CD69 limits early inflammatory diseases associated with immune response to *Listeria monocytogenes* infection

Javier Vega-Ramos[1,*] and Elisenda Alari-Pahissa[1,*], Juana del Valle[1], Eugenio Carrasco-Marín[2], Enric Esplugues[3], Miquel Borràs[4], Carlos Martínez-A[5] , Dirk H. Busch[6], and Pilar Lauzurica[1,6]

[1]From the Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Barcelona; [2]Servicio de Inmunología, Hospital Universitario Marqués de Valdecilla, Servicio Cántabro de Salud, Santander; [3]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520; [4]Unitat de Toxicologia Experimental i Ecotoxicologia, Parc Científic de Barcelona, Universitat de Barcelona, Barcelona; [5]Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, [6] .Institue for Medical Microbiology, Immunology and Hygiene, Technical University Munich, Germany; and [6]Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

* These authors contributed equally to this work

Corresponding author: Pilar Lauzurica, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda a Pozuelo Km. 2.2, 28220 Madrid, Spain, Phone (+34) 91/822-3718, Fax (+34) 91/509-7966, e-mail: lauzurica@isciii.es

Running title: CD69 limits immune response to infection

Summary

Mouse infection with intracellular bacteria induces a potent inflammatory response that requires protective mechanisms to avoid infection-induced immune pathology. CD69 is expressed in all leukocytes during activation following infection with a wide range of microbial pathogens. This study explores the way in which CD69 affects cell activation following *Listeria monocytogenes* (Lm) infection and its effects on host protection. We show that infectivity and bacterial clearance capability are unaltered in $CD69^{-/-}$ peritoneal macrophages, bone marrow-derived macrophages and dendritic cells. We found no major altered cell populations in splenocytes of Lm-infected $CD69^{-/-}$ mice. However, an increase in the expression of Th1 cytokines was observed after infection, with increased production of types I and II interferon (IFN). In addition, $CD69^{-/-}$ splenocytes showed increased apoptosis, consistent with IFN enhancement of lymphocyte apoptosis in response to Lm infection. $CD69^{-/-}$ mice showed liver and spleen damage, and greatly increased susceptibility to Lm infection, compared to wild-type controls. However, $CD69^{-/-}RAG2^{-/-}$ mice were more efficient at controlling Listeria infection. Therefore, although Lm-specific T cells was decreased in $CD69^{-/-}$ mice, the fact that the difficulties in the control of listeriosis arise as early as day one post-infection point to that the defect in the response in CD69-/- mice is mediated by lymphocytes. These results highlight a role for CD69 in preventing infection-induced immunopathology..

Key words: Bacterial Infection; Knockout; Rodent; T Cells

Introduction

Immunity to infection is controlled by fine interplay among distinct cell programs. Cytokines are critically involved in microbicidal function, activating both macrophages and NK cells, and linking innate and adaptive immune responses. Cytokine effector mechanisms are crucial for host protection and resolution of otherwise potentially fatal disease processes, but can induce immune-mediated pathology if not correctly regulated. Cytokines with immunosuppressive properties, such as IL-10 and TGF-β, are thus produced to prevent infection-induced immune pathology. IL-10 production by dendritic cells and macrophages has been demonstrated in response to infection by *Bordetella pertussis* and Lm [1-4]. Other mainly pro-inflammatory cytokines, such as TNFα and IFNγ, also have a regulatory role in the response to intracellular infection by controlling inflammation-induced damage [5,6]. In addition, strategies have been identified that sabotage protective Th1 responses and prolong pathogen persistence; for example, the intracellular bacteria Lm induces apoptosis during early stages of the immune response to infection by inducing type I IFN, while the inability to respond to type I IFN in mice greatly improves control of bacterial challenge [7-10]. Induced apoptosis associated with pathologic or ineffective immune responses to infection by other bacteria and protozoa has also been reported [11,12]. We analyzed CD69-regulated protection against intracellular bacteria using an experimental model of Lm infection in mice, due to its well-studied immune response (reviewed in Pamer[13]). Lm is a facultative, gram-positive bacterium that invades phagocytes efficiently. Infection is restricted principally to liver and spleen, where it infects mainly macrophages and hepatocytes. After Lm infection, phagocytosis and proinflammatory cytokine release are induced in resident macrophages. TNFα, IL-1β and IL-12 release induces NK and T cells to produce IFN-γ, thus activating macrophages to become bactericidal, and inducing Th1 lymphocyte differentiation.

CD69, a broadly expressed leukocyte receptor [14] with rapid kinetic onset after activation, is a disulfide-linked homodimeric membrane type II C-type lectin [15]. Previous studies showed that CD69 regulates the immune response by modulating the expression of various cytokines. $CD69^{-/-}$ mice show increased anti-tumor and autoimmune responses caused at least in part through increased pro-inflammatory cytokine and chemokine production [16,17]. CD69 targeting by a non-depleting anti-CD69 antibody similarly increases anti-tumor responses by enhancing natural killer (NK) cell activity and treatment of NK cells with this antibody results in increased cytotoxic activity and IFNγ production [16,18]. CD69 thus regulates anti-tumor immune responses by modulating the expression of various cytokines, including TGFβ and IFNγ.

Although the role of CD69 has been studied extensively, its function in the immune response against infective intracellular pathogens has not yet been elucidated. This study examines the role of CD69 in various cell types in response to Lm infection and compares the immune response of $CD69^{-/-}$ and WT mice to Lm. $CD69^{-/-}$ mice develop an intense tissue-inflammatory response and die shortly after Lm challenge. Increased susceptibility to infection was found to be associated with increased number of bacteria in target organs and elevated type I and II IFN cytokines. Our results show that CD69 is an important regulator of immune response-mediated pathology.

Material and methods

Mice

Mice were bred at the Centro Nacional de Biotecnología (Madrid, Spain) and at the University of Barcelona under specific pathogen-free conditions. The experiments were performed using mice on the C57BL/6 genetic background, except when Balb/c and RAG2-/- Balb/c mice were used as indicated. $CD69^{-/-}$ mice were backcrossed on the C57BL/6 and the Balb/c backgrounds at least nine times[19]. OT-I and CD45.1 mice were bred under specific pathogen-free conditions at the mouse facility at the Technical University Munich. All procedures involving animals and their care were approved by the University of Barcelona Ethics Committee and were conducted according to institutional guidelines in compliance with local (Generalitat de Catalunya decree 214/1997, DOGC 2450; and the government of Bavaria) and international (Guide for the Care and Use of Laboratory Animals, NIH 85-23, 1985) laws and policies.

In vivo experiments

Lm strain EGD was stored as 30% glycerol stocks at -80ºC and diluted into pyrogen-free saline for injection into mice. Lm was injected intravenously at a dose of 7.5×10^3 Lm/mouse, except where indicated. To determine organ Lm burden, spleen and liver were homogenized in RPMI-1640 medium without antibiotics plus 0.05% Triton X-100. Serial dilutions of homogenate were plated on brain-heart infusion (BHI) agar, and bacterial colony-forming units (CFU) were assessed after overnight growth at 37ºC. Small portions of spleen and liver were also fixed in 3% paraformaldehyde and stained with hematoxylin/eosin (H&E). In some instances, mice were observed for mortality for 10 days after challenge.

For the study of the specific CD8+ T cell response, mice were infected with recombinant ovalbumin-expressing *Listeria monocytogenes* (Lm-Ova, kindly provided by H. Shen, Philadelphia, PA) intravenously[19]

Flow cytometry

Spleen cells (1 x 10^6) were stained (30 min, on ice) with FITC- or PE-conjugated antibodies or with biotinylated antibodies followed by streptavidin-PE or -PE–Cy5 (Becton Dickinson). The following antibodies and multimers were used: anti-DX5 (DX5), -B220/CD45 (RA3–6B2), -CD4 (GK1.5), -CD8 (53–6.7), -CD11b (M1/70), -CD11c (HL3), -CD25 (PC16), -CD43 (1B11), -CD69 (H1.2F3), -IFNg (XMG1.2), -TNFa (MP6-XT22) and –IL-10 (JES5-16E3), all from BD Biosciences) and H2-Kb/SIINFEKL or H2-M3/fMIGWII multimers (Streptamers; IBA, Germany)[20]. Cells were washed and analyzed on a FACScan flow cytometer (Becton Dickinson), counting 10^4 target cells using CELLQuest software (Becton Dickinson). For intracellular cytokine staining, spleen cells were collected 1 day after intravenous infection with Lm (1 x 10^6) and cultured in the presence of brefeldin A (10 mg/ml; Sigma), alone or together with phorbol 12-myristate 13-acetate (25 ng/mL) and ionomycin (1 μM) for 5 h. Cells were then surface-labeled, fixed and permeabilized with cytofix/cytoperm (Becton Dickinson), stained with anti-IFNγ-FITC, anti-TNFα-PE or anti-IL-10-PE antibody (BD Pharmingen) and analyzed by flow cytometry as above.

Adoptive transfer experiments.

CD3+ CD8+ CD44 low cells were purified from blood of CD69+/+ and CD69-/- OT-I mice by FACS sorting (MoFlo XDP, Dako) with anti-CD3 (145-2C11, BD Bioscience),

anti-CD8-PE (5H10, Caltag) and anti-CD44-FITC (IM7, BD Biosciences). The purity was greater than 99%. 100 naïve CD8+ OT-I T cells were transferred intraperitoneally.

Preparation of bone marrow-derived myeloid dendritic cells (BMDC), bone marrow-derived macrophages (BMDM) and peritoneal elicited macrophages (PEM)

Bone marrow was collected from femurs of mice and cultured to generate BMDM and BMDC. For BMDM, cells were cultured for 7 days in complete RPMI 1640 containing 20% heat-inactivated FCS (HI-FCS), and 20% culture supernatant from L929 cells as a source of granulocyte-colony stimulating factor (G-CSF). After day 7, cells were washed and cultured in the above medium without L929 supernatant and without antibiotics in a 96-well plate. To generate BMDC, cells were cultured for 7 days in 10 ml RPMI 1640/10% HI-FCS and 20 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) replacing half of the media for fresh media every 3 days. After day 7, non-adherent BMDC were collected and cultured in medium without GM-CSF and without antibiotics in a 96-well plate.

Resident peritoneal elicited macrophages (PEM) were obtained by peritoneal lavage, plated (10^5 cells/well in 100 μl) in RPMI 1640 / 10% HI-FCS without antibiotics on 96-well plates, and incubated (overnight, 37ºC). Cells were washed three times in antibiotic-free complete media. The remaining adherent cells were typical peritoneal macrophages.

***Ex vivo* Lm infection**

To assess intracellular Lm growth in BMDM, BMDC and PEM, cells were plated in 96-well plates in antibiotic-free complete medium. For infection, medium was removed, Lm (multiplicity of infection (MOI) of 5, except where indicated) were added to each well and incubated (30 min, 37ºC), washed and incubated for an additional 15 min. Medium

was then aspirated and replaced with medium containing gentamicin (10 μg/ml, Sigma), capable of killing any remaining extracellular Lm but unable to affect growth of intracellular bacteria. Plates were maintained at 37ºC throughout the assay. Time 0 was taken after 30 min incubation with gentamicin. At various times post-infection, cells were washed with antibiotic-free Dulbecos's Phosphate Buffered Saline (DPBS) (Gibco), and lysed in 100 μl DPBS containing 0.05% Triton X100; serial dilutions of lysates were plated on BHI agar plates, incubated (24 h, 37ºC), and bacterial colonies were counted. To quantify and locate Lm within peritoneal macrophages, 1 x 10^5 PEM were plated on coverslips in 24-well plates, and Lm infection was performed as above. At different times post-infection, cells were washed in DPBS and fixed with DPBS containing 2% paraformaldehyde (20 min, room temperature). For immunofluorescent staining, coverslips were washed again in DPBS and cells permeabilized with DPBS containing 20 mM glycine and 0.05% saponin (15 min). Cells were stained with rabbit anti-Lm serum (Difco) diluted in DPBS containing 20 mM glycine, 1% BSA (Sigma) and 0.025% saponin (1 h), then washed in DPBS with 20 mM glycine and 0.025% saponin. Secondary staining was performed with Alexa 488 goat anti-rabbit (Molecular Probes, Eugene, OR) and Alexa 594 phalloidin diluted in DPBS with 20 mM glycine, 1% BSA and 0.025% saponin. Phalloidin staining identifies only Lm in host cell cytosol, as Lm within this compartment polymerize host cell actin on their surface [21]. Cells were then washed and analyzed directly on an LSC microscope (Compucyte Corp, Cambridge, MA) to quantify bacterial fluorescence. To compare data from four different experiments, each time point is represented as the percentage of the maximum fluorescence obtained in each experiment. Alternatively, cells were washed and mounted in Aqua Poly/Mount (Polysciences, Warrington, PA) to localize cytosolic Lm in a Fluoview confocal laser scanning microscope (Olympus, Japan).

Quantitative real-time RT-PCR analyses

Total RNA was extracted from fractionated splenocytes using Tri Reagent (Sigma-Aldrich) as recommended. The enriched lymphocyte fraction was obtained by depletion of adherent cells by adhesion to plastic followed by depletion of B cells by panning with anti-rat IgM (Dako). The cells remaining were highly enriched in T lymphocytes. The adherent cells obtained before panning with anti-rat IgM were used as the adherent fraction. Real-time PCR was performed in a rapid thermal cycler system (Lightcycler; Roche Diagnostics) with primers from different exons that generated products of ~200 bp. Results for each cytokine are normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression and measured in parallel in each sample.

Cell death assays

Splenocytes from infected mice (3 d, 7.5 x 10^3 Lm) were collected and cultured for 24 h and cell death was determined by annexin V-FITC (BD) and PI staining. Flow cytometric analysis was performed on a FacScan cytometer.

Statistical analysis

Data were plotted using Prism software (Graphpad, Inc.). A Mann-Whitney unpaired test with two-tailed p-values and 95% confidence intervals was used for all statistical analyses except for a 2-way anova test that was used to compare cell death between WT and CD69-/- splenocytes ex vivo and a Log rank test that was used to compare survival among $CD69^{+/+}$ and $CD69^{-/-}$ mice.

Results

Major leukocyte populations are not altered in Lm-infected CD69$^{-/-}$ mice

To study the role of CD69 in the immune response to Lm infection, we compared various parameters of listeriosis in CD69$^{-/-}$ and WT mice. To determine whether Lm infection altered leukocyte populations in CD69$^{-/-}$ compared to WT mice, we performed flow cytometry using specific antibodies that define major spleen cell populations. In mice infected with 5 x 10^3 Lm, we detected no differences at one or three days post-infection in B cells, CD4 T cells, CD8 T cells and NK lymphocyte percentages or in lymphocyte subpopulations, including CD4$^+$ T cells expressing the activation markers CD25 or CD43, or CD11b$^+$ cells (Table I). There were also no major differences between CD69$^{-/-}$ and WT mice infected with 10^6 Lm, although we found a small increase in B220$^+$ cells at this dose, correlating with a minor decrease in CD4$^+$ and CD8$^+$ T cells. Cell subtype proportions were thus similar in CD69$^{-/-}$ and WT mouse spleens following Lm infection. Myeloid cells are both Lm targets and key cells for total Lm clearance. To test the ability to kill intracellular Lm and Lm infectivity in CD69$^{-/-}$ and WT macrophages, we performed *in vitro* infections of peritoneal elicited macrophages (PEM). PEM were infected at a MOI of 5 or 1, and intracellular bacteria were quantified by counting bacteria CFU (Fig. 1A) or by measuring the mean fluorescence intensity of macrophages after immunofluorescence staining of intracellular Lm (Fig. 1H). WT and CD69$^{-/-}$ PEM were infected with similar efficiency and had similar Lm growth kinetics (Fig. 1A, H). CD69$^{-/-}$ and WT macrophages showed identical intracellular location of Lm, as indicated by Lm co-localization with actin (Fig. 1B-G). Moreover, we recovered similar numbers of Lm from infected BMDC and BMDM in CD69$^{-/-}$ and WT mice (Fig. 1I, J), although high CD69 levels are expressed in WT BMDM 6 h after infection (Fig. 1K). The results

indicate that there is no alteration in infectivity or bacterial clearance in CD69$^{-/-}$ myeloid cells.

Increased expression of Th1 cytokines in infected CD69$^{-/-}$ splenocytes

Our previous work supports a role for CD69 as a negative regulator of proinflamatory cytokine production in autoimmune and anti-tumor responses [16,17]. To test whether CD69$^{-/-}$ mice control expression of key cytokines following Lm infection, we quantified cytokine mRNA levels in spleen lymphocyte and adherent cell fractions. IFNα and IFNβ expression were increased in lymphocytes and adherent cells from spleens of infected CD69$^{-/-}$ mice compared to WT mice, with a two-fold increase in IFNβ mRNA induction in CD69$^{-/-}$ adherent cells and an important increment of this cytokine in lymphocytes at 1 and 3 days post-infection (Fig. 2). IFNα mRNA expression was markedly increased in mouse T lymphocytes at 3 days after infection, with a seven-fold increase, and a two-fold increase in T lymphocytes at 1 day post-infection, while a 30 to 50% increase in adherent cells was observed at 1 and 3 days post-infection. IFNγ also showed a considerable increase at 3 days post-infection in spleen lymphocytes and adherent cells (Fig. 2). We also found significant differences in IFNγ but not TNFα expression at 1 day post-infection in splenocytes of mice infected with a high Lm dose (Fig. 3A). The increase in IFNγ is predominant in CD8 T lymphocytes of infected CD69$^{-/-}$ mice, both in lymphocytes reactivated in vitro or in lymphocytes incubated only with brefeldin A (Fig. 3A,B), pointing to a role for CD69 in the CD8 response in innate immunity. We also analyzed the expression of immunosuppressive cytokines that are induced following Lm infection as part of the Lm-evasion mechanism. We observed a small increase in TGFβ mRNA at 1 day post-infection (Fig. 2), and a non-significant increase in IL-10 intracellular protein (Fig. 3A). The mRNA level of other

proinflammatory cytokines such as IL-1β and the chemokine CCL5 was not affected in infected CD69$^{-/-}$ splenocytes (data not shown). CD69 thus modulates cytokine production during the response to Lm infection, with the absence of CD69 leading to exacerbated IFN production.

Increased tissue damage in infected CD69$^{-/-}$ mice

Production of type I IFN in Lm-infected WT mice is associated with apoptosis induction in lymphocytes, which impairs the anti-Lm response; we therefore studied whether the increased production of type I IFN observed in Lm-infected CD69$^{-/-}$ mice further increased lymphocyte death. We found that at 3 days post-infection CD69$^{-/-}$ mice showed increased apoptotic lesions compared to WT counterparts, corresponding to an overall increase in liver and spleen damage (Fig. 4). We observed centro-nodular depletion and disorganization of spleen white pulp in all CD69$^{-/-}$ mice, compared to the well-preserved white pulp in WT mouse spleens (Fig. 4A, B). The liver showed increased lymphoid infiltration foci and an increased number of apoptotic hepatocytes in CD69$^{-/-}$ mice, while only small numbers of apoptotic foci were found in WT mice (the comparative degree of severity, according to a histologist's subjective scoring from 0 - no damaged tissue - to 5 - highly damaged tissue- was 1 for WT and 3 for CD69$^{-/-}$ mice) (Fig. 4C, D). In accordance, we observed an increase in Annexin^{+} PI^{+} cells when splenocytes obtained 3 day post-infection were cultured *in vitro* for an additional 24 h, with medium alone or with antibiotics (penicillin and streptomycin) (Fig. 4E).

Increased susceptibility of CD69$^{-/-}$ mice to Lm infection

Since the anti-Lm immune response in CD69$^{-/-}$ mice is altered by increased proinflammatory cytokine production, augmented apoptosis, we studied listeriosis

susceptibility and survival in CD69$^{-/-}$ and WT mice. Following a primary Lm infection using a sublethal dose of bacteria, the number of Lm in CD69$^{-/-}$ mouse spleens was 10-50 times greater than in WT mice at 3 days post-infection (Fig. 5A); only small, although significant differences were found as soon as at 1 day after infection (Fig. 5A). Greater differences were observed when we compared bacteria recovered from livers of Lm-infected CD69$^{-/-}$ and WT mice 3 days post-infection, with 10^2 to 10^3 times more bacteria in CD69$^{-/-}$ mouse livers (Fig. 5A). Greater recoveries of Lm in CD69-/- mice were observed when mice were infected intravenously or intraperitoneally with different sublethal doses, independent of the mouse strain used (BALB/c or C57BL/6) (data not shown and Supplementary figure 1). CD69$^{-/-}$ mice had thus an impaired anti-Lm immune response associated with increased tissue damage.

The consequence of increased Lm growth and early damage to spleen and liver in infected CD69$^{-/-}$ animals was a lower survival rate compared to WT mice (Fig. 5B). Only a 20% of CD69$^{-/-}$ mice survived an Lm dose that was sublethal in WT mice.

Augmented innate response but defective specific response in infected CD69$^{-/-}$ mice.

The fact that CD69 deficiency contribute to defective response to Lm as early as day post infection indicate an involvement of innate component in the diseases progression. To analyze the contribution of innate immune response to the defective control of infection by CD69 -/- mice, infections were performed in CD69-/- and CD69+/+ in a RAG2-/- background. CD69 deficient RAG2-/- had not impaired capacity to control Lm infection but, on the contrary, showed lower levels of listeriosis than CD69+/+ mice (Fig. 6A). Therefore, it seems that there is an inadequate interaction between lymphocytes and innate immune cells.

Even if differences, start arising at early time points, given that $CD69^{-/-}$ mice start to dying on day 4, we check whether CD69 deficiency affects the listeriosis at later time points, when the initial specific response start to contribute to the disease progression. Results at 5 days post infection, using a low dose of Listeria ($5x10^3$), showed even more increased listeriosis in the $CD69^{-/-}$ surviving mice (75%) compare to CD69+/+ counterparts (Fig.6B). To study the specific T cell response to Lm, we analyzed splenocytes of mice at seven days post-infection with Lm-OVA, reactive with H2-Kb/SIINFEKL or H2-M3/fMIGWII multimers. As shown in Figure 6C, CD69-/- mice presented lower frequencies of H2-Kb/SIINFEKL multimer stained CD8+ T lymphocytes (Fig. 6C and D) In contrast, the frequencies of H2-M3/fMIGWII multimer stained CD8+T cells were not different (Fig. 6E). However, the diminished lower frequency of specific CD8+ T cells observed in CD69-/- mice cannot be explained by a defect intrinsic to the T cells, since transferred $CD69^{-/-}$ OT-I CD8+ T lymphocytes were not recovered in lower frequencies from the spleens of 7 and 12 days LM-OVA infected mice than CD69+/+ OT-I CD8+ T lymphocytes. Interestingly, they neither had impaired IFNγ production(Supplementary figure 2).

Thus, CD69 deficiency enhance innate response to Lm, but has detrimental effects in the specific and unspecific lymphoid function in response to the infection.

Discussion

We studied the role of CD69 in protective immune responses to the intracellular pathogen Lm, and show that CD69 is associated with control of the immune response to Lm infection. Bacterial clearance capability of CD69-/- innate myeloid cells is unaltered, as demonstratred by in vitro experiments and by the fact that CD69-/- RAG2-/- mice can control the infection even better than the CD69+/+ counterparts. However, potent cell-mediated protective immunity was induced in $CD69^{-/-}$ mice with increased type I and II IFN production. In $CD69^{-/-}$ mice, the uncontrolled Th1 inflammatory response is in agreement with increased damage to liver and spleen, increased susceptibility to Lm infection, and rapid death. Cytokines induced during the innate phase of infection, such as type I IFN, sustain expansion and survival of antigen-activated T cells [10,22-25]. However, IFNα/β impairs the anti-Lm immune response by making lymphocytes and macrophages more sensitive to the pathogen's listeriolysin-O (LLO) mediated toxicity [8-10,26,27]. Actually, it is being speculated that, even if the pathways activated by Lm and viruses are the same, the lower type I IFN levels secreted in response to Lm is probably to restrict massive LLO-induced of apoptosis.

In our study, the $CD69^{-/-}$ T cells, under the observed higher IFNα/β levels, might be exposed to increased activating signals, probably reflected by the observed increased IFNγ production by $CD8^{+}$ T cells. In this CD8+ T cells, the INFγ production at such an early time point as one day post-infection must be due to unspecific activation of CD44 high memory T cells, which have been seen to react to innate cytokines such as INFα[28]. LLO-mediated toxicity of activated sensitized cells may be responsible for the increase in cell death followed by tissue necrosis observed in Lm-infected $CD69^{-/-}$ mice. One possible scenario is that in Lm-infected CD69-/- mice, antigen presenting cells produce

an increased INFα/β response, sustained in vivo by other factors. Indeed IFNγ may support in vivo feed back regulation of INFα/β production [29].

Therefore, we consider the INFγ, which is increased during Lm infection in CD69-/- mice, as an important player in cell death induction. In addition to the functions of stimulating phagocyte bactericidal activity and the development of the Th1 response, INFγ also mediates apoptosis by the induction of the expression of many proapoptotic molecules, including Caspase 1, PKR, DAPs, cathepsin D, and surface expression of Fas and TNFα receptors [30-37]. IFNγ is required for direct activation-induced death of T lymphocytes, as was demonstrated *in vitro* [6]. In addition, IFNγ-activated macrophages indirectly trigger CD4 T cell apoptosis during mycobacterial infection, where adherent cells are needed to mediate this effect [5]. Splenocytes from Lm-infected $CD69^{-/-}$ mice showed a considerable increase in IFNγ mRNA levels in spleen lymphocytes and adherent cells at 3 days post-infection; we also found an important increase in IFNγ protein expression in NK and $CD8^{+}$ T cells from Lm-infected $CD69^{-/-}$ mice.

In Lm-infected $CD69^{-/-}$ mice, increased IFNγ production in the early phase may induce a stronger T cell activation that, in the presence of infected apoptotic macrophages, may lead to increased apoptosis of T cells and tissue necrosis.

CD69 deficiency also induced a detrimental adaptive immune response to Lm infection, which could contribute to the deficient control of the infection from day three onwards[38], when newly primed specific T cells play a major role in infection control. However, this diminished response is not due to a defect in the capacity of CD69-/-CD8+T cells to respond to Lm infection, because they function similarly to CD69+/+CD8+T cells when transferred to infected CD69+/+ mice. It has been shown similar cross-presentation capability between CD69-/- and CD69+/+ mice in adoptive transfer experiments (Alari E., 2010 unpublised data).

A diminished specific respone could contribute to the deficient control of the infection, but it cannot explain the already increased listeriosis at day one post-infection. Since a defective innate response is neither the reason for the increased susceptibility in CD69-/- mice, given that CD69-/- RAG2-/- mice better control the infection, we hypothesize that a defective interaction between innate cells and lymphocytes contribute to the defective infection resolution.

The regulation of T cell activation and homeostasis is thus of major importance in the control of Lm pathogenesis, and CD69 may control Th1 immune activation by controlling the expression of type I and II IFN, restricting T lymphocyte number and activation during intracellular Lm infection. These mechanisms pertain to those that control expanded effector cells aimed to maintain T cell homeostasis [39].

In summary, we demonstrate a role for CD69 in the control of Th1-mediated inflammation during Lm infection. Our studies in $CD69^{-/-}$ mice show that CD69 contributes to the regulation of type I and II IFN, and has an important role in T cell homeostasis. Increased pro inflammatory responses can also induce pathological damage in autoimmune and inflammatory disorders, and indeed, CD69 has been implicated in autoimmune disease development [17]. Our previous studies in models of autoimmune and anti-tumor responses showed the anti-inflammatory/immune properties of CD69. Here, we provide evidence that CD69 is a critical negative regulator of immune activation during intracellular bacterial infection, protecting infected mice from lethal tissue damage.

Acknowledgments

We thank Dr. Hans-Willi Mittrücker (Berlin, Germany) for providing the Lm EGD used in this study. We are grateful to Dr. Dirk Busch (Munich, Germany) for providing H2-

Kb/SIINFEKL and H2-M3/fMIGWII multimers (Streptamers; IBA, Germany). We are grateful to Dr. Roy R. Lobb and Catherine Mark for excellent critical reading of the manuscript. J. Vega, and E. Alari were supported by predoctoral fellowships from the Spanish Ministry of Education and Science. This work was supported by grants (SAF2002-02895 and SAF2005-04876) from the Ministerio de Educación, Ciencia y Tecnología. The Department of Immunology and Oncology was founded and is supported by the Spanish National Research Council (CSIC) and by Pfizer.

References

1. Fleming SD, Campbell PA. Macrophages have cell surface IL-10 that regulates macrophage bactericidal activity. J Immunol. 1996;156:1143-1150.
2. Flesch IE, Kaufmann SH. Role of macrophages and alpha beta T lymphocytes in early interleukin 10 production during Listeria monocytogenes infection. Int Immunol. 1994;6:463-468.
3. Boyd AP, Ross PJ, Conroy H, Mahon N, Lavelle EC, Mills KH. Bordetella pertussis adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. J Immunol. 2005;175:730-738.
4. Redpath S, Ghazal P, Gascoigne NR. Hijacking and exploitation of IL-10 by intracellular pathogens. Trends Microbiol. 2001;9:86-92.
5. Dalton DK, Haynes L, Chu CQ, Swain SL, Wittmer S. Interferon gamma eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. J Exp Med. 2000;192:117-122.
6. Refaeli Y, Van Parijs L, Alexander SI, Abbas AK. Interferon gamma is required for activation-induced death of T lymphocytes. J Exp Med. 2002;196:999-1005.
7. Jiang J, Lau LL, Shen H. Selective depletion of nonspecific T cells during the early stage of immune responses to infection. J Immunol. 2003;171:4352-4358.
8. Auerbuch V, Brockstedt DG, Meyer-Morse N, O'Riordan M, Portnoy DA. Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. J Exp Med. 2004;200:527-533.
9. Carrero JA, Calderon B, Unanue ER. Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection. J Exp Med. 2004;200:535-540.
10. O'Connell RM, Saha SK, Vaidya SA, et al. Type I interferon production enhances susceptibility to Listeria monocytogenes infection. J Exp Med. 2004;200:437-445.
11. Monack DM, Mecsas J, Bouley D, Falkow S. Yersinia-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. J Exp Med. 1998;188:2127-2137.
12. Luder CG, Gross U. Apoptosis and its modulation during infection with Toxoplasma gondii: molecular mechanisms and role in pathogenesis. Curr Top Microbiol Immunol. 2005;289:219-237.
13. Pamer EG. Immune responses to Listeria monocytogenes. Nat Rev Immunol. 2004;4:812-823.
14. Testi R, D'Ambrosio D, De Maria R, Santoni A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol Today. 1994;15:479-483.
15. Sanchez-Mateos P, Sanchez-Madrid F. Structure-function relationship and immunochemical mapping of external and intracellular antigenic sites on the lymphocyte activation inducer molecule, AIM/CD69. Eur J Immunol. 1991;21:2317-2325.
16. Esplugues E, Sancho D, Vega-Ramos J, et al. Enhanced antitumor immunity in mice deficient in CD69. J Exp Med. 2003;197:1093-1106.
17. Sancho D, Gomez M, Viedma F, et al. CD69 downregulates autoimmune reactivity through active transforming growth factor-beta production in collagen-induced arthritis. J Clin Invest. 2003;112:872-882.
18. Esplugues E, Vega-Ramos J, Cartoixa D, et al. Induction of tumor NK-cell immunity by anti-CD69 antibody therapy. Blood. 2005;105:4399-4406.

19. Schiemann M, Busch V, Linkemann K, Huster KM, Busch DH. Differences in maintenance of CD8+ and CD4+ bacteria-specific effector-memory T cell populations. Eur J Immunol. 2003;33:2875-2885.
20. Knabel M FT, Schiemann M, Wulf A, Villmow B, Schmidt B, Bernhard H, Wagner H, Busch DH. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. Nat Med. 2002;8:631-637.
21. Tilney LG, Portnoy DA. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes. J Cell Biol. 1989;109:1597-1608.
22. Curtsinger JM, Schmidt CS, Mondino A, et al. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J Immunol. 1999;162:3256-3262.
23. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol. 2005;174:4465-4469.
24. Valenzuela JO, Hammerbeck CD, Mescher MF. Cutting edge: Bcl-3 up-regulation by signal 3 cytokine (IL-12) prolongs survival of antigen-activated CD8 T cells. J Immunol. 2005;174:600-604.
25. Havenar-Daughton C, Kolumam GA, Murali-Krishna K. Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection. J Immunol. 2006;176:3315-3319.
26. Carrero JA, Calderon B, Unanue ER. Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes. J Exp Med. 2006;203:933-940.
27. Stockinger S, Materna T, Stoiber D, et al. Production of type I IFN sensitizes macrophages to cell death induced by Listeria monocytogenes. J Immunol. 2002;169:6522-6529.
28. Marzo AL, Vezys V, Williams K, Tough DF, Lefrancois L. Tissue-level regulation of Th1 and Th2 primary and memory CD4 T cells in response to Listeria infection. J Immunol. 2002;168:4504-4510.
29. Trinchieri G. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). Curr Opin Immunol. 1997;9:17-23.
30. Tamura T, Ishihara, M., Lamphier, M. S., Tanaka, N., Oishi, I., Aizawa, S., Matsuyama, T., Mak, T. W., Taki, S., Taniguchi, T. . DNA damage-induced apoptosis and ice gene induction in mitogenically activated T lymphocytes require IRF-1. Leukemia 1997;11:439-440

31. Villa P KS, Earnshaw WC. Caspases and caspase inhibitors. Trends Biochem Sci 1997 22:388-393.
32. Yeung MC, Liu, J., Lau, A. S. . An essential role for the interferon-inducible, double-stranded RNA-activated protein kinase PKR in the tumor necrosis factor-induced apoptosis in U937 cells Proc Natl Acad Sci USA 1996;93:12451-12455.
33. Lee SB EM. The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology. 1994;199:491-496.
34. Inbal B CO, Polak-Charcon S, Kopolovic J, Vadai E, Eisenbach L, Kimchi A. DAP kinase links the control of apoptosis to metastasis. Nature;390:180-184.
35. Deiss LP, Feinstein, E., Berissi, H., Cohen, O., Kimchi, A. Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death Genes Dev. 1995;9:15-30.

36. Xu X, Fu, X. Y., Plate, J., Chong, A. S. . IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression Cancer Res 1998;58:2832-2837.
37. Tsujimoto M, Yip, Y. K., Vilcek, J. Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor J. Immunol. 136,2441-2444. J Immunol 1986;136:2441-2444.
38. Zinkernagel RM, Blanden RV, Langman RE. Early appearance of sensitized lymphocytes in mice infected with Listeria monocytogenes. J Immunol. 1974;112:496-501.
39. Grossman Z, Min B, Meier-Schellersheim M, Paul WE. Concomitant regulation of T-cell activation and homeostasis. Nat Rev Immunol. 2004;4:387-395.

Figure 1. Similar intracellular growth of Lm in WT and CD69$^{-/-}$ myeloid cells. (A) Peritoneal elicited macrophages (PEM) were obtained and infected *in vitro* with a MOI of 1 or 5 as indicated. At the times indicated, macrophages were lysed and the number of intracellular Lm was quantified. One representative experiment is shown of four with similar results. (B-G) Intracellular location of Lm in PEM from WT (B, C and D) or CD69-/- (E, F and G) was detected by staining actin (B and E) and intracellular Lm (C and F); yellow color indicates co-localization (D and G). (H) The mean fluorescence of stained intracellular Lm was quantified at the times indicated. The graph shows data grouped from four independent experiments (see Methods). (I) BMDC obtained from WT or CD69$^{-/-}$ mice were infected *in vitro* at a MOI of 5. At the times indicated, cells were lysed and the number of intracellular Lm quantified. (J) BMDM obtained from WT or CD69$^{-/-}$ mice were infected *in vitro* at the indicated MOI. At the times indicated, cells were lysed and the number of intracellular Lm quantified. The experiment with BMDC is representative of four while the experiment with BMDM is representative of three with similar results. (K) BMDM were stained with anti-CD69-PE and F4/80-FITC at the times indicated after *in vitro* infection with Lm.

Figure 2. Increased Th1 cytokine and chemokine expression in CD69$^{-/-}$ mice after Lm infection. Mice were infected with a sublethal dose of Lm and cytokines from adherent and T lymphocyte (LT)-enriched splenocytes were measured by real time RT-PCR at 1 and 3 days post-infection. All data were normalized with HPRT. Pools of three mice were used for quantification. One experiment representative of two with similar results.

Figure 3. Increased IFNg protein expression in CD69-/- mice. (A, B) Splenocytes from mice infected intravenously with a high Lm dose (10^6) for 24 h were cultured with brefeldin-A (BFA) (B) or phorbol myristate acetate (PMA), ionomycin (Io) and BFA (A) for 4 h, then stained for IFNγ, TNFα and IL-10 and cell surface markers. Bars represent mean ± SD; *n* = 4 (WT), *n* = 6 ($CD69^{-/-}$). *p<0.05 **p<0.01

Figure 4. Increased cell death and tissue damage in infected $CD69^{-/-}$ mice. (A-D) Spleen (100X magnification) and liver (40X magnification) sections obtained one and three days after infection with a sublethal Lm dose (7.5 x 10^3) were hematoxylin/eosin stained. White pulp is clearly observed in WT spleen sections (A), while centro-nodular depletion and disorganization of white pulp is found in $CD69^{-/-}$ sections (B). In the liver, WT sections are well preserved (C), while $CD69^{-/-}$ sections show increased lymphocyte infiltration and apoptotic foci (D). Sections shown are representative of two experiments with similar results; *n* = 3 per group per experiment. (E) Splenocytes obtained 3 days after infection with a sublethal Lm dose (7.5 x 10^3) were cultured for 24 hours alone or with antibiotics (penicillin/streptomycin), then stained with Annexin V and PI to detect cell death. Bars represent the mean ± SD; *n* = 3 (WT), *n* = 4 ($CD69^{-/-}$). p < 0,05 between Wt and CD69-/- mice by 2-way Anova analysis.

Figure 5. Increased susceptibility of $CD69^{-/-}$ mice to Lm. (A) Mice were infected with 7.5 x 10^3 CFU/mouse and colony counts in the spleen were determined at 1 and 3 days post-infection. Colony counts in the liver were quantified after 3 days. Bars represent the mean ± SD. Spleen samples: *n* = 7 (3 dpi), *n* = 4 (1 dpi). One experiment representative of seven with similar results. Liver samples: *n* = 3 (WT), *n* = 4 ($CD69^{-/-}$). One experiment representative of four with similar results. *p<0.05 **p<0.01. (B) WT (filled

triangles) and CD69$^{-/-}$ mice (empty squares) were inoculated i.v. with 7.5 x 10^3 CFU/mouse and monitored for survival. $p = 0.0135$ by the log rank test; $n = 5$ per group. One experiment representative of five with similar results.

Figure 6. Adaptive versus innate response contribution to response to Lm infection in CD69-/- mice. (A) CD69 deficiency does not impair the innate immune response to Lm in RAG2-/- mice**.** RAG2-/- CD69 +/+ and CD69-/- mice in Balb/c background were infected with 5x10 3 CFU and 4 days later c.f.u. were determined in the spleen. *n= 3* for the WT group (not considering the 1 mouse that had succumbed on day 4) and *n=4* for the CD69-/- group. Experiment representative of two similar experiments with similar results. (B) Increased listeriosis in CD69-/- at later time points in the acute infection. Mice were infected with 5x10^3 CFU and spleen and liver colony counts were determined after 5 days. *n= 8* for the WT group and *n= 6* for the CD69-/- group (2 mice being found dead at 5dpi). *** p<0.001. One experiment representative of two similar experiments with similar results**.** (C) Decreased adaptive immune response in infected CD69$^{-/-}$ mice. CD69$^{-/-}$ or WT mice were infected with 5 x 10^{3}^{3} Lm-OVA, and 5 days later splenocytes were collected and analysed for specific CD8 T cells. Representative dot plots showing splenocytes stained with PE multimers detecting SIINFEKL specific CD8+ T cells. (D) Percentage of SIINFEKL-multimer positive CD8+ T cells gated on live splenocytes or CD8+ cells. (E) Graph showing percentage of fMIGWII-multimer positive H2-M3 restricted CD8 T cells gated on live splenocytes or CD8+ cells. *p < 0,05. *n* = 8 for WT group and *n = 11* for CD69-/- group.

Table I. Distribution of main subpopulations in Lm-infected mice

Lm dose:	7.5×10^3		7.5×10^3		1×10^6	
	1 dpi[1]		3 dpi[2]		1 dpi[3]	
	WT	**CD69$^{-/-}$**	**WT**	**CD69$^{-/-}$**	**WT**	**CD69$^{-/-}$**
B220+	63.05 ± 3.97	65.35 ± 4.36	71.93 ± 3.33	68.55 ± 7.42	**62.95 ± 2.77**	**68.34 ± 2.01**
CD4+	19.56 ± 0.99	19.35 ± 3.76	18.68 ± 4.92	20.02 ± 5.75	**23.56 ± 2.72**	**19.33 ± 1.41**
CD8+	10.76 ± 1.8	9.44 ± 1.76	9.81 ± 3.16	10.38 ± 3.56	**9.60 ± 0.87**	**8.15 ± 0.94**
DX5+	2.12 ± 0.41	2.07 ± 0.43	2.88 ± 0.14	3.25 ± 0.60	2.03 ± 0.49	2.12 ± 0.21
CD11b+	2.20 ± 0.25	2.41 ± 0.66	4.24 ± 1.98	3.88 ± 0.58	ND	ND
CD11c+	ND	ND	5.00 ± 0.60	4.06 ± 0.91	ND	ND
CD4/CD25	12.85 ± 0.52	14.25 ± 1.78	16.44 ± 1.36	14.97 ± 1.43	4.60 ± 0.16	4.25 ± 0.26
CD4/CD43	ND	ND	8.13 ± 1.77	7.96 ± 1.72	ND	ND
CD69+	5.77 ± 0.83	0.16 ± 0.04	22.36 ± 11.46	0.32 ± 0.24	ND	ND

[1] n = 4 mice/group

[2] n = 7-11 (WT) and 9-13 (CD69$^{-/-}$), except for CD11b, DX5 and CD43, where n = 4 (WT) and 4-6 (CD69$^{-/-}$) mice

[3] Mice infected i.v. with a high dose of Lm (10^6), n = 4 (WT) and 6 (CD69$^{-/-}$) mice

Data in bold $p<0.05$

Fig 1

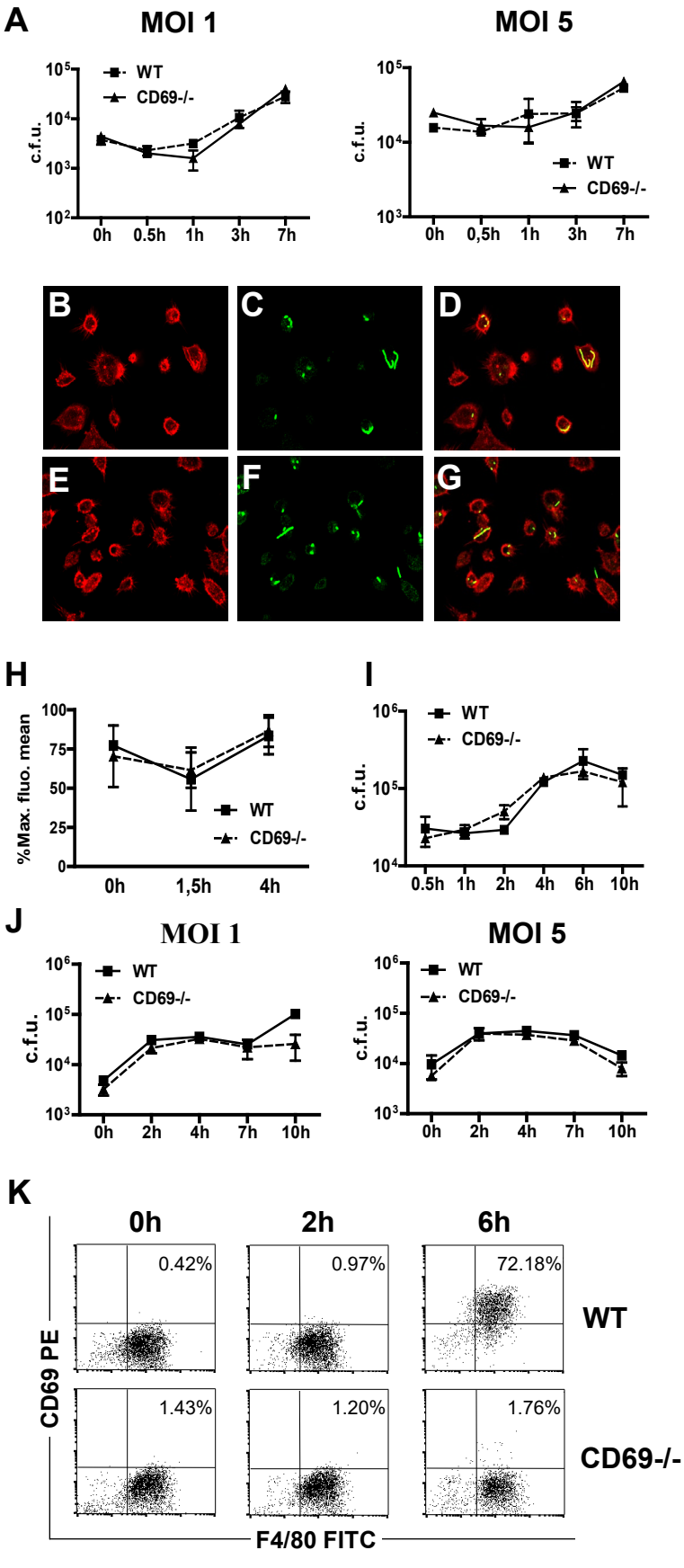

A
MOI 1
MOI 5
WT
CD69-/-
c.f.u.
0h
0.5h
1h
3h
7h
0,5h
B
C
D
E
F
G
H
%Max. fluo. mean
1,5h
4h
I
2h
6h
10h
J
K
0h
2h
6h
0.42%
0.97%
72.18%
1.43%
1.20%
1.76%
CD69 PE
F4/80 FITC

Fig 2

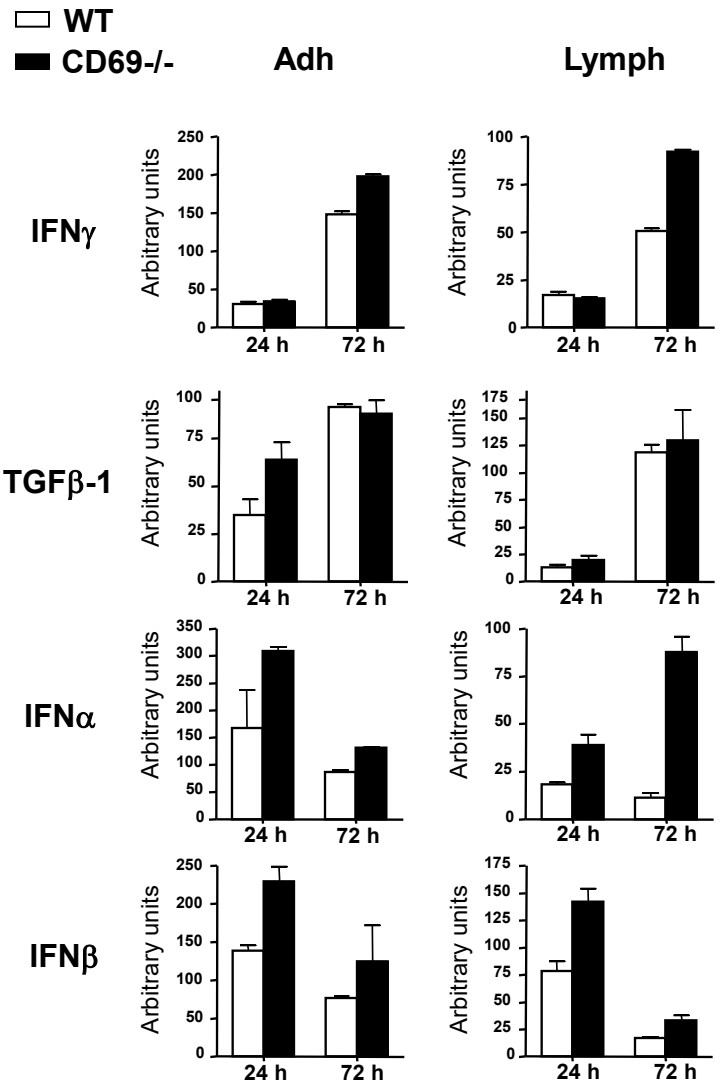
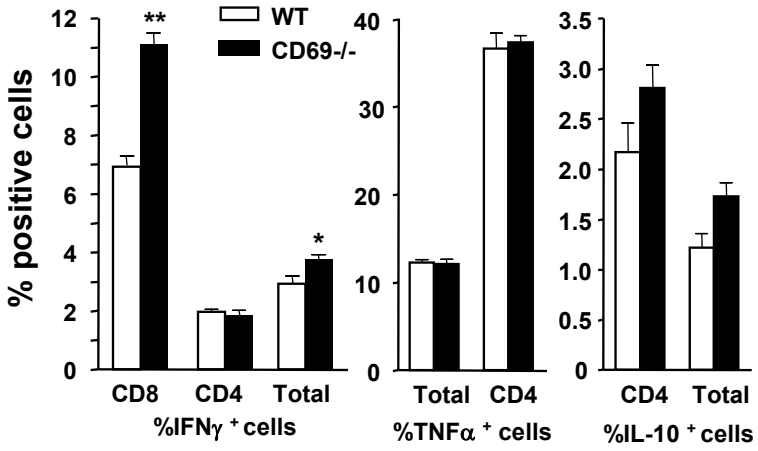

WT
CD69-/-
Adh
Lymph
IFNγ
TGFβ-1
IFNα
IFNβ
Arbitrary units
24 h
72 h

Fig 3

A


WT
CD69-/-
% positive cells
**
*
CD8
CD4
Total
%IFNγ + cells
Total
CD4
%TNFα + cells
CD4
Total
%IL-10 + cells

B

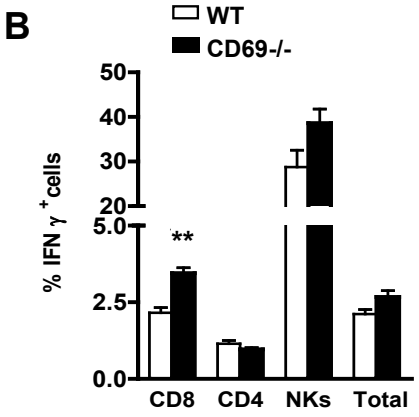
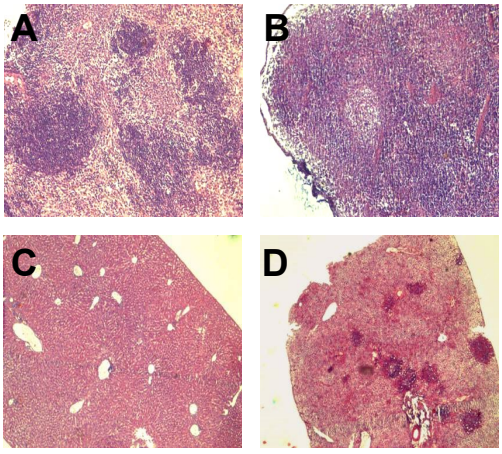

WT
CD69-/-
% IFN γ + cells
**
CD8
CD4
NKs
Total

Fig 4


A
B
C
D

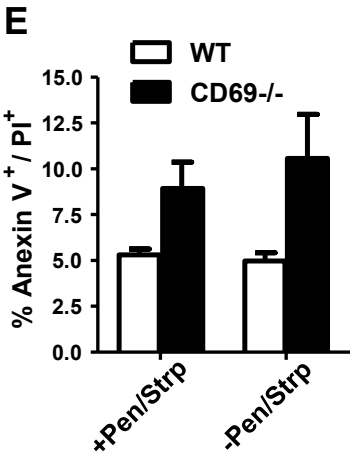

E
WT
CD69-/-
% Anexin V+ / PI+
15.0
12.5
10.0
7.5
5.0
2.5
0.0
+Pen/Strp
-Pen/Strp

Fig 5

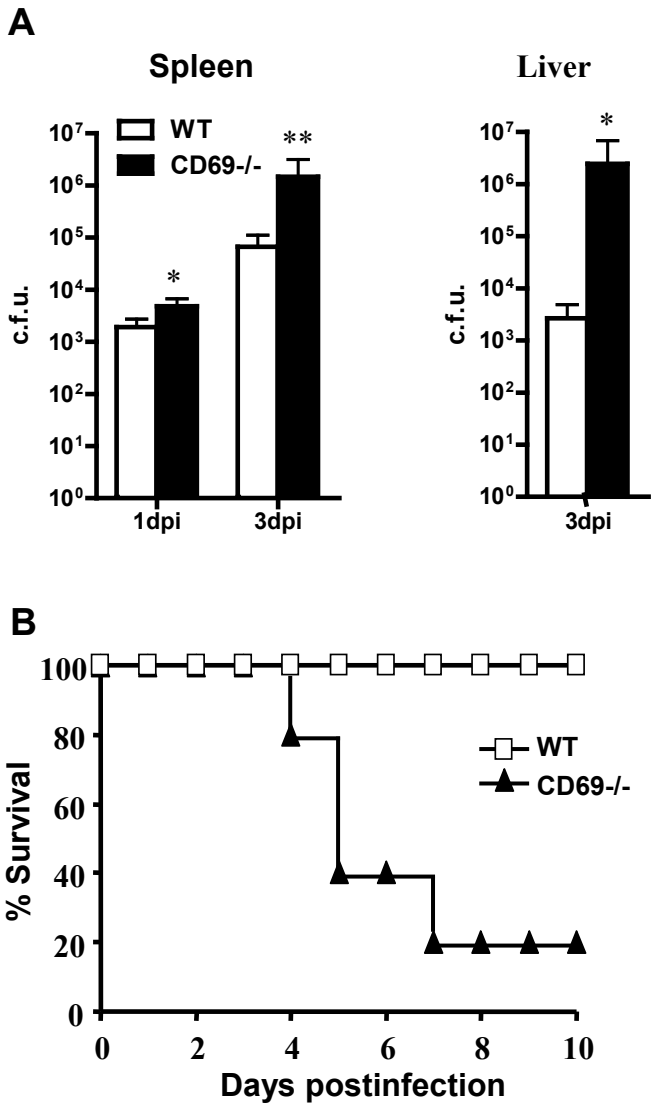

A
Spleen
Liver
WT
CD69-/-
c.f.u.
1dpi
3dpi
3dpi
B
% Survival
Days postinfection
WT
CD69-/-

Fig 6

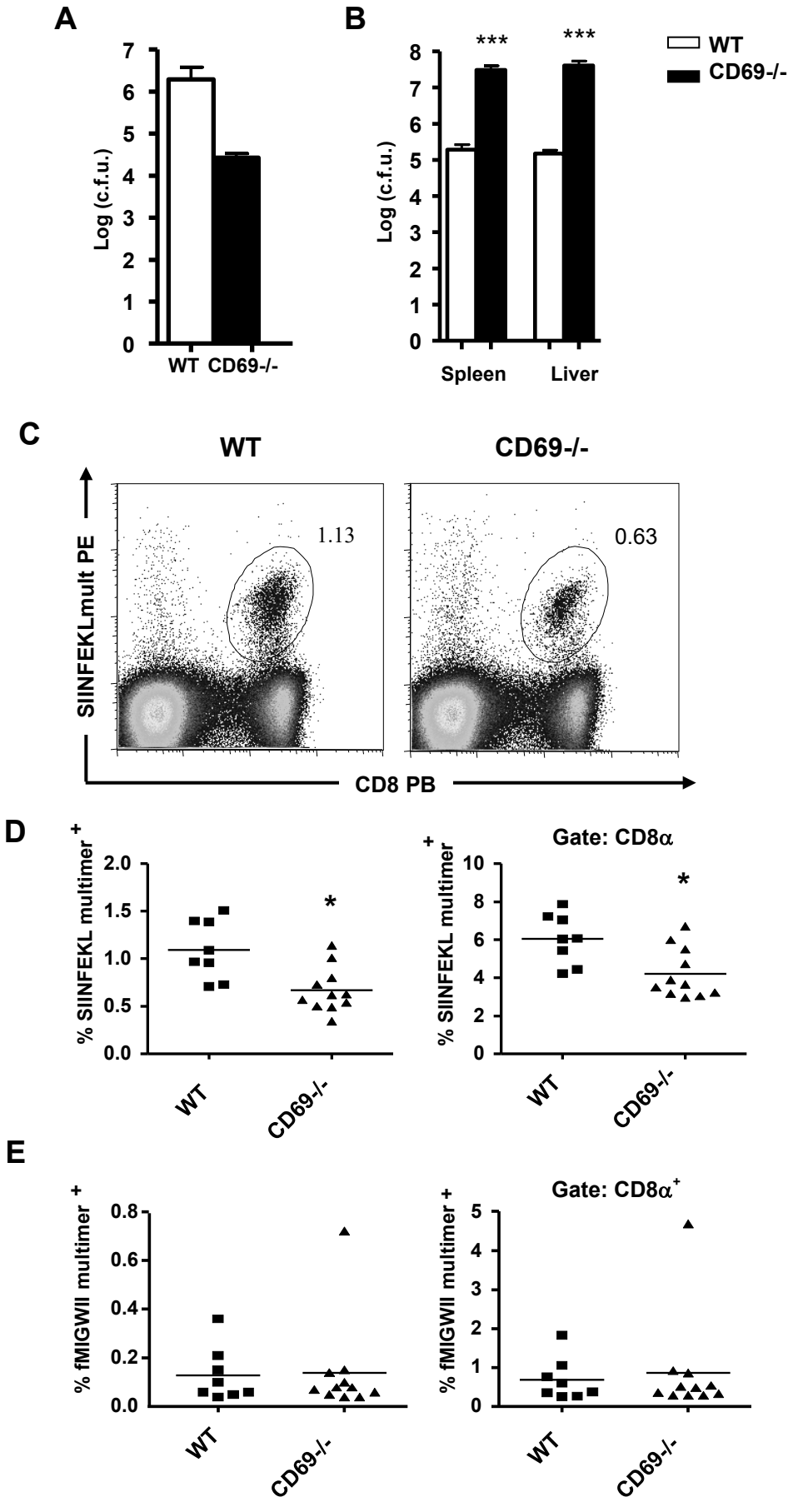
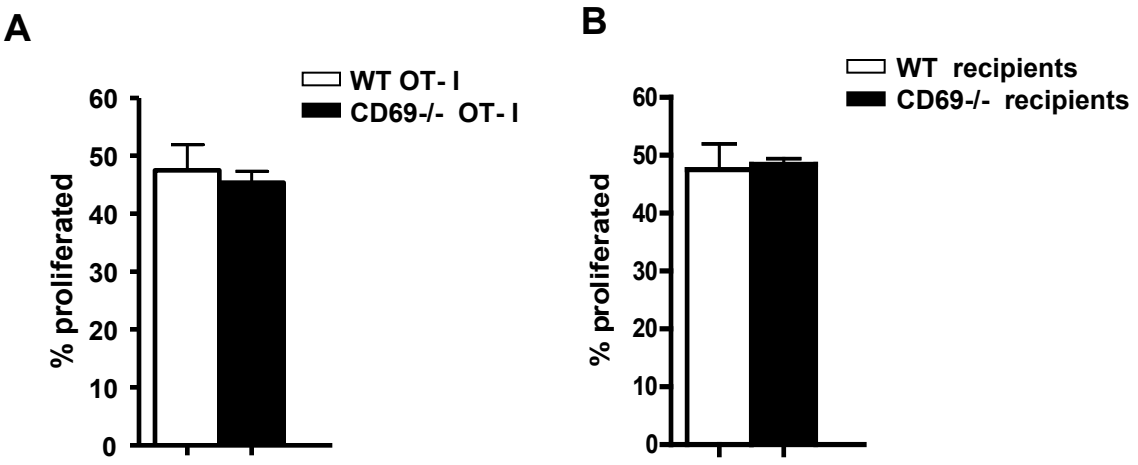
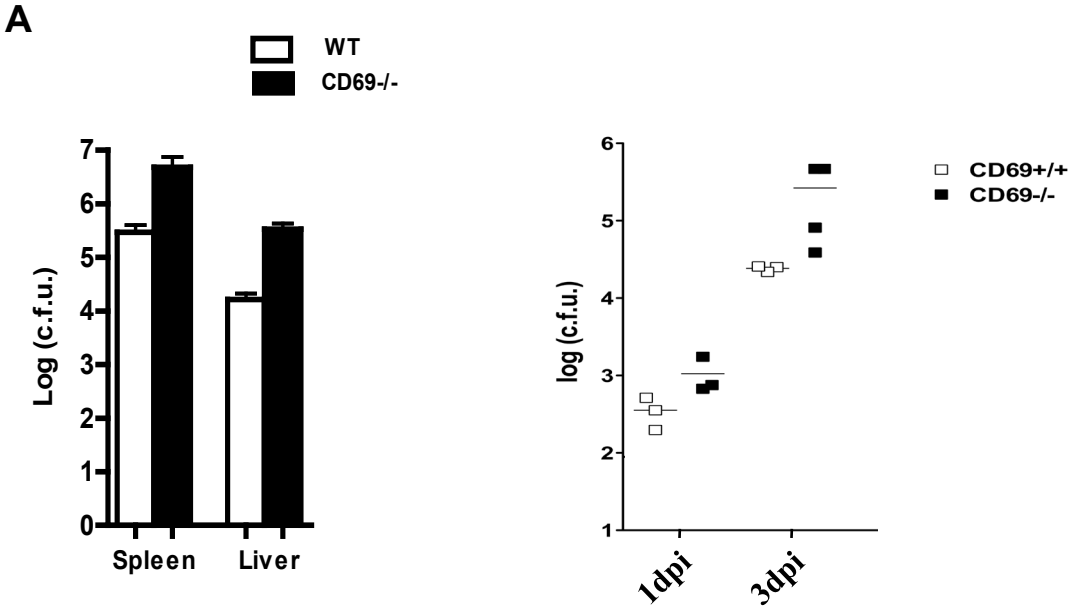

A
Log (c.f.u.)
WT
CD69-/-
B
***
***
WT
CD69-/-
Log (c.f.u.)
Spleen
Liver
C
WT
CD69-/-
1.13
0.63
SIINFEKLmult PE
CD8 PB
D
% SIINFEKL multimer+
Gate: CD8α
E
% fMIGWII multimer+
Gate: CD8α+

Fig 7


A
WT OT- I
CD69-/- OT- I
% proliferated
60
50
40
30
20
10
0
B
WT recipients
CD69-/- recipients
% proliferated
60
50
40
30
20
10
0

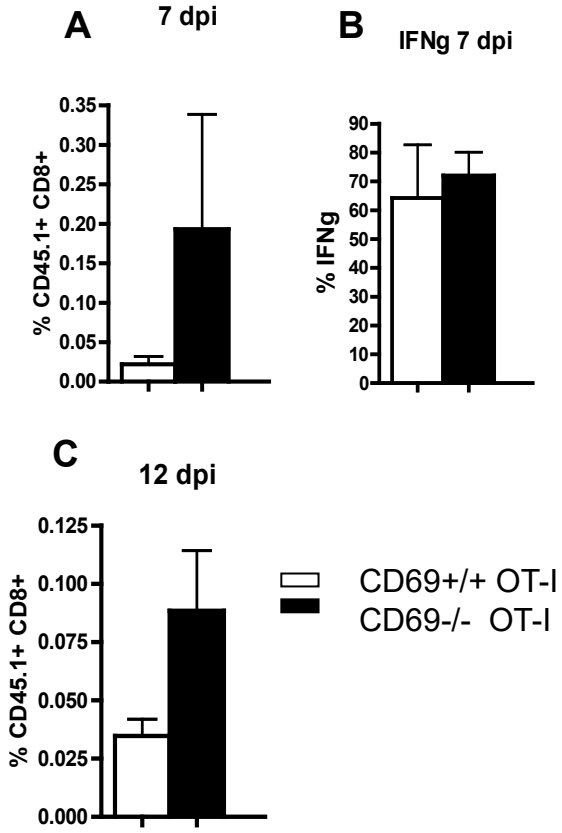
Suplementary figure 1



Supplementary figure 3. **Increased susceptibility of Balb/c CD69-/- mice to Lm**

(A) Balb/c mice were infected with $5x10^3$ CFU/mouse and 4 days after infection colony counts were determined in the spleen and liver. *n= 3* mice per group. (B) Mice were infected intraperitoneallywith 7.5 x 10^3 CFU/mouse and colony counts in the spleen were determined at 1, 3 and 7 days post-infection. *n* = 3 - 4 per group per time point, except for CD69-/- at 7dpi, when one of 3 mice died and is not shown. One experiment representative of at least three similar experiments with similar results. WT (empty squares) and CD69-/- mice (filled squares)

Suplementary figure 2



Supplementary figure 1. CD69 deficiency on CD8+ T cells does not reduce their frequency during the specific response to Lm. CD69+/+ or CD69-/- naïve OT-I CD8+ T cells were intraperitoneally transferred into CD45.1 recipients (100 cells per mouse) which were subsequently infected with 5x103 Lm-OVA CFU. (A) Graph showing the frequency of CD45.2+ CD8+ cells in the spleens 7 days after infection. *n= 3* mice per group. (B) Percentage of IFNg producing cells within the CD45.2+ CD8+ transferred cells. (C) Frequency of transferred cells 12 days after infection. *n=4* mice per group.