to severe disease in order to analyse pathological features of the infection.

Experiments in chapters 5 and 6 were conducted in order to evaluate whether high levels of proinflammatory cytokines and in particular IL-6, might affect host immune responses and the clinical course of infection. Mice were treated with LPS or mice expressing with high levels of IL-6 were infected with pdmH1N1 2009 (CAT09) simultaneously. In the case of LPS exposure, results showed that clinical signs and weight loss were directly influenced by LPS in CAT09 infected. However, no differences in viral load of lungs from infected mice were detected upon LPS exposure, indicating that LPS treatment was not affecting viral replication *in vivo*. IL-6 secretion upon LPS exposure correlated with body weight loss and higher pathology.

The role of IL-6 in influenza infection was addressed by inducing IL-6 in mice prior CAT09 infection. Again, IL-6 levels correlated with weight loss. Surprisingly, viral replication was not affected by high levels of IL-6 since viral load did not exhibit significant differences when both infected groups were compared, although high level of IL-6 in infected animals correlated with sooner viral clearance than the CAT09 infected animals. A strong antibody response was detected in infected animals, being only CAT09 infected mice without IL-6 treatment, the ones with the highest hemagglutinin inhibiting titers. IL-10 levels correlated with IL-6 levels in serum and lungs in the first days after infection. Finally, histopathological lesions were more severe in mice with high levels of IL-6 and CAT09 infected.

From the onset of the 2009 pandemic, oseltamivir resistance (OsR) mutations have been described on circulating pdmH1N1 2009 viruses. In chapter 7, *in vitro* and *in vivo* experiments in which two strains (R6 and R7) of OsR pandemic virus were compared. There were kinetics differences in both virus *in vitro* that finally were reflected in the pathogenesis of infection *in vivo*. The results obtained in the *in vitro* analysis showed different fitness in viral replication in the virus studied in comparison with a oseltamivir sensitive virus (F), being F>R6>R7. On the following *in vivo* experiment, both OsR strains produced a fatal outcome although with different magnitude and kinetics, R6-infected group experimented a 40% of lethality and R7-group a 20% at 4 dpi. However, at 7 dpi the percentatge of survival was a 50% in both OsR-infected groups. Viral replication detected in lungs from OsR-infected groups had higher but not statistically different values for R7 than for R6. There was a strong antibody response at 14 dpi on both infected groups for each virus but no cross- reactive antibodies. Interestingly, high levels of IL-6 were detected in serum from R7-mice with significant differences. Surprisingly, levels of IL-6 in lungs of R6, R7 and control animals were similar at all time-point with no statistical differences. Serum and lung IL-10 had also slightly higher values in R7-mice when compared with controls at 3 and 5 dpi respectively. Histopahological findings showed more severe lesions on R7-mice at 5 dpi.

To analyze possible virulence differences in viral fitmess, ferrets were infected with two contemporary pdmH1N1 2009 viruses from two patients without known co-morbid conditions, one that became fatal (F) while the other only showed mild (M) respiratory disease. These were studied in chapter 8. Ferrets developed different degree of clinical signs severity that did not correlate with the origin of the virus used in the infection, exhibiting severe (S) or non severe (NS) pathology. A significant decrease in body weight was detected in S animals compared to NS animals at 4 to 7 dpi. Clinical progress of the infection correlated directly with histopathological findings. The analysis of the acute phase proteins showed that the concentrations of haptoglobin (HP) and serum amyloid A (SAA) increased in both groups after 2 dpi. Virus titres in all tissues were higher in ferrets belonging to S group when compared to ferrets belonging to NS group at 4 dpi. Animals infected with both virus showed a strong hemagglutinin inhibiting antibody response in sera to both viruses at 10 and 14 dpi. Ferrets with a severe progress of the clinical infection showed slightly higher antibody responses and higher viral titers after infection.

RESUMEN

La gripe es un problema de salud pública en todo el mundo, siendo una de las enfermedades infecciosas más comunes y una patología de las vías aéreas altamente contagiosa. Desde abril de 2009, un nuevo virus de influenza A H1N1, con origen porcino dio lugar a la aparición de brotes en todo el mundo siendo declarada posteriormente como una situación de pandemia. Hoy en día el virus pdmH1N1 de 2009 continúa en circulación, generalmente provocando infecciones leves y autolimitadas. Sin embargo, un pequeño porcentaje de pacientes requieren hospitalización y atención especializada en la Unidad de Cuidados Intensivos (UCI). Es digno de mención comentar que se ha identificado un aumento en la producción de citoquinas proinflamatorias en pacientes de UCI. Esta observación sugiere la hipótesis de que la heterogeneidad en el resultado de la infección por virus de la gripe pdmH1N1 de 2009 podría ser debido no sólo a la diferencia entre fitness /virulencia de las diversas cepas de virus pandemico circulantes, sino también por el medio ambiente inmune del huésped que pueden contribuir a la patogénesis respiratoria grave, probablemente por una respuesta inmune exacerbada asociada a hipercitoquinemia.

Para probar esta hipótesis el presente trabajo se divide en cuatro estudios que describen los experimentos realizados en ratones y hurones con los virus pdmH1N1 de 2009 aislados durante el brote de 2009 de pacientes que mostraron grados de enfermedad desde leve a severa con el fin de analizar las características patológicas de la infección.

Los experimentos en los capítulos 5 y 6 se llevaron a cabo con el fin de evaluar si los altos niveles de citoquinas proinflamatorias y, en particular IL-6, podrían afectar a la respuesta inmune del huésped y al curso clínico de la infección. Los ratones fueron tratados con LPS o ratones que expresaron altos niveles de IL-6 fueron infectados con el virus pdmH1N1 de 2009 (CAT09) simultáneamente. En el caso de la exposición a LPS, los resultados mostraron que los signos clínicos y la pérdida de peso fueron directamente influenciadas por LPS en los animales infectados con CAT09. Sin embargo, no se detectaron diferencias en la carga viral de los pulmones de los ratones infectados con exposición de LPS, lo que indica que el tratamiento con LPS no estaba

afectando a la replicación viral *in vivo*. La secreción de IL-6 después de la exposición a LPS correlacionó con pérdida de peso corporal a mayor grado de patología.

El papel de la IL-6 en la infección por influenza se abordó mediante la inducción de IL-6 en ratones antes de ser infectados con CAT09. Una vez más, los niveles de IL-6 se correlacionaron con la pérdida de peso. Sorprendentemente, la replicación viral no fue afectada por altos niveles de IL-6 debido a que la carga viral no mostró diferencias significativas cuando se compararon los dos grupos infectados, aunque los animals infectados con alto nivel de IL-6 aclararon el virus antes que los animales infectados con CAT09. Se detectó una fuerte respuesta de anticuerpos en los animales infectados, siendo los ratones infectados sólo con CAT09 sin tratamiento con IL-6, los que obtuvieron los mayores títulos de inhibición de hemaglutinación. IL-10 correlacionó con los niveles de IL-6 en el suero y en los pulmones en los primeros días después de la infección. Finalmente, las lesiones histopatológicas fueron más graves en los ratones con altos niveles de IL-6 e infectados con CAT09.

Desde el inicio de la pandemia de 2009, mutaciones de resistencia a oseltamivir (OsR) se han descrito en virus circulante pdmH1N1 de 2009. En el capítulo 7 estudios *in vitro* e *in vivo* en el que se compararon dos cepas (R6 y R7) de virus pandémico OsR. Hubo diferencias *in vitro* en la cinética de ambos virus que finalmente se reflejó en la patogénesis de la infección *in vivo*. Los resultados obtenidos en el análisis *in vitro* mostraron diferencias en el fitness en la replicación viral de los virus estudiados respecto a un virus sensible a oseltamivir (F), siendo F > R6 > R7. En el siguiente experimento *in vivo*, ambas cepas OsR produjeron un resultado fatal aunque en diferente magnitud y cinética; el grupo infectado con R6 experimentó un 40 % de letalidad y el grupo R7 un 20 % a los 4 dpi. Sin embargo, a 7 dpi el porcentaje de supervivencia fue de un 50 % para ambos grupos infectados con virus OsR. La replicación viral detectada en los pulmones de los grupos infectados con virus OsR obtuvo los valores no estadísticamente diferentes para R7 que para R6. Hubo una fuerte respuesta de anticuerpos a los 14 dpi en ambos grupos infectados para cada virus, pero no anticuerpos de reacción cruzada. Curiosamente, se detectaron altos niveles de IL-6 en el suero de los ratones R7 con diferencias significativas. Sorprendentemente, los niveles de IL-6 en los pulmones de R6, R7 y animales control fueron similares a todos los tiempos sin diferencias estadísticas. Niveles de IL-10 de suero y pulmón mostraron

valores ligeramente más altos también en ratones R7 en comparación con los controles a los 3 y 5 dpi, respectivamente. Hallazgos histopatológicos mostraron lesiones más severas en ratones R7 a 5 dpi.

Para analizar las posibles diferencias de virulencia, se infectaron hurones con dos virus pdmH1N1 de 2009 de dos pacientes sin condiciones comórbidas conocidas, uno con consecuencia fatal (F), mientras que el otro sólo mostró enfermedad respiratoria leve (M). Este estudio está comprendido en el capítulo 8. Los hurones desarrollaron diferente grado de severidad de signos clínicos que no se correlacionaron con el origen del virus utilizado en la infección, exhibiendo patología severa (S) o no severa (NS). Se detectó una disminución significativa en el peso corporal en los animales S en comparación con los animales NS a 4 a 7 dpi. La evolución clínica de la infección se relaciono directamente con los hallazgos histopatológicos. El análisis de las proteínas de fase aguda mostró que las concentraciones de haptoglobina (HP) y amiloide A sérico (SAA) incrementaron en ambos grupos después de 2 pi. Los títulos de virus en todos los tejidos fueron más altos en los hurones pertenecientes al grupo S en comparación con los hurones pertenecientes al grupo de NS a 4 dpi. Los animales infectados con ambos virus mostraron una fuerte inhibición de la hemaglutinina de la respuesta de anticuerpos en el suero de ambos virus a 10 y 14 dpi. Los hurones con un progreso severo de la infección clínica mostraron respuestas de anticuerpos ligeramente más altos y los títulos virales más altos después de la infección.

LIST OF ABBREVIATIONS

2. LIST OF ABBREVIATIONS

A

A/ CastillaLaMancha/RR5661/2009- M

A/Baleares/RR6121/2009- R6

A/CastillaLaMancha/RR5911/2009- F

A/Catalonia/63/2009- CAT09

A/Madrid/RR7495/2011- R7

Acute phase proteins- APP

Alveolar macrophages- Mf

Antibody-dependent cell-mediated cytotoxicity- ADCC

Antigen presenting cells- APC

Avidin-biotin-peroxidase- ABC

B

Biosafety level 3- BSL3

Bovine serum albumin- BSA

Broncheo alveolar lavage- BAL

C

C57BL6/JOlaHsd- C57BL6

Centers for Disease Control and Prevention- CDC

Chronic obstructive pulmonary disease- COPD

Conventional DCs- cDCs

Cytophatic effect- CPE

Cytotoxic T lymphocytes- CTL

D

Days post infection- dpi

Dendritic cells- DCs

Diaminobenzidine tetrahydrochloride- DAB

Dulbecco's Modified Eagle Medium- DMEM

E

Enzyme-Linked Immunosorbent Assay- ELISA

F

Fetal bovine serum- FBS

G

Granulocyte colony-stimulating factor- G-CSF

Granzymes- Gr

H

Haematoxylin eosin- HE

Haptoglobin- Hp

Hemagglutination inhibition- HAI

Hemagglutinin- HA

Highly pathogenic avian influenza- HPAI

Human immunodeficiency virus- HIV

I

Influenza virus- IV

Instituto de Salud Carlos III- ISCIII

Intensive care unit- ICU

Interferon alpha- IFN-a

Interferon delta: IFN-δ

Interferon gamma- IFN-γ

Interferon gamma-induced protein 10- IP-10

Interleukin 2- IL-2

International Organization of Epizooties- OIE

Intranasally- IN

Intraperitoneally- IP

Intravenously- IV

Ion channel- M2

K

Knock out- KO

L

Lipopolysaccharide- LPS

M

Macrophage inflammatory proteins 1 beta- MIP-1β

Madin-Darby Canine kidney- MDCK

Major histocompatibility complex- MHC

Matrix protein- M1

Minimum Essential medium Eagle- MEM

Monocyte chemoattractant protein-1- MCP-1

Multiplicity of infection- MOI

Murine IL-6- mIL-6

N

National Center for Biotechnology Information- NCBI

National Influenza Centre- NIC

Natural cytotoxicity receptors- NCR

Natural killer cells- NK

Neuraminidase inhibitors- NAIs

Neuraminidase- NA

Nitric Oxide Synthase 2- NOS2

NOD-like receptor family pryin domain containing 3- NLRP3

Non severe- NS

Non-structural protein 1- NS1

Nuclear export protein- NEP; also known as NS2

Nucleoprotein- NP

O

Oseltamivir resistance- OsR

P

Pandemic A (H1N1) 2009- pdmH1N1

Pathogen-associated marker pattern- PAMP

Pattern-recognition receptors- PRRs

Phosphate Buffer Saline- PBS

Plaque Forming Unit- PFU

Plasmacytoid DCs- pDCs

Plasmid psDNA3.1+-mIL6- pIL-6

Polymerase complex- PB1, PB2 and PA

R

Real time polymerase chain reaction- RT-PCR

Recombinant mouse IL-6- rmIL-6

Regulatory T cells- Tregs

Retinoic acid inducible gene-I- RIG-I

S

Serum amyloid A- SAA

Severe- S

Sialic acid- SA

Specific pathogen free- SPF

Swine IV- swIV

T

T cells- Tregs

T helper 17- Th17

T helper- Th

Tissue Culture Infective Dose- TCID50

Toll like receptors- TLRs

Tuberculosis- TB

Tumor Necrosis Factor alpha- TNF- α

V

Vascular endothelial growth factor- VEGF

Viral RNA- vRNA

W

White blood cells- WBC

Wild type- WT

INTRODUCTION

2. INTRODUCTION

I. Introduction to influenza

Before Influenza virus (IV) took his place as etiological agent, the term influenza was established in Italy in the XV century. The concept derived from the former Italian expression *ex influentia colesti* used to refer to its mysterious origin in which the stars influenced the course of the disease. Previously, in 412 BC Hippocrates, the father of medicine, described a flu-like disease for the first time at Perinthus in North Greece [1].

Influenza is one of the most common infectious diseases and a highly contagious airborne pathology with potentially fatal outcomes. It is characterized by symptoms that include fever, headache, cough, nasal congestion, sneezing, and whole-body aches [2]. Despite the availability of inactivated vaccines derived from the current circulating strains, every year large segments of the human population are affected by influenza infection, because of frequent natural variation of the hemagglutinin (HA) and neuraminidase (NA) envelope proteins of the virus. This variation allows the virus to escape neutralization by preexisting circulating antibody in the blood stream, present as a result of either previous natural infection or immunization [2]. IVs are unique in their ability to cause both recurrent annual epidemics and more serious pandemics that spread rapidly and may affect all or most age-groups. The size of epidemics and pandemics, and their relative impact, reflects the interplay between the extent of antigenic variation of the virus, the amount of protective immunity in populations, and the relative virulence of the viruses [3].

II. Influenza viruses

i. Classification and antigenic types

The IV belongs to the family of *Orthomyxoviridae*, defined by viruses that have a negative-sense, single-stranded, and segmented RNA genome. IVs are divided into types A, B, and C on the basis of variation in the nucleoprotein antigen. In types A and B the HA and NA antigens undergo genetic variation, which is the basis for the emergence of new strains; type C is antigenically stable [4].The influenza A viruses are further subdivided on the basis of antigenic differences between the HA and NA surface proteins. There are now 18 different HA (H1 to H18) and 11 different NA (N1 to N11) subtypes for influenza A viruses, being the recently designated as H18N11 novel influenza A virus described in a flat faced fruit bat (*Artibeus planirostris*) from Peru[5].

ii. Structure of the influenza virus

IVs are spherical or filamentous enveloped particles 80 to 120 nm in diameter. The helically symmetric nucleocapsid consists of a nucleoprotein (NP) and a multipartite genome of single-stranded antisense RNA in seven or eight segments (Figure 1). The envelope carries the HA attachment protein and the NA. The virus binds to host cells via HA. Transcription and nucleocapsid assembly take place in the nucleus. Progeny virions are assembled in the cytoplasm and bud from the cell membrane, killing the cell. IV genome comprises eight viral RNA (vRNA) segments. Each segment of the genome encodes a single virus polypeptide: PB2, PB1, PA, HA, NP, NA, M1, or NS1 [6, 7]. Transcripts of the M1 and NS1 genes produce M2 and NS2 as splicing variants [8] . Until then, it was thought that the influenza viral genome encode 10 viral proteins in total. However, further studies of the IV genome identified exception of a segment that encodes two proteins by alternative splicing. In 2001, a viral protein, PB1-F2, was discovered as a second polypeptide made from the PB1 mRNA [9]. Later, a third major polypeptide PB1-N40 was also identified as synthesized from the PB1 mRNA [10]. More recently, the novel influenza A virus protein PA-X was discovered [11] and in 2013 Muramoto and colleages [12] identified small proteins produced from the PA segment and

identified the translation initiation codons of these proteins on the PA mRNA by use of mutational analysis; the nature of these proteins has remained unclear. Currently, the identification of novel influenza virus proteins is receiving considerable interest and influenza segment PA has now been shown to encode as many as four proteins, PA, PA-X, PA-N155 and PA-N182 [12]. All the studies together demonstrated that the eight segments of the influenza viral genome can encode up to 16 proteins.

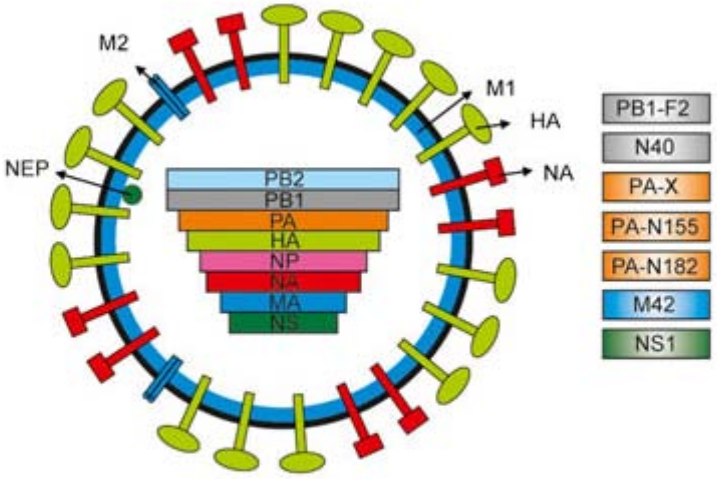


Figure 1. Influenza A virus particle. Schematic representation of Influenza A virus particle and gene segments. The influenza genome consists of eight single-stranded RNAs. The non-structural proteins and/or newly identified proteins with unknown function are depicted in the rectangles. The hemagglutinin (HA), neuraminidase (NA), and M2 proteins are inserted into the host-derived lipid envelope. The matrix (M1) protein underlies the lipid envelope. A nuclear export protein (NEP/NS2) is also associated with the virus. The viral RNA segments are coated with nucleoprotein and are bound by the polymerase complex. Imagen adapted from Schrawen et al [13].

iii. Genetics of the influenza virus

Genetic evolution of IV is given by key elements such as his segmented nature coupled to the error-prone RNA polymerase transcription and replication of the viral genome. Therefore, IV through the accumulation of mutation (antigenic drift) and/or reassortment (antigenic shift) oftentimes resulted in enhanced pathogenesis and expanded host range [2].

Antigenic drift is caused by point mutations and it is defined as the minor gradual antigenic changes in the HA or NA protein. Influenza displays a high mutation rate due to the error-prone nature of the viral polymerase, so mutant viruses are easy to isolate [7]. Mutations on the human virus HA or NA amino acid sequence occur at a frequency of less than 1% per year. Nonetheless, antigenic drift variants can cause epidemics and often prevail for 2–5 years before being replaced by a different variant [2]. The antigenic sites of HA are all located in HA1 at or near the top of the molecule and mostly are found in protein loops. Similarly, antigenic drift has been found for NA and the sites of antigenic drift mapped to specific regions of the NA atomic structure [2].
An important mutation that may occur in some IV strains is the H275Y mutation on the NA protein which confers to the virus Oseltamivir resistance (OsR).

Antigenic shift, as a consequence of antigenic shift, IV develops a "reassortment" which is the switching of individual viral RNA gene segments during mixed infections with different IVs. Viruses resulting from such genetic exchanges are called reassortant viruses. Although reassortment occurs for influenza A, B, and C viruses, it does not occur among the different types [2,7]. Antigenic shift leads to high infection rates in an immunologically naïve population and is the cause of influenza pandemics through the introduction of a new human virus [2]. The emergence of new pandemic strains of influenza A virus usually results from such a reassortment. There is a great deal of evidence for the reassortment of RNA segments between human and animal viruses *in vivo* and among human viruses in nature [2].

iv. Host receptors

One of the important factors conducting the tissue or cellular tropism of the virus is the specific binding of its surface glycoprotein HA to sialic acid (SA) receptors on the target cell surface [4, 14]. HA binds to SA possessing either α2,3- or α2,6-linkage to galactose. Interestingly, receptor preferences are thought to define host range [15]. For example, in avian species α2,3-linked receptors are most abundant in the digestive tract, the primary site of influenza infection in avians, in human both α2,6- and α2,3- linked SA can be found in cells within the respiratory tract, but in different locations: α2,6-linked SA are preferentially expressed in cells in the human upper respiratory tract, whereas α2,3-linked receptors are found in cells deeper in the lungs [16]. Ferrets develop

similar clinical signs than human after infection with influenza virus, likely in part because the distribution of α2,6- and α2,3-linked SA receptors in the ferret respiratory tract resembles that observed in humans [17,18]. Recently, it has been shown that α2,6-linked SA receptors are more abundant than α2,3-linked receptors throughout the ferret respiratory tract [19].

Moreover, the presence of α2,6- and α2,3-linked SA receptors in swine tracheal epithelial cells allows transmission of both avian and human viruses to pigs [20]. This supports the hypothesis that these animals can serve as a mixing vessel for the reassortment of novel influenza A viruses and their subsequent transmission to humans.

III. Immune response to Influenza infection

When a infectious pathogen as influenza virus invade a host, the immune system response with the innate immune system that provides an immediate, but non-specific response followed by and adaptive immune response. On a second encounter, the immune system improved its recognition of the pathogen and adapts its response to defence the host against the pathogen. Both, innate and adaptive immune responses will be described in more detail on the following pages, a schematic picture adapted from Crisci et al. 2012 [21] describe both immune responses in Figure 2.

i. Innate immunity

The innate immune system forms the first line of defense against influenza virus infection. It consists of components (e.g. mucus and collectins) that aim to prevent infection of respiratory epithelial cells. In addition, rapid innate cellular immune responses are induced that aim at controlling virus replication [22].

Influenza A virus infection is sensed by infected cells via pattern-recognition receptors (PRRs) that recognize viral RNA, the main pathogen-associated marker pattern (PAMP) of influenza A viruses. The PRRs are toll like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) and the NOD-like receptor family pryin domain containing 3 (NLRP3) protein [23]. TLR7 binds single-stranded viral RNA (especially in plasmacytoid dendritic cells) and TLR3 and RIG-I bind double-stranded viral RNA (in most other infected

cells). Studies performed in mice showed that signalling of these receptors leads to production of proinflammatory cytokines and type I interferons [24].

ii. Cells involved on innate immune response to influenza virus infection

Alveolar macrophages (Mϕ)

Once macrophages become activated in the lungs during IV infection, they phagocytose (apoptotic) influenza virus-infected cells to limit viral load [25,26]. Activated macrophages also spread Nitric Oxide Synthase 2 (NOS2) and Tumor Necrosis Factor alpha (TNF-α) two molecules that have been identified as contributors to influenza virus induced pathology [27]. These two distinct and competing functions of alveolar macrophages in the immune response against influenza virus infection emphasize the importance of a balanced response.

Dendritic cells (DC)

Dendritic cells (DC) play an important role as professional antigen-presenting cells during an influenza infection. The conventional DCs (cDCs), monitor the airway epithelial lumen to detect and opsonise (neutralized) virions and apoptotic bodies from infected cells but can also be infected themselves [22]. Mice experiments demonstrated that DC present influenza virus derived antigens, the immuno-peptides (epitopes), by Major histocompatibility complex (MHC) class I or class II molecules to activate a T cells response [28].

Other DC subtype that has been investigated are the plasmacytoid DCs (pDCs), highly specialized in sensing viruses cell that readily secreting Interferon alpha (IFN-α) after exposure to swine IV (swIV) [29].

Natural killer cells (NK)

NK are important effector cells of the innate immune response. They can recognize antibody-bound influenza virus infected cells and lyse these cells, a process called antibody dependent cell cytotoxicity (ADCC). These cells can recognize influenza virus-infected cells with their cytotoxicity receptors (NCR) NKp44 and NKp46. Upon binding to the IV HAs the receptors trigger the human NK cell to lyse the infected cell [30].

γδ T cells

Myeloid cells such as monocytes, Mϕ, neutrophils, and myeloid DCs, clearly display innate characteristics, while lymphoid lineage B and αβ T cells represent the classical adaptive response. γδ T cells, however, display characteristics of both. γδ T cells, while sharing αβ T cell functions, also perform immune surveillance of an innate character and are the only major set of tissue-resident T cells [31]

The antiviral activities of γδ T cells have been demonstrated in different models [32]. In the mouse model, γδ T cells were shown to contribute to recovery from influenza pneumonia [33], but no data are available on the contribution of γδ T cells at early stages of influenza virus infections. Activated mouse γδ T cells showed profound cytotoxicity against hemagglutinin (H1 or H3) expressing target cells in a non–major histocompatibility complex–restricted manner [34]. In human, γδ T cells could efficiently kill macrophages infected with human (H1N1) and avian (H9N2 and H5N1) demonstrating the antiviral activity and the capacity of this cells to inhibit virus replication against influenza A viruses [35].

iii. Adaptive immune system

The adaptive immune system forms the second line of defense against influenza virus infection. It consists of humoral and cellular immunity mediated by virus-specific antibodies and T cells respectively (Figure 2).

iv. Humoral immunity

IV infection induces virus-specific antibody responses[36]. Presences of specific antibodies that recognize HA and NA have been correlated with protective immunity when the efficacy of current human influenza vaccines was tested [37]. The HA-specific antibodies provide protection when they are in direct correlation with the virus that causes the infection. HA-specific antibodies are capable to neutralize the virus by binding to the HA, inhibiting virus attachment and entry in the host cell of elderly and adults vaccinated individuals [38]. Also, antibodies to the NA have protective potential. By binding NA, antibodies do not directly neutralize the virus but they inhibit enzymatic activity that finally limits virus spread. Furthermore, NA-specific antibodies also facilitate ADCC and also may contribute to clearance of mice virus-infected cells [39]. NP is an important target for protective T cells. Also, NP-specific antibodies in mice and human may contribute to protection against influenza virus infection [40]. The leading antibody isotypes in the influenza specific humoral immune response are IgA, IgM and IgG. Mucosal or secretory IgA antibodies are produced locally and transported along the mucus of the respiratory tract. They can afford local protection from infection in airway epithelial cells. These antibodies are also able to *in vitro* neutralize IV intracellularly [41]. Serum IgAs are produced rapidly after IV infection in human patients and the presence of these antibodies is indicative of a recent IV infection [42]. Serum antibodies of the IgG subtype predominantly transudate into the respiratory tract and afford long-lived protection on IV infected children [43]. In mice, IgM antibodies initiate complement mediated neutralization of influenza virus and are a hallmark of primary infection [44].

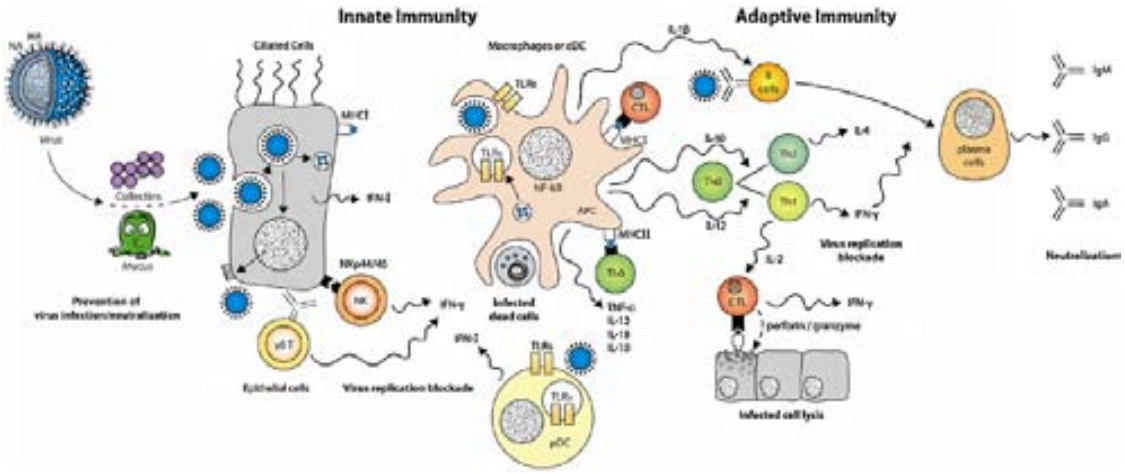


Figure 2. Immunity during influenza virus infection. The innate immune system forms the first line of defence against influenza infection. In the course of the innate immune response, cells like macrophages, dendritic cells, natural killer and γδT cells are recruited with the objective of controlling and blocking virus replication and dissemination. These cells secrete different types of chemical mediators such as cytokines that will activate the T cells and induces their differentiation or elicit an adaptive response with the production of specific IV-antibodies responses. Adapted from Crisci et al., 2013 [21].

v. Cellular immunity

Upon infection with IV, CD4+ T cells, CD8+ T cells and regulatory T cells (Tregs) are induced. CD4+ T cells play an important role in the immune response to this pathogen through the secretion of antiviral cytokines, and by providing help to CD8+T cells and B cells by promoting the development of immunological response of mice [45,46]. CD4+ T cells also participate directly in viral clearance through the secretion of antiviral cytokines [45,47]. CD4+ T cells are activated after recognizing virus-derived MHC class II-associated peptides on Antigen presenting cells (APC) that also express co-stimulatory molecules. In human immunodeficiency virus (HIV) infected patients, it was observed that some CD4+ T cells display cytolytic activity to infected cells [48]. However, the most important phenotype of these cells is T helper (Th) cells. Naive CD4+ cells can differentiate into T helper 1 (Th1) cells that are characterized by the production of Interferon gamma (IFN-γ), Interleukin 2 (IL-2) and TNF-α. Alternatively, antigen signalling in the presence of IL-4 induces the naive CD4+ cell population to develop into Th2 effectors secreting IL-4, IL-5 and IL-13[49]. Viral infections are known to predominantly induce Th1 or Type 1 immunity that promotes the activation of CD8+ T

cells and macrophage functions and drives B cell differentiation. In addition, regulatory T cells (Tregs) and T helper 17 (Th17) cells have been identified that regulate the cellular immune response to influenza virus infection. In contrast, in an inflammatory environment, Th17 cells improve T helper responses by producing IL-6 which inhibits Treg function [50].

On the other hand, cytotoxic CD8+ T cells move to respiratory sites where virus replication is localized to eliminate infected cells. The main function of virus-specific CD8+ T cells is that of cytotoxic T lymphocytes (CTL). Upon influenza virus infection these cells are activated in the lymphoid tissues and recruited to the site of infection. There, they recognize and eliminate influenza virus infected cells and thus prevent production of progeny virus. Their lytic activity is mediated by the release of perforin and granzymes (Gr) (e.g. GrA and GrB). Perforin permeabilizes the membrane of the infected cells and subsequently Grs enter the cell and induce apoptosis. Recently, in human and mice experiments it was shown that even in the absence of GrA and GrB influenza virus-specific CTL were able to lyse target cells in vivo [51]. Furthermore, CTL produce cytokines that improve antigen-presentation by stimulating MHC expression and that display antiviral activity. Post-infection virus-specific CTL are found in the lymphoid organs and in circulation. The reactivity and affinity of the memory CTL during a secondary infection depends on the co-stimulation they received during their initial differentiation phase [52]. Human CTL induced by IV infection are mainly directed against NP, M1 and PA proteins [53,54]. These proteins are highly conserved and therefore CTL responses display a high degree of cross-reactivity, even between different subtypes of influenza A virus.

IV. Pathogenesis and clinical signs during Influenza Infection

Influenza in adults and adolescents typically presents with an abrupt onset of fever and chills, accompanied by headache and sore throat, myalgias, malaise, anorexia, and a dry cough. Fever (38–40°C) peaks within 24 h of onset and lasts 1–5 days. Physical signs include the appearance of being unwell, hot and moist skin, flushed face, injected eyes, hyperaemic mucous membranes, and a clear nasal discharge. Although several of the symptoms of influenza are common to all age-groups, a review of published reports of influenza in children, adults, and elderly adults shows that the proportion of patients in whom these complaints are noted varies by age [55].

i. Genes involved on influenza A pathogenicity and virulence

HA, plays a critical role in adaptation to certain hosts through its affinity for receptors differentially expressed between species [15,20]. HA receptor preference appears to affect transmission by controlling the anatomical site of viral replication. The presence of HA is initially expressed as a precursor of HA0 and then cleaved into HA1 and HA2, forming a disulfide bond-linked complex. Structural data show that a loop structure exists in the cleavage site between HA1 and HA2, and this flexible loop is crucial for the efficient cleavage of HA0 [56]. Cleavage susceptibility of HA0 correlates well with the pathogenicity of highly pathogenic avian influenza (HPAI) viruses in poultry [57]. In mice, the multi-basic cleavage site was required for H5N1 virulence and viral spread to the mouse brain following intranasal infection [58].

PB2, numerous substitutions within the PB2 subunit have been shown to alter host range and virulence. H5N1 viruses with a PB2 E627K mutation cause a lethal, systemic infection in mice, but become nonpathogenic for mammals if this residue remains a glutamic acid [58]. A PB2 K627E mutation reduces transmission of human IVs in the guinea pig, presumably because of reduced replicative ability in the upper respiratory tract [59]. A PB2 D701N mutation is also associated with increased influenza virus virulence in mammals by increases viral replication [60]. Interestingly, pdmH1N1 2009 have neither the PB2 627K nor the 701N mutations that are associated with high pathogenicity, although second-site compensatory mutations in PB2 (590S and 591R) have been identified [61].

PB1-F2, various studies have suggested that this protein plays an important role in virulence of primary IV infection and in promoting secondary bacterial infection [62]. PB1-F2 was shown to contribute to the pathogenicity of the 1918, 1957, and 1968 pandemic strains, as well H5N1 HPAI viruses [63,64]. When an S66N mutation was incorporated into the PB1-F2 proteins of H5N1 and 1918 viruses, they became attenuated in mice [63]. In addition, viruses with an N66S mutation caused increased disease severity, lung titers, and cytokine production in mice [62]. Interestingly, pdmH1N1 2009 express only a truncated, 11–amino acid PB1-F2 protein, although introduction of PB1- F2, either with 66N or 66S, into recombinant pdmH1N1 2009 virus did not substantially enhance its virulence in mice or ferrets or predispose mice to secondary bacterial infection with Streptococcus pneumonia [65].

NS1, prevents activation of transcription factors that induce IFN-β by blocking recognition of influenza PAMPs through RIG-I [65,66]. The NS1 proteins of H5N1 HPAI viruses are associated with the induction of proinflammatory cytokines in the infected host [66]. Likewise, an H5N1 P42S change results in a substantial increase in virulence in the mouse model and reduced levels of IFN-αβ production *in vitro* [67]. L103F and I106M mutations in the NS1 of H5N1 viruses, which increase NS1 binding to the cellular pre-mRNA processing protein cleavage and increase in vitro viral replication, presumably by suppressing expression of IFN-α/β mRNAs [68].

NA, optimal influenza virus replication requires a functional balance between HA sialic acid binding affinity and receptor destroying, enzymatic activity of NA [69]. This balance can be perturbed by a number of events, such as reassortment, introduction into a novel host, and antiviral therapy. The earliest human isolates of the 1957 H2N2 pandemic viruses paired an NA with preference for α2,3-linked substrates with an HA that bound well to α2,6- SA receptors. Over the years, this N2 gradually acquired the ability to cleave both α2,3 and α2,6 linkages, adapting to meet the receptor specificity of the HA [70]. This likely provided a selective advantage by allowing progeny virions to be released more efficiently from the cell surface. Antiviral drugs can also influence the adaptation of influenza viruses.

V. Influenza Pandemics

Pandemics are rare events that occur every 10–50 years and cause a colossal loss of human lives. The lack of experience of the human immune system to identified and solved an influenza infection from a newly strain, could turn a seasonal IV in a pandemic virus with the efficient and sustained ability to be transmitted human-to-human and finally, spreading globally. Over the human health history, historical record have been preserved as an evidence as how a pandemic of influenza could affect human population not only due to the high morbidity and mortality of the disease, but also for the high societal costs reflected on absenteeism, reduction and even paralysis of many sectors as schools or businesses, saturation of health services by the large number of patients that need medical attention and in the worst, loss of a family`s primary breadwinner.

It is impossible to know with certainty the first time an IV infected humans or when the first influenza pandemic occurred. However, many historians have speculated that the year 1510 a.d. (500 years ago) marks the first recognition of pandemic influenza while there are historical records describing a pandemic outbreak of influenza-like disease in Europe. Mentioned influenza-like disease was characterized by a "gasping oppression" with cough, fever, and a sensation of constriction of the heart and lungs began to spread, apparently everywhere [71].

i. Pandemics in the XX century

During the last XX century, three pandemics of influenza affected the human population. The involved influenza subtypes were the followings: H1N1 (1918-1919), H2N2 (1957-1963) and H3N2 (1968-1969) [1]. Each subtype was originated as a consequence of reassortment. In order of appearance, the first one was the so called "Spanish" influenza in 1918 and 1919, with an HA related to those of swine viruses or H1 subtype viruses. Viruses of this subtype circulated until 1957, when viruses of the H2N2 subtype (Asian strains) were isolated. The H2 subtype HA has little or no cross-reactivity with the H1 HA. In addition to containing an H2 HA, the Asian strains had a new NA (N2). For 11 years, the H2N2 strains of influenza virus spread and changed

until the next pandemic in 1968 with the introduction of a new H3 subtype (Hong Kong strains). These drastic antigenic changes came about from the reassortment of previously circulating human viruses and IVs of animal origin.

The H1N1pandemic of 1918–1919 "Spanish influenza": on 1918 the world witnessed the worst IV outbreak in recorded history; the named "Spanish influenza" ("Spanish Flu"), publicity and sanitary strategies to try to control dissemination could not stop virus spread (Figure 3). The virus was spread all over the global population in three waves between 1918 and 1919 carrying 20 million to 50 million of human lives [72]. It was especially dangerous to young adults [73] with unusually high numbers of deaths in young and healthy people aged 15 to 35 years [74]. It has been estimated that about 25 per cent of the world's population was infected. During this period, development of "Spanish Flu" spread was strongly influenced by the First World War (1914-1918). In fact, global dissemination and severity were directly linked to the war and the movement of troops [75]. Studies focusing on the HA protein have found that the HA gene contributed to efficient viral replication and high virulence of the 1918 virus in mice [26,76]. Recently, the genome of the 1918 pandemic IV was completely sequenced [77], and the virus was reconstructed using reverse genetics [26]. The reconstructed 1918 virus caused a highly pathogenic respiratory infection in mice [26] and macaque models that culminated in acute respiratory distress and a fatal outcome [78]. It was shown that mice vaccinated with the monovalent pdmH1N1 2009 vaccine were completely protected in a lethal challenge model with the 1918 influenza virus. Because the 2009 pandemic H1N1 virus contains the HA gene derived from the classical swH1N1 lineage, it is antigenically very similar not only to classical swH1N1 viruses, but also to the 1918 virus [73]. Also, ferrets immunised with DNA vaccines encoding proteins of the original 1918 H1N1 pandemic virus exhibited protective cross-reactive immune responses against infection with a 1947 H1N1 virus and a recent 1999 H1N1 virus [79]. Consequently, seroepidemiologic studies had demonstrated cross-protective immunity in the population, primarily in people >60 years [80].

Figure 3. The Mysterious Stranger: a cartoon in the *Dallas News* during the 1918–1919 influenza pandemic. Source: How to fight Spanish influenza. Literary Digest 1918 Oct 12;59:13. The cartoon is attributed to Knott of the *Dallas News* [81].

The H2N2 pandemic of 1957-1963 "Asian influenza": it was named after the first identification in Guizhou, a province in south-central China. Investigations to elucidate the origin of the Asian circulation strain, placed the novel pandemic H2N2 as an avian-human reassortant. Unlike "Spanish Flu", the principal target of this virus was centred on the elderly population but also on infants, with about 1 to 2 million of deaths worldwide [82]. Although the proportion of people infected was high, the illness was relatively mild compared to the Spanish flu. In December of 1957 when it was believed controlled, a second wave struck at the beginning of 1959, to suddenly disappear given rise to the next pandemic [83,84]. H2N2 stopped circulating in the human population in 1968. However, strains of H2 subtype still continue to circulate in birds and occasionally in pigs and they could be reintroduced into the human population through antigenic drift or shift.

The H3N2 pandemic of 1968 "Hong Kong influenza": the last pandemic of the XX century was a new of Asian origin. Influenza A viruses of the H3 subtype caused the 1968 Hong Kong pandemic, the HA gene being introduced into humans following a reassortment event with an avian virus [85]. The pandemic began in Hong Kong and deaths hovered all over the world. It is believed that 1 to 2 million of people died [83]. Since 1968, H3N2 has been one of the most prevalent seasonal influenza virus circulating in human and swine population [86]. In a cross-reactive immunity experiment, it was demonstrated that a DNA vaccine, based on the HA and NA of the 1968 H3N2 pandemic virus, induced cross-reactive immune responses against a recent 2005 H3N2 virus challenge in ferrets [79].

ii. 2009 Swine influenza pandemic: the first pandemic of XXI century.

The first pandemic of the XXI century have been originated by an swine-origin influenza A H1N1 virus, characterized by a novel combination of gene segments "triple reassortant", that had not been identified among human or swIV. As mentioned before, pigs are considered logical candidates for reassortment because they can be infected by either human or avian viruses as they possess both SA receptor (SAα2,6 and SAα2,3) in the cells of the respiratory system [20]. In addition, pigs are known to be involved more frequently in interspecies transmission of influenza A viruses than other animals [87].

iii. Origin of pandemic A (H1N1) 2009 virus

On April 15 and April 17, 2009, the first two human cases of influenza caused by a new influenza strain were confirmed. A 10-year-old children from southern California was the first infected identified; two days later, the Centers for Disease Control and Prevention (CDC) confirmed a second case of infection with the same virus in a 9-year-old girl from an adjacent county in California [88]. During the subsequent 2 weeks, additional cases of infection with this new virus were detected in Mexico, California, Texas, and other states [88]. That unique combination of gen segments had not been

previously indentified. The CDC distributed information confirmed that these cases were caused by the same new swine strain of influenza A (H1N1) virus, also CDC described that the generation of the circulating strain derived from a triple reassortment of human, swine and avian viruses [89]. Phylogenetic analyses of pdmH1N1 2009 virus isolates revealed a great homogeneity of genomic sequences. The virus was antigenically distinct from human seasonal influenza viruses but genetically related to three viruses that circulated in pigs. The pdmH1N1 2009 virus has therefore inherited virus gene segments of all three sources: swine, human and avian origin [90,91]. CDC released the genomic sequences of vRNAs from 6 swine flu isolates from California and Texas on 29 April 2009 [88]. The samples of the infected patients were genetically analysed and the NA (N1) and M genes of the pdmH1N1 2009 virus were shown to have different origins, from the "avian-like" Eurasian swine H1N1 lineage, which emerged in Europe in 1979 after reassortment between a classical swine and an avian H1N1 virus [92]. The virus then spread through Europe and Asia [93], displacing the classical swine H1N1 virus from Europe and generating new reassortants in swine with different influenza A viruses of human origin [94]. Finally, the PA, PB1 and PB2 genes of the pdmH1N1 2009 virus are from the North American H3N2 "triple-reassortant" lineage, which was first isolated from pigs in America in 1998 in which it showed unusual pathogenicity [95]. The pdmH1N1 2009 virus has therefore inherited virus gene segments of all three sources: swine, human and avian origin (Figure 4).

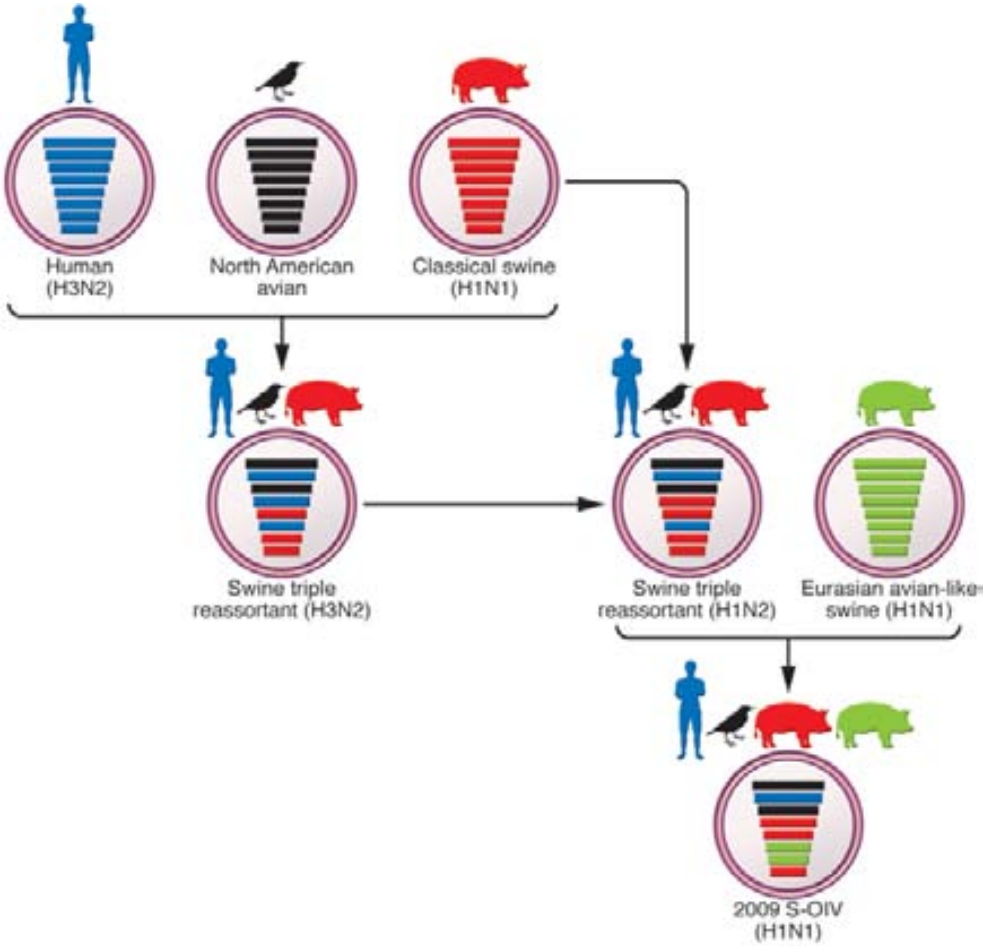


Figure 4. Diagrammatic representation of the evolutionary history of pdmH1N1virus. Reassortment of North American swine H3N2 and H1N2 triple-reassortant viruses (of North American avian, human [H3N2], and classical swine [H1N1] origin) with Eurasian avian-like swine viruses (H1N1) resulted in the pdmH1N1 2009 virus. Each gene segment of avian, human, or swine origin corresponds to a characteristic feature on the surface of the schematic viral particle. Adapted from Tscherne et al [96].

iv. Epidemiology of A (H1N1) 2009 pandemic

The emergence of the pdmH1N1 2009 influenza virus in humans in early April came as a total surprise. The pdmH1N1 2009 strain quickly spread worldwide through human-to-human transmission. On December 30th, 2009 the number of countries that reported laboratory-confirmed pdmH1N1 2009 virus cases in humans was 208 and more than 214 on April 18th, 2010 [97]. Studies have shown that pdmH1N1 2009 virus was circulating in the environment three months prior to the outbreak [98].

The transmissibility of the pdmH1N1 2009 virus in house of infected patients was lower than that seen in past pandemics [99]. The mean time between the onset of symptoms in a patient case and the onset of symptoms in the house contact infected by that patient was 2.6 days (2.2–3.5). A characteristic feature of the pdmH1N1 2009 was that it disproportionately affected children and young adults as compared to the older age groups [100]. In most countries, the majority of pdmH1N1 2009 virus cases have occurred in younger age groups, with the median age estimated to be 12–17 years in Canada, USA, Chile, Japan and the UK. On 2009, of the 272 patients with pdmH1N1 2009 virus infection who were hospitalized in the USA in a three month period, 45% were under the age of 18 years, whereas only 5% were 65 years of age or older [101].

This age distribution suggested partial immunity to the virus in older population [102]. This hypothesis was supported by subsequent studies which showed that 33% of humans over 60 years of age had cross-reacting antibodies to pdmH1N1 2009 virus by hemagglutination-inhibition test and neutralization tests [88,92,103].

It should be noted that while the highest rate of severe disease leading to hospitalization has been in patients less than 5 years of age, the highest case fatality rate was recorded in the 50–60-year old population [97]. More than 3 years after the emergence of the 2009 pdmH1N1 virus, the associated global mortality remains unclear. Of 18.500 laboratory-confirmed pdmH1N1 2009 virus-associated deaths identified during April, 2009, to April, 2010 worldwide, less than 12% were reported from Africa and southeast Asia, although these regions are home to more than 38% of the world's population [104]. Spain was one of the first European countries to inform pandemic influenza cases[105]. Nowadays, epidemiological survilance of the pdmH1N1 2009 virus indicated that we are in a postpandemic period which does not mean that the pdmH1N1 2009 virus has gone away, in fact this pathogen is still on circulation.

v. Virulence markers pdmH1N1 2009 virus

As well as the pathogenicity, the virulence of the IVs can be measured using parameters of morbidity and mortality within animal models. IV pathogenicity is considered multigenic and it is determined by the variety of genes within a particular IV strain within a specific host [54,91]. Genetic mutations in IV proteins, including HA and NA [54], NS1 [106] and PB1-F2 [63], and the polymerase complex, occur during viral host adaptation and result in enhanced virulence. The particular genetic mutations related to specific characteristics can enhance various aspects of the viral life cycle, including virus binding and entry, genome transcription and translation, virion assembly and release, and evasion of innate immune responses have been identified (Table 1) [96].

		Pathogenicity determinant	
Protein	Function	High	Low
PB2	Polymerase cofactor, binds most mRNA caps	627K	627E
PB2	Polymerase cofactor, binds most mRNA caps	701N	701D
PB1-F2	Proapoptotic	66S	66N
HA	Membrane glycoprotein, binding, and fusogenic functions	Multi-basic cleavage site	Single-basic cleavage site
NA	Membrane glycoprotein, sialidase	274Y	274H
NS1	Evasion of host immune response	92E	92D
NS1	Evasion of host immune response	C-terminal E-S-E-V motif	C-terminal deletion

Table 1. Influenza genes involved on pathogenicity and virulence. Adapted from Tscherne et al, 2011 [96].

vi. Pathogenesis and clinical signs during pdmH1N12009 infection

In general terms, pdmH1N1 2009 virus infection is mostly a mild, self-limiting upper respiratory tract illness with (or for some patient groups, without) fever, cough and sore throat, myalgia, malaise, chills, rhinorrhea, conjunctivitis, headache and shortness of breath. Up to 50% of patients present with gastrointestinal symptoms including diarrhea and vomiting. The spectrum of clinical presentation varies from asymptomatic cases to

primary viral pneumonia resulting in respiratory failure, acute respiratory distress, multi-organ failure and death [101].

The pdmH1N1 2009 virus replicates in the cells of the upper and lower respiratory tract, the incubation period appears to range from 2 to 7 days, but most patients probably shed virus from day 1 before the onset of symptoms through 5–7 days after [107]. The median period during which the virus could be detected with the use of real time polymerase chain reaction (RT-PCR) in quarantined patients was 6 days (range 1–17), whether or not fever was present [108].

vii. Clinical risk factors of pdmH1N1 2009 virus infection

Influenza infection in immunocompromised hosts may prolong the illness longer than normal, and the virus may replicate for an extended period of time. Approximately, one quarter to one half of patients with pdmH1N1 2009 virus infection who were hospitalized or died had no reported coexisting medical conditions [109,110]. Underlying conditions that are associated with complications from seasonal influenza also are risk factors for complications from pdmH1N1 2009 virus infection. Chronic obstructive pulmonary disease (COPD), asthma, cardiovascular disease, hypertension and diabetes have been reported as risk factors for critical illness following infection with the pdmH1N1 2009 virus [101,111,112]. In addition, during the 2009 pandemic, pregnancy [113,114] obesity [114,115] and smoking [111] were identified as risk factors for severity. Therefore, basal immune alterations and/or the presence of a previous pro-inflammatory state favoured by the presence of medical conditions may impact the normal development of specific immune responses against the pdmH1N1 2009 virus, increasing the risk of developing severe forms of the infection [116]. The patients in the high risk groups need to be cared more and treated at priority as compared to low risk groups to prevent the loss of life.

Pregnancy: It is linked to a number of induced changes in the immune system of the mother, aimed at tolerating the fetus. In pregnant women, the balance between pro- and anti-inflammatory factors seems to be crucial [117]. In addition, changes in the peripheral levels of immune mediators such as Interferon delta (IFN-δ), TNF, Vascular endothelial growth factor (VEGF), Granulocyte colony-stimulating factor (G-CSF), eotaxin, and Monocyte chemoattractant protein-1(MCP-1) may impact the proper performance of the immune response [118]. Although these changes in the immune system are not fully understood, it is believed that they may increase the severity of some infections [117,119]. Previous 1918 and 1957 pandemics reported as well as the risk to the pregnant woman, the risks to the fetus; increased rates of miscarriage, stillbirth, and premature birth [3]. The increased mortality detected in pregnant female mice infected with the pdmH1N1virus was associated with increased infiltration of neutrophils and macrophages in the lungs of these animals. Also, pregnant mice showed higher levels of chemokines and pro-inflammatory cytokines, lower respiratory epithelial regeneration and poorer fetal development than nonpregnant mice [120,121]. Although pregnant women represent only 1 to 2% of the population, among patients with pdmH1N1 2009 virus infection, they have accounted for up to 7 to 10% of hospitalized patients,[101] 6 to 9% of ICU patients, [111] and 6 to 10% of patients who died [122,123].

Pulmonary complications: Primary viral pneumonia is associated with a high mortality rate. It begins within 24 h of the onset of febrile illness with a dry cough that later becomes productive of bloody sputum accompanied by tachypnoea, diffuse fine rales, progressive cyanosis, and respiratory failure [3]. IVs can lead to an acute exacerbation of chronic bronchitis in people with chronic obstructive pulmonary disease or cystic fibrosis and to wheezing in patients with asthma [124]. Results of pdmH1N1 2009 virus experiments with cell cultures and mouse models have reported that a high level of cytokines could itself prevent the development of and appropriated immune response against viruses, affecting dendritic cell function along with HLA-II mediated antigen presentation[125,126]. In a mouse model of COPD, animals infected with influenza virus showed an exacerbated inflammatory response to infection [127]. In humans, previous studies demonstrated that chronic respiratory diseases such as COPD and asthma during a pdmH1N1 2009 influenza infection are characterized by a basal predisposition to the release of inflammatory mediators [128].

Metabolic complications: Among patients with severe or fatal cases pdmH1N1 2009 virus infection as severe obesity or morbid obesity has been reported at factors of higher pathogenicity than in the general population [111,122]. In addition to the risks associated with obesity, such as cardiovascular disease or diabetes, immunological alterations in the obese may contribute to its role as a risk factor in pdmH1N1 2009 infection [129]. Obese mice infected with the pdmH1N1 2009 virus exhibited significant higher morbidity and mortality compared to non-obese mice [73]. Adipocytes have structural similarities with the immune cells and perform certain functions related to them, such as the release of inflammatory mediators. Furthermore, differentiation of macrophages in the adipose tissue is conditioned by the metabolic environment and immune cells in turn are able to control lipid and glucose metabolism, suggesting an immune metabolic axis. Then, a chronic caloric excess could interfere with the mechanisms of the immune response [130].

viii. Immunopathogenesis during pdmH1N1 2009 influenza infection

The immune system is designed to protect and maintain homeostasis and the ability of an organism to adapt to the environment. Therefore, it plays a key role in viral clearance, as explained in the section "Immune response to Influenza infection" (section III). Human autopsy studies of pdmH1N1 2009 infected patients have pointed out the contribution of excessive acute inflammatory responses to death following severe influenza infection, including influx of innate cells into the lungs and overproduction of cytokines (Figure 5) and chemokines that culminate in life-threatening pulmonary immunopathology [131].

Hypercytokinemia: a first report published in December 2009 revealed that severe disease caused by the pdmH1N1 2009 virus was characterized by the presence of high systemic levels of cytokines, chemokines and other immune mediators from the early stages of the disease [125,132,133]. Infection by the influenza pdmH1N1 2009 virus induced the secretion of antiviral defense-related chemokines (interferon gamma-induced protein 10 (IP-10)), macrophage inflammatory proteins 1 beta (MIP-1β), MCP-1 and IL-8). These chemotactic molecules mobilize T lymphocytes, monocytes, macrophages and neutrophils to the site of infection to fight the infection [134]. However, an accumulation of these cells may contribute to inflammatory-mediated damage to the infected tissue. Infected patients also exhibited elevated levels of other pro-inflammatory immune mediators that stimulate T Th1, IFN-δ, TNF-α, IL-15, IL-12p70. On the other hand, Th1 cytokines may, as chemokines, contribute to tissue injury. Studies on cytokine profiles also revealed elevation of two Th17 related cytokines (IL-9, IL-6) in the early course of the severe cases of pneumonia caused by pdmH1N1 2009 virus. However, a beneficial role of IL-17 in lethal influenza has been previously proposed [132,133]. Additionally, G-CSF, which has been described as interfering with the synthesis of IL-17 [135] has been reported to be directly associated with the risk of death in critically ill patients.

Regarding IL-6, there is a fairly broad consensus in the literature that this cytokine could be a potential biomarker for severe pdmH1N1 2009 infection, in both human and in mouse studies [125,132,136,137]. Elevated systemic levels of IL-6 were strongly associated with ICU admission and with fatal outcomes. Furthermore, in animal and clinical studies, global gene expression analysis indicated a pronounced IL-6-associated inflammatory response [137,138]. In addition, IL-6 has been implicated in the cytokine storm following avian influenza A H5N1 and in severe acute respiratory syndrome infection [139]. It has also been associated with severe cases of seasonal influenza infection [140]. Cases of severe pandemic influenza disease were also marked by high levels of two immunomodulatory cytokines (IL-10 and IL-1ra).

Hypercytokinemia persisted in the most severe cases, which could have perpetuated the inflammatory damage and, in consequence, the respiratory failure observed in these patients (Figure 5) [133,138]. Similarly, other clinical studies demonstrated that high plasma levels of IL-6, IL-8 and MCP-1 correlated with the extent and progression of pneumonia [141]. The most severe cases also showed persistent viral shedding, again indicating poor control of viral replication.

Antibodies reponse: one of the most striking features of the 2009 pandemic was the low proportion of elderly individuals infected by the new virus, compared to seasonal influenza [101,111,112]. In addition, severe illness caused by the new variant predominated in young patients, with 90% of deaths occurring in patients <65 year old [136,142] which is contrary to the normal trend in seasonal influenza. It is believed that adults born after 1956 have suffered previous exposures to antigenically related influenza viruses, developing in consequence cross-reactive antibodies with the ability to recognize the 2009 strain [80,143]. Studies on antibody prevalence show the presence of cross-reacting antibodies in as much as in 33% of the over-60 population [144]. This result is consistent with the fact that young adults admitted to the ICU during the 2009 pandemic lacked protective antibodies in the early stages of the disease, as revealed by hemagglutination inhibition (HAI) and micro-neutralization assays [132,138]. However, the absence of early HAI and neutralization activity was the rule in young patients, independent of disease severity and outcome. It is important to note that most of these critical patients were able to mount specific antibody responses against the pandemic virus, regardless of severity [138]. This suggests that factors other than the development of specific antibodies contribute to the pathogenesis of severe pandemic influenza.

Cellular immune responses: cross-reactive T CD4+ Th lymphocytes and T CD8+ CTLs established by vaccination campaigns or natural infection by the seasonal influenza A virus have been reported to contribute to clearance of the pdmH1N1 virus from the lungs[145,146]. Even in the absence of protective antibody responses, individuals vaccinated against seasonal influenza A may still benefit from pre-existing cross-reactive memory CD4+ T cells thus reducing their susceptibility to the influenza pdmH1N1 2009 virus [146]. T CD4+ effector cells are essential for virus clearance, but in turn, they may contribute to the hypercytokinemia observed in the most severe cases caused by influenza pdmH1N1 2009 virus infection. In fact, a study in a murine model demonstrated that depletion of T cells prevented immunopathology, although with decreased viral clearance [147]. In turn, CD8+ T cells are known to release cytotoxic molecules (granzyme and perforin) and antiviral cytokines (TNF-α and IFN-δ), which are essential for mediating the elimination of infected cells. A small report on human autopsy tissues documented diffuse alveolar damage, haemorrhage and necrotizing

bronchiolitis in the lungs of patients who died from influenza pdmH1N1 2009 virus infection. Immunohistological examination revealed an aberrant immune response associated with marked expression of TLR3 and IFN-δ and a large number of CD8+ T cells and Gr B+ cells within the lung tissue [148], highlighting the role of cellular immune responses in the immunopathology of pdmH1N12009 influenza infection. T cells from influenza pdmH1N1 2009 infected patients presenting with a severe clinical course have been described as resulting in impaired effector cell differentiation and as failing to respond to mitogenic stimulation [126]. In addition, T cell anergy is observed during the severe acute phase of the infection [126]. The adaptive immune response of pdmH1N1 2009 virus infected patients has been reported to be characterized by decreases of CD4- lymphocytes and of B-lymphocytes and by increases in T-regulatory lymphocytes [149]. The latter cells may suppress the development of specific responses against the virus. Critical pandemic influenza illnesses coursed with lower expression in the white blood cells (WBC) of a group of genes key to the development of antigen presentation and adaptive immune response [138]. Deficiencies in the cellular immunity occurred in severe cases of influenza pdmH1N1 2009 infection could help explain the poor control of the virus observed in these patients, and the increased risk of these patients to suffer from bacterial over-infections.

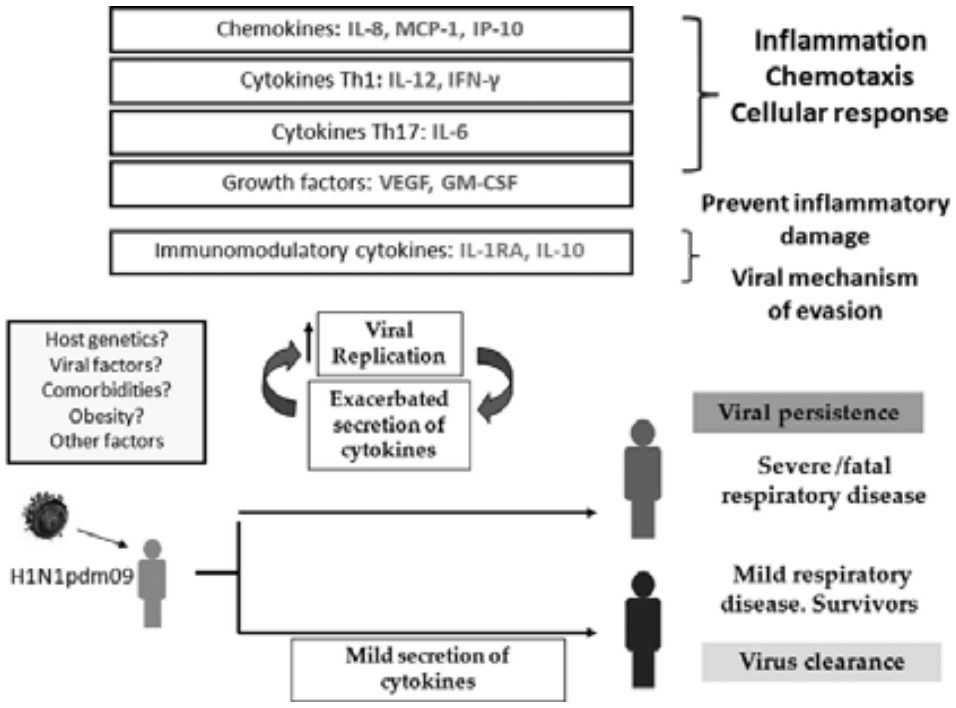


Figure 5. Hypercytokinemia as a host response signature in severe pandemic influenza. Adapted from Almansa et al, 2012 [116]

VI. Oseltamivir resistance (OsR)

There are two neuraminidase inhibitors (NAIs) licensed globally for the treatment and prevention of influenza, Relenza (zanamivir) was the first in this class [150] followed by Tamiflu (oseltamivir) [151]. Oseltamivir is one of the most common antiviral treatments used on influenza infection [152] and it is classified as NAI because it acts by inhibiting NA surface protein, which in turn reduces the ability of the virus to infect other respiratory cells [153]. When used for treatment of influenza, these drugs only reduce the duration of illness by a day or two, this may be highly significant (beneficial) in infections of high pathogenicity viruses [154]. Oseltamivir binds to the active site of NA present at the surface of an infected cell [155], preventing it from removing SA residues and causing virus aggregation. In clinical trials with oseltamivir, an NA H274Y resistance mutation in the background of a seasonal H1N1 virus was identified, but deemed clinically unimportant because of its expense on virus fitness [156]. Surprisingly, during the influenza season of 2007–2008, Oseltamivir resistance (OsR) H1N1 viruses with the H274Y mutation began to predominate in the circulating H1N1 population. It was observed that the antiviral treatment with oseltamivir was efficacious if initiated within 48 h of the onset of signs and symptoms, beeing the clinical cure rates higher [157]. Resistance to the NAIs in patients under treatment has been found to be relatively low. OsR emerged both in challenge studies and in naturally acquired infections, with resistant virus isolated from 1 to 4% of oseltamivir-treated adult patients [158]. The most commonly observed resistance neuraminidase mutation in OsR influenza viruses as described below, depend on the strain involved on the infection.

Seasonal H1N1: There was a minimal use of oseltamivir in Norway when in late 2007, several seasonal H1N1 viruses with an H274Y mutation were isolated from patients with none history of drug exposure. While H274Y is the primary mutation seen in N1 viruses, another example in a seasonal H1N1 virus is the I222V mutation that was also

detected in surveillance of community isolates from untreated patients [159]. This mutation conferred reduced susceptibility to oseltamivir.

Pandemic influenza A (H1N1): There are numerous reports of the emergence of resistant viruses among immunocompromised patients undergoing oseltamivir treatment or prophylaxis, which is not unexpected due to the longer periods of therapy [120,160]. There are also reports of transmission of resistant viruses in hospitalized settings among immunocompromised patients [161]. During the pandemic period, in addition to the H274Y mutation, both H1N1 viruses (seasonal and pandemic) had a common I222V (I223V in N1 numbering) mutation, suggesting possible human–human spread [88]. Also, a I222R mutations have been reported and the variants emerged in two immunocompromised patients, one treated sequentially with oseltamivir than zanamivir and a second treated with oseltamivir [162]. Besides, one virus had both I222R and H274Y mutations [163]. Oseltamivir has also been reported to have lower clinical efficacy in children infected with influenza B compared with influenza A [164].

Finally, like the seasonal and pdmH1N1 2009 virus strains, H274Y mutations have been seen in H5N1 isolates from infected patients treated with oseltamivir [165].

VII. Animal models for influenza studies

The ability of an IV to infect a specific host species is determined by the ability of the virus to attach to, replicate in, and release from cells of that specific host. Attachment to SA receptors is dependent upon the influenza HA, while internal viral proteins and various host factors are critical for viral replication. The NA functions in the release of infectious progeny virus through the removal of SA from the cell surface [166]. As mentioned before, receptor specificity is a primary determinant of viral attachment and infection of host cells. SA receptor distribution varies between species and among tissues and cell types within the same host [167].

There are many animal models that have been used to study the influenza virus infection as guinea pig, Syrian hamster, chinchilla, hedgehog, chicken, and rat [168]. However, experimental pathogenicity of the pdmH1N1 virus is generally reproduced in mice, ferrets and nonhuman primates [169]. Another interesting animal model proposed to be use for influenza study are pigs that shared immunological similarities with human [21,170,171].

Mice: mice are not a natural host of IVs. However, mice are among of the most commonly used mammalian models for evaluating influenza infection [166]. Mouse small size and low cost allow us to conduct studies on a larger scale. However, mice´s small size also increases the difficulty of readily observing the clinical signs of the disease [168]. Inasmuch as the receptor specificity to IV, commonly used laboratory strains of mice express both SAα2,6 and SAα2,3 receptors in their respiratory tract, making them susceptible to both human and avian influenza viruses. The specific distribution of these receptors is still being studied and may vary by mouse strain. Expression of both SAα2,6 and SAα2,3 receptors in multiple organs of BALB/c mice, including trachea, lungs, cerebellum, spleen, liver, and kidney was recently reported, while studies with C57BL/6J mice reported a lack of SAα2,6 expression in the lungs, but SAα2,3 receptors were demonstrated in ciliated airway and type II alveolar epithelial cells [172].
Pathogenicity patterns of pdmH1N1 2009 virus in mice showed a more efficient replication in the lungs of infected mice, generating earlier bronchitis and alveolitis, when compared with current seasonal strain. It also elicited markedly increased production of IL-10, IFN-γ, IL-4 and IL-5 [97].Genetic variations that exist between mouse strains in the many host genes involved in viral replication, and the innate and adaptive immune response, can greatly influence the outcome of the disease [173].

Ferrets: Ferrets are an attractive mammalian model for these studies owing to their relatively small size and the fact that they mimic numerous clinical features associated with human influenza disease. The high susceptibility of ferrets to influenza virus and the ability of sick ferrets to transmit virus to healthy animals was first demonstrated in 1933 by Smith et al [174] when they isolated influenza A virus from this animal model. From that time onwards, the use of the ferret model has been indispensable in experimental studies to elucidate virus-host interactions following IV infection [175,176].

Ferrets and humans share similar lung physiology, and human and avian IVs exhibit similar patterns of binding to SAs, which are distributed throughout the respiratory tract in both species [17,177]. The distribution of α2,6 SA and α2,3 SA receptors in the ferret respiratory tract was reported to resemble that of humans, with α2,6 SA receptors in the upper respiratory tract extending into the lower respiratory tract as far as the bronchioles and α2,3 SA receptors in the lower respiratory tract distal to the respiratory bronchioles [17]. However, in a recent study, a predominance of α2,6 SA receptors was reported throughout the respiratory tract of ferrets, including the lower respiratory tract [19]. Receptor specificity plays a critical role in IV transmission, which is one the primary reasons why ferrets are often used for studying IV transmission [178].

A transmission and pathogenesis study on ferrets reported that pdmH1N1 2009 virus was also more pathogenic, replicating to higher titers in the trachea and lung and causing more severe bronchopneumonia with prominent viral antigen expression in the peribronchial glands and alveolar cells than human seasonal H1N1 viruses [179], while showing much less pathogenicity for the animal than the HPAI H5N1 virus [18,180]. The pdmH1N1 2009 influenza virus RNA was also detected in the intestinal tract of inoculated ferrets, consistent with the occurrence of gastrointestinal symptoms in many human pdmH1N1 2009 influenza cases [181].

As with any other laboratory animal model, there are also some disadvantages to use of the ferret. The initial cost of the animal is higher compared with some other models. Ferrets require more housing space than do mice [168]. Another drawback to the use of ferrets is the lack of availability of inbred, KO and specific pathogen–free ferrets. Because ferrets are not inbred, they do not all respond the same way to a particular strain of virus. Ferrets from standard vendors to be used for studies of influenza should first undergo testing to confirm that they do not have positive titres for the strain of influenza that is being studied. Finally, another disadvantage of using the ferret is the amount of labor required to care for them, especially as young animals.

Others: Nonhuman primate model, have been less extensively used in influenza research but are often thought to best imitate human infectious diseases. In general, human influenza A viruses infect and replicate in the upper respiratory tract of macaques, causing either asymptomatic or mild clinical infections in these animal [182]. However, there are limitations to using this model, such as availability, the need for

trained personnel, cost for caging and maintenance, additional health risks as tuberculosis (TB), and the regulations associated with procuring and maintaining primates.

Similarly, the virus induced elevated fever, severe lung lesions with oedematous exudates and inflammatory infiltrates and high antigenic loads in pneumocytes in nonhuman primates, similar to what was reported for HPAI H5N1 influenza viruses [183]. This may be related to the affinity of the virus for 2,3α SA receptors in the lower respiratory tract [184].

The pdmH1N1 2009 virus replicates efficiently in the lungs and other respiratory organs of infected non-human primates [169]. The pdmH1N1 2009 virus caused more severe histopathologic lesions in the lungs than seasonal influenza [78,185]. However, mortality was not reported in non-human primates infected with pdmH1N1 2009 influenza virus, suggesting that disease is still not as severe as that seen with 1918 or HPAI viruses [169]. Additionally, non-human primates may be used for evaluation of innate and adaptive immune responses [182].

Domestic pigs, are closely related to humans anatomically, genetically and physiologically, and represent an excellent animal model to study various microbial infectious diseases. Indeed, experiments in pigs are much more likely to be predictive of therapeutic treatments in humans than experiments in rodents [21]. Based on the same subtypes that infect pigs and human and the similarities between clinical diseases, pathogenesis and tissue tropism of IV infections in pigs and humans, pig presented numerous advantages to study different aspects of influenza infections [171]. Various studies have demonstrated the pathogenesis and transmission of pdmH1N1 2009 virus in swine [21,170,186].

During a swine pdmH1N1 2009 infection, it was observed that NP- and H1-specific antibodies could be detected 7 dpi and CD4+ T cells became activated immediately after the infection [186]. Both CD4+ and CD8+ T lymphocytes expanded from 3 to 7 dpi coinciding with clinical signs [186]. Subsequently, Brookes et al [187] used a similar virus that in infected pigs produced clinical signs, viral pathogenesis restricted to the respiratory tract, infection dynamics consistent with endemic pig strains, virus transmissibility between pigs and virus-host adaptation events [187]. On the other hand, the pandemic virus did not cause any signs in miniature pigs; viral titers were detected in lungs at 3 dpi [169].

HYPOTHESIS AND GENERAL OBJECTIVES

3. HYPOTHESIS AND GENERAL OBJECTIVES

Hypothesis

Given the heterogeneity in the outcome of pdmH1N1 2009 influenza virus, we hypothezised that that the differential outcome of pdmH1N1 2009 influenza virus infection could be due not only to differential fitness/virulence of the virus but also to the host immune environment.

General objectives

1. To evaluate how high levels of proinflammatory cytokines and in particular IL-6, might affect host immune responses and the clinical course of infection one infecting mice with one pdmH1N1 2009 influenza virus.

2. To characterize and compare *in vitro* and *in vivo* in mice viral fitness of two oseltamivir sensitive pdmH1N1 2009 viruses named R6 (RR6121) and R7 (RR7495).

3. To study the dynamics of infection in ferrets of two different strains of pdmH1N1 2009 influenza virus isolated from a mild and a fatal case.

5. FIRST STUDY:

ROLE OF INFLAMMATION IN INFLUENZA A pdmH1N1 2009 VIRUS

I. Introduction

A subset of pdmH1N1 2009 reported cases required hospitalization and mechanical ventilation support. Previous studies performed by our collaborators from "Hospital Clínico de Valladolid" evaluating the role of proimmflamatory cytokines during infection showed a significant increment of some of them in patients who needed mechanical ventilation support [138].

Lipopolysaccharide (LPS) could be found in Gram-negative bacteria. As previously described in the section related to immune responses, LPS is recognized by TLRs, especially TLR2 that recognizes Lipoproteins and Lipoarabinomannan as LPS, as well as TLR4 [49]. It has long been recognized that systemic exposure of mammals to relatively small quantities of purified LPS leads to an acute inflammatory response. The mechanism for this response has been described to act via TLRs on macrophages that recognizes LPS and elicits a variety of proinflammatory cytokines [188]. Cytokines induced by LPS included TNF-α and various other interleukins (IL-1α, IL-1β, IL-8, IL-12) among which is IL-6.

To get insight into the possible role of that the inflammatory environment might play in the immune response to influenza infection, mice stimulated with LPS were infected with a pdmH1N1 2009 strain beeing the progress and pathology of disease followed in comparison with pdmH1N1 2009 infected mice without LPS treatment.

II. Hypothesis and specific objectives

Hypothesis

Patients severely affected by pdmH1N1 2009 virus might present an imbalanced immune response characterized by an exacerbated inflammatory state previous to infection that could affect negatively the progress of the disease.

Specific objectives:

- To establish a mice animal model able to reproduce a proinflammatory state using LPS in which infection could be develop.
- To infect mice with a strain of pdmH1N1 2009 virus (A/CATALONIA/63/2009 (pdmH1N1) (CAT09).
- To evaluate IL-6 levels in serum and lungs of infected animals in relation with IL-6 levels in controls animals.
- To evaluate the pathology and viral load in lungs of CAT09 infected animals with or without LPS treatment.

III. MATERIALS AND METHODS

i. Cell line

Madin-Darby Canine Kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Walkesville, USA) supplemented with 5% Fetal Bovine Serum (FBS) (Euroclone, Sziano, Italy), 100UI/ml penicillin and 100μg/ml streptomycin (Invitrogen ®, Barcelona, Spain), 2mM glutamine (Invitrogen ®, Barcelona, Spain).

ii. Viral load

Viral quantification was determined by two standard methods: 50% Tissue Culture Infective Dose ($TCID_{50}$) and plaque assay determining Plaque Forming Units (PFU).

$TCID_{50}$ determination: Virus titers were measured using the $TCID_{50}$ assay on MDCK cells. MDCK cells were seeded in 96-well plates and inoculated by virus culture supernatants diluted 10-fold serially. MDCK cells were infected with 20 μL of diluted virus sample and then viruses were adsorbed to MDCK cells for 1 hr. After incubation, infected cells were cultured with post-infection medium prepared with DMEM supplemented with 1μg/ml of porcine trypsin type IX (Sigma-Aldrich SA, Madrid, Spain). Infected cells were incubated at 37°C during 7 days and then observed to determined cytophatic effect (CPE) when comparing to uninfected cells. The $TCID_{50}$ was determined using the Reed and Muench method [189].

PFU determination: MDCK cells plated in 12-well tissue cultures plates were inoculated with 0.1 ml of 10-fold dilutions to determine viral load (10^1–10^6 pfu), dilutions were performed in Dulbecco's Phosphate Buffer Saline (PBS) (AttendBio Research, S.L, Barcelona, Spain) and 1% bovine serum albumin (BSA) (Sigma-Aldrich SA, Madrid, Spain) at room temperature. Samples were adsorbed to MDCK cells for 1 hr. Then, inoculums were aspirated and cells were washed once with Phosphate Buffer Saline (PBS) (Lonza, Walkesville, USA). Wells were overlaid with 1.4 % noble agar (Becton Dickinson, France) mixed 1:1 with Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich SA, Madrid, Spain) supplemented with 100 UI/ml penicillin and 100ug/ml streptomycin (Invitrogen ®, Barcelona, Spain) and 0.5 μg/ml of bovine trypsin (Sigma-Aldrich SA, Madrid, Spain). Plates were inverted and incubated for 4 days. Cells were

fixed for 20 min using 10% formalin (Sigma-Aldrich SA, Madrid, Spain) and then overlaid with 1% crystal violet (Anorsa, Barcelona, Spain). Cells were then washed with water to visualized plaques. Plaques were counted and compared to uninfected cells.

iii. pdmH1N1 2009 Catalonian virus

A human pandemic Influenza A virus, A/Catalonia/63/2009 (CAT09), isolated in 2009 (GenBank accession numbers GQ464405-GQ464411 and GQ168897) was used for infection of mice [170]. It was propagated following standard procedures by infecting MDCK cells. CAT09 was passaged in MDCK two times and the viral stock had a titre of 10^5 PFU/ml.

iv. Ethics statement

All mice experiments developed during the progress of the thesis were performed following the animal use protocol approved by "Comissió d'Ètica en l'Experimentació Animal I Humana de la Universitat Autònoma de Barcelona". Seven weeks-old C57BL6/JOlaHsd (C57BL6) female mice (Harlan Laboratories, Barcelona, Spain) were housed in groups on experimental isolation cages at the biosafety level 3 (BSL3) facilities of the Centre de Recerca en Sanitat Animal (CReSA, Barcelona, Spain). Once mice were separated into different groups they were kept for one week in acclimation. Animals were kept in standard housing cages and provided with commercial food pellets and tap water *ad libitum* throughout the experiment.

v. Mice treatment and infection

C57BL6 female mice were used to determined doses and route for LPS (Sigma-Aldrich SA, Madrid, Spain) treatement and influenza infection (route and dosis for LPS treatment and/or CAT09 infection will be described on results paragraph).

vi. Sampling

Mice were observed daily to record changes on body weight and clinical signs. At 0, 1, 3, 5 and 10 days post infection (dpi) serum and necropsies were performed. All blood collections were obtained under % 5 isofluorane anaesthesia in order to minimize suffering. Animals were euthanized with IP inoculation of penthobarbital and tissue samples of lung and spleen were collected at necropsy.

vii. IL-6 by Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples and supernatant of macerated lungs were respectively assayed using the Mouse IL-6 DUOSET ELISA Kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

viii. Determination of viral load in tissues

Tissue samples were collected, snap frozen on dry ice and stored at –80°C until further processing. Tissue samples were weighed, homogenized and centrifuged briefly. pdmH1N1 2009 infectivity virus was determined by previously described plaque assay in MDCK cells.

ix. Histopathology

All lung mice samples of the present thesis work were histopatologically analyzed by a complete necropsy of all animals immediately after euthanasia. Lung samples were collected for macroscopical and histological examination. Tissue samples were fixed for 48h in neutral-buffered 10% formalin. They were then embedded in paraffin wax, sectioned at 3 µm, and stained with haematoxylin and eosin (HE) for histopathological assessment.

x. Statistical analysis

All statistical analysis developed along the fourth studies that compose this thesis were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). For all analyses, mice (chapters 5, 6 and 7) and ferrets (chapter 8) were used as the experimental unit. The significance level (α) was set at 0.05 with statistical tendencies reported when $P<0.10$. The Shapiro Wilk´s and the Levene test were used to evaluate the normality of the distribution of the examined quantitative variables and the homogeneity of variances, respectively. Any continuous variable was detected having a normal distribution. Thus, a non-parametric test (Wilcoxon test) using the U Mann-Whitney test to compare each pair of values was used to compare the different values obtained for all the parameters (weight loss, survival, clinical score, acute phase proteins, IL-6 and IL-10 cytokines, antibody response and viral load) between groups (composed depending with each study) at all sampling times. All statistical analysis and graphs were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

IV. Results

i. Mice Pilot experiments

Pilot experiments (A and B) were performed to set up an animal model able to reproduce a proinflammatory state by LPS treatment in C57BL6 mice (Table 2). Two different experiments were performed in order to determine the appropriate route (intranasal-IN or intraperitoneal-IP) and the dose of LPS adequate to induce inflammation.

Protocol	Stimuli	Doses	Sampling
Pilot experiment A: LPS IN route vs IP route	LPS (IN) LPS(IP)	10 μg/mouse 200 μg/mouse 10 μg/mouse 200 μg/mouse	Serum
Pilot experiment B: LPS High IP dose vs Low IP dose	LPS (1dose) LPS (3 daily dose)	150 μg/mouse 200 μg/mouse 150 μg/mouse 200 μg/mouse	Serum Lung

Table 2. Pilot experiments for LPS stimulation in C57BL6 mice describing the route and doses used.

1. Pilot experiment A: Route of LPS administration

Five groups of mice were named according to LPS dose and route used for cytokine induction, a control group was treated with PBS IN and IP. The rest of the groups were treated as described on table 2 for Pilot experiment A. Animals were observed and weighed for 6 days. Clinical signs as decreased activity levels, ataxia and kyphosis were observed on animals with highest IP dose (250 μg/mouse) from day 1 to 3 post LPS treatment (data not shown). Percentage of weight loss on mice treated with the highest

IP dose (250 µg/mouse) was around 9.5% at 1 day post-LPS treatment and 8.5% at 2 days post-LPS treatment, there was a statisticall significant decrease of body weight in 250 µg/mouse IP-group when compared with both intranasally inoculated groups at 1 dpi ($p < 0.001$). At 2 dpi animals from 250 µg/mouse IP-group showed a significant decrease of body weitght when compared with the rest of the LPS inoculated group and also with control animals ($p < 0.0001$). At 3 days post-LPS treatment, IP inoculated animals at 250 µg/mouse dose recovered their normal weight until the end of protocol. The rest of the LPS-treated mice showed a similar pattern than control mice from day 1 to 5 post-LPS treatment for weight loss. Groups of animals treated with the highest and the lowest IN dose of LPS and mice treated with the lowest IP dose did not show loss of body weight at the first 5 days of the experiment. At day 6 post LPS treatment IN inoculated groups lost about 9.5% of weight (Figure 6).

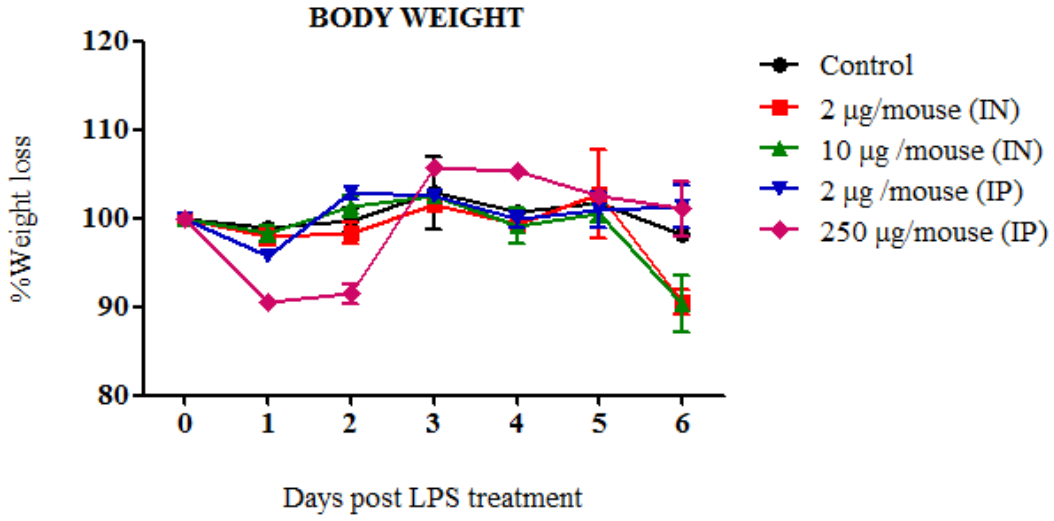
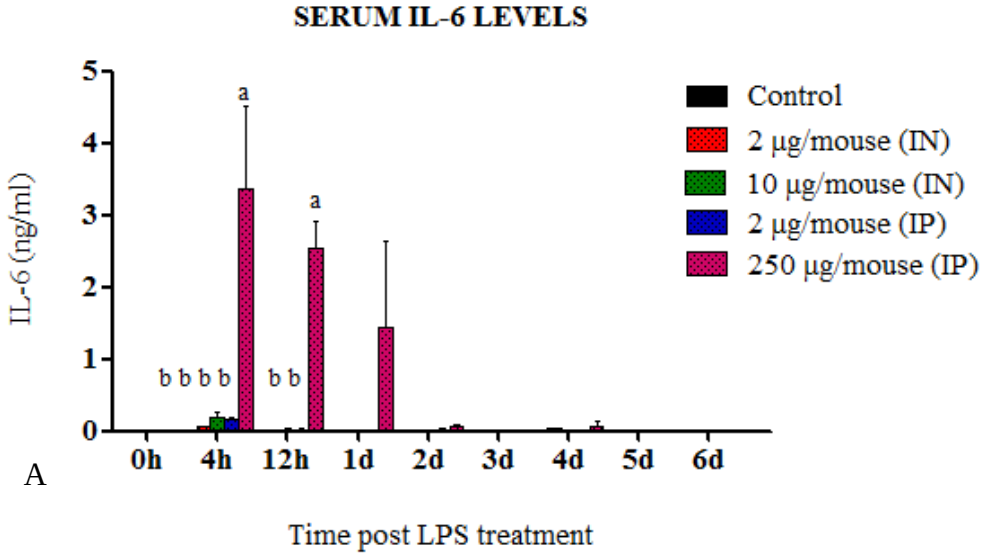


Figure 6. Influence of LPS treatment on body weight on IN vs IP LPS treated mice. Groups of 20 mice were distributed as follows: low dose IN (2 µg/mouse); high dose IN (10 µg/mouse); low dose IP (2 µg/mouse); high dose IP (250 µg/mouse). All values are the mean ± SEM of one experiment.

a. IL-6 secretion on serum and lung

Samples of lung and serum were collected to determine IL-6 levels at 0, 4, 12 hours post-LPS treatment and at 1, 2, 3, 4, 5 and 6 days post-LPS treatment. In sera, large concentrations of IL-6 were observed at 4, 12 and 24 hours post-LPS treatment in animals treated with 250 μg/mouse, significant increase of IL-6 levels were observed at 12 hours post-LPS treatment in 250 μg/mouse-treated group when compared with LPS-treated mice and control mice; at 1 day post-LPS treatment we also observed a significant increase of IL-6 in 250 μg/mouse-treated group when compared with animals treated with the lowest doses of LPS IN and IP. Low concentration to undetectable levels of IL-6 levels on sera of the remaining animals were only observed at 4 hours post-LPS treatment during the rest of the protocol (Figure 7 A). Lung IL-6 concentrations were higher than levels detected on sera. Levels of IL-6 were detected in lungs of LPS inoculated animals along the 6 days of the experiment. Large amounts of this cytokine were found on lungs of animals treated with 250 μg/mouse, particularlly at 4 hours post-LPS treatment (9.2 ng/gr) and at 12 hours post LPS treatment (3.6 ng/gr) (Figure 7 B). Values of IL-6 on lungs of animals treated with doses of LPS below 250 μg/mouse were lower than 1.5 ng/gr.



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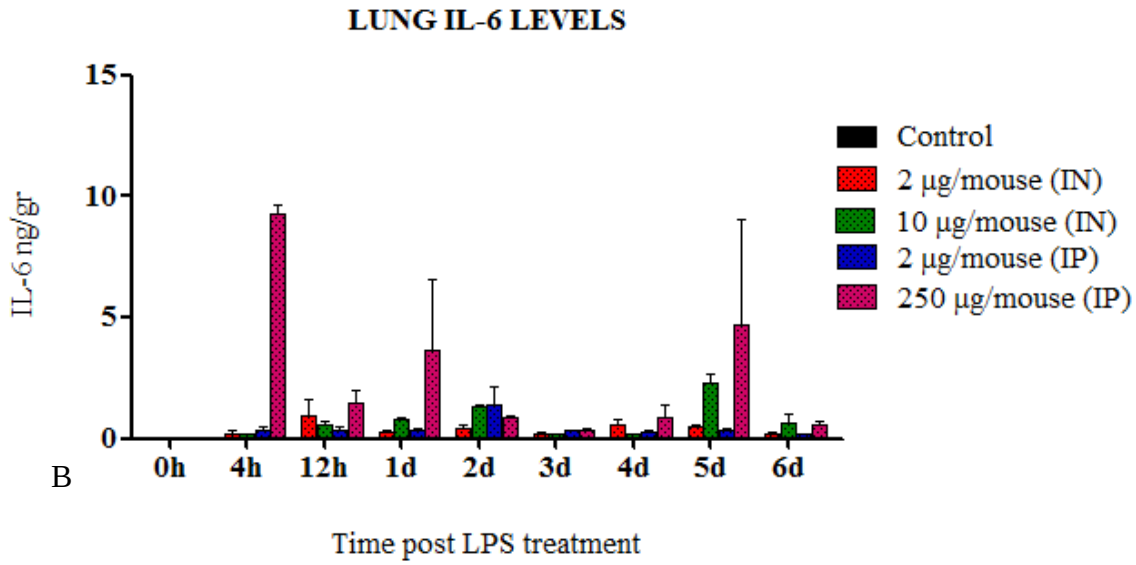


B

Figure 7. IL-6 concentration on serum (A) and lung (B) of IN vs IP LPS-treated mice. Groups of 20 mice were distributed as follows: control group; low dose IN (2 µg/mouse); high dose IN (10µg/mouse); low dose IP (2 µg/mouse); high dose IP (250 µg/mouse). At 0, 4 and 12 hours (h) post-LPS treatment and at 1, 2, 3, 4, 5, 6 days (d) post-LPS treatment levels of IL-6 of 5 animals per time-point were measured. All values are the mean ± SEM of one experiment.

In summary, the results obtained after LPS stimulation by two different routes indicated that LPS inoculated IP was able to stimulate high levels of IL-6 in mice. Also, the best IL-6 induction was obtained when animals were treated with the highest dose of LPS (250 µg/mice). However, those animals presented some undesirable clinical signs (ataxia, kyphosis, and decreased locomotor activity). For this reason, a second pilot experiment was performed in order to adjust the appropriate LPS concentration dose.

2. PILOT EXPERIMENT B: LPS dose

Five groups of mice were intraperitoneally inoculated and groups were named as described on table 2 for Pilot experiment B. A daily dose was given to two groups of mice during three days, maintaining the dose in all inoculations; the other two groups recived a single dose at the beginning of the experiment. Animals were daily observed and weighed for 4 days. No weight loss was observed on control animals. The percentage of weight loss of all LPS treated groups was about 10 to 15% at day 1 post-

LPS treatment, specifically animals belonging to control group showed a significant decreased of body weight when compared with each LPS treated group during the entire experiment (1 and 2 dpi $p<0.001$; 3 and 4 dpi $p<0.01$). At 1 dpi mice from 150 µg/mouse single dose-group presented a significant decrease of body weight when compared with 200 µg/mouse daily dose-group. At 2 dpi both groups that received a daily dose of LPS showed a significant decrease of body weight when compared with 150 µg/mouse single dose-group ($p<0.001$). Mice from 150 µg/mouse single dose-group presented a significant decreased of body weight at 3 and 4 dpi when compared with the group with the highest daily treatment of LPS ($p<0.001$). Animals that received a daily dose of LPS during the tree following days continued losing 18 to 25 % of body weight from day 2 to 4 LPS treatment. Single dose regime induced a decrease of body weight in mice at 1 dpi that was recovered on the following days (Figure 8).

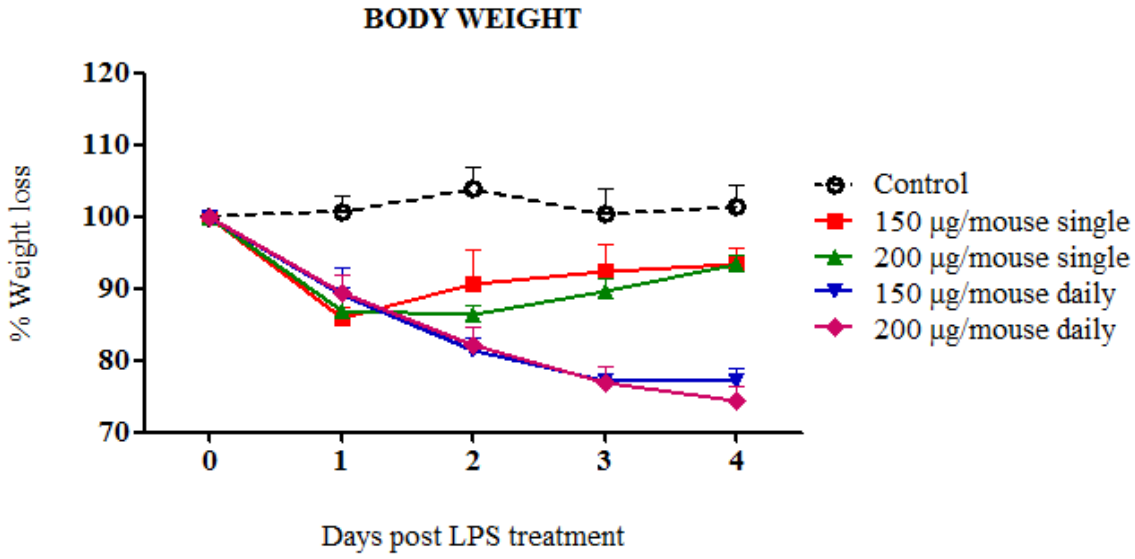
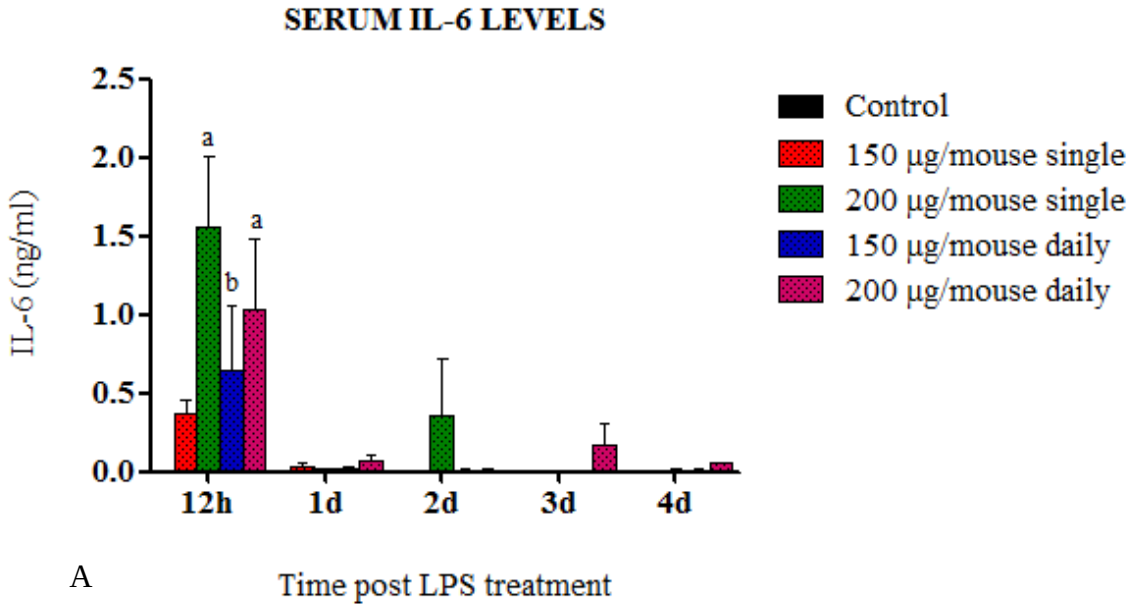


Figure 8. Influence of LPS treatment on body weight on single and daily doses were determined. Groups of 20 mice were distributed as follows: low single dose IP (150 µg/mouse); high single dose IP (200 µg/mouse); low daily dose IP (150 µg/mouse); high daily dose IP (200 µg/mouse). Daily dose was administrated at same time during 3 days. All values are the mean ±SD of one experiment.

a. IL-6 secretion on serum and lung

Samples of lungs and serum were collected to study IL-6 levels at 12 hours and at 1, 2, 3 and 4 days post-LPS treatment. The highest concentrations of IL-6 were observed at 12 hours post-LPS treatment on sera of LPS inoculated groups (0.3 to 1.5 ng/ml), there was a statistically significant increased of IL-6 in both 200 µg/mouse-groups (single and daily dose) when compared with animals from 150 µg/mouse single dose. IL-6 on control group was under detection levels (Figure 9A). At day 1 post LPS treatment levels of IL-6 decreased dramatically and at day 2 post LPS treatment only the highest single dose of LPS (200 µg/mouse) was able to induce IL-6 excretion (0.3 ng/ml). On the contrary, lung IL-6 concentrations above background were detected during the 4 days of the experiment, decreasing over time from 12 hours to 4 days post-LPS treatment. Similarly to the results in serum, levels of IL-6 on lung reached its maximum level at 12 hours post-LPS treatment (1.3 ng/gr) on the group of animals receiving a single 200 µg/mouse dose. In summary, all doses used in this study were able to induce lung IL-6 secretion in mice. Excluding the group that received a single 200 µg/mouse, the rest of the animals secreted less than 0.8 ng/gr of IL-6 on the first 24 hours (Figure 9B).



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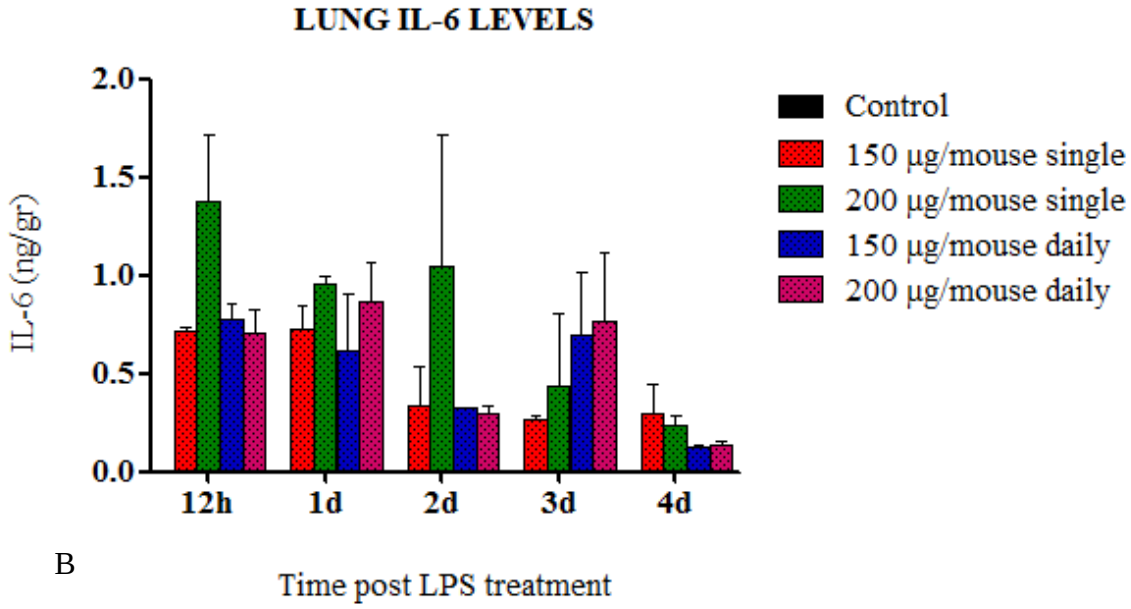


B

Figure 9. IL-6 concentration on serum (A) and lung (B) of single and daily doses LPS treated mice was determined. Groups of 20 mice were distributed as follows: Control group; low single dose IP (150 µg/mouse); high single dose IP (200 µg/mouse); low daily dose IP (150 µg/mouse) and high daily dose IP (200 µg/mouse). At 12 hours post LPS treatment (h), and at 1, 2, 3, 4, 5, 6 days post LPS treatment (d) levels of IL-6 of 5 animals for time-point were measure. All values are the mean and ± SEM of one experiment.

In conclusion, IL-6 production in serum of LPS-treated animals reached a detectable IL-6 systemic stimulation even at 2 days post-LPS treatment in animals treated with the highest single daily dose (200 µg/mouse), even when IL-6 was not detected on the other treated groups. Local responses in lungs were induced when 200 µg/mouse daily dose uas used, inducing the highest amount of IL-6 when compared with the rest of the groups. Beside, the 200 µg/mouse dose of LPS stimulated an inflammatory environment without altering animal clinical signs. In agreement with our previous results in which IP route was determined as the appropriated one for LPS treatment, the conditions to be used for futher experiments were determined as 200 µg/mouse following IP route in order to reproduce an inflammatory state on mice.

ii. Role of LPS-derived inflammation on pdmH1N1 2009 virus infection

Once the dose and route of LPS treatement was established, mice were infected with pdmH1N1 2009 virus in presence or absence of LPS. C57BL6 seven weeks-old female mice were divided into four groups of 48 mice each, distribution was done as follows: control group (C), LPS treated-group IP inoculated with 200 μg/mouse of LPS (LPS), CAT09 infected-group IN infected with 50 μl (10^4 PFU/mice) of pdmH1N1 2009 virus (CAT09) and CAT09 infected and later treated with LPS group, using the same doses described above (CAT09+LPS). Firstly, at 0 hours C group was IP inoculated with 200 μl of PBS and IN inoculated with 50 μl of PBS to reproduce both LPS treatment and CAT09 infection. At the same time, CAT09-group and CAT09+LPS-group were IN infected with 50 μl (10^4 PFU/mouse) of CAT09. Twelve hours after infection LPS-group and CAT09+LPS-group were IP treated with 200 μg/mouse of LPS resuspended in a volume of 200 μl of PBS.

In the course of the 10 days of the experiment mice were daily monitored and weighed. No body weight loss was observed in control mice, whereas LPS-treated mice and CAT09+LPS-infected mice had a peak of weight loss at 1 and 2 days after inoculation. Animals treated solely with LPS recovered their normal weight after day 3 pi. On the contrary, CAT09+LPS-mice showed a sustained loss weight during the entire experiment with a second peak of weight loss at 7 and 8 dpi. Mice that were only infected with CAT09 virus without LPS treatment, showed a slight decrease on body weight from days 2 to 9 dpi which was recovered at day 10 pi (Figure 10). Mice only treated with LPS showed a significant decrese of body weight when compared with controls and CAT09-infected mice at days 1 and 2 pi ($p < 0.0001$). Excluding days 0 and 10 pi, animals in the CAT09+LPS-group showed a statistically significant decrease of body weight ($p < 0.0001$) between days 1 to 9 pi, when compare with the other experimental groups (Figure 10). Even though the percentage of body weight on CAT09-infected animals was not as dramatic as the one in animals from CAT09+LPS-group, CAT09-infected mice showed a significant decrease of body weight ($p < 0.0001$) from days 3 to 5 pi when compare with control animals and with LPS-treated mice at 1 dpi ($p < 0.0001$) (Figure 10).

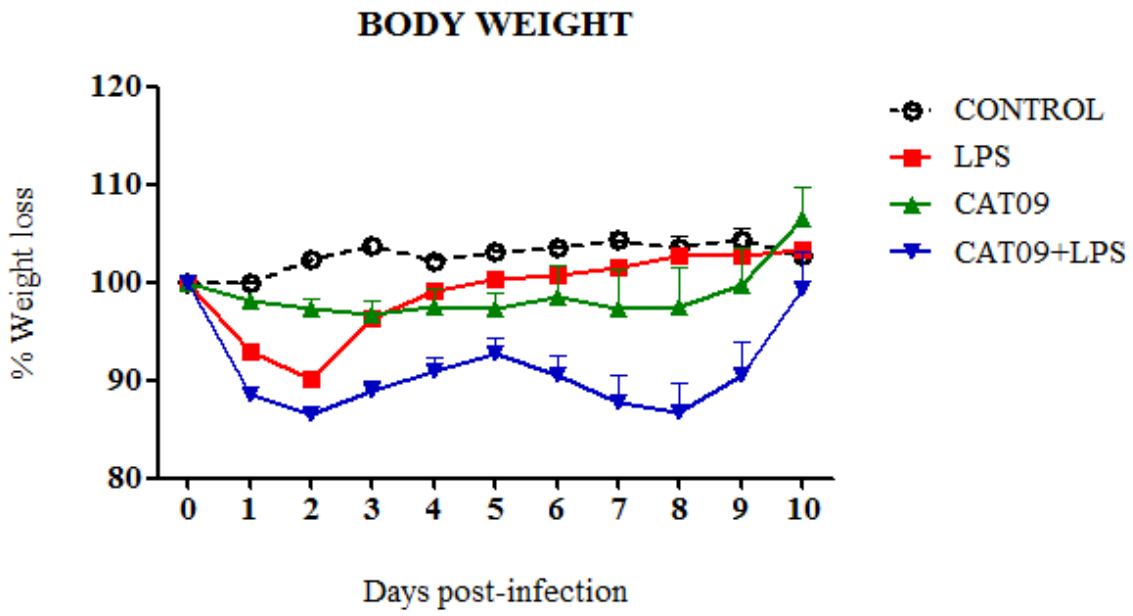


Figure 10. Influence of on body weight on CAT09 infection on LPS treated mice. Groups of 48 mice were distributed as follows: Control group; LPS (200 μg/mouse); CAT09 (10^4 PFU/mouse) and CAT09 (10^4 PFU/mouse) high daily dose IP (200 μg/mouse) + LPS (200 μg/mouse). All values are the mean and ± SEM of one experiment.

iii. Virus Replication

Lung samples of six infected animals were used to determine viral titers at 1, 5 and 10 dpi (Figure 11). At 1 dpi virus titer reached its maximum value but no statistically significant differences were observed when infected groups were compared. Similar viral levels on both infected groups were also found at 5 dpi and by day 10 pi no infectious virus was detectable.

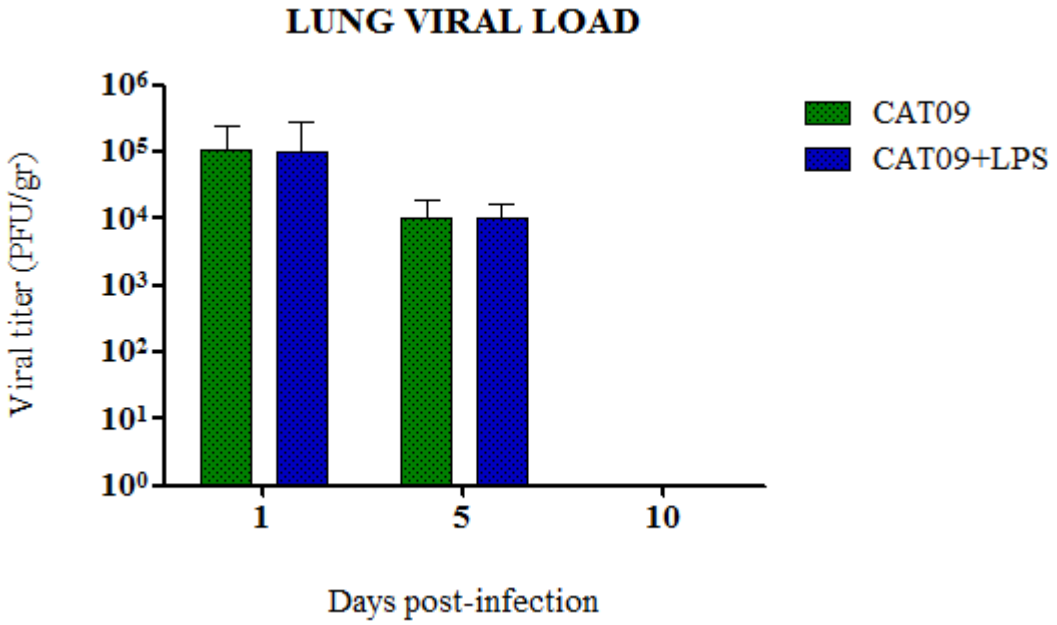
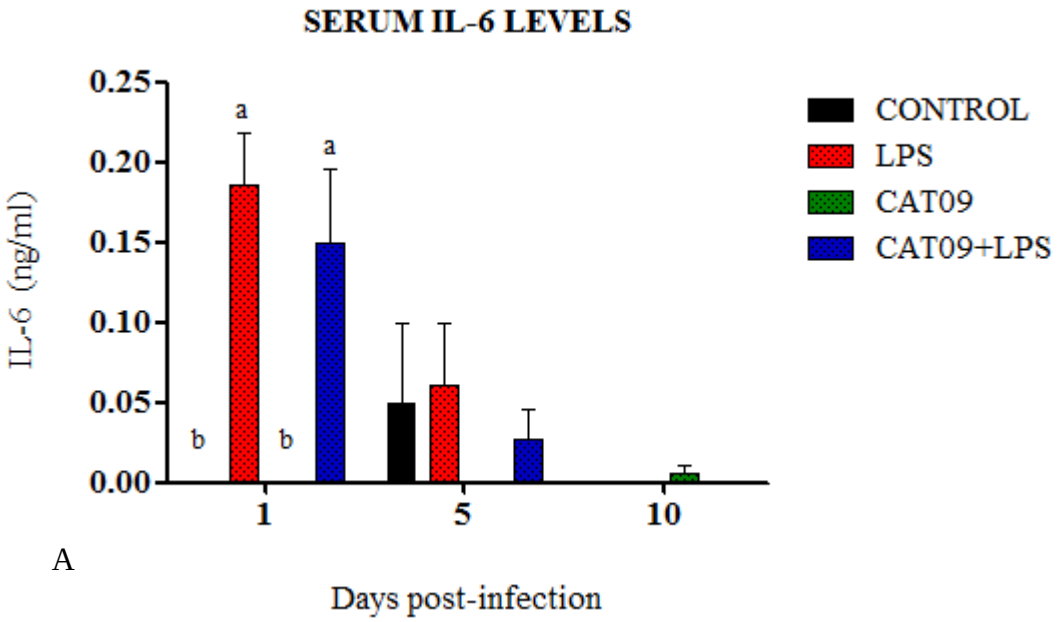


Figure 11. Viral load quantification at 1, 5 and 10 dpi on supernatants of macerated of infected lung were performed. PFU of five lung of each group: CAT09 and CAT09+LPS was determined. All values are the mean and ± SD of one experiment.

iv.IL-6 concentrations on serum and lung of treated animals.

At days 1, 3, 5, and 10 pi, six animals from each group, including controls, were sampled to collect blood to test IL-6 secretion. In sera, a statistically significant higher concentration of this cytokine ($p<0.05$) at 1dpi in LPS (0.18 ng/ml) and in CAT09+LPS (0.14 ng/ml) animals were detected when compared with control mice and CAT09-group (Figure 12 A). On the following two time-points, IL-6 concentration on serum was almost undetectable in all groups. Interestingly, CAT09-infected mice secreted low amount of IL-6 at day 5 and 10 pi (Figure 12 A). No significant differences within groups were found at days 5 and 10 pi when levels of IL-6 in serum were compared. To measure IL-6 concentration on lungs necropsies were performed at day 1, 5 and 10 pi. At day 1 pi a significant difference in lung IL-6 concentration ($p<0.05$) was detected in CAT09-group (8.1 ng/gr) when compared with animals in the C group, LPS-group (4.6 ng/gr) and CAT09+LPS-group (4.1 ng/gr). A significant increase of IL-6 ($p<0.05$) was detected on supernatants of lungs from day 5 pi in CAT09 infected animals (10.5 ng/gr) when compare with C animals and LPS-treated mice (3.0 ng/gr), however no

differences were found when values from CAT09 infected animals were compared with CAT09+LPS-group (7.4 ng/gr). At day 10 pi all groups showed IL-6 concentrations similar to control animals with no significant differences within groups (Figure 12 B).



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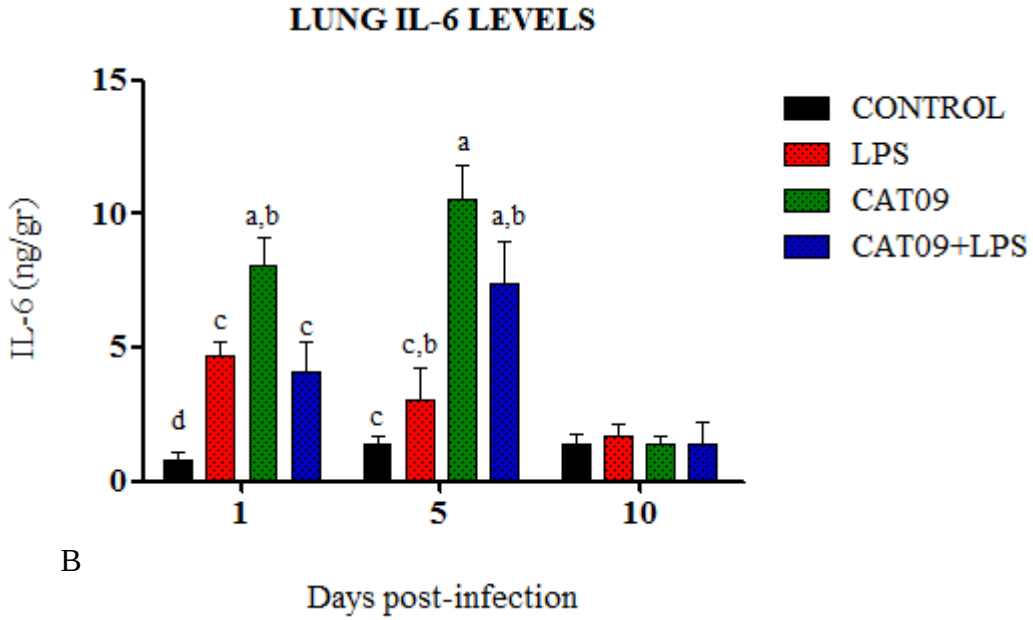


B

Figure 12. IL-6 concentrations on serum (A) and lung (B) was determined by ELISA. Groups of 48 mice were distributed as follows: Control group; LPS (200 μg/mouse); CAT09 (10^4 PFU/mouse) and LPS (200 μg/mouse) + CAT09 (10^4 PFU/mouse). All values are the mean and ± SEM of one experiment; a, b, c, d indicates significant differences ($p<0.05$).

v. Histopathology

Lung tissues were histopathologically examined. Control animals did not present any histopathological lesions. At 1 and 3 dpi, treated and/or infected animals did not present hispathological lesions. On animals treated with LPS mild to severe necrotizing bronchiolitis were observed at 5 and 10 dpi. CAT09-infected animals also presented mild to severe necrotizing bronchiolitis at 5 and 10 dpi. However, mice from CAT09+LPS-group presented interstitial pneumonia characterized by an acute inflammation with large inflammatory infiltration in the alveoli at 10 dpi (Figure 13).

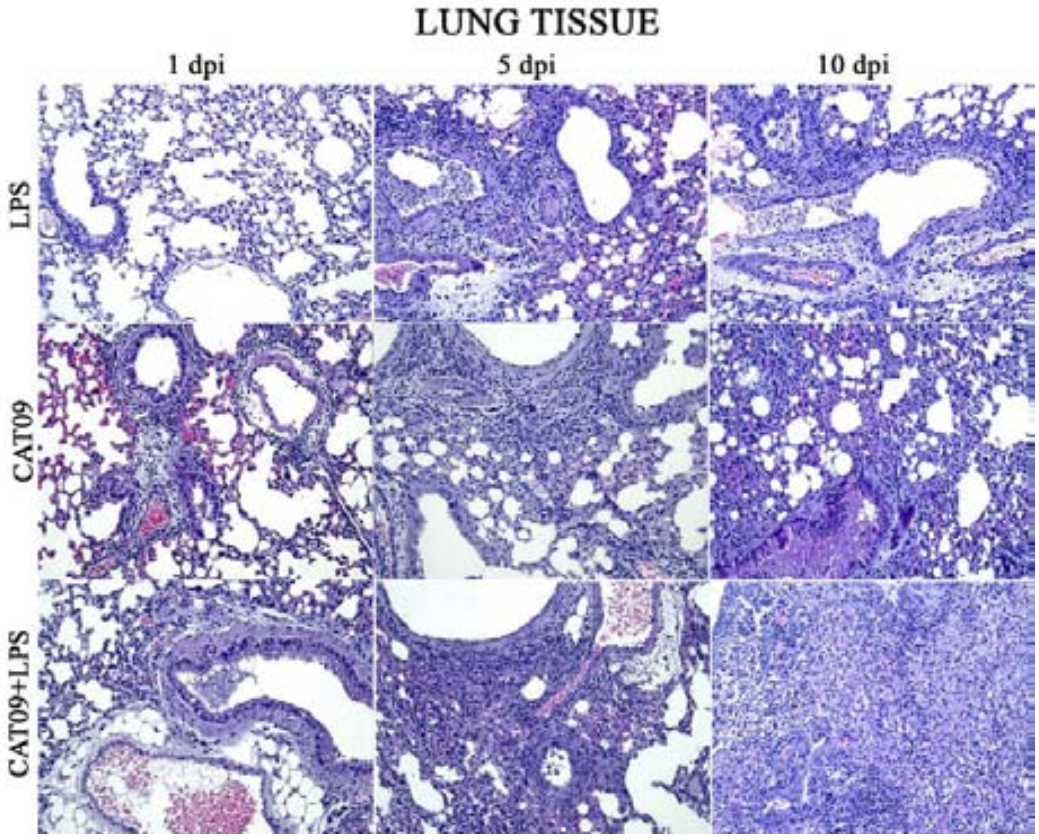


Figure 13. Histopathology of mice belonging to LPS, CAT09 and CAT09+LPS groups. No histopathological lesions were observed in any group at 1 dpi. Mild to severe necrotizing bronchiolitis were observed at 5 and 10 dpi in all groups. At 10 dpi CAT09+LPS-group presented interstitial pneumonia characterized by an acute inflammation with large inflammatory infiltration in the alveoli; LPS and CAT09 groups presented minor lesion of necrotizing bronchiolitis. Hematoxilin/Eosin stain.

All in all, the results obtained in this chapter where the inflammatory response of infected mice, by means of LPS treatment, was analysed in the course of pdmH1N1 2009 infection in mice showed that parameters such as clinical signs and weight loss were directly influenced by LPS treatment, in particular the first 2 days post infection/treatment (Figure 10). However, LPS treated mice but not infected were able to recover its normal weight at 3 dpi, conversely, mice infected with pdmH1N1 2009 virus and treated with LPS maintained a constant weight for 9 days. pdmH1N1 2009 infected mice without LPS treatment showed a lower percentage of weight loss than animals belonging to the CAT09+LPS-group. Additionaly CAT09 infected mice recovered its normal weight at day 10 pi (Figure 10). Interestingly, viral load of lung of both infected groups did not showed any difference, indicating that LPS treatment is not affecting viral replication *in vivo* (Figure 11). Concentration of IL-6 on serum and lung of both LPS treated groups were similar at the first 24 hours post infection indicating that virus replication did not affect IL-6 secretion when was associated to LPS treatment. Nevertheless, we found significantly higher levels of IL-6 on lung of pdmH1N1 2009 infected mice that were not treated with LPS (Figure 12). Finally, more severe histopathological lesions were observed at 10 dpi on animals treated with LPS and infected with pdmH1N1 2009 virus than animals only infected (Figure 13).

6. SECOND STUDY:

ROLE OF IL-6 ON pdmH1N1 2009 VIRUS INFECTION IN MICE

I. INTRODUCTION

As mentioned on the previous chapter, pdmH1N1 2009 clinically severe patients developed a proimmflamatory cytokines state during influenza infection, characterized by an increase of cytokines such as IL-6 together with IL-8, IL-10 y G-CSF in pdmH1N1 2009 virus infection. This was particularly relevant in patients who needed mechanical ventilation support [138]. However, a recent study the role of IL-6 using IL-6 KO mice demonstrated that the absence of IL-6 had not significant major clinical repercussions, suggesting that IL-6 does not play any essential non-redundant role in pdmH1N1 2009 infection in mice [137]. Thus, there is a controversy on the role of IL-6 in the case of pdmH1N1 2009 infection. Results in chapter 5 indicated that there was a different course of pdmH1N1 2009 infection in mice when LPS was administered in infected mice. However, a large number of cytoquines are induced in LPS treated mice, including IL-6. In addition, while KO models addressed the scenario of absence of IL-6, there is no evidence from animal studies on the potential protective or deletereous role of an exhuberant IL-6 response in the course of influenza infection.

Nevertheless, the question of whether high IL-6 levels had any role during pdmH1N1 2009 infection remains unexplored. Therefore, the aim of this study was analysing the course of pdmH1N1 2009 virus infection when IL-6 levels were experimentally induced on C57BL6 mice.

II. HYPOTHESIS AND SPECIFIC OBJECTIVES

Hypothesis

Our hypothesis was that different individuals might be able to induce variable levels of IL-6 upon pdmH1N1 2009 virus infection. IL-6 overproduction during influenza infection could be a consequence of a fail of the adaptive immune response to control de virus. Thus, in a situation when induced IL-6 levels were higher than average, this cytokine would play a role in pdmH1N1 2009 pathogenensis or it could mediate inflammatory driven tissue damage.

Specific objectives

- To establish a mice animal model able overproducing the proinflammatory cytokine IL-6.
- To infect the IL-6 overproducer mice with a strain of pdmH1N1 2009 Influenza A virus (A/CATALONIA/63/2009 (pdmH1N1) (CAT09).
- To evaluate the pathology, antibody response, IL-6 levels and viral load in serum and lungs of infected animals with or without IL-6 treatment.

III. MATERIALS AND METHODS

i. Cell line and virus preparation

MDCK cells were cultured in order to performed virus propagation and supernatant virus titration as previously described in chapter 5.

ii. pdmH1N1 Catalonian virus

pdmH1N1 virus A/CATALONIA/63/2009 (CAT09) was used for mice infection as previously described in chapter 5.

iii. IL-6 plasmid

The plasmid pcDNA3.1^{+}-mIL-6 (pIL-6) was design to overexpress mouse IL-6 (mIL6) (Gene ID: 16193) in mammals cells. The mIL6 coding sequence was inserted between the restriction sites AflII and EcoRI of the expression vector pcDNA3.1^{+} (Invitrogen, California, USA) (detailed design of the recombinant multicloning site in Figure 14). The inserted sequence had a synonymous substitution (T to A) at position 273, thus the sequence of the mIL6 protein was not changed. The generation of the recombinant plasmid and the production of a first stock of purified, endotoxin free plasmid with and without the mIL6 were performed by GenExpress (GenExpress Gesellschaft für Proteindesign mbH).

The mIL6 plasmid was used to transform chemiocompetent E.coli cells by thermic shock. Transformed cells were first screened on a selective Luria-Bertani Agar (LB) (Sigma-Aldrich SA, Madrid, Spain) plate with 100 mg/mL ampicillin (Izasa, Barcelona, Spain) and after propagated and scaled up in the same selective broth media. The plasmid was purified following the manufacture instruction of the EndoFree Plasmid purification Kit Maxi (Qiagen, Hilden, Germany). The identity of the produced plasmid was checked by sequencing with primers *T7 promoter* and *BGH rev* and the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Byosystems, California, USA).

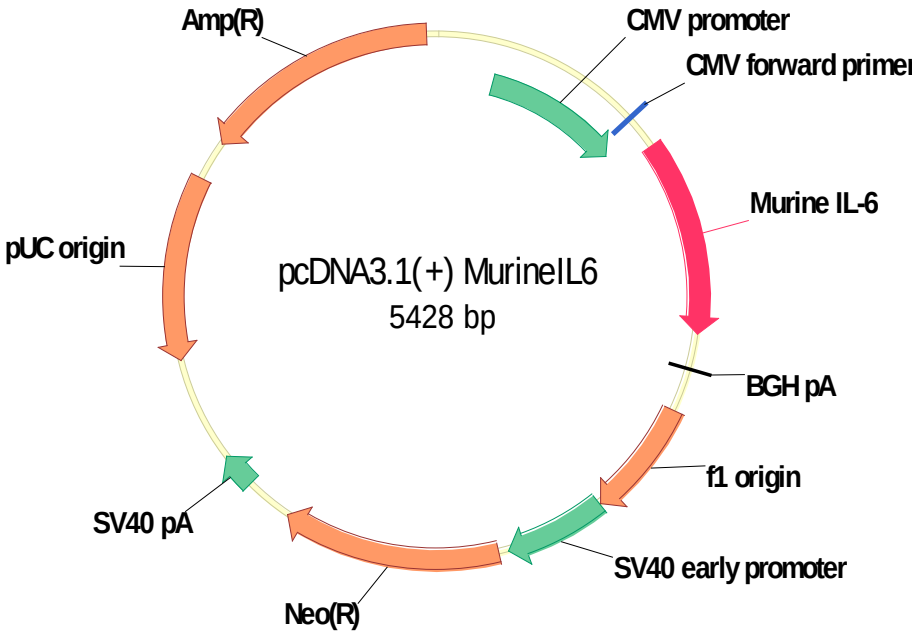


Figure 14. Genome organization of IL-6 plasmid performed using VectorNTI (Invitrogen)

iv. *In vitro* plasmid IL-6 transfection

The plasmid pcDNA3.1^{+}-mIL6 was transfected in African green monkey cells (Vero) according to manufacture instruction of FuGENE HD (Promega Biotech, Madrid, Spain). Vero cells were transfected in media composed of MEM supplemented with mM of L-Glutamine and FBS. Secretion of IL-6 was detected in the cells supernatant at 24, 48 and 72 hours post-transfection by mouse IL-6 DUOSET ELISA Kit.

v. Immunofluorescence assay

Briefly, cells were fixed in 100% methanol for 15 min and permeabilized with TRITON X-100 (Sigma-Aldrich Quimica, S.A., Spain) for 15 minutes at room temperature, and then 3% BSA in PBS was used for blocking for one hour. Later, fixed cells were incubated with the primary IL-6 detection antibody (from Mouse IL-6 DUOSET ELISA Kit) diluted 1:100 on PBS 3% BSA, at 4°C overnight. After whasing four times, cells were incubated with Streptavidin:TRITC (AbD Serotec, North Carolina, USA) for 1 hr at room temperature. After four washes, cells were stained with Hoechst 33258 (Sigma-Aldrich Quimica, S.A., Spain), to label nucleus DNA.

vi. Ethics staments

As described previously in chapter 5.

vii. Mice treatment and infection

C57BL6 seven weeks-old female mice were used to determine doses and route for Recombinant Murine IL-6 (rmIL-6) (Immunotools, Friesoythe, Germany) or pIL-6 treatement and influenza infection.

viii. Sampling

Samples were obtained as described in chapter 5.

ix. IL-6 detection by ELISA

Serum samples and supernatant of homogenated lungs were assayed using Mouse IL-6 DUOSET ELISA Kit kit described in chapter 5.

x. IL-10 detection by ELISA

Serum samples and supernatant of macerated lungs were respectively assayed using the Mouse IL-10 READY-SET-GO! Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

xi. Determination of viral load in tissues

Lung samples were collected and viral load was determined as described in chapter 5.

xii. Hemagglutination Inhibition (HI) Assay

Antibodies against IV were measured by HI assay using chicken red blood cells (RBC) and four hemagglutination units of pdmH1N1 2009 virus. Before the assay, sera were treated overnight at 37 ºC with four volumes of Receptor Destroying Enzyme (RDE) (Sigma-Aldrich SA, Madrid, Spain) solution (100 U/ml) to remove non-specific inhibitors of hemagglutination. Next day, serum samples were incubated for 30 min at 56ºC after the addition of five volumes 1.5% sodium citrate. Finally, one volume of a 50% suspension of RBC was added and incubated for 1 h at 4ºC. Control positive and negative sera were used as controls. HI titres of >40 were considered positive.

xiii. Histopathology

All mice tissue samples were histopatologically analyzed as described in chapter 5.

xiv. Statistical analysis

It was performed as described in chapter 5.

IV. RESULTS

i. Pilot experiments

Two pilot experiments (A and B) were performed to establish serum and lung IL-6 overproduction in mice by several approaches. In the first pilot experiment, mice were inoculated with rmIL-6 (Pilot A) at different doses (Table 3). In a second pilot experiment (Pilot B), three groups of animals were intravenously (IV) and IP inoculated with pIL-6 at different doses (Table 3)

Protocol	Stimuli	Doses	Sampling
Pilot experiment A: rmIL-6 inoculation	rmIL-6 pdmH1N1 2009 (CAT09)	0.5 μg/mouse (IP) 1 μg/mouse (IP) 2 μg/mouse (IP) 10^4 PFU/mouse (IN)	Serum Lung
Pilot experiment B: pIL-6 inoculation	pIL-6 (plasmid)	9 μg/mouse (IP) 10 μg/mouse (IV) 12.5 μg/mouse (IV)	Lung

Table 3. Pilots experiments for IL-6 overproduction in C57BL6 mice, to each protocol the table above describe route and doses used for stimulation.

ii. PILOT EXPERIMENT A: rmIL-6 inoculation

To induce overproduction of IL-6 in C57BL6 mice, animals were IP inoculated with rmIL-6 at different doses as described on table 3 for Pilot A experiment, a control group inoculated IP with PBS was also included on the protocol. Body weight was daily monitored for 9 days. No loss of body weight was observed in mock-inoculated mice whereas CAT09-infected group had a peak of weight loss at 2 dpi. No percentage of weight loss was observed during the 9 days of protocol on control animals and/or rmIL-6 treated mice. Percentage of weight loss on CAT09 infected mice was around 8.9% at 2 dpi to recover their normal weight on the following days (Figure 15).

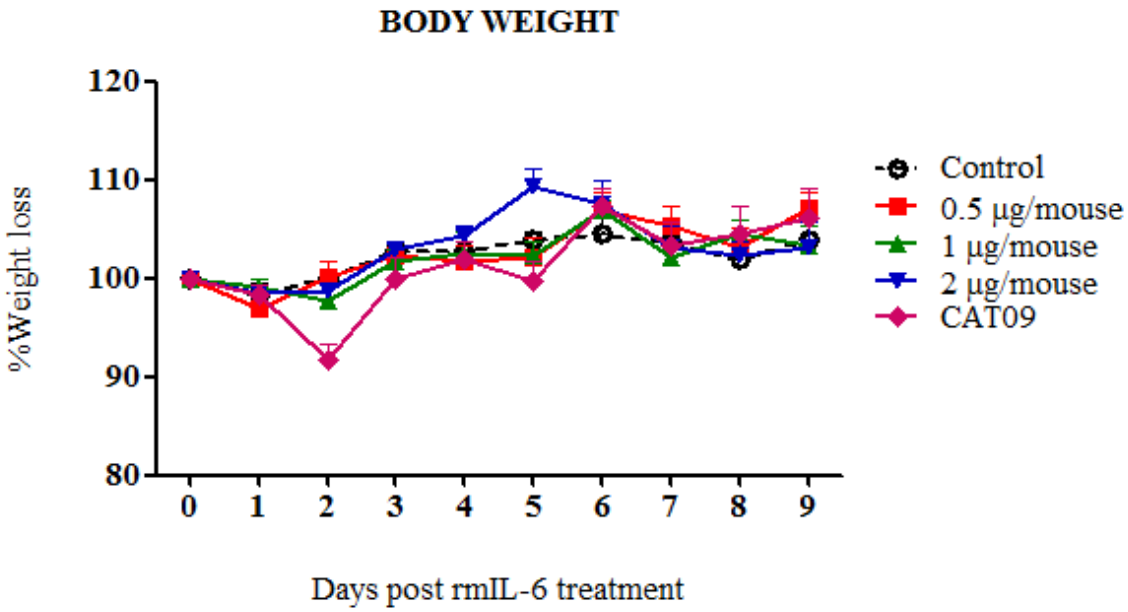


Figure 15. Influence of rmIL6 treatment on body weight at different doses was determined. Groups of 10 mice were distributed as follows: low dose IP (0.5 µg/mouse); intermedium dose IP (1 µg/mouse); high dose IP (2 µg/mouse) and CAT09 infected-mice. All values are the mean ± SEM of one experiment.

a. Virus Replication

Viral titers on lungs of infected mice were measured and the highest viral titers were found at 3 and 5 dpi (Figure 16). At 9 dpi presence of virus was still detectable on lungs of CAT09-infected mice.

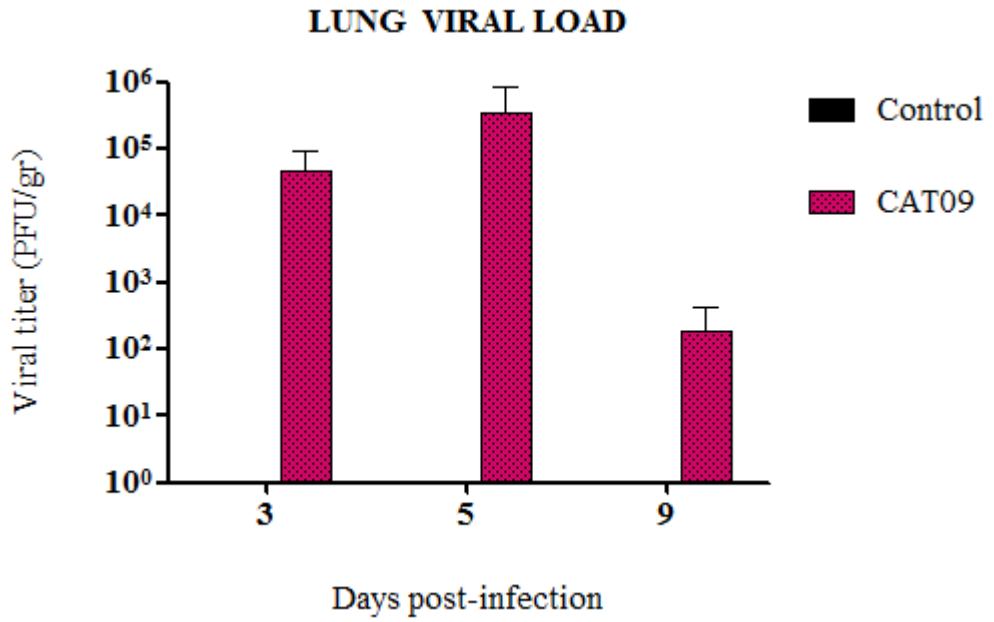
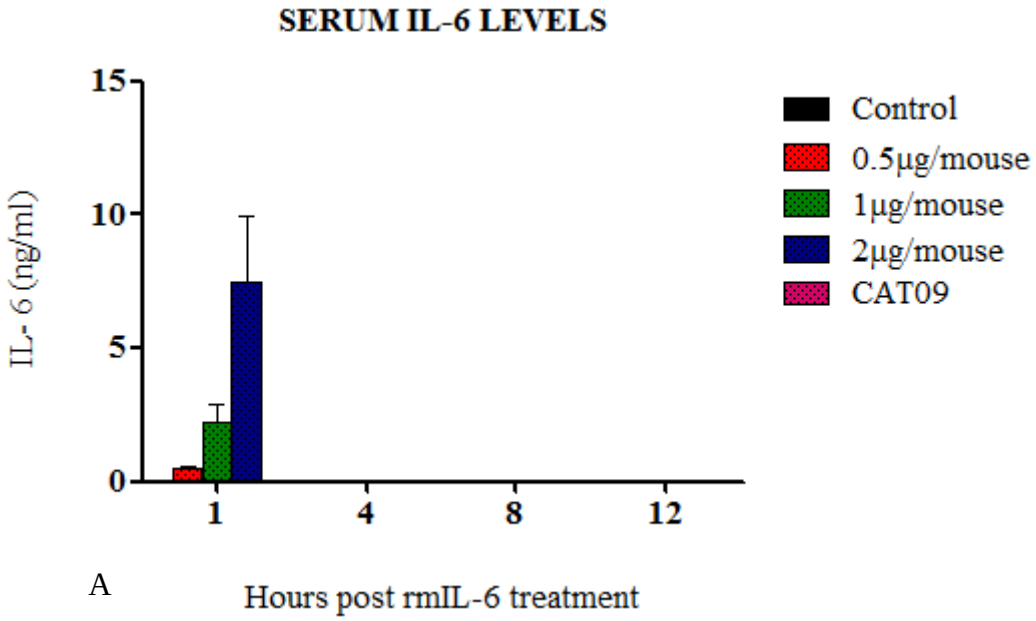


Figure 16.Viral load at 3, 5 and 9 dpi of infected lung. PFU of five lung of Control group ■ CAT09 and CAT09 ▩ were determined. All values are the mean and ± SD of one experiment.

b. IL-6 expression in rmIL-6-inoculated mice and in CAT09 infected-mice

IL-6 expression on sera of rmIL-6-inoculated mice at 1, 4, 8, 12 hours post rmIL-6 treatment was analysed. Induction of IL-6 was only observed at 1 hour post-rmIL-6 treatment to undetectable levels from 4 to 12 hours post-rmIL-6 treatment on sera of rmIL-6 inoculated mice. Levels of IL-6 were also determined on sera (1, 4, 8, 12 hpi) and in lungs at 3, 5 and 9 dpi) of CAT09-infected mice (Figure 17). No IL-6 concentration was detected on serum of infected animals during the first 12 hours after infection (Figure 17 A). The highest IL-6 concentration in lungs of infected mice was

observed between 3 and 5 dpi with values of 1.8 and 1.3 ng/gr to decrease to control levels at 9 dpi (Figure 17 B).



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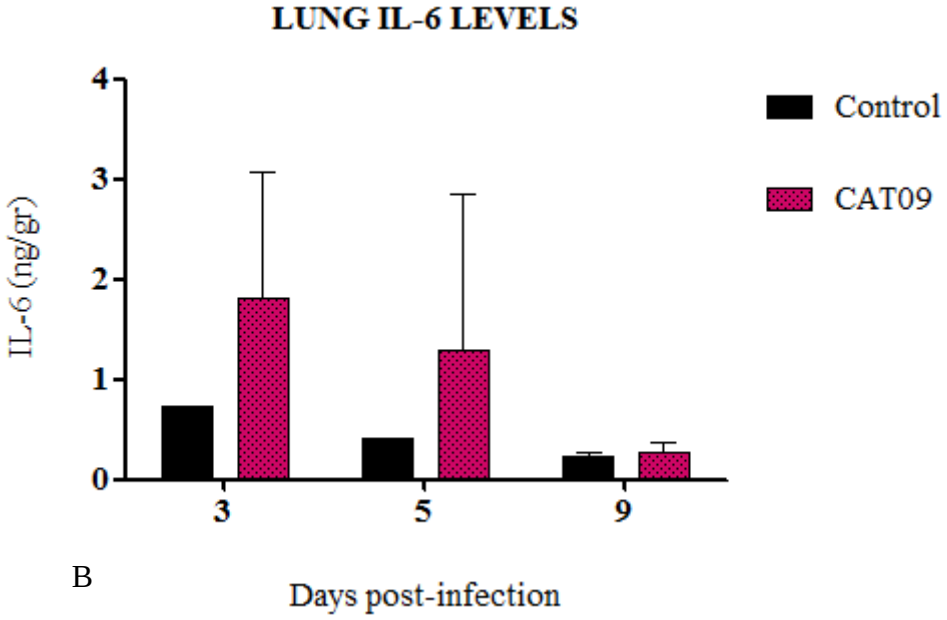


B

Figure 17. IL-6 concentration in serum (A) and lungs (B) was determined by ELISA. Groups of 20 mice were distributed as follows: Control group; low dose IP (0.5 μg/mouse); intermedium dose IP (1 μg/mouse); high dose IP (2 μg/mouse) and CAT09 infected-mice. At 1, 4, 8 and 12 hours post rmIL-6 treatment levels of IL-6 on serum were determined, and at 3, 5, 9 days post rmIL-6 treatment levels of IL-6 in five lungs for time-point were measured. All values are the mean and ±SD of one experiment.

In conclusion, rmIL-6 inoculation at all doses was poorly mantained over time. IL-6 was only detected at 4 hours post-rmIL-6 treatment and therefore this protocol cannot be considered for the purpose of this study. On the other hand, IL-6 production was detected in lungs from CAT09-infected mice at 3 and 5 dpi, in agreement with the data in figure 12 from chapter 5.

iii. *In vitro* pIL-6 transfection

1. IL-6 production on Vero transfected cells

In order to test whether our IL-6 plasmid was functional, Vero cells were transfected and supernatants were collected at 24, 48 and 72 hours post transfection (hpt) to study IL-6 secretion. Transfected and control cells were inmunostained as previously described to differentiate the nuclei from cytoplasm and for IL-6 secretion. Control cells did not exhibit IL-6 staining during the experiment (Figure 18 A). At 48 hpt, Vero-tranfected cells showed DAPI nuclei stain surrounded and punctuated by red points corresponding to IL-6 staining (Figure 18 B, C).

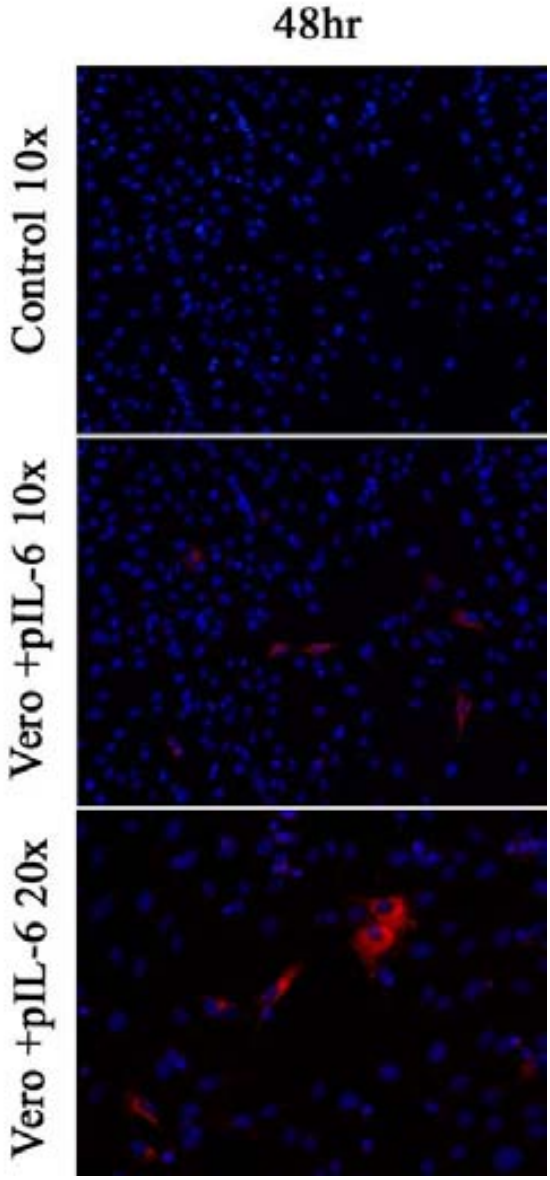


Figure 18. From the top to the base, Control cells (10X), Vero-transfected cells (10X) and Vero-transfected cells (20X). On blue DAPI nuclei stain; punctuated red dots correspond to IL-6 production on cytoplasm of Vero plasmid-transfected cells.

2. IL-6 production on supernatants of pIL-6 transfected-cells

IL-6 levels were studied in culture supernatant of transfected cells for cytoquine secretion. As early as 24 hpt, high levels of IL-6 were detected on supernatants of transfected cells, however the highest concentrations (>20 ng/ml) was produced at 72 hpt (Figure 19).

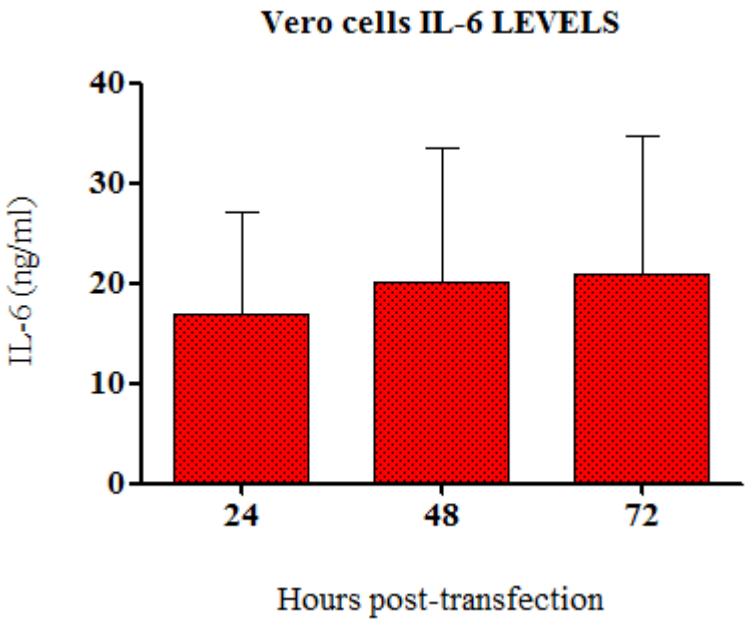


Figure 19. Vero cells were transfected with pIL-6 and supernatants collected at 24, 48 and 72 hpt for IL-6 analysis by ELISA. All values are the mean ± SEM of one experiment.

In conclusion, our IL-6 plasmid was able to induce IL-6 secretion when transfected to competent cells *in vitro*.

iv. PILOT EXPERIMENT B: *in vivo* IL-6 plasmid transfection

Once *in vitro* production of IL-6 was tested on transfected Vero cells when using our pIL-6, we investigated whether C57BL6 mice IP or IV inoculated with our pIL-6 were able to secrete high levels of IL-6 *in vivo*. Animals distributed as described on table 3 for pilot B experiment were tested in order to determine the adecuate dose for IL-6 induction. Necropsies were performed and lungs were collected at 8 and 24 hpt. IL-6 was detected on both time-points at all different concentrations of plasmid. At 8 hpt animals treated with 9 µg/mouse (IP) of pIL-6 induced 7.5 ng/gr of IL-6; at 10 µg/mouse (IV) of pIL-6 treated mice secreted 3.8 ng/gr of IL-6 and the highest IL-6 concentration was detected at 8 hpt (25 ng/gr) in the 12.5 µg/mouse (IV) pIL-6 treated mice. When IL-6 levels were measure at 24 hpt, animals treated with 9 µg/mouse (IP) of pIL-6 induced 3.8 ng/gr, the ones treated with 10 µg/mouse (IV) of pIL-6 induced 4.0 ng/gr and the ones treated with 12.5 µg/mouse (IV) induced 9.4 ng/gr (Figure 20).

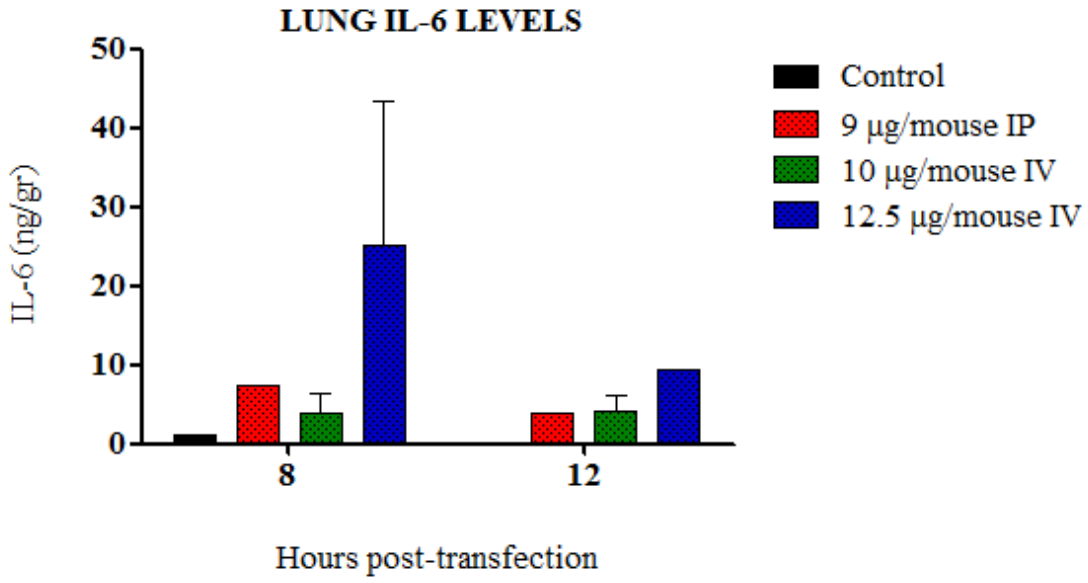


Figure 20. IL-6 concentration in lungs of pIL-6 treated mice at 8 and 12 hpt. Groups were distributed as follows: Control group; low dose IP (9 µg/mouse); intermedium dose IV (10 µg/mouse) and high dose IV (12.5µg/mouse). All values are the mean ± SEM of one experiment.

In conclusion, all doses used to stimulate animals with pIL-6 were able to induce secretion of IL-6 in lungs. The highest doses used in this experiment (12.5 μg/mouse) induced an irregular secretion of IL-6 when comparing 8 hpi with 12 hpi. Considering these results we decided to inoculate mice with the intermedium dose of 10 μg/mouse in the following experiments.

v. Role of IL-6 on pdmH1N1 2009 virus infection in C57BL6 mice

1. Mice and pdmH1N1 2009 infection

Female C57BL6/JOlaHsd mice (aged 7 weeks) obtained from Harlan Laboratories were injected with pIL-6 DNA by using a hydrodynamic-based transfer technique [190]. Briefly, 10 μg/mouse of pIL-6 were diluted in 2.0 ml of PBS and injected speed via tail vein using a 27-gauge needle and syringe within a time period of 5 to 8 seconds. DNA injection was completed in less than 5 seconds. Serum samples were collected a different time-point and lungs were dissected from dead animal using the standard surgical procedures.

Animals were divided into four groups of 48 mice each, distribution was done as follows: untreated control group (C); plasmid *in vivo* IL-6 transfected-group (pIL-6); pdmH1N1 2009 infected-group (CAT09) and CAT09 infected and later *in vivo* IL-6 transfected-group (CAT09+pIL-6). C-group remains untreatred. Firstly, at 0 hours, pIL-6-group and CAT09+pIL-6-group were IV treated with 10 μg/mouse of IL-6 plasmid resuspended in a volume of 2 ml of PBS. After plasmid inoculation CAT09-group and CAT09+pIL-6-group were intranasally infected with 50 μl (10^4 PFU/mouse) of CAT09. To induce overproduction of IL-6 mice were IV inoculated with 10 μg/mouse of pIL-6, based on pilot experiment B results. Body weight was daily monitored for 10 days. No loss of body weight was observed on untreated C mice. At 1 dpi and/or transfection, pIL-6 treated mice and CAT09+pIL-6-group showed a peak of body weight decrease that was statistically significant ($p<0.0001$) when compared with C animals and CAT09-infected mice. In the case of the animals from the pIL-6 treated group, mice recovered their normal weight at 3 dpt, following a similar pattern than control mice during the rest of the experiment. On the contrary, CAT09+pIL6 mice showed a sustained weight loss from day 1 pi until the end of the protocol, which was statistically

significant ($p<0.0001$) when comparing it with C mice and pIL-6 treated mice. However, when CAT09+pIL6 mice were compared with CAT09 mice no statistically significant differences were observed; it was worth noticing that body weight decrease on animals only infected with CAT09 started 2 days later (3dpi) than CAT09+pIL6 mice, being the later faster and more dramatic during the first tree days after infection. In mice belonging to the CAT09-infected group we also observed a belated peak of weight loss at day 7 pi. Weight loss on CAT09-infected was statistically significant ($p<0.0001$) from day 3 to 10 pi when compared with C animals and pIL-6 treated mice (Figure 21).

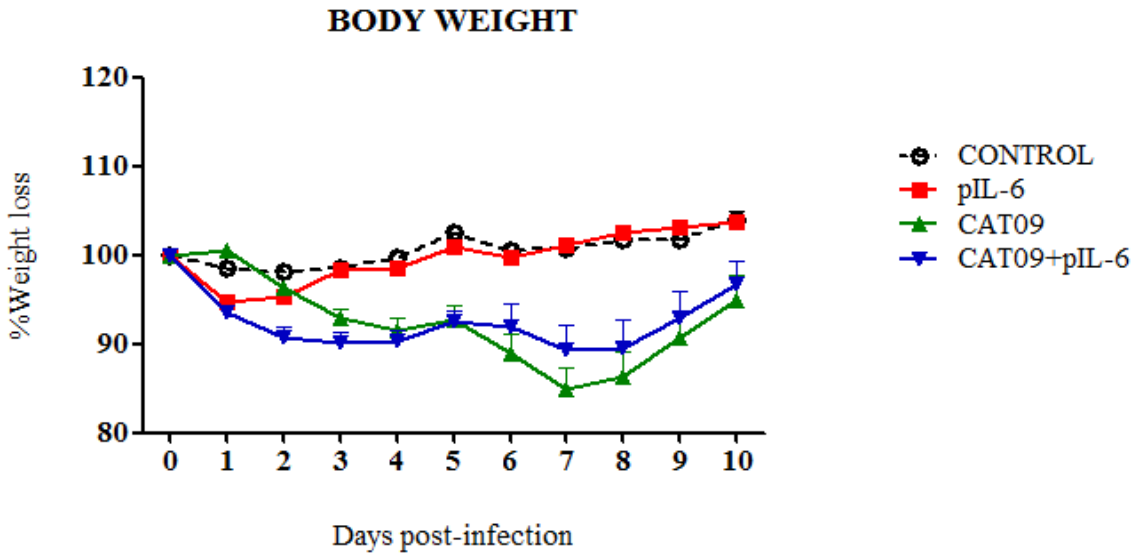


Figure 21. Influence of on body weight on CAT09 infection on pIL-6 treated mice. Groups of 48 mice were distributed as follows: Control group; pIL-6 (10 μg/mouse); CAT09 (10^4 PFU/mouse) and pIL-6 (10 μg/mouse) + CAT09 (10^4 PFU/mouse). All values are the mean and ±SEM of one experiment.

2. Viral replication

Viral titers on lungs of infected mice were measured at different times post-infection. Only at 1 and 3 dpi, viral levels were detected (Figure 22) on CAT09 infected mice. Animals transfected with pIL-6 and infected with CAT09 showed detectable viral titers only at 1 dpi. On the contrary animals only infected with CAT09 virus but not treated with the pIL-6 showed detectable virus particules at days 1 and 3 pi. No statistically significant differences were found at any time-point when data from animals in infected groups were compared (Figure 22).

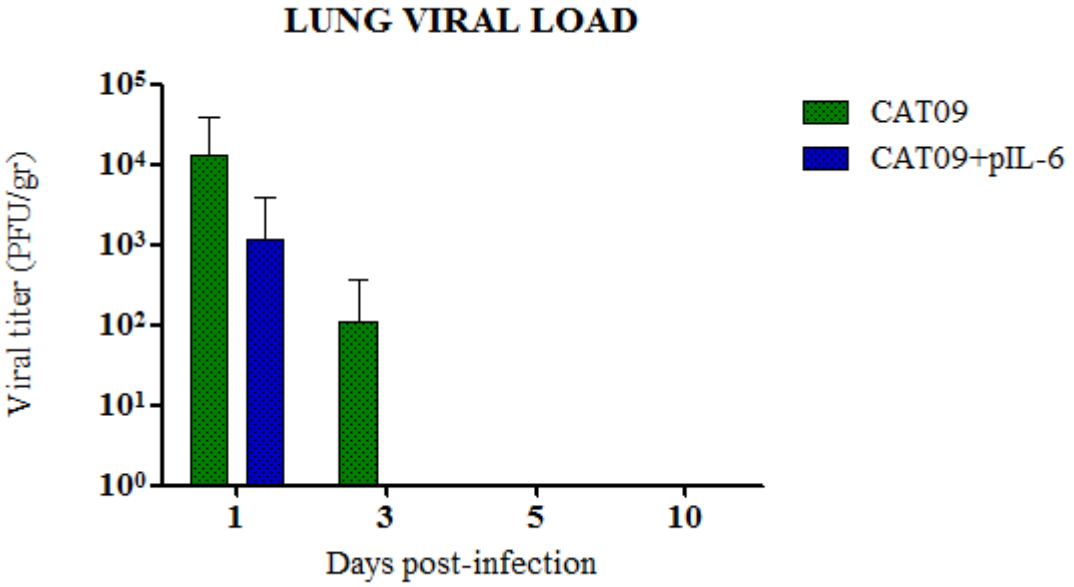


Figure 22. Viral load quantification at 1, 3, 5 and 10 dpi in infected lung. PFUs of six lung of each group from ■ CAT09 and ■ CAT09+pIL6 groups were determined. All values are the mean and ± SD of one experiment.

3. Antibody response

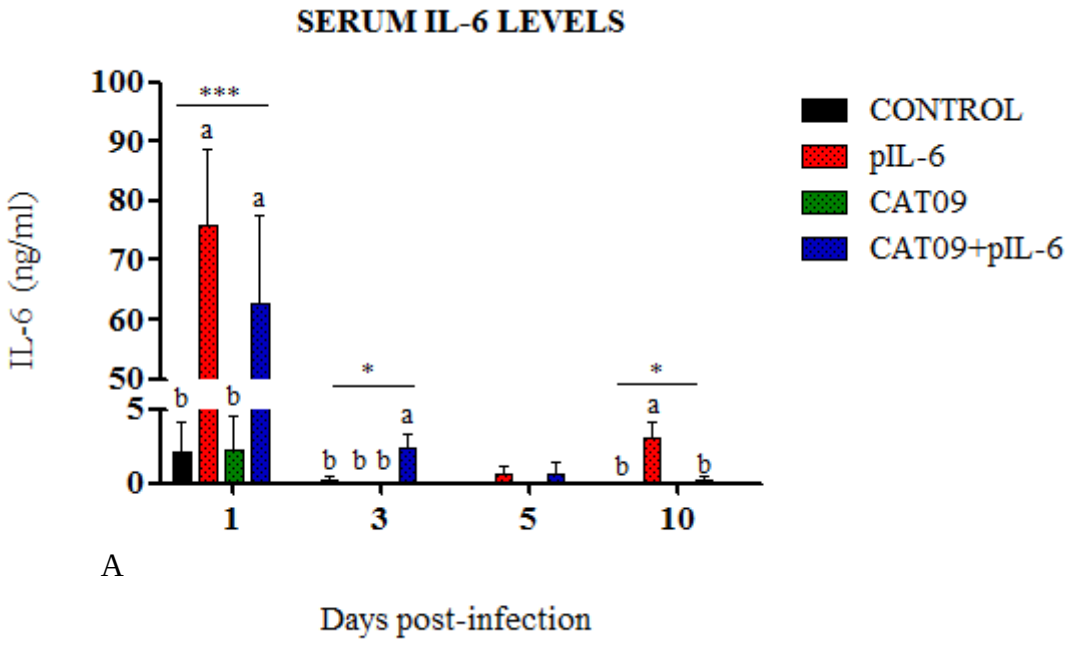
Antibody response to CAT09 virus was determined by hemagglutination inhibition (HAI) assay. Serum from day 10 pi was tested. Untreated mice were seronegative for HAI. Six animals of each infected group were tested and all mice from both groups proved to be positive at day 10 pi. There was a strong HI antibody response on both infected-groups with higher titers in animals belonging to the CAT09-group when compared with the group CAT09+pIL6 (Table 4).

GROUP	ANIMAL NUMBER	A/CATALONIA/63/2009 (CAT09) HIA 10dpi
CONTROL	1	-
CAT09	2	<1280
	3	<640
	4	<640
	5	<160
	6	<320
	7	<1280
CAT09+pIL-6	8	<80
	9	<160
	10	<640
	11	<160
	12	<640
	13	<320

Table 4. Serum of six animals from CAT09-group and CAT09+pIL-6-group were collected at 10 pi and antibody response against CAT09 virus was measure by HIA.

4. IL-6 concentrations on serum and lung

At days 1, 3, 5, and 10 pi, six animals from each group, including controls, were sampled to collect blood to test IL-6 secretion. Results in sera showed a statistically significant ($p<0.0001$) higher concentration of this cytokine at 1dpi on CAT09+pIL-6 (62.8 ng/ml) and pIL-6 (75.7 ng/ml) groups when compare with C and CAT09-group. A slightly less concentration was detected in CAT09+pIL-6 mice (Figure 23 A). At day 1 pi no statistically significant differences were found when the two groups treated with pIL-6 were compared. IL-6 concentration in serum (2.3 ng/ml) of CAT09+pIL-6 mice was significantly ($p<0.05$) higher at day 3 pi when compared with the rest of the groups. At day 5 pi no statistically significant differences were found whithin groups. Animals only treated with pIL-6 secreted IL-6 (3.0 ng/ml) at 10 dpt that was significantly higher ($p<0.05$) than levels in C animals and in CAT09-mice. For IL-6 concentration in lungs, necropsies were performed at day 1, 3, 5 and 10 pi. Levels of IL-6 in lungs were measured, exhibiting the highest IL-6 concentration (> 10 ng/gr) at 1 dpi. Mice in pIL-6, CAT09 and CAT09+pIL-6 showed similar levels and mice from C group also secreted IL-6 albeit at lower levels than the rest of the groups (6.8 ng/gr). On the following days, IL-6 in lungs from all groups showed no statistically significant differences whitin groups at any time-point (Figure 23 B).



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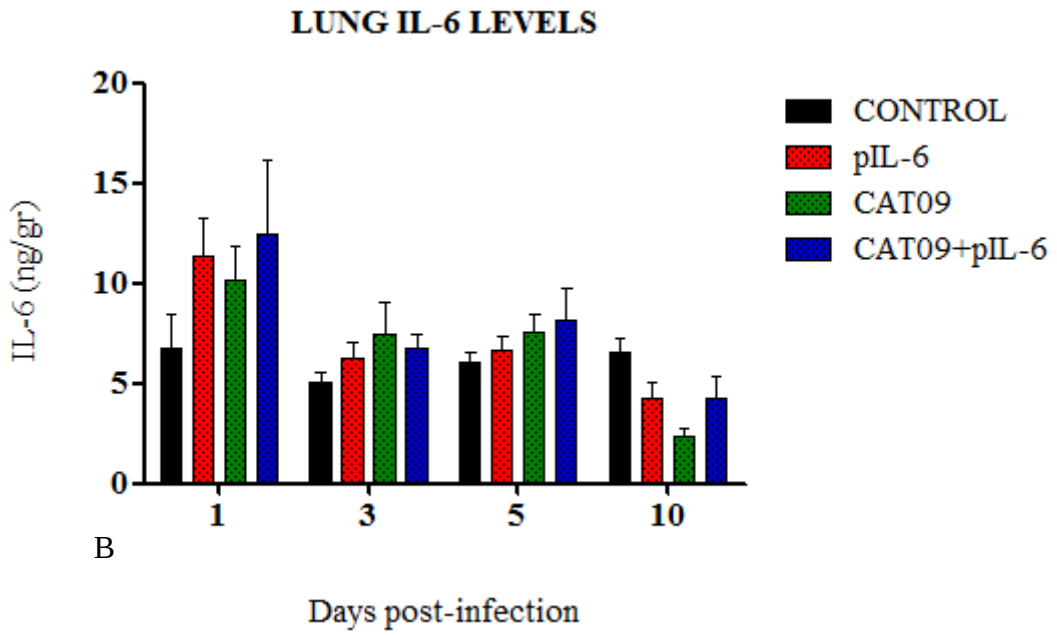
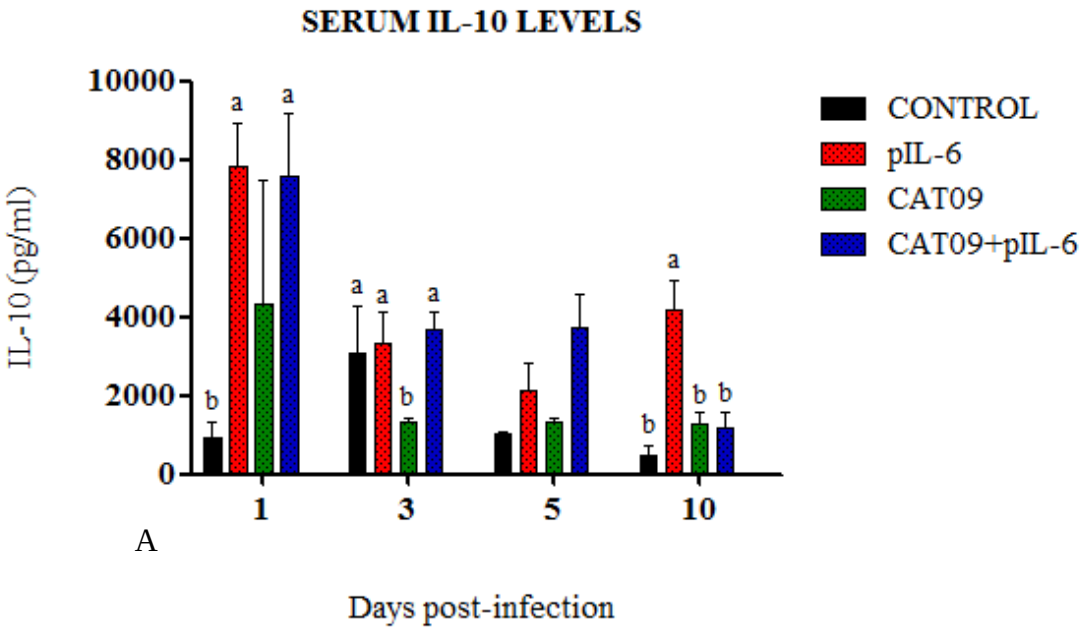


Figure 23. IL6 concentration on sera and lungs of CAT09 infected mice in presence or absence of pIL-6 treatment. At 1, 3, 5 and 10 dpi IL-6 concentration was determined. Groups were distributed as follows: Control group; pIL-6; CAT09 and CAT09+pIL-6. All values are the mean ± SEM of one experiment; a, b indicates significant differences *($p<0.05$) and *** ($p<0.0001$).

5. IL-10 concentrations on serum and lung of treated animals.

At days 1, 3, 5, and 10 pi, six animals from each group, including controls, were sampled to collect blood to test IL-10 secretion. On sera from day 1 pi, the data showed a similar concentration of IL-10 at 1 dpi on CAT09+pIL-6 mice (7572.3 pg/ml) and in pIL-6 mice (7849.9 pg/ml). At 1 dpi there was a statistically significant higher levels of IL-10 on CAT09+pIL-6 and pIL-6 ($p<0.05$) groups when compare with control group (Figure 23 A). At day 3 pi both IL-6 plasmid treated groups showed statistically significant ($p<0.05$) higher concentration of IL-10 when compared with CAT09-group. No statistically significant differences whitin groups were found at day 5 pi. IL-10 concentration on serum from day 10 pi of pIL-6 treated mice was significantly ($p<0.05$) higher when compared with the rest of the groups (Figure 24 A). To measure IL-10 concentration on lungs, necropsies were performed at days 1, 3, 5 and 10 pi. Levels of IL-10 in lungs were measured but no statistically significant differences were found at

days 1 and 3 pi. At day 5 pi, pIL-6-treated mice showed significantly ($p<0.05$) higher levels of IL-10 when compared with CAT09+pIL-6-group. Mice from both pIL-6-treated and control groups secreted statistically significant ($p<0.05$) higher levels of IL-10 at day 10 pi when compared with CAT09 animals (Figure 24 B).



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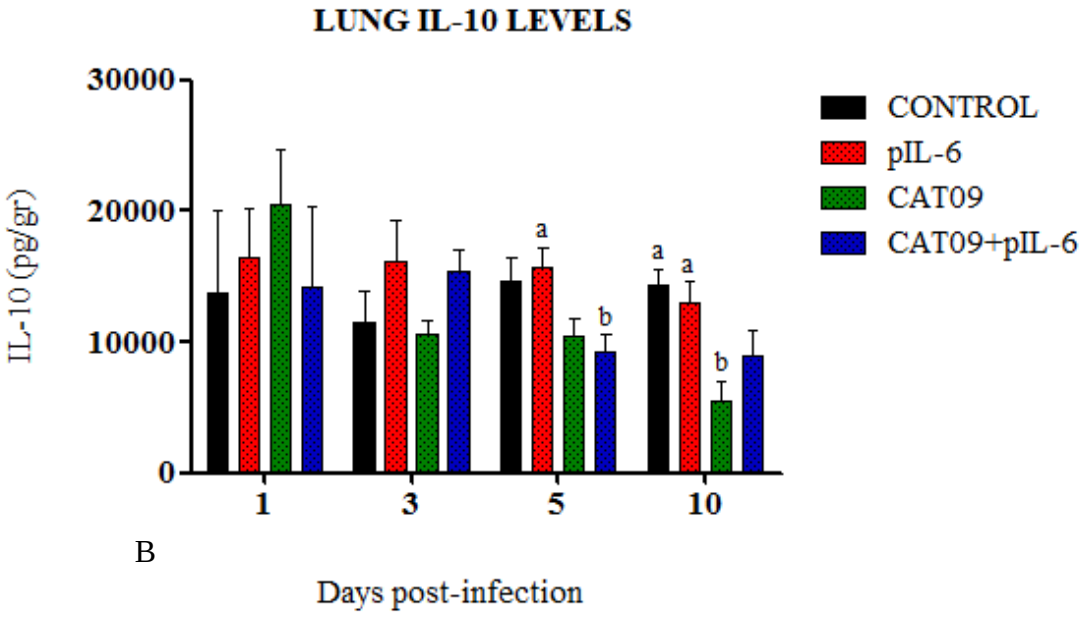


B

Figure 24. IL-10 concentration on sera and lung of CAT09 infected mice in presence or absence of pIL-6 treatment. At 1, 3, 5 and 10 dpi IL-6 concentration was determined. Groups were distributed as follows: Control group; pIL-6; CAT09 and CAT09+pIL-6. All values are the mean ± SEM of one experiment; a, b indicates significant differences ($p<0.05$).

6. Histopathology

Lung tissues were histopathologically examined. Control animals did not present any histopathological lesions. At 1 dpi treated and/or infected groups did not present hispathological lesions. At 3 dpi, only 3 animals (n=5) of the CAT09-infected presented mild necrotizing bronchiolitis. At 5 dpi both infected groups presented mild to severe necrotizing bronchiolitis. No histopathological lesions were observed in animals only treated with pIL-6 at any time-point. At 10 dpi all CAT09-infected animals presented severe interstitial pneumonia whereas animals both infected and treated with pIL-6 presented lesions that went from a mild necrotizing bronchiolitis (1 in 5) to an interstitial pneumonia (2 in 5) and 2 animals from CAT09+pIL-6 did not presented any lesion (Figure 25).

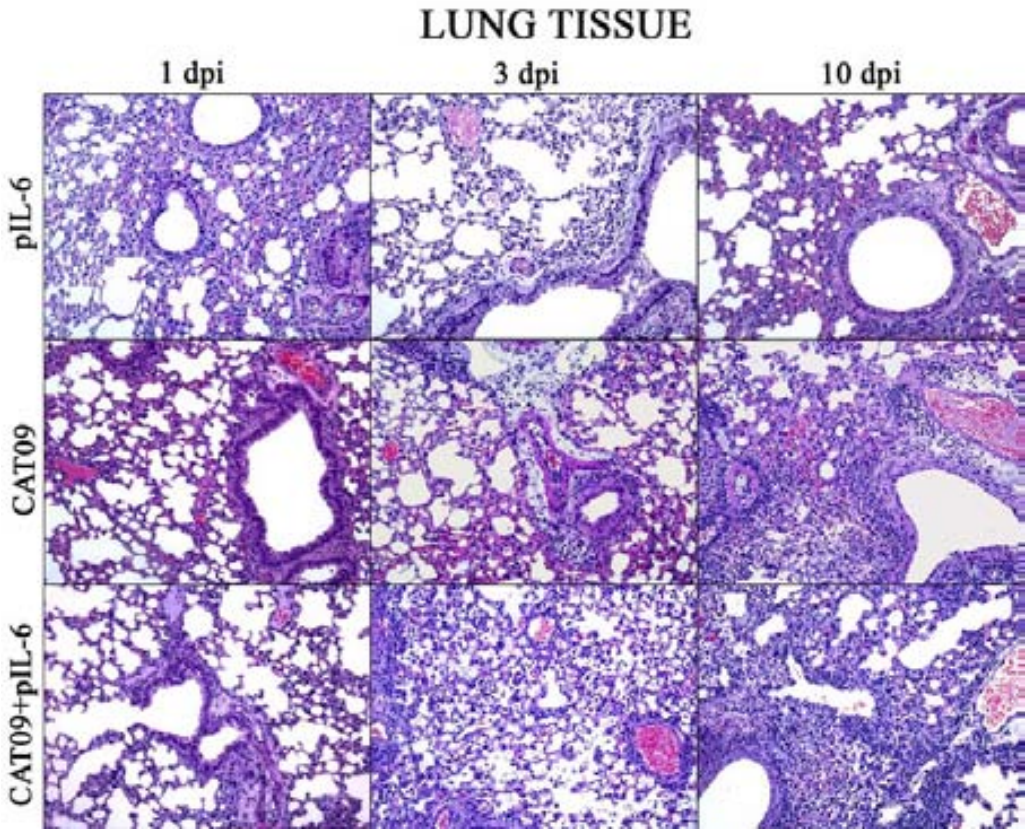


Figure 25 Histopathology of mice belonging to pIL-6, CAT09 and CAT09+pIL-6 groups. No histopathological lesions were observed in any group at 1 dpi. At 5 dpi both infected groups presented mild to severe necrotizing bronchiolitis. No histopathological lesions were observed in animals only treated with pIL-6 at any time-point. At 10 dpi all CAT09-infected animals presented severe interstitial pneumonia whereas mice from CAT09+pIL-6 group presented lesions that went from a mild necrotizing bronchiolitis to an interstitial pneumonia. Hematoxilin/Eosin stain.

All in all, the role of IL-6 during a pdmH1N1 2009 virus infection investigated on this study through inoculation of pIL-6 in mice. Results showed that the use of pIL-6 affected weight loss (Figure 21). Interestingly, when viral load from both infected groups were compared, animals treated with pIL-6 and infected with the CAT09 virus showed viral replication only at 1 dpi, whereas CAT09 mice exhibited detectable viral levels at day 1 and 3 pi (Figure 22). There was a strong antibody response on infected animals, being mice only CAT09 infected without pIL-6 treatment the ones with the highest HI titers (Table 4). Once a viral infection is established, the immune system released a cocktail of cytokine in orden to fight the pathological process. During the experimental influenza infection performed on this study, two important cytokines as IL-6 and IL-10 were measured. IL-6 levels were highly induced after pIL-6 treatment in serum, which were not that dramatic in lungs (Figure 23). Interestingly, IL-10 levels paralleled IL-6 levels in serum and lungs (Figure 24). Histopathological lesions were more severe in CAT09+pIL-6 group (Figure 25).

7. THIRD STUDY:

IN VITRO AND *IN VIVO* STUDIES ON pdmH1N1 2009-OSELTAMIVIR RESISTANT VIRUS IN MICE

Between the varieties of NAIs, Oseltamivir corresponds to the most widely used agent to treat influenza disease. The action mechanism of the drug disables the virion progeny release [191]. In general term, cases of oseltamivir-resistance pdmH1N1 2009 infection have been characterized by an uncomplicated, mild respiratory disorder, although in some reports, the pathogenicity of these viruses has been described higher or comparable than that of seasonal influenza viruses in mice [192] and humans [193]. During the 2009 pandemic outbreak, several cases of Oseltamivir resistance viruses characterized by a H275Y mutation were observed, becoming a risk for human health [193]. Previous studies on H275Y mutation of influenza viruses suggested that resistant virus variants with the same neuraminidase mutation may differ in fitness [156, 194]. However differences between pdmH1N1 2009 Oseltamivir-resistance viruses are not well established. Therefore, a better understanding of circulating strains of pdmH1N1 2009 Oseltamivir-resistance might be crucial to improve patient treatment.

II. HYPOTHESIS AND SPECIFIC OBJECTIVES

Hypothesis

We hypothesize that oseltamivir resistance viruses on circulation presented different fitness that might confer them different levels of virulence depending on the strain. Two viruses with the H275Y mutation were isolated and *in vitro* and *in vivo* studies were performed.

Specific objectives

- To compare *in vitro* viral growth of the oseltamivir-sensitive and resistant pdmH1N1 2009 viruses
- To infect mice with the two strains of oseltamivir-sensitive and resistant pdmH1N1 2009 viruses for:
    - Evaluation of mortality, pathology clinical signs and viral load in lung of infected animals at different time-points.
    - Comparison of antibody response in infected animals and cytokine profile (IL-6 and IL-10)

III. MATERIALS AND METHODS

i. Cell line and virus propagation

MDCK cells were cultured for virus propagation and virus titration as previously described in chapter 5.

ii. OsR viruses

Two different strains of pdmH1N1 2009 Oseltamivir resistance viruses were used. They were isolated at the National Influenza Centre (CNM, ISCIII) from respiratory samples sent by the Spanish Influenza Surveillance System for virological characterization. Both viruses possessed the H275Y mutation and were named as follows: A/Baleares/RR6121/2009 (R6) and A/Madrid/RR7495/2011 (R7). R6 was isolated from a leukemic patient who died after influenza infection and R7 was isolated from a clinically severe patient who survived the infection. Oseltamivir resistance viruses were passaged fourth times on MDCK cell. R6 and R7 viral titer was $10^{7.8}$ $TCID_{50}$/ml and $10^{7.3}$ $TCID_{50}$/ml respectively. By plaque assay viral titers were 10^5 PFU/ml and 10^6 PFU/ml respectively. The pdmH1N1 2009 virus called F (A/CastillaLaMancha/RR5911/2009) had a viral titer of 10^5 PFU/ml and $10^{7.1}$ $TCID_{50}$/ml.

iii. *In vitro* infection

MDCK cells were infected at 0.01 MOI (multiplicity of infection) with oseltamivir resistant pdmH1N1 2009 viruses R6 (RR6121) and R7 (RR7495). At 0, 6, 12, 24 and 48 hours post-infection supernatants were collected and PFU viral load was determined.

iv. In vivo infection

a. Ethics statement

As described previously in chapter 5

b. Mice infection and sampling

Sixty female C57BL6/JOlaHsd mice (7 weeks old) (Harlan Laboratories, Barcelona, Spain) were distributed in 3 groups of 20 animals each one. Distribution was done as follows: control group (C), R6 infected-group (R6) and R7-infected-group (R7). Each virus was inoculated separately whereas control group remain uninfected. Intranasal inoculation using a dose of 10^3 PFU/ml was performed. Serum and lung samples were collected at 0, 4, 7 and 14 dpi. Lungs were dissected from dead animal using the standard surgical procedures. Samples were obtained as described in chapter 5.

c. Cytoquine detection by ELISA

Serum samples and supernatant of homogenated lungs were assayed using IL-6 and IL-10 ELISA kit described in chapter 5.

d. Determination of viral load in tissues

Lung samples were collected and viral load was determined as described in chapter 5.

e. Hemagglutination Inhibition (HI) Assay

HI assay technics was performed as previously described in chapter 6.

f. Histopathology

All mice tissue samples were histopatologically analyzed as described in chapter 5.

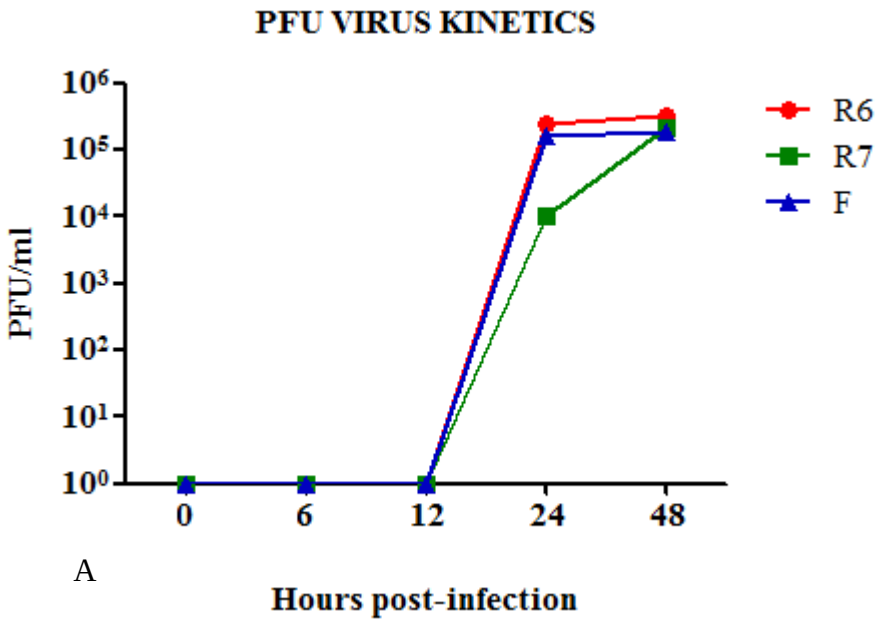
g. Statistical analysis

It was performed as described in chapter 5.

IV. RESULTS

i. *In vitro* viral growth of oseltamivir-sensitive and resistant pdmH1N1 2009 viruses

OsR R6 virus (A/Baleares/RR6121/2009), R7 virus (A/Madrid/RR7495/2011) and F virus (A/CastillaLaMancha/RR5911/2009) as a representative of pdmH1N1 2009 without H275Y mutation, were used in the experiments. Cultures of MDCK cells were infected with R6, R7 and F at low multiplicity (0.01 MOI) of infection and viral titers were determined at different hpi. Viral replication kinetics for R6, R7 and F viruses was examined by PFU plaque assay (Figure 26 A and B). At 24 hpi, supernatants of infected cells showed presence of infective viral particles at similar levels in the case of R6 and F virus. R6 viral titers ($2.4x10^5$ PFU/ml) and F ($1.6x10^5$ PFU/ml) were higher when compare with R7 ($1x10^4$ PFU/ml) (Figure 26 A). No differences were observed in supernatants of infected cells at 48 hpi. When viral titres were measure by $TCID_{50}$ assay (Figure 26 B) supernatants of F-infected cells showed an early cytophatic effect at soon as 6 hpi. The progeny from R6 virus was detected as early as 12 hpi. At 12 hpi F virus showed higher viral titers ($10^{4.6}$ $TCID_{50}$/ml) when compared with R6 ($10^{3.3}$ $TCID_{50}$/ml). At 24 hpi, higher viral titers were observed on R6 ($10^{6.9}$ $TCID_{50}$/ml) and F ($10^{6.8}$ $TCID_{50}$/ml) infected cells when compare with R7 ($10^{5.8}$ $TCID_{50}$/ml) (Figure 26 B). Viral production was undetectable before 24 hpi on R7-infected cells. However, both OsR viruses (R6 and R7) reached similar titers at 48 hpi, $10^{6.1}$ $TCID_{50}$/ml and $10^{6.8}$ $TCID_{50}$/ml respectively. In conclusion, R6 virus seems to replicate faster than R7 virus but lower than F virus.



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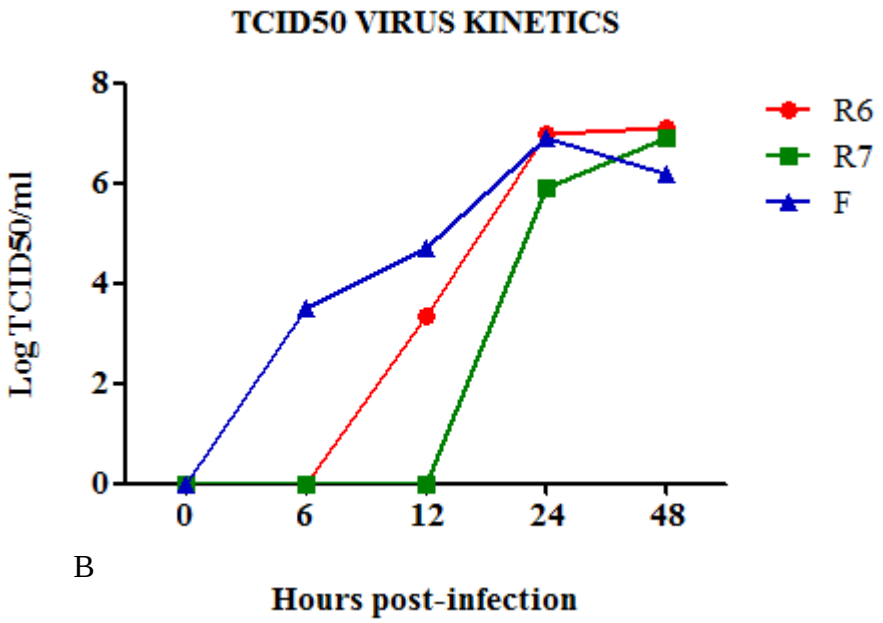

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Figure 26. *In vitro* virus kinetics of pdmH1N12009 Oseltamivir resistance viruses (R6 and R7) were compared with a non resistance pdmH1N1 2009 virus (F) and viral titer was determined at 0, 6, 12, 48 hpi by plaque assay (A) and by $TCID_{50}$ (B).

ii. R6 and R7 infection in Mice

Based on to the fact that R6 virus replication was faster than R7 virus in cell cultures, we examined the *in vivo* relevance and the consequences of this difference in mice. C57BL6 mice were intranasally infected with 10^3 PFU of R6 or R7 viruses and a control group remained untreated. Survival and body weight were monitored daily for 14 days. No loss of body weight was observed in untreated mice whereas R6 infected mice had a peak of weight loss at day 4 pi and R7 infected mice experimented a similar peak but three days later, at day 7 pi. R6-infected mice showed a statistically significant ($p<0.05$) higher percentage of weight loss during the first 2 dpi when compare with R7-infected mice. Both infected groups showed a statistically significant higher percentage of weight loss from days 3 to 7 pi when compared with control animals ($p<0.05$). The statistical analysis of days 8 to 14 pi did not showed any differences whithin groups; however, it is important to highlight that the recovery of body weight was slower in R6 group whereas R7 infected mice started to increase body weight at 7 dpi (Fig. 27 A). Importantly, 40% lethality on R6-infected mice was observed a 4 dpi in comparison with 20% lethality on R7-infected animals. On the following 2 dpi R6-infected maintained the 40% lethality and R7-infected maintained the 30% lethality. From day 7 to 14 pi both infected groups was showed a 50% of lethality. Percentatge of survival was a statistically significant ($p<0.0001$) on control animals when compared with OsR virus from day 4 pi until the end of protocol. All untreated mice survived along to the protocol (Fig. 27 B).

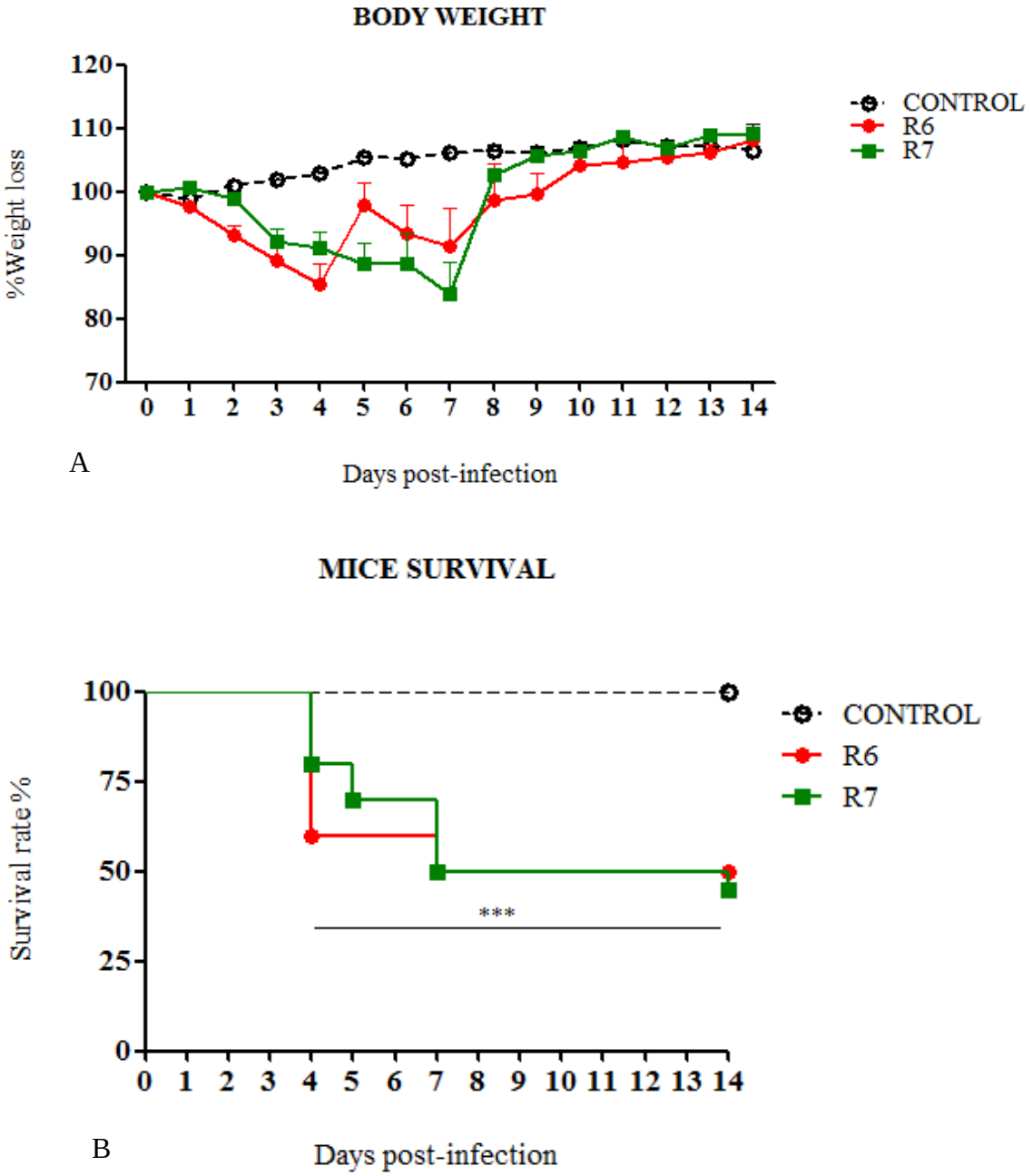


Figure 27. Influence on body weight (A) and survival (B) of OsR-infected mice. Groups of 20 animals were distributed as follows: Control group, R6-infected mice and R7 infected-mice. All values are from (A) are the mean ± SEM of one experiment. (B)*** Indicates significant differences within OsR-infected groups and control mice.

iii. Virus Replication

Since IV primarily infects lungs, samples of lungs from five infected animals were used to determine viral titers at different time-points. At 7 dpi virus titer reached its maximum value on R7-infected mice. At all time-points a higher virus load was detected in lungs from R7-infected mice; however, no statisticall significant differences were observed whitin infected groups (Figure 28). The presence of virus was increased gradually in the lungs from 3 to 7 dpi in R7 mice and for R6 mice viral load was reduced at 7 dpi, by 14 dpi no infectious virus was detectable on both infected groups (data not shown).

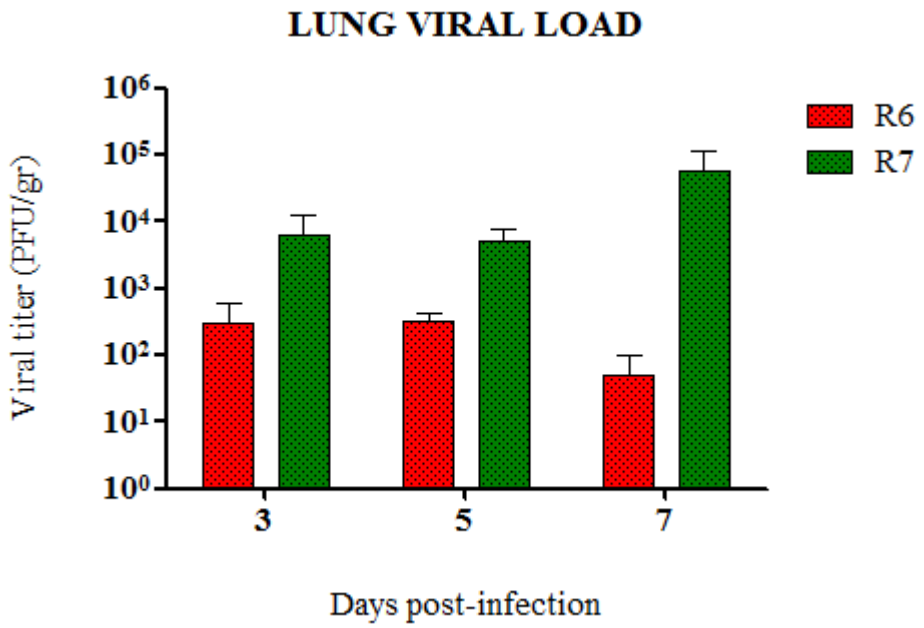


Figure 28. Virus load in lungs from pdmH1N1 Oseltamivir resistance-infected mice. Viral load of lung of six infected-animals per group was determined (R6-infected mice and R7-infected mice). All values are the mean and ± SEM of one experiment.

iv. Antibody response

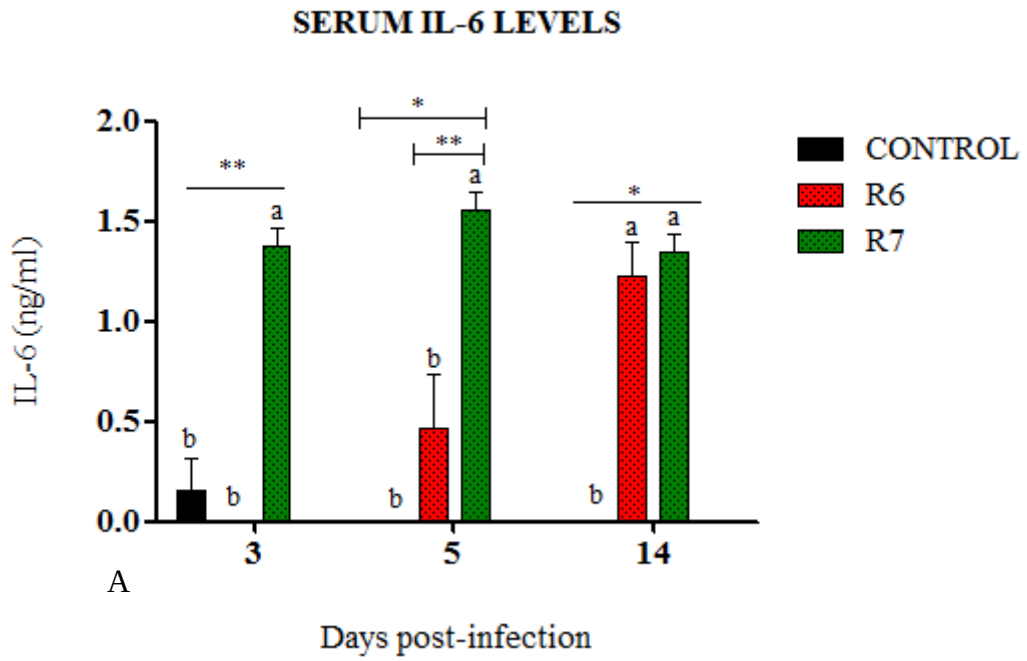
Antibody response to R6 and R7 viruses was determined by HAI assay. Sera from days 7 and 14 pi were tested. Uninfected C mice were seronegative at all time-points. Two animals from R6 group and three animals from R7 group revealed an early presence of hemagglutinin inhibiting antibodies as soon as 7 dpi. There was a strong HI antibody response on both infected-groups towards the virus used for infection in each group; however, no crossreactive antibodies were detected at 14 dpi (Table 5).

GROUP	ANIMAL NUMBER	A/Baleares/RR6121/2009 (R6)		A/Madrid/RR7495/2011 (R7)	
		7dpi	14dpi	7dpi	14dpi
CONTROL	1		-		-
R6	2	<160		<10	
	3	<80		<10	
	4		<160		<40
	5		<80		<40
	6		<320		<40
	7		<80		<40
R7	7	<10		<20	
	8	<10		<40	
	9	<10		<40	
	10	<40		<160	
	11		<40		<80
	12		<80		<320

Table 5. Sera from six R6 and R7- infected animals were collected and at days 7 and 14 pi and antibody response against OsR viruses was measure by HIA.

v. IL-6 levels on OsR virus infected mice

IL-6 levels in lungs and serum of C57BL6 infected mice (n=5) were measured to investigate whether there might be any correlation with this cytokine (Figure 29). Interestingly, higher values of this cytokine were observed at all time-point in sera from R7-infected animals when compared with R6-infected mice. From the statistical point of view there was a significant higher amount of IL-6 in serum from R7-infected mice at days 3 and 5 pi. At day 3 pi R7 infected mice exhibited 1.3 ng/ml of IL-6 vs R6-infected mice that secreted levels of IL-6 similar to C samples (0.1 ng/ml). Different amounts of IL-6 were found also at day 5 pi when animals from R7-group exhibited 1.5 ng/ml and their counterparts had levels of 0.4 ng/ml. Absence of IL-6 or very low levels were detected on C animals at any time-point (Figure 29 A). Conversely, lungs from infected animals showed similar concentration of IL-6 than control animals without statistical significant differences within groups at any timepoint (Figure 29 B).



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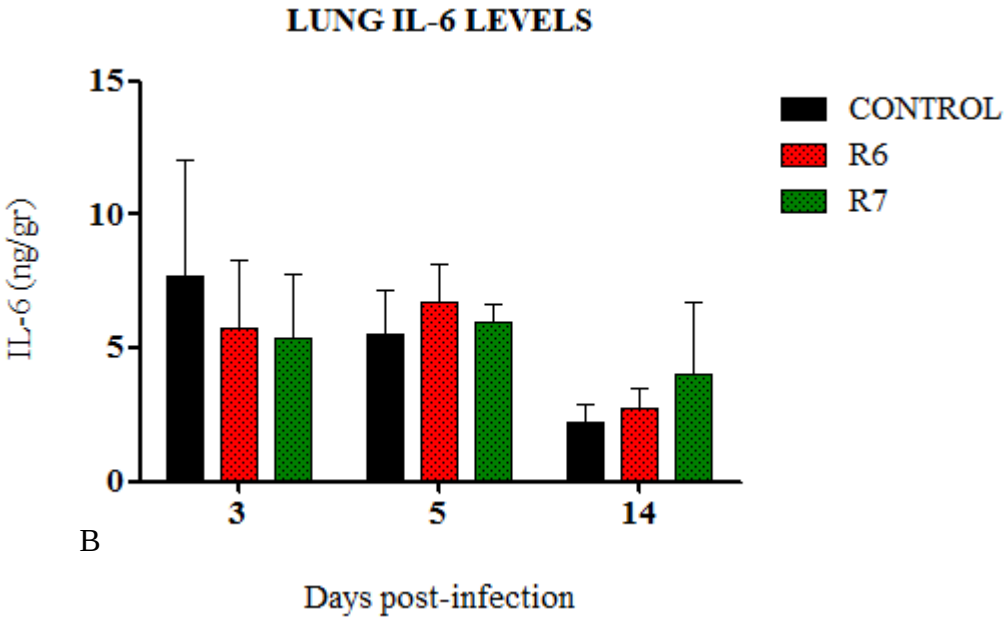


Figure 29. IL-6 levels in serum (A) and lungs (B) from OsR-infected mice. Groups of 20 mice were distributed as follows: Control group; R6-infected mice and R7-infected mice. All values are the mean and ±SEM of one experiment; a, b indicates significant differences *($p<0.05$), **($p<0.001$).

vi. IL-10 levels on OsR virus infected mice

IL-10 analysis in lungs and serum of C57BL6 infected mice (n= 5) were performed (Figure 30). Statistically significant ($p<0.05$) highest concentrations of IL-10 were detected in sera from R7-infected mice (2086.03 pg/ml) when compared with C animals (1684.1 pg/ml) at day 3 pi (Figure 30 A). At 14 dpi, a statistical tendency ($p<0.1$) was found when R7-group was compared with samples from C mice (Figure 30 A). No statistical differences where found between infected groups were compared at day 14 dpi. Regarding local responses in lungs from infected mice, statistically significant ($p<0.05$) higher concentration of IL-10 was detected at day 5 pi on R7-infected animals (28855.1 pg/gr) when compared R6- infected mice (13901.2 pg/gr) (Figure 30 B). Lung of controls animals showed higher concentration of IL-10 than at all time-points with no statistical differences.

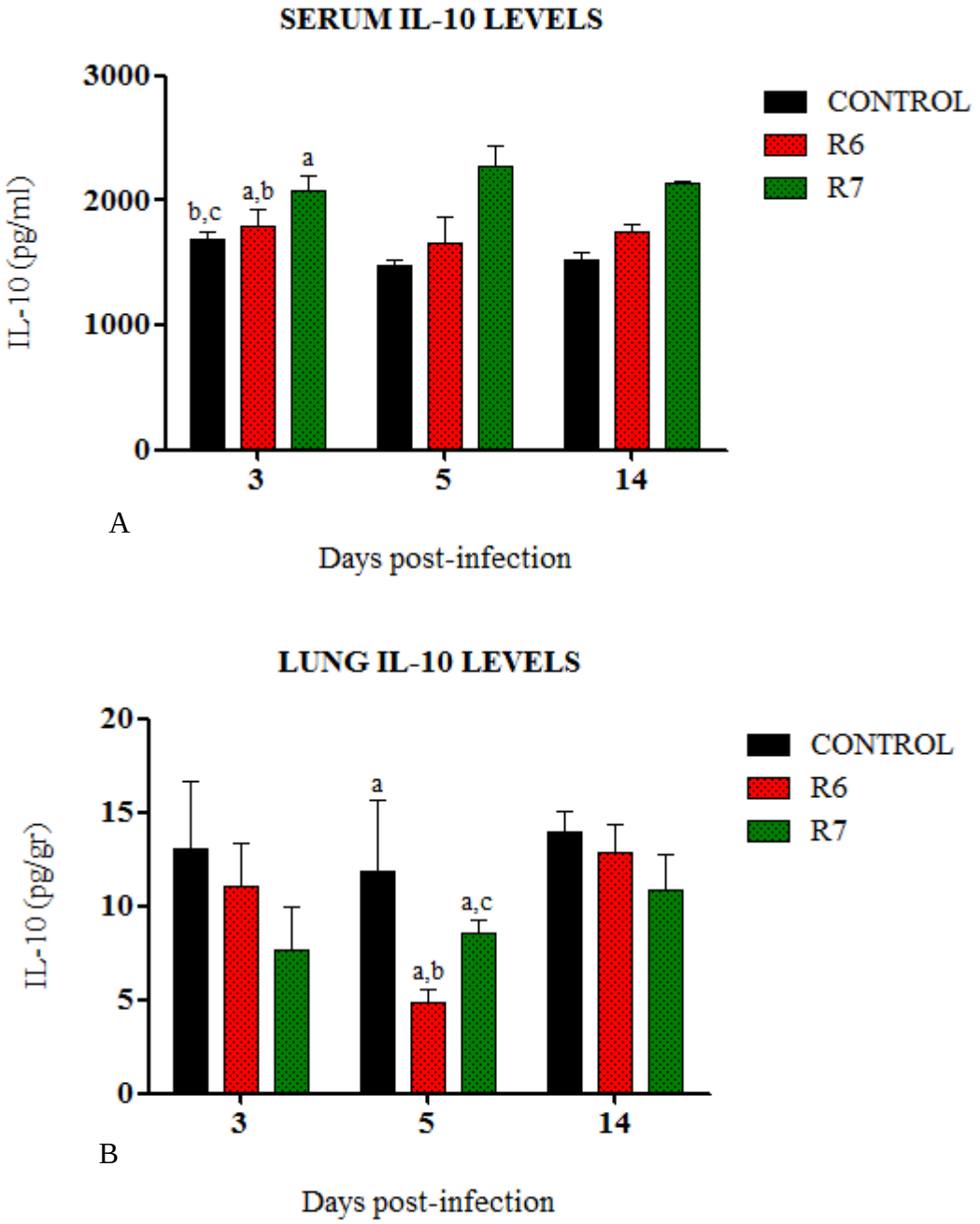


Figure 30. IL-10 levels in serum (A) and lungs (B) from OsR- infected mice. Groups of 20 mice were distributed as follows: Control group; R6-infected mice and R7-infected mice. All values are the mean and ± SEM of one experiment; a, b, c indicates significant differences *($p<0.05$).

vii. Histopathology

Lung tissues were histopathologically examined. Control animals did not present any histopathological lesions in the lungs during the experiment at any time-point. At 3 and 14 dpi all infected animal did not present histopathological lesions. All R6-infected animals presented severe interstitial pneumonia at 5 dpi whereas in the group of R7-infected animals, four mice presented interstitial pneumonia and three animals exhibited necrotizing bronchiolitis at 5 dpi. At 7 dpi both R6 and R7-infected mice mainly presented interstitial pneumonia (Figure 31).

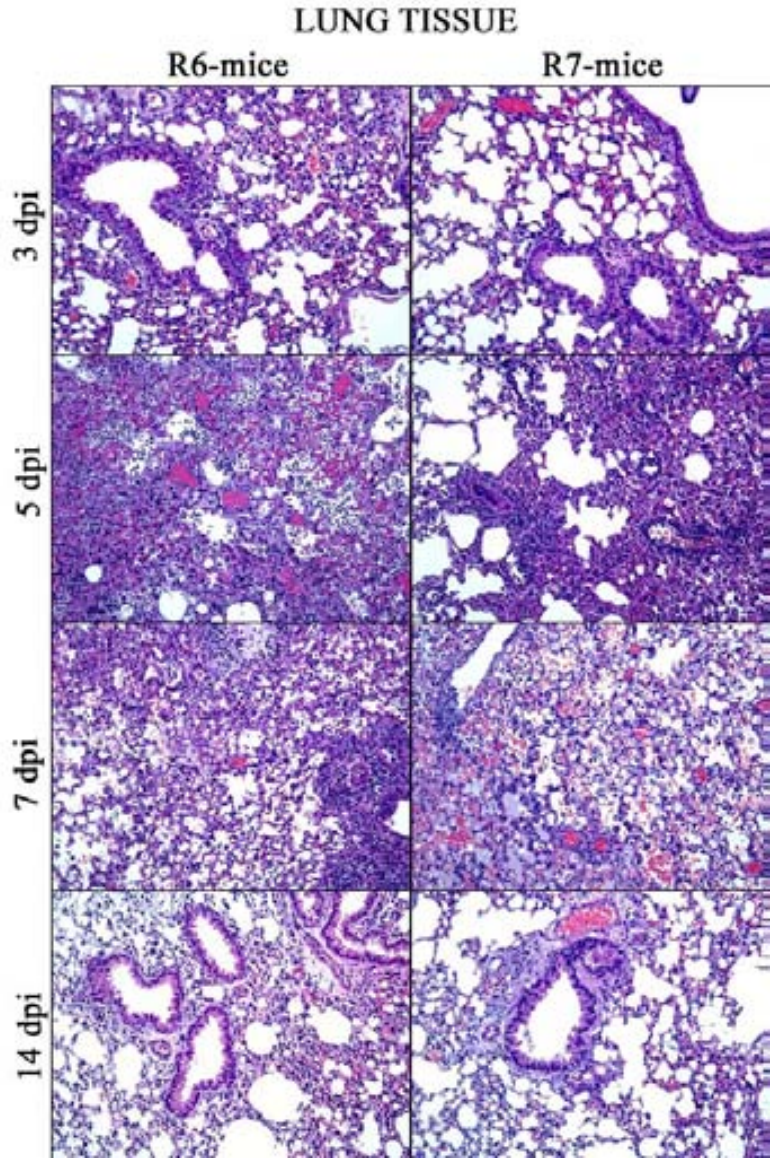


Figure 31 Histopathology, lung tissues were histopathologically examined. Control animals did not present any histopathological lesions at any time-point. At 3 and 14 dpi all infected animal did not presented histopathological lesions. At 5 dpi all R6-infected animals presented severe interstitial pneumonia. Lesion in lung of R7-group were from interstitial pneumonia to necrotizing bronchiolitis at 5 dpi. Hematoxilin/Eosin stain.

In summary, results obtained *in vitro* showed a different fitness in viral replication in the three virus studied, being F>R6>R7 (Figure 26). When these virus were studied *in vivo* both OsR strains produced a fatal outcome although on different magnitudes and kinetics (Figure 27). R6-infected group experimented a 40% of lethality and R7-group a 20% at 4 dpi. However, at 7 dpi the percentage of survival was a 50% for both OsR-infected groups. Viral replication detected in lungs from OsR-infected groups had higher values for R7 than for R6, however not statisticall differences were observed within infected groups (Figure 28). An early antibody response was detected at 7 dpi in 2 animals from R6-group and 3 animals from R7-group by HIA assay. There was a strong antibody response at 14 dpi on both infected groups for each virus but no crossreactive antibodies (Table 5). To go further insight into the immunological response towards OsR viruses, levels of IL-6 and IL-10 at 3, 5 and 14 dpi were studied. Interstingly, high levels of IL-6 were detected in serum from R7-mice with significant differences at days 3 and 5 pi when compared with serum from R6-mice and controls. Surprisingly, levels of IL-6 in lungs of R6, R7 and control animals were similar at all time-point with no statistical differences (Figure 29). Serum and lung IL-10 had also slightly higher values in R7-mice when compared with controls at 3 and 5 dpi respectively (Figure 30), levels of IL-10 in serum were similar in both OsR groups, in lung there was a statistically significant higher amount in R7 mice when compared to R6 mice. Histopahological findings showed more severe lesions on R6-mice at 5 dpi (Figure 31).

8. FOURTH STUDY:

pdmH1N1 2009 INFLUENZA INFECTION IN FERRETS FROM

A MILD AND FATAL CASE

The majority of pdmH1N1 2009 IVs caused mild symptoms in most infected patients; however, few cases required more exhaustive medical care including hospitalization and specialized attention in intensive care units (ICUs). During seasonal influenza, the risk group associated to severe patient is focused in the extreme ages of life (children and the elderly); nevertheless, in the case of pandemic severe affected patients, age ratio was observed in healthy young adults and children without comorbid conditions [110,112].

Pandemic influenza infected ferrets present clear clinical signs from mild to severe, including fatal outcome and therefore, ferrets can be considered a good model to mimic the immunopathogenesis observed on human pandemic infected patients [195]. Several previous studies support the use of this animal model to reproduce the variability of clinical signs found in the human population [175,196]. Previous information in *in vitro* and *in vivo* studies in mice and experimental infection in mice showed a difference on pathogenicity/virulence when IV strains from a fatal and a mild case were compared [197]. Altogether, actual data indicate that strains of pdmH1N1 2009 virus with enhanced pathogenicity circulated during the 2009 pandemic.

II. HYPOTHESIS AND SPECIFIC OBJECTIVES

Hypothesis

The majority of pdmH1N1 2009 influenza viruses caused mild symptoms in most infected patients, however, a greater rate of severe disease was observed in healthy young adults and children without comorbid conditions. The purpose of this work was to study two contemporary different strains of pdmH1N1 2009 virus in ferrets from two patients without known co-morbid conditions, one with fatal consecuences (F) and other who only showed mild respiratory disease (M).

Specific objectives

- To infect ferrets with two strains of pdmH1N1 2009 virus from a mild (M) and fatal (F) case.
- To perform a broad clinical score to evaluate clinical aspects of pdmH1N1 2009 in ferrets.
- To measure acute phase proteins (Haptoglobin and Serum Amyloid A) levels in serum and lungs from control and pdmH1N1 2009 infected animals.
- To evaluate antibody responses, viral load in lungs, trachea and broncheoalveolar lavage in pdmH1N1 2009 infected animals.
- To asses histopathological changes during pdmH1N1 2009 infection.

III. MATERIALS AND METHODS

i. Cell line and virus propagation

MDCK cells were cultured for virus propagation and virus titration as previously described in chapter 5.

ii. Viral Load

$TCID_{50}$ and PFU determination was performed as previously described in chapter 5.

iii. Virus

All virus strains obtained from pdmH1N1 2009 infected patients were isolated using embryonated specific pathogen free (SPF) and subsequently multiplied on MDCK following the procedures of International Organization of Epizooties (OIE). All viral stocks were stored at -80ºC at the BSL3 facilities at CReSA.

Two distinct pdmH1N1 2009 influenza viruses named A/CastillaLaMancha/RR5661/2009 (M) and A/CastillaLaMancha/RR5911/2009 (F), were isolated at the National Influenza Centre (CNM, ISCIII) from respiratory samples sent by the Spanish Influenza Surveillance System for virological characterization. Virus M was isolated from a patient who showed mild clinical signs of influenza and virus F was isolated from patient who developed an infection with fatal consequence, neither patient presented previous pathology at the moment of infection. Both viruses were thoroughly described in Rodriguez et al. [197]. Genetic characterization of M and F viruses was performed by our collaborators from Instituto de Salud Carlos III through ultrasequencing of purified virion RNAs obtained after two passages in MDCK cells. The consensus sequences obtained for the viral genomes are described on Table 6 from Rodriguez et al. [197]. Influenza viruses M and F were expanded in MDCK three times. M virus had a titre of $10^{8,3}$ $TCID_{50}$/ml and F virus had a titre of $10^{8,2}$ $TCID_{50}$/ml.

Segment	M	F	Consensus
PB2	221A	221T	221A (1007) 99,7%
PA	269K	269R	269R (1004) 96,94%
	328K	328R	328K (1014) 99,4%
	529D	529N	529D (1014) 99,9%
NP	400K	400R	400K (1023) 96.2%
HA	38E	38K	38K (1909) 99,8%
	127S	127L	127S (1909) 99,7%
	226N	226K	226K (1909) 99%
NA	274F	274Y	274Y (1553) 99,9%

Table 6. Amino acid differences between M and F viruses. Residues found in M or F viruses are represented in blue or red respectively. ‘‘Consensus’’ represents an amino acid sequence obtained using the influenza virus resource database from NCBI and including around one thousand pdmH1N1 2009 viruses isolated in the time frame of one month before and after the isolation date of M and F viruses. Numbers in parenthesis represent the number of examined sequences, followed by the percentage of the corresponding amino acid present in these sequences. Adapted from Rodriguez et al. [197].

iv. Ethics statement

All experiments were performed under a reviewed and approved protocol (nº 1976) by “Comissió d’Ètica en l’Experimentació Animal I Humana de la Universitat Autònoma de Barcelona”. Ferrets were housed in groups on experimental isolation rooms at the biosafety level 3 facilities of the Centre de Recerca en Sanitat Animal (CReSA, Barcelona, Spain). All ferrets were daily monitored for clinical signs and any animal determined to be in a moribund state, was ethically euthanized.

v. Animals and infection

Fourteen adult ferrets (*Mustela putorius furo*) under 24 month-old were numbered from 1 to 14 were randomly selected from a stable, purposely bred colony (Isoquimen, Spain). Ferrets were randomly assigned to different experimental groups and those groups separated into experimental isolation rooms and then kept for one week in acclimation. Animals were kept in standard housing cages and provided with commercial food pellets and tap water *ad libitum* throughout the experiment. Animals were divided into three groups, Control group (1) included two ferrets, number 1(male) and number 2 (female) and they were inoculated intratracheally with PBS. Group 2 including animals infected with M strain numbered from 3 to 8, included 4 males (ferrets 3 to 6) and two females (ferrets 7 and 8). Group 3 included animals infected with F strain numbered from 9 to 14 and all animals from this group were males. Ferrets were intratracheally inoculated with 10^6 $TCID_{50}$/ml of the corresponding virus. All ferrets were proven seronegative at 0 dpi by ID ScreenH Influenza A Antibody Competition ELISA (ID VET, France).

vi. Clinical score

Ferrets were monitored daily for clinical signs which were recorded according to parameters defined in Table 7. Changes in rectal temperature and body weight were measured at approximately the same time each day, around 10-11am. Any animal losing 25% of its day 0 body weight and/or exhibiting a scoring of more than 20 points or determined to be in a moribund state was humanely euthanized.

Clinical score	0	1	2	3
Playful	normal	mild apathy	apathetic	
Arched back	normal	arched		
Bristly hair	no bristly	bristly tail	bristly tail and back	all body bristly
Sneezing	no	sporadic	frecuent	
Nasal secretion	no secretion	low	abundant	
Conjunctival secretion	no secretion	low	abundant	
Breathing rate	normal (40-50)	50-70	70<	
Auscultation	normal	anomalous		
Lost weight	no lost weight	(1-5%)	(5-10%)	(>10)
Temperature	normal (37,5-39,4)	hyperthermia (39.5-40°C)	(>40)	<37
Mucous	normal	congestive		
Heart rate	<250	250-300	300 <	

Table 7. Clinical Score. Animals were monitored daily at the same time for clinical observations

vii. Sampling

Samples of nasal swabs, oral swabs, blood and serum were collected at 0, 2, 4, 7, 10 and 14 dpi. Two animals of groups 2 and 3 were euthanized at 4, 7 and 14 dpi and samples of lungs, trachea and nasal turbinates were collected.

viii. Blood collection

All the ferrets were sedated with 0.5 mg/kg Butorphanol administered subcutaneously for blood collection. Two ml of blood were taken from Cava Cranial Vein at 0, 2, 4, 7, 10 and 14 dpi. Samples were collected into a 1ml blood-heparine tubes for Acute Phase Protein (APP) determination. Heparin blood samples were centrifugated at 3000rpm for 10 min at 4 Cº to separate plasma sample. EDTA blood samples were analyzed the same day of extraction.

ix. Acute phase proteins

Acute phase proteins were determined by commercial ELISAs according to the manufacturer's recommendation (Haptoglobin Assay and Multispecies Serum Amyloid A Immunoassay, both from Tridelta Development Ltd, County Kildare, Ireland). Serum samples were tested in duplicate.

x. Determination of viral load in tissues

Tissue samples were collected, snap frozen on dry ice and stored at –80°C until further processing. Tissue samples were weighed, homogenized and centrifuged briefly. pdmH1N1 2009 virus infectivity was determined as previously described plaque assay in MDCK cells in chapter 5.

xi. Hemagglutination Inhibition (HI) Assay

Antibodies against IV were measured using a HI assay as described in chapter 6.

xii. Histopathology and Immunohistochemistry

A complete necropsy was performed on all animals immediately after euthanasia. The following tissues were collected for histological examination: right lung, nasal turbinate, trachea and mesenteric lymph node. Lung samples were taken in a standardized way. Tissue samples were fixed for 48 h in neutral-buffered 10% formalin. They were then embedded in paraffin wax, sectioned at 3 μm, and stained with haematoxylin and eosin (HE) for histopathological assessment.

Influenza A virus antigen detection was performed in tissues stained with a primary antibody against the influenza A nucleoprotein (NP). Briefly, paraffin-embedded samples were sectioned at 3 μm thick, dewaxed and treated with 3% H_2O_2 in methanol to eliminate the endogenous peroxidase. Then, sections were treated with protease at 37°C for 10 min and blocked with 2% bovine serum albumin (85040C, Sigma-Aldrich Quimica, S.A., Spain) for one hour. Later, tissues were incubated with the primary monoclonal antibody anti-NP Influenza A virus (ATCC, HB-65, H16L-10-4R5) diluted 1:250, at 4°C overnight. After being rinsed, samples were incubated with biotinylated goat anti-mouse IgG secondary antibody (Dako, immunoglobulins AS, Glostrup, Denmark), followed by incubation with avidin-biotin-peroxidase complex (ABC) (Thermo Fisher Scientific, Rockford, IL, USA). The reaction was developed with 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (brown colour) (Sigma-Aldrich, Madrid, Spain) at room temperature, followed by counterstaining with Mayer's haematoxylin. Swine lung sections from pig experimentally infected with Influenza A virus, were used as positive controls. Same sections in which the specific primary antibodies were substituted with PBS were used as negative controls.

xiii. Statistical analysis

It was performed as described in chapter 5.

IV. RESULTS

i. Clinical score

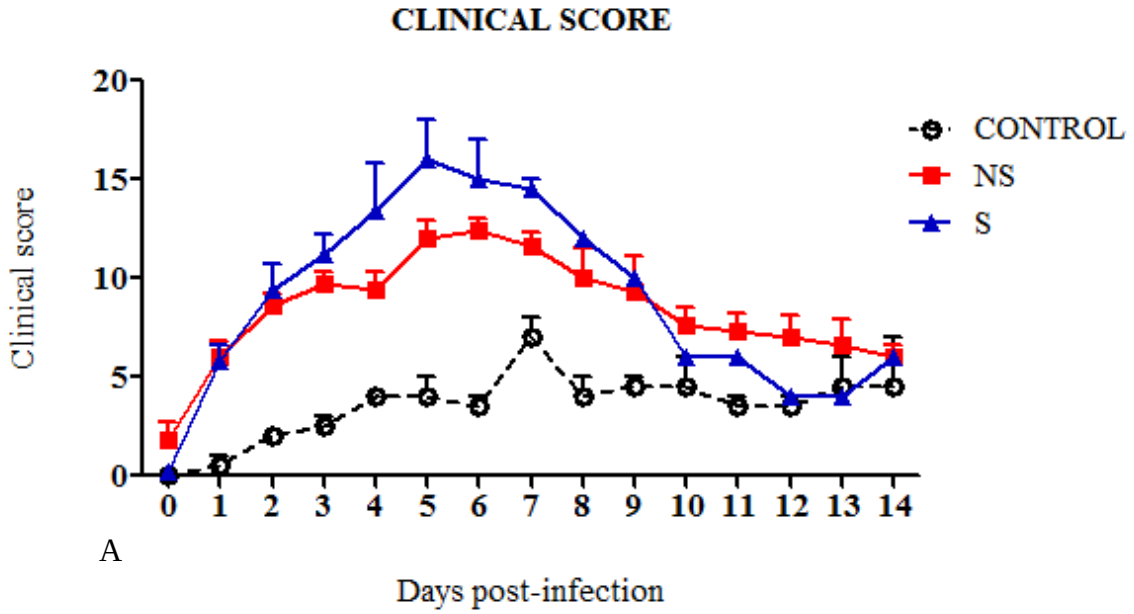
Clinical observations scores were daily recorded according to symptoms described in Table 7 during the course of the experiment. Infected ferrets started to show clinical signs as decrease in activity levels, nasal discharge and/or sneezing at 2 dpi which extended over the following 10 days in both infected groups. As outbreed animals, ferrets from both infected groups showed high variability in the progress of the clinical infection. In order to analyse the data related with the course of the infection in each individual ferret, animals were classified according to each clinical score, in other words, ferrets with less than 4 points were considered as control, ferrets scoring within 6 to 11 points were classified as non severe (NS) and animals scoring within 12 to 19 were classified as severe (S). Regardless of the virus used as inoculum, animals distribution was established as follows: control group (C) included two ferrets, number 1(male) and number 2 (female); non severe group (NS) included seven ferrets, numbers 3, 10, 11, 12, 13, 14 (males) and number 8 (female) and the severe group (S) included 5 ferrets, number 4, 5, 6, 9 (males) and number 7 (female) (Table 8).

INOCULUM	ANIMAL NUMBER	CLINICAL SCORE 4dpi	GROUP
MOCK	1	4	CONTROL
	2	4	CONTROL
A/CastillaLaMancha/RR5661/2009 **(M)**	3	6	NON SEVERE
	4	19	SEVERE
	5	13	SEVERE
	6	17	SEVERE
	7	13	SEVERE
	8	11	NON SEVERE
A/CastillaLaMancha/RR5911/2009 **(F)**	9	14	SEVERE
	10	11	NON SEVERE
	11	11	NON SEVERE
	12	10	NON SEVERE
	13	6	NON SEVERE
	14	11	NON SEVERE

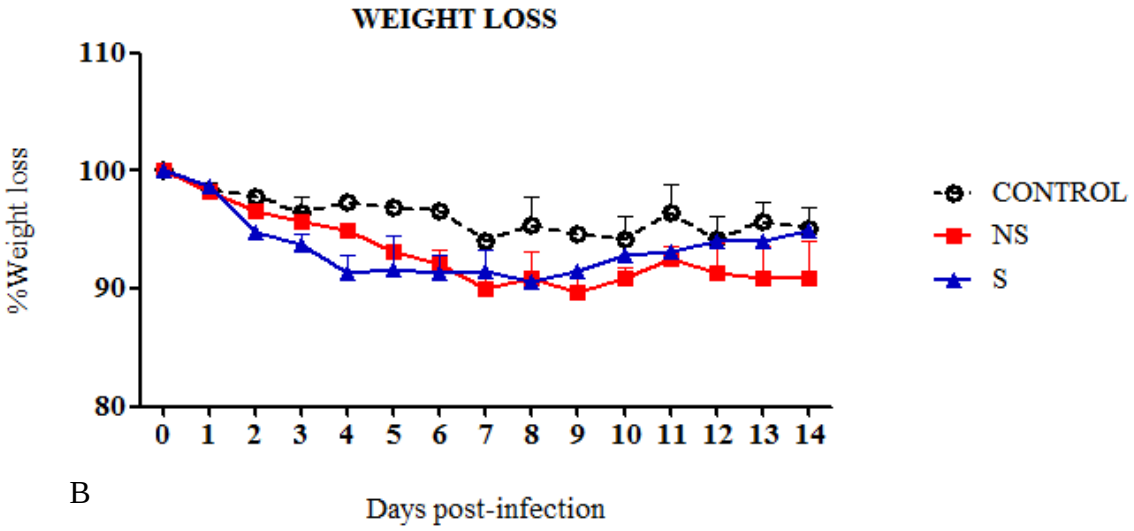
Table 8. Clinical score classification per animal. Animals were classified according to each individual clinical score. Ferrets with less than 4 points remained as control (C), ferrets scoring within 6 to 11 were classified as non severe (NS) and animals scoring within 12 to 19 were classified as severe (S).

ii. Clinical observations in pdmH1N1 2009 infected ferrets

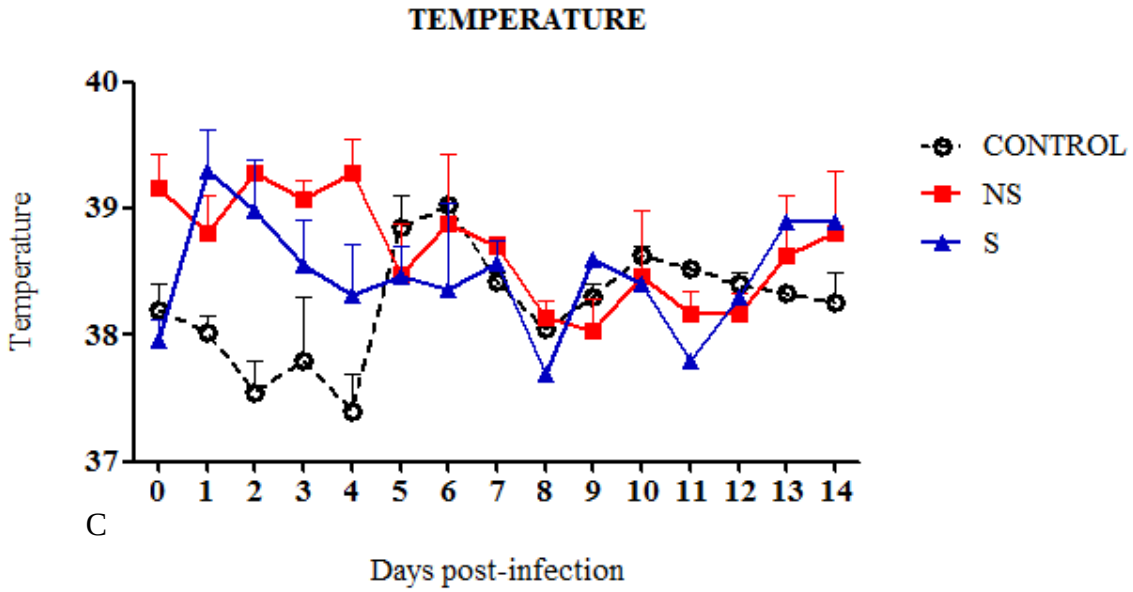
At 4 dpi one ferret infected with M virus and one ferret infected with F virus lost around 25% of body weight and scored more than 20 points. Both animals were humanely euthanized. Clinical conditions were significantly affected on infected-ferrets throughout infection. Animals belonging to NS showed a statistically significant clinical score when compared with control animals at 2, 3 and 4 dpi ($p<0.05$) (Figure 32 A). A statistical tendency ($p<0.10$) in higher clinical score was observed in S animals when compared with control group at 2, 3 and 4 dpi. On the following days: 5, 6 and 7 dpi, a tendency persisted within animals from NS when compared with control ferrets (Figure 32 A). Ferrets belonging to the group with S clinical signs had a significant decrease on body weight ($p<0.05$) when compared to NS at 2 dpi. At 4 dpi, there was a tendency ($p<0.10$) reflected on higher percentage of weight loss on S animals when compared with NS animals. Ferrets with non severe clinical score showed a significant decrease on body weight when compared with control group at 4 dpi (Figure 32 B). Body temperature was not statistically representative by infection with any virus (Figure 32 C).


CLINICAL SCORE
Clinical score
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection
CONTROL
NS
S

A


WEIGHT LOSS
%Weight loss
110
100
90
80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-infection
CONTROL
NS
S

B



C

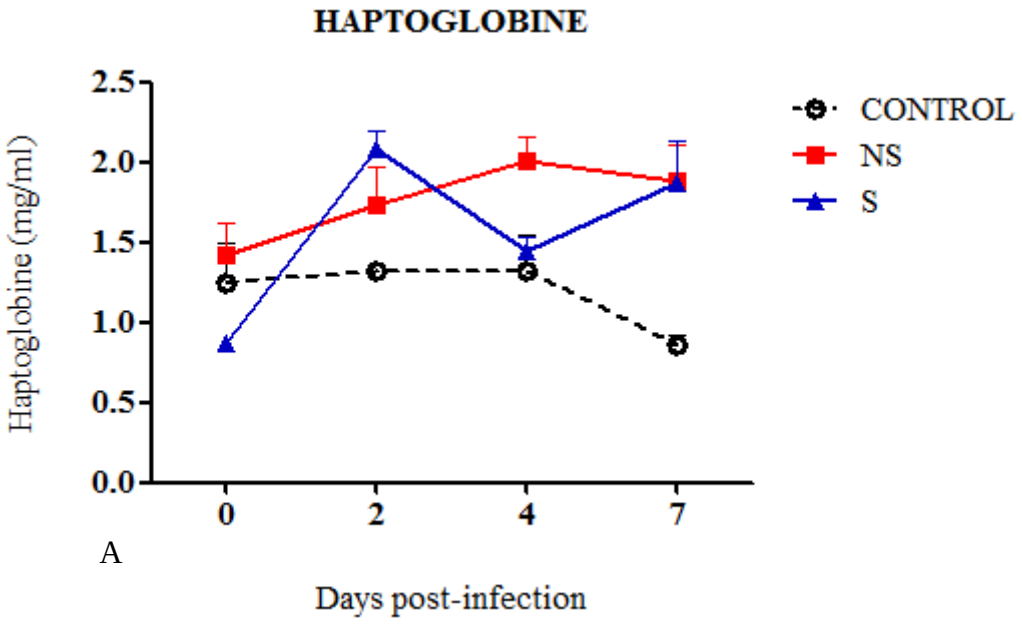
Figure 32. Average clinical signs of disease in ferrets following infection with M or F viruses. Animals were monitored daily for clinical observations using a specific scoring system (Table 7). (A) Clinical score in ferrets during the experimental infection. Changes were exhibited by animals in NS group showing a statistically significant severe clinical score when compared with control animals at day 2, 3 and 4 post-infection ($p<0.05$) (B) Percentage of weight loss during experimental infection. NS ferrets showed a significant decrease when compared with control group at day 4 post-infection. (C) Temperature was not affected during infection experiment with any virus. All values are the mean and ± SEM of one experiment.

iii. Acute phase proteins (APP)

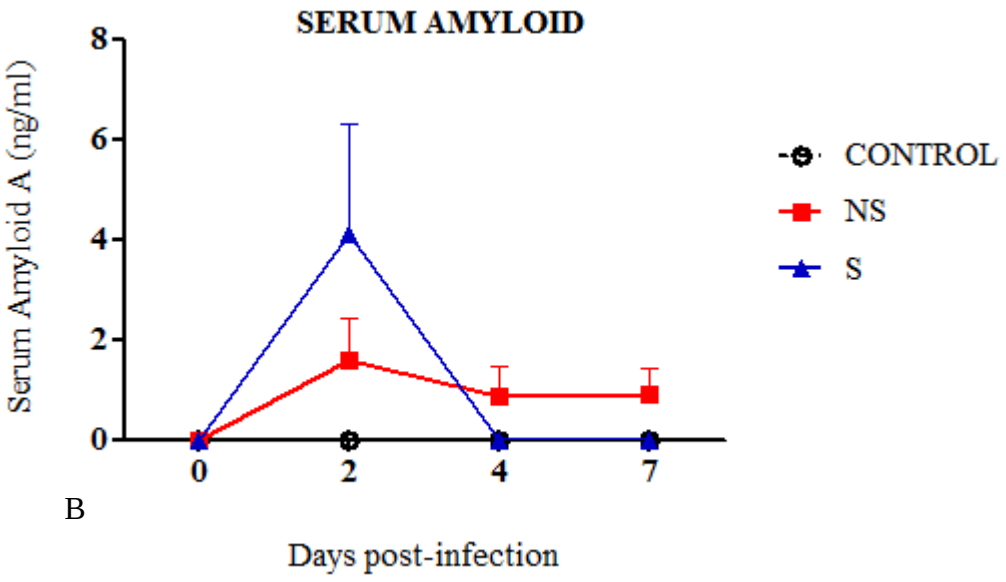
Haptoglobin (Hp) being an APP, might be prone to be altered in any inflammatory process like IV infection, which may increase plasma levels of Hp. Indeed, an increase in Hp levels was reported during influenza in pigs caused by pdmH1N1 2009 virus [198]. In ferrets, pre-infection individual levels of Hp were found to be below 1.94 mg/ml. The highest individual level after infection reached 2.44 mg/ml (at 2 dpi) in an animal presenting severe symptoms (S) of the disease. The mean concentrations of Hp increased from 0.87 to 2.08 (at 2 dpi) and from 1.42 to 2.01 (at 4 dpi) in the NS and S, respectively (Figure 33 A).

Serum amyloid A (SAA) is another important APP that has also been reported to increase in serum from humans and horses after influenza infection [198]. In ferrets, an increase of SAA after infection with pdmH1N1 2009 was detected when compared to their control counterparts only at 2 dpi. Large variations in SSA concentrations were observed between animals presenting severe symptoms of the infection at 2 dpi. At this point in time, the highest mean peak level reached 4.1 ng/ml. SAA levels decreased to background levels in the group presenting severe symptoms at day 4 dpi whereas in the NS group levels had stagnated (Figure 33 B).

No significant differences were detected for both APP studied, probably due to individual differences and a low number of individuals in some experimental groups.



A



B

Figure 33. Concentrations of (A) Hp and (B) SAA in serum from two groups of ferrets presenting different levels of disease (S and NS) before and at various time points after intratracheal infection with pdmH1N1 2009 virus. All values are the mean ± SEM of one experiment.

iv. Antibody response

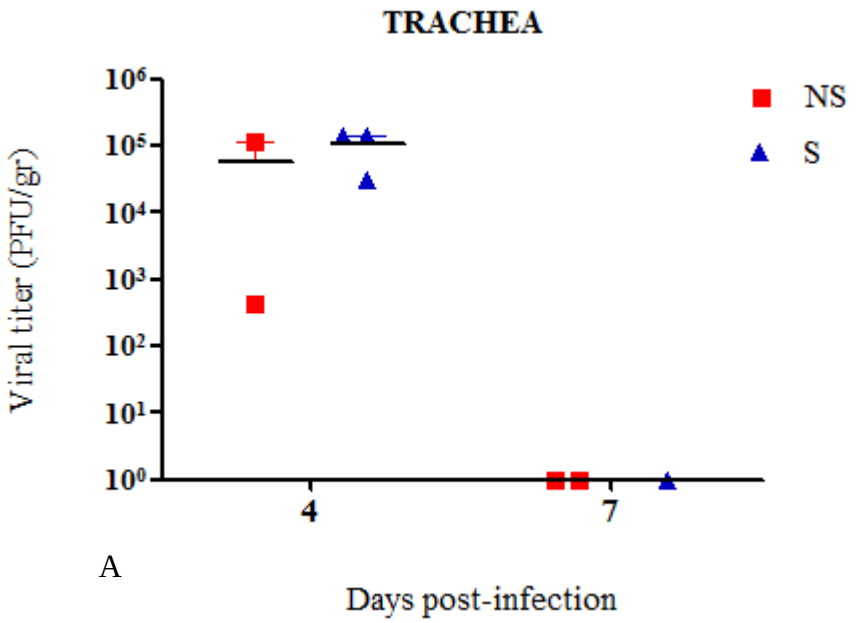
Sera from 0, 10 and 14 dpi were examined for the presence of specific antibodies against influenza NP. Antibody response against HA from M and F viruses was determined by HAI assay in sera from 2, 4, 7, 10 and 14 dpi. All infected animals showed a positive antibody response against NP at 10 and 14 dpi (Table 9). Control ferrets were shown to be seronegative at all timepoints. Animals belonging to NS and S revealed an early presence of HA inhibiting antibodies for M and F viruses as soon as 7 dpi. There was a strong HI antibody response not only to the virus used in infection but also there were crossreactive antibodies in sera of infected-groups at 10 and 14 dpi (Table 9). Ferrets from NS and S appeared to exhibit higher antibody responses to F virus, with higher HI titres detected in sera samples when compared with antibody responses to M virus; however, there was not significant differences within infected-groups. Control group showed statistically significant differences ($p<0.05$) with infected groups at all time points.

Group	Animal number	VIRUS	ELISA NP		A/CastillaLaMancha/RR5911/2009 (F)					A/CastillaLaMancha/RR5661/2009 (M)				
			D10	D14	D2	D4	D7	D10	D14	D2	D4	D7	D10	D14
C	1	mock	-	-	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40
	2	mock	-	-	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40
NS	3	M	+	+	<160	<160	<10,240	<5,120	<10,240	<80	<40	<5,120	<10,240	<20,480
	10	F	+	+	<320	<320	<10,240	<10,240	<10,240	<80	<160	<10,240	<20,480	<20,480
	11	F	+	+	<320	<320	<5,120	<10,240	<5,120	<160	<160	<5,120	<20,480	<5,120
S	4	M			<160	<320				<80	<160			
	5	M	+	+	<320	<320	<10,240	<10,240	<10,240	<80	<160	<10,240	<10,240	<20,480
	9	F			<320					<80				

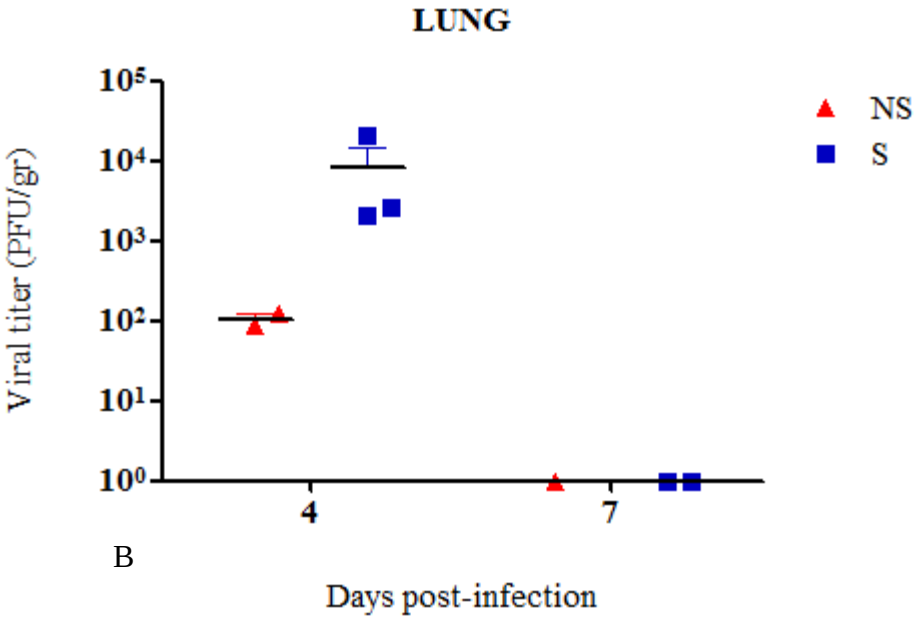
Table 9. At days 10 and 14 serum samples were collected to determine positive Influenza A samples by NP ELISA. Antibody response against F and M viruses was determined by HIA analizing serum samples from 2, 4, 7,10 14 dpi.

v. Viral load

To test the presence of infectious virus particles, samples of trachea, lung and broncheo alveolar lavage (BAL) were collected at 4 and 7 dpi. Control animals did not showed any viral titer at any time-points. Viral titers from trachea, lungs and BAL were analyzed as a set including the tree different tissues. As a result, animals with severe clinical signs (S) at 4 dpi showed higher viral load in compared to NS ferrets. This differences were statistically significant ($p<0.05$). There was not statistically significant differences when compared tissues of trachea, lung and BAL among themselves. However, titers of trachea showed a higher viral titers (Figure 34 A) tendency ($p<0.10$) when compared with lung titers at 4 dpi (Figure 34 B). Viral titers in tissues under study showed great variability. No virus was detected in trachea, lung or BAL at 7dpi.


TRACHEA
Viral titer (PFU/gr)
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
4
7
NS
S
Days post-infection

A


LUNG
Viral titer (PFU/gr)
10⁵
10⁴
10³
10²
10¹
10⁰
4
7
NS
S
Days post-infection

B

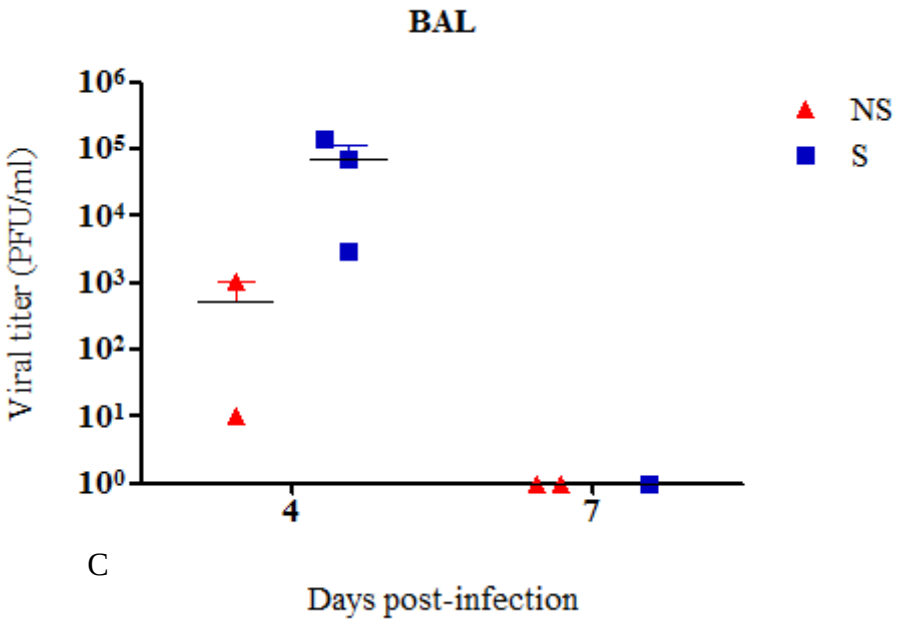


Figure 34. Viral load from ferrets in NS and S group at 4 and 7 dpi. Ferrets were intratracheally inoculated with 10^6 TCID/ml with M virus, F virus or PBS. At day 4 and 7 post-infection samples of trachea, lung and BAL were collected to measure PFU viral titers. Data of trachea, lung and BAL was analyzed as a set including the tree different tissues. (A) Virus titers in trachea. (B) Lung titers (C) Viral titers in BAL. No virus was detected in any tissue sample on day 7 after infection. All values are the mean ± SEM of one experiment.

vi. Histopathology and Immunohistochemistry

The most significant lesions were observed in lungs from infected animals. No major lesions were observed macroscopically in any organ at necropsy. Interstitial pneumonia (Figure 35 A) characterized by an acute inflammation with large amounts of neutrophils, macrophages and hyaline membranes filling the alveolar lumen was observed in lungs of ferrets presenting severe clinical symptoms (S). Mild bronchopneumonia (Figure 35 B) or bronchitis, consistent with a mild exudative lesion with suppurative or lymphoplasmacytic infiltration and bronchial epithelial necrosis was observed in animals which reported only mild clinical signs (NS). No histological abnormalities were found in control ferrets. (Fig. 35 C)

Presence of anti-NP viral antigen detected by immunohistochemistry (IHC) was only observed in animals sacrificed at 4 days post infection (data not shown). Presence of viral antigen correlated with the grade of pathological lesion observed. Animals which presented more severe pathological lesions showed higher amounts of viral antigen by IHC. In these animals, viral antigen was mainly observed in the bronchiolar epithelium, in the surface and glandular epithelium in the bronchi and interstitially in the alveolar septa and alveoli, mainly located in the alveolar epithelium. Animals which presented mild pathological lesions showed a lesser amount of anti-NP positive cells or did not show any positive cells at all; in these animals positivity was restricted to bronchiolar epithelial cells.

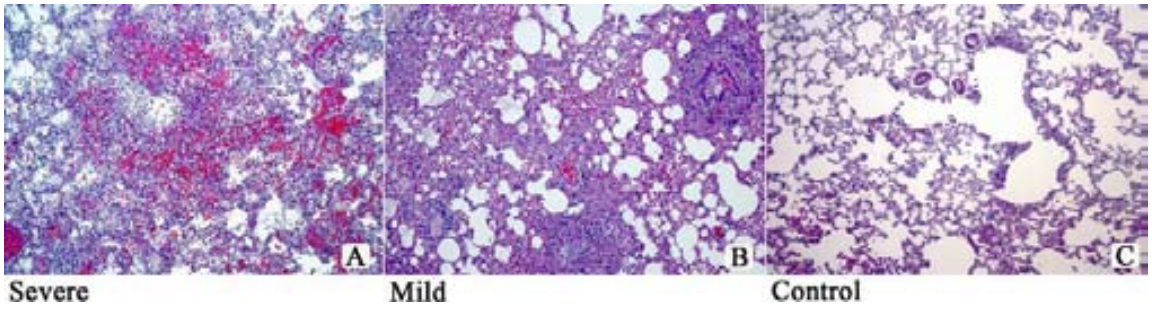


Figure 35. Histopathology and immunohistochemestry of ferrets belonginf to NS or S group of animals according to their clinical symptoms. (A) Histopathology of a ferret infected with F virus which presented severe clinical signs and interstitial pneumonia. Detail of the alveolar interstitium showing diffuse alveolar damage with presence of hyaline membranes, edema, large macrophagic infiltrate and hemorrhage. Hematoxilin/Eosin stain. (B) Histopathology of a ferret infected with M virus which presented mild clinical signs and bronchopneumonia. Bronchiolar-alveolar junction showing mild lymphoplasmacytic infiltration and bronchial epithelium slough. Hematoxilin/Eosin stain. (C) Histopahtology of a control ferret. Hematoxilin/Eosin stain.

Therefore, as consequence of the experimental pdmH1N1 2009 virus infection isolated from a mild and a fatal outcome on human, ferrets developed different degree of clinical signs severity that did not correlate with the origin of the virus used in the infection. Non severe group of ferrets (NS) consisted in two animals infected with M and five animals infected with F virus whereas severe group of ferrets (S) consisted in four animals infected with M and one ferret infected with F virus (Table 8). Severe infected animals showed a significant decrease in body weight compared to non severe infected animals at 4 to 7 days post-infection. Clinical progress of the infection correlated directly with histopathological findings (Figure 32). The analysis of the acute phase proteins showed that the concentrations of haptoglobin (HP) and serum amyloid a (SAA) increased on both groups after 2 dpi, however it was not statistically significant (Figure 33). Virus titres in all tissues were higher on ferrets belongin to S group when compared to ferrets belonging to NS group at 4 dpi ($p<0.01$) (Figure 34). All infected ferrets showed a strong hemagglutinin inhibiting antibody response in sera with crossreactive antibodies at 10 and 14 dpi (Table 9). Severe progress of infection correlated with high antibody responses, higher viral titres and higher histological damage.

9. DISCUSSION

9. DISCUSSION

Ferrets and mice animal models have proven a useful tool for studying pandemic influenza viruses, due to its utility to in measuring infectivity and pathogenicity. In an effort to elucidate how the heterogeneity in the outcome of pdmH1N1 2009 influenza virus could be due not only to differential fitness/virulence of the virus but also to the host immune environment *in vivo* experiments were conducted in which mice and ferrets were infected with different strains of pdmH1N1 2009 influenza virus to analyze the immunological response after infection in varied conditions. Results obtained along the thesis were divided into four chapters (chapters 5-8).

Chapters 5 and 6

Invading viruses are rapidly sensed by the host innate immune system; once innate immune cells are activated the production of cytokines begins. A dramatic increase of proinflammatory cytokine upon pdmH1N1 2009 infection were detected when studies focused in the immunological response of clinically severe patients were performed suggesting an important role in the pathogenesis and disease development [199]. Severe patients have shown increased levels of mediators which stimulate Th1 responses (IFN-γ, TNFa, IL-15, IL-12p70) and Th-17 ones (IL-8, IL-9, IL-17, IL-6) [132]. Additionally, among the the different cytokines analized, sera levels of IL-6 and IL-10 were significantly up-regulated on severe pdmH1N1 2009 patients [199]. Up-regulated levels of IL-6 have been reported in critically ill pdmH1N1 2009 infected patients [88], [136], [111], [132].

In mammals, invasion of pdmH1N1 2009 influenza virus into the lung tissues induces the production of pro-inflammatory cytokines with consequent development of pneumonia. Recent studies performed in mice described the increased production of several cytokines where higher levels of IL-10 in lungs of pdmH1N1 infected mice were found at day 6 pi with the pdmH1N1 2009 virus compared with a seasonal H1N1 virus [169]. However, Belser and colleges did not observe substantial differences in mouse cytokine production among pdmH1N1 2009 viruses isolated from patients in the early stages of the pandemic [196]. Related to IL-6, a previous study performed by Paquette et

al. demonstrated increased levels of this cytokine in pdmH1N1 2009 infected mice. However, infection of IL-6 -/- mice resulted in disease indistinguishable from that in IL-6 wildtype mice, as measured by survival, weight loss, viral load, and pathology [137]. They suggested that IL-6 does not play an essential non-redundant role in the host response to pdmH1N1 2009 infection in mice. Nevertheless, pathological response to pdmH1N1 2009 virus infection in mice overexpressing proinflammatory cytokines as IL-6 had not been previously investigated.

Taken on mind the situation described above, the main aim of these investigations was to establish an animal model characterized by an exacerbated inflammatory state prior to infection. On the first study we studied the role of LPS-derived inflammation on pdmH1N1 2009 virus infection; in order to compare the basal inflammatory state with LPS induction, parameters as changes on body weight, levels of IL-6 on serum and lungs, lung virus replication and histopatological studies were measured. Results obtained after LPS treatment encourage us to investigate the specific role of IL-6 in H1N1 2009 infection. Thus, we inoculated mice with pIL-6 following a similar protocol that on LPS experiment, in these experiments, IL-10 in serum and lungs was also evaluated as well as antibody responses by HIA.

To our knowledge, no information has been described so far about how external stilmulis used in both experiments (LPS or pIL-6) affected pdmH1N1 2009 influenza infection. In accordance with previous studies [181], [200] we did not observe mortality when animals were infected with an IN dose of 10^4 PFU/mouse with the pdmH1N1 2009 virus (CAT09) either. Clinical signs as decrease of body weight were observed on both experiments either when animals only infected or infected and treated with external stimuli such as LPS or IL-6. However, the results obtained of body weight on CAT09 infected animals showed a different pattern within the two experiments. In both experiments, mice showed similar characteristics of breed, sex and age; we also followed the same infection protocol: route, dose and virus stock used for infection. Weight loss of CAT09 infected mice without treatment with LPS or pIL-6 showed a significant decrease of body weight when compare with controls, however the percentage of weight loss was lower and for a shorter period of time in the experiments using LPS (Figure 10) than the body weight pattern in the experiment using pIL6 (Figure 21) for the same group (CAT09-mice). Besides, in the experiment using LPS,

the CAT09 infected animals recover their normal weight at the end of the protocol whereas in the experiment using pIL-6, CAT09 infected animals did not recover their normal weight during the experimental time. Mice body weight was less affected by treatment with pIL-6 than with LPS treatment during the first 2 dpi; LPS treated animals showed a higher percentatge of weight loss than pIL-6 mice. As LPS is envolved in TLR mechanisms that elicits a variety of proinflammatory cytokines as TNF-α and various interleukins (IL-1α, IL-1β, IL-8, IL-12) among which IL-6 has been reported as a possible biomarker for inflammation in influenza infection [188] it was expected that LPS treated animals to show more clinically affected than pIL-6 treated mice. Groups that were infected and treated with LPS or with pIL-6 showed a higher percentatge of body weight than the rest of the groups at the first 5 dpi; even though we detected more significant changes on body weight on LPS-treated+CAT09 animals than in pIL-6+CAT09 treated animals. In conclusion, CAT09 infected animals and treated with LPS presented more signs of severity, measured as body weight loss, than CAT09 infected animals and pIL-6 treated.

To measure the severity of the viral infection viral titer in lungs of infected animals were measured. Viral load in CAT09 infected mice without external stimuli (LPS or pIL-6) was detected between days 1 and 5 pi in the case of the LPS experiment and between days 1 and 3 pi in the pIL-6 experiment. LPS treatment did not affect viral replication since viral titers did not present any differences within infected groups (with or without LPS external inoculation). It is well established that infection with influenza A virus facilitates secondary bacterial disease. However, there is a growing body of evidence that the microbial context in which influenza A virus infection occurs can affect both innate and adaptive responses to the virus. In a secondary bacterial infection, LPS stimulates the immune system through TIRAP-MyD88/TRAM-TRIF pathways producing an uncontrolled cytokine release that might induce tissue damage allowing viral replication. Recently, it has been shown that exposure to bacterial ligands like LPS reduces the ability of influenza A virus to primary human monocyte-derived macrophages [201]. However, this was not the case in the lungs from animals CAT09 infected and LPS treated (Figure 11). Also, Shinya et al have reported that prestimulation of the TLR4 pathway with LPS protects mice from lethal infection with H5N1 influenza virus by the TLR4-TRIF pathway and their data suggest that the TLR4-

TRIF axis has an important role in stimulating protective innate immunity against H5N1 influenza A virus infection [202]. Conversely, our data suggested that there is no protective effect of LPS when pdm H1N1 2009 is infecting mice. This apparent discrepancy could be due to different activation mechanisms of the innate immune system between H5N1 and pdmH1N1 2009.

On the other hand, the possible role of IL-6 in pdmH1N1 2009 viral load was studied. Animals treated with the pIL-6 and CAT09 infected showed viral load in lungs only at 1 dpi whereas CAT09 only infected mice had virus replication at day 1 and 3 pi. This data suggested a IL-6 protective role in pdmH1N1 2009 infected mice. It was worthnoticing that IL-6 levels correlated with weight loss and pathology in those mice but not with viral burden.

Concentration of IL-6 on serum and lung of both LPS treated groups were similar at the first 24 hours post infection indicating that virus replication did not affect IL-6 secretion when was associated to LPS treatment. Nevertheless, we found significantly higher levels of IL-6 in lungs of pdmH1N1 2009 infected mice that were not treated with LPS. As expected, pIL-6 induced significant increased IL-6 levels in serum and lungs of pIL-6 treated mice when compared with animals only infected with CAT09 virus.

Antiviral immune responses play as a double edged sword in resolution of infection and pathogenesis of acute lung injury caused by infection with influenza virus. IL-10 is known to be an anti-inflamatory cytokine produced by several different cell types [203]. Studies performed in a murine model by McKinstry and collaborators [204] revealed that IL-10 inhibits development of Th-17 responses during influenza infection, correlating with compromised protection. Markedly increased production of IL-10 toghether with IFNγ, IL-4 and IL-5 were detected after infection of mice, ferrets and non-human primates [169]. IL-10 is induced by several mechanisms, including inflammation and IL-6 [205]. Given the important role of IL-10 in controlling immune responses, this interleukin was measured in pIL-6 treated mice.

Results obtained at 1 and 3 dpi in the pIL6 treated mice indicated that higher levels of IL-10 correlated with higher levels of IL-6, exhibiting significantly higher concentrations than untreated animals and only CAT09 infected animals (Figure 24 A). Interestingly, mice only treated with the plasmid secreted significantly higher amounts of IL-10 than CAT09 infected mice at 5 dpi and at 3 dpi, pIL-6 treated animals secreted significant more IL-10 than controls. Results from pdmH1N1 2009 infection probably reflected a host response to minimize over-exuberant pulmonary inflammation and promote tissue repair in mice infected. In fact, IL-10 could play a protective role by controlling levels of proinflammatory cytokines.

In agreement with previous work [206], the 2009 H1N1 viruses tested here produced pulmonary pathology similar to those of other influenza A viruses and included mild to moderate bronchiolitis and alveolitis. Necrosis was not a common feature, and neutrophilic responses were typically mild [169], [200]. Congruently with observations described in human by other authors [132,199] we observed that infection with pdmH1N1 2009 virus is capable to induce an intensive production of IL-10 and IL-6. However, we did not observed a direct correlation between cytokine levels and severity of pandemic infection outcome in C57BL6 mice.

Chapter 7

Since pandemic of 2009 virus arrival, numerous cases of the emergence of resistant viruses [207] among patients undergoing oseltamivir treatment or prophylaxis have been reported [120,160]. There are also reports of transmission of resistant viruses in hospitalized settings among immunocompromised patients [161], some cases related to H274Y mutation, suggesting possible human–human spread [88]. The study of virulence and fitness of the OsR strains is a key factor in determining the long-term usefulness of antiviral therapy in particular to those patients that need especial assistance.

The investigation developed in the chapter 7 has been focused on characterizing and comparing *in vitro* and *in vivo* viral fitness of two OsR pdmH1N1 2009 viruses (R6 and R7).

The fitness of a virus describes its relative ability to produce infectious progeny in a host [208]. In the literature, fitness of OsR virus is still controversial. Whereas some studies reported a statistically significant impairment in viral growth *in vitro* [209], other groups did not detect significant differences in terms of replicative capacity between the pdmH1N1 2009 WT virus and its H275Y variant [210]. Evaluation of the growth fitness of three viruses was performed using two OsR virus (R6 and R7) and one Oseltamivir sentisite virus (F), as a representative of pdmH1N1 2009 without H275Y mutation. The results showed a different fitness in viral replication, being F>R6>R7. We also observed reduced viral plaque areas in cells infected with OsR virus that was previously associated to the H274Y mutation [211]. Nevertheless, the fact that the OsR pdmH1N1 2009 virus has altered kinetics when compared with the WT counterpart indicates some fitness alteration due to the H275Y mutation, as it has been previously reported [212]. Thus, our results from the *in vitro* growth assay were in accordance with previous studies in which WT H1N1 virus grew at higher titers than H1N1 viruses with H274Y mutation [211].

Subsequently to the *in vitro* study, mice were inoculated with 10^3 PFU/mouse of oseltamivir resistant pdmH1N1 2009 viruses to compare their relative growth fitness and pathogenesis in mice. Results from this study showed that both OsR strains produced a fatal outcome although on different magnitudes and kinetics. R6-infected animals experimented a 40% of lethality and R7-group a 20% at 4 dpi. However, at 7 dpi the percentatge of survival was a 50% for both OsR-infected groups (Figure 27). It is worthnoticing that no lethal outcome was observed when animals were infected with CAT09 virus when using ten times more virus (10^4 PFU/mouse) in previous experiments (Chapters 5 and 6).

To address clinical signs observed in infected mice, weight loss was measured. R6 infected mice had a peak of weight loss at day 4 pi and R7 infected mice exhibited a similar peak but three days later, at day 7 pi. R6-infected mice showed a statistically significant ($p<0.05$) higher percentage of weight loss during the first 2 dpi when compare with R7-infected mice. It is important to highlight that body weight recovery was slower in the R6-infected mice whereas R7-infected mice started to increase body

weight at 7 dpi (Fig. 27 A). Hammelin and collaborators observed that animals infected with WT pdmH1N1 2009 showed a slightly less pronounced weight loss that the mutant H274Y pandemic strain [213]; our observations supported this results since we also observed that CAT09 virus used for infection in chapters 5 and 6 was less effective in inducing weight loss than the OsR viruses (Figures 10 and 21). When weight loss induced by both OsR virus in infected animals were compared, R6-infected mice showed a statistically significant ($p<0.05$) higher percentage of weight loss during the first 2 dpi when compare with R7-infected mice. Both infected groups showed a statistically significant higher percentage of weight loss from days 3 to 7 pi when compared with control animals ($p<0.05$). These important clinical results correlated with slightly more severe histopathological changes observed in lungs of R6 mutant infected mice compared to R7 infected mice in particular at 5 dpi (Figure 31).

Viral fitness of the H274Y mutants and WT of the pdmH1N1 2009 virus has been previously addressed in BALB/c mouse model. Hamelin at al reported that the H274Y pdmH1N1 2009 mutant virus is clearly as fit as the WT virus; being replication of both isolates equally efficient in the lower respiratory tract in BALB/c mouse [213]. In accordance to this previous report, in chapter 5 and 6 we described a successfull infection in using C57BL6 mice with the pandemic WT virus (CAT09) with similar levels in viral replication (Figures 11 and 22) that the ones obtained from the OsR virus infected C57BL6 mice (Figure 28). Viral replication detected in lungs from C57BL6 OsR-infected mice seems to have higher values for R7 than for R6, however not statisticall differences were observed within OsR infected groups (Figure 28). A recent report on H275Y mutation for pdmH1N1 2009 indicated that the WT and H275Y viruses induced comparable mortality rates, weight loss, and lung titers in mice [212]. Our results are in some agreement with this statement but we have observed different kinetics in mortality rates and weight loss when two OsR virus were compared.

An early antibody response against HA was detected at 7 dpi in 2 animals from R6-group and 3 animals from R7-group by HIA assay. There was a strong antibody response at 14 dpi on both infected groups for each virus but no cross recative antibodies. Likewise, R6-infected mice seems to have higher titers of HA antibodies that the ones from R7-infected mice (Table 5)

Induction of IL-6 after influenza infection has been reports on ferrets [213,214] and after OsR virus infection in mice [213]. The immunological response towards OsR viruses by means of IL-6 and IL-10 at 3, 5 and 14 dpi was explored. Interstingly, high levels of IL-6 were detected in serum from R7-mice with significant differences at days 3 and 5 pi when compared with serum from R6-mice and controls. Surprisingly, levels of IL-6 in lungs of R6, R7 and control animals were similar at all time-point with no statistical differences. Serum and lung IL-10 had also slightly higher values in R7-mice when compared with controls at 3 and 5 dpi respectively. Altogether, those results suggest that the H274Y (R7) pdmH1N1 2009 mutant isolate stimulated a more important and prolongued inflammatory response in mice compared to R6 virus which could be due to rapid induction of IL-6 with the corresponding induction of compensatory secretion of IL-10. This mechanism could explain the sustained body wheight exhibited by R7-infected mice as compared with the R6-infected mice (Figure 27).

In conclusion, we observed that faster *in vitro* replication of OsR pdmH1N1 2009 virus was reflected by a faster mortality rate in mice, however, it did not correlated with viral load in lungs of infected mice. High levels of IL-6 and IL-10 could be responsible for a prolonged pathogenesis in OsR infected mice.

A previous report has shown that the drug-resistant (mutant) virus was at least as virulent as the drug-susceptible (wild-type) virus in mice [213]. The results presented in this study also support this idea. Based on these data, H274Y pdmH1N1 2009 mutant strains have the potential to disseminate in the population and to eventually replace the susceptible strain, a phenomenon that has been already observed with seasonal A/Brisbane/59/2007-like (H1N1) viruses. These data indicated that surveillance for possible H274Y pdmH1N1 2009 mutant strains must be a priority for sanitary authorities in order to detect possible H274Y pdmH1N1 2009 mutant/variant strains acquiring higher fitness. Consequently, the potential emergence and dissemination of such variants should be carefully monitored.

Chapter 8

Patients affected by a pdmH1N1 2009 infection develop a whole range of clinical features, most patients infected by the virus experienced uncomplicated illness, however, a small subset of patients developed a severe disease which may be related to their individual susceptibilities. Ferrets are an attractive mammalian model for influenza studies owing to their relatively small size and the fact that they mimic numerous clinical features associated with human influenza disease. In this study, ferrets were infected with two strains of pdmH1N1 2009 virus, one isolated from a patient exhibiting mild clinical signs of infection and the other one from a patient with a fatal outcome. During the course of the experiment, ferrets from both infected groups showed a clinical score severity that was independent from the virus used for infection, animals infected with M virus present mild to severe clinical signs and one of the ferrets presented a critical condition and was euthanized for humanitarian reasons. Similar outcome was present in ferrets infected with F virus. These findings were not in agreement with the results obtained in our group in a previous work in which the same two virus strains were used to infect mice. The experiments using mice indicate that F virus was more pathogenic than the M one, as indicated by the morbidity and mortality rates observed in the F-infected mice [197]. These apparent discrepancies can have two explanations. The first one could be the fact that mice were inbreed whereas ferrets were outbreed, exhibiting changes in the population than could be similar to the ones affecting humans. Secondly, mice are not a natural host of IVs. However, mice are among of the most commonly used mammalian models for evaluating influenza infection. These two reasons can both apply to explain the results from these experiments.

Due to the extensive clinical observation registered during infection of ferrets, the data from each ferret was analysed individually, allowing us to divide all animals by the clinical score. Changes on weight, body temperature and activity are the most common parameters used to evaluate the clinical profiles associated with influenza infection in ferrets. For example, the activity scoring in infected ferrets are based on the scoring system described by Reuman et al [215] and Zitzow et al [216]. In order to perform a more comprehensive evaluation, an amplified score was recorded including a whole range of

clinical signs. Data showed that the clinically severe animals (S) showed a pattern of clinical signs similar to that commonly founded on ferrets infected with a HPAI and the animals who presented mild clinical signs (NS) response similar to those infected with a seasonal virus [217] (Figure 32). Also, animals belonging to the NS group presented mild histological lesions generally characterized by mild bronchopneumonia or bronchitis. On the contrary, the lung pathology observed in S ferrets showed signs of acute inflammation as diffuse alveolar damage and inflammatory infiltrate, similar to the histological lesions presented on deceased human patients infected with the pandemic 2009 [206].

Recent studies performed infecting pigs with IV showed interesting data of the immune responses by means of serum concentrations of acute phase protein as C-reactive protein, haptoglobin and serum amyloid A in response to infection. In these studies, positive correlations were found between serum concentration of Hp and SAA and lung scores, and between clinical score and concentrations of SAA in IV infected pigs [198]. Presently, there is no information about APP after IV infection in ferrets. The results on the present study suggested that there was an increase of Hp and SAA at day 2 pi, however no statistically differences were found, probably due to the low number of animals per group. These results pave the way for analysing further APP responses to influenza infection in ferrets.

In relation to the antibody response, animals showed seroconversion from 10dpi by ELISA and HIA in both infected groups. Interestingly, crossreactive antibodies were generating, indicating that similar regions of each HA molecule was recognized by the immune system in ferrets. Noteworthy, HA sequence has only three changes at the aminoacid level when comparing M and F virus (Table 6). On the other hand, antibody immune responses against HA was not equal against F and M virus. Ferrets from NS and S appeared to have increased antibody response to F virus. This result might be associated not only with the individual immune state of the animals but also with different virulence of specific influenza strains as it was observed in the murine model [197].

Viral replication in the respiratory tract of infected ferrets was directly associated with pathology. As expected, animals belonging to S group showed higher viral titer (Figure 34) wich also correlated with higher tissue damage (Figure 35).

Our results suggest that the severity in the progress of infection was independent from the virus used for infection, which might be associated to the host immune response. The severity in the progress of infection was independent from the virus used for infection suggesting that the host immune response was determinant in the outcome of the infection in ferrets. This diversity in ferrets mimicked the variability observed in the human population.

10. CONCLUSIONS

10. CONCLUSIONS

1. A proinflammatory state was satisfactory induced after intraperitoneally inoculation of 200 μg/mouse of LPS in C57BL6. LPS alone produced mild clinical signs as body weight decrease at 1 dpi but at 2 dpi LPS treated mice recovered their normal weight.

2. Pandemic H1N1 2009 infection has been successfully performed with the A/CATALONIA/63/2009 strain (CAT09) in C57BL6 mice without prior adaptation.

3. Overproduction of IL-6 was successfully achieved by the administration of 10 μg/mouse of a plasmid expressing murine IL-6 (pIL-6) in C57BL6 mice.

4. Viral load did not correlate with LPS treatment; however, there was a decrease in viral load when IL-6 was overexpressed.

5. High levels of histopathology and IL-6 secretion correlated with LPS treatement in CAT09 infected mice.

6. Presence of IL-6 in CAT09 infected mice induced a faster decrease in body weight.

7. High levels of IL-6 correlated with levels of IL-10, lower histopathology lesions and lower titer in antibodies against HA in CAT09 infected mice.

8. Different fitness in *in vitro* viral replication in two OsR virus (R6 and R7) was observed when compared with one oseltamivir sensitive virus (F), being F>R6>R7.

9. OsR strains produced a fatal outcome in C57BL6 infected mice although with different kinetics, which correlated with their *in vitro* fitness.

10. Viral fitness *in vitro* did not correlate with viral replication in lungs from R6 or R7 infected mice, exhibiting higher values for R7 infected animals.

11. There was a strong HA antibody response at 14 dpi in both R6/R7 infected groups towards each virus but no cross reactive antibodies.

12. Lower levels of IL-6 and IL-10 correlated with viral clearance in lungs and a faster recovery in OsR infected mice.

13. Two groups of ferrets were successfully infected with two strains of pdmH1N1 2009 virus from a mild (M) and fatal (F) case.

14. Clinical pathology in infected ferrets did not correlate with the virus used as inoculum. Animals were classified according to their clinical score in the non severe group (NS) and severe group (S).

15. Severe infected animals showed a significant decrease in body weight compared to NS infected animals at 4 to 7 days post-infection.

16. Clinical progress of the infection correlated directly with histopathological findings.

17. The analysis of the acute phase proteins showed that the concentrations of haptoglobin (HP) and serum amyloid A (SAA) increased on both groups after 2 dpi. No statisticall differences were observed when both infected groups were compared.

18. Virus titres in all tissues were higher in ferrets belonging to S group when compared to ferrets belonging to NS group at 4 dpi ($p<0.01$).

19. Animals infected with M or F virus showed a strong hemagglutinin inhibiting antibody response in sera to both viruses at 10 and 14 dpi. Ferrets with a severe progress of the clinical infection showed slightly higher antibody responses and higher viral titres after infection.

11. OTHER PUBLICATIONS

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2011 “CHIMERIC CALICIVIRUS-LIKE PARTICLES ELICIT SPECIFIC IMMUNE RESPONSES IN PIGS”

Crisci E, Fraile L, Moreno N, Blanco E, Cabezón R, Costa C, Mussá T, Baratelli M, Martinez-Orellana P, Ganges L, Martínez J, Bárcena J, Montoya M

2012 “HIGHLY VIRULENT PANDEMIC H1N1 INFLUENZA VIRUS IN A FATAL CASE WITH HOMOZYGOUS CCR5Δ32 MUTATION”

Ana Falcon; Ariel Rodriguez: Maria Teresa Cuevas; Francisco Pozo; Susana Guerra; Blanca García-Barreno; Pamela Martinez-Orellana; Pilar Pérez-Breña; Maria Montoya; Jose Antonio Melero; Manuel Pizarro; Juan Ortin; Inmaculada Casas; Amelia Nieto.

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