UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENICAS QUÍMICAS

TESIS DOCTORAL

Función de CD69 en progenitores hematopoyéticos y la infección por el virus Vaccinia

Function of CD69 hematopoietic progenitor cells and in Vaccinia infection

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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El trabajo presentado como tesis doctoral con el título: "**Function of CD69 in hematopoietic progenitor cells and in Vaccinia virus infection"** por Doña Laura Notario Muñoz, ha sido realizado bajo la dirección de la Doctora Pilar Lauzurica Gómez, en el Departamento de Activación Inmunológica del Instituto Salud Carlos III, y tutorizada por la Dra. María Belén Yélamos López.

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A mi familia, por todo.

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ABBREVIATIONS

Abbreviations

mAb: monoclonal antibody

APC: Antigen-presenting cell

BAC: Bacterial artificial chromosome

BFA: Brefeldin A

BCR: B cell receptor

BM: Bone Marrow

Bp: Base pair

BrdU: Bromodeoxyuridine

BSA: Bovine serum albumin

CAIA: Collagen II (CII)-antibody induce arthritis

CD: cluster of differentiation

CFU: Colony forming units

Chip: Chromatin Immunoprecipitation

CIA: Collagen-induced arthritis

CLP: Common myeloid progenitor

CNS: conserved non-coding sequences

COX-2: Cyclooxygenase-2

CTLD: C-Type Lectin Domain

DAMP: damage-associated molecular patterns

DC: dendritic cell

DSS: dextran sodium sulphate

FACS: Flow cytometry

FTY720: Fingolimod

G-CSF: Granulocyte-colony stimulating factor

GM: geometric mean

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GRZ-B: Granzyme B

HIF: Hypoxia-inducible factor

HSC: Hematopoietic stem cell

HSPC: Hematopoietic stem progenitor cell

IFN: Interferon

Igs: immunoglobulins

IL: Interleukin

ILC: innate lymphoid cells

Ion: Ionomycin

i.p: intraperitoneal

i.v: intravenous

Kb: Kilobase

KO: knockout

KSL: c-Kit Sca Lin- cells

LCMV: lymphocytic choriomeningitis

LN: lymph nodes

LPS: Lipopolysaccharides

LT-HSC: Long-term HSC

MDSC: Myeloid-derived suppressor cells

MFI: Mean fluorescence intensity

MPP: Multipotent progenitors

MSC: Mesenchymal stem cell

NK: Natural Killer

NOS-2: Nitric Oxide Synthase 2

OVA: ovoalbumin

PAMP: pathogen-associated molecular patterns

PKC: Protein kinase C

PMA: Phorbol 12-myristate 13-acetate

PT: peritoneum

PTH: Parathyroid hormone

PTK: protein tyrosine kinase

RAG: Recombination Activating Gene

RT-PCR: Real Rime PCR

S1P: Sphingosine-1-phosphate

S1P1: Sphingosine-1-phosphate receptor 1

S1P3: Sphingosine-1-phosphate receptor 3

S1P5: Sphingosine-1-phosphate receptor 5

SCF: Stem cell factor

SDF-1: Stromal cell-derived factor 1

SLO: secondary lymphoid organs

ST-HSC: Short term HSC

TCR. T cell receptor

TF: Transcription Factor

TFBS: Transcription Factor Binding site

TGFβ: Transforming growth factor beta

Th1: T-helper 1

Th17: T helper 17

Th2: T helper 2

TLR. Toll-like receptor

TNF: Tumour Necrosis Factor

Treg: Regulatory T cells

UTR: untranslated region

VACV: Vaccinia Virus

VCAM: Vascular cell adhesion protein 1

VLA-4: Very Late Antigen-4

WT: Wild type

SUMMARY

CD69 is early expressed upon leukocyte activation and controls lymphocyte trafficking and cytokine secretion. In this thesis, the function of CD69 was studied in hematopoietic progenitor cells and in Vaccinia virus infection.

During the host response to viral infection, the transmembrane CD69 protein is highly upregulated in all immune cells. We have studied the role of CD69 in the murine immune response to Vaccinia virus (VACV) infection, and we report that the absence of CD69 enhances protection against VACV at both short and long times postinfection in immunocompetent and immunodeficient mice. Natural killer (NK) cells were implicated in the increased infection control, since the differences were greatly diminished when NK cells were depleted. This role of NK cells was not based on an altered NK cell reactivity, since CD69 did not affect the NK cell activation threshold in response to major histocompatibility complex class I NK cell targets or protein kinase C activation. Instead, NK cell numbers were increased in the spleen and peritoneum of CD69-deficient infected mice. That was not just secondary to better infection control in CD69-deficient mice, since NK cell numbers in the spleens and the blood of uninfected $CD69^{-/-}$ mice were already augmented. CD69-deficient NK cells from infected mice did not have an altered proliferation capacity. However, a lower spontaneous cell death rate was observed for $CD69^{-/-}$ lymphocytes. Thus, our results suggest that CD69 limits the innate immune response to VACV infection at least in part through cell homeostatic survival.

As we have just shown above, total splenic cell counts were found increased in $CD69^{-/-}$ mice at steady state and after infection, and these results are in agreement with previous results of our laboratory [1]. As well, CD69 interacts with S1P1 which mediates leukocytes trafficking. Thus, we studied the possible role of CD69 targeting in leukocyte mobilization. As result, we described for the first time the capacity of anti-CD69 monoclonal antibodies (mAbs) to induce a rapid and massive egress of mature leukocytes as well as hematopoietic stem and progenitor stem cells (HSPC) from bone marrow, both in wild type mice treated with anti-mCD69 mAb and in transgenic huCD69 mice in $CD69^{-/-}$ background treated with anti-huCD69 mAb. A single dose of anti-human CD69 mAb induced a decrease in total leukocytes number in bone marrow and an increase in spleen and blood. These effects on cell distribution were already apparent at 4 hours after treatment and could be observed for at least 6 days. The coadministration of FTY720 and anti-CD69 mAbs impaired the decrease in bone marrow cellularity observed by anti-CD69 treatment, demonstrating the role of S1P1 in mobilization of bone marrow leukocytes. The treatment also upregulated S1P1 and CXCR4 surface levels and induced mTOR signaling. Importantly, the treatment led to the proliferation and expansion of HSPC. CD69 targeting

and AMD3100 (inhibitor of CXCR4), induced similar mobilization, but anti-CD69 produced increased proliferation and the quantity of HSPC were higher in anti-CD69 than in AMD3100-treated mice both in BM and spleen. The combined treatment with CD69 targeting and AMD3100 do not have neither a synergic effect nor a clear additive effect on BM mobilization. We propose the use of anti-human CD69 as a new mobilizer of hematopoietic stem cell (HSCs). The mobilization of HSC is used for treatment for people who have reduced cell counts as a result of diseases or due to chemotherapeutic agents.

Since CD69 targeting increased mobilization of bone marrow cells to periphery, resembling $CD69^{-/-}$ mice and due to that the absence of CD69 improved the immune response to Vaccinia virus infection, we analyzed the influence of human and mouse CD69 targeting on Vaccinia virus infection. We reported that the increase of leukocyte number in SLO (secondary lymphoid organs) previously described for human and mouse CD69 targeted mice both in immunocompetent and immunodeficient mice (Chapter II) remained augmented during the infection and provided an improved capacity to clear the viral infection. CD69 targeting increased NK cells reactivity and augmented number and percentage of IFNγ-producing NK cells. In human CD69 targeted mice was examined the humoral mediators and found increased IL17a, IL17b, IL17f, IL-1α, IL-1β, Lymphotoxin α (LTα), Lymphotoxin β (LTβ), IFNγ, CCL2 and CCL12. Together these results suggested the possibility of using CD69 targeting as a treatment for enhancing anti-Vaccinia response, opening the possibility to use this treatment to increase vaccination responses.

When analyzing CD69 overexpression, it was found an increased numbers of single positive T cells in thymus and a higher increase in T regulatory cells, leading to a high reduction in T lymphocytes and even higher reduction of Treg cells in SLO. Likewise, B cells were moderately reduced in SLO, although an unexpected augment in NK cell number were found in bone marrow, thymus and spleen. In addition, a huge increase of IL17a, IL17b, IL17f, IL21, IL-1α, IL-1β, IL7, CCL2, CCL12, CCL7 and CCL8 was found together with a moderated increase of TNF and IFNγ in CD69 overexpressing mice. Thus, these results are in agreement with a major function of CD69 in controlling humoral mediators production, in addition to retention of B lymphocytes in bone marrow, T lymphocytes in thymus and T and B cells in lymph nodes. Overall, the overexpression of CD69 reinforces the role of CD69 influencing in NK, B and T cell development and its influence in lymphocyte homeostasis and trafficking. During VACV infection, SLO cell expansion occurred in WT mice whereas was impaired in the overexpressing mice. Thus, the difference in the number of cells between WT and CD69 overexpressing mice is even higher. CD69 overexpressing mice showed a better control of VACV infection in spite of the strong reduction

of T and B cells and the vast reduction of number of IFNγ-producing NK and T cells in SLO. However, no differences in the number of infiltrating cells were observed in non-lymphoid organs. Besides, we found a better response to Vaccinia virus infection both analyzed in ovaries after intraperitoneal infection, in lung after intranasal infection and in spleen, lymph nodes, kidney, lung and liver after 24 hours of intravenously infection. Overall, we hypothesize that innate cells of CD69 overexpressing mice must have an augmented activity producing high humoral mediators levels such as IL17 and other cytokines found in this mice. More works are needed to know the mechanism implicated in this response.

RESUMEN

CD69 se expresa de manera temprana tras la activación leucocitaria y controla la migración linfocitaria y la secreción de citoquinas. En esta tesis, se ha estudiado la función de CD69 en la células hematopoyéticas progenitoras y en la infección por el virus Vaccinia.

Durante la respuesta inmune frente a la infección viral, la proteína transmembrana CD69 es expresada en todas las células inmunitarias. En este trabajo, se ha estudiado el papel de CD69 en la respuesta inmune frente a la infección por el virus Vaccinia (VACV) en ratones, y se ha observado que la ausencia de CD69 mejora la protección contra VACV tanto a corto como a largo plazo en ratones inmunocompetentes e inmunodeficientes. Las células Natural Killer (NK) han sido involucradas a lo largo del tiempo con una mejor respuesta frente a la infección y en nuestro trabajo vemos que el aumento de células NK en ausencia de CD69 está relacionado con un aumento del control de la infección, ya que en estudios de eliminación de células NK, las diferencias no solo fueron abolidas sino que en este caso los ratones $CD69^{-/-}$ controlaban peor la infección. Este papel de las células NK no se basó en una reactividad alterada de las células NK, ya que CD69 no afectó al umbral de activación de las células NK en respuesta a MHC tipo I o a la activación de la proteína quinasa C. En cambio, el número de células NK aumentó en el bazo y peritoneo de ratones infectados deficientes para CD69. Esto no fue sólo secundario a un mejor control de la infección en ratones deficientes para CD69, ya que el número de células NK en bazo y sangre de ratones $CD69^{-/-}$ no infectados ya estaban aumentados. Las células NK deficientes en CD69 de ratones infectados no tenían una capacidad de proliferación alterada. Sin embargo, se observó una menor tasa de muerte celular espontánea para los linfocitos de ratones $CD69^{-/-}$. Por lo tanto, nuestros resultados sugieren que CD69 controla la respuesta inmune innata a la infección por VACV, al menos en parte a través de una mayor supervivencia celular.

Como acabamos de mencionar, los recuentos totales de células esplénicas se encontraron aumentados en ratones $CD69^{-/-}$ en estado basal y tras infección, y estos resultados están de acuerdo con los resultados anteriores de nuestro laboratorio [1] y podrían estar relacionados con la interacción de CD69 con S1P1. Además, hemos descrito que el tratamiento anti-CD69 simula en múltiples modelos la ausencia de CD69. Por lo tanto, se estudió el posible papel del tratamiento anti-CD69 en la movilización de leucocitos. Como resultado, describimos por primera vez la capacidad de los mAb anti-CD69 para inducir una salida rápida y masiva de leucocitos maduros, así como de HSPC de la médula ósea, tanto en ratones normales tratados con anti-mCD69 como en ratones que contienen el transgén de CD69 humano en fondo genético $CD69^{-/-}$ (huCD69), tratados con anti-huCD69 MAb. Una única

dosis de anti-CD69 humano disminuyó el número total de leucocitos de la médula ósea y aumentó los del bazo y la sangre en más del 20%. Estos efectos ya eran evidentes a las 4 horas después del tratamiento y pudieron observarse durante al menos 6 días. El hecho de que la disminución de la celularidad de médula ósea no se observase cuando los anticuerpos anti-CD69 se coadministraron con FTY720, indica que la redistribución observada, se debe a la movilización de leucocitos desde la médula ósea y que esto depende de la función de S1PR. El tratamiento, también aumentó la expresión en superficie de S1P1 y CXCR4 e indujo la señalización de mTOR. Es importante destacar que el tratamiento llevó a la proliferación y expansión de HSCP. CD69 y AMD3100 (inhibidor de CXCR4), indujeron una movilización similar, pero anti-CD69 produjo una proliferación incrementada y la cantidad de HSPC fue más alta en tratamientos con anticuerpos anti-CD69 que con AMD3100, tanto en médula ósea como en bazo. El tratamiento combinado con anti-CD69 y AMD3100 no tienen ni efecto sinérgico ni un claro efecto aditivo sobre la movilización de células en la médula ósea. Proponemos el tratamiento con anticuerpos anti-CD69 humano como un nuevo movilizador de HSCs. La movilización de HSC se utiliza para el tratamiento de personas que tienen reducidos los recuentos celulares como resultado de enfermedades o debido a agentes quimioterápicos.

Debido a que el tratamiento con anticuerpos anti-CD69 aumentó la movilización de células de médula ósea hacia la periferia y debido a que la ausencia de CD69 mejoró la respuesta inmune frente a la infección por el virus Vaccinia, se analizó la influencia de los tratamientos anti-CD69 humano y de ratón en la infección por el virus Vaccinia. El aumento del número de leucocitos en SLO previamente descritos para ratones tratados con anti-CD69 humano y de ratón tanto en ratones inmunocompetentes como inmunodeficientes (Capítulo I y II) permanecieron aumentados durante la infección y proporcionaron una capacidad mejorada para eliminar la infección viral. El tratamiento con anti-CD69 indujo una mayor reactividad de las células NK y un número aumentado de células NK productoras de IFN. En ratones tratados con anti-CD69 humano se analizaron los mediadores humorales y se encontraron aumentados IL17a, IL17b, IL17f, IL-1α, IL-1β, Lymphotoxin α (LTα), Lymphotoxin β (LTβ), IFN, CCL2 y CCL12. Juntos, estos resultados sugieren la posibilidad de utilizar anticuerpos anti-CD69 como tratamiento para mejorar la respuesta anti-Vaccinia, abriendo la posibilidad de utilizar este tratamiento para aumentar las respuestas de vacunación.

Cuando se analizó la sobreexpresión de CD69, se encontró un aumento del número de células T maduras en timo y un mayor aumento de células T reguladoras, lo que condujo a una alta reducción de los linfocitos T y una mayor reducción de las células Treg en los órganos linfoides secundarios. Del

mismo modo, las células B se redujeron moderadamente en SLO, aunque un aumento inesperado en el número de células NK se encontró en médula ósea, timo y bazo. Además, se ha encontrado un elevado incremento de la expresión de IL17a, IL17b, IL17f, IL21, IL-1α, IL-1β, IL7, CCL2, CCL12, CCL7 y CCL8 junto con un moderado incremento de TNF e IFNγ en ratones que sobreexpresan CD69. Por tanto, estos resultados están de acuerdo con una función de CD69 en el control de la producción de mediadores humorales y en la retención de linfocitos B en la médula ósea, linfocitos T en el timo y células T y B en nódulos linfáticos, además de un papel de CD69 en el control de IL17 y otras citoquinas. Durante la infección con Vaccinia, en el ratón WT ocurre una gran expansión del bazo aumentando mucho su número de células y esta capacidad esta anulada en el ratón que sobreexpresa CD69. De esta forma, la diferencia en el número de células es incluso mayor entre estos ratones. Sin embargo, la sobreexpresión de CD69 mostró un mejor control frente a la infección por el virus Vaccinia a pesar de la fuerte reducción de células T y B y la reducción del número de células NK y T en SLO productoras de IFN. A pesar de esto, las células infiltrantes en los órganos no linfoides fueron similares entre el ratón WT y el que sobreexpresa CD69. Se encontró una mejor respuesta a la infección por el virus Vaccinia tanto en ovarios después de la infección intraperitoneal, en pulmón después de la infección intranasal y en bazo, ganglios linfáticos, riñón, pulmón el hígado después de 24 horas de infección intravenosa. En conjunto, en este ratón, las células inmunes deben tener muy aumentada su actividad dada la gran cantidad de citoquinas producidas. Se necesita un mayor trabajo para conocer los mecanismos involucrados.

INTRODUCTION

1. HEMATOPOIETIC SYSTEM

The hematopoietic system is originated in bone marrow during adult life, where stem cells will give rise to the progenitors responsible for development of the different hematopoietic lineages that subsequently will originate all mature blood cells and will migrate to the lymph, secondary lymphoid organs and tissues. In Figure 1 is schematized how this pluripotent hematopoietic stem cells will give rise to the hematopoietic cells. A common myeloid progenitor produces the progenitor of megakaryocytes and erythrocytes and the progenitor of macrophages and granulocytes. The progenitor of megakaryocytes and erythrocytes will originate megakaryocytes that in blood will appear as platelets and erythroblasts that will mature to erythrocytes. The progenitor of macrophages and granulocytes will give rise to polymorphonuclear leukocytes such as neutrophils, eosinophils, basophils, monocytes and dendritic cells. Monocytes will reach tissues where they will mature to mast cells, macrophages and dendritic cells. On the other hand, the pluripotent hematopoietic stem cells will also generate a common lymphoid progenitor, which will give rise to the NK, T and B lymphocytes which after maturation process will be developed in activated NK cells, T lymphocytes and plasma cells respectively. While B lymphocytes differentiate into bone marrow, T lymphocytes do so in thymus. Both, from these locations, they will migrate through the bloodstream to the secondary lymphoid organs and lymph where they will mature. After encountering an antigen, they will be able to develop an effective immune response through

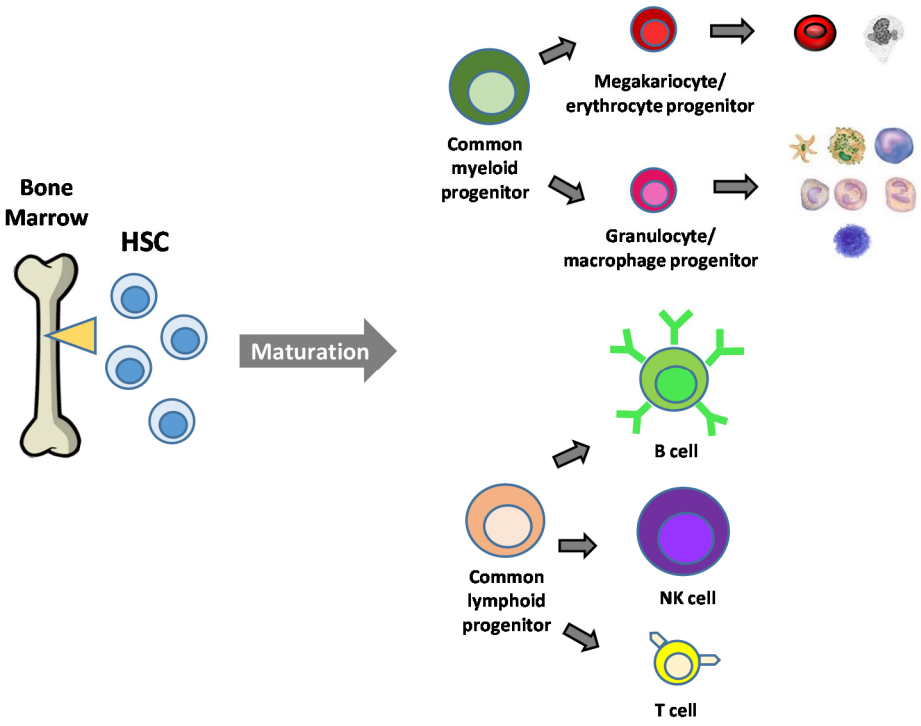


Figure 1. Development of hematopoietic system. *All blood cells of the hematopoietic system come from a common progenitor, named hematopoietic stem cell, which will originate all the lymphoid and myeloid cells of the immune system.*

differentiation into effector cells capable of combating the infection. Preserving homeostasis in hematopoiesis is essential for the proper development of the immune system that will ensure the maintenance of the substantial numbers of the different types of blood cells.

1.1 Hematopoietic progenitor and stem cells and their niche

The study of stem cells has been a breakthrough thanks to the studies that have been carried out for approximately 20 years, when they began the first trials. The importance of these cells led to the development of new effective tools that can induce a rapid mobilization of HSC into the circulation and later they can be collected through non-invasive techniques such as blood extraction. HSC therapy represents one of the most successful advances in cell-based therapies applied in hematological disorders. However, new advances must be performed to ensure a greater number of HSC cells. Hematopoietic stem cells (HSC) are developed in bone marrow microenvironment throughout adult life where they differentiate. Hematopoietic stem cells are defined by their ability to self-renew to differentiate, giving rise to the rest of the cells of the hematopoietic system and letting reconstitution in animals that have been irradiated [2]. Bone marrow offers the suitable environment for the long-term survival, functionality and cell integrity of these cells. The stem cell niche sends signals that support HSC properties, including self-renewal capacity and long-term multilineage repopulation ability. HSC are responsible for the development of the different blood cells during the process of haematopoiesis while some undifferentiated stem cells are retained at basal levels within bone marrow [3]. The number of these cells increases during processes of cellular damage and signals of stress [4]. This fact allows preserving hematopoietic stem cell homeostatic conditions capable of inducing maturation of hematopoietic cells if was necessary due to pathogen attack.

Stem cells represent a very small percentage of the entire bone marrow and are contained within Lin- (negative lineage) HSC compartment (Fig.2). Lin- cells are constituted by hematopoietic cells that do not express markers of mature hematopoietic lineages such as T and B lymphocytes, NK cells, dendritic cells and myeloid cells and within Lin- cells we can distinguish two subpopulations according to Sca-1 and c-kit expression which do not express markers for mature hematopoietic lineages. Sca-1 is defined as the common biological marker used to identify HSC along with other markers and c-kit is also known as stem cell factor receptor and is expressed on HSC including multipotent hematopoietic stem cells, progenitors committed to myeloid and/or erythroid lineages, and T and B cell precursors and the interaction with its ligand is critical for the development of hematopoietic stem cells. Two

subpopulations can be distinguished according to Sca-1 and c-kit expression: KSL cells (c-Kit^{hi} Sca+ Lin-) and CLP cells (Common lymphoid progenitors) as shown in figure 2. The image displays the different markers that can be used to differentiate the different stages of maturation of HSC cells of human and mouse. KSL cells are an early form of hematopoietic stem cells whereas CLP cells are descendants of the pluripotential hematopoietic stem cell (pHSC) which are capable of generating all of the cell types of the complete blood cell system.

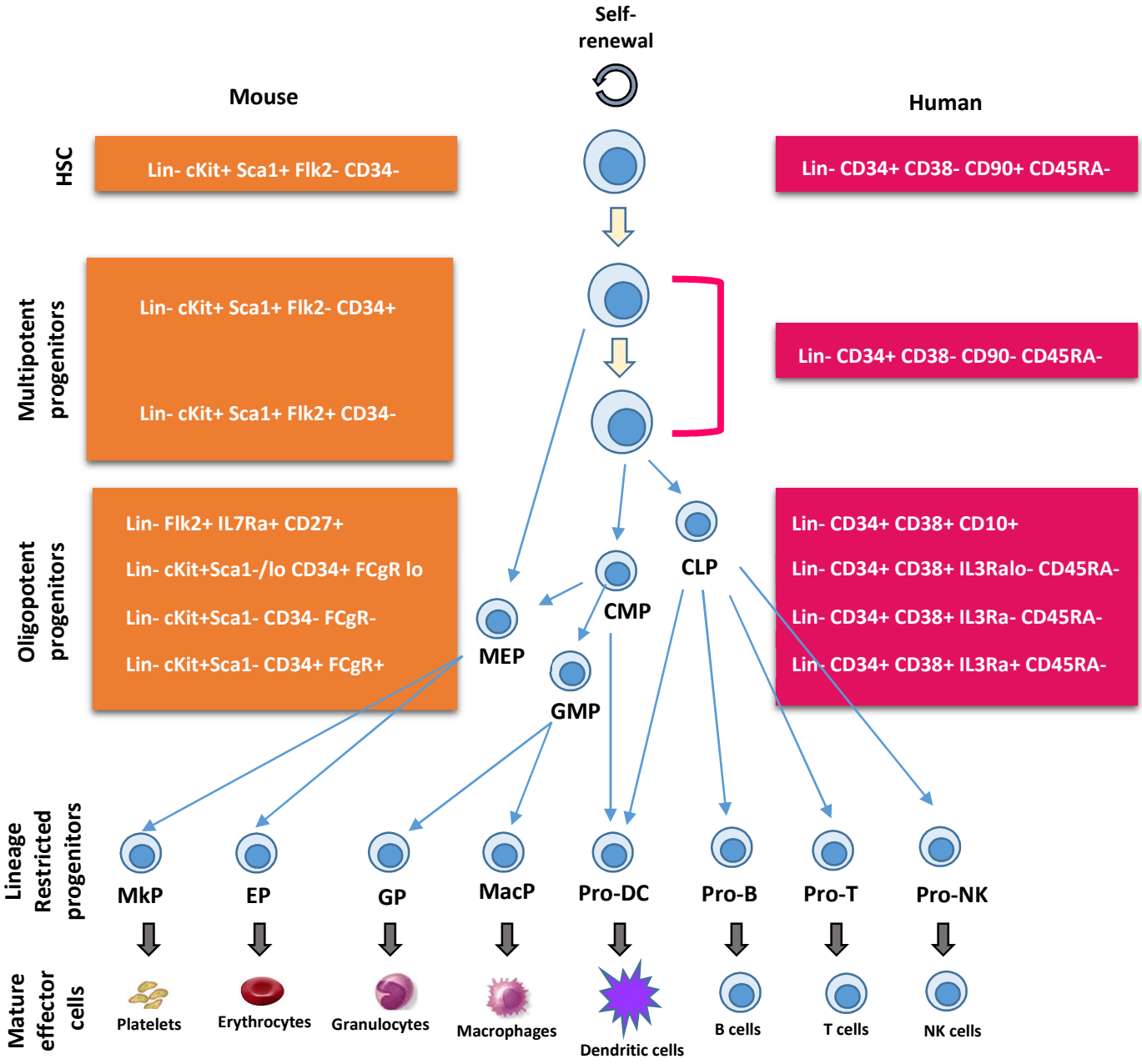


Figure 2. Different hematopoietic stem cell development between mice and humans. *HSC are characterized by self-renewal capacity. Throughout differentiation, stem cells from mice and humans express different surface markers and define the different subsets of HSC hierarchy.*

Besides, three functionally HSC subpopulations can be identified within KSL cells according to CD34 and FLT3 expression: LT-HSC o *long term* HSC (KSL $CD34^{neg}$ $FLT3^{neg}$), ST-HSC o *short term* HSC (KSL $CD34^{+}$ $FLT3^{neg}$) o MPP or multipotent progenitors (KSL $CD34^{+}$ $FLT3^{+}$) [5] [6-8]. LT-HSCs have life-long and self-renewing potential through a process of asymmetric cell division [9], and give rise to ST-HSCs and MPP with more restricted self-renewing capacity. ST-HSCs are lineage-restricted progenitors and undergo extensive proliferation and differentiation to produce differentiated and functional hematopoietic cells. MPP or multipotent progenitor cells are less primitive cells and have low or no self-renew capability. ST-HSCs or multipotent progenitors (MPPs) are only able to sustain hematopoiesis in the short term, while LT-HSCs must persist for the lifespan of the organism to perpetually replenish the hematopoietic system. HSC transplants will be performed by the transfer of LT-HSCs in irradiated animals, which as we have described have greater capacity for engraftment and self-renewal. Suitable antibody combinations of these markers may allow the analysis of all these cell populations and their isolation by sorting technique. Purification method starts with isolation of bone marrow or blood cells where these cells can be found. Once extracted, HSCs can be collected by magnetic cell separation (MACS) or single-cell sorting based on cell surface markers expression described above [10]. The previously mentioned markers can be used to differentiate HSC subpopulations in mice, however, in humans there are other markers that allow us to differentiate stem cells and purify them. The main cell surface marker of HSC in humans is CD34+ cells which is down-regulated when cells are differentiated into more abundant mature cell [11] and, unlike mice, they do not express Sca-1 and other markers described in mouse hematopoietic progenitors.

The vast majority of HSC resides in bone marrow though HSC can be also found in different tissues including spleen, liver and blood. Most HSCs appear to be located in an area proximal to the bone called endosteal niche [12], where osteoblasts provide an environment that supports maintenance and survival of the HSCs [13]. A second progenitor niche in bone marrow is proximal to sinusoidal endothelium named perivascular niche [14]; which has been suggested to contain higher fraction of cycling HSCs and thus comprising more differentiated population of stem cells.

The output of progenitor cells from bone marrow is dependent on mechanisms that influence cell adhesion and migration to mediate the egress of these cells into the bloodstream (Fig.3).

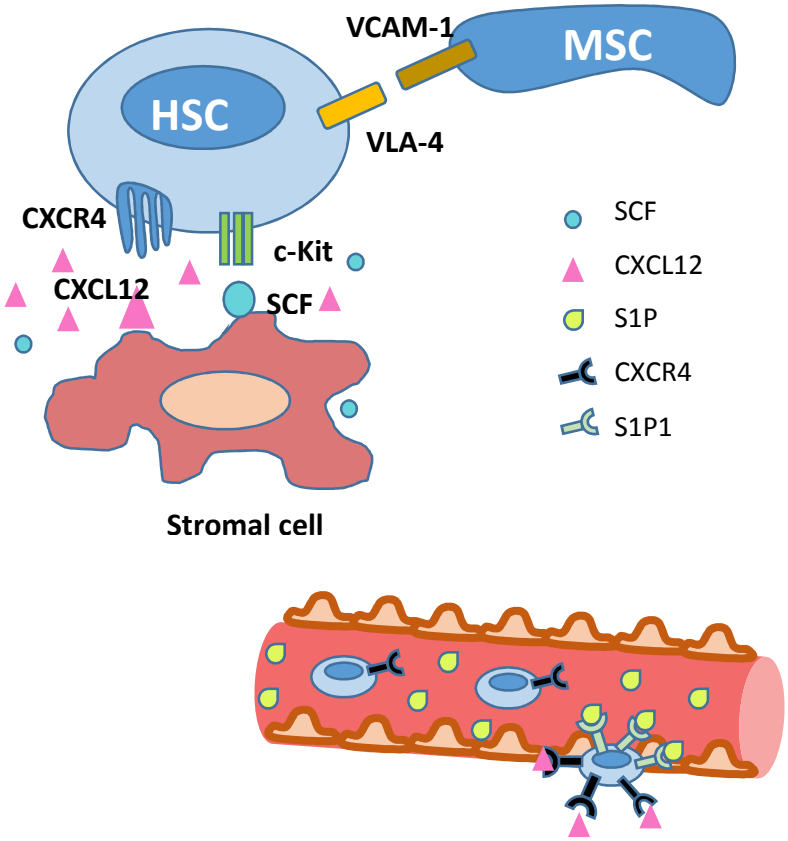


Figure 3. Different mechanism of stem cell mobilization.

CXCL12 (SDF-1, stromal cell-derived factor-1) is the principal chemoatracttant of HSC and is expressed by bone marrow osteoblasts, endothelial cells and stromal cells [15]. The interaction of CXCL12 with its receptor CXCR4, is the key to regulate the retention or egress of HSC. CXCL12 also plays an active chemotactic role in mobilization, and an increase of CXCL12 levels in plasma is sufficient to induce bone marrow cell mobilization [16]. The balance between CXCL12 and CXCR4 plays a crucial role in the retention and release of HSC within bone marrow [17, 18]. The migration of HSC also depends on Sphingosine-1-phosphate (S1P)/Sphingosine-1-phosphate receptor 1 (S1P1) axis. Cells expressing S1P1 respond to an increase of S1P concentration; S1P is low expressed in bone marrow and high in plasma. HSCs also express S1P receptors allowing the mobilization from non-lymphoid peripheral tissues to draining lymphatics [19] through interaction with S1P1. Increase plasma S1P levels and S1P1 expression in bone marrow progenitor cells generate a gradient from bone marrow to blood, so that the increase of S1P levels in plasma enhances HSC egress from bone marrow [20] The up-regulation of S1P1 in HSC and the increase of S1P will result in the mobilization of HSC. S1P signaling is essential for stem cell mobilization and these levels are transiently elevated in the plasma and BM during

AMD3100 treatment, as well as G-CSF-induced mobilization [4]. Although the increase of S1P could be related with the increase of CXCL12, an evidence direct not has been demonstrated [21]. It has been described that under homeostatic conditions, there is a balance between CXCL12 and S1P, while CXCL12 is held elevated to keep stem cells in a quiescent state in bone marrow, S1P is expressed in small amounts to reduce the output of bone marrow cells. By the other hand, in stress situations, the expression of S1P in blood and bone marrow increases, while CXL12 decreases to facilitate the outflow of progenitor cells [22]. The use of S1P/S1P1 axis disruptors, such as FTY720, or S1P1-deficient mice had reduced HSC output from bone marrow, demonstrating the contribution of S1P/$S1P_1$ axis to the egress of HSCs/HPCs [4]. The binding of Stem cell factor (SCF) and its ligand c-Kit also influences in precursors and stem cells mobilization[23]. SCF has been shown to increase the survival of HSCs *in vitro* and contributes to the self-renewal and maintenance of HSCs in-vivo. SCF promotes cell survival, proliferation, differentiation, adhesion and functional activation [24]. SCF has been shown to increase adhesion and thus may play a large role in ensuring that HSCs remain in the niche [23]. C-kit is expressed by HSC cells and is considered as a potent chemoatracttant. C-Kit downregulation leads to a lower binding of SCF to c-Kit and to a stem cell mobilization. Finally, the vascular cell adhesion molecule 1(VCAM1)/ vascular leukocyte antigen 4 (VLA4) axis have been also described as major factors of HSC cell mobilization. VLA-4 is a receptor for vascular cell adhesion molecule-1 and fibronectin [25]. It has a crutial role in lymphopoiesis, inflammatory recruitment of leukocytes, and other situations that require cell adhesion to the vascular endothelium due to mediate cell–cell and cell–matrix interactions in a wide range of biological contexts [26, 27]. The expression of VLA-4 and VCAM-1 are related to cell adhesion, whereas VLA-4 is expressed on hematopoietic precursor cells and most of leukocytes including monocytes, lymphocytes, eosinophils and basophils, VCAM-1 is expressed on mesenchymal stem cells (MSC).

Originally, HSC were extracted from the bone marrow of big bones [21] but currently the use of mobilizers that act on any of the mechanisms mentioned above are the treatment of choice to achieve a high number of these cells from blood to be transplanted in individuals who present leucopoenia as a consequence of the course of some diseases or as a result of chemotherapeutic treatments [28-30]. Some of these agents with mobilization effects are presented below:

Granulocyte colony stimulating factor (G-CSF): G-CSF is induced by infection and other stress stimuli to be produced by osteoblasts and induces the differentiation and maturation of HSC cells [30]. The mechanism of action by which induces this increase in output of stem cells is produced by: (1)

secretion of proteases (MMP-9, NE and CG), (2) depletion of osteoblasts, (3) inhibition macrophage stimulation of MSCs, resulting in reduced production and expression of CXCL12, (4) increase of the sympathetic tone in the BM microenvironment, (5) alteration of chemotactic gradients of S1P and CXCL12 [31] and (6) upregulation of S1P1 [4].The mobilization induced by G-CSF is mainly mediated by CXCL12, inducing a transient increase of CXCL12 followed by a decrease [4]. Treatment with G-CSF is based on a daily treatment for 5 days at doses of 10 µg/kg/day administered by subcutaneous injection and apheresis starts to be performed at the end of this schedule. The number of apheresis that should be carried out in patients depends on the efficiency of mobilization. G-CSF is the most widely used mobilizer agent and currently in clinical procedures only G-CSF alone or the combination with one of the mobilizers mentioned below (AMD3100) are used.

Granulocyte-macrophage colony stimulating factor (GM-CSF): Although GM-CSF can also be used as a mobilizer of bone marrow HSC cells, its effect is more reduced than that induced by G-CSF even with higher doses [30]. GM-CSF has side effects of toxicity, such as fever, hypoxemia, and first-dose effect [32].

AMD3100: (Mozobil, Plerixafor) is a peptide that blocks the CXCL12 interaction with CXCR4 in bone marrow. CXCR4 is expressed by mesenchymal stem cells. AMD3100 was initially developed for the treatment of HIV infection, since CXCR4 is a coreceptor for virus entry in CD4 T cells and its role in stem cell mobilization was later discovered [33]. AMD3100 is extremely specific in its affinity and acts as antagonist for the CXCR4 receptor. The inhibition of CXCR4 by AMD3100 produces a rapid increase in circulation of hematopoietic progenitors and HSC [34]. Treatments with AMD3100 increase white blood cell (WBC) counts which reach a peak approximately 6 hours after injection of AMD3100 in human and after 1 hour in mice. AMD3100 is administered subcutaneously at 160–240 µg/kg on day 4 or 5 after G-CSF, 6 h prior to apheresis. The use of AMD3100 is only employed in patients with a serious risk for mobilization failure and it is approved to be used in combination with G-CSF for stem cell mobilization in patients with myeloma and lymphoma [35, 36]. AMD3100 is able to induce a faster mobilization than G-CSF, however, the ability to mobilize HSC is more limited than G-CSF, while the joint treatment increases the output of HSC higher than the treatment of each one separately. The combination regimen mobilized up to 50% more CD34+ cells and reduced the number of apheresis procedures, however, AMD3100 has a high cost.

Stem cell factor is also known as SCF, KIT-ligand, KL, or steel factor. Stem cell factor is a hematopoietic growth factor produced by endothelial cells and perivascular stromal cells in the bone marrow niche [30]. It has particular importance in the mast cell and erythroid lineages, but also acts on multipotential stem and progenitor cells, megakaryocytes, and a subset of lymphoid progenitors. SCF-induced mobilization of stem cells and progenitor cells from the bone marrow into the blood may be mediated by binding to c-Kit or alterations in the interactions of hematopoietic cell integrins with VCAM-1 or fibronectin [37]. A recombinant human SCF used in combination with G-CSF has been showed to increase stem cell yield in poor mobilizers [38-41]. Clinical studies showed that treatment with SCF increases the numbers of progenitor cells of many types [42].

Sphingosine-1-phosphate (S1P) agonists. S1P is a bioactive phospholipid produced by peripheral blood mainly by erythrocytes [43, 44], activated platelets [45] as well as by endothelial cells [46-48]. The administration of S1P agonist one hour prior to AMD3100 treatment induced dose-dependent HSC mobilization and this mobilization was increased adding G-CSF to the treatment [49]. Therefore, we can conclude that the treatment with agonist of S1P agonists will improve HSC mobilization.

VCAM1/VLA4 inhibitors: VLA-4 regulates HSC adhesion to vascular cell adhesion molecule-1 (VCAM-1) within bone marrow stroma [50]. VCAM1/VLA4 axes are the major factors of HSC retention together with CXCL12/CXCR4. In preclinical studies, blockade of VLA4 expressed on HSCs resulted in mobilization of HSC progenitors into the bloodstream [51] through preventing interaction with its ligand, VCAM1 [52, 53]. One of these treatments is Natalizumab, a monoclonal VLA4 antibody which is used for the treatment of multiple sclerosis and Crohn's disease and was found to induce a modest increase in peripheral CD34+ cell count that was found higher in combination with AMD3100 or with AMD3100 plus G-CSF [54, 55]. Combination of the three drugs caused a 17-fold increase in HSC number compared with G-CSF alone [56]. These data are promising and further studies are needed to explore the role of VLA4 inhibition in stem cell mobilization.

Parathyroid hormone (PTH): is a hormone secreted by the parathyroid gland involved in the regulation of calcium and phosphorus metabolism and besides presents regulatory effects on bone formation and bone resorption [25]. Activation of the receptor for PTH and PTH-related protein (PTHrP) (PPR) in osteoblasts have been associated to a significant expansion of the pool of HSC in the adult bone marrow and to their increased mobilization into the bloodstream [57]. PTH accelerates differentiation

and activation of osteoclasts at least in part by upregulation of macrophage-colony stimulating factor (M-CSF). PTH treatment induced an increase in plasma HSC similar to that produced by G-CSF treatment [58-60]. It is necessary to carry out more studies to know the role of PTH in the mobilization of HSC.

Proteasome inhibitors. Bortezomib has been shown to be capable of inducing mobilization of HSC [61]. Bortezomib blocks the activation of nuclear factor-κB by preventing proteasomal degradation of IκBα [62]. However, these results were found when the treatment was performed in mice deficient for VLA-4. Therefore, it means that both pathways must be involved so that the mobilization can be developed. On the other hand, studies were also conducted on treatments combined with Bortezomib with G-CSF or AMD3100, and the result was a greater induction of stem cell mobilization.

Growth-regulated protein β (Groβ)*:* also called CXCL2, belong to CXC chemokine family which binds to the CXCR2 receptor that is expressed in bone marrow microenvironment [63]. CXCL2 is expressed by HSCs and lymphoid progenitors of bone marrow and regulates the mobilization of these cells including short-term and long-term progenitor cells. Studies in monkeys revealed that the mobilization peak occurred within one hour to the 4 hours whereas in mice it take place within the first 15 minutes to 30 minutes and persists for 90 minutes. Transplantation with HSCs in Groβ treatment resulted in faster neutrophil and platelet recovery in mice, with enhanced engraftment and repopulation activity [64]. Besides, mobilization included all classes of short term progenitor cells. Combined treatment with a single dose of G-CSF and Groβ obtained the same results as the 5-day treatment with G-CSF [65, 66]. Therefore, Groβ treatment is postulated as a good mobilizer alone or with G-CSF whose rapid mobilization enhances homing and engraftment properties.

Stabilization of hypoxia-inducible factor (HIF): HIF has a significant role in BM HSC quiescence and self-renewal. In HSCs niche, HSC proliferation and function is plainly affected by oxygen since HSCs with long-term reconstitution ability reside in hypoxic areas of the bone marrow [67, 68]. HIFs are being described as crucial regulators of the stem cell phenotype [69]. Also, *in vitro* studies culturing human bone marrow HSCs under hypoxic conditions (1.5% O2) promoted their ability to engraft and repopulate the hematopoietic compartment of immunodeficient NOD/SCID mice[70]. HIF induce CXCL12 promoting homing to tissues and HIF-1α influence in VEGF-A in the BM sinusoids, leading to vasodilation and enhancement of HSC mobilization [71]. Besides, it has been demonstrated that the

treatment combined with G-CSF and AMD3100 led to an increase in mobilization of HSCs in mice when compared with a combination of G-CSF and AMD3100 alone [72].

Therefore, there are described different treatments that can induce the mobilization of stem cells from bone marrow to bloodstream and the combination of two or more of these treatments is important to significantly increase the output of HSC from bone marrow. However, it is necessary to develop more drugs that have more advantages than the previous ones, reducing significantly the number of apheresis that must be performed with a greater capacity of mobilization. In this thesis, there will be presented results in a new treatment that mobilize HSC based in CD69 as target.

2. IMMUNE SYSTEM

The immune system exists to protect the host from infection induced by viruses, bacteria, fungi and parasites. The development of an effective immune response is mediated by the cells and soluble factors of the immune system which are mobilized through the blood and lymphatic system to the different organs to block the spread of pathogens. The immune system is composed of several biological processes that let to establish a response against external aggressions and sort out the reaction against the own. The immune response occurs in two phases: innate immune response and adaptive immune response. The first line of defense against infection comprises the epithelia formed by internal and external surfaces of the body and phagocytes that lie beneath all epithelial surfaces and engulf and digest invading microorganisms. The ***innate immune response*** is constituted by innate mechanisms that act immediately with performed elements and are followed by an "early induced response" that induces inflammation trigger by local infection captured by phagocytes which recruit and activate other effector cells. The innate immunity depends on germline-encoded receptors to recognize features that are common to many pathogens. With these receptors, the cells of the innate immune response can detect in the pathogens a variety of danger signals called molecular patterns associated with danger (DAMP) or the presence of pathogen-associated molecular patterns (PAMP). These receptors are used by the innate immunity to discriminate very efficiently between host cell and pathogen and identify broad classes of pathogen. The soluble mediators of the innate immunity such as cytokines are different to distinct pathogens and in this way the innate response contributes to the induction of an appropriate adaptive immune response.

Epithelia is protected by many kinds of chemical defenses, including antimicrobial peptides and enzymes. The phagocytes residing beneath the epithelia, act both in the direct killing of microorganisms and in producing in cytokines and chemokines that induce an inflammatory response and recruit new phagocytic cells and circulating effector cells to the site of infection. The humoral innate immunity is composed, in addition, by cytokines, chemokines and soluble circulating defensive proteins named complement system, constituted by plasma proteins that are activated by microorganisms promoting their removal by phagocytic cells. Cytokines produced by macrophages such as TNF-α, IL-l, and IL-6, in addition to the important local effects, have long range effects being the most important the initiation of the acute-phase response. Within a day or two, the acute-phase response provides to the host of several proteins with functional properties of antibodies capable to recognize a broad range of pathogens. However, unlike antibodies, acute-phase proteins have not structural diversity. The interferons produced by viral infected cells also contribute to host defenses in several ways. Induced resistance to viral replication in all cells, increases antigen presentation and activate NK cells to kill virus infected cells.

This early induced response is also constituted by NK lymphocytes which are activated by interferons and macrophage-derived cytokines to serve as an early defence against virus and other intracellular infections. NK cells differentiate and mature in bone marrow, circulate into the blood and lymph and can be found in lymph nodes, spleen, tonsils, and thymus. They present cytotoxic granules and through their expression of a range of germ line-encoded receptors, they are able to recognize virus-infected cells either by direct recognition of viral proteins or by sensing of infection-induced reductions in major histocompatibility complex class I (MHC-I) levels and expression of stress molecules. The NK cells also eliminate tumour cells as they downmodulate MHC molecules. Upon this recognition, NK cells become activated, proliferate, and use a range of effector mechanisms to eliminate target cells. These include the production of cytokines, such as the expression of death-inducing ligands of the tumor necrosis factor (TNF) superfamily of receptors [73] and gamma interferon (IFN-γ) [74, 75], which represent a key player in antiviral defense; the secretion of granzyme B (GrzB) and perforin-containing cytotoxic granules in the immune synapse with the infected cells. NK cells can be also stimulated to secrete IFN-γ by IL-12 produced by dendritic cells and macrophages or by IL-18 produced by activated macrophages acting in synergy with IL-12 [76, 77]. Finally, the innate immune system is also constituted by a subset of immune cells named innate lymphoid cells (ILCs) whose functions are

related to resistance to pathogens, regulation of autoimmune inflammation, tissue remodeling, cancer and metabolic homeostasis [78].

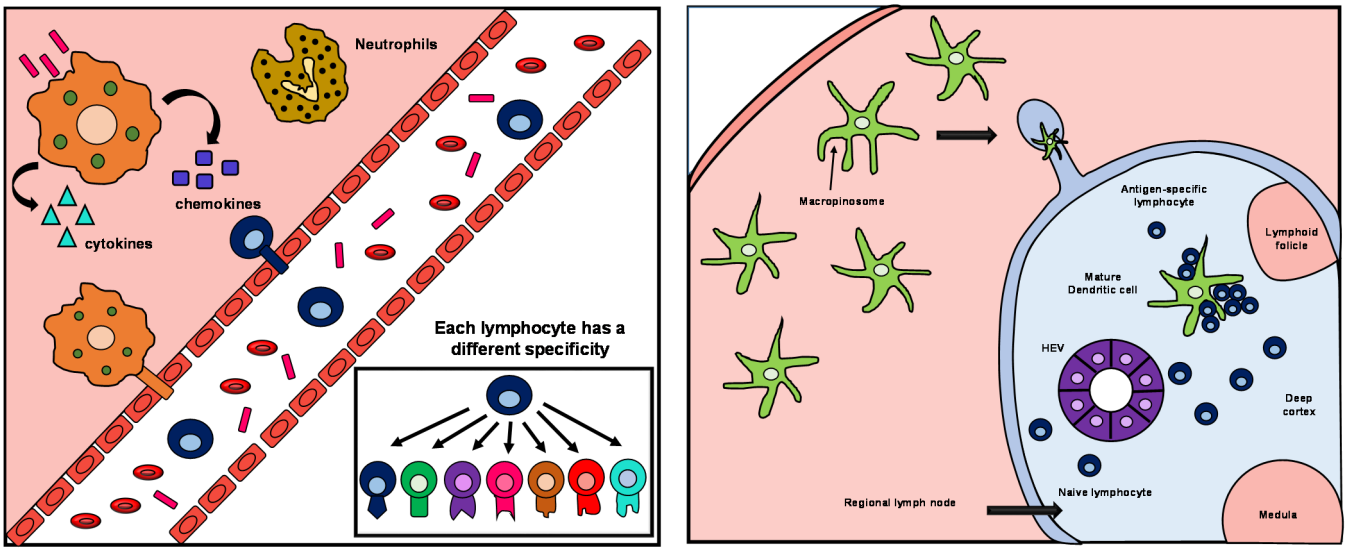


Figure 4. Activation of immune response. *Left, Activation of inflammatory response. Macrophages are triggered after encountering pathogen and cytokines and chemokines are released to increase the permeability of blood vessels. Neutrophils and monocytes migrate to the site of infection. Right, adaptive immune response is imitated by dendritic cells that after pathogen recognition migrate to lymph nodes and induce clonal expansion of antigen-specific lymphocytes. Adapted from "Immunobiology" by Janeway.*

Only if an infectious organism can breach the innate defence, the ***adaptive immune response*** have to arise. This immune response is characterized by the generation of antigen specific effector cells, that target the specific pathogen, and memory cells which prevent reinfection with the same organism. Relevant players in the adaptive immune response are B cells with a B cell antigen receptor (BCR) and T cells with a T cell antigen receptor (TCR) which are produced along life in bone marrow and thymus respectively. During T and B cell development in each lymphocyte is generated one unique antigen by a sophisticated gene rearrangement process. At any time, one person has approximately 10^{10} lymphocytes between T and B cells subset and each lymphocyte has a different receptor that recognizes a distinct epitope (Fig.4). Thus, the adaptive immune system uses a large repertoire of receptors that recognize a huge variety of antigens. The adaptive immune response is activated when mature dendritic cells process the pathogen and migrate to SLO to present the antigen to T lymphocyte (Fig. 4). B and T lymphocytes that circulate by SLO are characterized for their high variety of receptors. The recognition of soluble antigens by B cells is performed by the BCR. When a T cell that recognize part of the pathogen encounter the migrated DC, stop the migration and proliferate and mature to

arm effector T cells. If they are CD8 T cells would be cytotoxic cells which destroy host cells infected with the invading pathogen. If they are CD4 T cell could differentiate to T helper cells Th1, Th2, Th17 and regulatory T cells (Treg) which perform different effector functions through different cytokines production. Th1 cells, through the production of IFNγ, activate macrophages to respond more efficiently to destroy engulf pathogens. The function of Th2 cells is the activation and differentiation of B cells to recognize specifically the pathogen through the production of IL4 and IL5 cytokines. Once differentiated, B cells mature to improve the fighting of its receptor in pathogen recognition and later will end differentiated in plasma cells that secrete its BCR as Igs. These Igs disseminate through the body and once bound to the pathogen activate effector mechanism such as the complement system that makes holes in the pathogen. Also, the Igs, once bound to the antigen, link to phagocytes by Fc of the Igs to engulf the pathogen. However, the humoral immune response will be effective through the contribution of cytokines which will allow the clonal expansion of B lymphocytes in response to a specific antigen. In addition to Th1 and Th2, a third population of T lymphocytes, Th17, has been seen to mediate in immune and inflammatory processes. Th17 has been characterized by the secretion of IL17. The signaling pathway used by the vast majority of cytokines is mediated by JAKs and STATs [79]. Treg cells also contribute to adaptative immune response regulating the proliferation of effector cells to modulate immune response and to avoid exacerbated reactions. Treg function is performed by the production of anti-inflammatory cytokines such as TGF-β and IL10 [80, 81]. Of all lymphocytes activated in response to an antigen, the majority of effector cells die, however 10% remain as long-living memory cells constituting the main reservoir of pathogen specific-T cells and are named memory T cells [82]. These cells constitute a cellular subpopulation crucial to improve the immune response against a pathogen presented for a second time. We can differentiate three types of memory T cells according to their circulation pattern and resident tissues and according to surface markers expression and cytokines production [83] and they are: central memory T cell (TCM), effector memory T cells (TEM) and tissue resident memory T cells (TRM). TRM cells express CD69 and CD103 and are retained on tissues.

3. CD69

3.1 Structure

CD69 is also named AIM (activation inducer molecule), EA-1 (early activation antigen), MLR-3 or Leu-23. CD69 is a disulfide-linked homodimeric membrane type II C-type lectin. CD69 is formed by a polypeptide of 199 aa (225Kb) containing a single N-linked glycosylation site in its extracellular domain and differences in glycosylation results in differences in size ranging from 28 to 32 kD [84-89]. CD69 is constitutively phosphorylated at the serine residues (Ser18 and Ser30) and non-glycosylated dimers can be also found [90, 91].

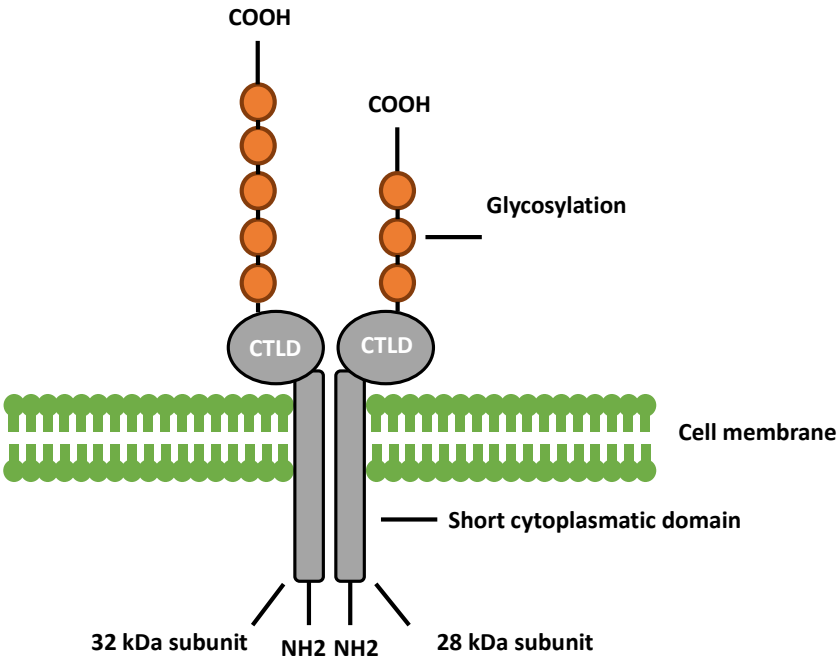


Figure 5. CD69 gene structure.

3.2 Gene structure and regulation

CD69 gene is clustered in the NK gene complex located on distal mouse chromosome 6 and human chromosome 12 [92-94]. CD69 mRNA gene contains 5 exons with a short 5'UTR (untranslated region) end and a long 3'UTR ends [92]. The 3'UTR presents sequences involves in post transcriptional degradation of CD69 mRNA [95]. The expression of CD69 is strongly regulated by transcriptional mechanisms [96]. In human and mouse CD69 promoters, it has been described the existence of a TATA element 30 base pairs upstream to the transcription start site (TSS) which guides transcription in resting and stimulated cells. Proximal to promoter region, it has been detected cis-elements that interact with transcription factors such as Erg-1, Erg-3, ATF-3/CREB and Ap-1 upon stimulation [97, 98]. A -78 to +16 region of human CD69 gene has been reported to be responsible of basal CD69

transcription. In this region, it has been also showed the binding of Sp1 at position -56 and NFKB motif at position -223 of the human CD69 promoter required for induction of CD69 in response to TNFα [92].

Comparison between different species of CD69 gene showed the existence of a promoter and four conserved noncoding sequences (CNS), CNS1, CNS2, CNS3 and CNS4 located 5'upstream of the promoter [96].

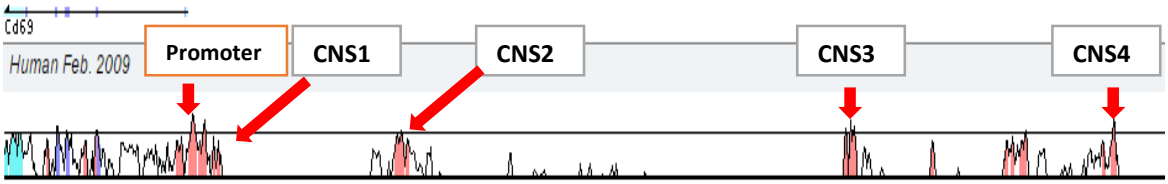


Figure 6. Vista Genome Browser of CD69 gene. *CD69 is constituted by a promoter and four conserved non-coding sequences (CNS1-CNS4). VISTA plot of conservation human (base) to mouse sequences, where curve shows percentage of conservation; red zones are conserved non-coding sequences (CNSs).*

In addition, a non-conserved hypersensitivity site (HS) has been located within the first intron of the CD69 gene that binds a huge number of transcription factors and polymerase II [99]. CNS2 and CNS4 have been described as potent enhancers of CD69 activity whereas CNS1 and CNS3 do not seem to influence in this activity. In a transgenic model, it was analyzed CD69 expression in combinations of the different CNSs with the promoter using hCD2 as reporter [96]. The addition of CNS1 and CNS2 caused suppression of CD69 expression whereas further addition of CNS3 and CNS4 supported development-stage and lineage-specific regulation in T cells but not in B cells.

3.3 Expression

CD69 is broadly expressed in bone marrow-derived cells. CD69 is expressed in all leukocytes upon activation in inflammatory, infectious or stressful processes and is constitutively expressed in platelets and not expressed in erythrocytes [91, 100-103]. In non-infected and healthy mice, the expression of CD69 is detected in small proportions in the main leukocytes subsets of SLO [104]. CD69 is slightly expressed in bone marrow B cell precursors [105]. Also, CD69 is low expressed in immature thymocytes and highly expressed in mature thymocytes [1]. Tissue resident NK cells in liver and NKT cells in thymus, spleen and liver, also known as type I innate lymphoid cells (ILC1), express CD69[106]. In addition, CD69 is expressed in all infiltrating leukocytes in non-lymphoid organs such as tissue

resident memory T cells and epidermal Langerhans cells. CD69 is early expressed in lymphocytes 2-3 h after treatment with anti-CD3 and anti-CD28 [87] but is expressed at later times in myeloid cells. In myeloid populations, CD69 expression reaches its maximum in about 24 h after treatment with PMA [107]. In bone marrow derived murine macrophages, lipopolysaccharide (LPS) is a potent inducer of CD69 [107]. CD69 is expressed as response to cytokines such as IL-2, TNFα and IFNα/β cytokines [91, 108]. Induction of CD69 can be produced upon antigen-specific TCR and BCR stimulation with anti-CD3/CD28, anti-CD2/CD28, anti-IgM and anti-CD16 mAb [109]. Human peripheral eosinophils acquire CD69 expression after GM-CSF or IL-13 stimulation [110]. CD69 has been detected within T cells at high levels in synovial fluid and synovial membrane from chronic rheumatoid arthritis patients [111]. Studies in patients with chronic active viral hepatitis, CD69 expression has been identified in liver-infiltrating CD69+ lymphoid cells both in portal tracts and in periportal areas [112]. Also, it has been described CD69 expression in NK cells and T lymphocytes in tumour infiltrates [113]. Bronchoalveolar eosinophils from patients with asthma and eosinophilic pneumonia [110] patients with B-cell lymphocytic leukaemia also express CD69 [114].

3.4 CD69 signaling

The need of PMA, in addition of anti-CD69, to produce IL-2 induction, suggests that CD69 is not capable of activating protein kinase C (PKC) signaling alone [94, 115]. CD69 activation involves calcium-dependent signaling pathways through activation of PKC [116].The role of GTP binding proteins as transducer molecules during T cell activation [117, 118] and the association of CD69 to GTP binding protein such as Ras, Raf and Vav have also been described [115, 119]. Other studies reported that CD69 induces AP-1 activity which has been involved in early T-cell activation processes [120]. AP-1 activation is related to the activation of ERK which can also influence in cytokine production, cell proliferation and activation of phospholipase A2 [121-123]. The relation between CD69 and ERK members of MAPK family has been described. CD69 induces activation of ERK, which promotes granulocyte exocytosis in different cell types [122, 124, 125]. CD69 leads to ERK phosphorylation and stabilizes TGF-β on cell surface of CD69+ CD4+ CD25- T cells [126]. Other studies have described the ability of CD69 to initiate the PTK-dependent signaling pathway in activated NK cells mediated by PCL-2 and VAV-1 [127]. CD69 also induces the activation of Syk. In the association of CD69 cytoplasmic tail with Jak2, Jak3, Stat1 and Stat5, it has been demonstrated the importance of Jak3 and Stat5 in Th17 differentiation [128]. It has been showed that CD69 regulates through STAT5 and ERK signaling

pathways the suppression activity of CD4+FOXP3+ CD69+ Treg cells [129]. Thus, in CD69$^{-/-}$ Tregs, Stat5 phosphorylation was inhibited whereas phospho-erk was enhanced. CD69 deficiency inhibits Stat5 phosphorylation, BIC/mRNA155 transcription and increase suppressor of cytokine signaling 1 (SOCS1) expression [130]. Also, in thymic CD4+ FOXP3+ Treg cells, CD69 has been showed to be associated with the transporter complex LAT1-CD98 together with an enhanced L-Trp uptake and promoting IL22 secretion [131].

3.5 Function

The *in vivo* role of CD69 has been studied in CD69 knockout mice model and in targeting of CD69 with anti-CD69 mAbs using different animal models of human diseases [100]. The vast majority of these works have demonstrated the role of CD69 as negative regulator of immune response through the production of a variety of cytokines and leukocyte trafficking [132]. In contrast, early *in vitro* studies pointed to role of CD69 as an activation molecule [91].

a. In vitro

MAb CD69 induced the activation of intracellular signals in different cell types. A co-culture with CD69 crosslinking and phorbol esters induced the expression of IL-2, IFNγ, CD25, TNFα synthesis [133] and an increased proliferation [101]. MAb CD69 stimulates glycolysis, synthesis of diacylglycerol and an increase of intracellular Ca2+ levels [134]. In platelets, CD69 crosslinking generates Ca2+ influx, degranulation, and platelet aggregation [103] while in monocytes induced the production of NO [101]. Cross-linking of CD69 mediates extracellular Ca2+ influx [109, 135, 136]. In a different setting, anti-CD69 seemed to block the ability of T cells to activate macrophages by cell contact [137]. On the other hand, anti-CD69 induces apoptosis of monocytes or eosinophils [138, 139]. Also, anti-CD69 mediates inhibitory signals on IL-1 receptor (IL-1R) or CD3-mediated T-cell proliferation [88]. All these data indicate that CD69 behaves *in vitro* as a co-stimulatory receptor although it could vary depending on the cellular context.

b. *In vivo*:

CD69 knockout mouse model was generated and described for the first time by Lauzurica et al in the year 2000 [105] and in this part of this thesis is named KO1, whereas other CD69 knockout mouse

model was published by Nakayama et al. two years later and here will be named KO2 [140]. Whereas exon II and III was deleted in the KO1, promoter and exon I was eliminated in the KO2.

CD69 deficient mice (KO1) showed greater antitumor activity against ***NK-sensitive tumors*** (RMA-S lymphoma and RM-1 prostate carcinoma) [1]. This higher response was found to be dependent on NK cells and T lymphocytes and associated with an increased production of MCP-1 and IL-1β and a reduced secretion of TGFβ. Importantly, an increase of lymphocytes in basal and anti-tumor conditions was found in peritoneum and spleen. It was found a reduced apoptosis in T lymphocytes and NK cells. In addition, increased recruitment of NK cells and T lymphocytes to the tumour site was observed. The absence of CD69 favoured an increase of the cellularity in spleen and peritoneum. These data demonstrated for the first time the role of CD69 as negative regulator of the immune response and show the possibility for immune therapy using CD69 as target.

The role of CD69 in infection has been studied in $CD69^{-/-}$ (KO1) mice model in Listeria monocytogenes model [141]. In ***Listeria monocytogenes*** infection, the immune response in the absence of T and B lymphocytes in $CD69^{-/-}$ $RAG2^{-/-}$ mice was augmented accompanied by an increased Lm elimination. However, $CD69^{-/-}$ mice showed an increased IFNα, β and γ expression without differences in TGF-β expression whereas an increased liver and spleen damage and an increased susceptibility to Lm infection were found compared to WT mice. $CD69^{-/-}$ mice shortly die after Lm challenge in spite of the improved control observed in $CD69^{-/-}$ $RAG2^{-/-}$ mice. Since, it was reported that Lm Listeriolysin induces apoptosis of activated lymphocytes as evasion mechanism and that $CD69^{-/-}$mice have an increased activation, the increased activated number of lymphocytes in $CD69^{-/-}$ mice led to an enhanced lymphocytes elimination and a lesser control of Lm burden.

The role of CD69 has been studied in ***collagen induced arthritis type II (CIA)*** in $CD69^{-/-}$ mice which is mediated by a T-cell response and the production of antibodies against collagen type II [142]. CD69 knockout mice (KO1) had exacerbated the T and B cell immune response to type II collagen and therefore the disease. In addition, there was a greater production of IgG2c and IgG2b isotypes and the IgG3 isotype and a locally increased T-cell proliferation. TGF-β levels were reduced and an enhancement of local cytokine production such as IL-1β, RANTES, MIP-1α and MIP-1β was detected. Collagen-induced arthritis model showed that CD69 decreases the autoimmune reactivity and inflammation response.

Collagen II (CII)-antibody induce arthritis (CAIA) has been developed with the two different CD69 knockout mice model with opposite results. In this model, the arthritis is induced with a mixture of anti-CII monoclonal antibody followed by Lipopolysaccharides (LPS). In $CD69^{-/-}$ (KO1) mice model [143], this arthritis was not grossly reduced but the increase in local proinflammatory cytokines and a decrease in joint of TGF-β level were also observed. Whereas using $CD69^{-/-}$ (KO2) mice [144], it was reported a markedly reduced inflammatory response, suggesting that CD69 plays an activating role in neutrophil function.

The **atherosclerosis** development in $ApoE^{-/-}$ and double deficient $ApoE^{-/-}$ $CD69^{-/-}$ were studied in $CD69^{-/-}$ (KO1) mice [145]. In the absence of CD69, the production of IFN and IL10 were elevated in activated T cells. These mice were fed with control or a high-fat diet to explore the role of CD69 on both spontaneous and diet-induced atherosclerosis and it was observed no differences in size and composition of atheromas between presence and absence of CD69. Therefore, though the production of cytokines are elevated in atherosclerotic $ApoE^{-/-}$ $CD69^{-/-}$ mice, the absence of CD69 does not influence in the formation of atheromas.

Using OT-II TCR transgenic mice with CD4 T cells OVA-specific $CD69^{-/-}$ (KO1) mice, it has been studied the role of CD69 in **differentiation of CD4 T cells to Th17 cells** [128, 146]. It has been demonstrated *in vitro* that CD69 deficient CD4 T cells activated with anti-CD3 and anti-CD28 had an increase of IL-17 and IFNγ production whereas IL17 but not IFNγ expression was augmented after OVA challenge. When CD4T cells were differentiated under specific *in vitro* conditions to Th1, Th2 and Th17 linages, IL17 was found increased in all of them and CD69 deficient mice immunized with type II collagen showed a higher proportion of antigen-specific Th17 subpopulation in the draining lymph nodes than WT mice. *In vitro* experiments showed that CD69 is associated with Jak3/Sta5 in regulating Th17 differentiation. For all this, CD69 reduces IL17 production and T-cell differentiation to Th17.

The influence of CD69 has also been studied in an ***albumin-induced asthmatic*** model in both two models of CD69 knockout mice, KO1 and KO2. KO1 mice showed an exacerbated allergic airway inflammation [147] and this response was associated with an increase of Th2 and Th17 cytokines. In the lungs was found a higher expression of VCAM-1 and the bronchoalveolar lavage showed a greater number of macrophages and eosinophils together with an increase in IL-4, IL-5, IL-13, IL-17, Eotaxin and IgE. In addition, an increase of oxazolone-induced skin contact hypersensitivity accompanied by

the increase of TNFα, IFNγ, IL-1β, IL-6 and IL-17 was showed. On the contrary, KO2 mice developed allergic asthma but in attenuated form compared to WT mice and the observed pattern of cytokines was different to that found in the $CD69^{-/-}$ (KO1) mice [148].

Experimental autoimmune ***myocarditis*** was also developed in $CD69^{-/-}$ mice (KO1) by immunizing animals with β-myosin [149]. This disease is associated with an increased infiltration of inflammatory cells into the heart myocardium followed by a cascade of physical and chemical changes leading to heart failure. These mice developed an exacerbated Th17 and IFNγ response in lymph nodes and spleen accompanied by an increased leukocyte infiltration in cardiac tissue, a higher increase in heart size in relation to the body and a developed necrosis of cardiac muscle. Thus, newly an increased autoimmune response to myosin challenge was showed in this autoimmune model.

In vivo **skin contact sensitization** model showed an increased inflammatory response in the absence of CD69 associated with an increase of DC in skin. Dendritic cells of CD69 deficient mice are more efficient in migrating to draining lymph nodes and this effect is S1P/S1P1 axis-dependent [150]. This increased recruitment leads to an enhanced immune response.

In a model of **peritoneal fibrosis** induced by dialysis fluid exposure, in normal or uremic status, it was found that the absence of CD69 (KO1) exhibited an enhanced inflammation and fibrosis [151]. The effluents showed an induction of proinflammatory and profibrotic cytokine production such as IL17, IL6, TGFβ and IL1β and chemokines such as GM-CSF, MIP1α, MIP1β and MCP-3 at 20 days after PDF exposure whereas at 40 days was induced a higher recruitment of T CD4 and T CD8 lymphocytes and an augmented Th17 and a smaller increase of FOXP3 CD4 T cells in the absence of CD69. This ratio of Th17 and Treg was increased in CD69 knockout mice. In addition, mix bone barrow from $CD69^{-/-}$ and $RAG2^{-/-}\gamma c^{-/-}$ transplanted in WT mice had a similar response than the observed in $CD69^{-/-}$ mice.

Recently, a role of CD69 in an autoimmune model of **psoriasis** induced by IL-23 injection has been described in KO1 mice [131]. The absence of CD69 leads to a reduced expression of IL-22 and STAT-3 and a lower inflammatory response in the psoriasis model. CD69 did not affect to IL17 secretion by γδ-T cells. However, CD69 controls the secretion of IL22 produced by γδ-T cells and this control is dependent on the aryl hydrocarbon receptor (AhR). In this work was reported that CD69 is associated

with the transporter complex LAT1-CD98 and enhances L-Trp uptake. L-Trp is a metabolic precursor of AhR ligand that promotes IL22 secretion.

CD69 regulates the **homing of CD4 T cells to the intestinal tissues** *in vivo.* Using an *in vivo* competitive homing assay with CD4 T cells of CD69$^{-/-}$ (KO2) mice [153], it was found that their migration to intestinal tissue was increased compared with WT CD4T cells. CD4 T cells of CD69$^{-/-}$ mice had increased chemokines expression such as CCL1, CXCL10 and CCL19 and chemokines receptors.

Crohn's colitis model has been performed by two groups using KO2 mice. Mice were treated with dextran sodium sulphate (DSS) in both groups. Radulovic. et al. found local increased cell accumulation and expression of chemokines and sever intestinal inflammation. Hassegawa et al. using the same CD69$^{-/-}$ (KO2) mice and the same **DSS induced colitis model** [152], found intestinal inflammation attenuated. Overall, the results in cytokines and chemokines expression are different in both studies, indicating the complex analysis of the mucosa inflammatory response.

Several **regulatory T cell** subsets have been studied based on the expression of CD69 molecule (review below).

In the mucosal inflammatory response, the effect of CD69 in **Treg differentiation** was studied using KO2 mice model in an antigen-specific transfer **colitis model**. Colitis was induced by adoptive transfer of T CD4 CD69$^{+/+}$ and T CD4 CD69$^{-/-}$ cells in Rag$^{-/-}$ mice [153]. This cell transfer showed an increased induction of colitis and impaired oral tolerance in cell adoptive transferring from CD69$^{-/-}$ mice. In this model, T CD4 CD69$^{-/-}$ cells had reduced potential to differentiate into FOXP3 regulatory T cell *in vivo* and *in vitro* [153]. The absence of CD69 in TCD4 lymphocytes increased the production of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-21 and decreased production of TGF-β1. This work showed for the first time that the absence of CD69 impaired differentiation and suppressor function of Treg cells [129].

Also, Cortes et al showed that **Treg** expressing CD69+FOXP3+ have higher suppressor activity than the classical FOXP3+CD25+ Treg cell population by adoptive transfer experiments. WT CD4+FOXP3+ CD69+ Treg cells reduced more efficiently OVA-induced immune response and the inflammatory response in CD69$^{-/-}$ (KO1) mice. The characterization of this peripheral FOXP3+CD69+ Treg subset compared

with WT CD4+FOXP3+ CD69- subset, showed a higher expression of FasL, IFNγ, IL2, IL4 and ICOS and chemokines such as CXCL5 and CXCL10 Treg but similar high levels of CTLA-4, CD38 and GITR molecules and secretion of TGFβ. In this work, it was demonstrated that the suppression activity of CD4+FOXP3+ CD69+ Treg cells is regulated by STAT5 and ERK signaling pathways. In $CD69^{-/-}$ Tregs, Stat5 phosphorylation was inhibited whereas phospho-erk was enhanced.

On the other hand, in thymic **FOXP3+ Tregs cells**, CD69 deficiency (KO1) inhibits Stat5 phosphorylation and BIC/mRNA155 transcription and increases suppressor of cytokine signaling 1 (SOCS1) expression. Also, CD69 deficiency, in FTOC cultures impairs thymic Tregs development in embryos, adults and also, severely inhibits development of thymus Tregs in $Rag2^{-/-}$ $\gamma c^{-/-}$ hematopoietic chimeras reconstituted with $CD69^{-/-}$ stem cells. These results point to CD69 as regulator of Treg development.

Other studies about the influence of CD69 in **regulatory T cells** were described in **tumor infiltrating leukocytes**. A new subset of regulatory T cells that supress T cell proliferation through membrane bound TGF-β1 was described as CD69+ CD4+ CD25- T cells infiltrating tumors [126]. They do not express FOXP3, secrete IL10, TGFβ, IL2 and IFNγ. Importantly, these tumor derived CD69+ regulatory T cells induced in tumor macrophages the ability to produce high amount of IDO protein [154]. CD69+ T cells induce considerable amounts of indoleamine 2, 3-dioxigenase (IDO) in tumor associated macrophages leading to downregulation of inflammatory immune response.

The role of CD69 was revealed in leukocyte migration and retention in the study of CD69 knockout mice.

CD69 was described as decisive in regulation of T cell trafficking through S1P1 expression which can physically interacts with CD69. S1P1 is a G-coupled receptor which binds sphingosine 1-phosphate (S1P) and belongs to a sphingosine-1-phosphate receptor subfamily comprising five members (S1PR1-5). Each one of these receptors is predominantly expressed in a cell type. The role of S1P1 has been performed *in vivo* models with S1P1 conditional and knockout mice model and with agonist or antagonist of S1P1 receptor which can deregulate the S1P receptor inducing lymphocyte retention in lymph nodes [155]. At steady state, when CD69 has not been expressed, lymphocytes are continually recirculating to preserve normal concentrations of leucocytes in lymphoid organs and bloodstream, necessary for immune surveillance [156]. The cells expressing S1PR are attracted by increased gradients of its ligand, S1P, which is highly expressed in plasma. The expression of CD69 leads to S1P1 downregulation and retention of lymphocytes in lymph nodes, so that in the course of

the immune response can be developed effector functions at the site of inflammation (Fig.7). In innate and B and T lymphocytes resident in non-lymphoid tissues, CD69 acts retaining these cells preventing its egress to circulation. CD69 forms a complex with S1P1 which is negatively regulated and this interaction has been deeply characterized [157, 158]. Works of Cyster lab demonstrated that S1P1 controls lymphocyte egress from thymus, spleen and lymph nodes and that CD69 modulates trafficking S1P1-dependent [158-160].

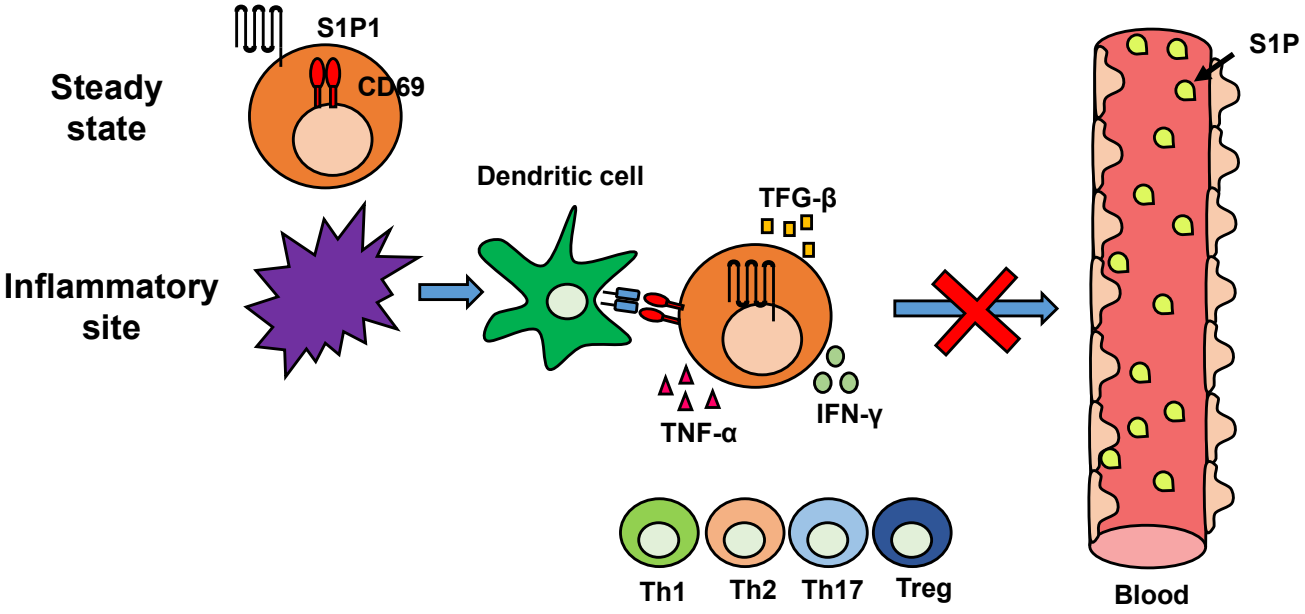


Figure 7. CD69 and S1P1 are mutually downregulated. Adapted of Shiow et al. Nature.

It was showed that CD69$^{-/-}$ T and B cells were poorly retained in lymph nodes after treatment with poly: IC or infection with lymphocytic choriomeningitis (LCMV). In LCMV infection, in the adoptive transfer of CD69$^{-/-}$ and CD69$^{+/+}$ T and B cells, CD69 deficient cells were less efficiently retained in lymph nodes and were found higher increased in lymph 18 hours after infection [108]. Correspondingly, mice overexpressing CD69 in thymus had increased proportion of single positive T CD4 and T CD8 cells compared to WT [140, 161]. Furthermore, S1P1 has been considered to contribute in immature B-cell egress from bone marrow to lymphoid secondary organs [162, 163] and in homing of plasma cells to bone marrow or in retaining in secondary lymphoid organs [164]. Thus, forced expression of CD69 in bone marrow immature B cells also reduces the number of immature B cells in the blood. The influence of S1P1 in regulatory T cells is established in development, differentiation and function of this subpopulation both in thymus and periphery [130, 165]. However, the control of NK migration depends essentially on S1P5, although at least in part it also depends on S1P1 [166]. As well, it has

been demonstrated that CD69 modulates skin dendritic cells migration through S1P1 [150] in a model of skin sensitization.

Furthermore, the function of CD69 in promoting the retention of **effector and resident memory T cells** (TEM and TRM) has been defined.

The role of CD69 has been analyzed in Tissue-resident memory CD8 T cells (TRM) using a combination of infection of herpes simplex virus infection (HSV) along with adoptive transfer of CD8 T cell specific for this pathogen [167]. It was found that CD69 and CD103 are necessary for the optimal formation and survival of CD8 TRM cells in the skin using CD69 deficient (KO1) antigen specific T cells, it was shown a reduction in TRM cell numbers and this reduction occurs shortly after the entry of T cells into the skin.

Also, in an infection model of Influenza virus using $CD69^{-/-}$ (KO2) mice [168], memory specific CD8 T cells (TRM) and effector memory CD8 T cells (TEM) response were measured in the lung. The accumulation of T cells in lung were found reduced using $CD69^{-/-}$ mice. Although specific niches for lung-resident memory CD8 T cells allow CD69-independent maintenance. These cells that express CD69 and persist in bone marrow are characterized by being associated with IL-7 of stromal cells. In addition, CD69 regulates the formation of CD4 T-helper memory cells. The absence of CD69 (KO2) induces fewer effective memory immune response and as a consequence produces deficiency of the memory Th cell to produce high-affinity antibodies. Accordingly with these studies, CD69+ thymic memory CD8 T cells contain two subsets with distinctive recirculation potential. CD69 is required for optimal formation of CD103+ TRM cells but CD69 expression alone did not identify permanently resident T cells [169]. In addition, CD69 expression was associated with a subset of virus-specific memory CD8 T cells in SLO with phenotype markers associated with TRM that express CD69 [170].

Mackay et al also showed that CD69 regulates effector T cell egress from peripheral tissues and prolongs T cell retention and favors memory formation. In CD8 T cells, the signaling pathways leading to activation coincides with the transcriptional downregulation of S1P1. Therefore, CD69 expression interferes with S1P1 function [171].

c. *In vivo* CD69 targeting

Anti-mouse CD69 2.2 and 2.3 and anti-human CD69 2.8 antibodies only recognize mouse CD69 and human CD69 respectively and were produced in CD69 knockout mice injecting cells expressing both molecules. These antibodies were generated in our lab and were used in most of the above described models. Anti-mouse CD69 2.2 (IgG1) induced an internalization of CD69 and not the elimination of CD69 positive cells [172] and thus not activate complement or bind Fc receptors. Anti-CD69 2.3 (IgG2a) activates complement and binds FC receptor and therefore depletes CD69+ cells. Anti-CD69 2.8 (IgG1) actuates as control of anti-CD69 2.2. Other laboratories use the anti-mouse CD69 generated in hamster distributed commercially.

In **NK cell dependent tumor model**, the targeting of CD69 showed reduced tumor growth, increased NK cells cytotoxicity and a reduction in the production of TGF-β, similar to the result obtained in CD69 deficient mice. However, CD69 targeting increased production of IFNγ unlike CD69 deficient mice. Also, therapeutic administration in healthy animals generated NK cell cytotoxicity activity in the absence of tumor priming. In addition, *in vitro*, anti-CD69 triggers NK cell function on resting NK cell and a reduction of TGF-β, and an increase of IFNγ production [1].

Also, it was studied the targeting of CD69 in **collagen-induced arthritis (CIA)** through two different types of antibodies: anti-CD69 2.2 and anti-CD69 2.3. The difference between both treatments is that anti-CD69 2.2 induces the internalization of CD69 and the second depletes CD69 positive cells. The first antibody led to an exacerbated response of the disease, reproducing the phenotype found in CD69 knockout mice whereas the treatment with anti-CD69 2.3 was able to reduce the disease due to a reduction in the proinflammatory cytokine production, lymphocyte proliferation and frequency of CII-specific T cells producing IFN-γ [173].

In **atherosclerosis** mice model, similar to the results obtained in $CD69^{-/-}$ (KO1) mice, CD69 targeting with anti-mouse CD69 2.3 did not display different atheroma development compared to non-treated mice in fat-fed $ApoE^{-/-}$ mice [145].

In an **ovoalbumin-induced allergic asthmatic** model, *in vivo* treatment with the anti-CD69 2.2 antibody produced similar results to CD69 knockout mice. Martin et al. described a greater inflammatory

response in the treated mice along with an increase in number of eosinophils [147]. Wang et al. showed that CD69 targeting using commercial antibody prevented the development of airway hyper-responsiveness while the analysis of bronchoalveolar fluid revealed an increase in eosinophil infiltration, mucus production, and a significant reduction of IL-5 [174].

Peritoneal fibrosis, induced by dialysis fluid exposure, it was found that the CD69 targeting exhibited an enhanced inflammation and fibrosis similar to that found in the absence of CD69 [151].

In **psoriasis**, the treatment with anti-CD69 2.2 similarly to the absence of CD69 reduced the uptake of aminoacid through Lat-1 [131].

Colitis: The anti-CD69 treatment also inhibited the induction of DSS induced colitis. IL10 expression was found increased in the colon DSS CD69 treated mice [152].

Tregs, CD69+ FOXP3+ Tregs secrete higher amounts of TGF-β and have a potent suppressor activity which is impaired by treatment with anti-CD69 2.2 [129]. The use of anti-mouse CD69 2.2 inhibited tTreg development in FOCT cultures of embryonic thymuses [130].

Ligand, Anti-CD69 2.2 binding to CD69 blocked interaction with Galectin-1 that was carbohydrate dependent [175].

3.6 Ligands

Currently, it has been described Galectin-1 as a ligand for CD69 that is expressed mainly by tolerogenic dendritic cells and activated T and B cells but not resting, and significantly upregulated in activated macrophages and T regulatory lymphocytes [176]. Selective binding of CD69 to Galectin-1 in DCs modulates differentiation of T lymphocytes to Th17, confirming that CD69 acts as a negative control of pro-inflammatory responses [177]. Galectins have a carbohydrate recognition domain with affinity for beta-galactosides [178] and it has been described that galectin-1 but no other galectins binds to extracellular domain of CD69 through sites of N-glycosylation present in CD69 [179]. Galectin-1 promotes cell apoptosis which may contribute to the immunosuppressive effects of CD69 protein

[180]. Functional assays of both mouse and human T cells demonstrated the role of CD69 in the negative effect of Galectin-1 on Th17 differentiation. Galectin-1 has not been the only ligand associated with CD69. Also, it has been described S100A8/S100A9 as an endogenous ligand expressed in myeloid cells. The interaction between CD69 and S100A8/S100A9 is carbohydrate dependent leading to Treg differentiation, regulating TGF-β and IL-4 and inhibiting STAT3 pathway [181]. S1P1 interacts with CD69 and this interaction occurs between proximal regions of CD69 membrane and transmembrane helix 4 of S1P1 [157] promoting a high affinity binding. CD69 binds to S1P1 on surface of lymphocytes mediating its internalization, preventing the outflow of lymphocytes [108, 182], whereas the absence of CD69 promotes S1P1 expression on the surface and lymphocyte outflow at the circulation. So, we can say that CD69 and S1P1 are downregulated mutually. In addition, early biochemical studies showed glucidic ligands for CD69 [183]. Immunoprecipitation and sequencing identified calreticulin as a CD69 interacting protein at surface of lymphocytes [184]. The implication of these potential ligands, however, has not been deeply explored. CD69 cytoplasmic tail interacts with Jak3/Stat5 and this interaction impairs Th17 differentiation [128].

4. VACCINIA VIRUS

4.1 Description

Vaccinia virus (VACV) is a member of the Poxviridae family belonging to Orthopoxvirus genus and was used as a vaccine to eradicate the Variola virus from the same family. Other orthopoxviruses include Variola virus (VAR), cowpox virus (CPV), monkeypox virus (MPV), ectromelia virus (ECT), camelpox virus (CMPV), raccoonpox virus and tatera poxvirus. The use of Vaccinia virus has continued over time because it is an exceptional model for studying the interactions that are established between virus and host and because it can be used as a vector for the expression of foreign genes. It is a large DNA virus with a linear double-stranded DNA genome that encodes many of their own enzymes for transcription [185] and DNA replication [186]. It is considered unique within DNA viruses because they only reproduce in the cytoplasm of the host cell, outside of the nucleus [187].It has a broad cellular tropism and infects almost all cell lines in culture. The virus codifies in its genome several proteins that give resistance against the action of interferons. Members of this virus family do not usually establish persistent or latent infections and have a low mutation rate [188]. VACV infection is initially controlled

by the innate immune response, but can only be eradicated by adaptive immunity being B and T cells important since *Rag*$^{-/-}$ mice will finally succumb to the infection [189].

The viral particle is a structure surrounded by a lipoprotein envelope. The replication cycle of Vaccinia virus begins with the entry of virus and ends with the assembly of complex macromolecular structures to form an infectious particle that induces cellular infection with secretion of early mRNAs and proteins (Fig. 8).

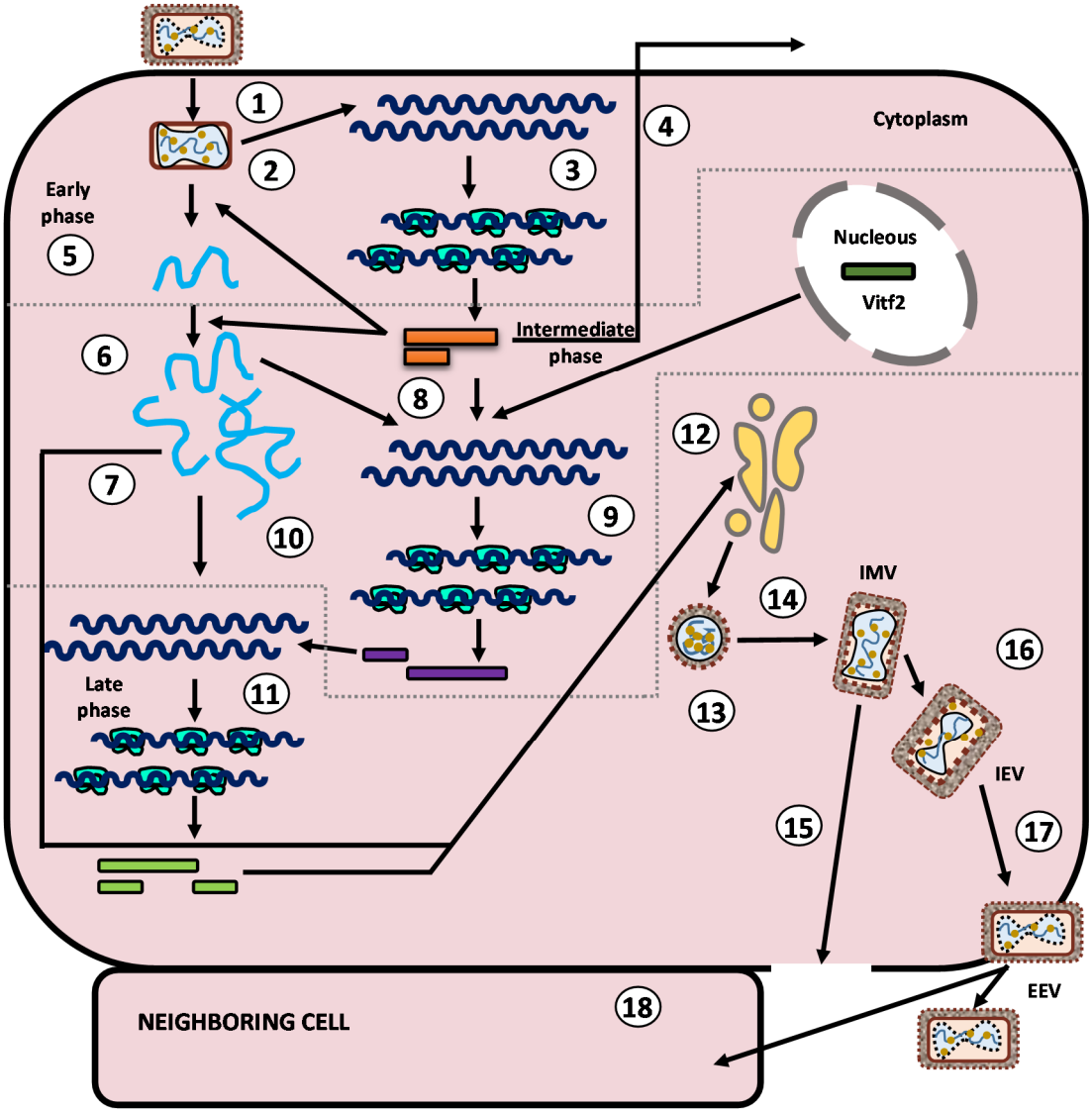


Figure 8. Vaccinia virus replication. Adapted from Harrison SC et al. PNAS.

DNA replication begins a few hours later from infection. Vaccinia virus induces the formation of 4 different types of virions: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). These virions differ according to their abundance, structure, location and the role that they play in the viral replication

cycle. IMV is the most abundant form of virus and is responsible for transmitting infection between hosts [190, 191]. IEV is formed by wrapping of IMV with intracellular membranes, and allows viral dissemination to the cell surface on microtubules. CEV is important for cell-to-cell spread. Lastly, EEV mediates the long-range dissemination of virus in cell culture and, probably, *in vivo* [192, 193]. Vaccinia virus is the only one that produces two infectious virus forms: IV and EV being EV related to promoting virus spread and to supporting mechanisms of immune virus evasion of antibody [194, 195] and complement [191].

4.2 Use of Vaccinia virus as vector expression

The technology of using recombinant viruses as live vaccines by introducing a heterologous gene of another microorganism began to be used more than 10 years ago [196, 197] and are also potent tools for gene therapy. They are effective vaccines to induce both humoral immunity by the production of specific antibodies, and cellular immunity, without the risk of exposure in the recipient animals [198-201]. Currently, Vaccinia virus vectors are being evaluated in many clinical trials although viral attenuation is necessary to reduce the adverse effects. Vaccinia virus is used to construct directed targets for vaccines candidates against various diseases, such as HIV-1 [202], hepatitis [203] influenza [204], malaria [205], tuberculosis (TB) [206], and even in oncogenic diseases of both human and domestic animals [207] and display high immunity.

4.3 Immune response to Vaccinia virus infection

In immune response to Vaccinia virus infection, both innate and adaptive immune response are involved. Next, we will detail the immune cells that have been described as important in this response. The innate immune response in Vaccinia virus infection include macrophages, dendritic cells, NK cells and production of cytokines, chemokines, complement and TLR. An increased severity infection was found in ectromelia virus infection in components of the complement system deficient mice [208].

The importance of macrophages has been also described in Vaccinia virus infection. It was observed that the depletion of the **alveolar macrophages** induced a higher viral replication and an exacerbation of the disease [209]. In addition, as a consequence of the lack of alveolar macrophages, a recruitment

of inflammatory cells into the lungs is induced, which produces a worse control of the infection [210] and links with a decrease in the weight of mice and an increase in temperature corporal.

Myeloid derived suppressor cells (MDSC) are a population of immature myeloid cells that regulate the immune system [211] and include myeloid progenitor cells, immature macrophages, immature dendritic cells (DCs), and immature granulocytes. Following to Vaccinia virus infection, these cells accumulate at the site of infection and regulate the activity of NK cells. The depletion of these cells induced a reduction in viral titre but an increase in mortality associated with an increased IFN production and therefore a poor control of infection by Vaccinia virus [212].

Dendritic cells are important in generating an immune response against poxvirus *in vivo*. The infectivity of Vaccinia virus is superior in immature than in mature cells. It has been found that Vaccinia prevents the maturation of dendritic cells and induces their apoptosis. In order to be able to carry out an immune response against Vaccinia, it is necessary that some DCs survive to the infection and initiate T-cell response or that may have matured before infection since they are less susceptible. Cross-presentation is another mechanism to induce a T cell response to Vaccinia virus [213]. Poxvirus infections can induce rapid and systemic release of IL-1β, IFN-α, GM-CSF and TNF-α [214, 215], all of which could affect DC maturation from precursors [216-218].

NK cells are crucial players in the first line of defense in innate immune response with a critical role in antiviral responses. NK cells were also critical for clearance in NK cell depleted mice in Vaccinia virus showing a higher viral titre [219]. They represent an essential tool in the fight against poxviruses like Vaccinia virus. VACV-infected cells have increased sensitivity to NK cell lysis. NK cells are activated by IFNα and β, IL-12 and IL-18 and its function is regulated by activator and inhibitory receptors [220]. Inhibitory receptors recognize MHC I and impede the response against self-antigens whereas the activators are related to NK cells activation [220] such as NKG2D which has been shown to be necessary for the function of NK cells [219, 221, 222] The production of type I IFN facilitates viral clearance. Another strategy for activation of NK cells is mediated via TLR-2 stimulation by Vaccinia virus in NK cells through PI3K-ERK pathway [221]. STAT1 is also involved in the activation of NK cells which was critical for the elimination of infection and whose signaling in dendritic cells promotes the expression of NKG2D ligands [223]. In NK cell depletion assays, $Stat1^{-/-}$ mice resulted in an increase in host susceptibility and a lower survival to VACV infection [224].

Also, the immune response against Vaccinia virus infection is mediated by IFNs which are a potent antiviral tool in Vaccinia virus removal [225-227]. **IFNs** are a group of glycoproteins that are classified into: **Type I** including IFN-alpha (IFNα) and IFN-beta (IFNβ) and others such as IFN-kappa, IFN-delta, IFN-omega and their activity is mediated by Type I IFN receptor which are ubiquitously expressed, **Type II** which includes IFNγ and is majorly secreted by activated T cells and NKs cells that induce macrophage activation and promote a Th1 response [228, 229] and **Type III** that includes IFN lambda which also acts in viral response but has a more limited distribution.

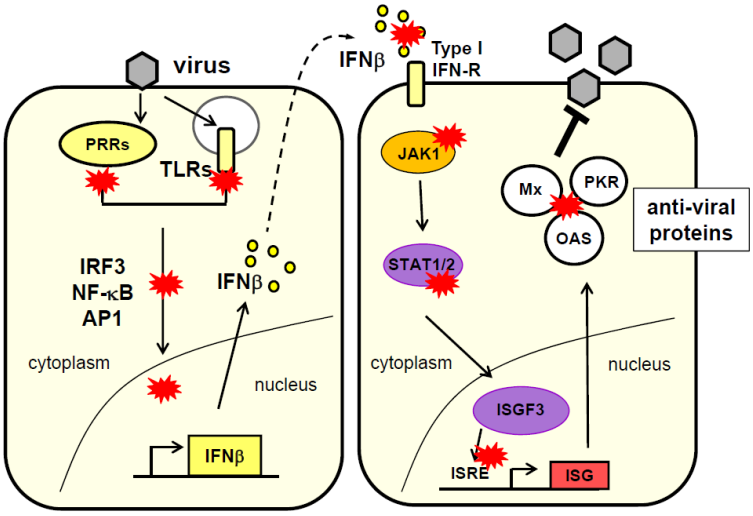


Figure 9. IFN signaling and mechanisms of immune evasion of Vaccinia virus against IFN production. "Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity" by Smith et al. Journal of general virology.

To test the importance of IFNγ, an IFNγ-deficient model has been used, which has demonstrated a significant increase in mortality after ECTV infection and an increase in viral titers compared to control mice suggesting a role for IFNγ in controlling early virus replication and recovery from ECTV infection [224]. In the same mouse model, it has been observed that IFNγ also contributes to the best anti-Vaccinia immune response in a respiratory VACV infection by restricting viral dissemination and promoting survival [230]. The influence of type I IFNs has also been demonstrated by the development of IFNαβR$^{-/-}$ mice. IFNαβR$^{-/-}$ NK cells failed to produce effector molecules indicating that type I IFN was critical for NK cell activation in response to Vaccinia virus infection. IFNαβR$^{-/-}$ mice displayed higher levels of viral titre compared to WT mice. Therefore, type I IFN control immune response to Vaccinia virus infection [219].

In addition to IFN, there are other cytokines that influence in anti-Vaccinia immune response to be effective and regulated. Fundamentally we are talking about TNF, IL-18, IL23 and IL17, which influence in the innate and adaptive immune response. **TNF** mediates protection against Vaccinia virus by inducing apoptosis of infected cells. The major cells expressing TNF are macrophages and activated T cells. The anti-Vaccinia effect of TNF was studied in $TNF^{-/-}$ mice, which significantly had more weight loss than WT and a significantly higher VACV titer compared to WT controls. TNF, like IFN, plays a protective role in controlling Vaccinia virus infection [231]. **IL-18**; also called IFN-inducing factor, is a proinflammatory cytokine [232] that regulates the innate and adaptive immune response. IL-18 is expressed by activated monocytes and macrophages and its activation begins with binding to its receptor which induces IFN synthesis and activation of NK and CTL cells [232, 233] as well as proliferation and cytotoxicity of these cells [234-237]. Administration of recombinant IL18 in mice has demonstrated its antiviral activity [77, 233] whereas IL18 deficient mice exhibited less ability to control VACV infection. IL18 can act synergistically with IL12 to promote Th1 responses, which has an essential role in defense against pathogens through IFN production [76]. Therefore, the joint action of both cytokines is essential for viral clearance and the generation of both specific and non-specific anti-Vaccinia responses. **IL2**, in spite of its importance in the development of a correct cytotoxic anti-viral immune response, the absence in mice of IL2 had a similar or slightly reduced anti-Vaccinia response to WT mice [238, 239]. The Th2 immune response is characterized by **IL4** production that suppresses T CD8 response. In the immune response to Vaccinia virus, the absence of IL4 may increase the cytotoxic capacity of CD8 T cells and may improve viral clearance, but since CD8 is not the only cell capable of removal Vaccinia virus, the response in mice deficient for IL4 was similar to control mice [240]. **IL12 e IL23** have been also described to regulate Th1-mediated immune responses. Whereas IL12 promotes the differentiation of IFN-γ-producing Th1 cells and generates an important antiviral response, IL23 stimulates production of IFN-γ increasing the cytotoxic capacity of CTLs. The study of anti-Vaccinia immune response indicates that IL-23, but not IL-12, is essential for controlling Vaccinia virus infection and for enhancing host defense [241]. **IL17** has also been implicated in the response to Vaccinia virus infection. It is produced by Th17 cells and induces the production of proinflammatory cytokines, chemokines, and adhesion molecules in some cell types. IL-17-deficient mice were less resistant to infection than WT mice [241]. In addition, treatment with an anti-IL-17 mAb in VV-IL-23-infected IFN-gamma-deficient mice resulted in a significant increase in viral titers compared to non-treated mice.

The recruitment of activated leukocytes to the site of infection is mediated by **chemokines** [242, 243], which play an important role in the response to pathogens [244]. They are expressed on cell surface and bind to their ligand by establishing gradients that allow the induction of cell attraction to the needed sites. One of the chemokines that has been described its role in immune response to Vaccinia virus infection is CCL2, which recruits monocytes, NK cells and T lymphocytes at the site of infection. In addition, CCL2 expression is mediated by type I Interferon. CCL2 deficient mice showed a failure to recruit NK cells and T lymphocytes to the lung after intranasal Vaccinia virus infection [245].

Together, all mechanism of the innate response initiate a powerful adaptive immune response essential for proper elimination of Vaccinia virus infection. All mechanisms previously described and the adaptive immune response are summarized in Figure 10.

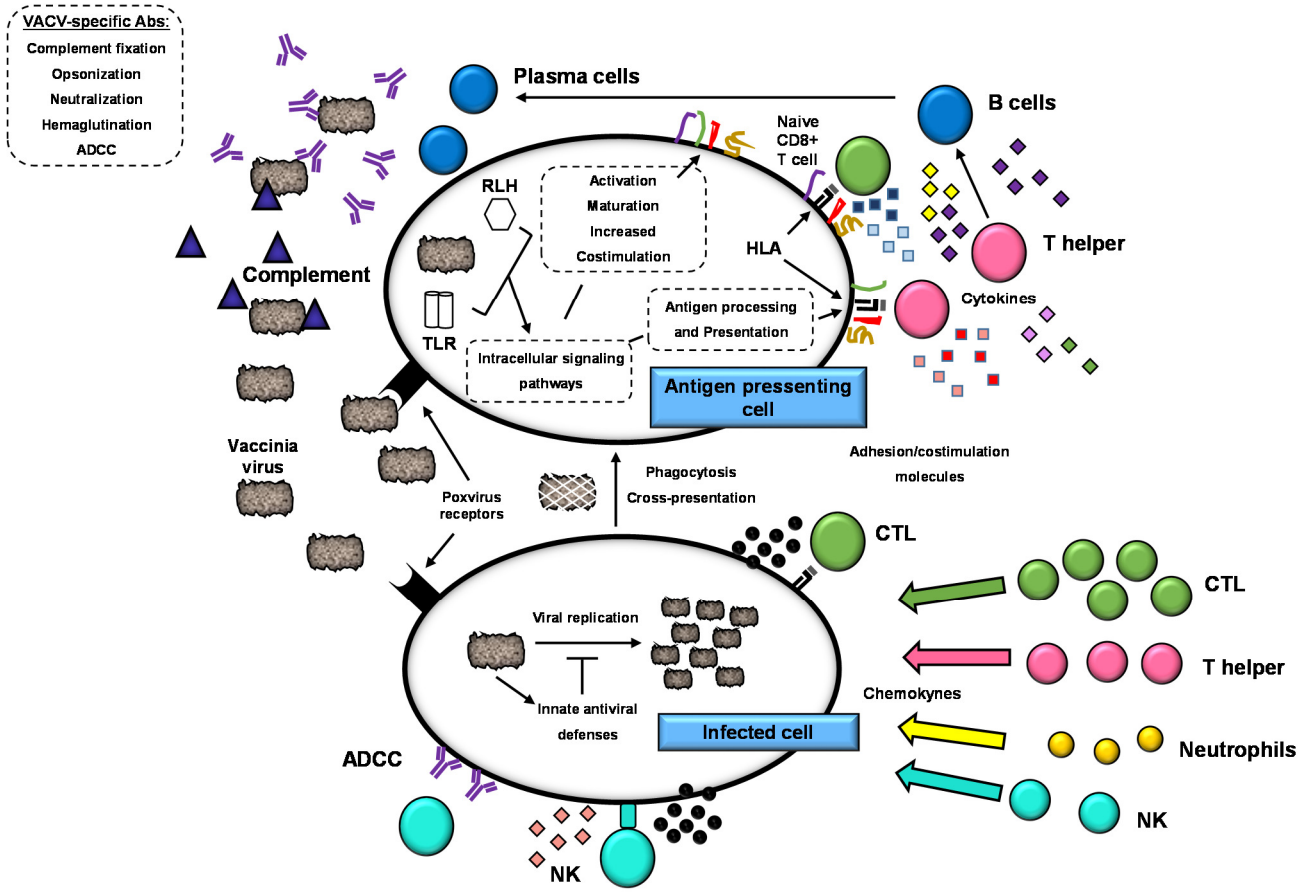


Figure 10. Immune response in Vaccinia virus infection. Adapted of "The immunology of smallpox vaccines". Curr Opin Immunol.

In adaptive immune response, **TCD4 cells** have been described as important in the anti-viral response [246]. They are the cells responsible for the differentiation of B cells giving rise to production of specific antibodies against Vaccinia virus and for inducing specific T CD8 virus responses. Activated T CD4 cells

are able to produce IFN, express CD127 and IL2 and proliferate [247, 248]. They have two effective mechanisms, the first is mediated by Fas/FasL to induce apoptosis of infected cells [249] and second is the transfer of cytotoxic granules, which contain perforins and granzymes, to the host cell. Mice deficient for CD4 lymphocytes in Vaccinia virus infection exhibited less effective protection against Vaccinia virus infection and an increased mortality [250, 251] and showed a reduced number of CTLs.

VACV infection, similar to other viruses, results in the generation of an adaptive immune response with the development of virus-specific **CD8 T cells** that reduce virus dissemination and complete clearance of virus. CD8 T cells contribute to this protective immunity via the release of interferon gamma (IFN-γ) and thanks to members of the Tumor Necrosis Factor (TNF) [252]. Spriggs et al. [253] demonstrated that CD8 deficient mice had similar survival to control mice when they had been infected with high doses of the virus and their rate of virus clearance was similar.

The protection induced by **humoral immunity** has been shown to have a great importance in the resolution of Vaccinia virus infection since a large number of viral proteins are recognized by immunoglobulins [254, 255]. In response to a viral infection, the antibodies bind directly to the virus, inducing aggregation and preventing infection of the host cell. In addition, they induce phagocytosis by complement activation. And finally, they can act activating cytotoxic cells once bound to the infected cell. Therefore, VACV-specific B cells are crucial in Vaccinia virus protection but CD4 and CD8 T cells can help to avoid the mortality in B cell deficient mice in VACV infection [255].

Finally, the formation of a **memory immune response** is important for proper protection in reinfection. Levels of Vaccinia-specific memory B cells are maintained over a long time being able to develop an efficient immune response upon re-infection [256]. CD4 deficient mice showed an impaired ability to form CD8 memory cells in Vaccinia virus infection, and thus preventing the control of Vaccinia infection compared to WT mice [257].

In spite of all the cellular mechanisms existing in the body to fight against Vaccinia virus infection, Vaccinia is able to develop multiple mechanisms of immune evasion to prevent its removal. Some of these mechanisms are summarized in the following table (Fig.11) [258].

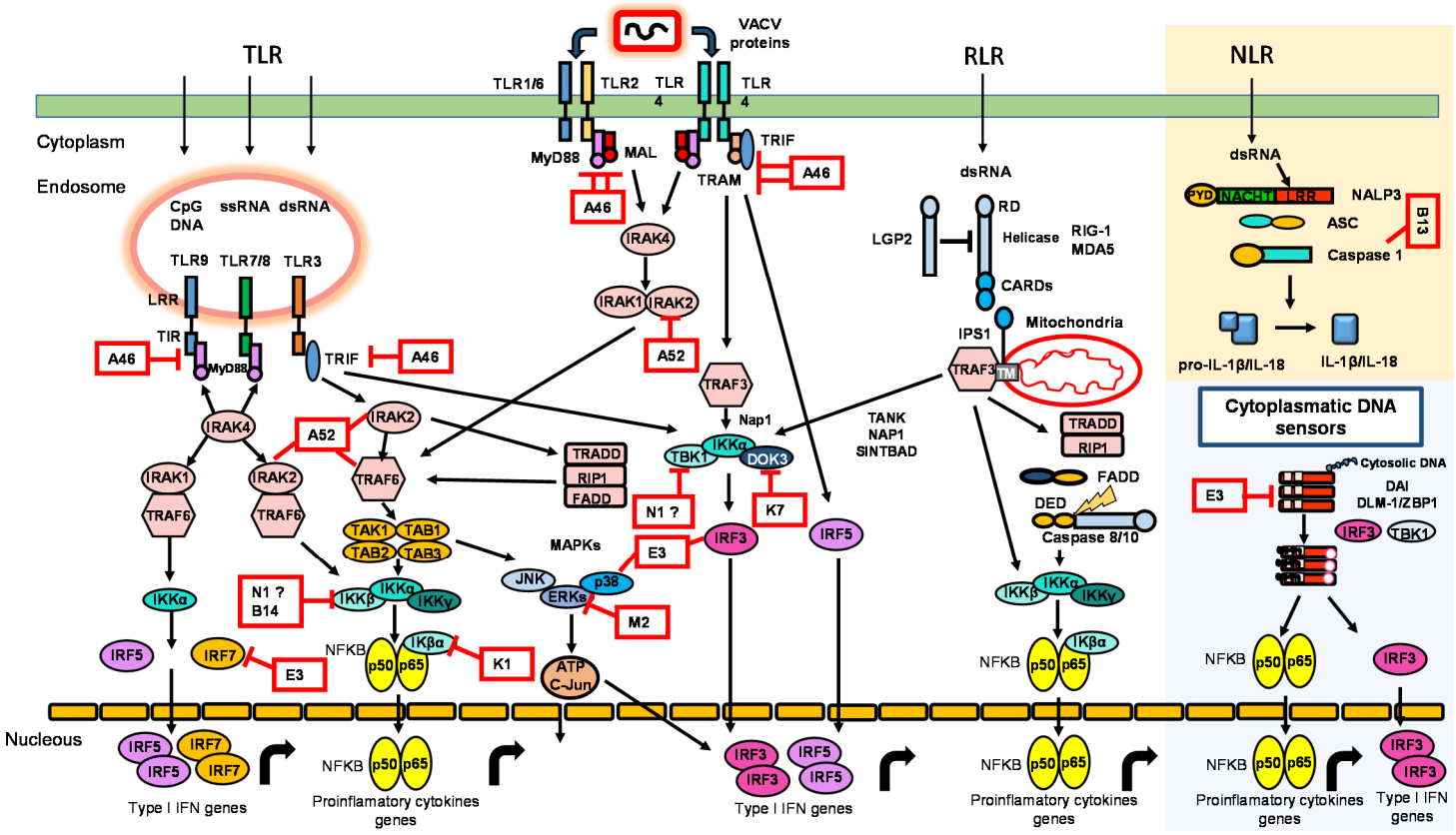


Figure 11. Signaling pathways and patterns recognition receptors in Vaccinia virus infection. Adapted from "The interferon system and Vaccinia virus evasion mechanisms" by Mariano Esteban. Journal of interferon and cytokine research.

OBJECTIVES

OBJECTIVES

The study of the role of CD69 in bone marrow leukocyte egress and on the course of VACV infection has been approached.

1. Analysis of CD69 absence and targeting in bone marrow leukocytes and HSC mobilization and its contribution in the increased number of peripheral leukocytes.

2. CD69 is rapidly expressed in viral infections; its role in the immune response to these infections has never been studied to date. We aimed at analyzing the effect of CD69 on the course of VACV infection.

    a. Analysis of CD69 deficiency in the immune response against Vaccinia virus infection.

    b. Examine the role of CD69 targeting in the immune response to Vaccinia virus infection.

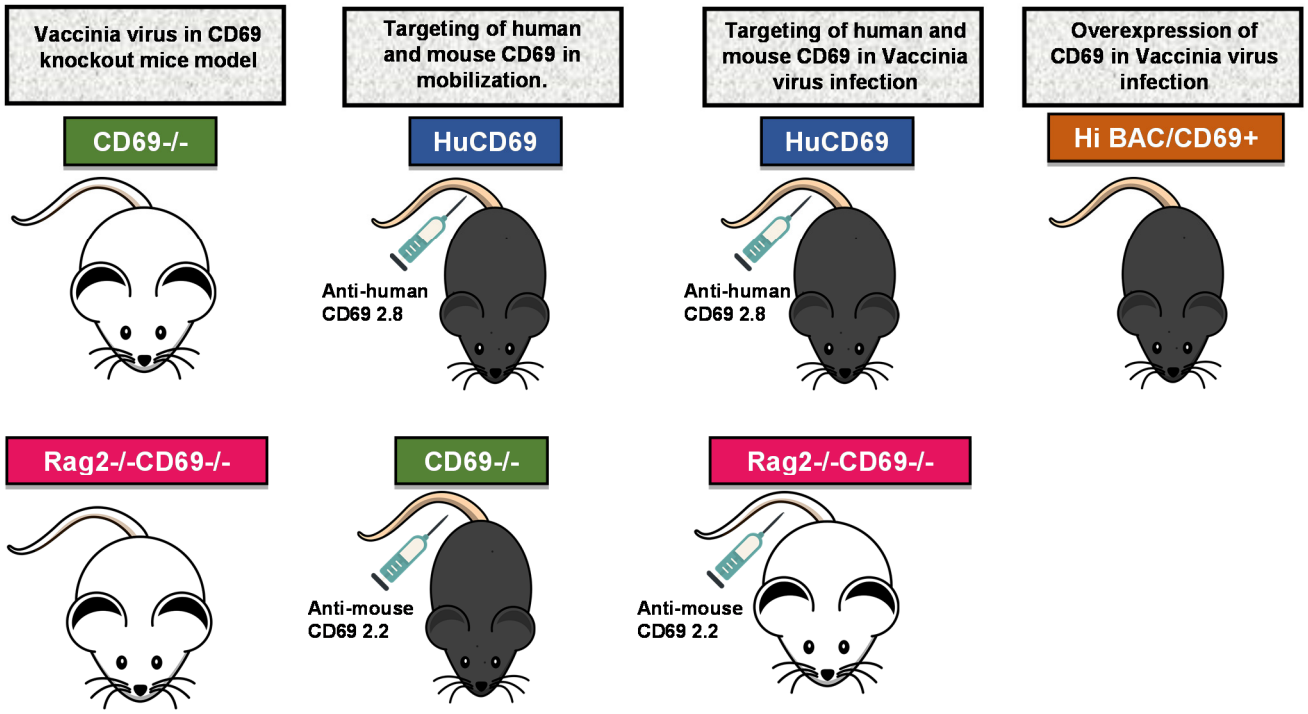
    c. Analysis of the effect of CD69 overexpression in the immune response to Vaccinia virus infection.

MATERIALS AND METHODS

MATERIALS AND METHODS

Mice

C57BL/6, Balb/c, Rag2$^{-/-}$ Balb/c mice, all CD69$^{+/+}$ or CD69$^{-/-}$, HuCD69 C57BL/6, CD69.BAC GFP C57BL/6 mice were bred and housed under specific pathogen free conditions in the animal facilities of the Instituto de Salud Carlos III (ISCIII), Madrid. HuCD69 mice were obtained by transgenesis of human CD69 BAC containing 100kb of CD69 gene by Bristol Meyers and were kindly provided by Dr. Robert Graziano. HuCD69 mice have three copies of human CD69 BAC in a mouse CD69 knockout background. CD69.BAC GFP mouse lines were generated by Dra. Teresa Laguna by a pronuclear injection of mouse CD69 BAC containing 100kb of CD69 gene in mouse oocytes by the Animal Facility of CNB (National biotechnology center, Madrid, Spain) in C57bl/6 mice. These mice were crossed in heterozygosis with wild type mice. GFP positive mice were used as mice that had incorporated CD69 BAC and GFP negative mice were employed as control mice. All mice used in this study were between 6 and 12 weeks of age, males and females. CD69$^{-/-}$ mice had been backcrossed on the C57BL/6 and the Balb/c backgrounds at least nine times. All procedures involving animals and their care were approved by the *ISCIII* Ethics Committees and were conducted according to institutional guidelines. The following image shows used mice and procedures.



In vivo treatments

HuCD69 mice were treated with anti-human CD69 2.8 and controls were treated with PBS or Isotype control anti-mouse CD69 2.2 intravenously whereas C57BL/6, $Rag2^{-/-}$ Balb/c mice were treated with anti-mouse CD69 2.2 and anti-human CD69 2.8 or PBS were used as control . Two treatments were especially used, 500 µg of anti- CD69 24 hours before sacrificing or 200 µg of anti-CD69 in two doses separated for one week. The anti-mouse CD69 2.2 mAb and anti-human CD69 2.8 mAb (IgG1 isotype) were generated in our laboratory [172] by the fusion of NS-1 myeloma cells with spleen cells from a $CD69^{-/-}$ mouse previously immunized three times with mouse 300–19 pre-B cells. The Ab were purified from concentrated hybridoma supernatants using protein G columns (GE Healhtcare, Piscataway, NJ, USA), dialyzed extensively against PBS, further purified by Zeba Spin desalting columns (*Thermo Scientific*) and stored at –80°C. The IgG1 isotype control antibody was likewise produced and purified. The resulting antibody preparations were tested on $CD69^{-/-}$ bone marrow-derived DC (BMDC) cultures at 10 µg/ml and were unable to upregulate CD80 or CD86 expression levels on these cells. *In vitro* cultures were performed in complete DMEM supplemented with 10% FCS, 50 µM 2-mercaptoethanol, and 2 mM L-glutamine at 37°C.

In AMD3100 assays, mice were mobilized with AMD3100 (CXCR4 Antagonist I - CAS 155148-31-5 – Calbiochem) dissolved in distilled water with 5mg/kg intraperitoneally and one hour after in experiment comparing with anti-human CD69 or 24 hours after treatment in BrdU proliferation assay comparing with anti-human CD69.

In experiments of internalization of S1P1, the inhibitor of S1P receptors, FTY720 (Fingolimod hydrochloride purchased from Sigma-Aldrich) has been used at dose of 1mg/kg for mouse intraperitoneally 24 hours before sacrificing dissolved in PBS1x.

In BrdU assays, all mice were injected intraperitoneally with 1 mg of BrdU intraperitoneally. Three hours after, mice were sacrificed, and splenocytes and bone marrow cells were collected and stained with fluorescent antibodies for cell surface markers.

NK cells were ablated by a single intravenous (i.v.) injection of 100 µg of anti-Asialo GM1 (eBiociences, San Diego, CA) or 50 µg of anti-Asialo GM1 (Wako, Chemicals USA, Richmond, VA) in 200 µl one day before infection. Control mice received the same_dose of rabbit IgG (Sigma-Aldrich) by the same schedule. Two days after infection mice were sacrificed and analyzed. Completeness of NK depletion was determined by the absence of $NKp46^{+}$ cells in the spleen and blood.

For neutralization of IFN-γ, mice were administrated with a single injection i.p. of 250 µg of anti- IFN-γ (clone xmg 1.2) in 400 µl PBS 1x. The same day, mice were infected i.p. with $1x10^6$ pfu or $1x10^7$ pfu of VV in $Rag2^{-/-}$ and $Rag2^{+/+}$ mice respectively and sacrificed 48 hours after infection for analysis.
Peritoneal macrophages were elicited by intraperitoneal injection of 2.5 mL of 3% thyoglycolate in distilled water. Cells were seeded at 1 x $10^6/cm^2$ in RPMI medium containing 10% FBS. Non-adherent cells were removed 6 h after seeding by extensive washing with medium. Macrophages were stimulated with LPS (Ultrapure 0111:B4 lipopolysaccharide from *Escherichia coli* purchased from InvivoGen) or without stimulation. 6 hours after stimulations, cells were collected for real time PCR and 24 hours later for western blot.

Cell Isolation

Cells were collected from Bone marrow (two femurs of each mouse), Blood collected (in 2 mM EDTA PBS to avoid coagulation), Spleen, Thymus and Lymph nodes (inguinal, maxillary, mandibular, brachial and axillary). Cells from tissues were released by mechanical disaggregation, washed twice with 1x PBS and pelleted. Bone marrow cells were extracted by flushed with 1 ml of PBS 1x from femurs of both legs. White blood cells (WBC) counts were determined after red blood lysis with ACK solution (0.15 M NH4Cl, 1mM KHCO3, 1mM Na2-EDTA, pH 7.4). Blood cells number shown are white blood cell counts per 1 ml of blood. Thymus and spleen tissues were disaggregated, and cells were washed in PBS. Leukocytes were labeled and analyzed by flow cytometry.

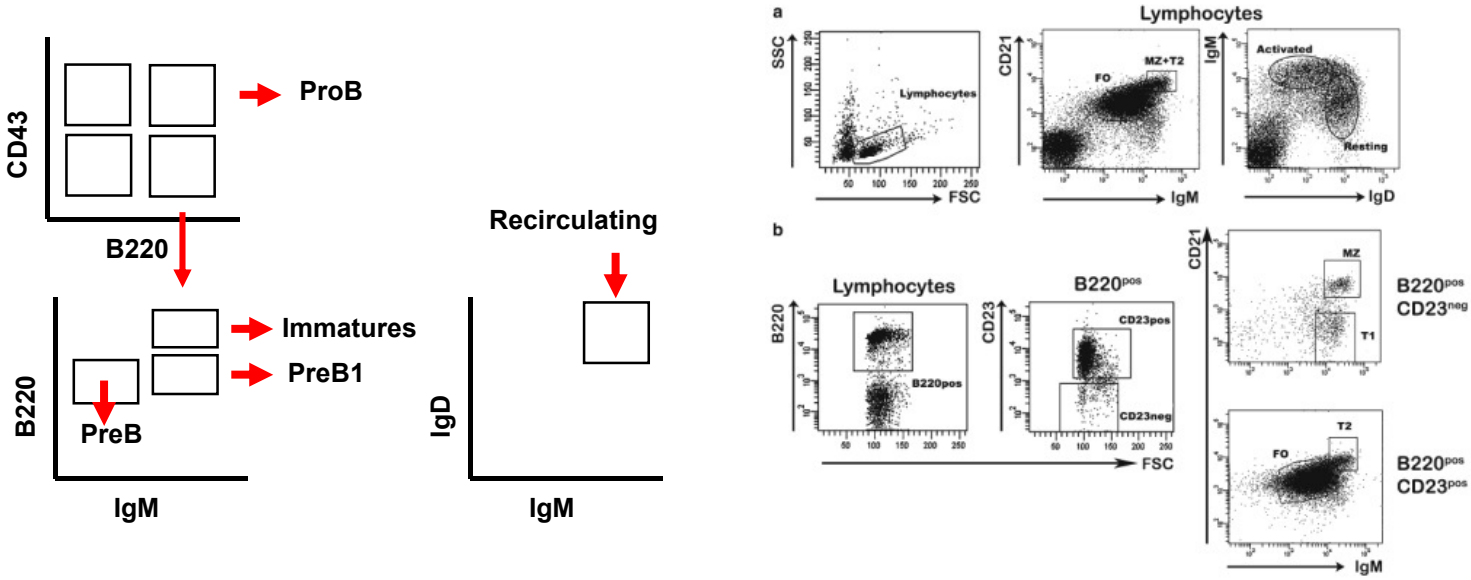
Abs and flow cytometry

Bone Marrow, Blood, Spleen, Thymus and Lymph nodes cells were incubated with anti-CD16/32 (Fc-block 2.4G2; BD Biosciences, Franklin Lakes, NJ, USA). The following antibodies used against mouse intracellular and surface antigens were purchased from eBioscience (San Diego, CA): anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7 or clone Ly-2), anti-CD11b (clone M1/70), anti-CD11c (clone N418 or clone HL3), anti-CD19 (clone eBio1D3), CD49b (clone DX5), anti-CD25 (clone 3C7), anti-CD69 (clone H1.2F3), anti-human CD69 (clone FN50), anti-CD107a (clone eBio4A3), anti-CD117 (clone 2B8), anti-CD122 (clone TM-b1), anti-F480 (clone BM8), anti-GR1 (clone RB6-8C5), anti- CD43 (clone R2/60), anti-IgM (clone RMM1), anti-IgD (clone 1126c), anti-CD21 (clone R4E3), anti-CD23 (clone B3B4), anti-B220 (clone RA3-6B2), anti-CXCR4 (clone 2B11), anti-CD3 (clone 17A2), anti-CD34 (clone RAM34), anti-FLT3

(clone A2F10), anti-Sca (clone D7), anti- 117 (clone 2B8) and anti-mouse VLA-4 (clone R1-2), anti-IFN (clone XMG 1.2), anti-NKp46 (clone 29A1.4), and anti-TNF (clone MP6-XT22). Expression of $S1P_1$ on BM, blood and spleen cells was detected using polyclonal rabbit anti–mouse $S1P_1$ Ab (Anti-EDG1 antibody (ab11424) purchased from Abcam) followed by donkey anti–rabbit-PE Ab (Jackson ImmunoResearch Laboratories). To assess intracellular production of IFN-γ and TNF-α, $2x10^6$ spleen cells were incubated in the presence of brefeldin A (BFA) (5 µg/ml) for 4 h at 37ºC and washed. Alternatively, cells were restimulated with 10 ng/ml PMA (*Phorbol* 12-myristate 13-acetate) and 1 µg/ml ionomycin or RPMI only in the presence of BFA (5 µg/ml) for 4 hours at 37ºC. Following incubation, cells were stained for surface molecules, fixed with 4% paraformaldehyde, washed, and incubated with anti-IFNγ mAb, anti-TNF and Granzyme B mAb in the presence of 0,75% saponin in PBS1x and 3% of FBS for 20 min at 4°C. The values shown are derived from subtracting the values of unstimulated wells (media alone) from the restimulated ones. To assess intracellular production of 4E-BP1 (clone V3NTY24) and IL-2 (clone JES6-5H4), cells were fixed with paraformaldehyde 4% (Electron Microscopy Sciences) for 12 minutes at room temperature in darkness and permeabilized with 1% of Saponin (Sigma-Aldrich) for 20 min at 4°C. For intranuclear staining with anti-mouse T-bet (clone eBio4B10) and FOXP3 (clone NRRF30), cells were fixed and permeabilized with the FoxP3/ Transcription Factor Buffer Set (BD). BrdU staining was performed using a BrdU Flow kit (Beckton Dikinson) and anti-mouse BrdU (clone B44) according to the manufacturer's instructions. Cells were analyzed with a FACScanto (Becton Dickinson, Franklin Lakes, NJ, USA), using BD FacsDiva software (Becton Dickinson), and data were analyzed with FlowJo (Tree Star Inc., Ashland, OR, USA).

B cells development.

Bone Marrow cells and spleen cells were analyzed to evaluate the different stages of development in B cells. Bone Marrow cells were gated according to B220, CD43, IgM and IgD staining as it's shown in the following image (left) and by the other hand, spleen cells were classified as described by M. Manuela Rosado et al. (right) according to B220, IgM, CD21 and CD23 staining [259].



Vaccinia virus and titers assays

The Western Reserve strain of VACV (kindly provided by Dr. Daniel Lopez) was grown in CV1 cells cultured in D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 5 µM β-mercaptoethanol. The titer was determined by plaque assay on CV1 cells, and the viral stock was stored at −80°C in PBS until use. 1×10^6 pfu were injected into $Rag2^{-/-}$ mice, and 1×10^7 were injected into immunocompetent mice intraperitoneally in 0.2 ml PBS. For survival studies, Balb/c mice were inoculated with 1×10^5 pfu intranasally and were weighed over time. CD69 BAC mice were injected with 1×10^7 pfu and the route of administration was different depending on the organ analyzed. Analysis of the viral titre in ovaries was performed by intraperitoneal infection while viral titration in spleen, lung, kidney, lymph nodes and liver was assayed at 24 hours after infection by intravenous injection. Experiment of survival was realized through weight measures over time and mice were infected with 1×10^5 pfu of VACV-WR by intranasal inoculation.

Viral load was measured by plaque-forming assay. In brief, female mice were sacrificed at the indicated times and ovaries and spleen were harvested and stored at −80°C in 0.5 ml of PBS until use. Ovaries and spleen from individual mice were first homogenized and freeze-thawed three times. Serial dilutions were plated on confluent CV1 cells. After one day of culture at 37ºC, plates were stained with crystal violet and the plaques were counted.

Gene expression analysis

Total RNA was isolated from cells with RNeasy Mini Kit (Qiagen) according to the manufacturer´s protocol. Quantitative RT-PCR was performed using the ABI 7500 (Applied Biosystems) with SYBR Green PCR Master Mix. All samples were analyzed comparing with the expression of the housekeeping gene B2m (Beta 2-microglobulin), used as an endogenous control for normalization of the expression level of target genes. Primer used for quantitative PCR sequences were as follows:

ACTB_F: AGCCATGTACGTAGCCATCC

ACTB_R: CTCTCAGCTGTGGTGGTGAA

S1P1_F: GTGTCCACTAGCATCCCGGAGGTTAAAGCTCTCCGCAGCTCA

S1P1_R: CCCAACAGGGGTAGCAGGAAGACCCC

TGFb_F: GGTGTVAGAGCCTCACCGCG

TGFb_R: AGAGCGGGAACCCYCGGCAA

For peritoneal assays, each sample was run in duplicate, and all samples were analyzed in parallel for the expression of the housekeeping gene 36B4 (acidic ribosomal phosphoprotein P0), which was used as an endogenous control for normalization of the expression level of target genes. Fold induction was determined from mean replicate values. Primer sequences are available on request.

NOS2_F: TGGAGCCAAGGCCAAACACAG

NOS2_R: TCCACCAGGAGATGTTGAAC

COX-2_F: CAAGGGAGTCTGGAACATTG

COX-2_R: ACCCAGGTCCTCGCTTATGA

TNFα_F: GCCTCTTCTCATTCCTGCTTG

TNFα_R: CTGATGAGAGGGAGGCCATT

IFNγ_F: TCAAGTGGCATAGATGTGGAAGAA

IFNγ_R: TGGCTCTGCAGGATTTTCATG

Expression of inflammatory genes was evaluated with the mouse RT^2*Profiler* PCR Inflammatory Cytokines and Receptors Array (SABiosciences) and expression of apoptosis genes was evaluated with the mouse RT^2*Profiler* PCR apoptosis (SABiosciences). RNA was obtained from bone marrow and spleen in mice treated vs. not treated through RNeasy Mini Kit and for cDNA synthesis First Standard kit (SABiosciences) was used. The RT^2*Profiler* array was probed according to the manufacturer's protocol,

using the Profiler PCR Array System and SYBR Green/Fluorescein qPCR master mix (SABiosciences) in an ABI 7500 Fast sequence analyzer (Applied Biosystems). Gene expression was measured with (http://www.superarray.com/pcr/arrayanalysis.php) web-based software package for the PCR Array System, which automatically performs all ΔΔCt based fold-change calculations from the specific uploaded raw threshold cycle data.

mRNA flow cytometry

Cells from bone marrow, spleen and blood were isolated and blood cells were lysed. CXCL12 in blood was analyzed by flow cytometry using FlowRNA II Assay kit (Affymetrix eBioscience) according to manufacturer's protocols. Samples were fixed for 30 min at 2-8 °C with PrimeFlow RNA Fixation Buffer I (Affymetrix), permeabilized with PrimeFlow RNA Permeabilization Buffer with RNAse inhibitors (Affymetrix), fixed again with PrimeFlow RNA Fixation Buffer II for 60 min at room temperature. For target probe hybridization for CXCL12, samples were incubated for 2 h at 40 °C. Next, samples were treated to signal amplification with PrimeFlow RNA PreAmp mix 1.5 hours at 40ºC, PrimeFlow RNA Amp mix 1.5 hours at 40ºC and last, samples were incubated with 1 hour at 40ºC with PrimeFlow RNA Label Probes. Finally, they were resuspended in Storage Buffer and acquired on a FACS Canto II (BD Biosciences).

Bone Marrow Chimeras

C57BL/6 recipient mice (CD45.1) were lethally irradiated with a dose of 1050 rads. $CD69^{+/+}$ (CD45.1/.2) and $CD69^{-/-}$ (CD45.2) donor mice were sacrificed, and bone marrow from femurs, tibiae and humeri was collected. Cells were passed through a 70-mm nitex mesh. A mixture of $5x10^6$ cells of each donor bone marrow was injected intravenously through the tail vein into irradiated recipient mice. After 6-8 weeks, BM (tibiae and femurs) and spleen cells were harvested and NK cell numbers ($NKp46^+$) and macrophages ($F4/80^+$) were analyzed.

Cell Death Assay

Splenocytes from non-infected mice were cultured in 24-well plates ($1x10^6$ cells/ml), and cell death was assayed at different times after culture by staining with propidium iodide (PI) followed by flow cytometric analysis.

CXCL12 ELISA

Plates coated with CXCL12 were purchased to R&D systems. CXCL12 levels were determined in plasma and supernatants of bone marrow, spleen and lymph nodes. Plasma was extracted in EDTA 2M-PBS. Supernatants of bone marrow (femurs), spleen and lymph nodes were collected in 500 ul of PBS1x. After centrifugation, the supernatant was kept and frozen. Samples were used undiluted and the protocol was realized according to manufacturer's protocols. Optical density was determined with a microplate reader set at 450 nm and wavelength correction at 570nm and the analysis reveal the CXCL12 expression calculated according to the amount of protein.

Western Blot

For Western Blot. cells were lysed at 4°C with 0.2 mL buffer A (0.5% Chaps, 10mM Tris pH 7.5, 1mM Cl_2Mg, 1mM EGTA, 10% Glycerol, 5 mM β) and protease inhibitor cocktail (Sigma). Protein content was assayed with the Bio-Rad protein reagent. All cell fractionation steps were performed at 4°C. Protein extracts were subjected to SDS-PAGE (10–15% gels) and blotted onto polyvinylidene difluoride membranes, which were incubated antibodies specific for NOS-2, COX-2 and β-actin. After incubation with HRP-conjugated secondary antibody, protein bands were revealed with an enhanced chemiluminescence kit (GE Healthcare). β-actin was used as a loading control. After treatment with 100 mM β-mercaptoethanol, 2% SDS in TBS and heating at 60°C for 30 min, blots were sequentially probed with other antibodies.

Immunohistochemistry

Immunohistochemistry was carried out on 10 µm-thick serial spleen sections of uninfected $Rag2^{-/-}$ $CD69^{-/-}$ and WT mice or mice at 2 days after infection with 10^5 pfu of VACV. Sections were first stained with anti-CD45 or anti-Ki67 and were subsequently stained with haematoxylin.

Statistical analysis

All data were plotted and statistically analyzed using Graphpad Prism software. Graphs show the means and standard errors of the mean (SEM). Statistical significance was performed using an unpaired two-tailed *t*-test. * $p<0.05$, ** $p<0.01$, *** $p<0.005$. A $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

CHAPTER I:
IMMUNE RESPONSE TO VACCINIA VIRUS INFECTION IN CD69 KNOCKOUT MICE.

RESULTS

Enhanced *in vivo* anti-VACV Activity in CD69$^{-/-}$ Mice mediated by NK cells.

During viral infection expression of CD69 is induced in all leukocytes. To evaluate the role of CD69 in the host response to Vaccinia virus CD69$^{+/+}$ and CD69$^{-/-}$ mice were intranasally infected with 10^5 pfu of VACV and their weight and appearance were monitored over time. CD69$^{-/-}$ mice showed reduced weight loss relative to their WT counterparts (Fig. 1A). Moreover, we observed that at 10 days post-infection all CD69$^{+/+}$mice developed lumbar pustules, whereas CD69$^{-/-}$ mice did not (Fig. 1B). CD69 WT and CD69$^{-/-}$ mice were also infected intraperitoneally and viral titers were analyzed in the ovary. At 7 dpi, CD69$^{-/-}$ mice had significantly lower titers than did WT mice (Fig. 1C). The difference was already significant 1 day after infection (Fig. 1C), which suggested that CD69 is involved in the early anti-viral immune response. Indeed, CD69$^{-/-}$ Rag2$^{-/-}$ mice also had lower viral titers than did CD69 WT Rag2$^{-/-}$ at 2 and 6 dpi (Fig. 1D), and this trend was already observable at 1 dpi (Fig. 1D). No significant differences were observed in viral load in spleen at 1 dpi when compared either CD69$^{+/+}$ with CD69$^{-/-}$ mice or Rag2$^{-/-}$ CD69$^{+/+}$ with Rag2$^{-/-}$ CD69$^{-/-}$ mice (Fig. 1E). Therefore, increased control of viral infection was observed in CD69$^{-/-}$ mice, also in the absence of adaptive immunity.

Previous studies showed that NK cells are crucial in VACV clearance *in vivo* in WT mice [260]. To investigate a possible contribution of NK cells to the enhanced anti-viral response observed in CD69$^{-/-}$ mice, CD69$^{-/-}$ and WT mice were treated with the NK cell-depleting anti-Asialo MAb one day before being infected with VACV, and virus titers were analyzed 1 dpi.

The anti-Asialo treatment eliminated the significance of the differences in viral load between CD69$^{-/-}$ and WT mice (Fig. 2A). In the Rag2$^{-/-}$ background the depletion of NK cells not only eliminated the advantage of CD69$^{-/-}$ mice but rendered them more susceptible to the infection than CD69$^{+/+}$ mice (Fig. 2B) pointing to a differential effect of CD69 deficiency on NK cells and other leukocytes present in Rag2$^{-/-}$ mice. Altogether these results show that the increased early control of viral infection observed in CD69$^{-/-}$ mice is majorly mediated by NK cells.

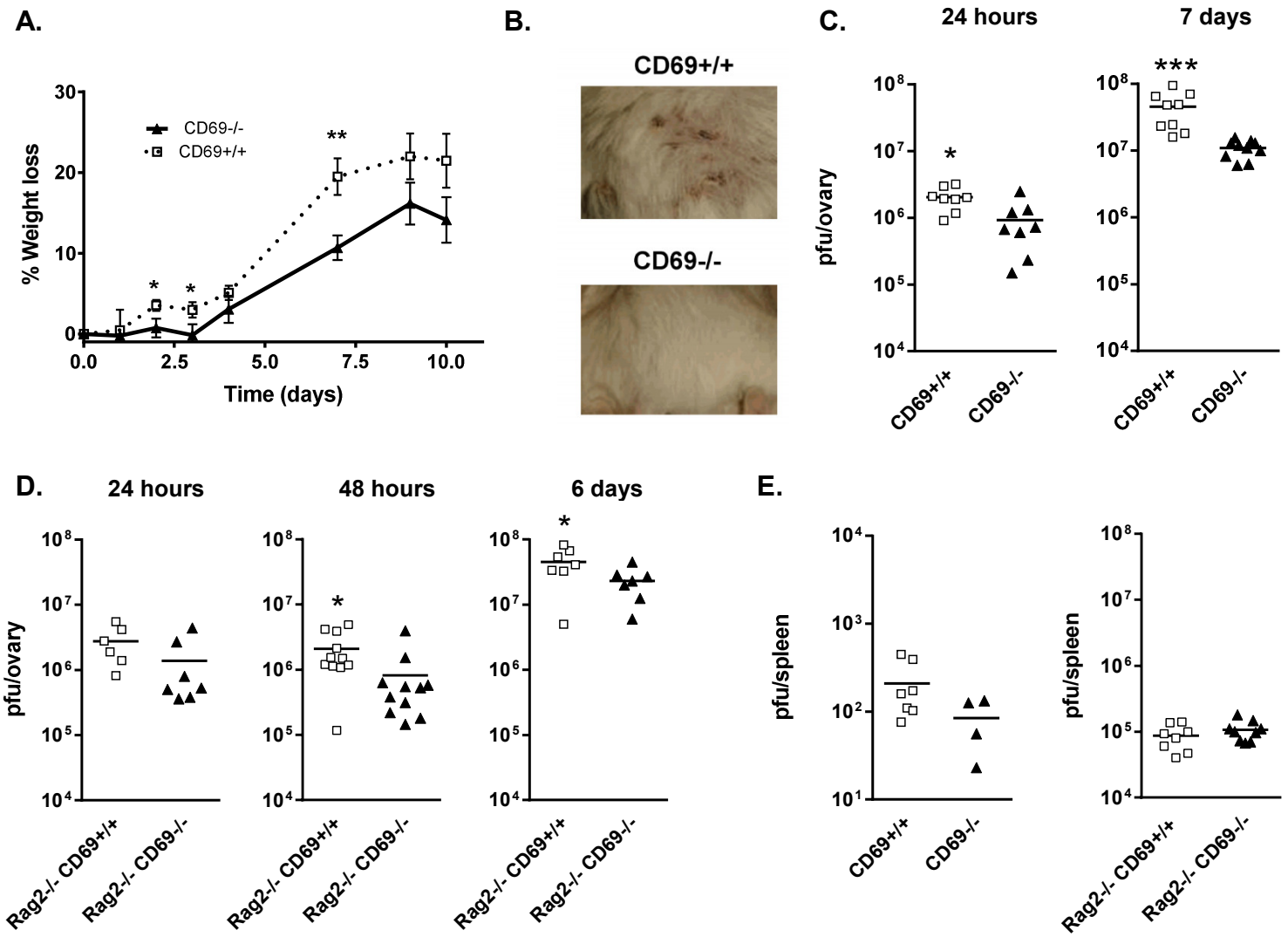


Figure 1. CD69$^{-/-}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice are more resistant to VACV infection than their CD69$^{+/+}$ counterparts. A, CD69$^{+/+}$ or CD69$^{-/-}$ immunocompetent mice were infected intranasally with 10^5 pfu of VACV-WR. Weight loss was evaluated over 10 days. B, At 10 days after intranasal infection, CD69 mice had developed pustular lesions in the lumbar region whereas CD69$^{-/-}$ mice were free of them. C, CD69$^{+/+}$ and CD69$^{-/-}$ mice were infected with 10^7 pfu of VACV-WR intraperitoneally. Ovaries were collected and analyzed for viral load at 24 hours and 7 days after infection. D, Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$CD69$^{-/-}$ mice were infected with 10^5 pfu of VACV WR and ovaries were collected at 24 hours, 2 days and 6 days after infection. E, CD69$^{+/+}$ and CD69$^{-/-}$ mice (left) and Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$CD69$^{-/-}$ mice (right) were infected with 10^6 pfu of VACV-WR intraperitoneally and splenic viral titers were measured one day post infection. Data shown are representative of one experiment (A and B) or a pool of two independent experiments (C-E).

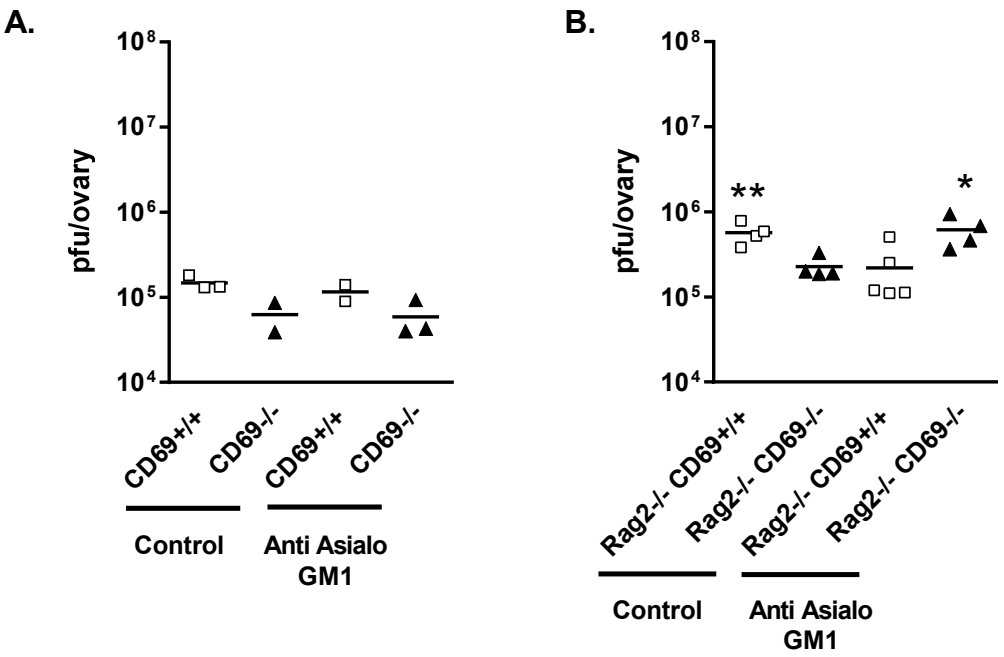


Figure 2. NK cells mediate the increased resistance to VACV-WR in CD69$^{-/-}$ mice and Rag2$^{-/-}$ Cd69$^{-/-}$ mice. CD69$^{+/+}$ and CD69$^{-/-}$ mice (A) and Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$CD69$^{-/-}$ mice (B) were treated with 50 µg of NK cell-depleting anti-Asialo GM1 or of rabbit serum control IgG i.v. one day before infection. Mice were then infected with 1×10^6 pfu of VACV-WR i.p. Ovaries were harvested and assayed for viral load one day post-infection. A, Data shown are pool from two independent experiments and representative of four independent experiments. B, Data shown are from one experiment and representative of three independent experiments.

Similar NK cell reactivity but increased NK cell numbers in VACV infected mice CD69$^{-/-}$ mice

We proceeded to analyze NK cell function in CD69$^{-/-}$ mice. For this, we analyzed cytokine and GrzB production and degranulation of NK cells (NKp46$^+$ cells) from infected mice. At early times of infection and in uninfected mice, percentages of IFNγ$^+$ and TNFα$^+$ cells within NK cells were equivalent in CD69$^{-/-}$ and WT splenocytes after PMA restimulation (Fig 3A) as well as in Rag2$^{-/-}$ CD69$^{-/-}$ and Rag2$^{-/-}$CD69$^{+/+}$ splenocytes after PMA or RMA-S restimulation (Fig 3B and data not shown), pointing to that CD69 does not affect the cytokine production capacity nor the activation threshold of NK cells. Thus, the NK-cell mediated increased viral resistance observed in the CD69$^{-/-}$ mice does not appear to be due to increased NK cell reactivity. By contrast, differences were observed in the numbers of reactive, cytokine-producing NK cells. Spleens of infected CD69$^{-/-}$ mice had significantly augmented numbers of

IFN and TNF producing NK cells (Fig. 3C), and a tendency to increased NK cell numbers in general (Fig.3D). Percentages of IFNγ^{+} and TNFα^{+} cells within CD4^{+} and CD8^{+} T cells were also unaffected by CD69 deficiency (Fig. 4A), but increased numbers of IFNγ^{+} and TNFα^{+} CD4^{+} and CD8^{+} T were present in the spleens of CD69$^{-/-}$ mice 24 hours post-infection (fig. 4B). Thus, similarly to NK cells, the reactivity of non-cognate, innately-responding T cells is unaltered in CD69$^{-/-}$ mice, but they are increased in number.

The increased NK cell numbers were due not to an augmented proportion of NK cells in the spleens of CD69$^{-/-}$ mice but to increased total cell counts (Fig. 3D and data not shown). At 24 hours post-infection, CD69-deficient mice had higher numbers of total spleen, blood and peritoneum cells (Fig. 3D, 4C). All the splenic and peritoneal main leukocyte subtypes, (CD4^{+} and CD8^{+} T cells, B cells (CD19^{+}), dendritic cells (DC) (CD11c ^{hi}) macrophages (CD11b ^{hi}, F480+, CD11c-), monocytes (CD11b ^{hi}, F480-, CD11c-, Gr1 ^{int}, SSC ^{low}), neutrophils (CD11b ^{hi}, F480-, CD11c-, Gr1 ^{hi}, SSC ^{int}) and eosinophils (CD11b ^{hi}, F480-, CD11c-, Gr1 ^{low}, SSC ^{hi}), were increased in number (Fig. 4D, E), but no major differences were found in their percentages (data not shown). At 7 days after infection, CD69$^{-/-}$ mice had approximately 40% more leucocyte splenic cells than did WT mice (Fig. 3D), and numbers of all the main lymphocyte subsets were also increased to the same extent (Fig. 4F).

Likewise, at 2 and 6 dpi, Rag2$^{-/-}$ CD69$^{-/-}$ mice also had increased spleen leukocyte numbers relative to Rag2$^{-/-}$ CD69$^{+/+}$ mice (Fig. 5A), which was translated to an increased splenic leukocyte density, as seen by immunohystochemical staining with anti-CD45 MAb (Fig. 5B). Numbers of NK cells were also significantly higher in CD69$^{-/-}$ Rag2$^{-/-}$ mice (Fig. 5A) but their percentage was unaltered (data not shown). Upon *ex vivo* restimulation the number of IFNγ^{+}, CD107^{+} and GrzB^{+} NK cells was also higher in CD69$^{-/-}$ Rag2$^{-/-}$ spleen cells (Fig. 5C, D). We also detected a tendency to increase the numbers of NK cells producing TNFα, even though this was not statistically significant (Fig. 5C). The numbers of other main leukocyte subtypes found in Rag2$^{-/-}$ were also increased in the spleens of CD69$^{-/-}$ Rag2$^{-/-}$ mice (Fig. 5E). These data reveal a positive correlation between viral infection control and effector NK cell numbers in CD69$^{-/-}$ mice.

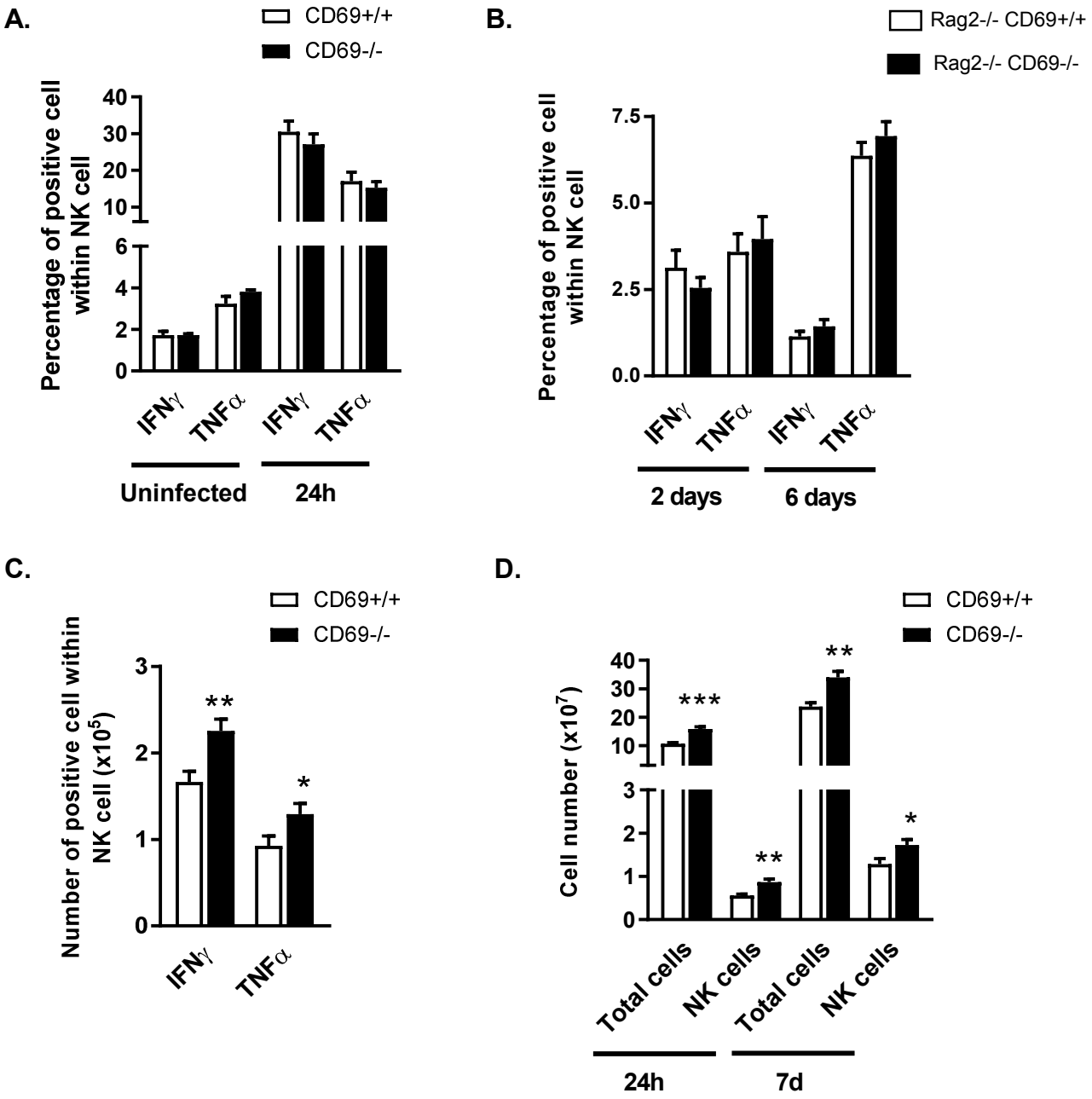


Figure 3. Unaltered NK cell activation in CD69$^{-/-}$ and Rag2$^{-/-}$CD69$^{-/-}$ mice infected with VACV. A, CD69$^{-/-}$ and WT mice were infected with 1 × 10^7 pfu i.p of VACV-WR or left uninfected and were sacrificed 24 hours after infection. Spleen cells were cultured with PMA/ionomycin and BFA for 4 hours and stained for cell surface markers and intracellular cytokines. Percentages of NK cells producing IFNγ or TNFα are shown. B, Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$ were infected with 1 × 10^5 pfu i.p of VACV-WR. Spleen cells were collected at 2 and 6 days after infection and re-stimulated with RMA-S cells over 4 hours; intracellular IFN-γ and TNFα were measured in NK cells and percentages of cytokine-producing cells are plotted. C, Number of IFNγ and TNFα-producing NK cells in CD69$^{-/-}$ and CD69$^{+/+}$ mice 24 hours after infection were measured as in A. D, Total cell and NK cell number in spleen of CD69$^{+/+}$ and CD69$^{-/-}$ mice 24 hours and seven days after infection. A-D, Data are a pool of two independent experiments.

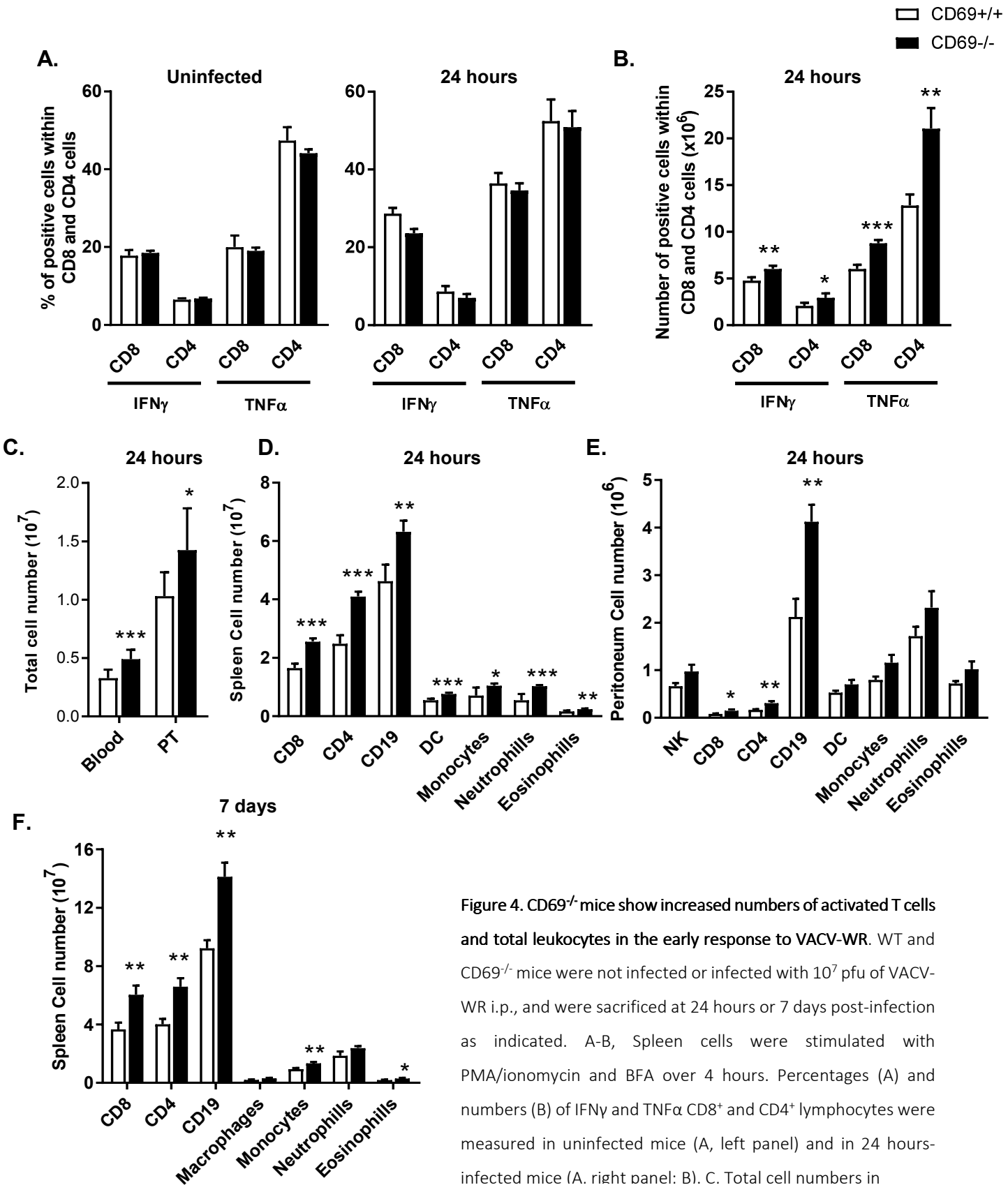


Figure 4. CD69$^{-/-}$ mice show increased numbers of activated T cells and total leukocytes in the early response to VACV-WR. WT and CD69$^{-/-}$ mice were not infected or infected with 10^7 pfu of VACV-WR i.p., and were sacrificed at 24 hours or 7 days post-infection as indicated. A-B, Spleen cells were stimulated with PMA/ionomycin and BFA over 4 hours. Percentages (A) and numbers (B) of IFNγ and TNFα CD8$^+$ and CD4$^+$ lymphocytes were measured in uninfected mice (A, left panel) and in 24 hours-infected mice (A, right panel; B). C. Total cell numbers in peritoneum and blood 24 hours after infection. D-E, Numbers of the indicated splenic (D, F) and peritoneal (E) lymphoid and myeloid subpopulations at 24 hours (D, E) and 7 days (F) after infection. A-F, Pool of two independent experiments.

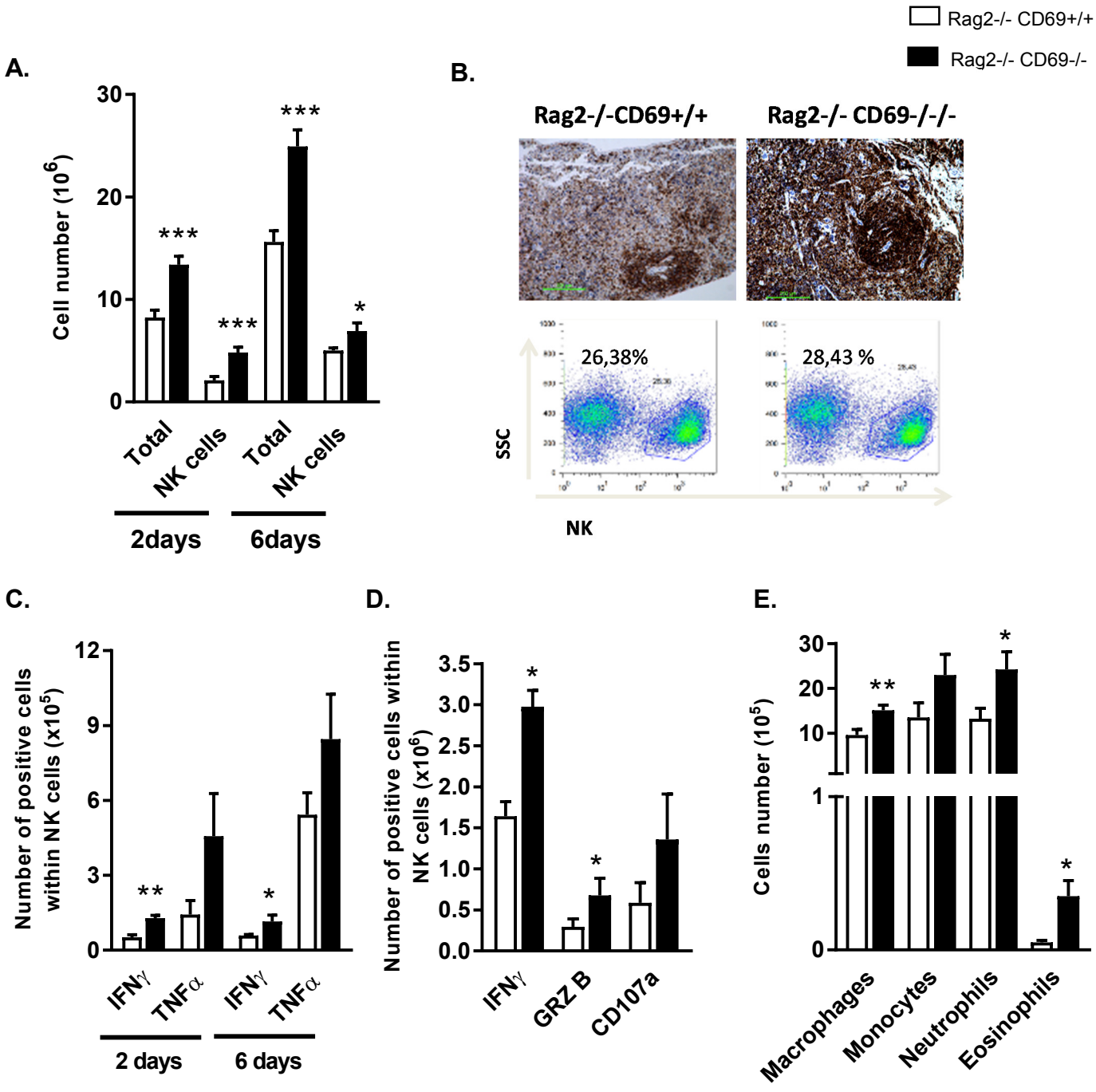


Figure 5. VACV-infected Rag2$^{-/-}$ CD69$^{-/-}$ mice show increased accumulation of splenocytes and reactive NK cell numbers. A-D. Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice were infected with 1 × 10^5 pfu of VACV i.p. and were sacrificed at days as indicated postinfection. A, Graph representing the splenic total cell and NK cell (DX5$^+$) numbers at 2 and 6 days postinfection. B, (Top) Immunohistochemical analysis of spleen by staining with anti-CD45 mAb and (bottom) representative plots of NK cell stainings (DX5$^+$) 1 day after infection. C, Spleen cells were collected at 2 and 6 days after infection and restimulated with RMA-S cells over 4 hours; numbers of IFN-γ and TNFα-positive NK cells are shown. D, Spleen cells were collected at 24 h post-infection and cultured with PMA/ionomycin and BFA over 4 hours. Numbers of IFN-γ, GrzB and CD107a-positive NK cells are shown. E, Graph representing the numbers of different splenic myeloid subpopulations. A, C, D and E, Pool of two independent experiments. B, One staining representative of seven mice.

Similar NK cell proliferation rate in CD69$^{-/-}$ Rag2$^{-/-}$ and CD69 WT Rag2$^{-/-}$ mice

The observation that Rag2$^{-/-}$ CD69$^{-/-}$ mice presents an increased accumulation of NK cells in the spleen led us to assess whether CD69 is associated with control of NK proliferation in anti-viral immunity. The proliferation activity of splenic NK cells of uninfected and VACV infected Rag2$^{-/-}$ CD69$^{-/-}$ and Rag2$^{-/-}$ CD69$^{+/+}$ mice was determined based on their capacity to incorporate BrdU after 2 days of infection. Similar BrdU^{+} percentages were found in splenic NK cells (Fig. 6A), although the number of BrdU^{+} NK cells was increased in the spleens of Rag2$^{-/-}$ CD69$^{-/-}$ mice (Fig. 6B). Equivalent results were obtained when proliferation of splenic NK cells from infected mice was analyzed with an *ex vivo* pulse of BrdU (data not shown). Furthermore, histological spleen sections two days after infection were stained by immunohistochemistry for Ki67, a nuclear protein associated with ribosomal RNA transcription, and a slight increase in Ki67 staining in mouse CD69$^{-/-}$compared to WT was observed, consistent with increased numbers of cycling cells (Fig. 6C). NK cells were analyzed for the expression of CD122 (IL-2Rβ) and CD25 (IL-2Rα) in spleen (Fig. 6D) and in bone marrow (Fig. 6E), but no differences were found in the percentages of NK cells expressing these components of the IL-2 receptor. These results show that CD69$^{-/-}$ NK cells proliferate at a similar rate as CD69$^{+/+}$ NK cells but that increased numbers of proliferating NK cells are found in CD69$^{-/-}$ mice.

Increased NK cell numbers in uninfected CD69-/- mice

The differences in cellularity between CD69$^{-/-}$ and CD69 WT mice at such early time points of infection may be indicative that the cellularity is already altered before the infection. Therefore, we analyzed uninfected mice and we observed that total cell and NK cell numbers in spleen were already increased in both CD69$^{-/-}$ and CD69$^{-/-}$ Rag2$^{-/-}$ mice (Fig. 7A). Moreover, spleens of CD69 WT and CD69-/- mixed bone marrow chimeras at steady state had higher proportions of CD69$^{-/-}$ NK cells than CD69$^{+/+}$ NK cells within total donors-derived NK cells, pointing that increased NK cell numbers in CD69$^{-/-}$ mice are due to an NK cell intrinsic effect. An equivalent observation was not done for other cell types, since, for example, the proportions of CD69$^{/-}$ F4/80^{+} cells were even lower than those of CD69$^{+/+}$ F4/80^{+} within total donors-derived F4/80^{+} cells (Fig.7B). An NK cell-intrinsic effect of CD69 deficiency is substantiated by the fact that CD69 is expressed on all NK cells of all the organs analyzed, even in the earliest maturation stages (Fig. 7C, D).

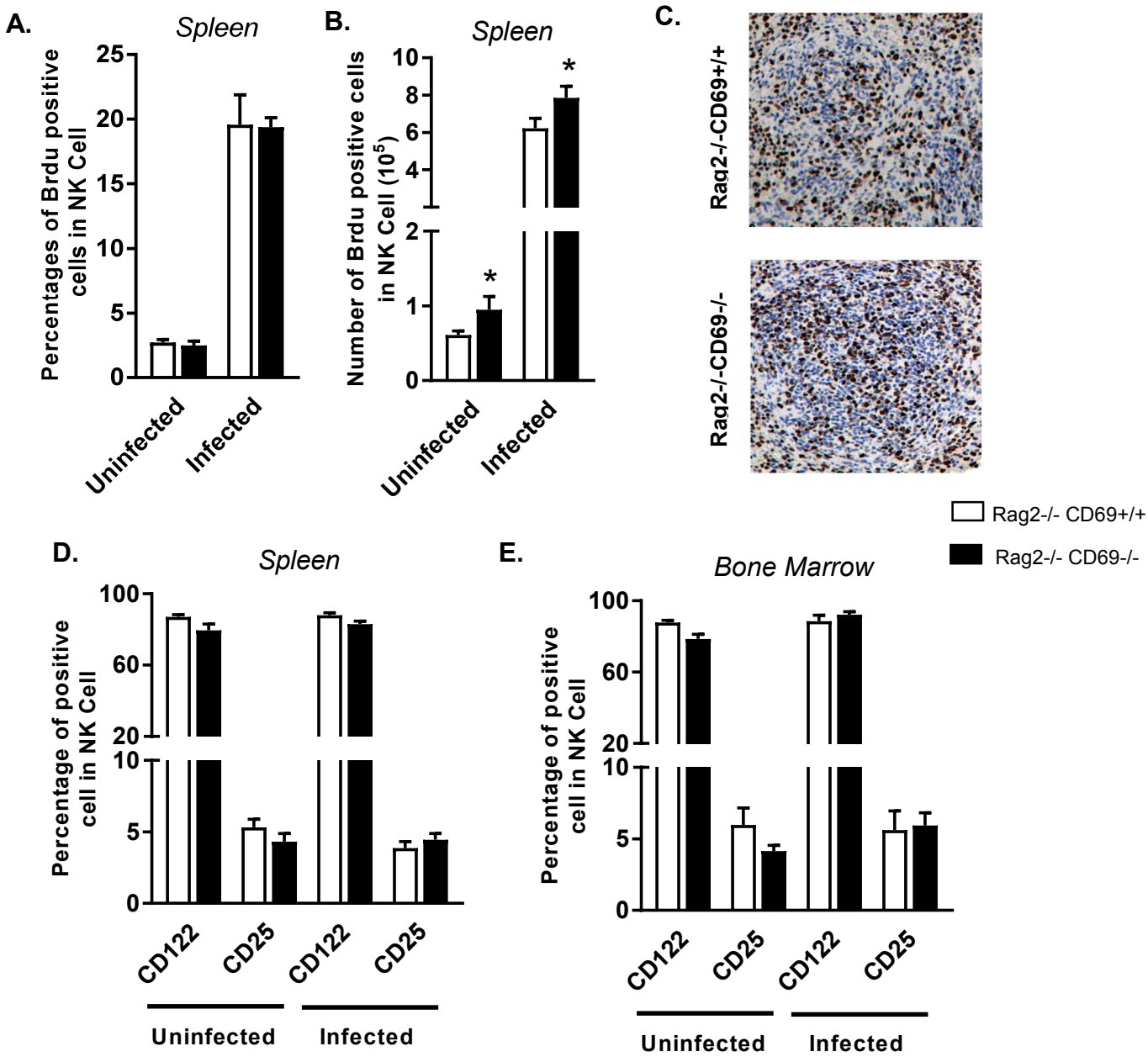


Figure 6. Unaltered NK cell proliferation rate but increased numbers of proliferating NK cells in VACV-infected Rag2$^{-/-}$ CD69$^{-/-}$ mice. A and B, Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$ mice were infected with 1x10^{6} pfu of VACV-WR i.p or not infected. Two days after infection, mice were injected i.p with 1mg of BrdU for 3 hours and then they were sacrificed. Spleen was collected and stained. Percentages (A) and numbers (B) of BrdU+ cells within NK cells (NKp46^{+}) in spleen. C, After 2 days of infection, histologic sections of spleen were stained for anti-mouse Ki67 by immunohistochemistry. D and E, Percentages of splenic (D) or bone marrow (E) NK cells positive for alpha and beta chain of IL-2 receptor were determined in infected and non-infected mice. A and B, Uninfected mice, pool of two independent experiments and infected, one experiment. C, One staining representative of seven mice. D and E, Pool of two independent experiments.

Steady state NK cell numbers can be affected by NK cell recirculation. S1P5 is a member of the sphingosine phosphate receptors family and T-bet-dependent S1P5 expression has been reported to control NK cell recirculation through mediating egress from lymphoid organs, finally affecting splenic NK cell counts [166]. We reasoned that CD69 could have an effect on NK recirculation through altered T-bet expression, and we characterized T-bet expression in bone marrow and spleen NK cells. However, we did not observe differences in the expression of T-bet in either site at 1 day after infection (Fig. 7E). Thus, CD69 deficiency does not alter NK cell number through altered T-bet expression.

Spontaneous cell death rate is reduced in CD69$^{-/-}$ NK lymphocytes

We also tested whether an increased immune cell survival was contributing to the observed greater number of NK cells in CD69$^{-/-}$ mice. Cell death is difficult to test *in vivo* because dead cells are rapidly eliminated. Thus, we cultured Rag2$^{-/-}$ CD69$^{-/-}$ and Rag2$^{-/-}$ CD69 WT splenocytes *in vitro* and analyzed the spontaneous cell death in NK cells by PI staining at different times of culture. No significant differences were found at early time points. However, we observed significantly reduced percentages of dead cells in Rag2$^{-/-}$ CD69$^{-/-}$ after 60 hours of culture (Fig.8A). These data suggests that the lymphocyte accumulation observed in the spleen is contributed by a lower spontaneous cell death rate in the CD69$^{-/-}$ mouse and that CD69 has a role in regulating NK cell survival.

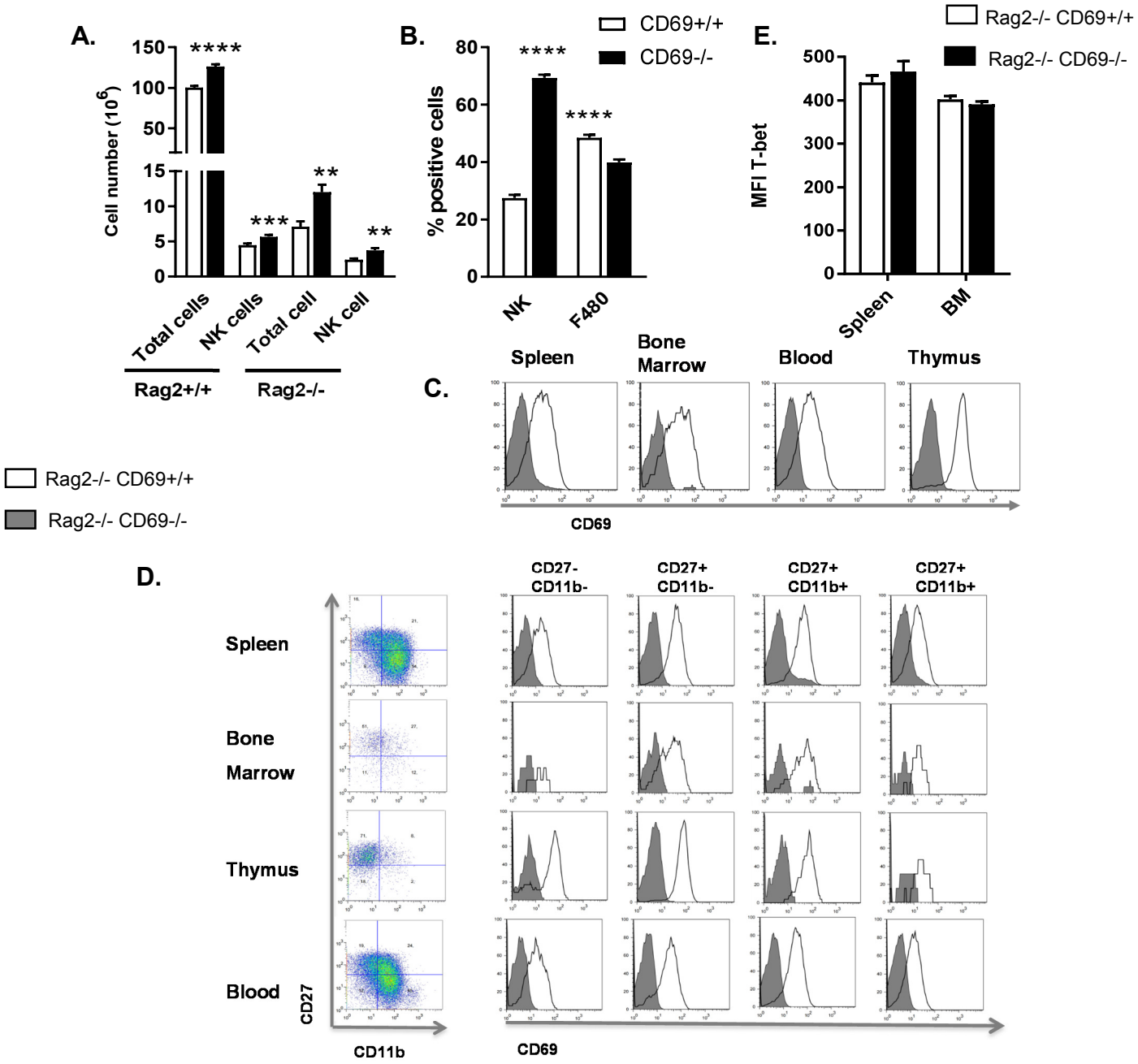


Figure 7. NK cell distribution and CD69 expression in NK cells. A, Total cell and NK cell numbers in spleens of unmanipulated $Rag2^{+/+}$ and $Rag2^{-/-}$ mice. B, C57BL/6 recipient mice (CD45.1) were reconstituted with a mixture of wild-type (CD45.1/.2) and $CD69^{-/-}$ (CD45.2) bone marrow cells. The values represent percentage of $CD69^{+/+}$ and $CD69^{-/-}$ NK cells or macrophages mice within total donors-derived NK cells or macrophages, respectively. C and D, Spleen, BM, thymus and blood cells from unmanipulated $Rag2^{-/-}$ $CD69^{+/+}$ and $CD69^{-/-}$ were collected and stained for NKp46, CD27, CD11b and CD69. Overlays of CD69 profiles gated on total NK cells (C) or NK cells in the different maturation stages (D) from $Rag2^{-/-}$ $CD69^{+/+}$ (solid line) and $Rag2^{-/-}$ $CD69^{-/-}$ (grey-filled, background control) mice. E, $Rag2^{+/+}$ and $Rag2^{-/-}$ Mice were infected with $1x10^6$ pfu of VACV-WR ip. One day after infection, spleen and bone marrow cells were analyzed for T-bet expression on NK cells ($NKp46^+$). The graphs show the MFI (Mean Fluorescence Intensity) of intranuclear T-bet staining in spleen and BM NK cells. A, B and E, Pool of two experiment. C and D, One experiment representative of two experiments.

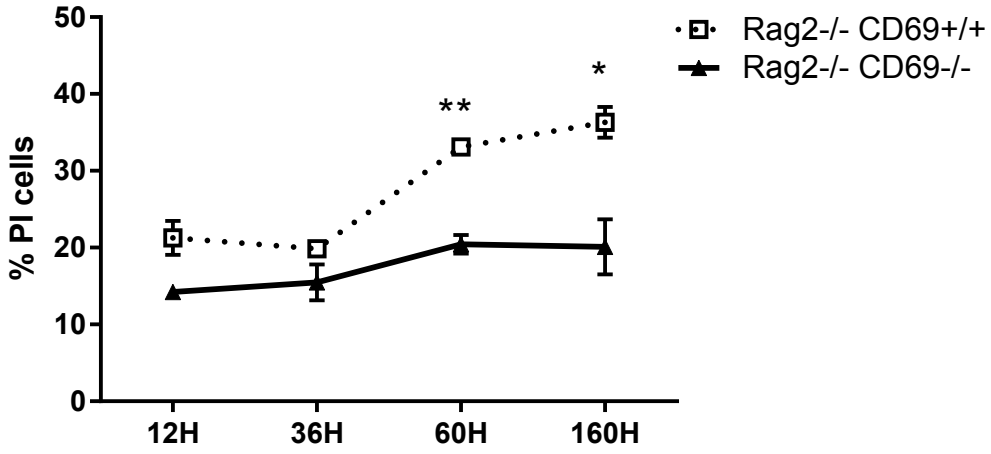


Figure 8. Attenuation of spontaneous cell death in Rag2$^{-/-}$CD69$^{-/-}$. Cell survival was assessed by PI staining of unfractionated splenic cells of uninfected Rag2$^{-/-}$CD69$^{-/-}$ and Rag2$^{-/-}$CD69$^{+/+}$ mice at different times of culture. One experiment representative of two experiments.

DISCUSSION

In this work, we show an early NK cell-mediated increased VACV infection control in $CD69^{-/-}$ mice consistent with increased NK cell numbers in peripheral sites in these mice. Two observations point to that the increased activated NK cell numbers in the $CD69^{-/-}$ spleens are not secondary to a differential infection evolution. On one hand, increased numbers of splenic NK cells were already present in uninfected mice, and this was translated to higher numbers of cytokine-producing cells upon *in vitro* restimulation. On the other hand, spleen viremia is not significantly different between $CD69^{+/+}$ and $CD69^{-/-}$ mice. However, it has been suggested that lymphoid organs are not sites of VACV replication but rather of viral particles drainage. Unlike spleen, ovaries are the organs that sustain the highest virus replication rate in systemic VACV infection [261]. The infection induces recruitment of NK cells into the ovary, among other leukocyte types [261].

The higher NK cell counts in $CD69^{-/-}$ mice do not seem to be due to increased NK cell proliferation capacity but rather by, at least in part, a lower rate of spontaneous cell death.

CD69 has a negative regulatory role in a number of autoimmune diseases and in anti-tumor immune response. In the first report of its role in the immune response to infection, we showed that CD69's-mediated immune-regulation was beneficial in the course of *Listeria monocytogenes* (Lm) bacterial infection, CD69 deficiency led to an impaired control of Lm infection, starting at early time points, and this was related to an enhanced production of type I and II IFNs and increased leukocyte apoptosis [141]. In contrast, we show here that negative immune modulation by CD69 is detrimental in the case of the viral infection with VACV in different mouse strains. This differential effect may be due to the different interaction of the pathogen with the augmented immune response given in the $CD69^{-/-}$ mouse. On one hand, Lm exploits the immune activation to induce massive lymphocyte apoptosis. Through the secretion of listeriolysin toxin, it induces apoptosis of lymphocytes sensitized by type I IFN. Upon phagocytosis of large amounts of apoptotic lymphocytes, the macrophages secrete IL-10, inducing immune-suppression and favoring bacterial outgrowth. In contrast to Lm, VACV virus employs large part of its genome to avoid immune recognition and activation [258]. Thus, the enhanced immune response of the $CD69^{-/-}$ may be detrimental in one case and beneficial in the other.

We have previously shown that $CD69^{-/-}$ mice had NK-cell mediated augmented anti-tumor immune response [1]. Similarly to the present results, NK cell reactivity was also unaltered in the absence of CD69, but the enhanced anti-tumor response was mediated by increased NK cell accumulation. Higher

numbers not only of NK cells but also of total leukocytes were reported already at steady state. Thus, it is likely that CD69 is also involved in the homeostatic regulation of NK cells as well as of other leukocyte subsets. The fact that the early effect of CD69 deficiency on viral control depends on NK cells is likely due to that these cells are of chief importance in the early control of this type of infection [260]. An effect of CD69 deficiency on the role of other cell types is not excluded, especially at later time points of infection.

Previous results of our group showed that CD69 deficiency did not affect the percentage of VACV-specific CD8+ T cells at 7 days post-infection, implicating that CD69 affects neither the priming nor the expansion rate of virus-specific cells [104]. However, total cell numbers were not recorded then, and, considering that steady state T cell numbers are increased in the $CD69^{-/-}$ mouse, it is expectable that this will be translated to increased VACV-specific $CD8^{+}$ T cell numbers during the primary adaptive immune response, which will likely contribute to the lower viral titers observed at day 7.

We have not observed any differences in the percentages of IFN and TNF producing NK cells upon *ex vivo* restimulation with PMA or the MHC-I deficient cell line RMA-S. The strong activation with PMA overrides NK cell activation thresholds set by the process of NK cell tuning [262] and thus the results with PMA may just reflect an equivalent capacity of CD69 WT and CD69-/- NK cells to produce cytokines. Instead, stimulation with MHC-I deficient targets does not override those thresholds [262]. Therefore, the equivalent cytokine production upon RMA-S restimulation reflects that, even if CD69 is expressed constitutively on NK cells at different maturation stages as well as in infection, it does not participate in NK cell tuning [263].

Altogether, this work points to a role for CD69 in the resolution of viral infection through a role in homeostatic control of NK cells.

CHAPTER II:

INFLUENCE OF HUMAN CD69 TARGETING IN MOBILIZATION OF PROGENITOR CELLS.

RESULTS

CD69$^{-/-}$ mice show decreased bone marrow cellularity and increased leukocyte counts in the periphery.

In the previous work, we have shown that CD69$^{-/-}$ mice (in Rag2$^{-/-}$ background) infected with Vaccinia Virus have increased splenic cell numbers of all the main leukocyte subtypes. Total splenic cell counts were actually also found increased in these mice at steady state [1] and this was related with a decreased cell death rate upon *ex vivo* culture. Nevertheless, given the role of CD69 in regulating the S1P1-mediated egress of T and B cells from lymph nodes and of T cells from the thymus, and the fact that S1P1 contributes to some extent to leukocyte egress from the BM [132], we wondered whether an increased hematopoietic cell output from the bone marrow could contribute to the greater cellularity observed in the absence of CD69. For this, we quantified total leukocyte and NK cell numbers in bone marrow, spleen, blood and thymus of CD69$^{-/-}$ mice and CD69$^{-/-}$ Rag2$^{-/-}$ mice. Total cell counts were not altered or decreased in the bone marrow of CD69$^{-/-}$ mice and CD69$^{-/-}$ Rag2$^{-/-}$ mice, respectively, but were increased in secondary lymphoid organs and blood of CD69$^{-/-}$ mice compared to CD69$^{+/+}$ counterparts in both backgrounds (Figure 9A-B). This was consistent with increased spleen weight in CD69$^{-/-}$ and CD69$^{-/-}$ Rag2$^{-/-}$ mice (data not shown). NK cells were decreased in the BM, and consistently with our recent publication, they tended to be or were significantly increased in the spleen of CD69$^{-/-}$ and CD69$^{-/-}$ Rag2$^{-/-}$ mice, respectively (Fig. 9C-D). This accumulation of leukocytes at peripheral sites and, in some strains, this relative depletion of BM leukocytes seen in CD69 knockout mice are consistent with a role of CD69 in leukocyte retention in the bone marrow.

Targeting of mouse CD69 induces bone marrow mobilization of hematopoietic cells from primary lymphoid organs to the periphery.

In view of the results explained above, we reasoned that compensatory mechanisms could be normalizing bone marrow (BM) cells numbers while still in the presence of an increased BM output, and that a role of CD69 in regulating cell egress from BM could be better appreciated when acutely

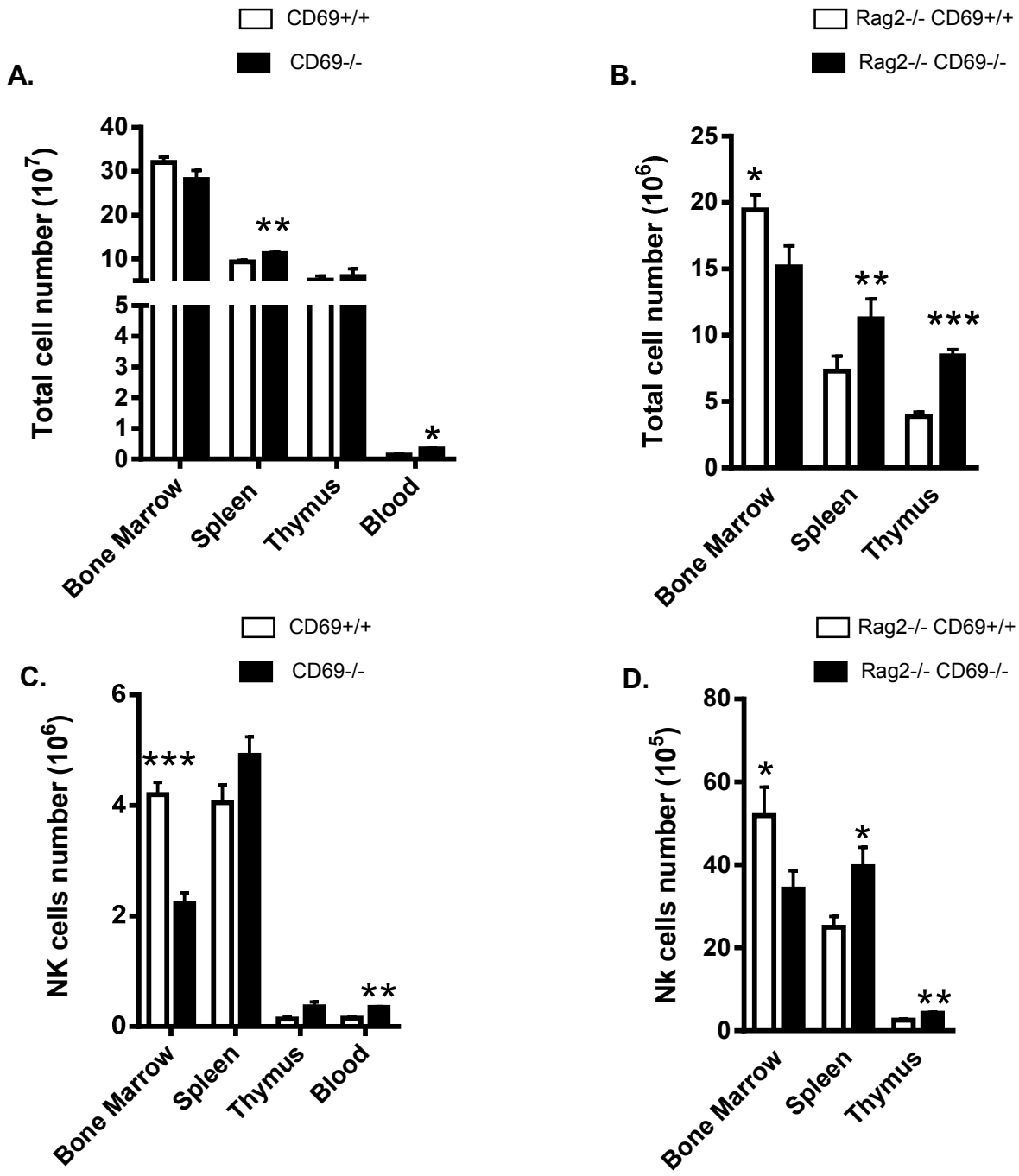


Figure 9. The absence of CD69 decreases the total cell number in bone marrow and increases in spleen and influences in NK cells number. A, Total cell number in Spleen, Bone Marrow, Thymus and Blood in Rag2+/+ mice. B, Total cell number in Spleen and Thymus and in Rag2-/- mice. C, NK cell number in Spleen, Bone Marrow, Thymus and Blood in Rag2+/+ mice and D, in Bone Marrow, Spleen and Thymus in and Rag2-/- mice.

affecting CD69 function with mAb targeting. Thus, we treated $CD69^{+/+}$ mice with anti-mouse CD69 MAb 2.2, and found that the treatment induces a 15% and 20% decrease of total leukocyte numbers in BM and thymus, respectively, and an increase of these numbers in the spleen and LN as soon as 24h after treatment, in comparison to PBS-injected controls (Fig. 10A). These findings are consistent with a decrease in thymus weight and an increase in spleen weight observed in anti-CD69 MAb-treated mice (data not shown). In control experiments, the treatment of $CD69^{-/-}$ mice with anti-mCD69 2.2 did not alter BM or spleen cell numbers, proving that the effect of the treatment was specific of mouse CD69 (data not shown). When analyzing the number of the main leukocyte subpopulations in the BM and the spleen we observed a sharp reduction in BM B cells and neutrophils, the most abundant cell types in this organ (Fig. 10B). In the spleen, the treatment induced a marked increase in B cell numbers, of about the same magnitude than the decrease observed in the BM (Fig. 10C). Splenic NK cell, CD8+ T cell, neutrophil and eosinophil numbers were also increased (Fig.10C). These results are consistent with anti-mCD69 2.2 treatment inducing mobilization of hematopoietic cells from primary lymphoid organs (bone marrow and thymus) and their accumulation in peripheral sites such as the spleen, being this effect evident in the most abundant leukocyte types in these organs (neutrophils in the BM and B cells in BM and spleen).

Anti- human CD69 MAb treatment of huCD69 transgenic mice induces mobilization cells from Bone Marrow.

With the aim of moving towards a more translational setting, we assessed the effect of anti-hCD69 2.8 treatment *in vivo*, with the use of the huCD69 transgenic mice in $CD69^{-/-}$ background. When kept in hemizygosity, the diploid cells of these mice contain 3 copies of a human CD69 BAC. After 24h, the anti-hCD69 2.8 treatment had induced similar decreases and increases in total leukocyte counts in BM and spleen, respectively, in the same direction than those induced by anti-mCD69 2.2 targeting, but of greater magnitude (Fig. 11A). Mobilization is usually assessed by the increase in blood cell counts shortly after treatment with mobilizing agents. This increase is ephemeral, and it has usually disappeared 24h post-treatment [264]. However, we were still able to find slightly increased white blood cell counts at 24h after anti-hCD69 treatment. In this work we assess more durable changes in cell redistribution between primary and secondary lymphoid organs. The presented numbers of BM

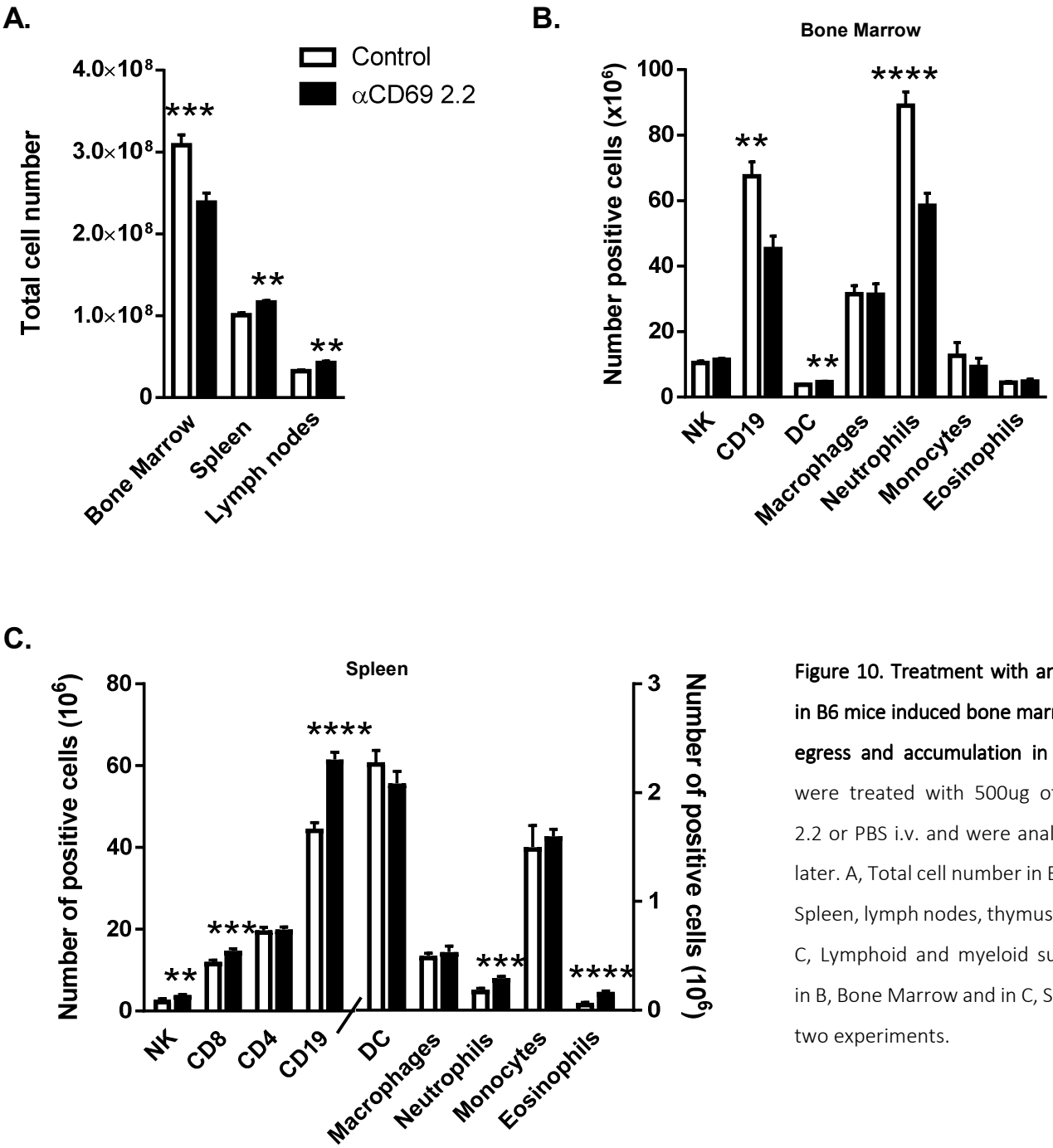


Figure 10. Treatment with anti-mCD69 2.2 in B6 mice induced bone marrow leukocyte egress and accumulation in spleen. Mice were treated with 500ug of anti-mCD69 2.2 or PBS i.v. and were analyzed one day later. A, Total cell number in Bone Marrow, Spleen, lymph nodes, thymus and blood. B-C, Lymphoid and myeloid subpopulations in B, Bone Marrow and in C, Spleen. Pool of two experiments.

cells are the sum of cells in the BM parenchyma and those that have already egressed and are in the sinusoids. Considering that white blood cell counts are still increased, we expect that if we quantified only cells in the BM parenchyma, we would find even greater differences. In control experiments, the treatment of $CD69^{+/+}$ mice with anti-hCD69 2.8 did not alter BM or spleen cell numbers, proving that the effect was specific of human CD69 targeting. CD69 expression was measured in thymus cells, due to some T cells express CD69 as a constitutive form, and we observed that mice that had been treated with anti-human CD69 had inhibited the expression of human CD69 respect non treated mice (Fig.

11B). When analyzing the main leukocyte subtypes, we observed a decrease in percentage of BM neutrophil and an increase in NK cells and dendritic cells (Supplementary Fig. 1A) whereas we found a decrease in BM neutrophils and B cell numbers (Fig. 11C). By the other hand, we observed a decrease in splenic percentage of CD8+ T, CD4+ T and dendritic cells and an increase in B cells and macrophages (Supplementary Fig. 1B) and we found an increase in splenic B cell, CD4+ T, CD8+ T cell, neutrophil, macrophage and eosinophil cell numbers (Fig. 11D). The fact that the treatment with anti-human CD69 affects the distribution of more cell types and with a greater magnitude could be due to an increased expression level of CD69 in most leukocyte subtypes in the huCD69 mouse model than in WT mice (Fig. 11E). If so, this would make this one a suitable model for the detection of small effects of CD69 targeting.

In a time course experiment, the greatest decrease in BM cellularity was observed at the earliest time point analyzed (a 30% decrease 4h after anti-hCD69 2.8 injection) and the BM cells counts gradually recovered until reaching the basal levels 9 days after treatment (Fig. 11F). Spleen cells counts were slightly augmented 4h after injection and gradually increased until a maximum at 3 days post-treatment, and had not reached basal levels by day 9 yet (Fig. 11G).

Altogether, these results suggest that targeting of human CD69 promptly induces egress of hematopoietic cells from the bone marrow and their accumulation in peripheral sites such as the spleen that the cell numbers are largely restored after about a week and that then, the sensitivity to a second dose is the same as to the first dose.

Anti-huCD69 treatment mobilizes B cells of various differentiation stages

The BM is site of B cell differentiation, selection and maturation. The release of immature B cells from the BM has been shown to depend on surface S1P1 expression [163] [162], which is down-modulated by CD69 [108]. To explore whether the egress induced by anti-hCD69 targeting affected preferentially B cells in certain maturation stages, we characterized the percentages and numbers of the different B cell subsets in BM and spleen 24h after treatment. We found that the numbers of all the B cell differentiation stage subsets (ProB cells, PreB cells, PreB1cells, immature and mature B cells) were decreased in the BM, but the percentage of reduction was greater the more differentiated subset (Fig. 12A) whereas percentages did not show changes except in B cell recirculating subpopulations

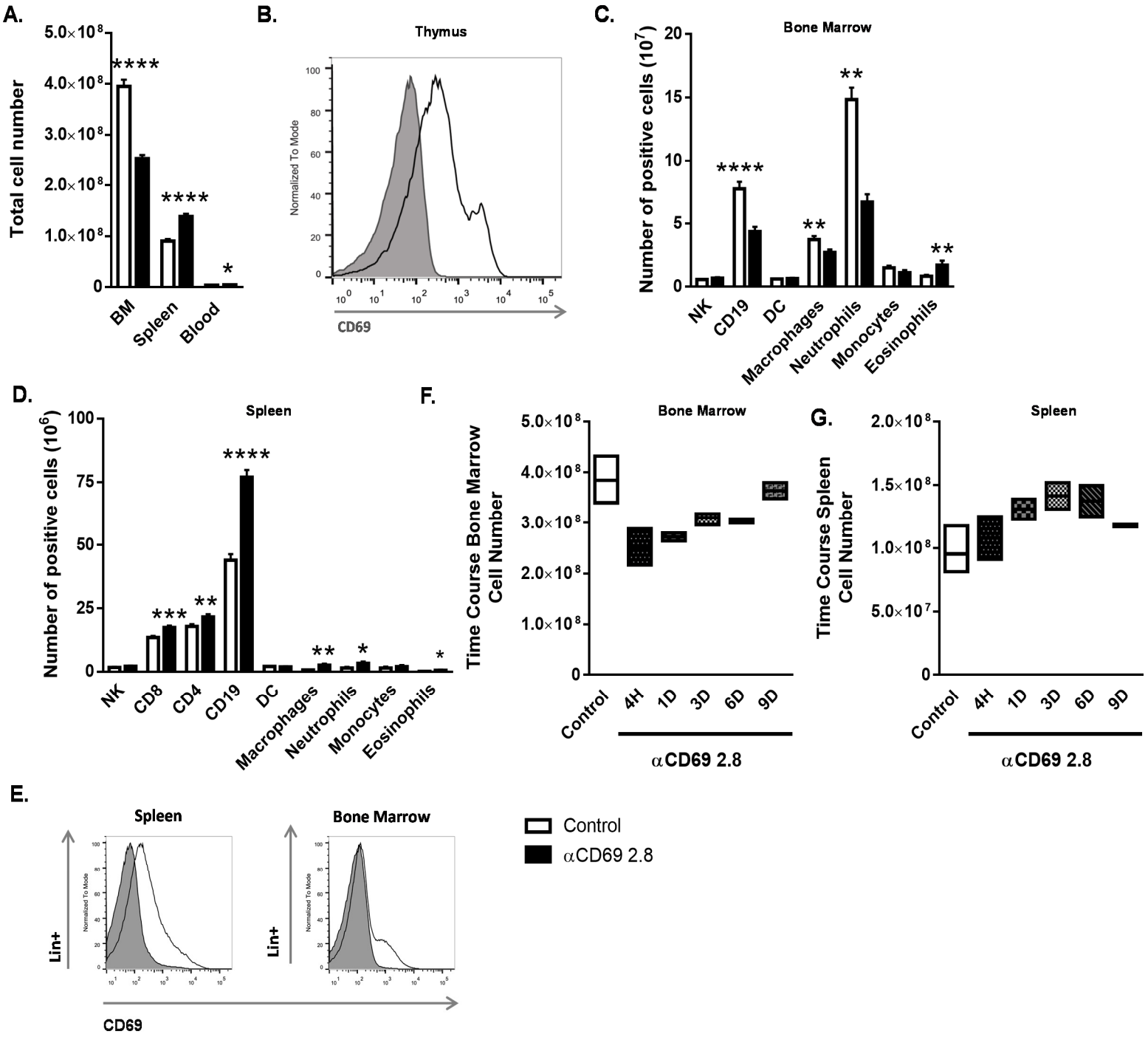


Figure 11. Targeting with anti-human CD69 2.8 in HuCD69 mice induced bone marrow leukocyte egress and accumulation in periphery. Mice were treated with 500ug of anti-huCD69 2.8 and were analyzed 1 day after treatment. A, Total cell number in Bone Marrow, Spleen and lymph nodes. B, CD69 expression in total thymocytes is shown. Staining of huCD69 (white) and anti-huCD69 treated mice (grey). C-D, Numbers of lymphoid and myeloid subpopulation cell in Bone Marrow. C, and in Spleen, D. E, Expression of CD69 in Lin+ cells in Spleen and Bone Marrow of $CD69^{-/-}$ and HuCD69+/- unmanipulated mice. F and G, Total cell number in F, Bone Marrow, and G, Spleen, where mice were sacrificed as indicated times. A and C-D, Pool of four experiments. B, One experiment representative of more than 10. E-G, One experiment.

where they were decreased (Supplementary Fig. 2A). This has been also reported in AMD3100-induced mobilization, and was related to a decreased motility of undifferentiated B cells [162].

In the spleen, we evaluated subsets of B cells according to CD23, CD21 and IgM marker expression within B220+ cells [26] dividing B cells between transitional (T1 and T2), marginal zone and follicular B cells. 24h after MAb treatment, the percentages and numbers of all these subsets were roughly augmented, except for those of transitional 2 B cells, which remained unaltered (Fig. 12B and Supplementary Fig. 2B).

Thus, CD69 targeting induces egress of all B cell subsets but preferentially of differentiated immature B cells from BM. However, this is not translated to a preferential accumulation of the recently recirculating, transitional B cell subsets in the periphery. Rather, the greatest increase is of follicular B cells, presumably naïve, suggesting a quick maturation of egressed B cells upon entering the spleen.

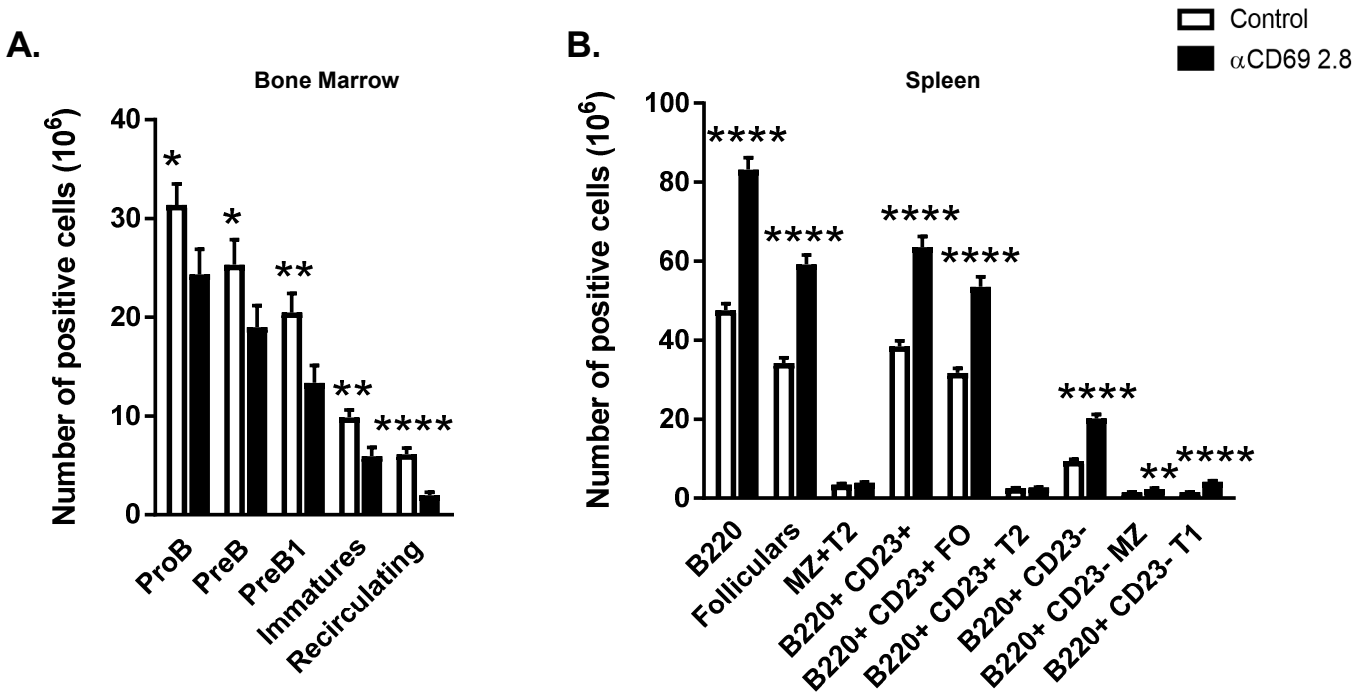


Figure 12. Targeting of CD69 in HuCD69 mice induced egress of immature B cells from bone marrow. Mice were treated with 500ug of anti-huCD69 2.8 and were sacrificed 1 day after treatment. A-B, Numbers of B cell subpopulations were measured in Bone Marrow in A and in spleen in B by flow cytometry. Pool of three experiments.

CD69 surface expression is not detected on all BM leukocyte subtypes mobilized by the anti-CD69 targeting.

In order to explore which cell types could be targeted by the antibody treatment, we analyzed CD69 surface expression in CD69 WT mice and huCD69 transgenic mice by flow cytometry using a stained sample of $CD69^{-/-}$ mice as background control staining. In the CD69 WT BM, the eosinophils showed considerable levels of constitutive surface CD69, whilst the rest of differentiated leukocyte subtypes had very low (B cells, monocytes) to practically undetectable levels (including neutrophils) (Fig. 13). As described, BM B cells had low levels of CD69 expression [162]. Like in previous works of the laboratory, [104], in the CD69 WT spleen, low levels of CD69 constitutive expression were detected on NK cells, DC and on subsets of T and B cells (Fig. 13 and data not shown).

In huCD69 transgenic mice, all the lymphocyte subtypes and dendritic cells of all the organs analyzed expressed surface CD69 at higher levels than WT mice (Fig. 13 and data not shown). Myeloid cells other than dendritic cells showed clearly detectable CD69 expression in the blood and LN (data not shown) but not in the BM and the spleen (Fig. 13). Thus, there is no direct correlation between the surface expression of CD69 and the extent of the effect on cell redistribution induced by anti-CD69 MAb targeting on a given leukocyte subtype. This suggests that CD69 targeting can induce cell egress indirectly.

CD69 targeting induces a mobilization of the same magnitude as AMD3100 and does not synergize with this drug.

The CXCR4 antagonist AMD3100 induces rapid mobilization of cells from the BM into the blood [265]. We aimed at comparing the effects of AMD3100 with the ones of anti-CD69 2.8 treatment in huCD69 mice, and also to analyze how they interacted upon co-administration. Importantly, anti-hCD69 2.8 induced a similar decrease in total BM leukocyte numbers at 24h post-treatment (Fig. 14A), and similar or even slightly greater decreases in all leukocyte subtypes numbers than AMD3100, except for

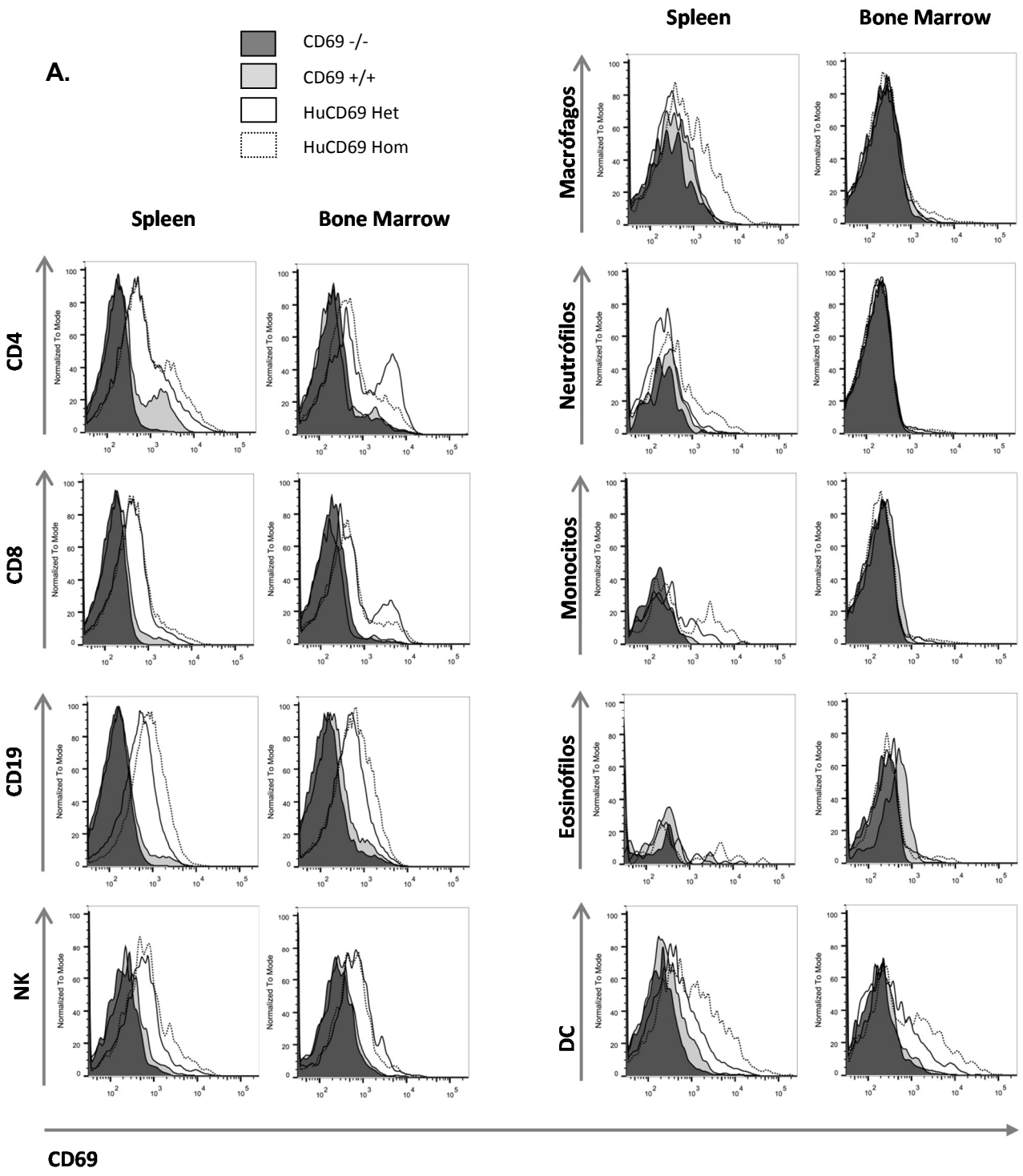


Figure 13. Expression of CD69 by the main cell subtypes in Spleen and Bone Marrow of huCD69 unmanipulated mice. Leukocytes from CD69$^{+/+}$, CD69$^{-/-}$, HuCD69+/-, and HuCD69+/+ mice were stained for CD69. Characterization of CD69 expression on CD4^{+} T cells (CD4^{+}), CD8^{+} T cells (CD8^{+}), B cells (CD19^{+},) NK cells (Nkp46+), DCs (CD11c hi SSC -) myeloid cells (CD11b hi), neutrophils (CD11b hi, GR1 hi SSC hi), monocytes (CD11b hi, GR1 lo SSC lo) and eosinophils (CD11b hi, GR1 lo SSC hi) . Results are representative of one experiment. The FACS staining was performed with a pool of three mice.

DC, which were more affected by AMD3100 (Fig. 14B). The co-administration did not further decrease BM leukocyte numbers. Curiously, in eosinophils, the co-treatment inhibited the decrease in BM cells counts (Fig 14B). AMD3100 treatment achieved higher white blood cell numbers than anti-CD69 2.8 treatment, and these were not additive when co-administered. In the spleen, though, the increase in cellularity was similar between the two treatments and, when co-administered, they achieved higher leukocyte numbers (Fig. 14A). These results show that, like AMD3100, anti-CD69 mAbs are potent and rapid BM leukocyte mobilizing agents. Taking into account that leukocyte numbers in the blood and spleen can be influenced by other processes, including homing into lymphoid organs and tissues and egress from lymphoid organs, we consider that the alteration in leukocyte numbers better reflecting leukocyte egress from bone marrow is the decrease observed in the bone marrow. Therefore, CD69 targeting and AMD3100 do not have a neither a synergic effect nor a clear additive effect on BM mobilization. This suggest that provided that the process is not saturated, the molecular mechanisms of mobilization are not independent, at least not their final steps.

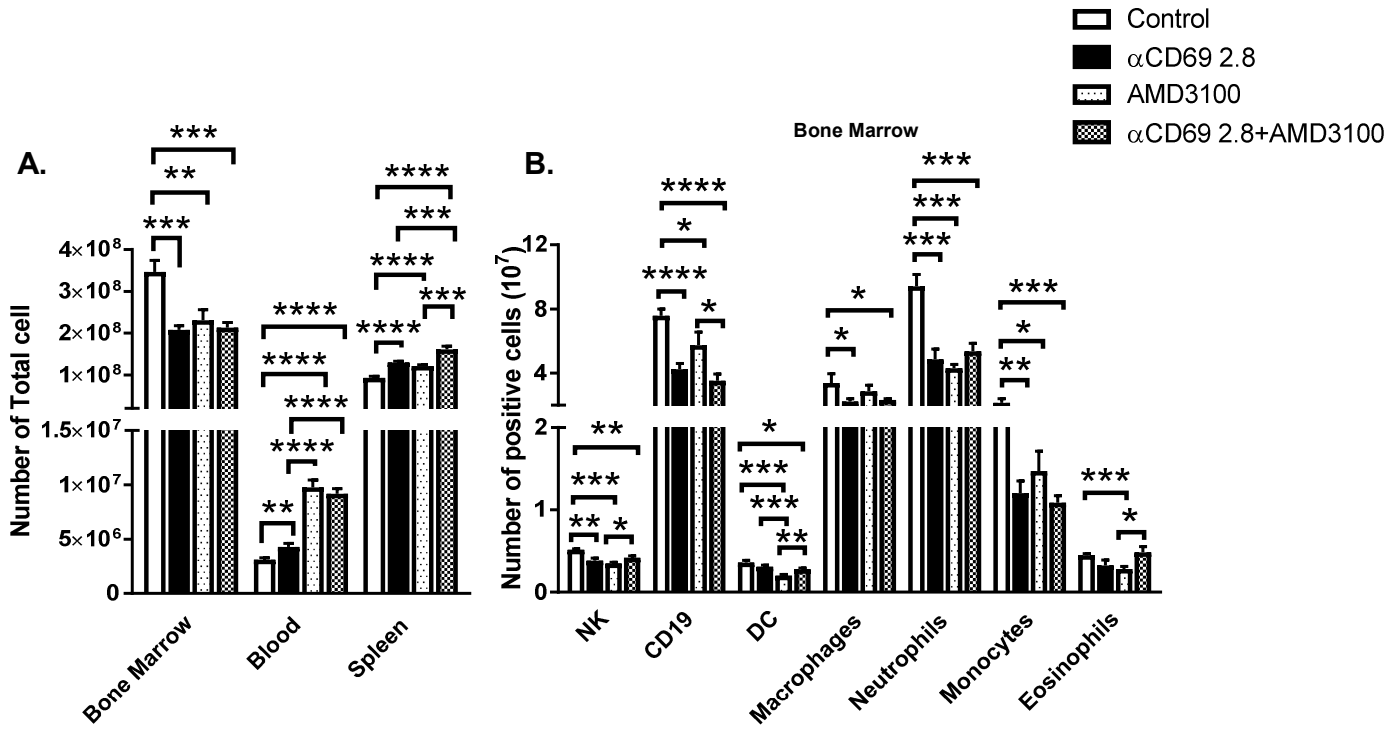


Figure 14. Combined treatment with anti-human CD69 and AMD3100 does not have a summative effect in the output of bone marrow cells. HuCD69 mice were treated with 500 µg of anti-hCD69 2.8 antibody one day before analysis or AMD3100 (150 µg/mouse) one hour before analyzing as indicated. A, Total cell number in bone marrow, spleen and blood. B, Number of lymphoid and myeloid subpopulations in bone marrow. A, Pool of two experiments.

CD69 targeting increases bone marrow CXCR4 expression

We further studied a possible implication of the CXCR4-CXCL12 axis. Petit et al. [3] describe that the mobilization induced by G-CSF is dependent on CXCL12 function and on elastase-mediated degradation of BM CXCL12. They show that CXCL12 BM levels rapidly but transiently increase 30-60 min post-injection, and later on (by 24h) decrease. Treatment with AMD3100 is also mediated by CXCL12 and also transiently increases BM CXCL12 levels even more rapidly (in 10 min), but by 1 hour they are decreased, whilst the ones in plasma are increased [264] [4]. We quantified CXCL12 in plasma and in supernatant of BM, spleen and LN cell suspensions at 30 min and 24h after anti-CD69 2.8 treatment. No differences were observed at 30 min (data not shown), but there was a tendency to increased CXCL12 levels in BM and decreased plasma, spleen and lymph nodes CXCL12 in treated mice at 24h (Fig.15A) and correspondingly RNA of CXCL12 in plasma was also observed decreased (Fig.15B). These results might be indicative of previous greater alterations.

Petit et al. also show that G-CSF treatment induces mobilization depending on CXCR4 function, that BM cell CXCR4 expression is reduced shortly after treatment (30-60 min) but increased after 24 hours, progressively increasing with repetitive daily treatments [3]. We analyzed CXCR4 surface expression on BM and spleen leukocytes, and found it increased in anti-CD69 2.8-treated hCD69 mice 24h after treatment (Fig 15C). However, after 5 days of a second dose administered 1 week after the first injection, there were no differences in the BM, while increased CXCR4 expression was still visible in the spleen (Fig 15D). Altogether, these results show that, like G-CSF, CD69 MAb targeting alters the expression of CXCR4-CXCL12 axis, at least the one of CXCR4, but suggest that it does it with a different kinetics.

CD69 targeting-induced mobilization depends on S1P receptors function

Nevertheless, S1P1 interacts with CD69 and they can mutually downregulate their surface expression [24]. We pursued to analyze the dependence on S1P receptors of the CD69-targeting induced mobilization by treating CD69 WT mice with anti-mCD69 2.2 with or without co-administration of FTY720. 24h after injection, the co-treatment with FTY720 almost completely inhibited the decrease

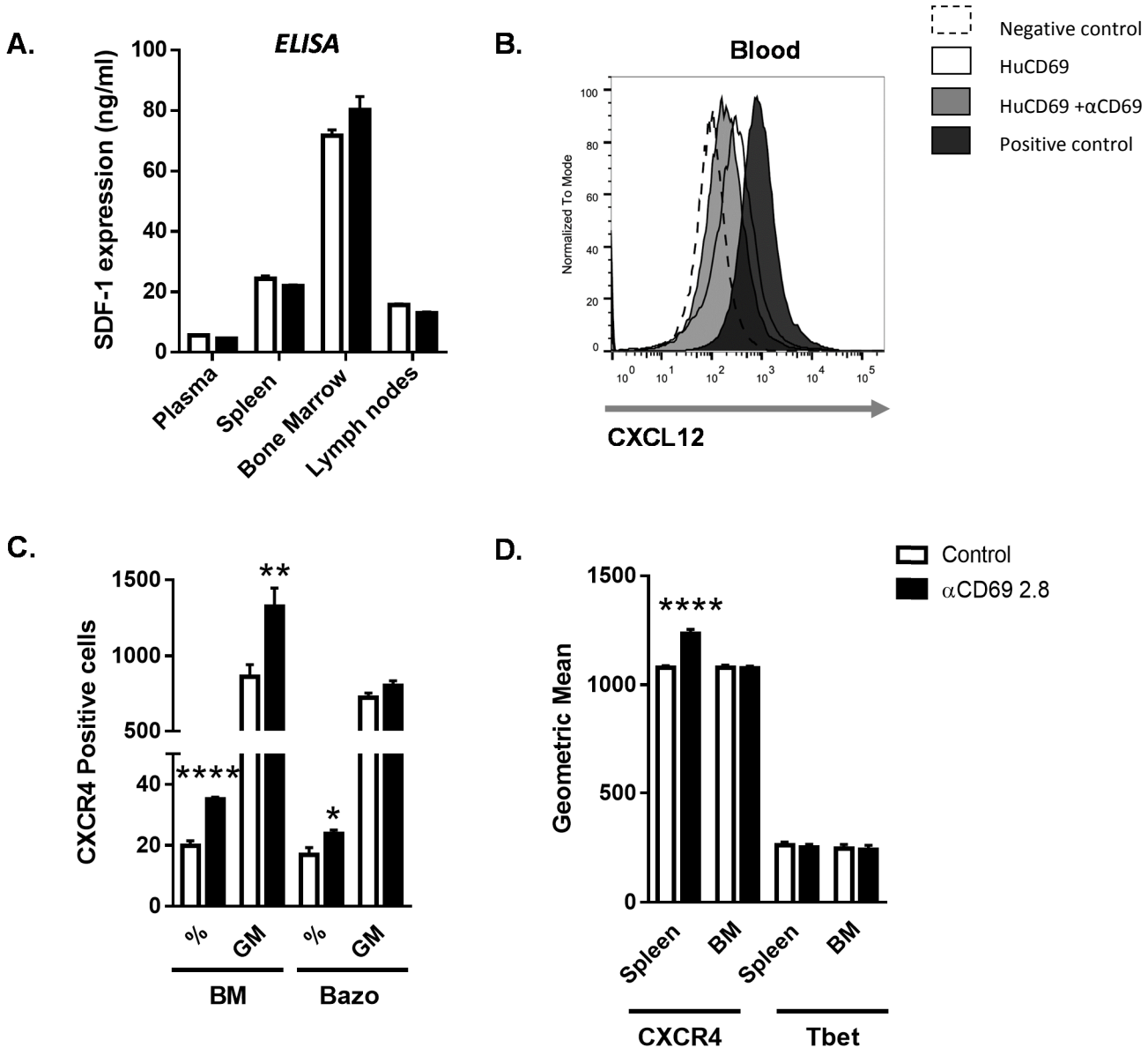


Figure 15. CXCL12 and CCXR4 expression in HuCD69 mice. A-C, HuCD69 were treated with 500 µg of anti-hCD69 2.8 antibody one day before analyzing .A-B, SDF-1 was measured by ELISA in A, in plasma, and supernatants of spleen, bone marrow and lymph nodes and in B, by RNA Primeflow in blood. C, Expression of CXCR4 in Bone Marrow and spleen 24 hours after treatment with anti-hCD69 2.8 was measured as percentage and geometric mean. D, Surface expression of CXCR4 and intranuclear expression of T-bet was measured after 5 days after second dose of 200 µg of anti-hCD69 2.8 antibody, both separated by a week. A, pool of two experiments. B, one experiment. C, Pool of three experiments. D, Pool of two experiments.

in total BM leukocytes induced by the anti-mCD69 2.2, and it also reduced, albeit to a lesser extent, the increase in spleen leukocyte numbers (Fig. 16A). As expected [4], FTY720 treatment induced a decrease in white blood cells counts (Fig. 16A). Altogether these results strengthen the interpretation

that the decreased and increased cellularity in BM and spleen, respectively, observed upon anti-CD69 treatment are predominantly due to an induction of leukocyte egress from the BM, and also show that CD69-targeting-induced mobilization of at least most BM leukocytes depends on S1P receptor function.

Of interest, the *in vivo* treatment augmented S1P1 surface levels in total BM and blood leukocytes (Fig. 16B-C). When analyzing S1P1 surface expression on the main lymphocyte subpopulations and DC, we found an increase of S1P1+ cells in bone marrow T and NK cells, but not B and DC (Fig. 16B). However, we found elevated S1P1 on spleen and blood B cells and spleen DC (Fig. 16C-D), opening the possibility that bone marrow B cells and DC that had upregulated S1P1 had trafficked from the bone marrow to the periphery. S1P1 surface levels were also elevated on blood T cells and spleen NK cells (Fig. 16C-D). Thus, anti-CD69 *in vivo* treatment increased S1P1 expression on leukocytes analyzed in bulk and on all the lymphocyte subsets and DC in at least one of the organs analyzed. We also incubated bone marrow, spleen and thymus cells from non-treated mice with anti-human CD69 2.8 *in vitro* (Fig. 16E), and measured S1P1 surface expression after two days. A significant increase in bone marrow and thymus but a decrease in spleen (Fig.16E) was observed after the culture, suggesting that CD69 targeting upregulates surface S1P1 in cells from primary lymphoid organs but not spleen cells, and that the increase in S1P1+ NK and DC cells in the spleen might be due to incoming S1P1+ NK and DC.

If CD69 targeting was blocking the interaction of CD69 with S1P1 and the downmodulation of surface S1P1, we would expect to find higher surface S1P1 levels on cells upon treatment, as we are finding. However, elevated S1P1 expression could also be due to other mechanisms, since, for example, G-CSF treatment also increases surface S1P1 levels.

CD69 targeting induces mTOR signaling

G-CSF induces increase of BM and plasma S1P levels and of BM cell surface S1P1 expression through mTOR signaling [4]. Since we found higher S1P1 expression upon anti-CD69 MAb treatment, we sought whether this could also be mediated by mTOR. This molecular complex signals through 4E-BP1 and phosphorylates it in Thr-37 and Thr-46 [131]. Thus, to assess mTOR activity we measured 4E-BP1 Thr-37 and Thr-46 phosphorylation on bone marrow and spleen cells 24 hours after treatment with anti-

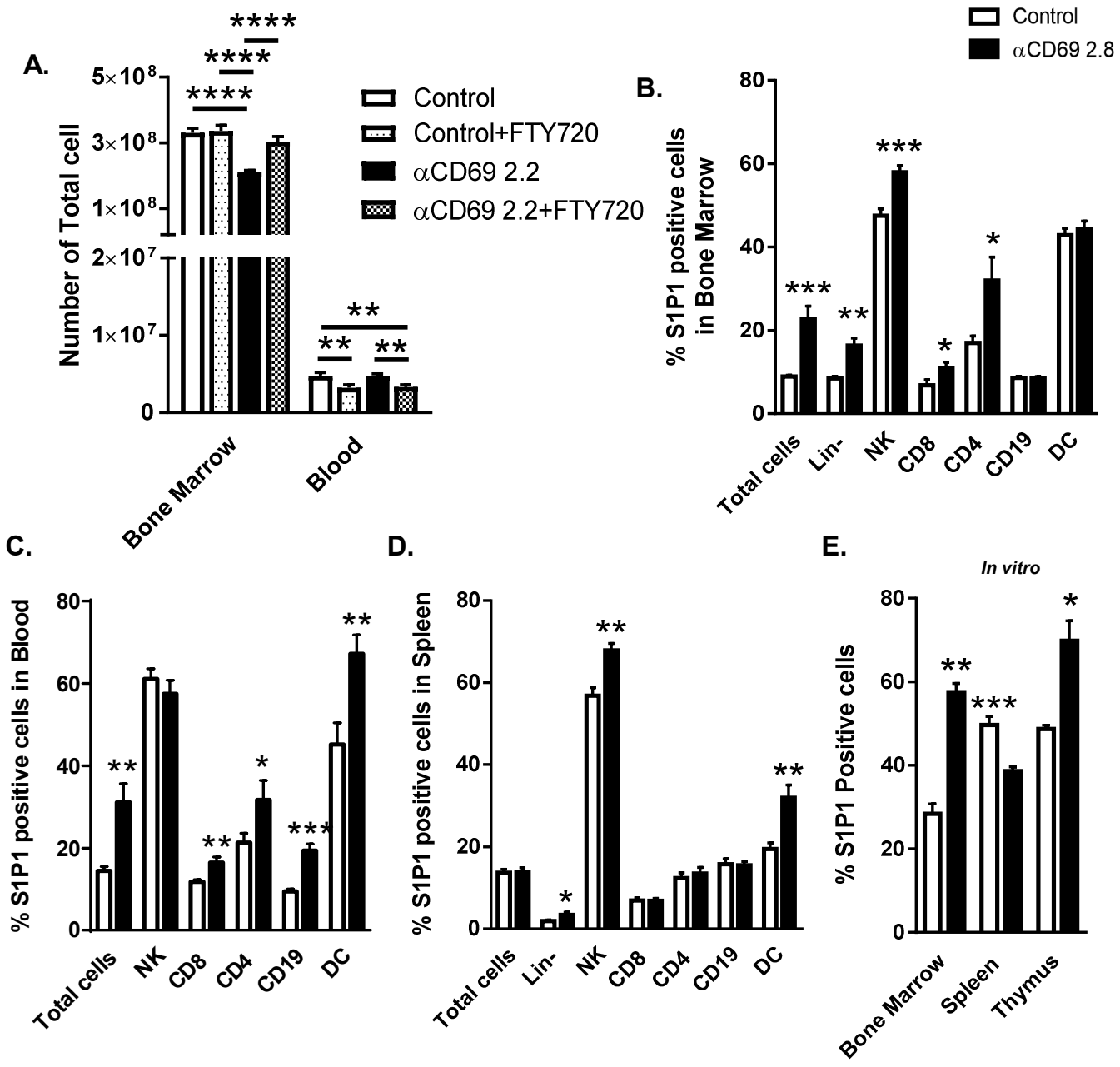


Figure 16. Treatment with FTY720 and anti-huCD69 impair bone marrow cell egress in HuCD69 mice. Mice were treated with 500ug of anti-huCD69 2.8 i.v or FTY720 (1mg/kg) i.p or both as indicate 24 hours before analyzing. A, Total cell number in bone marrow and blood. B-D, Mice were treated with anti-huCD69 2.8 i.v. S1P1 expression was measured by flow cytometry in B, bone Marrow, in C, blood and in D, spleen cells in indicated cell population. E, Bone marrow, spleen and thymus cells from HuCD69 mice were cultured *in vitro* with anti-huCD69 for two days and S1P1 expression was analyzed by flow cytometry. A, Pool of two experiments. B-D, Pool of two experiments. G, One experiment.

human CD69 by intracellular staining and observed that it was increased in both sites (Fig 17). This data shows CD69 targeting induces mTOR signaling, and this could be the mechanism by which it upregulates S1P1.

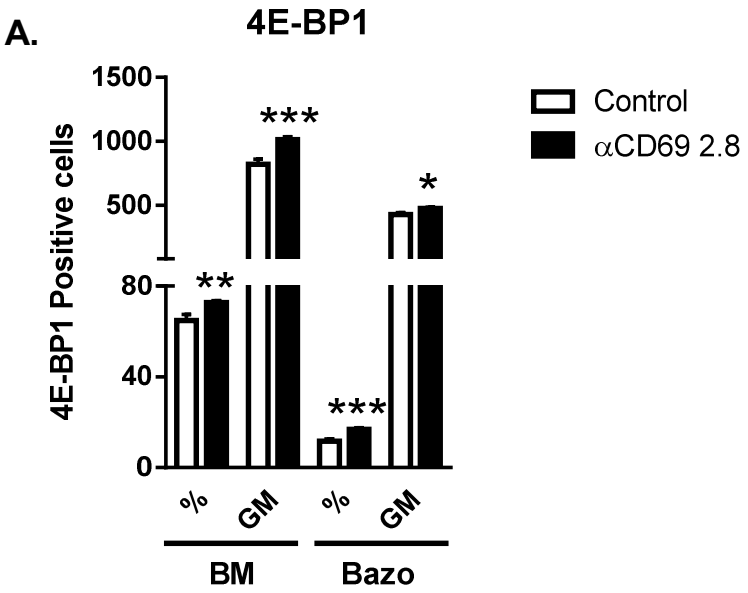


Figure 17. mTOR regulates anti-huCD69 2.8 mAb induced mobilization in Bone Marrow. A, Intracellular expression of mTOR was measured in mice treated with anti-huCD69 2.8 mAb 24 hours before analyzing versus non treated mice. Geometric mean and percentages were analyzed by flow cytometry. One experiment.

Anti-human CD69 increases VLA-4 expression in bone marrow

The retention of bone marrow cells has been also related with the interaction between VCAM-1/VLA-4. VLA-4 is an adhesion molecule expressed on HSPC and other bone marrow hematopoietic cells [266] [267] which binds, among other ligands,VCAM-1 expressed on bone marrow stroma. The disruption of this axis with VLA-4 inhibitors leads to HSC mobilization although the mobilization induced is less effective compared to G-CSF [268, 269]. Surface expression of VLA-4 has been reported to be downregulated upon G-CSF or AMD3100 treatment, and this decrease has been proposed to contribute to the loss of cell retention into the bone marrow [270] [271] [265]. To assess whether VLA-4 expression decrease could also participate in anti-CD69 2.8-induced mobilization we analyzed surface VLA-4 on bone marrow subpopulations 24h after anti-human CD69 treatment. We observed an increase in the percentage of VLA-4+ NK cells, T cells and B cells, a decrease in VLA-4+ monocytes and eosinophils, and no changes in dendritic cells, macrophages and neutrophils (Fig.18A). In the spleen cells, VLA-4 expression was slightly decreased only on CD4+ T cells (Fig.18B) while it was unaltered on the rest of cell subtypes. However, the number of VLA-4 expressing cells was decreased in total cells, NK cells, macrophages, neutrophils and monocytes (Fig.18A) whereas the number of

VLA-4 expressing cells in spleen was increased in total cells, NK cells, B cells, macrophages, monocytes and eosinophils (Fig.18B). Thus, anti-CD69 2.8 can induce bone marrow egress through VLA-4 downregulation in some cell subtypes.

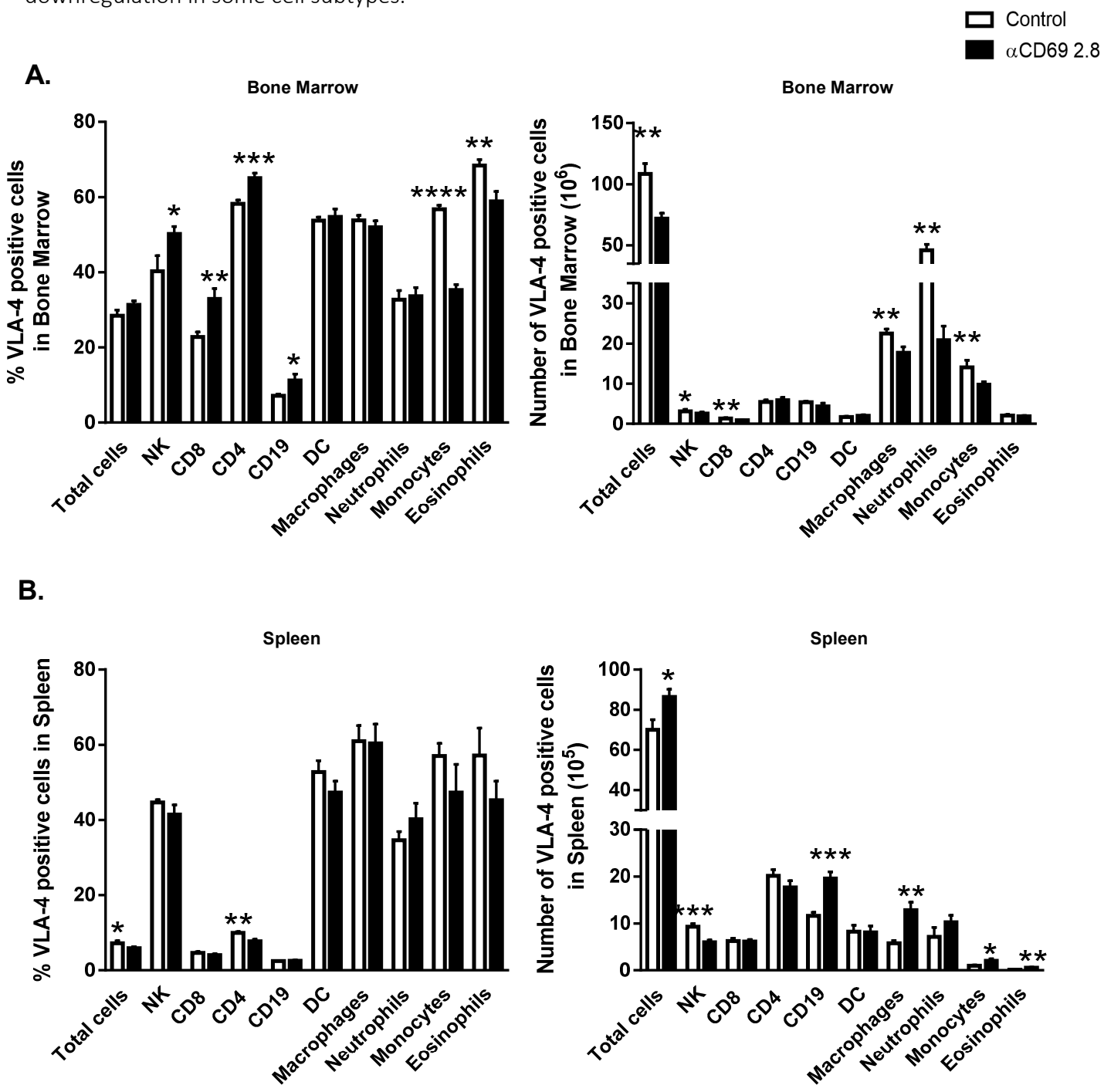


Figure 18. The treatment with anti-human CD69 decreased VLA-4 cell number in bone marrow and increased in periphery in HuCD69 mice. A and B, Mice were treated with anti-huCD69 2.8 i.v. and were analyzed 1 day after treatment. Surface VLA4 expression were measured by flow cytometric in lymphoid and myeloid subpopulations in Bone Marrow A, and Spleen B, as indicated in percentages (left) and numbers (right). One independent experiment.

Anti-huCD69 treatment induces proliferation and accumulation of primitive hematopoietic cell numbers in BM and spleen

Mobilizing treatments are especially interesting to induce egress of hematopoietic stem and progenitor cells into the blood, where they can be collected and used for therapeutic interventions. To study whether anti-CD69 treatment could be used to mobilize this kind of cells, we analyzed the effects of anti-human CD69 2.8 treatment on their numbers in BM and spleen. Lin- cells were analyzed for Sca-1 and c-Kit expression to determine the number of KSL ($Lin^{-}Sca1^{+}c\text{-}kit^{+}$). KSL cells were further divided into long term HSC (LT-HSC; KSL $CD34^{neg}$ $FLT3^{neg}$), short term HSC (ST-HSC; KSL $CD34^{+}$ $FLT3^{neg}$) and multipotent progenitors (MPP; KSL $CD34^{+}$ $FLT3^{+}$) [27, 28]. 24 h after the injection, anti-hCD69 2.8 treatment had induced a great increase in BM KSL cell numbers of all HSPC subtypes both in the spleen and the BM (Fig. 19A-B and supplementary Fig. 3A-B). Whilst such an accumulation of KSL in the spleen could be explained by an effect on mobilization, the increase in BM could not. When analyzing the effect on KSL cells, we observed that, 24h after injection, the administration of anti-CD69 to hCD69 mice led to higher BM and spleen KSL, LT-HSC, ST-HSC and MPP cell numbers than those observed in AMD3100 treatment, and that the co-treatment tended to further increased those numbers (Fig.19C-D). This could be due to the fact that AMD3100 tended to increase proliferation of BM KSL cells, and that this could be added to the effect on proliferation of CD69 targeting.

We wondered whether these higher KSL numbers were related to an increase in the proliferation rate, and measured BrdU incorporation by BM and spleen KSL cells. As seen in Fig. 20, at 24h post-treatment, the percentage of BrdU+ of all the KSL subtypes was much higher in anti-hCD69 2.8-treated hCD69 mice than in untreated controls, both in the BM and the spleen. 24h may seem a time too short for cell expansion. However, BM HSPC have been shown to proliferate *in vivo* in response to infection or treatment with adjuvants, and to be increased in number as soon as 24h post-challenge [272] [273]. Thus, the increase in BM HSPC number is likely due to an increase in their proliferation rate, while the increase in the spleen can be contributed by both HSPC mobilization and expansion. Actually, the quantity of BM and spleen HSPC tended to be even higher in anti-hCD69-treated mice than in AMD3100-treated mice both, and this was related to an induction of BrdU incorporation into HSPC by anti-hCD69 but not by AMD3100 treatment. Altogether, these results show that a single dose of anti-hCD69 induces not only mobilization of HSPC but also their proliferation and expansion both in the BM and the periphery.

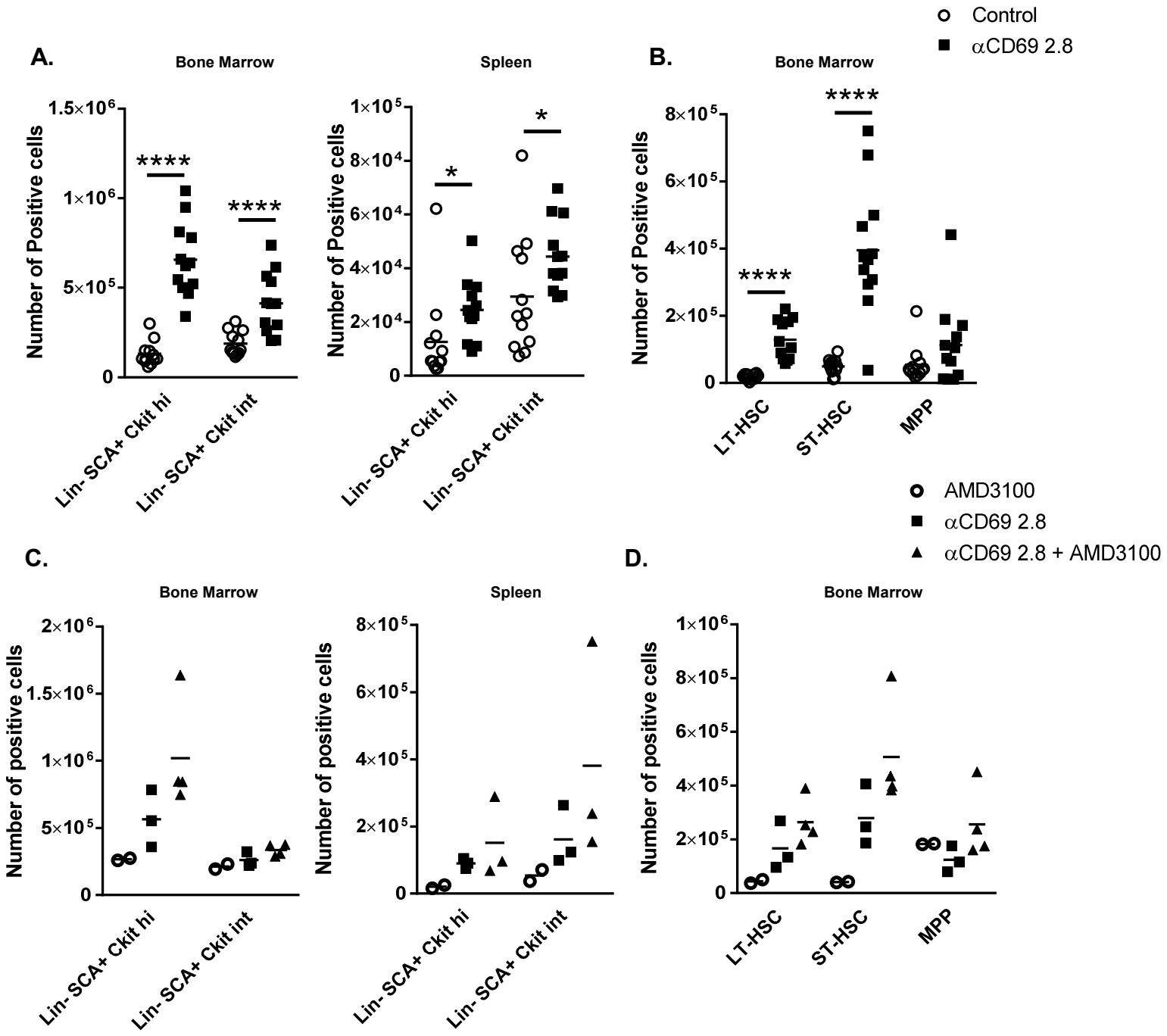


Figure 19. The treatment with anti-human CD69 increase HSC cells in Bone Marrow. A-B, Mice were treated with anti-human CD69 2.8 24 hours before scarifying. A and B, Lin- cells were stained by SCA+ and C-kit hi or C-kit int. Numbers in A, of KSL and CLP cells were measured in (left) Bone Marrow and (right) spleen. B, Cells CD34 and FLT3 were gated in Sca+, C-kit hi cells and numbers in B are shown in Bone Marrow. B, Cells CD34 and FLT3 were gated in Sca+, C-kit hi cells and numbers in B are shown in Bone Marrow. C-D, Mice were treated with anti-human CD69 2.8 i.v (24 hours), AMD3100 i.p (one hour), both treatments as indicated. Numbers in C, of KSL and CLP cells were measured in (left) Bone Marrow and (right) spleen. D, Number of LT-HSC, ST-HSC and MPP cells in Bone Marrow. A-B, Pool of three experiments. C-D, One experiment.

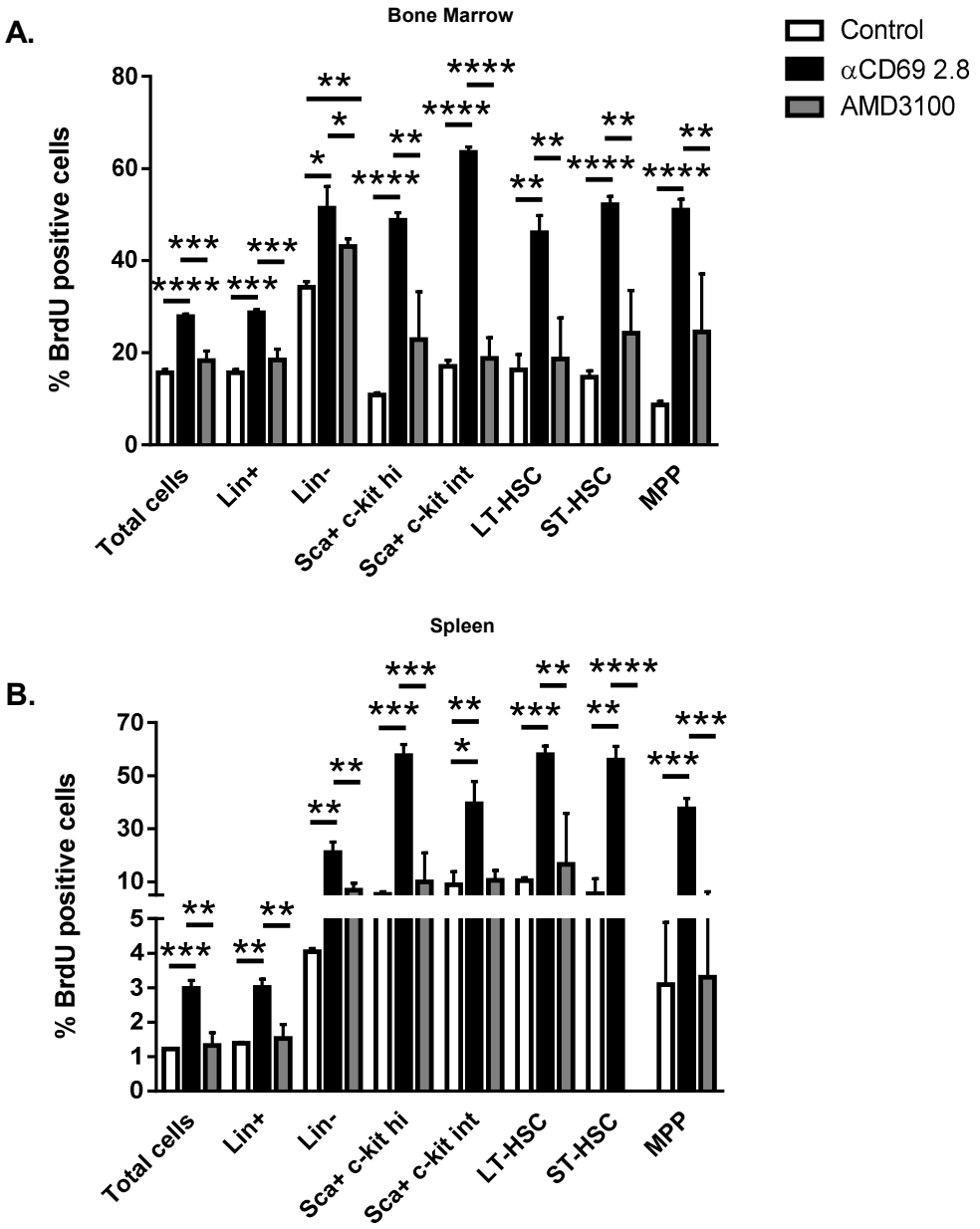


Figure 20. Treatment with anti-hCD69 2.8 induced an increase proliferation rate in bone marrow primitive cells compared to AMD3100. Mice were treated with anti-mCD69 2.8 i.v or AMD3100 i.p. as indicated and were analyzed one day later. Mice received 1 mg of BrdU intraperitoneally and three hours later, mice were analyzed. Bone Marrow and Spleen cells were collected and cell proliferation rate was assessed by BrdU incorporation by flow cytometry in total cells and subpopulations of Lin- in Bone Marrow in A, and in spleen in B. One experiment.

To assess whether anti-hCD69 2.8 could be influencing HSPC directly or indirectly, we analyzed CD69 expression on the subpopulations of BM and spleen HSPC of HuCD69 mice, using $CD69^{-/-}$ mice as control for background staining (Fig.21A). We found clear CD69 expression on KSL cells in bone

marrow. When KSL cells were divided in LT-HSC, ST-HSC and MPP, CD69 expression was detected on BM MPP cells. Of note, CD69 expression on human hematopoietic stem cells (CD34+) at steady state has been reported [274]. Thus, anti-hCD69 treatment also induces proliferation and expansion of HSPC subtypes with no detectable CD69 surface expression, suggesting that, at least on these subtypes, the antibody is acting indirectly.

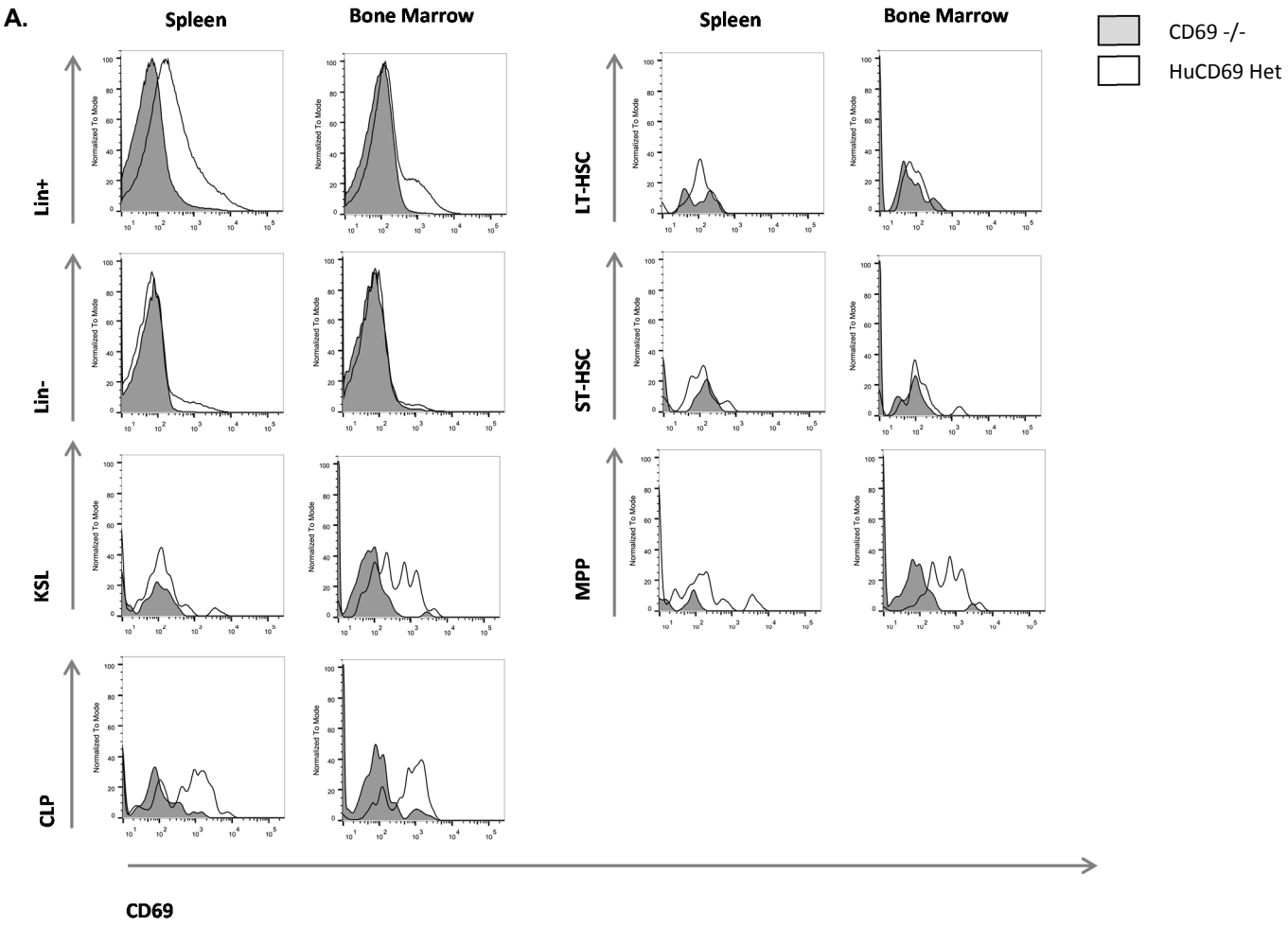


Figure 21. Expression of CD69 in hematopoietic stem cell in Spleen and Bone Marrow of unmanipulated mice. Cells from bone marrow and spleen of $CD69^{-/-}$ C57Bl6 and HuCD69 mice were stained for specific markers of hematopoietic stem cell. Results are representative of one experiment.

Targeting human CD69 increases proliferation rate of bone marrow mature leukocytes

Since we observed proliferation induction of HSPC and since previous data of the laboratory showed that CD69 targeting induces non-cognate T cell proliferation through upregulation of the IL-2 and CD25

[29], we wondered whether the antibody could also induce proliferation of mature leukocytes, which would help to explain the increase in spleen cellularity. Mice were treated with anti-human CD69 for 24 hours, injected with BrdU and sacrificed 3 hours after. The frequencies of BrdU positive cells were increased in bulk bone marrow leukocytes as well as in the major leukocyte subpopulations, except for eosinophils (Fig.22A). The higher percentages of BrdU+ T cells in distinctive subpopulations of precursors cells are consistent with previous results. This proliferation also correspond with the observed augmented frequencies of CD25+ and IL-2+ cells in bone marrow, spleen and blood (Fig. 23B).

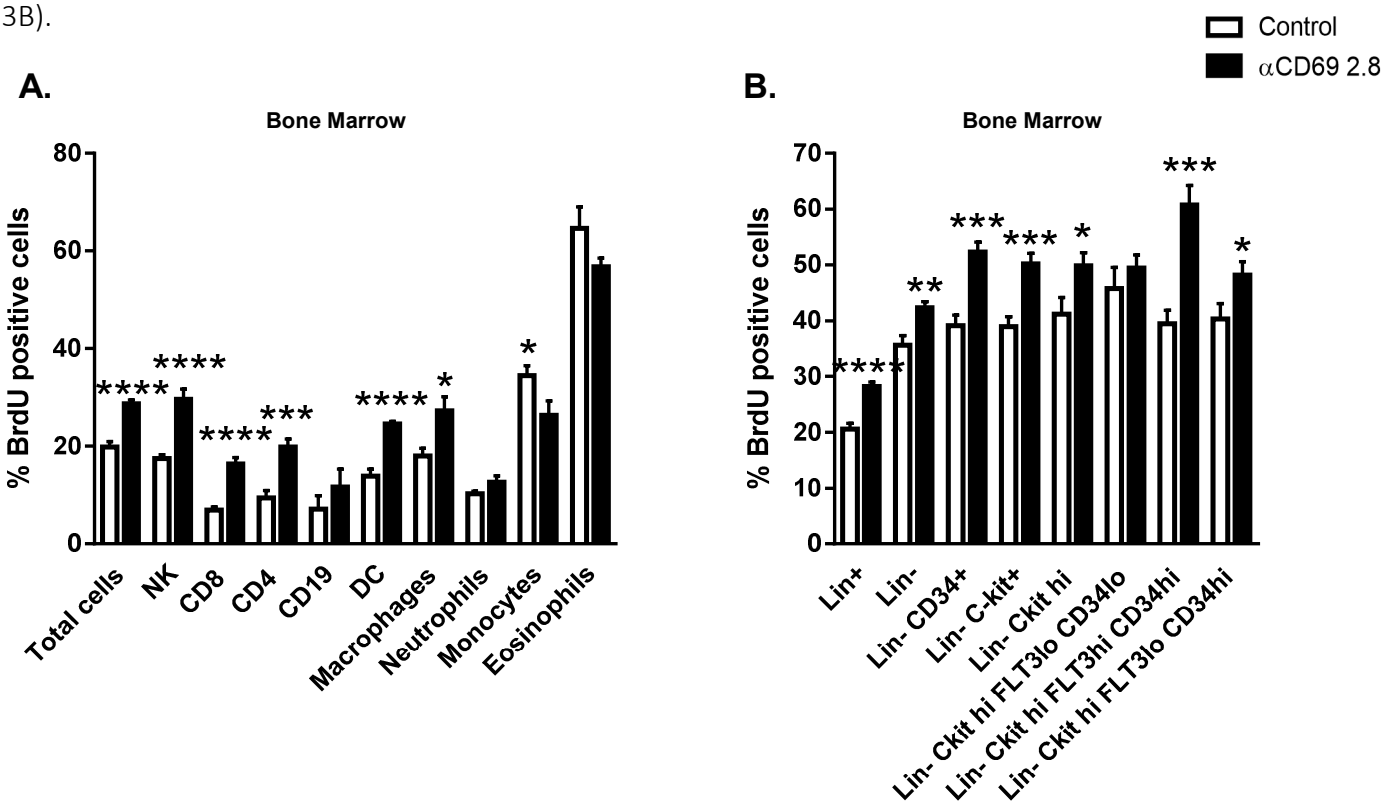


Figure 22. An increased proliferation rate in BM progenitor cells is observed in HuCD69 mice treated with antihuman-CD69 *in vivo*. Mice were treated or not treated with anti-huCD69 2.8 24 hours before BrdU injection. Mice received 1 mg of BrdU intraperitoneally and three hours later, mice were analyzed. Bone Marrow were collected and cell proliferation rate was assessed by BrdU incorporation. A, BrdU was measured by flow cytometry in lymphoid and myeloid subpopulations in Bone Marrow. B, BrdU was measured in precursor cells in Bone Marrow. A- B, pool of two experiments.

In previous works we have shown decreased spontaneous apoptosis of $CD69^{-/-}$ leukocytes [1] [275] associated with decreased TGFβ expression [1]. Thus, we also tested whether an increased immune cell survival could contribute to the higher spleen cellularity observed upon anti-CD69 2.8 treatment. Dead cells are quickly phagocytized *in vivo*. To circumvent this, we performed *ex vivo* cultures of spleen

cells from anti-human CD69 treated mice and analyzed the spontaneous cell death by PI staining at different times of culture. Significant differences were found as soon as 2 days of culture and this difference was increased over time (Fig.24A). Moreover, we found a 50% decrease in TGF-b expression in bone marrow and spleen cells of treated mice, measured by real time RT-PCR. (Fig.23A). Thus, like CD69 deficiency, CD69 targeting also led to enhanced cell survival and low TGFβ expression.

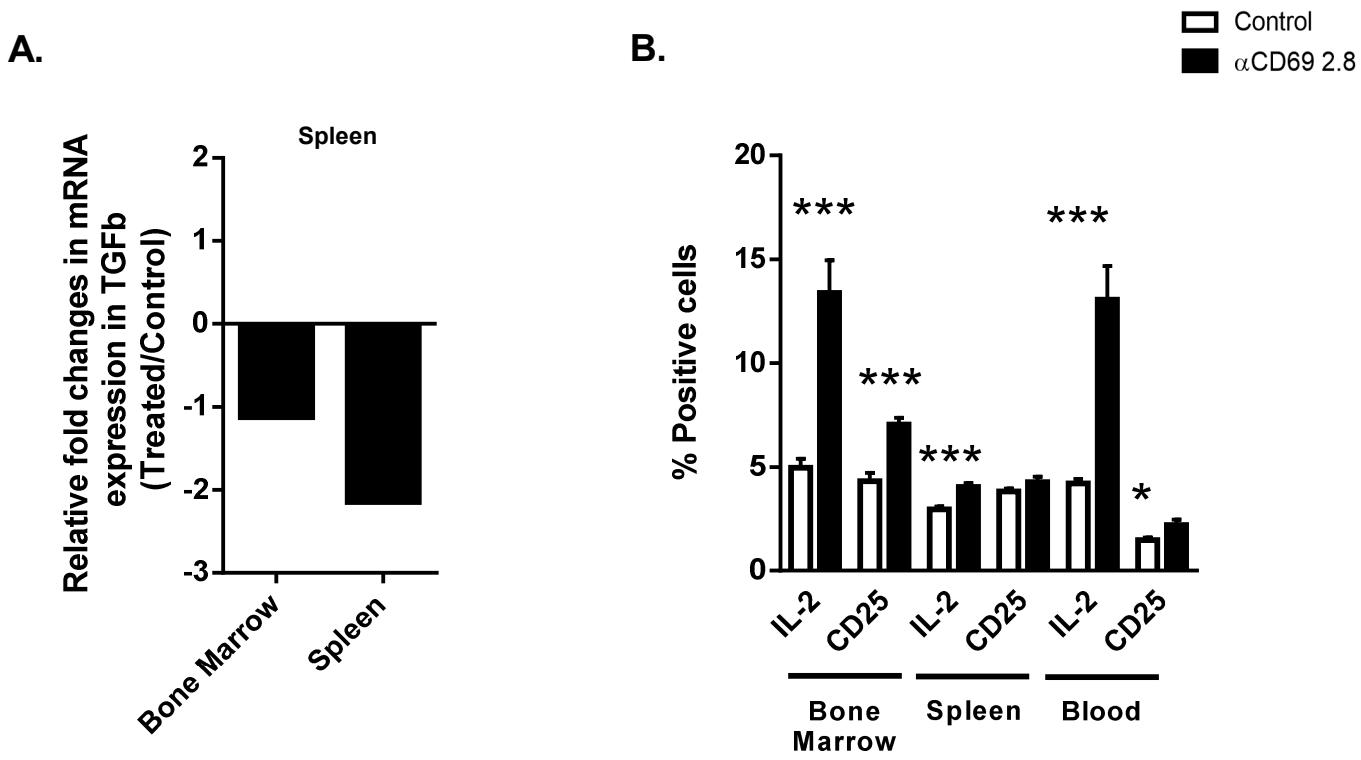


Figure 23. Targeting of human CD69 induce an increase IL-2 expression and reduce TGFβ. Mice were treated with 500ug of anti-huCD69 2.8 and were analyzed 1 day after treatment. A, mRNA of TGF-β was measured by Real Time PCR and was represented as relative fold change treated mice respect control mice B, Intracellular expression of IL-2 and CD25 surface expression was analyzed by flow cytometer in bone marrow, spleen and blood. A, One experiment of pool of samples from three independent experiments, each one of them with 4 animal for group. B, Pool of three experiments.

Altogether these data point to that the leukocyte accumulation observed in the spleen, especially that observed at 3 and 6 days post-treatment, could be contributed by the lower spontaneous cell death and increased proliferation rate observed in CD69-targeted mice.

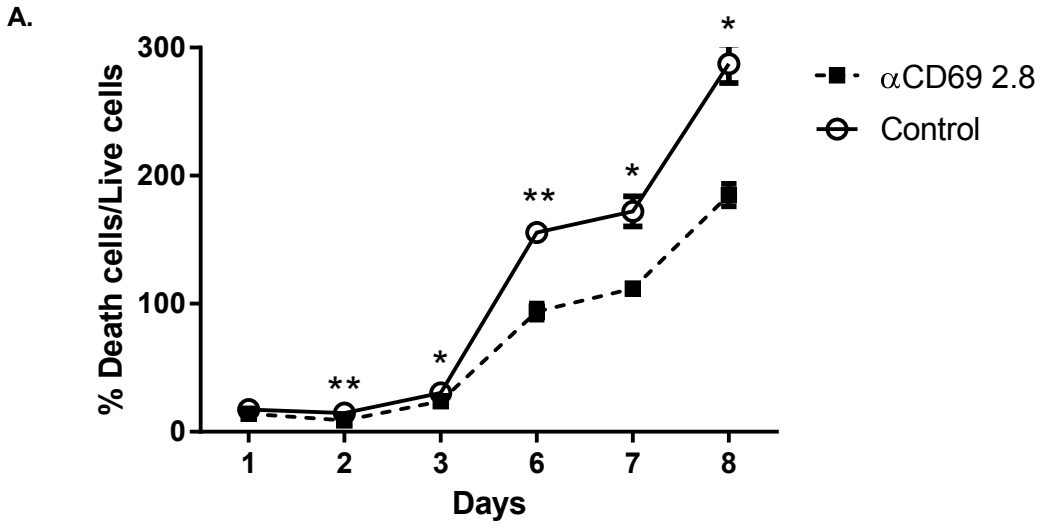


Figure 24. Attenuation of spontaneous cell death in targeting of human CD69. A, Survival was measured by PI staining of unfractionated splenic cells of uninfected HuCD69+ CD69+/+ mice in C57Bl/6 genetic background treated with anti-huCD69 2.8 mAb or Isotype control.

DISCUSSION

Previous results have demonstrated the importance of mobilizing HSC as a treatment for people who have reduced cell counts as a result of diseases or due to chemotherapeutic agents that cause destruction of many cells [276]. Currently, G-CSF is one of the most used mobilizers. In the present work we describe for the first time the capacity of anti-CD69 MAbs to induce a rapid and massive egress of mature leukocytes as well as of HSPC from the bone marrow, both in wild type mice treated with anti-mCD69 as well as in transgenic hCD69 mice in $CD69^{-/-}$ background treated with anti-hCD69 MAb. A single dose of anti-human CD69 decreased the number of total bone marrow leukocytes and increased those of the spleen and blood. The effects on cell redistribution were already apparent at 4 hours after treatment and could be observed for at least 6 days. The fact that the decrease in BM cellularity was not observed when anti-CD69 mAbs were co-administered with FTY720 points to that the observed redistribution is due to leukocyte mobilization from the bone marrow, and that this is dependent on S1PR function. The treatment also upregulated S1P1 and CXCR4 surface levels and induced mTOR signaling. Importantly, the treatment led to the proliferation and expansion of HSCP.

The mTOR pathway has been shown to have an essential role as mechanisms involved in the mobilization produced by G-CSF through activation of S1P. So in assays of inhibition of this route, the result is a defect in mobilization. We observed an increase of 4E-BP1 involved in mTOR signalling pathway, both in bone marrow and in spleen. In addition to the results already observed, in which the treatment with anti-human CD69 induces the mobilization of effector cells as well as hematopoietic stem cells. Finally, we required to compare the different mobilization between our treatment and AMD3100. Our data revealed that the mobilization induced by both treatments is similar in total cell counts but we observed a greater number of precursor cells in our treatment with anti-human CD69 or when we combined both treatments, being more reduced in treatment with AMD3100. In addition, human CD69 targeting induced a significant increase in proliferation rate higher than induced by AMD3100 in total cells and precursor cells.

Importantly, in humans, CD69 expression on human hematopoietic stem cells (CD34+) has already been described at steady state [274].

In the present results, CD69 targeting and CD69 deficiency affect bone marrow egress in the same sense but with different magnitude: CD69 deficiency has little or no effect on BM cellularity, depending on the mouse strain used, and a relatively small effect on cell counts of peripheral lymphoid organs

and blood. Instead, CD69 targeting induces massive mobilization from the BM. One possible explanation is that the effects of anti-CD69 targeting are not merely due to a blockade of CD69 function but rather, that the antibody is inducing, directly or indirectly, signaling pathways, at least that of mTOR, and that these pathways lead to cytokine production, cell proliferation, increased survival and, in general, the emulation of a stress situation finally leading to massive leukocyte mobilization. Actually, early *in vitro* studies of CD69 function showed that Ab crosslinking of CD69 on pre-activated T cells, B cells, NK cells, monocytes, eosinophils and basophiles increased their activity [87, 103, 109, 134-139]. Another possibility is that a putative sudden loss of CD69 function upon anti-CD69 treatment could activate signaling cascades leading to the observed effects, and that these were not detected in the constitutive deficiency of CD69 at steady state due to compensatory mechanisms.

Like that of CD69, S1P1 deficiency also had a modest effect on egress from BM at steady state, as shown by the normal [4] or nearly normal numbers of blood [49] and peripheral S1P1 KO B cell counts [158] and the small increase in immature S1P1 KO B cells in the B parenchyma [162]. Thus, the effect of CD69 on BM cell egress at steady state could be due, to its interaction with S1P1.

In contrast to the modest or absent effect of S1P1 deficiency on BM egress at steady state, this deficiency had more marked effects on G-CSF and AMD3100-induced mobilization [49][4]. This, together with the fact that treatment with FTY720 markedly reduced the egress induced by these agents, has led to the notion that S1P receptors mediate such mobilization. G-CSF and AMD3100 have been shown to increase plasma and BM levels of S1P through the induction of IgG-dependent complement cascade, which induces erythrocyte lysis and release of S1P [20, 45]. Like in these reports, we also observed inhibition of mobilization upon FTY720 co-treatment. FTY720 treatment desensitizes leukocytes to S1P-induced migration. Moreover, as well as S1P, it also increases endothelial barrier function by influencing actin and functional protein rearrangements [277]. Through either of the two functions, FTY720 could inhibit leukocyte translocation through the endothelium.

Considering the fact that leukocyte and HSPC mobilization from BM partly depends on surface S1P1 [49][4] and the fact that CD69 surface levels negatively regulate those of S1P1, one attractive explanation for this is that CD69 targeting is blocking its interaction with S1P1, allowing the accumulation of the latter on the cell surface and promoting its function in cell egress. Consistently with this possibility, anti-CD69 treatment leads to an increase of surface S1P1 levels. Of note, increase of S1P1 surface levels by transgenic overexpression in B cells was sufficient to increase B cell egress from the BM and B cell numbers in the periphery [162]. However, the fact that, for example, one of

the cell types undergoing higher mobilization, the neutrophils, does not express detectable surface CD69, not even in the huCD69 transgenic mouse, argues against that blockade of CD69 cis- interaction with S1P1 is, if anything, the main mobilizing force induced by CD69 targeting, at least not for all the cell subtypes, since that effect would be cell intrinsic. Nevertheless, a possible mechanism for a bystander effect of CD69 targeting could be that the egress CD69+ cells enhanced the egress of other CD69- cells. A similar phenomenon has been described for neutrophils, the first cells to be mobilized, whose egress paves the way for the mobilization of other cell types [278]. Since BM neutrophils are CD69-, the first mobilized cells in our case would be other cell types, likely B cells. Possibly related with this phenomenon, a positive feedback for cell mobilization can be envisaged from a recent publication showing that the increase in vascular permeability of the BM sinusoids induced by blockade of endothelial CXCR4 by AMD3100 enhances HSPC motility and egress through the influx into the parenchyma of ROS [279].

The fact that both anti-CD69- and G-CSF/AMD3100-induced mobilizations are inhibited by FTY720 could be simply due to a common bottleneck last step consisting in a need for the function of S1P receptors-mediated for massive cell egress in stress-simulating situations. However, a more complex crosstalk has been reported between CXCR4/SDF-1 axis and S1PR roles in mobilization: apart from the fact that G-CSF and AMD3100 increase plasma and BM levels of S1P, S1P also induces release of SDF-1 from stromal cells through ROS induced signaling [4][264]. SDF-1 can quickly diffuse from the BM towards blood and can promote chemotaxis and cell egress [264]. This crosstalk is compatible with the mentioned putative positive feedback mechanism by which an initial increased vascular permeability would promote diffusion of S1P from the plasma to the BM and of SDF-1 from the BM to plasma amplify mobilization.

CXCR4-mediated mobilization with AMD3100 of B cells was reduced in B-cell conditional S1P1 KO mice, [280]. Like in our results using anti-huCD69 MAbs, the mobilization rate was higher in immature B cells than in B cells in earlier developmental stages. This was related to a decreased motility of the latter [162]. This observation suggested that other factors apart from CXCR4/SDF-1 determine cell motility and migration capacity.

The effects of CD69 targeting and of AMD3100 in decreasing bone marrow cellularity were similar and were neither synergistic nor additive. If the process of bone marrow egress was not saturated, this would point to that the molecular mechanisms are not independent, that is, that at least the downstream molecular mediators are shared in both pathways. The synergy or additive effects

observed upon concomitant VLA-4 and CXCR4 blockades have been proposed as signs of independent molecular mechanisms of bone marrow mobilization [54] [56] [269]. However, a synergic effect of G-CSF with AMD3100 was observed on the increase of blood colony-forming units counts at 1h post-AMD3100 treatment, in spite of that G-CSF induces mobilization through CXCR4 and CXCL-12 [34]. In those experiments, G-CSF was administered 2-4 days before AMD3100 injection. It is possible, thus, that the G-CSF has had the time to induce bone marrow HSPC proliferation, and that these expanded HSPC are further mobilized by AMD3100 treatment, contributing to the synergic effect. In our experiments we administered anti-CD69 MAb and AMD3100 at the same time, and, in spite of the fact that anti-CD69 induces cell proliferation, we might not have allowed the time for significant leukocyte expansion and for such a synergic effect to be evident in total leukocyte.

Even if the mobilization by agents disrupting the SDF-1/ CXCR4 axis is also dependent on S1P receptors function, a synergy was observed in the chemotactic effects induced by SDF-1 and S1P. However, this was observed in *in vitro* studies, in which the roles in S1P in processes other than chemotaxis and that may have a more prominent effect *in vivo*, like the decrease of endothelial permeability, may be missed.

In spite of possible crosstalk at more upstream levels, a bottleneck effect of a common S1PR-dependent last step of cell egress could still explain the non-synergic nor additive effects of AMD3100 and anti-CD69 MAbs.

If the effect of CD69 targeting was solely based on direct blockade of CD69 interaction with S1P1 and upregulation of the S1P1 surface levels, it would act at the level of this final step of S1P1-dependent cell egress. This mechanism could explain the upregulation of surface S1P1 levels but not that of CXCR4 expression observed upon CD69 targeting. CD69 targeting-mediated S1P1 upregulation is not likely to be the cause of the increase of CXCR4 surface levels, since S1P1 deficiency in B cells had no effect on CXCR4 expression [280] and S1P1 overexpression even decreased CXCR4 expression on HSC [281]. Golan et al. report that S1P1 expression on LSK cells is upregulated by G-CSF and AMD3100 treatment, and that this is inhibited by co-treatment with FTY720. In the case of G-CSF, they show that S1P1 upregulation is dependent on mTOR signaling. [4]. These reports, together with the fact that we observe S1P1 upregulation also on cell types with non-detectable CD69 surface expression, point to that this upregulation is not based on the interaction in cis between CD69-S1P1 but rather on anti-CD69-induced mTOR signaling. Moreover, the fact that we observe upregulation of CXCR4 and that this has also been described upon G-CSF treatment [3], strengthen the point that a possible crosstalk

between anti-CD69 mAbs and CXCR4/SDF-1-induced mechanisms of mobilization at upstream steps should not be disregarded.

In conclusion, our data suggest that the treatment with anti-human CD69 could be used as a new mobilizer since it achieves a greater egress of bone marrow cells and a higher production of haematopoietic stem cells and this is mediated by S1P1, modulation of the axis SDF-1/CXCR4 and by the influence on mTOR. Furthermore, as previously described, it induces a significant increase proliferation rate than that induced by AMD3100. This results, attached with the benefit of not requiring a continuous dosage and due to the effects are seen at early times, makes it a good alternative to G-CSF. We propose the use of anti-human CD69 as a new mobilizer, alone or in combination with AMD3100 to support the mobilization of these cells and as an alternative to treatment with G-CSF.

CHAPTER III:

CD69 TARGETING EFFECT IN VACCINIA VIRUS INFECTION.

RESULTS

CD69 targeting induces an increase and proliferation of all leukocyte subsets

In this work, we aimed to know the effect of targeting of CD69 in VACV infection. For this, we have used the previously characterized mAb anti-CD69 2.2 that binds to mouse CD69 molecule and mAb anti-CD69 2.8 that binds to human CD69 molecule [172] (chapter II). Both were obtained from the same immunization in CD69 deficient mice, do not cross-react and not activate the complement system or Fc binding effector functions. We have reported that CD69 targeting induced cell mobilization affecting to all progenitors cells from bone marrow at 24 hours after treatment (chapter II). With the aim of ensuring that anti-CD69 treatment would be effective against a viral infection, we injected two doses of 200ug of anti-CD69 separated by a week and the cellularity of leukocyte subtypes were analyzed in BM and spleen 5 days after the second dose. Cellularity of main leukocyte subsets were observed decreased in BM and increased in SLO (Fig.25A-C). Similar pattern was found in total cell counts in bone marrow and spleen (Fig.25A) and the cell subsets profiles were largely similar to the ones seen 24 hours after the first treatment (chapter II), with the main difference in BM and spleen neutrophil and spleen macrophage numbers which were back to control levels (Fig.25B-C)

To better characterize the action of CD69 targeting in spleen cell proliferation, we treated with anti-CD69 2.8 mAb in transgenic mice that express human CD69, and analyzed BrdU incorporation in main spleen leukocytes subpopulations (Fig. 25D). As shown in figure 25, this treatment induced proliferation in almost all different subsets of splenic leukocytes.

As $CD69^{-/-}$ mice had increased cellularity in SLO and an augmented anti-Vaccinia immune response mediated by increased number of NK cells and that CD69 targeting in uninfected mice led to a similar pattern of increased leukocytes in peripheral lymphoid organs (Chapter II), we studied whether anti-CD69 treatment would increase the immune response against infection with VACV infection in WT mice.

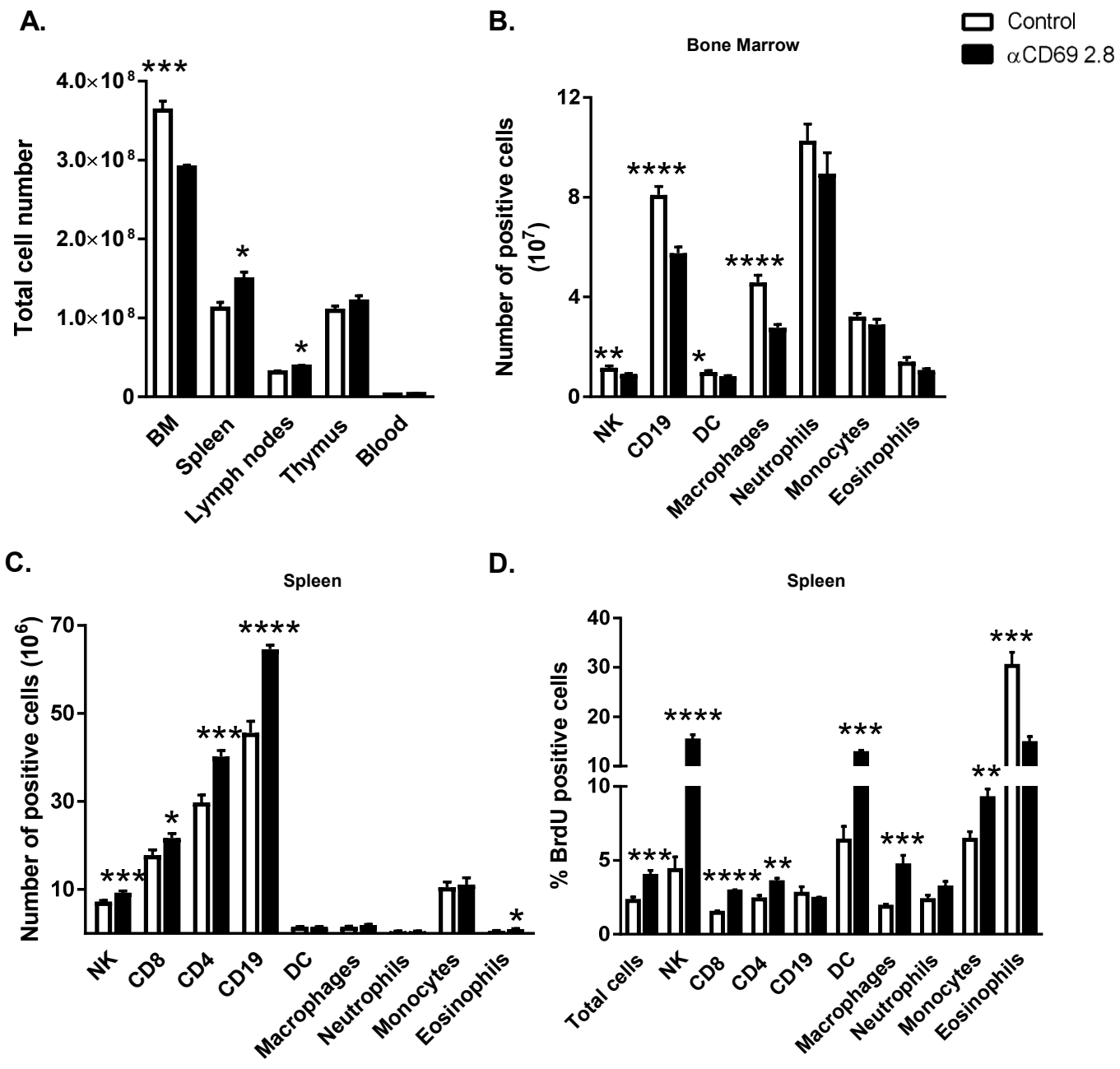


Figure 25. HuCD69 mice treated with anti-human CD69 *in vivo* showed an increase proliferation in spleen and a similar effect with two doses compared to one dose treatment. A-C, Mice were treated with two doses of 200ug of anti-huCD69 2.8 separated by a week and five days after second treatment, mice were analyzed. A, Total cell number in Bone Marrow, Spleen, thymus, lymph nodes and blood. B-C, Numbers of lymphoid and myeloid subpopulation cell in B, Bone Marrow and C, Spleen. D, Mice were treated or not treated with 500 ug of anti-huCD69 2.8 24 hours before BrdU injection. Mice received 1 mg of BrdU intraperitoneally and three hours later, mice were analyzed. Spleen cells were collected and cell proliferation rate was assessed by BrdU incorporation. BrdU was measured by flow cytometry in lymphoid and myeloid subpopulations. Pool of two experiments

Mouse CD69 targeting enhances immune response to VACV infection in Rag2$^{-/-}$ mice

In order to assess the importance of the innate immunity response against VACV infection in anti-CD69 treated mice, we infected Rag2$^{-/-}$ anti-mouse CD69 treated mice with 1x10^{6} pfu of VACV in the schedule shown in figure 26A and it was performed the analysis at two days after infection, when the early immunity was developed (Fig26A). Interestingly, the mice treated with anti-mouse CD69 2.2 exhibited less viral titers than control mice even in the absence of innate Tα/β and Tγ/δ cells (Fig. 26B). CD69 targeting induced a decreased and increased cell numbers in bone marrow and spleen respectively, compared to control mice (Fig. 26C). However, a significantly increase in total blood and thymus leukocyte counts was not detected. Bone marrow of anti-CD69 targeted mice had reduced numbers of all precursor cells as NK, DC and other myeloid cells and more primitive precursor cells as c-Kit+ cells compared to control mice (Fig. 26D and data not shown). Consistently, in spleen, CD69 targeting induced an augmentation of NK cells, monocytes and neutrophils (Fig. 26E). Remarkably, we have found a lower number of bone marrow leukocytes in steady state and infection in Rag2$^{-/-}$ CD69$^{-/-}$ mice compared to WT mice (Fig 26F) These results are consistent with the data previously shown of the increased splenic leukocyte number after infection of Rag2$^{-/-}$ CD69$^{-/-}$ mice (Figure 5A in chapter I). These results demonstrated that in Rag$^{-/-}$ mice, anti-CD69 treatment increases control of viral elimination and is accompanied by augmented number of leukocyte.

To assess whether activation was altered by CD69 targeting, we measured the production of effector cytokines such as IFNγ- and TNFα and the CD107a as marker of degranulation activity within NK cells. VACV infected mice treated with anti-CD69 2.2 had an increase of around 30 percentage of IFNγ- and TNFα-producing NK cells but CD107^{+} NK cells were increased although not significantly (Fig 27A). Since the number of NK cells were augmented in the CD69 targeted mice, the numbers of splenic IFNγ- and TNFα-producing NK cells reached more than double of the control mice and CD107^{+} NK cells were significantly increased (Fig. 27B).

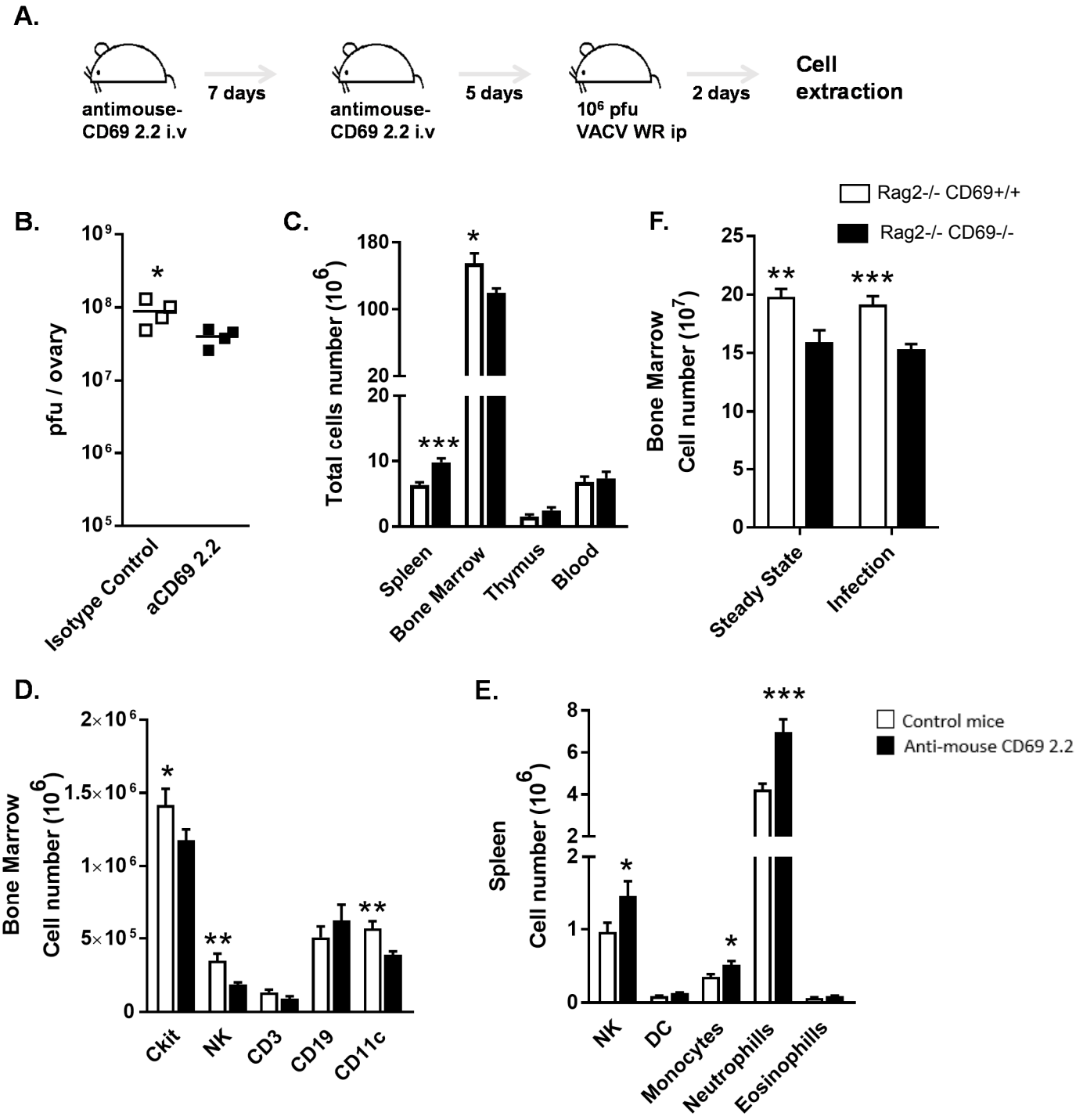


Figure 26. mAb anti-mCD69 2.2 treatment in Rag2$^{-/-}$ CD69$^{+/+}$ mice induced an increased accumulation of leukocytes. A, Mice were treated with anti-mCD69 2.2 or PBS with two doses separated by a week and 5 days after second treatment, mice were infected with 1x10^6 pfu i.p and two days after, mice were sacrificed. C-E, Cells were collected from treated and not treated mice, and subjected to flow analysis. B, Viral titers were analyzed in ovaries after two days of infection. C, Absolute cell numbers of spleen, thymus, bone marrow and blood in anti-mCD69 2.2 treated mice compared to untreated mice. D-E, Lymphoid and myeloid subpopulations cell numbers were analyzed in D, Bone Marrow and E, spleen. Pool of three independent experiments. (At least n=9 in each condition). B, One experiment representative of two independent experiments.

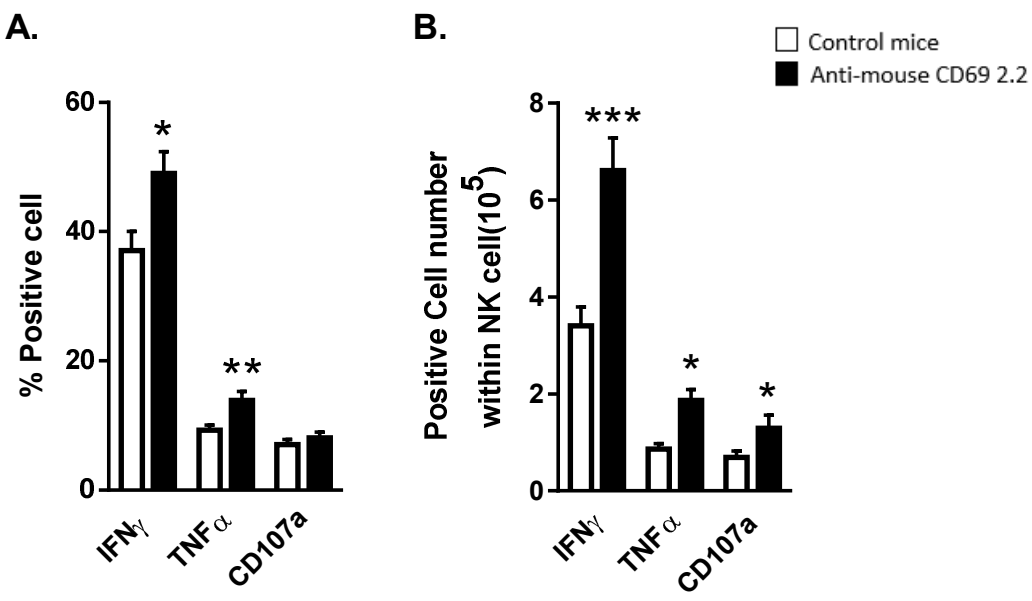


Figure 27. mAb anti-mCD69 2.2 treatment in Rag2$^{-/-}$ CD69$^{+/+}$ mice promoted NK cell activity. Splenocytes were assayed for intracellular production of IFN-γ, TNFα and CD107a in NK cells. A, percentages and B, cell number. A and B, pool of three independent experiments.

Together, these results showed that mouse CD69 targeting increases the innate immune response against VACV infection, increasing leukocyte counts, enhancing NK cell activity and increasing immune response to VACV infection. In a similar VACV infection setting in the absence of CD69 (chapter I), we found that leukocyte numbers were increased, including NK cells, that led to an increased VACV elimination though NK cells activity was not changed. These data support the idea that the increased production of TNFα, IFNγ and cytolytic exocytosis of NK cells observed during CD69 targeting upon infection may be due to signaling derived of the anti-CD69 engagement.

Mouse CD69 targeting enhances immune response to VACV infection in WT mice.

Since in immunocompetent mice, T lymphocytes play a pivotal role in VACV infection response, we followed the scheme shown in figure 28A in order to observe the effect of CD69 targeting when adaptive response has been developed. Thus, mice were treated with anti-mouse CD69 2.2 and infected with 1x10^7 pfu and analyzed seven days after infection (Fig.28A). We have found that mice

treated with anti-mouse CD69 2.2 were highly more efficient in removal Vaccinia virus infection than PBS-injected control mice (Fig.28B). The VACV infected CD69 targeted mice had decreased total leukocyte numbers in bone marrow and a strong increase of these numbers in the spleen, lymph node and blood compared to control mice (Fig.28C). When analyzing the main leukocyte subsets in the spleen, we observed a marked increase in cell numbers of all main subsets analyzed (Fig. 28D). Importantly, the number of TNFα and IFNγ cytokine producing cells were increased in splenic lymphocytes (Fig. 28E), specifically TNFα in T CD4 cells and IFNγ in CD4, NK and CD8 lymphocytes thought no major differences in the percentages of these subsets were observed (data not shown).

Thus, the targeting of mouse CD69 and later VACV infection resulted in accumulation of leukocytes including effector lymphocytes in peripheral sites and increased the ability to clear VACV infection.

Overall, these results showed that mouse CD69 targeting increases the innate and adaptive immune in response to VACV infection.

Anti- human CD69 mAb treatment of huCD69 transgenic mice induces enhanced anti-VACV response.

To approach a clinical application of anti-CD69 treatment in VACV responses, we treated human CD69 transgenic mice with anti-human CD69 2.8 mAb using the same schedule as before (Fig. 29A). At two days after VACV infection, mAb CD69 2.8 treated mice were more efficient in clearing the virus (Fig. 29B) and it was found an enhancement of total splenic cell number and blood (Fig. 29C). The number of all splenic subtypes analyzed was increased compared to control mice (Fig. 29D). When we analyzed the cytotoxic capacity of NK cells and CD8 and CD4 T lymphocytes at two days after infection, we observed that the treatment induced an increase in the number of TNFα-producing cells in all subpopulations and a significant increase in the number of IFNγ-producing cells in NK cells and CD4 cells, and a trend in CD8 cells (Fig. 29E). These results revealed that the innate immune response to Vaccinia virus infection is increased in the targeting of human CD69.

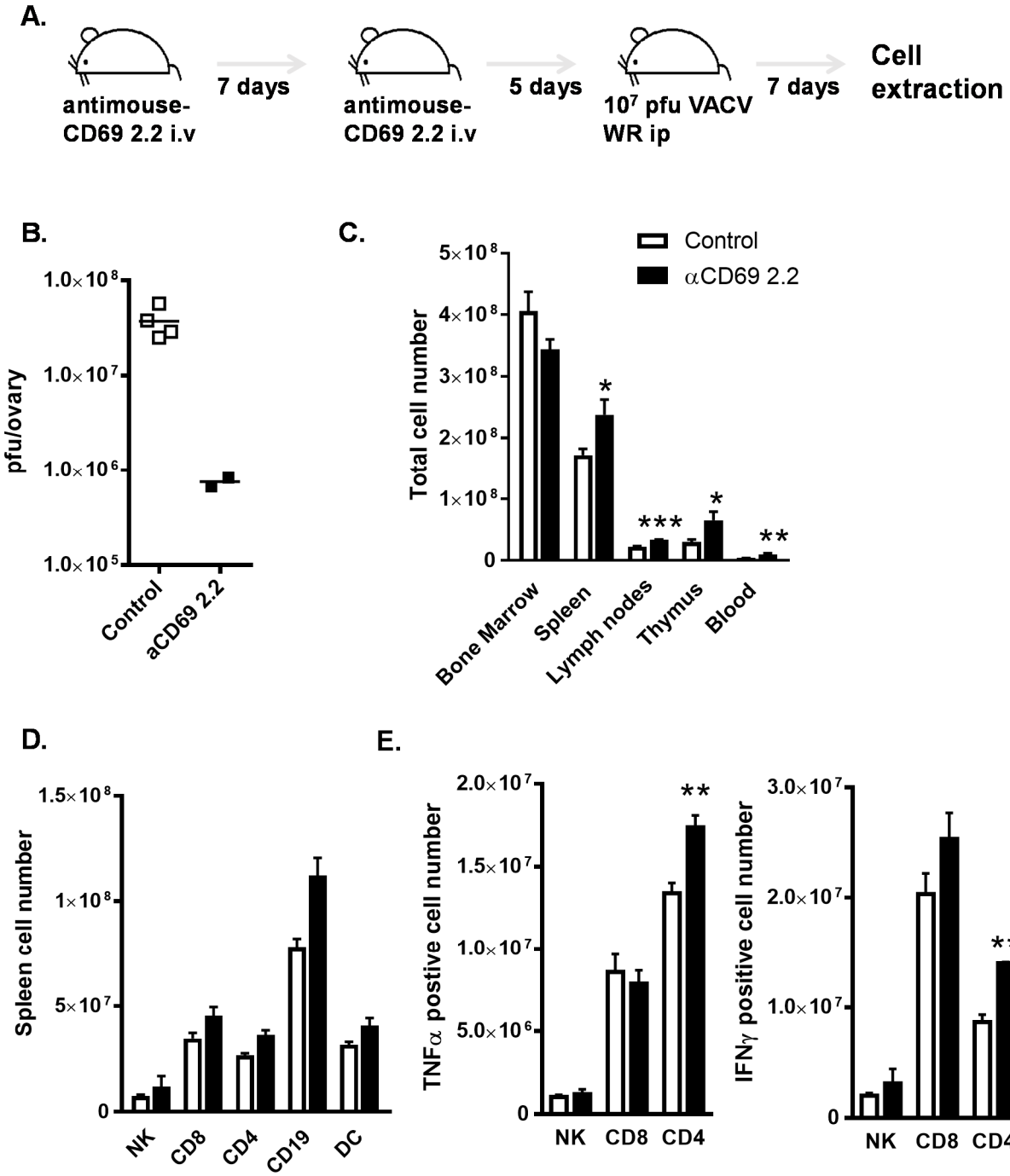


Figure 28. mAb anti-mCD69 2.2 treatment in CD69$^{+/+}$ mice also induced an increased accumulation of leukocytes in spleen. A, C57bl/6 mice were treated with PBS or 200ug of anti-mCD69 2.2 with two doses separated by a week and five days after second treatment, mice were infected with 1x10^7 pfu i.p and 7 days after mice were analyzed. Cells were collected from treated and not treated mice, and subjected to flow analysis. B, Ovaries were collected seven days after infection and viral titers were measured. C, Absolute cell numbers of spleen, thymus, bone marrow, lymph nodes and blood in anti-mCD69 2.2 treated mice compared to untreated mice. D, Number of different lymphoid and myeloid subpopulations in spleen. E, Numbers of IFNγ and TNFα-producing cells in spleen.

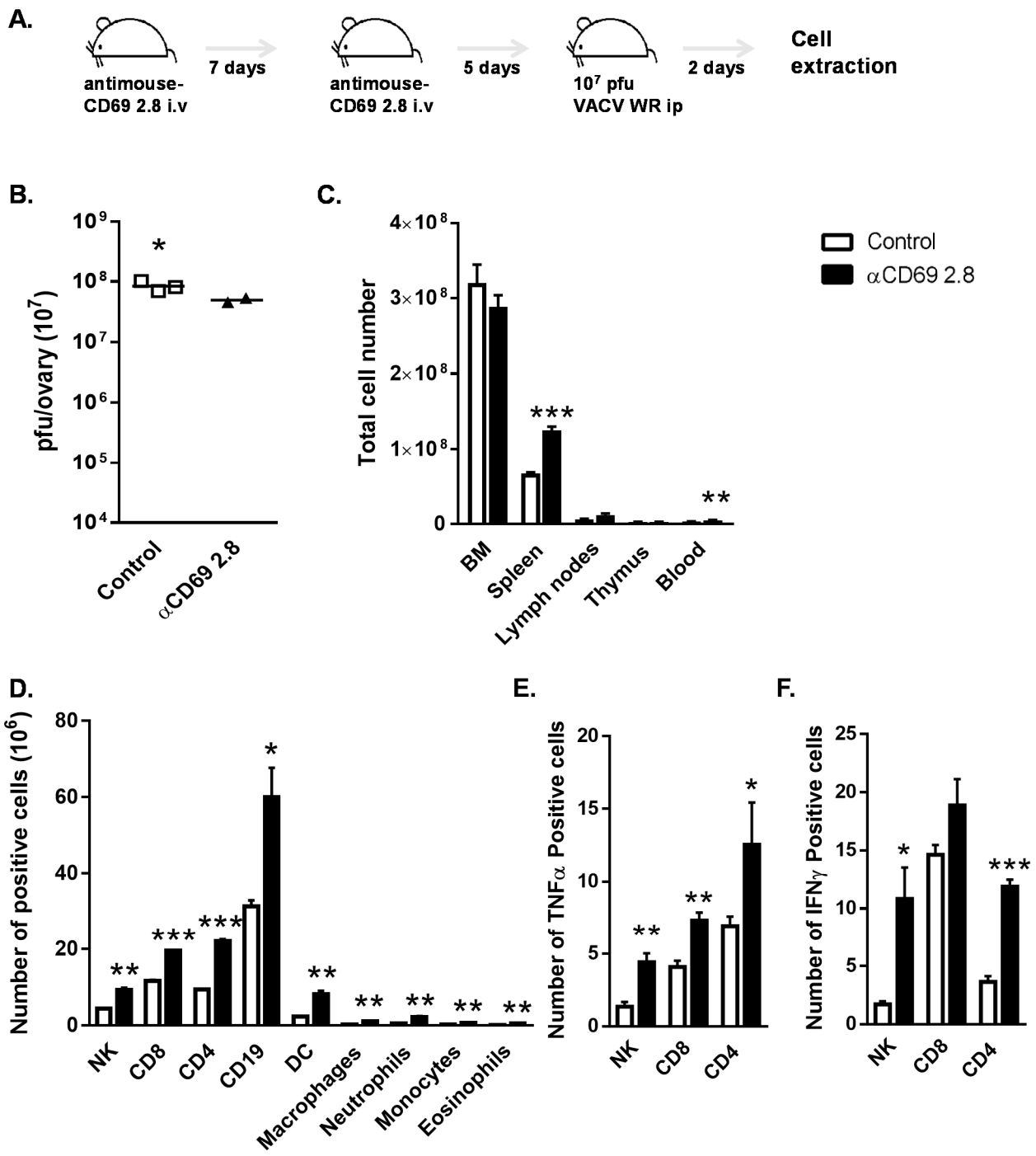


Figure 29. Early anti-VACV response upon Targeting with anti-human CD69 2.8 mAb promoted a better virus clearance and a concomitant accumulation of leukocytes two days after infection. A, Mice were treated with 2 doses of 200ug of anti-huCD69 2.8 or PBS i.v with a gap of 1 week and 5 days after second dose mice were infected with VACV and analyzed 2 days after infection. B, Viral titles in ovaries. C, Absolute cell numbers of spleen, bone marrow, lymph nodes, thymus and blood. D, Numbers of leukocytes subpopulations in the spleen. E-F, Numbers of IFNγ and TNFα-producing cells in spleen.

When analyzing HuCD69 mice after 7 days of infection (Fig. 30A), we observed that anti-human CD69 2.8 treatment cleared VACV infection more efficiently than control mice two days after infection (Fig. 30B). These mice had induced similar decreases and increases in total leukocyte counts in BM and spleen, respectively, than those WT mice treated with anti-mCD69 targeting, and a small increase in lymph nodes and blood leukocyte counts (Fig. 30C). In control experiments, the treatment of $CD69^{+/+}$ mice with anti-hCD69 2.8 did not alter BM or spleen cell numbers, proving that the effect was specific of human CD69 targeting (data not shown). In all main leukocyte subtypes, we observed a tendency to increased numbers whereas CD8 T cells, macrophages and eosinophils were increased significantly (Fig. 30D). The analysis of TNFα and IFNγ-producing cells showed an increase in both cytokines in CD4 T cells (Fig. 30E).

In summary, *in vivo* targeting of human CD69 mice with mAb 2.8 previous to VACV infection, increases viral elimination compared to non-treated mice both at early times, 2 and 7 days after infection and is accompanied by augmented cell number in peripheral lymphoid organs.

The effect of anti-huCD69 2.8 on cytokine and chemokine production.

To further investigate the mechanisms implicated in the increased anti-VACV response in treatment with mAb CD69.2.8, the expression of soluble immune mediators in splenic cells was evaluated. Q-PCRs were performed on splenic cells obtained from huCD69 mice 1 day after treatment with mAb huCD69.2.8 mAb (Fig. 31). The results showed a highly significant increase in IL-1α, IL-1β, Lymphotoxin α and Lymphotoxin β, IFNγ, IL-17b and IL-17f mRNA expression (Fig. 31A), and a huge increase in CCL2 and CCL12 in the mAb CD69.2.8–treated mice (Fig. 31B). Levels of IL-17a expression were unchanged (Fig. 31A). Because it is well documented that these cytokines and chemokines are important players in viral infection, these results are consistent with the augmented capacity of *in vivo* VACV elimination by mAb CD69.2.8.

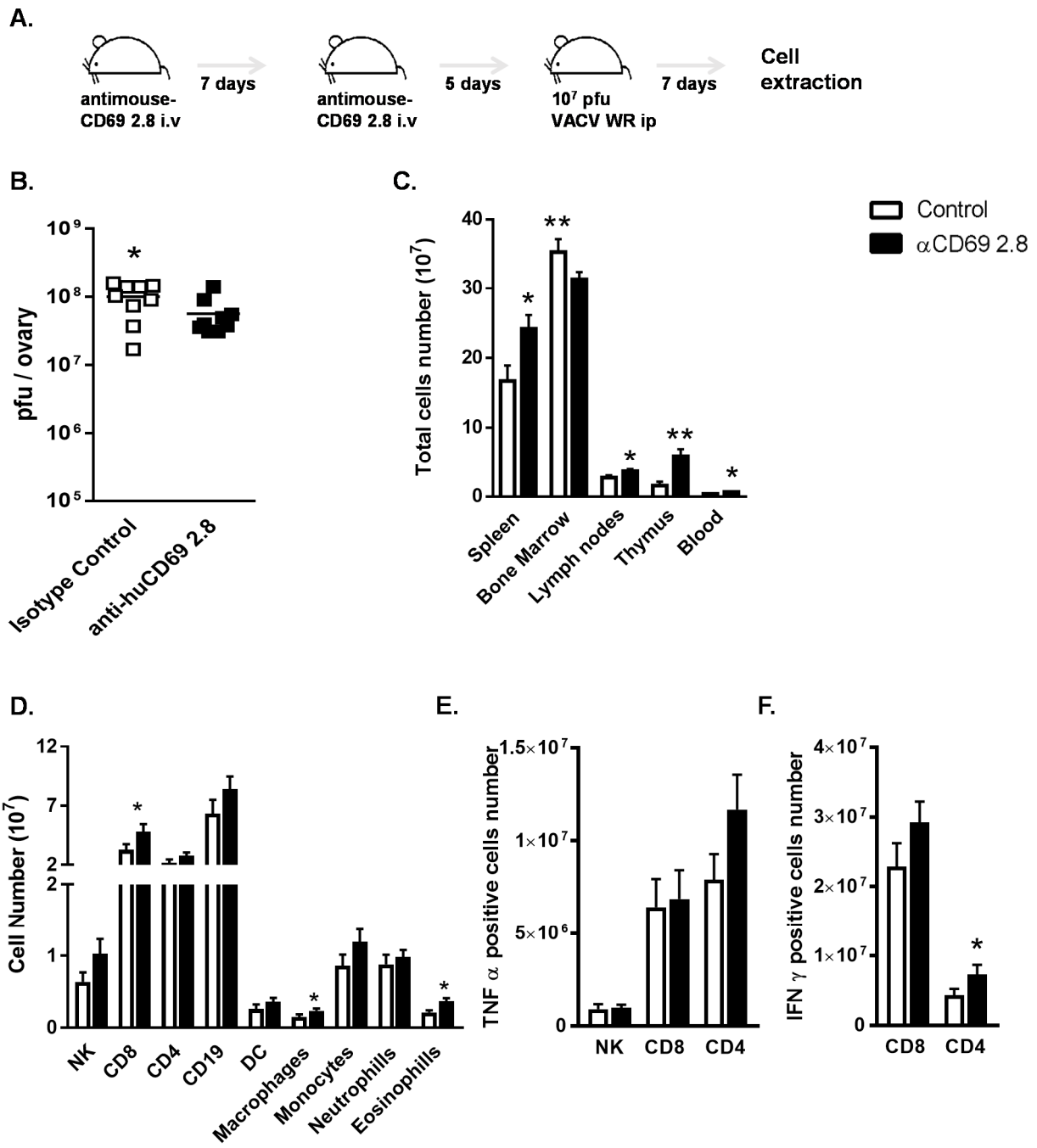
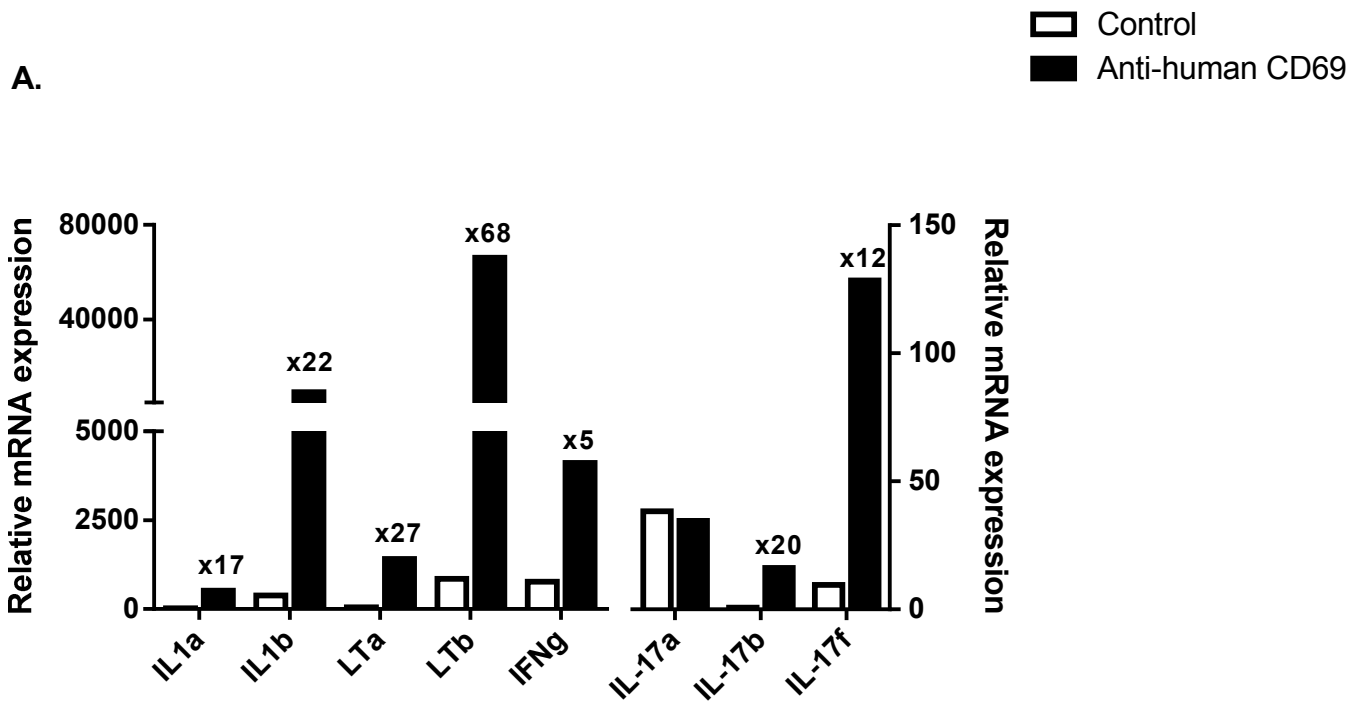


Figure 30. HuCD69 mice treated with mAb anti-huCD69 2.8 exhibited a higher egress of Bone Marrow cells in Vaccinia virus infection. A, Mice were treated with PBS or 200ug of anti-huCD69 28 with two doses separated by a week and five days after second treatment, mice were infected with 1x10^7 pfu i.p and 7 days after, mice were analyzed. B, Measures of viral titres in ovaries. C, Absolute cell numbers in spleen, bone marrow, lymph nodes and blood. D, Number of different lymphoid and myeloid cells in spleen. E-F, Numbers of IFNγ and TNFα-producing cells in spleen Pool of two experiments.



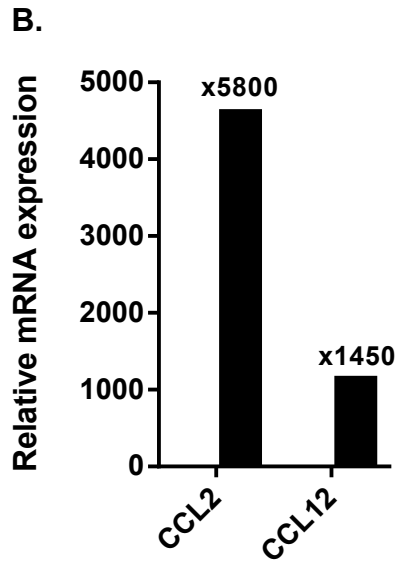


Figure 31. Increased induction of proinflammatory cytokines and chemokines production. Spleen cells were collected from anti-human CD69 treated mice and control mice. Q-PCR analysis of proinflammatory cytokines and its receptors was assayed. A, Data from IL-17a, IL-17b, IL-17f, IL1-α, IL-1β, LTa, LTb and IFNγ. B, Data from CCL2 and CCL12. Data are represented in a graph as fold-induction to β-actin.

DISCUSSION

Recent insights in CD69$^{-/-}$ mice have shown that CD69 molecule is a regulator of leukocyte egress from BM to periphery and is able to increase anti-VACV response (chapter I and chapter II). Both, anti-mouse CD69 named mAb 2.2 used in immunodeficient and WT mice and the novel anti-human CD69 named mAb 2.8 employed in transgenic mice expressing human-CD69, do not deplete CD69-expressing cells and have been associated with mobilization of bone marrow cells to peripheral lymphoid organs in steady state (Chapter II). Here we report that the murine anti-CD69 mAb 2.2 and the human anti-CD69 mAb 2.8 display a potent host anti-VACV responses similar to CD69$^{-/-}$ mice. These anti-VACV responses are a consequence of a maintained increased cellularity in SLO and reduced in BM. Furthermore, treatment of healthy animals expressing human CD69 with mAb CD69.2.8 induces an increase in leukocytes rate proliferation of SLO and an increase of cytokines expression such as IL-1α, IL-1β Lymphotoxin α and Lymphotoxin β, IFNγ, IL-17b and IL-17f mRNA and chemokines such as CCL2 and CCL12 in the absence of VACV infection, unveiling a new role for mAb CD69.2.8 in triggering cytokines and chemokines expression. Importantly, this occurs with a significant decrease of TGF-β levels (chapter II, Fig. 23). Here, we significantly improve the knowledge of the effect of pretreatment with anti-CD69 in cytokine production in healthy animals since in previous works only it has been described an effect in increasing IFNγ production in NK cells and an increase in IL-17 production in splenocytes from healthy animals. However, treatment with anti-mouse CD69 2.2 studied in different mouse models diseases had already revealed a high variety of different cytokines and chemokines production [129, 145, 147, 172, 173]. Thus, during VACV infection the effect of mouse CD69 targeting in Rag2$^{+/+}$ CD69$^{+/+}$ mice, but not in CD69 deficiency mice, was the increase in NK cell activity through the induction of TNFα and IFNγ production supporting that anti-CD69 treatment augmented cytokine production. The mechanisms involved associating CD69 with Th17 differentiation have been described to be related to Jak3 and Stat5 signaling pathway [128]. However, the mechanisms which induce the increase of cytokines and chemokines production by anti-human CD69 treatment remain to be elucidated. The reduction of TGF-β may explain these results, since the inhibition of regulatory cytokines mediating the increase immune response was reported to occur through blockade of TGF-β signaling in T cells or diminishing TGF-β presence on antigen-presenting cells. Reduction of TGF-β production has been reported in almost all model of diseases described in CD69$^{-/-}$ mice and mAb -2.2 treated mice [1, 126, 129, 141, 142, 172, 173]. In agreement with the increase in IL-2 production

reported in human CD69 targeting mice (chapter II) and the anti-CD69 induction of T cell proliferation through production of IL-2 by DC [104], the treatment with mAb CD69.2.8 induces leukocyte proliferation of all subset in SLO shown here and in BM (Chapter II).

The data presented here in anti-CD69 mAb–treated mice resemble the results obtained in $CD69^{-/-}$ mice in almost all levels examined, including increased capacity to eliminate VACV infection, increased leukocyte subsets cellularity in SLO, increased proinflammatory cytokines and decreased TGF-β expression. Importantly, we and other groups demonstrated that treatment with mAb CD69.2.2 internalizes CD69 and that mAb CD69.2.2–treated mice showed these findings few hours after mAb CD69 treatment [131, 172].

In conclusion, our observations indicate that mAb CD69.2.2 that reacts with mouse CD69 and mAb CD69 2.8 that reacts with human CD69 have similar effects, which are partially comparable to the absence of CD69. The properties of anti-CD69 mAb as adjuvant in immune response to infection may help in vaccination, since Vaccinia virus is widely used as viral vector, carrying heterologous antigens in most currently studies in vaccines clinical trials and is considered the best positioned candidate to be used in the priming phase when two different viral vector are used.

CHAPTER IV:
OVEREXPRESSION OF CD69 IN VACCINIA VIRUS INFECTION.

RESULTS

Altered leukocyte distribution by CD69 overexpression in mice.

Though it has been widely studied the influence of the absence of CD69, an extensive study of the effect of the overexpression of CD69 has not been approached. To study the effect of CD69 overexpression in the homeostasis and function of immune cells, we analyzed mice that carry 6 copies of BAC containing CD69 gene (herein after referred as Hi-CD69.BAC mice). As shown in figure 32 (and data not shown), all leukocyte subpopulations in bone marrow and lymphoid organs express CD69 at very high levels in Hi-CD69.BAC mice. Then, we analyzed at steady state the number of total leukocytes subsets in BM and lymphoid organs of Hi-CD69.BAC compared to WT mice (Figure 33A). Studying bone marrow, NK cell numbers were augmented but no significant differences were observed neither in total cell number nor in the number of any other subset analyzed (Figure 33A and B). NK cells were also increased in the thymus, although total cells were not altered by the overexpression of CD69 (Fig. 33A and C). In addition, it was observed an important increase in SP CD4 and SP CD8 thymocytes, concomitant with a decrease of CD4 CD8 DP and CD4 CD8 DN thymocytes (Fig. 33C). These results supported the reported function of CD69 as regulator of S1P1 expression, controlling thymocytes egress [158, 282] and are in accordance with the previous reports that analyzed overexpression of CD69 in thymocytes [140, 161]. In the spleen of Hi-CD69.BAC mice, total spleen cell number was decreased and lymphocytes numbers were reduced eleven-fold in CD8 T cells, three-fold in CD4 T cells and almost two-fold in B cells compared to WT mice (Figure 33A and D). Consequently, number of NKs and myeloid cells were found increased (Fig. 33D). Similarly to spleen, in blood of CD69 overexpressing mice, it was observed a decrease in total cell number, due to a drastically decreased of T and B cells compared to WT mice (Fig. 33A and 33E). However in lymph nodes the decrease in total cells number of 45 percent, corresponded to the decrease in T lymphocytes cell number but not in the number of B cells (Fig. 33A and 33F). Together these results showed that CD69 controls the exit of mature SPCD4 and SPCD8 cells in thymus and the release of B cells from the bone marrow [158, 163, 280, 283]. However, the increase in the NK cell numbers in bone marrow, thymus and spleen, cannot be explained by the possible interaction of CD69 and the known role described by S1PRs in these cells.

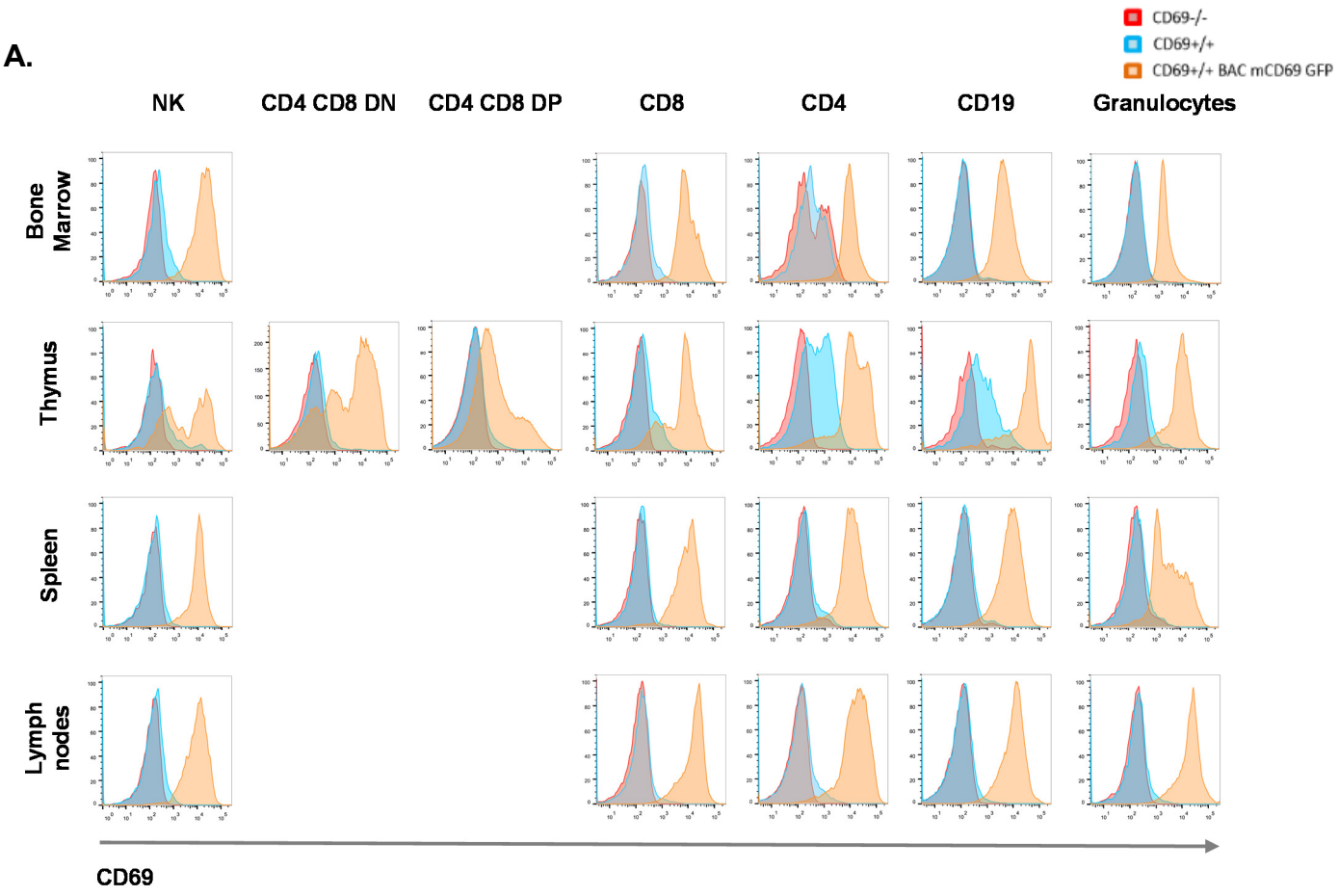


Figure 32. Hi-CD69.BAC mice overexpress CD69 in hematopoietic and immune cells. CD69 expression is shown in subsets of collected cells from thymus, spleen, bone marrow, and lymph nodes (brachial, axillary, maxillary, inguinal and popliteal) from Hi-CD69.BAC and control mice.

Regulatory T cells in thymus in overexpressing CD69 mice.

S1P1 has been described as an intrinsic negative regulator of thymic differentiation, peripheral maintenance and suppressive activity of Treg cells [284]. Because S1P1 is down-regulated by CD69 overexpression, regulatory T cells in Hi-CD69.BAC were compared to WT mice. Accordingly with the function of CD69 in retention of T lymphocytes in thymus, we found an increase in the cell numbers of the major regulatory T cell subpopulations in thymus, being the most prominent increase in the

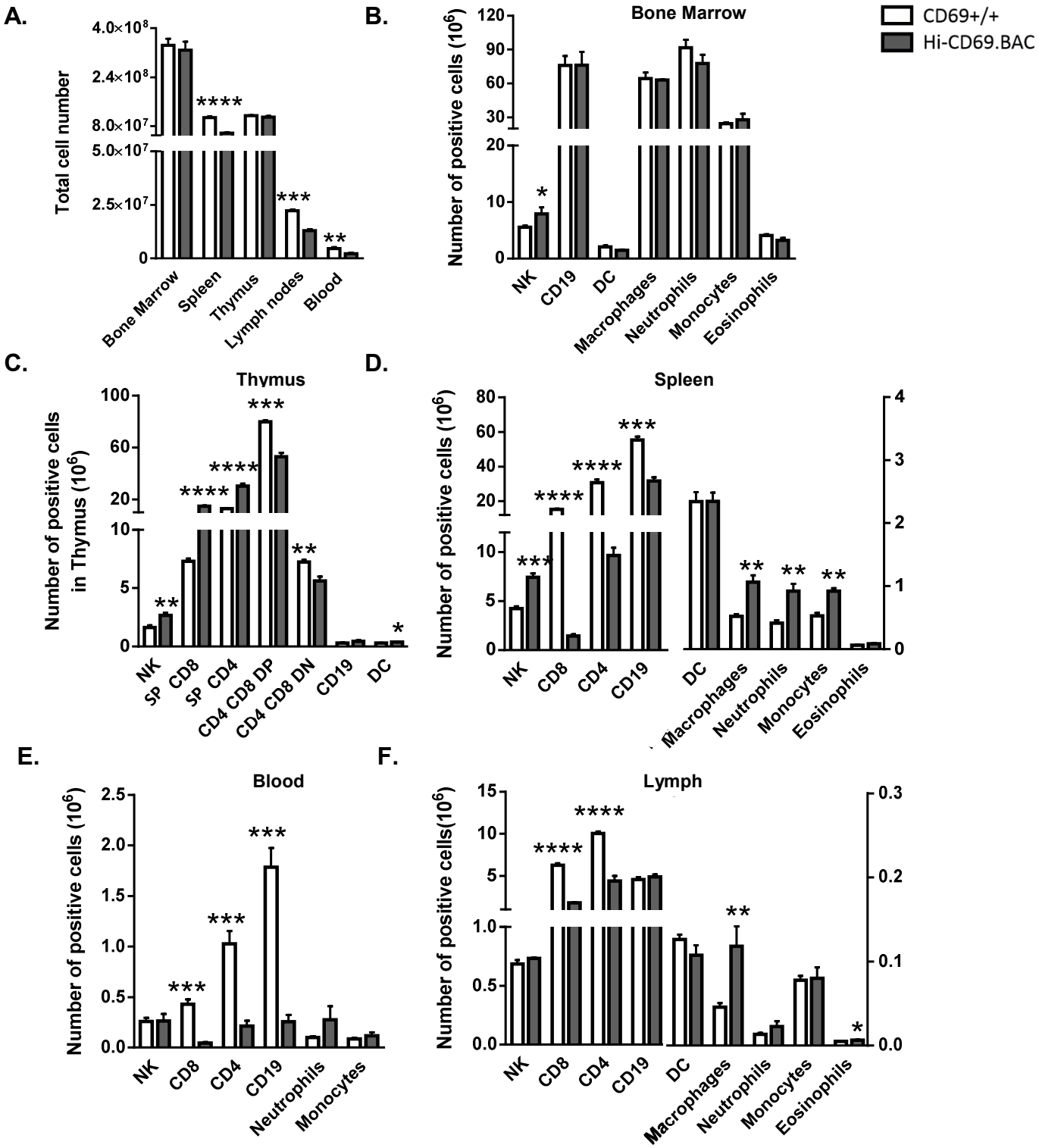


Figure 33. Hi-CD69.BAC mice showed deregulated leukocytes homeostasis. Bone marrow, thymus, spleen, lymph nodes and blood cells were collected from WT and transgenic mice and absolute cell number were counted in A. B-F, Numbers of lymphoid and myeloid subpopulations in B, bone marrow, in C, thymus, in D, spleen, in E, blood and in F, lymph nodes in WT mice compared to CD69+/+ BAC mCD69. One experiment representative of three.

FOXP3^{+} CD25^{+}CD4+ T cells of more than three-fold (Fig.34A). This increase is higher than the observed by SP CD4+ mature thymocytes that was about two-fold (Fig. 33C and 34A), agreeing with the role of CD69 as positive regulator of Treg differentiation. In spleen, a decrease of about 70% were observed in FOXP3+CD25^{+}CD4+ cells, a decrease proportional to the decreased observed in total CD4 T cells (Fig. 33D and 34C). On the contrary, in lymph node, the decrease of CD25+FOXP3+T cells is about 30% while the decrease of CD4 was more than 60% (Fig. 33F and 34B). These results point to a mechanism for selective retention of CD25+FOXP3+ Tregs in lymph nodes. Therefore, the high expression of CD69 in Hi-CD69.BAC mice can affect differentially to regulatory and non-regulatory T cells.

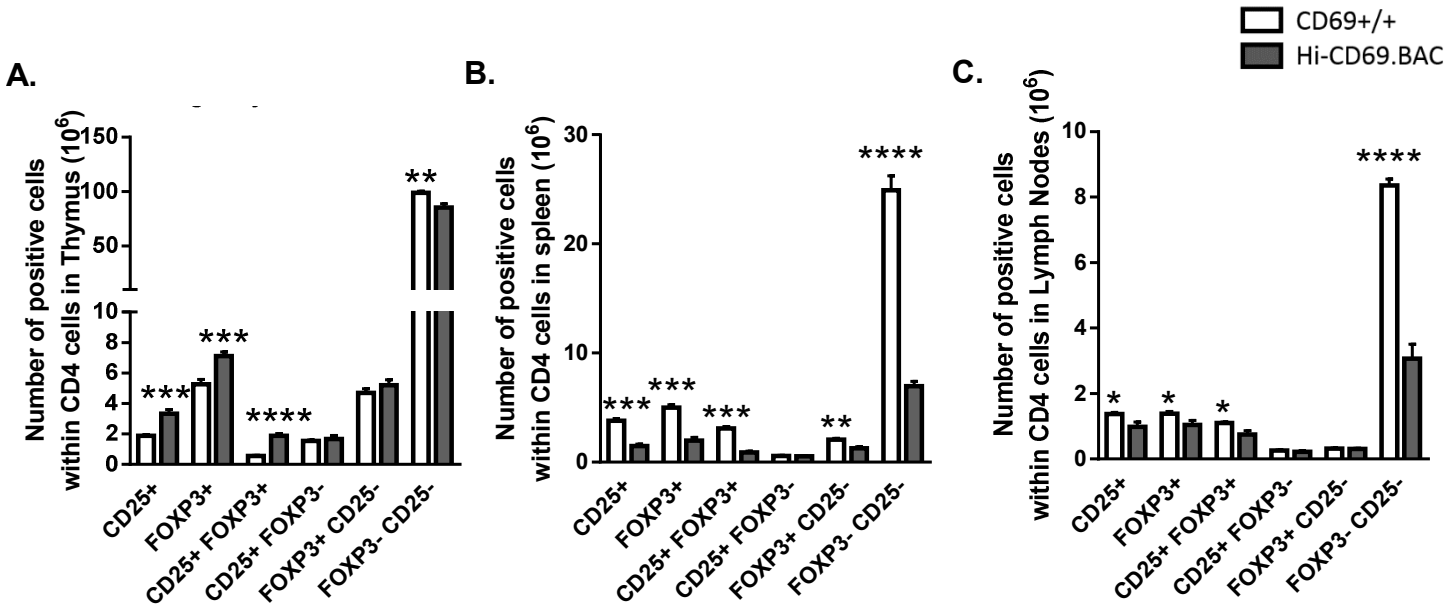


Figure 34. Influence of CD69 overexpression in Tregs distribution. Number of Tregs were analyzed in Hi-CD69.BAC mice and WT mice by flow cytometry according surface CD25 expression and Intranuclear FOXP3 expression in A, Thymus, B, spleen and C, Lymph nodes.

Innate and adaptive immune response to Vaccinia virus infection in Hi-CD69.BAC mice

To assess the implication of the overexpression of CD69 in the ability of the immune response, we analyzed the response to Vaccinia virus infection in Hi-CD69.BAC mice. Because in these mice the number of T and B lymphocytes were considerably diminished in peripheral lymphoid organs, we first evaluated this effect when the adaptive immune response has been developed. For this, we infected mice i.p. with 1x10^{7} pfu and analyzed them 7 days after infection. Surprisingly, we found that the

infection was equally resolved by Hi-CD69.BAC and WT mice (Fig. 35A). The analysis of total cell number showed that the overexpression of CD69 limits the expansion of the immune response in the spleen, because while Hi-CD69.BAC mice only expanded 20%, the splenocytes number in WT mice increased more than two-fold (Fig. 35B). Thus, in WT mice compared to Hi-CD69.BAC mice, at 7 days after VACV infection, dendritic cells had an increase of more than ten-fold, NK cells four-fold, B lymphocytes almost two-fold, CD8 T cells three-fold whereas CD4 T lymphocytes were found similar (Fig. 35C). Besides, the number of IFN-γ-producing (IFN-γ^+) and TNF-α-producing (TNF-α^+) cells within NK cells, CD8 cells and CD4 cells decreased in Hi-CD69.BAC mice compared to WT mice in similar proportion to NK, CD8 and CD4 cell number (Fig. 35D-E). Overall, these data discarded that Treg activity would be increased due the CD69 overexpression. Also, the analysis of the ratio of Treg to CD4 T cells in Hi-CD69.BAC compared to WT mice showed a similar proportion before and after infection (Supplementary figure 1). Together, these data rejected the hypothesis that the diminished number of Treg cells in Hi-CD69.BAC mice would be responsible for the increased anti-viral response observed in this mice.

Therefore, being the number of effector immune cells significantly decreased in infected Hi-CD69.BAC mice but the effectiveness of the adaptive immune response similar in Hi-CD69.BAC and WT mice, we reasoned that this could be due to an enhanced innate anti-VACV response supported by the increased NK cells and myeloid cell number found in the spleen at steady state. Accordingly, we infected Hi-CD69.BAC mice and WT mice and we analyzed the response two days after infection and we found that Hi-CD69.BAC mice removed VACV infection more efficiently than WT mice (Fig.36A). When analyzing total cells counts, an expansion about 10% and 40% of splenic cells were observed in the Hi-CD69.BAC mice and WT respectively (Fig.36B) that again point to the difficulties of CD69 overexpressing mice to recruit or expand cells in the lymphoid organ. Analysis of the leukocyte subpopulations in Hi-CD69.BAC mice compared to WT mice revealed a decrease in the number of T and B cells in spleen similar to that observed at steady state whereas NK cell number were similar (Fig.36C).

Detection of macrophages, neutrophils, B cells and T lymphocytes by histologic staining of lymphoid organs showed a similar results than that observed by FACS (Fig.37). In addition, histological staining of kidney and lung showed a low but equal level of infiltrating cells without any obvious differences between Hi-CD69.BAC and WT mice (data not shown) and therefore CD69 overexpression seemed

irrelevant for immune cell seeding of non-lymphoid organs. This data is consistent with the observation that TRM cells developed similarly in CD69-/- and WT mice [171].

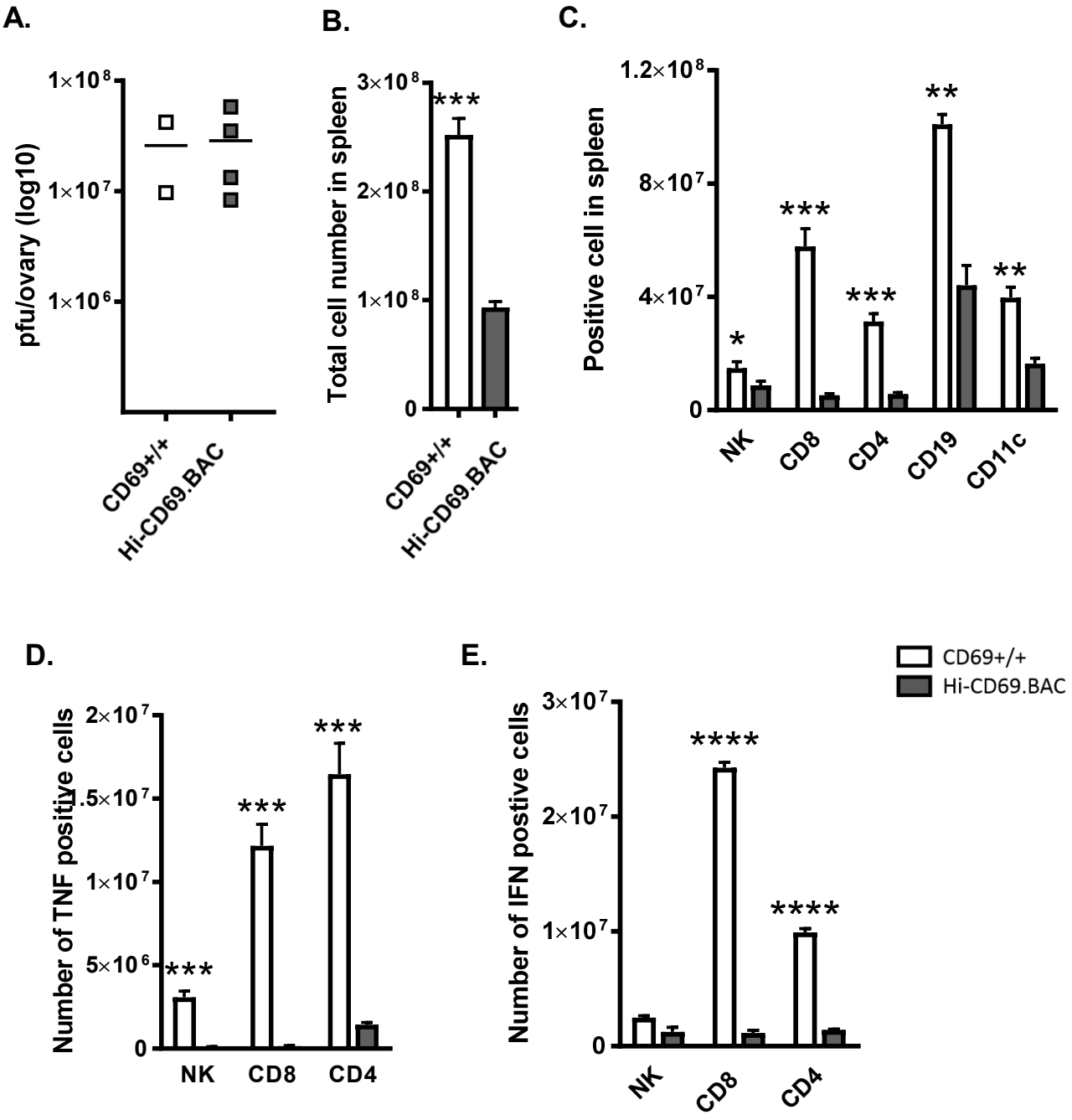


Figure 35. Reduced number of TNFα and IFNγ producing lymphocytes in SLO but similar VACV removal in Hi-CD69.BAC mice. Mice were infected with 1x10^7 pfu i.p and 7 days after infection, mice were sacrificed. Cells were collected from spleen and subjected to flow analysis. E, Ovaries were collected seven days after infection and viral titres were measured. B, Absolute cell numbers in spleen. C, Number of lymphoid subpopulations cells and dendritic cells in spleen. D-E, Number of TNF-α-producing cells in D and IFN-γ-producing cells in E, in spleen cells.

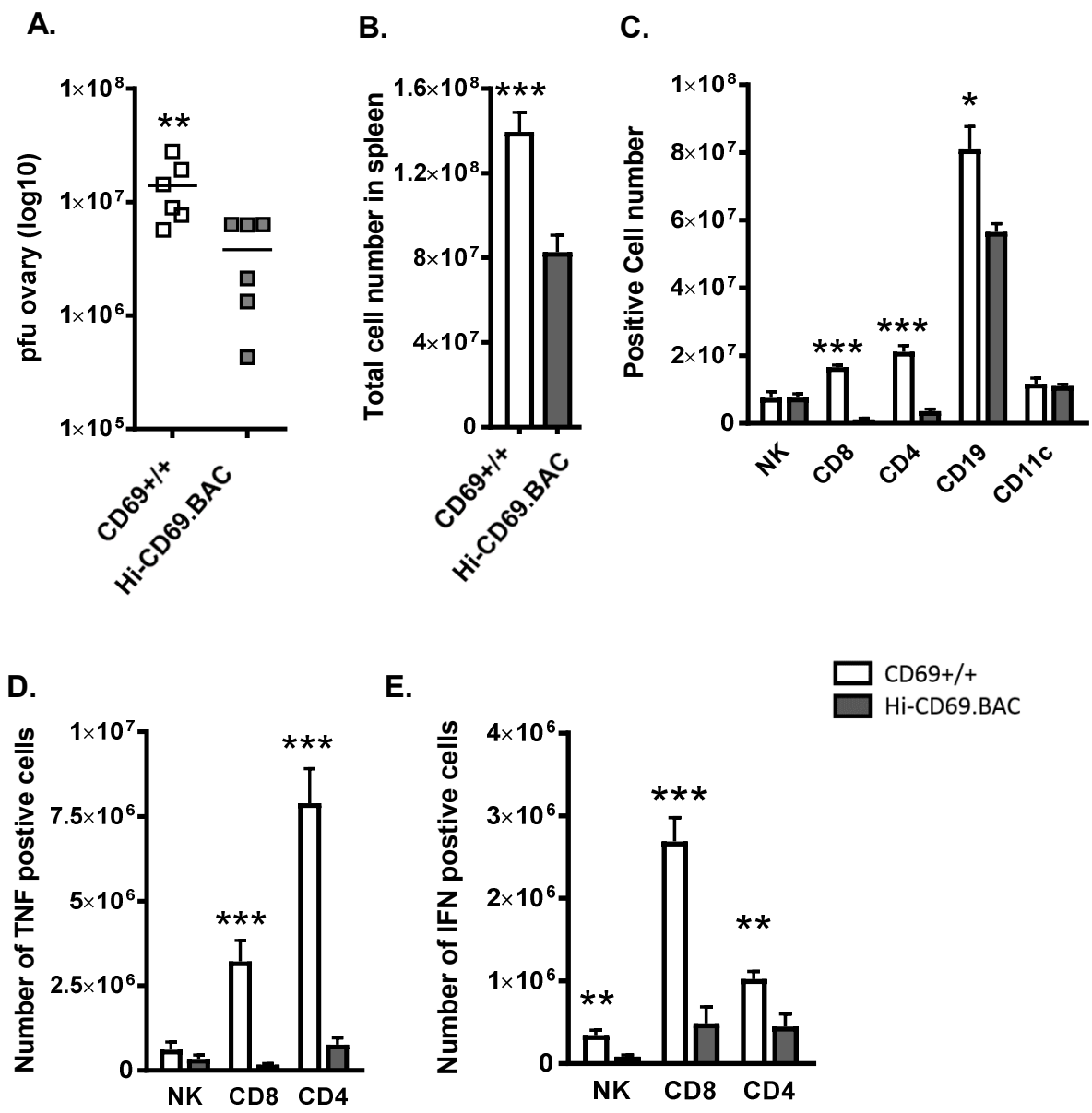


Figure 36. Reduced number of TNFα and IFNγ producing lymphocytes in SLO accompanied with an increased VACV elimination two days after infection in Hi-CD69.BAC mice. Mice were infected with 1x10^7 pfu i.p and 2 days after infection, mice were analyzed. A, Ovaries were collected two days after infection and viral titres were measured. B, Absolute cell numbers in spleen. C, Number of lymphoid subpopulations cells and dendritic cells in spleen. D-E, Number of TNF-α-producing cells in C and IFN-γ-producing cells in D, in spleen cells. Pool of two experiments.

Because, at steady stage, NK cells were augmented in Hi-CD69.BAC compared to WT mice in spleen, thymus and bone marrow and equal numbers in lymph node and blood, we speculated whether NK cells could be the responsible of the augmented anti-viral response. In fact, the augmented NK cells

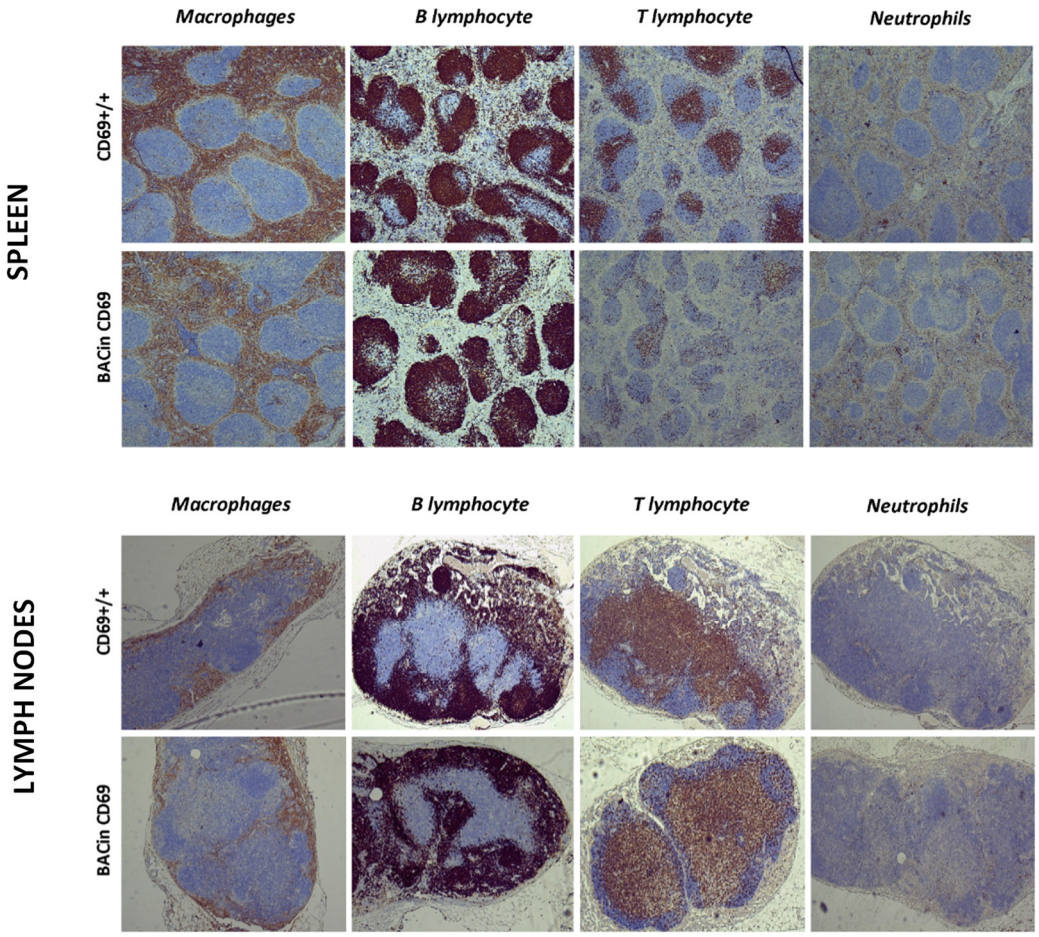


Figure 37. Immunohistochemichal analysis in Hi-CD69.BAC mice. Mice were infected intraperitoneally with 10^7 pfu of Vaccinia Virus and two days after infection, samples were collected from spleen, lymph nodes, kidney and lung and analyzed for macrophages, B lymphocytes, T lymphocytes and neutrophils staining. One experiment. The images are reprentive of three samples for group.

numbers in spleen and blood before infection corresponded to an increase in the number of NK cells in the last stage of maturation analyzed by the expression of CD27and CD11b in Hi-CD69.BAC mice (Fig. 38A-B). However, after VACV infection, a decrease in the TNF-α-producing (TNF-α^+) cells and IFN-γ-producing (IFN-γ^+) cells were observed within NK cells as well as in T lymphocytes in spleen of Hi-CD69.BAC mice when compared to WT mice (Fig.36D-E). Hence, the increased innate viral resistance observed in Hi-CD69.BAC mice at two days after infection, could be induced by increased activity of NK cells, but not due to the production of TNF-α and IFN-γ by these cells.

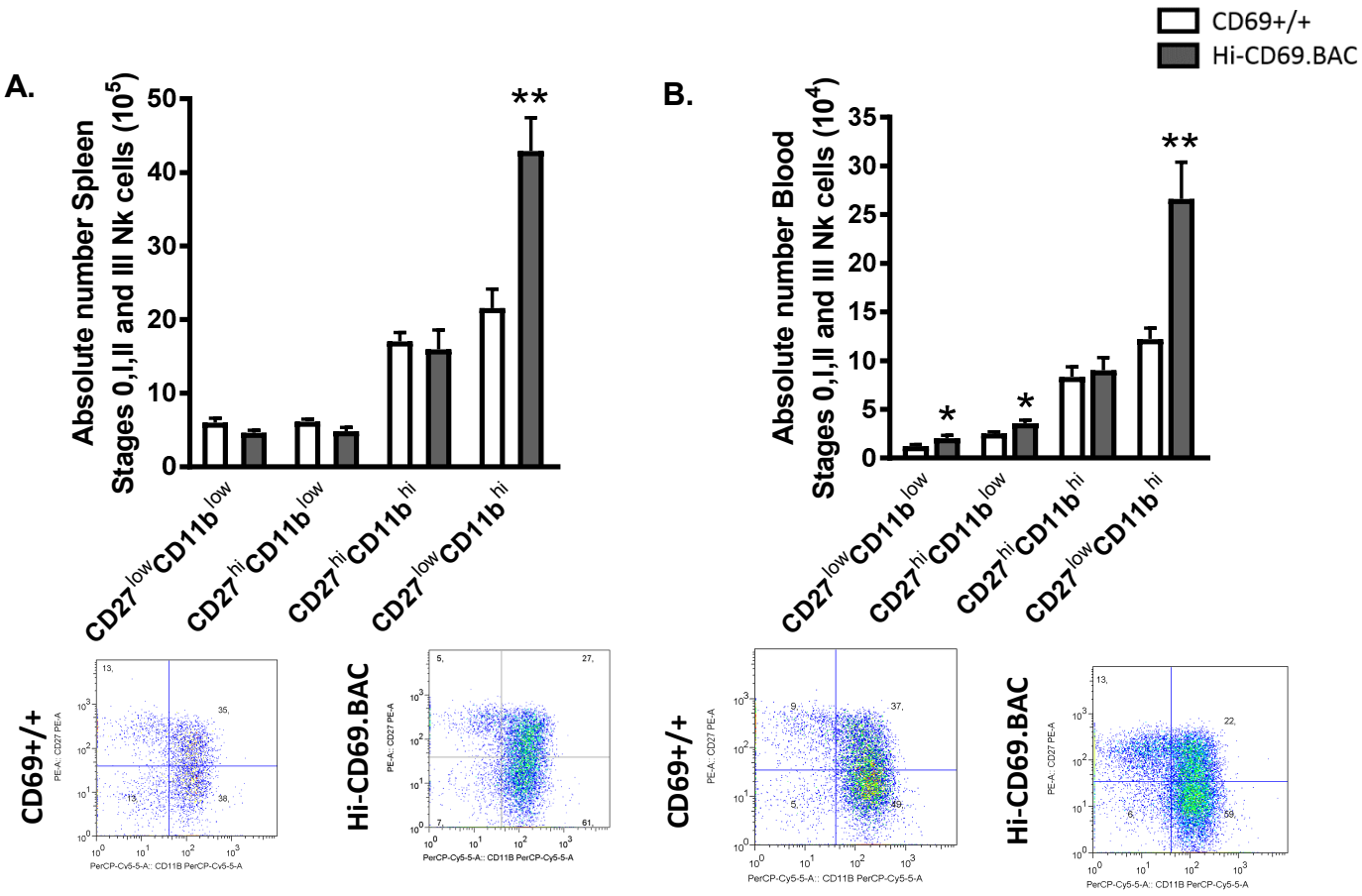


Figure 38. Induced increased proportion of mature NK cells in Hi-CD69.BAC mice. A-B, Cells from spleen and blood were analyzed according to CD11b and CD27 expression within NK cells in four subpopulations in this order of maturation: CD27lo CD11blo, CD27hi CD11blo, CD27hi CD11bhi and CD27lo CD11bhi. Numbers of these subpopulations were analyzed in A, spleen and in B, blood. Pool of two experiments.

Since CD69 have been described to be expressed in infiltrating leukocytes in many tissues including mucosa and skin regulating entrance and maintenance of T cell [171], we aimed to know whether the immunity in non-lymphoid organs, would be augmented against VACV in mice overexpressing CD69. Although the intraperitoneal pathway has been described as route of choice in Vaccinia virus infection with ovary as preferred site of virus replication where it remains for a long time, it has also described that the response could be evaluated in hematopoietic and non-lymphoid organs at short times upon intravenous injection. Therefore, we infected Hi-CD69.BAC and WT mice with Vaccinia virus intravenously and evaluated the viral titre in spleen, kidney, lung, lymph nodes and liver at 24 hours after infection (Fig.39). The results revealed a significantly better innate response to infection in spleen, kidney, lung and lymph nodes and the same tendency in the liver of Hi-CD69.BAC mice (Fig.39).

These results point to an increased immunity at day one after infection in non-lymphoid organs that might be mediated by infiltrating CD69 expressing immune cells. However, in the results shown in figure 36 and data not shown, the infiltration in lung and kidney after two days of infection was not different between CD69.BAC and WT mice. NK cell numbers in bone marrow, thymus and spleen were increased and thus, overexpression of CD69 deregulated NK cells. Though, it have been described an increased in numbers of NK cell in peripheral organs at steady stage, how the NK cells reach and are maintained in different peripheral tissues has been poorly investigated.

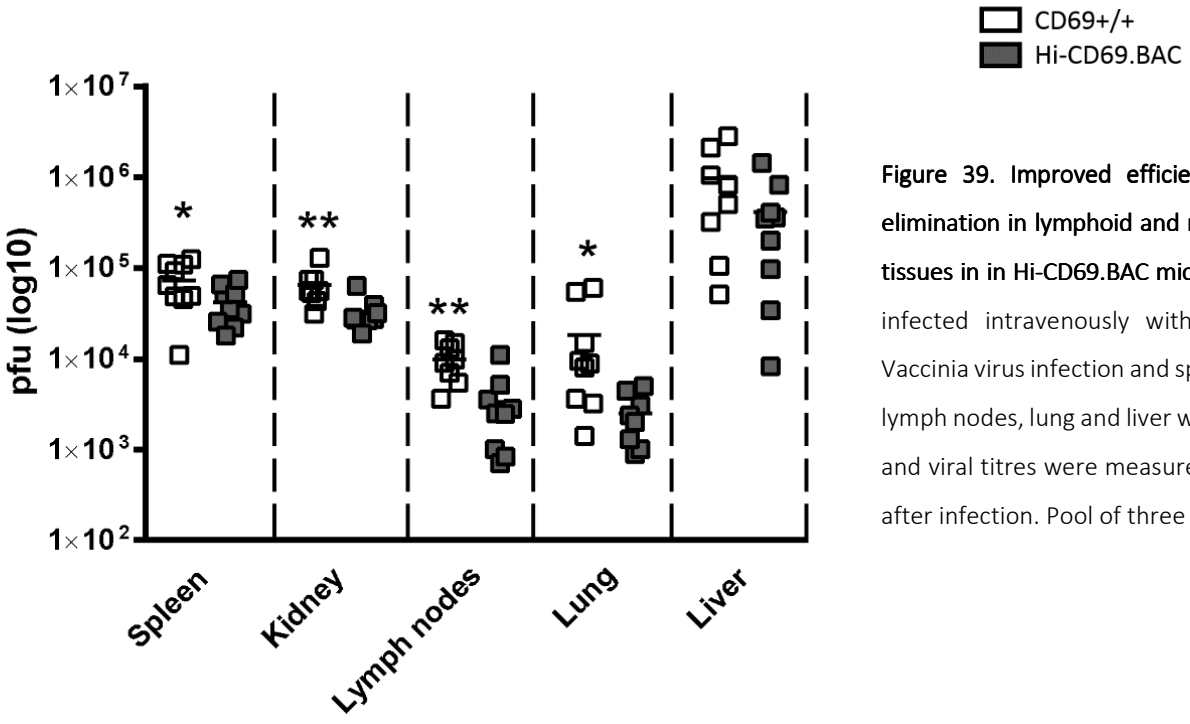


Figure 39. Improved efficiency in VACV elimination in lymphoid and non-lymphoid tissues in in Hi-CD69.BAC mice. Mice were infected intravenously with 10^7 pfu of Vaccinia virus infection and spleen, kidney, lymph nodes, lung and liver were collected and viral titres were measured at one day after infection. Pool of three experiments.

Besides, as in barrier tissues, most of the immune cells expressed CD69, we analyzed the local immune protection in the respiratory mucosal tissues by intranasal infection with 10^5 PFU of VACV in Hi-CD69.BAC and WT mice. We approached to study the survival to the infection by examining over time the mice weight. The response was similar in both mouse strains until 7 day where Hi-CD69.BAC mice showed a greater capability in removal the intranasal infection of Vaccinia virus (Fig.40A). At this point, the animals were sacrificed and the virus titre was analyzed in lung, which constitutes the principal virus retention site in intranasal infection. Correspondingly with the increased survival observed, the viral titer was higher in WT mice than in transgenic mice (Fig.40B). Therefore, also in mucosa, CD69 overexpression leads to a higher immune protective capability, although the difference in survival of

Hi-CD69.BAC mice compared to WT mice were not detected until 7 days post infection. In fact, 7 days are needed to produce specific effector and memory T cells which locally express CD69.

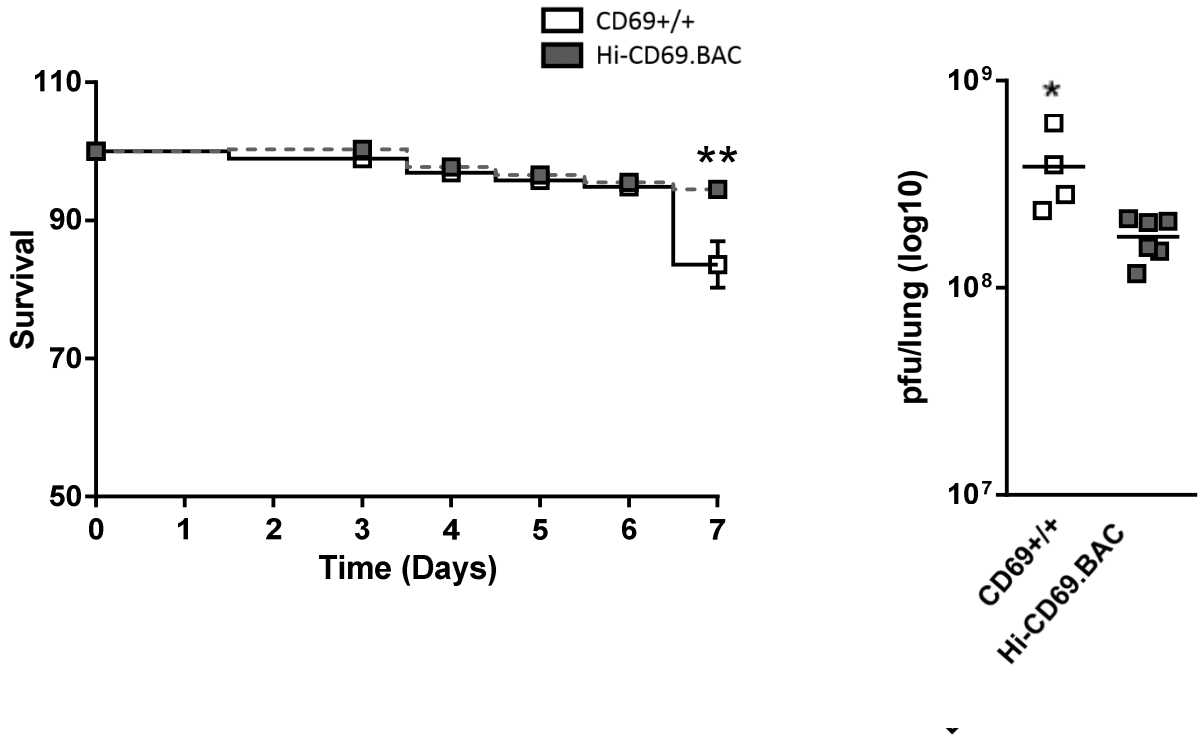


Figure 40. Increased survival in intranasal infection of Vaccinia virus in Hi-CD69.BAC mice. Mice were infected intranasally with 10^5 pfu of Vaccinia virus infection. A, Weight loss was evaluated over 7 days. B, At 7 days after intranasal infection, lung were collected and analyzed for viral load.

The effect of CD69 overexpression in cytokine and chemokine expression.

To investigate the mechanism that induces the increase in anti-VACV response in CD69 overexpressing mice, the expression of soluble immune mediators in total and NK purified splenic cells were analyzed by Q- PCR (Fig. 41A-B and data not shown). We found that these mice showed an increased production of IL17a, IL17b and IL17f in total spleen cells and IL17a in NK cells (Fig. 41A and data not shown). An increase in the expression of IL-7, CCL2, CCL7 and CCL8 was also observed (Fig. 41A-B), which have been described its role on the development and homeostasis of NK cells and in other leukocytes subsets [285]. Surprisingly, overexpression of CD69 also increased expression of proinflammatory cytokines such as TNF and IFNγ production and IL-1α, IL-1β and IL-21 (Fig. 41A). Finally, CCL2 and CCL12 expression were augmented in CD69 overexpressing mice whose role is associated with an increase in cell chemotaxis to the site of infection (Fig. 41B). This increase in inflammatory humoral mediators

reveal an important role of CD69 in controlling the immune response mediated by cytokines and chemokines. Due to the overall increase in VACV elimination, together, these results suggest the influence of cytokines and chemokines in the better antiviral response in spite of the reduced number of T and B cells found in SLO. Also, this increase of humoral mediators may influence in the number of NK cells observed in thymus, bone marrow and spleen. In addition, CCL2 and its homolog CCL12 may contribute to recruit leukocytes to other tissues different of SLO.

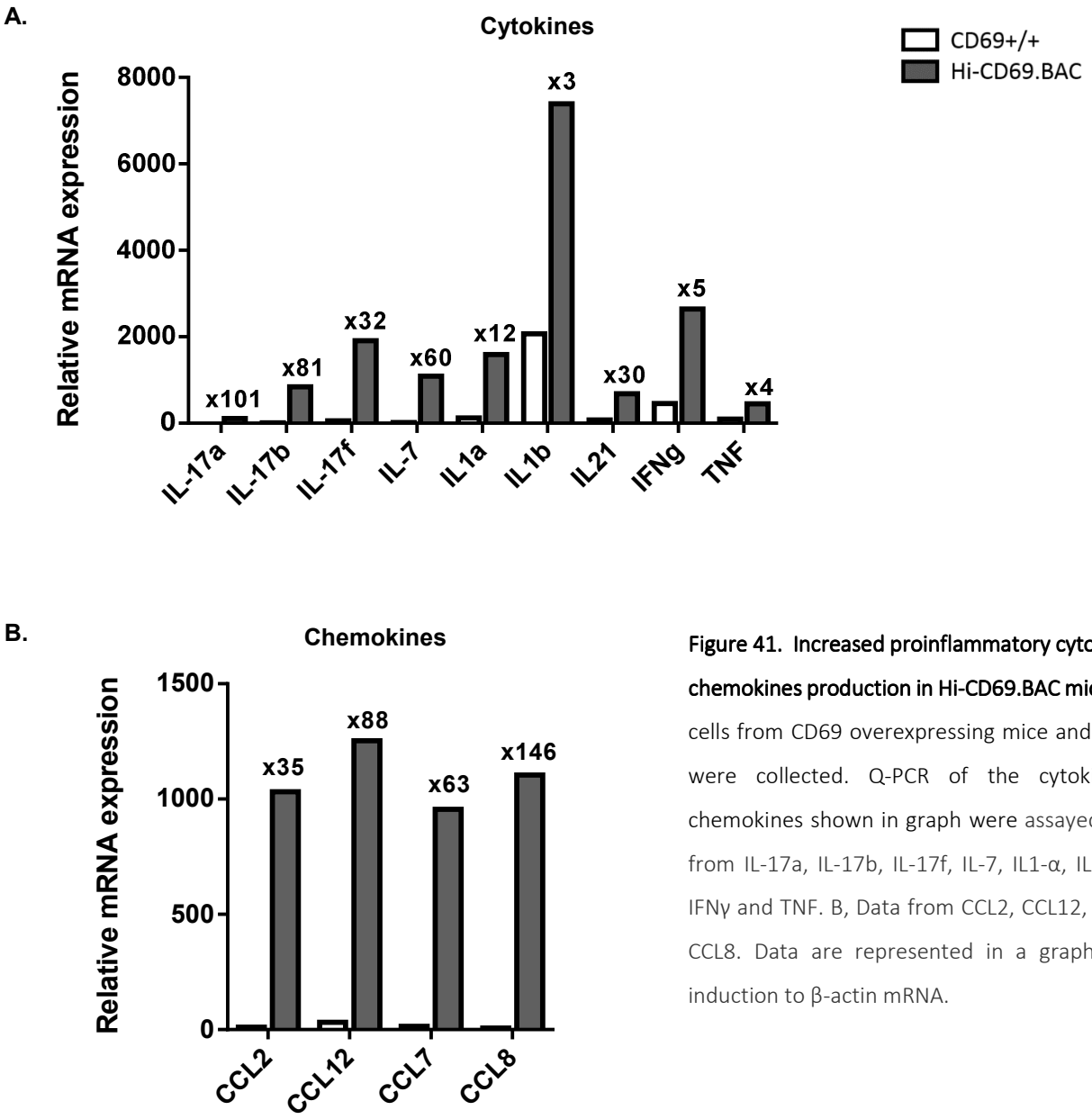


Figure 41. Increased proinflammatory cytokines and chemokines production in Hi-CD69.BAC mice. Spleen cells from CD69 overexpressing mice and WT mice were collected. Q-PCR of the cytokines and chemokines shown in graph were assayed. A, Data from IL-17a, IL-17b, IL-17f, IL-7, IL1-α, IL-1β, IL21, IFNγ and TNF. B, Data from CCL2, CCL12, CCL7 and CCL8. Data are represented in a graph as fold-induction to β-actin mRNA.

DISCUSSION

In this study, we evaluated the implication of CD69 overexpression in leukocyte circulation and function. It was found an increased numbers of single positive T cells in thymus and a higher increase in regulatory T cells, leading to a high reduction in T lymphocytes including Treg cells in SLO. Likewise, B cells were moderately reduced in SLO, although an unexpected augmentation in NK cell numbers were found in bone marrow, thymus and spleen. Nevertheless, CD69 overexpressing mice showed a better control of VACV infection in spite of the reduction of T and B cells in SLO compared to WT mice. On the other hand, humoral mediators CCL2, CCL12, CCL7, CCL8, IL-17, IL-1, IL-7 and IL-21 was highly augmented and TNF and IFNγ moderately increased.

In CD69 overexpressing mice, the increased number of SPCD4 and SPCD8 T cells in thymus, and the decreased T cells in SLO, is the result of CD69 interfering with S1P1 function, since S1P1 regulates egress of mature thymocytes from thymus in response to a S1P gradient in plasma [286-288]. Similar results were reported in transgenic mice overexpressing CD69 in thymic cells driven by an Lck promoter [140, 161]. Our data also support the function described for S1P1 of negative regulation of development, function and retention of Treg [284], since CD69 overexpression interfering with this function resulted in a stronger increased and decreased of Treg in the thymus and SLO respectively, compared to SP CD4 thymocytes. In CD69 deficient mice, it has been reported a decrease in SPCD4 thymocytes but a proportional decrease in CD4 CD25+FOXP3+ regulatory T thymocytes. Therefore, in $CD69^{-/-}$ mice, the differential function of S1P1 in the subset of Treg cells within the CD4 thymocytes was not appreciated [289]. B cell reduction in SLO of CD69 overexpressing mice is also in agreement with CD69 interfering with S1P1 function in B cell egress from bone marrow, although B cell progenitors in bone marrow were not altered [163, 280]. In agreement, similar altered distribution of B cells was appreciated in $S1P1^{-/-}$ mice [163] showing reduced numbers of B cells in spleen and blood and similar quantities in LNs.

In CD69 overexpressing mice, the described function for CD69 in retention of mature T and B cells in lymph nodes [140, 161] is appreciated, since in these organs, T and B cell number are less decreased than in spleen of CD69 overexpressing mice compared to WT mice. In NK cells, S1P1 facilitates egress from lymph node [290] reducing egress from BM [291]. If CD69 could interfere with this function, it would be expected an increase in NK cell numbers in these organs, however, in CD69 overexpressing mice, the increase in NK cell number was found in bone marrow, thymus and spleen. NK cells egress

from bone marrow and lymph nodes is mediated by S1P5 which does not interact with CD69 [166]. Therefore, the increased development of NK cells in CD69 overexpression mice is induced, at least partially, by a non-S1P related mechanism. The increased number of NK cells in bone marrow, thymus and spleen may be justified by the increased expression of CCL7, CCL8, IL7 and IL21 expression important for NK cell survival, development and homeostasis [292]. These huge increase of soluble activation and attractant factors might attract almost all types of leukocytes but since NK and myeloid cells are less strongly controlled by CD69 than T and B lymphocytes, they will be less retained than these cells in primary lymphoid organs, allowing a greater output to SLO [132].

To assess whether overexpression of CD69 could be involved in a differential immune response, we analyzed the impact of Vaccinia virus infection in this transgenic mice model. The study of innate and adaptive immune response in SLO of CD69 overexpressing mice during Vaccinia virus infection revealed that the number of leukocytes and their pattern of distribution were similar to that observed at steady state which may be explained by a limited recruitment of cells into SLO organs.

Vaccinia virus is characterized for its wide tropism, being capable of infecting any cell type. Despite of a reducing number of IFNγ producing cells found in VACV infected CD69 overexpressing mice, we found a better response to Vaccinia virus infection both analyzed in ovaries after intraperitoneal infection, in lung after intranasal infection and in spleen, lymph nodes, kidney, lung and liver after 24 hours of intravenously infection. Therefore, the overexpression of CD69 leads to a better immune response to removal Vaccinia virus infection by all routes analyzed, except the similar adaptive immune response observed between Hi-CD69.BAC mice and WT mice in response to intraperitoneal Vaccinia Virus infection. These data suggest that these mice have an increased response when the peripheral non-lymphoid or mucosa tissue are involved by any implicated route of entrance and a better adaptive immune response when a mucosa is involved as virus entrance. Since we did not observed by histological studies differences in the number of infiltrating cells in non-lymphoid organs of CD69 overexpressing compared to WT mice (figure 34 and data not shown), and most of infiltrating leucocytes expressed CD69, we suggested that overexpression of CD69 may help to retain myeloid and NK cells in these locations. In addition, it is well documented the function of CD69 in retention of T effector cells and memory local formation [171]. We reasoned that in the CD69 overexpressing mice during the immune response to intranasal VACV infection, the better innate response developed in the lung of these mice, seen during i.v. infection, would support the improved adaptive immune response observed in this mice mediated by the CD69 retention of T cells. The increased expression

of IL17a, IL17b and IL17f, IL-1 and IL-21 production in CD69 overexpressing mice between 10 and 100-fold compared to WT mice and 5-fold in TNF and IFNγ expression are agree with the increased resistance to Vaccinia virus infection in this mice. Also, TNF and IFNγ play a key role in the best response to Vaccinia virus infection. IL17 deficient mice showed to be more sensitive to VACV infection than WT mice [241]. The blockage of IL-1βR in Vaccinia virus led to an increase of IL-1β expression in murine and human antigen-presenting cells being responsible of an enhanced T CD8 memory T cells and resistance to VACV infection [293]. Recent studies have shown that IL-21 is required to sustain an antiviral function of T CD8, B and NK cells preventing disease progression and is considered as a good candidate for use as a Vaccinia adjuvant [294-297]. Thus, the increased expression of IL-17 and the proinflammatory cytokines TNF and IFNγ accompanied by the augmented number of NK cells probably resulted of the increase of CCL7, CCL8, IL-21 and IL-7 expression may be promoting the higher immune response found in CD69 overexpressing mice leading to a better control of VACV infection. In spite of the reduced number of T lymphocytes in SLO is compensated by the vast increased production of humoral mediators.

DISCUSSION

The results presented in this thesis show the importance of CD69 in cell distribution and in Vaccinia virus infection in the absence, targeting or overexpression of CD69. In the presented work, it has been found that the absence of CD69 displayed a better control of Vaccinia virus infection through an enhanced cellularity in spleen, TNFα and IFNγ-producing NK cell, NK cell number and a reduced spontaneous cell death. The targeting of mouse and human CD69 induced a decrease of bone marrow cells and an increase of spleen cells and this effect was maintained for 6 days. Anti-human CD69 showed a similar mobilization than AMD3100 and was non-summative in combined treatment. CXCR4 expression was augmented in bone marrow and spleen cells whereas CXCL12 expression was found increased in bone marrow and decreased in spleen, lymph nodes and plasma. FTY720 treatment inhibited bone marrow cell mobilization in anti-CD69 treated mice and an increase of S1P1 expression in the main leukocyte subtypes in bone marrow and blood was observed in targeted mice pointing to S1P1 increase as a mechanism of mobilization of bone marrow cells. Accordingly, anti-human CD69 also induced an increase of mTOR signaling. Besides, anti-human CD69 induced proliferation of progenitor and stem cells that was more a homeostatic proliferation since was higher than the observed after AMD3100 treatment. In agreement, the targeting of mouse and human CD69 resulted in an improved immune response against Vaccinia virus infection in immunodeficient and immunocompetent mice. Treatment with anti-human CD69 2.8 induced a significant increased expression of different cytokines and chemokines in HuCD69 mice. Moreover, overexpression of CD69 induced T cell and Treg cell retention in thymus, being more affected Treg cells, and a proportionally reduced output of these cell to periphery. B cell numbers were also diminished in SLO in less proportion whereas a significant increase of NK cell number was established in bone marrow, thymus and spleen. Also, IL17a, IL17b, IL17f, IL-1α, IL-1β, IL-7 and IL-21 were augmented between 10 and 100-fold, TNF and IFNγ around 5-fold and CCL2, CCL12, CCL7 and CCL8 between 40 and 140-fold compared to WT mice. The vast majority of these cytokines and chemokines were also augmented in CD69 human treated mice. Thus, this great augment of proinflammatory cytokines and chemokines, seems to overpass the effect of the reduced number of T and B lymphocytes found in the CD69-overexpressing mice since anti-Vaccinia immune response was found augmented in intraperitoneal, intravenous and intranasal infection.

The absence of CD69 enhances the host response to Vaccinia virus infection in immunocompetent and immunodeficient mice in both innate immune response and adaptive immune response. This is accompanied by an increased leukocyte number including NK cells and TNFα and IFNγ-producing NK

cells in the spleen and NK cell depleted mice exhibited a similar response in $CD69^{-/-}$ mice than WT mice but a significantly worse response in $Rag2^{-/-}CD69^{-/-}$ mice compared to $Rag2^{-/-}CD69^{+/+}$, pointing to NK cell numbers as the responsible of the best antiviral response. The increase of spleen cellularity observed in these mice after infection had already been detected in uninfected mice. Similarly, increased cell number were previously reported in CD69 deficient mice in a different genetic background at steady state and spleen and peritoneum in an anti-tumor response [1]. In previous works, CD69 knockout mice have been associated with an increase of IFNγ production and a reduced TGF-β expression [1]. In the presented work, the reduction of TGF-β has been also linked with an increased cell survival, which has been observed augmented in $Rag2^{-/-}$ CD69 knockout mice whereas no differences in proliferation rate were observed. Thus, the increase in spleen cell number may be, at least in part, the result of a reduced cellular apoptosis.

The role of CD69 has been studied in numerous models of autoimmune [131, 142, 144, 147-149, 153] and antitumor diseases [1], acting as negative regulator of the immune response. However, the influence of CD69 on anti-infectious immune response has been only described in the infection of Listeria monocytogenes [141] but not in antiviral immune response. The absence of CD69 in mice infected with Listeria monocytogenes led to an increased in lymphocyte numbers with an increased production of IFN type I and II. As it is described, an augmented interferon together with listeriolysin from the bacterial infection leads to an increase of cellular apoptosis and poorer infection control. However, in Vaccinia virus infection, similar to the results observed in the antitumor response, the increase of NK cells and other cell type number contribute to the best antiviral response observed.

In addition to the increased number of spleen cells in $CD69^{-/-}$ mice at steady state, we found a decreased cell number in BM. To analyze whether the absence of CD69 induced an augmented NK cell egress from BM, we assayed BM chimeras of $CD69^{-/-}$ and $CD69^{+/+}$ in $CD69^{+/+}$ recipient mice and found a higher proportions of $CD69^{-/-}$ NK cells than $CD69^{+/+}$ NK cells in spleen among total donor-derived NK cells. Also, previous works showed that CD69 targeting mice resemble the phenotype of $CD69^{-/-}$ mice [147, 172, 173], and then we used anti-CD69 antibodies to check if these mice had a similar phenotype in steady and infection setting than knockout mice and to know the mechanism involved. Targeting of CD69 mouse showed a greater cellular recruitment in periphery and decreased bone marrow cell count. We studied the effect of anti-human CD69 antibodies in a mouse model that expresses human CD69 to analyze the possible use of this treatment as mobilizer of hematopoietic bone marrow cells in humans. Human CD69 targeting decreased the number of the major cell populations of bone

marrow, and an increase of many spleen cell subtypes. CD69 targeting induced bone marrow cell mobilization similar than AMD3100 and both treatments did not have a summative effect. Besides an increase of CXCR4 expression in bone marrow and spleen and a reduction of CXCL12 expression in blood, spleen and lymph nodes and an increase in bone marrow was found in treated mice. Both CXCL12 and CXCR4 are potent chemoattractants and constitute one of main mechanism of HSC mobilization [18], but changes in the expression of these chemokines depended on the time analyzed [3]. Lapidot et al. demonstrated that CXCL12 expression increases in bone marrow after G-CSF-induced mobilization but decreases over time [3]. Anti-CD69 treatment acts on CXCL12/CXCR4 axis but we have not studied its expression dynamics. Importantly, it has been described that CD69 and S1P1 are mutually regulated [108] and that the involvement of S1P/S1P1 axis as one the main mechanism of hematopoietic cell mobilization [22]. In fact, an increased S1P1 expression is detected in CD69 knockout mice. In the presented work, CD69 treatment increased S1P1 expression in the major cell types of bone marrow and blood and treatment with anti-CD69 together with FTY720, an inhibitor of S1P1 expression, prevented the mobilization induced by anti-CD69 treatment implicating S1P1/S1P axis in CD69 targeting induced. On the other hand, we found an implication of mTOR cell signaling pathway in both spleen and bone marrow from CD69 treated mice measured as phosphorylation of 4E-BP1 and it has been also described that this pathway was implicated in CD69 signaling [131] and in bone marrow cell mobilization induced by G-CSF [4]. The importance of mobilizers lies in its use for hematopoietic stem cells mobilization, which were shown to be increased in bone marrow and spleen after treatment with human anti-CD69. This increase has been also related to a significant increase in proliferation rate of hematopoietic stem cells being significantly higher than AMD3100 treatment. Proliferation could be also mediated by the increase in IL2 and CD25 (IL2rα) production found in bone marrow, spleen and blood of CD69 treated mice and this corresponds with previous data of our lab where was described that anti-mouse CD69 treatment induces proliferation of IL2-mediated memory phenotype T cells and was inhibited by treatment with anti-CD25 antibodies [104]. As described above, CD69 knockout mice had been previously characterized by a reduction of TGFβ, which was also observed in CD69 treated mice reproducing CD69 knockout mouse phenotype [1]. Thus, anti-human CD69 can induce mobilization of mature and precursor cells through changes in CXCR4/CXCL12 axis and increasing S1P1 expression, mTOR signaling and proliferation rate.

Currently, G-CSF constitutes the main mobilizer of bone marrow progenitor cells and is combined in research studies with other treatments to improve mobilization efficiency [30]. More studies are

needed to approach the possible use of anti-CD69 antibodies together with G-CSF to improve current results.

Since we have seen an increased leucocyte cells numbers in peripheral organs in CD69-targeted mice similar to $CD69^{-/-}$ mice and we observed an augmented anti-VAV response in $CD69^{-/-}$ mice, the study of anti-VACV response in this mice was approached. To evaluate the response, we designed a treatment to keep reduced CD69 expression over time and similar results were found. Mice treated with anti-mouse CD69 resulted in a cellular mobilization in both $Rag2^{-/-}CD69^{+/+}$ mice and human CD69 mice. The treatment with anti-mouse CD69 in $Rag2^{-/-}CD69^{+/+}$ mice demonstrated increased viral clearance capacity two days after infection accompanied with an increase of leucocytes numbers and increased proportion and number of TNFα and IFNγ-producing NK cell. Similar results were found in human CD69 mice model two days after infection whereas a better response to Vaccinia virus infection was also found in mice treated with anti-human CD69 at seven days after infection but no changes were found in the NK cell activity. In addition, anti-human CD69 treated mice showed an enhanced production of proinflammatory cytokines such as IL17, IL1, IFNγ and Lymphotoxin and chemokines such as CCL2 and CCL12. Therefore, CD69 targeting reveals the possibility of targeting CD69 to manipulate the immune response against Vaccinia virus infection and its use for the treatment of this infection.

The effect of CD69 targeting has been linked to the NK cell-dependent antitumor model [172], to a collagen-induced arthritis model [173]and to an ovalbumin-induced asthma model [147]. To date, its role in an antiviral model was unknown.

Therefore, the absence and targeting of CD69 induced a higher cell numbers in spleen in uninfected mice and promoted a better antiviral immune response.

Finally, the transgenic mouse model that overexpressed CD69 expressed CD69 at basal conditions in all cell populations analyzed, demonstrating the influence of CD69 on cellular distribution, inducing retention of mature T lymphocytes, T CD4 SP and T CD8 SP, in thymus and a reduction in the numbers of these cells in SLO and peripheral blood. Also, overexpression of CD69 interferes to a greater extent in regulatory T cell subpopulation, inducing retention of FOXP3+ CD25+ CD4+ cells in thymus and a proportional reduction in the number of regulatory T cells in spleen and to a lesser extent in lymph nodes. This cell distribution is justified by the relation between CD69 and S1P1, whose interaction has been described for lymphocytes [108, 163, 171]. The absence of S1P1 impairs exit of T lymphocytes to

the periphery being incapable of leaving thymus and although we can find lymphocytes B in peripheral lymphoid organs, they are severely reduced in blood and lymph [158]. Number of B cells in CD69 overexpressing mice were also diminished in SLO although less than T lymphocytes. Overexpression of CD69 influences in NK cells numbers being increased in thymus, bone marrow and spleen. The significant increase of IL-21, CCL7, CCL8 and IL-7 in CD69 overexpressing mice, which can influence in NK cell homeostasis, could be responsible of the increased NK cell number observed in this mice. Because the immune response of CD69 overexpression in Vaccinia virus infection induced an increased innate immunity measured in ovaries after intraperitoneal infection and in spleen, lymph nodes, kidney, lung and liver after intravenous inoculation, despite of the small number of TNFα and IFNγ producing cells and augmented adaptive immune response measured in lungs after intranasal infection. The study of proinflammatory cytokines revealed a very significant increase in IL17a, IL17b and IL17f, IL-1α and IL-1β production in these mice and an increase of TNF and IFNγ expression. Increased TNF and IFNγ production may justify the best response found in mice overexpressing CD69 since both cytokines have been shown to play a key role in the best response to Vaccinia virus infection. On the other hand, the increase of IL17 could be related to an increase in differentiation to Th17 which establishes a balance with regulatory T cells. The increase of IL17 in transgenic mice could be justified by the reduction of regulatory T cells observed in SLO. The importance of CD69 in Th17 has been studied in an *in vitro* model of T CD4 lymphocytes differentiation to Th17 in CD69 knockout mouse, which showed increased levels of IL17 [128]. IL17 deficiency mice or treatment with anti-IL17 mAb resulted to be more sensitive to Vaccinia virus infection than control mice [241]. Thus, the best response of the CD69 overexpressing mice may be a result of the increase of IL-17 and the proinflammatory cytokines TNF and IFNγ.

In summary, our data reveal that the absence and targeting of CD69 show increased spleen cell recruitment due to an increased mobilization of bone marrow cells whereas CD69 overexpressing mice have a similar number of bone marrow cells, probably due to the reduced expression of S1P1, and a lower cellularity in SLO. And on the other hand, the absence, targeting and overexpression of CD69 showed a better response to Vaccinia virus infection.

CONCLUSIONS

CONCLUSION

In the immune response to Vaccinia Virus infection in CD69 knockout mice:

1. $CD69^{-/-}$ and $Rag2^{-/-}$ $CD69^{-/-}$ mice show an enhanced *in vivo* anti-Vaccinia activity mediated by NK cells.
2. Infected $CD69^{-/-}$ and $Rag2^{-/-}$ $CD69^{-/-}$ mice show an increased accumulation of splenocytes at early response in VACV.
3. The increased antiviral response in these mice is mediated by similar NK cell reactivity but increased NK cell number and similar NK cell proliferation rate.
4. In uninfected mice, $Rag2^{-/-}$ $CD69^{-/-}$ mice exhibit increased NK cells numbers and a reduced spontaneous cell death rate.
5. $CD69^{-/-}$ mice show decreased bone marrow cellularity and increased leukocyte counts in the periphery.

Influence of human CD69 targeting in mobilization of progenitor cell:

6. Targeting of mouse and human CD69 induce bone marrow mobilization of hematopoietic cell from primary lymphoid organs and accumulation in periphery.
7. Anti-human CD69 treatment mobilizes immature B cells from bone marrow.
8. The effect on mobilization of CD69 targeting is similar and non-additive to the one of AMD3100 (inhibitor of CXCR4).
9. Targeting of CD69 alters CXCR4/CXCL12 axis, increasing CXCR4 expression in bone marrow and spleen.
10. Treatment with FTY720 (inhibitor of S1P1) and anti-CD69 impairs bone marrow cell egress, demonstrating the role of S1P receptors in CD69 targeting-induced bone marrow mobilization.
11. CD69 targeting induces mTOR signaling and this mechanism links to the upregulation of S1P1.
12. Anti-CD69 treatment induces accumulation of primitive hematopoietic cell number in bone marrow and spleen.
13. Treatment with CD69 increases proliferation rate in mature and primitive hematopoietic cell in bone marrow and spleen and this proliferating rate is higher than AMD3100 treatment.

14. CD69 targeting induces an increase in IL2 and IL2-R expression and a decrease in TGF-β in bone marrow and spleen.
15. Targeting of CD69 with two doses induces an increase in number of leukocyte subsets in periphery and increase proliferation rate and humoral proinflammatory mediators in spleen.

In CD69 targeting effect in Vaccinia virus in $Ra2^{-/-}$ $CD69^{+/+}$, $CD69^{+/+}$ and human CD69 mice:

16. In these mice targeting of CD69 promotes better virus clearance and a concomitant accumulation of leukocytes in periphery and a decrease in bone marrow cell number.
17. During the innate immune response to Vaccinia virus infection analyzed in $Ra2^{-/-}$ $CD69^{+/+}$ mice, anti-CD69 treatment induces an enhanced activity of NK cells, increasing percentages of IFN-γ and TNFα-producing cells.

In overexpression of CD69 in Vaccinia virus infection:

18. Overexpression of CD69 alters leukocyte homeostasis resulting in a decrease of the main subsets in SLO and an increase in thymus of SP CD4 T cells, SP CD8 T being Tregs cells more affected. However, NK cell numbers were increased in bone marrow, thymus and spleen.
19. CD69 overexpressing mice induces a high increase of proinflammatory cytokines and chemokines.
20. In these mice, innate immune response was increased in different inoculation settings. Viral titres were found diminished in ovary by intraperitoneal infection and spleen, lymph nodes, liver, lung and kidney in intravenous inoculation.
21. In intranasal infection, removal of Vaccinia virus in lungs and survival were increased in overexpressing mice compared to WT mice.

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ANNEX

ANNEX CHAPTER I:

The increased viral control in $Rag^{-/-}CD69^{-/-}$ mice is dependent on IFNγ.

IFNγ-secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFNγ in the adaptive immune response. As the anti-VACV response in $CD69^{-/-}$ mice is augmented at day one after infection, to investigate the involvement of IFNγ in the control of anti-VACV response in $CD69^{-/-}$ mice, the interferon activity was blocked before VACV infection by treatment with anti- IFNγ blocking mAb. Injection with either control diluent or anti-IFNγ was performed in $Rag2^{-/-}$ $CD69^{-/-}$ and $Rag2^{-/-}$ $CD69^{+/+}$ mice, 6 hours before infection and spleen cell subpopulation and ovary viral load was analyzed after 2 days. IFNγ blockaded in $Rag2^{-/-}CD69^{-/-}$ mice lost efficiency in eliminating VACV reaching even lower competence than IFNγ blockaded in $Rag2^{-/-}CD69^{+/+}$ mice, reversing the phenotype observed in untreated mice (Figure 1B). The analysis of splenic cell subpopulations showed that the anti-IFNγ treatment reduced in $Rag2^{-/-}CD69^{-/-}$ mice the accumulation in spleen of macrophages and dendritic cells reaching similar numbers than in $Rag2^{-/-}CD69^{+/+}$ mice, however IFNγ blockade did not affect accumulation of NK lymphocytes that remained increased compared to $Rag2^{-/-}CD69^{+/+}$ mice as in NK-non-IFNγ blockade mice (Figure 1A and C). Therefore in IFNγ blockade in $Rag2^{-/-}CD69^{-/-}$ mice, the increased number of NK lymphocytes are not efficient in VACV elimination and thus IFNγ but no other mediators produced by NK cells account for viral elimination in $Rag2^{-/-}CD69^{-/-}$ mice. Accordingly with the effect that IFNγ has in leukocyte trafficking and viral elimination and to understand the role of IFNγ in other subpopulations of cells, IFNγ was blocked using $CD69^{-/-}$ and $CD69^{+/+}$ mice. The same treatment as $Rag2^{-/-}$ mice was carried out in and $CD69^{+/+}$ mice. The blockade of IFNγ in both mice leads to approx. 50% decrease in cell accumulation in the spleen (Figure 1D) and a significant viral load increase in of both infected mice (Figure 1E). However, an augmented accumulation of leukocytes (Figure 1F) and increase efficiency in viral elimination are observed in $CD69^{-/-}$ mice compared to WT mice. Since, as shown above, in anti-IFNγ treated in $Rag2^{-/-}$ $CD69^{-/-}$ and $Rag2^{-/-}$ $CD69^{+/+}$ mice there are no difference in viral elimination or accumulation of myeloid cells, then, in these mice, mediators different of IFNγ produced by cells not present in Rag mice, lymphocytes T and/or B, are responsible for the differences in cell accumulation and efficient viral elimination observed. Together the presented results point to a crucial role of IFNγ in the increased immune response against VACV seen in $Rag2^{-/-}$ $CD69^{-/-}$ mice, but other factors

produced by rearranged antigen based lymphocytes, seems to be relevant in the augmented early anti-viral response found in the CD69$^{-/-}$ mice.

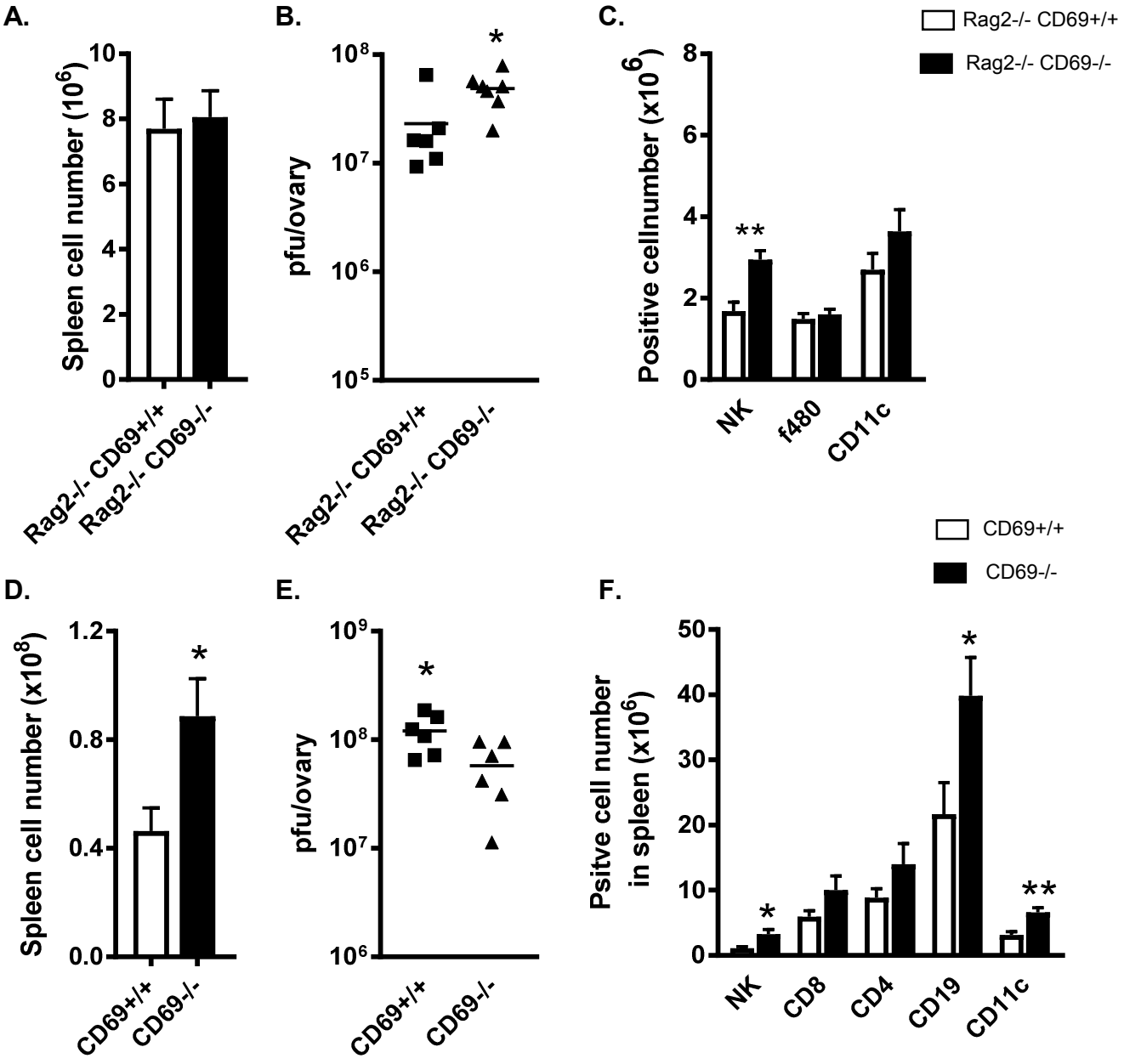


Figure 1. The increased viral control of Rag2$^{-/-}$CD69$^{-/-}$ NK cells is dependent on IFNγ whereas is not required to improve the antiviral response in immunocompetent mice. Mice were treated with 250 ug of Anti mouse IFN γ (clone XMG 1.2) 4 hours before infection with of VV i.p in immunodeficient mice and 1 × 10^7 pfu in immunocompetent mice. Two days after infection mice were analyzed. A and D, Total cell number of spleen. B and E, Ovaries viral load. C and F, number of splenic lymphoid and myeloid cell subpopulations. Pool of two experiments.

CD69 promotes M1 polarization in response to LPS.

Activation of macrophages is related to the immune response to viruses. M1 macrophages are mainly induced by IFNγ or upon stimulation with bacterial products such as LPS. To investigate whether CD69 contributes to the polarization to M1, we examined the levels of classical inflammatory mediators such as NOS-2, COX-2 and TNFα. Peritoneal macrophages from WT and CD69$^{-/-}$ mice were stimulated with LPS or not stimulated. As expected, the stimulation of WT macrophages with LPS generated high NOX-2, COX-2 and TNFα levels, whereas a deficient response was observed in the absence of CD69 measured by both real time PCR (Figure 2A) and western blot (Figure 2B).

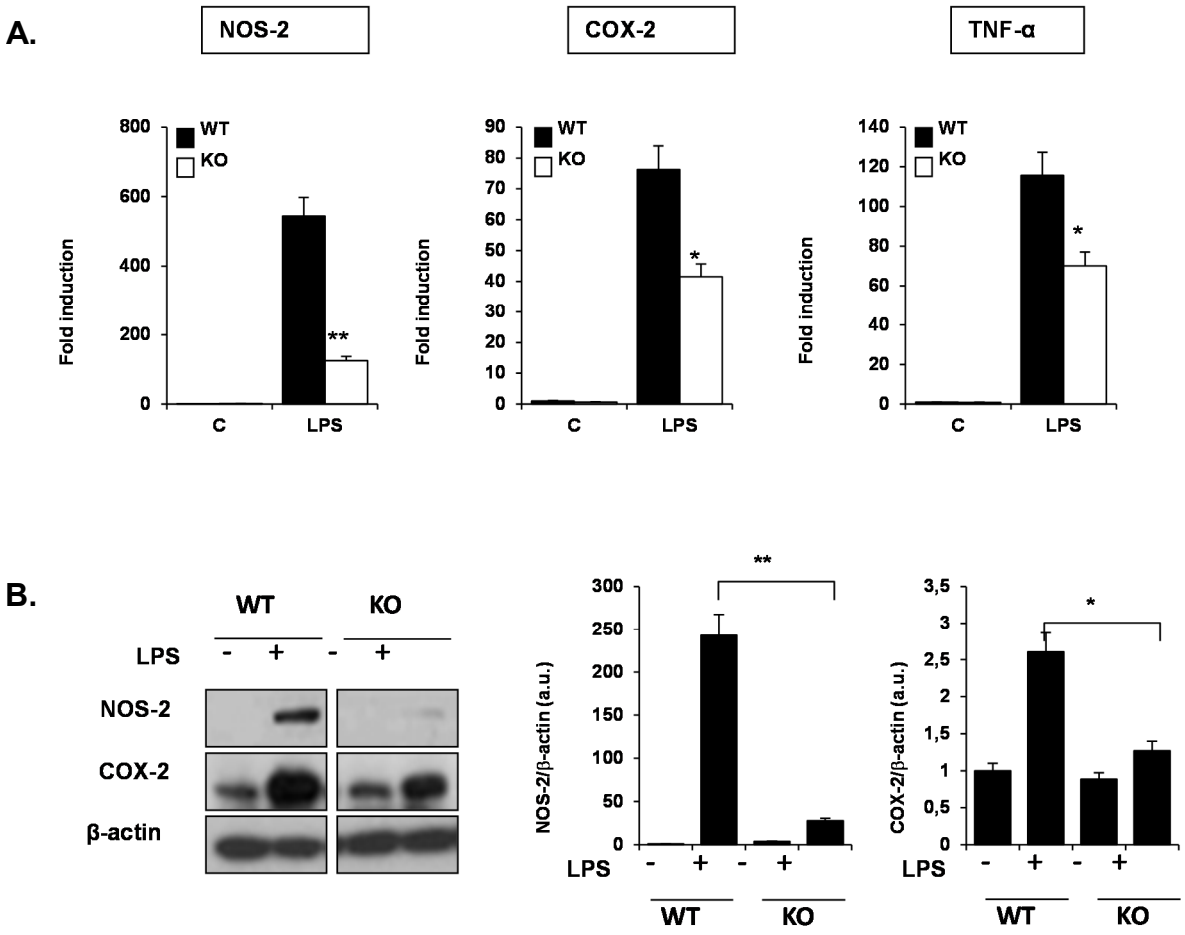
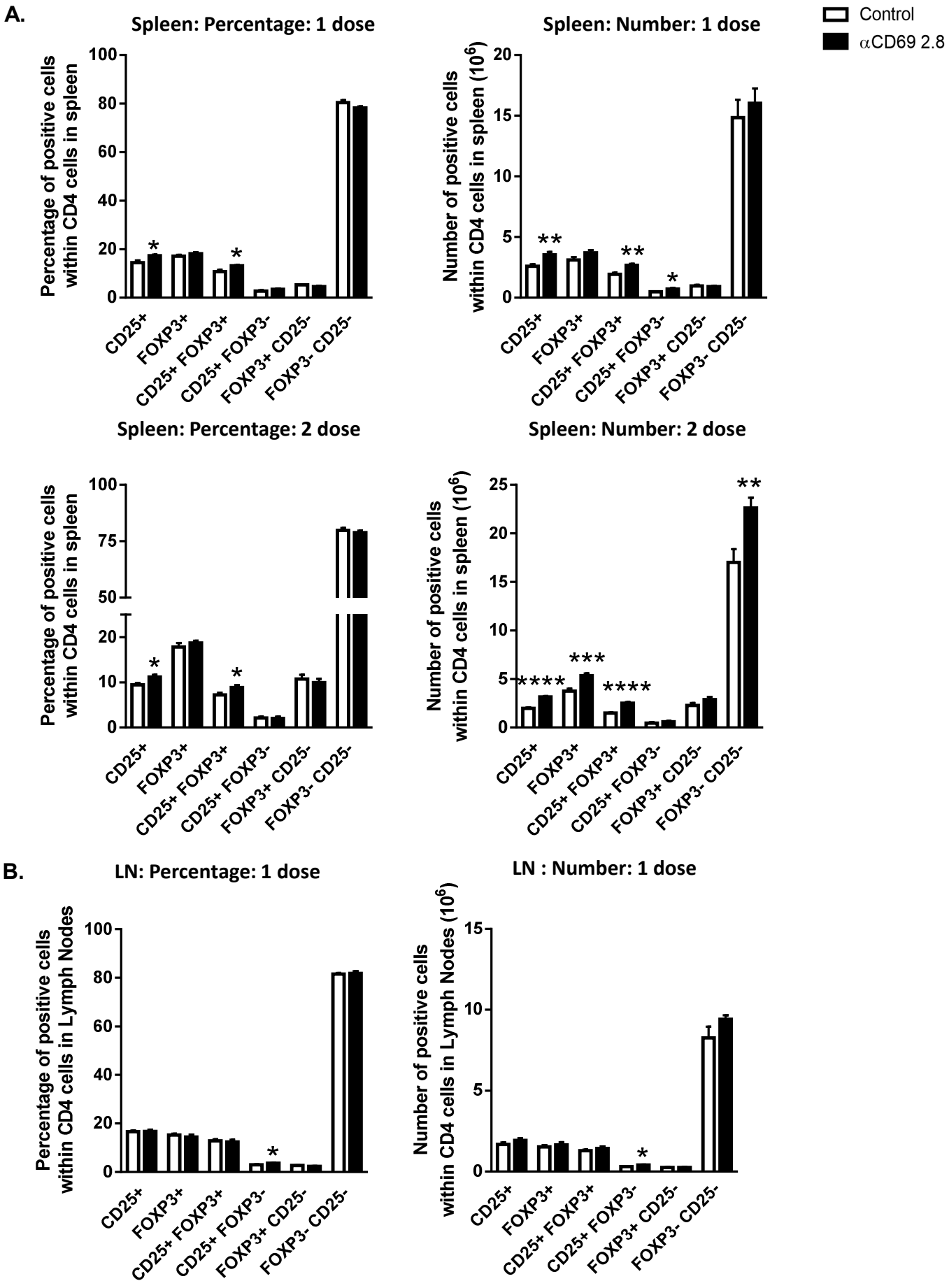


Figure 2. Impairment of inflammatory response in CD69-deficient macrophages. (A) Peritoneal macrophages from WT and CD69-deficient mice were activated for 6 hours with LPS (200 ng/ml) and expression of NOS-2, COX-2 and TNF- was evaluated by quantitative PCR. Results were obtained from three independent experiments carried out by duplicate. Data indicate Standard Deviation (S.D.). (B) Protein levels of NOS-2 and COX-2 were evaluated by Western blot after stimulation of peritoneal macrophages with LPS (200 ng/ml) for 24 hours. Western blots are a representative experiment out of three. Band intensity of Western blots was analyzed by densitometry, normalized to actin levels and represented as the mean S.D. of the fold change from LPS condition (n=3).

ANNEX CHAPTER II:

Mobilization of regulatory T regs with anti-human CD69

In addition, once observed that our treatment leads to significantly more mobilization of both mature and precursor cells, we decided to analyze the effect on regulatory T CD4 cells since previously had been demonstrated that AMD3100 produce an increase of mobilization of Tregs immediately after treatment [298] and this effect has also been shown with G-CSF [299]. Therefore, after inducing mobilization with anti-human CD69, we collect spleen, thymus and lymph nodes cells and analyzed the expression of FOXP3 and CD25 within CD4 cells. The classic Tregs CD4 CD25+FOXP3+ were augmented in percentage in spleen (Fig.1A) in both treatments whereas in lymph nodes and thymus (Fig.1B-C) no changes were observed in one dose treatment and an increase was detected in two doses treatment in lymph nodes and a decrease in thymus. Then we analyzed the number of Treg cells and observed that the spleen displayed a greater number of regulatory cells in treated mice (Fig.1A), the lymph nodes showed a trend at 24 hours but the differences were significant in the long treatment (Fig.1B) while in thymus the tendency was the opposite, there was a clear reduction at 24 hours whereas when we treated with two doses that decrease was not significant (Fig.1C). We also found changes in the number of other populations in spleen, such as CD4 CD25+, including FOXP3+ and FOXP3-, and CD4 FOXP3+, including CD25 + and CD25- (Fig.1A). Lymph nodes showed no changes when mice were treated at 24 hours except the CD4+ CD25+ FOXP3- population which was increased, whereas when the mouse was treated with two doses of anti-human CD69, all regulatory populations were increased. And lastly in the thymus, the numbers of regulatory cells do not present differences between treated and control mice when they received two doses of treatment, whereas at 24 hours we found a decrease of CD4 CD25+ (Fig.1C). Together, these results suggest that treatment with anti-human CD69 induces egress of regulatory T cells from thymus which were observed in spleen and lymph nodes to produce a compensatory effect that regulates cell mobilization and prevents the complete emptying of the bone marrow.


A.
Spleen: Percentage: 1 dose
Spleen: Number: 1 dose
Control
αCD69 2.8
Percentage of positive cells within CD4 cells in spleen
Number of positive cells within CD4 cells in spleen (10^6)
CD25+
FOXP3+
CD25+ FOXP3+
CD25+ FOXP3-
FOXP3+ CD25-
FOXP3- CD25-
Spleen: Percentage: 2 dose
Spleen: Number: 2 dose
B.
LN: Percentage: 1 dose
LN : Number: 1 dose
Percentage of positive cells within CD4 cells in Lymph Nodes
Number of positive cells within CD4 cells in Lymph Nodes (10^6)

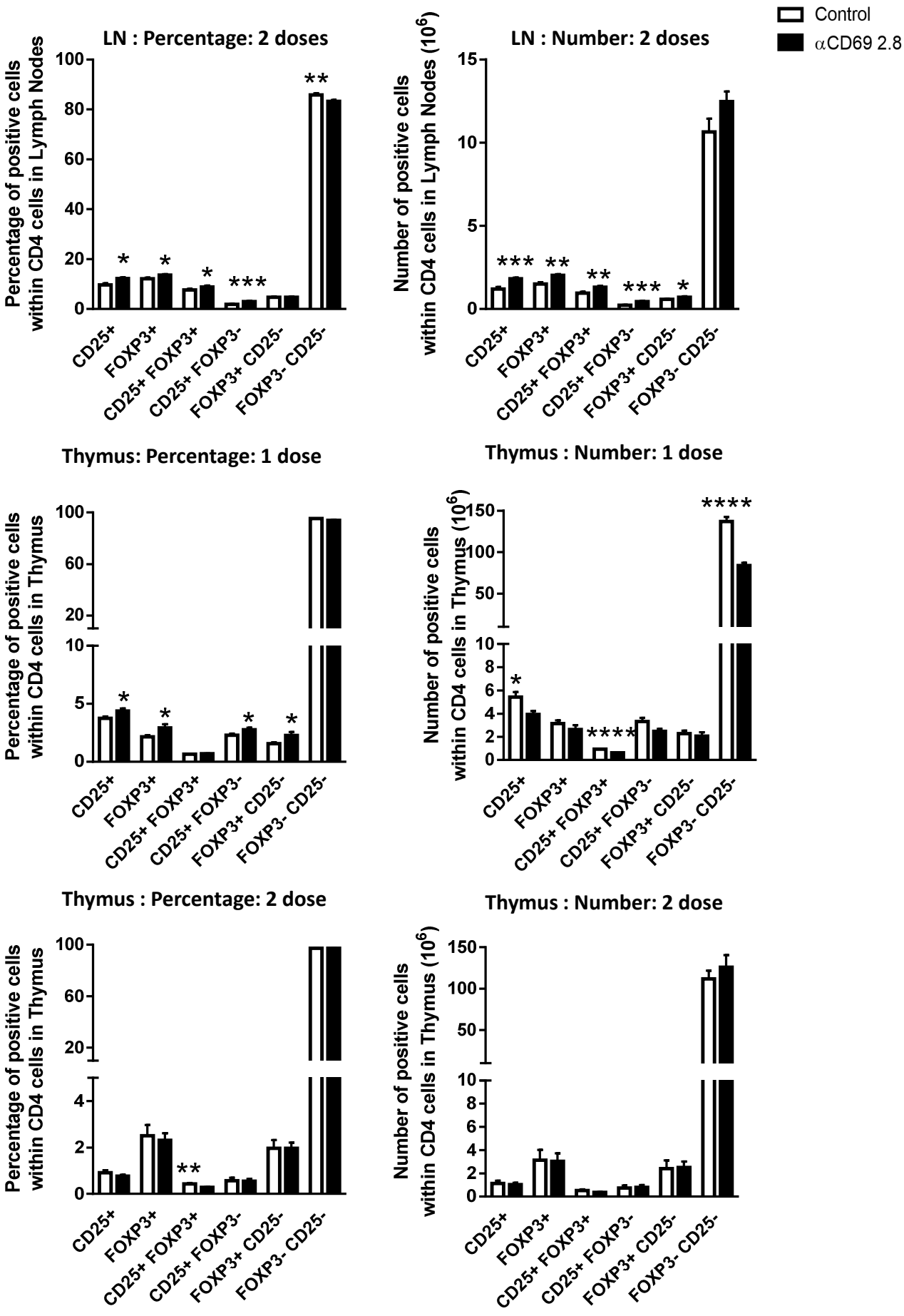
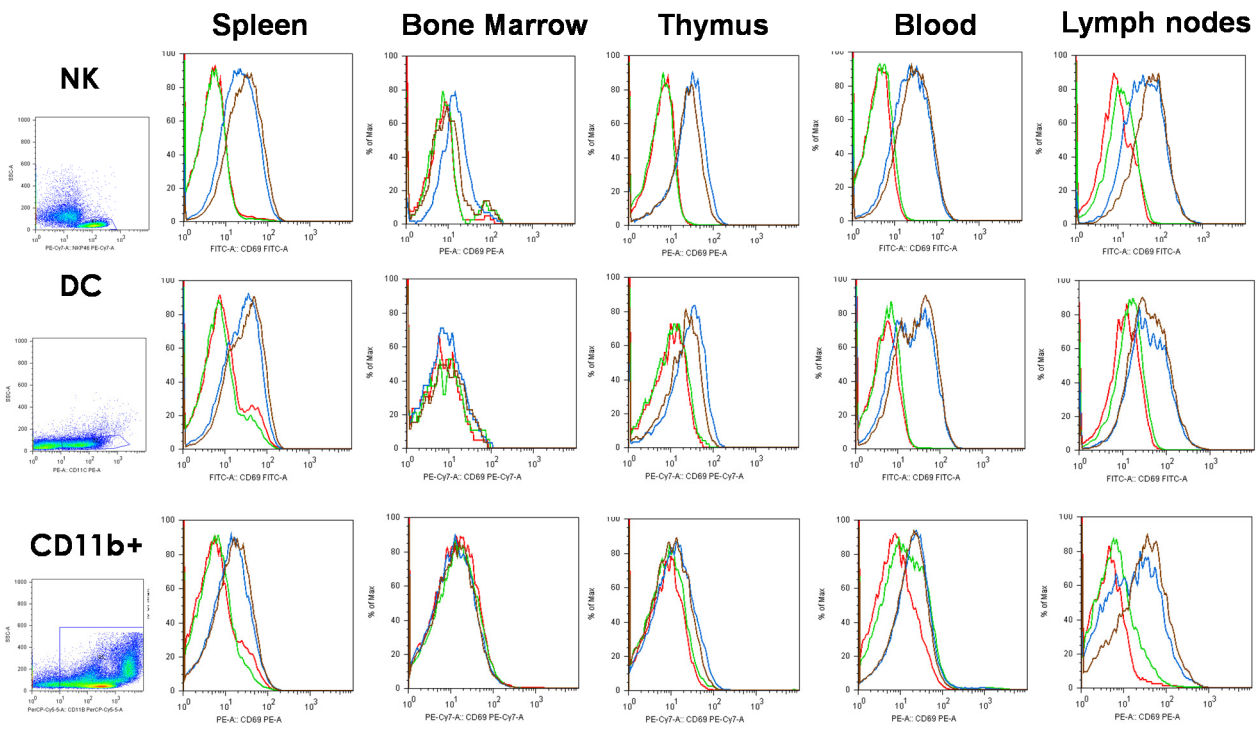

Control
αCD69 2.8
LN : Percentage: 2 doses
Percentage of positive cells within CD4 cells in Lymph Nodes
LN : Number: 2 doses
Number of positive cells within CD4 cells in Lymph Nodes (10^6)
C.
Thymus: Percentage: 1 dose
Percentage of positive cells within CD4 cells in Thymus
Thymus : Number: 1 dose
Number of positive cells within CD4 cells in Thymus (10^6)
Thymus : Percentage: 2 dose
Thymus : Number: 2 dose
CD25+
FOXP3+
CD25+ FOXP3+
CD25+ FOXP3-
FOXP3+ CD25-
FOXP3- CD25-

Figure 1. Targeting of human CD69 induce peripheral CD4+ Treg cells. Mice were treated with 2 doses of 200ug of anti-huCD69 2.8 or PBS i.v with a gap of 1 week and 5 days after second dose mice were analyzed or with 1 dose of 500ug of anti-huCD69 2.8 or PBS i.v and one day after mice were analyzed. Tregs were analyzed by flow cytometry according surface CD25 expression and Intranuclear FOXP3 expression in A, spleen, B, Lymph nodes and C, Thymus left t in percentages and right in numbers within CD4 T cells. Pool of two experiments.

ANNEX CHAPTER III:

CD69 expression in Rag2$^{-/-}$ at steady-state.

To assess CD69 expression levels at steady-state in the different bone marrow, spleen, thymus blood and lymph nodes cell subtypes, we stained samples from Rag2$^{-/-}$ CD69$^{+/+}$ and Rag2$^{-/-}$ CD69$^{-/-}$mice for different cell subsets (Figure 1). NK cells showed a constitutive CD69 expression in spleen, thymus, blood and lymph nodes while NK cells from bone marrow not changes were observed. Similar result was observed in CD69 expression in dendritic cells. CD11b+ cells also presented a constitutive expression in spleen, thymus and lymph nodes. Macrophages, MSDC, granulocytes and B cells did not show CD69 expression in samples analyzed except granulocytes from lymph nodes that also expressed CD69 as a constitutive form. Thus, most cell subtypes show a certain expression of CD69 at steady-state, but this is especially remarkable for NK and dendritic cells.



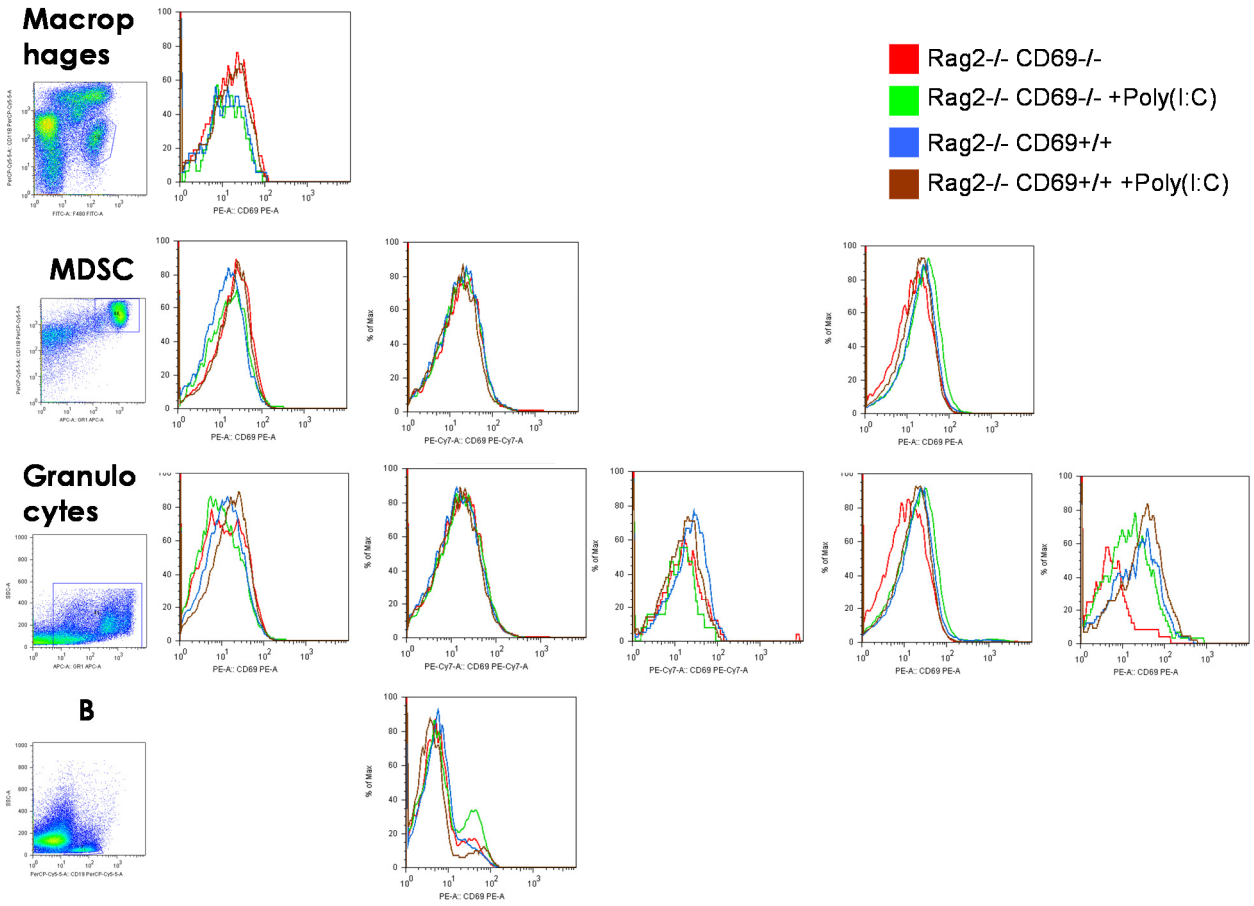


Figure 1. CD69 expression in different cell subpopulations in Rag2-/- CD69-/- mice. Mice were treated with poly i:c or PBS and Spleen, Bone Marrow, Thymus, Blood and lymph nodes were collected and CD69 expression was measured in the indicated subpopulations. Red lines correspond to $Rag2^{-/-}CD69^{-/-}$ mice, Blue lines in $Rag2^{-/-}CD69+/+$ mice, green line in $Rag2^{-/-}CD69^{-/-}$ mice treated with poly i:c and brown lines in $Rag2^{-/-}CD69^{+/+}$ mice treated with poly i:c. One experiment.

ANNEX CHAPTER IV:

Overexpression CD69 also influenced in NK cell distribution.

As we mentioned in the corresponding chapter, CD69 influences in cell distribution through its interaction with S1P1. Here, we examine the influence of CD69 overexpression on the proportion of different lymphoid and myeloid populations. Accordingly, we observed that the percentage of single-positive CD4 and CD8 were significantly augmented in thymus (data not shown) and a reduction was observed in the percentage of these cells in spleen, blood and lymph nodes (Fig. 1A-C). However, we found that the overexpression of CD69 not only influenced in proportion of T cells, but also affected the distribution of NK cells and myeloid cells being found augmented in spleen, blood and lymph nodes. This increase in the proportion of NKs but also of myeloid populations may be related to the increase production of humoral mediators modulating the immune response of transgenic mice to Vaccinia virus infection.

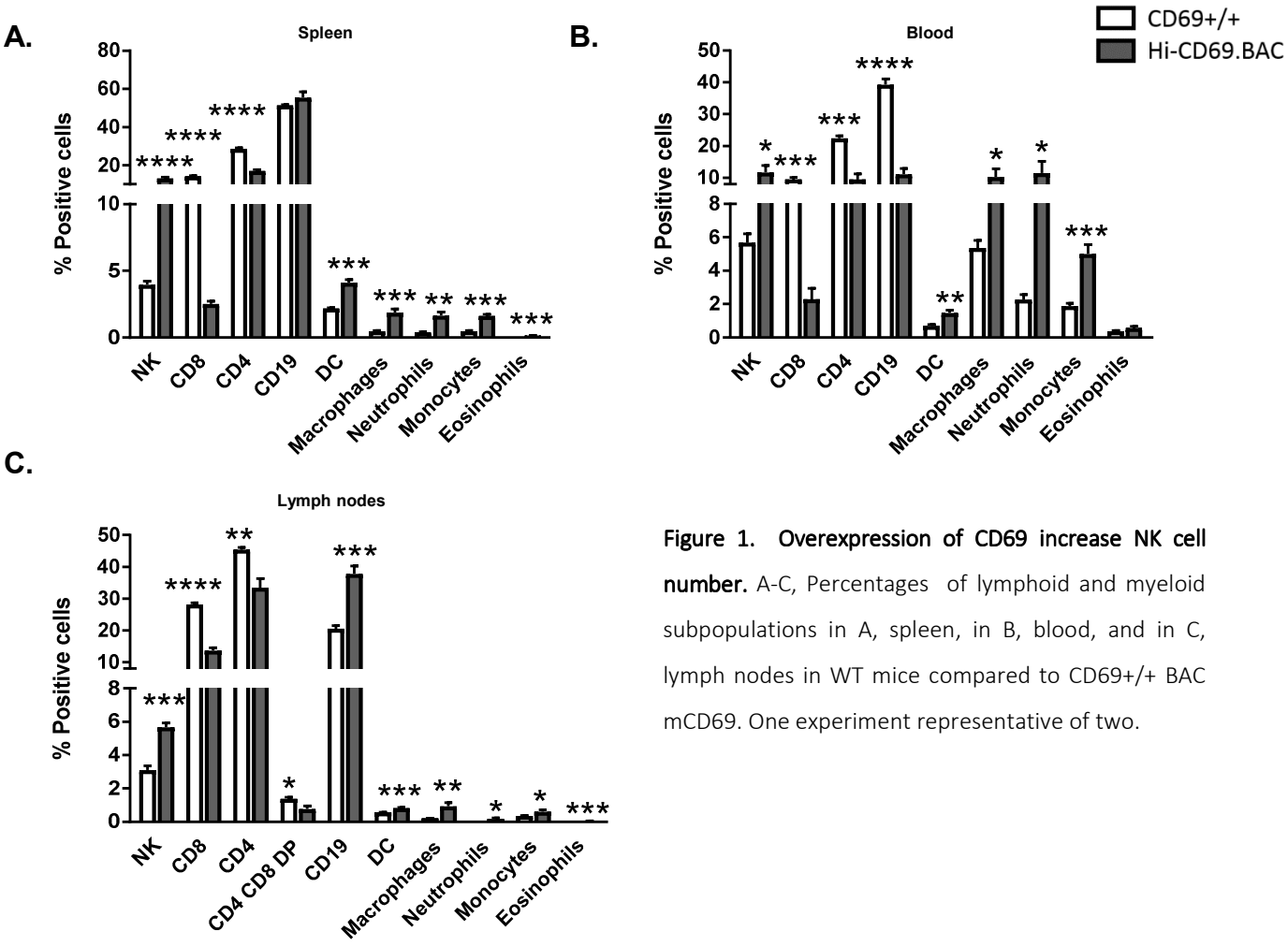
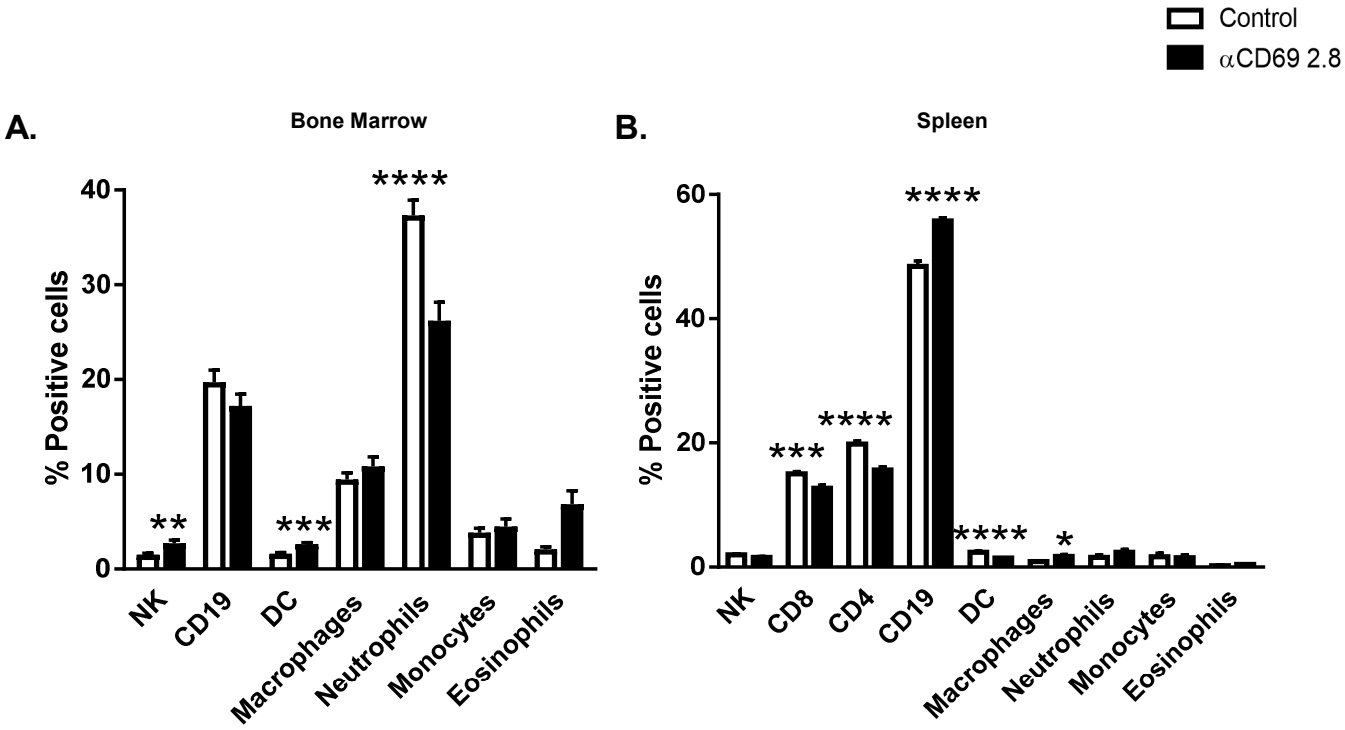


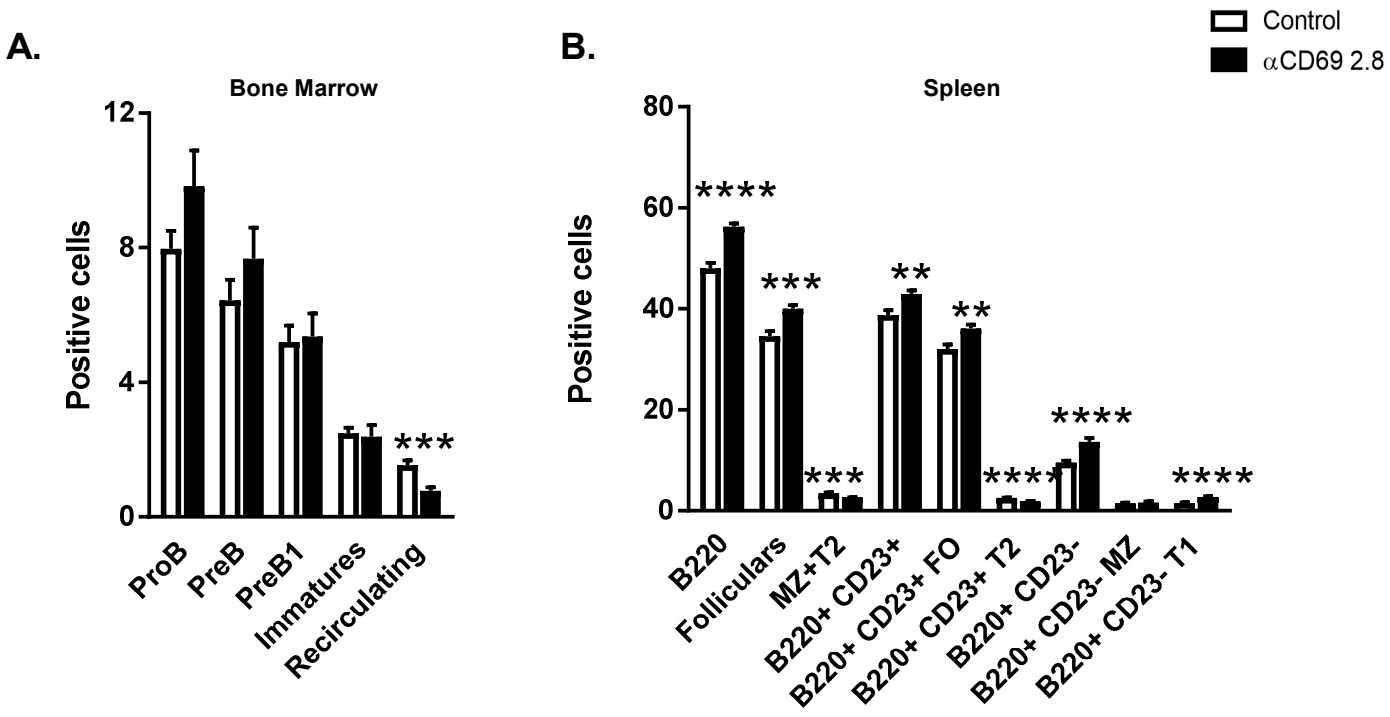
Figure 1. Overexpression of CD69 increase NK cell number. A-C, Percentages of lymphoid and myeloid subpopulations in A, spleen, in B, blood, and in C, lymph nodes in WT mice compared to CD69+/+ BAC mCD69. One experiment representative of two.

SUPPLEMENTARY DATA

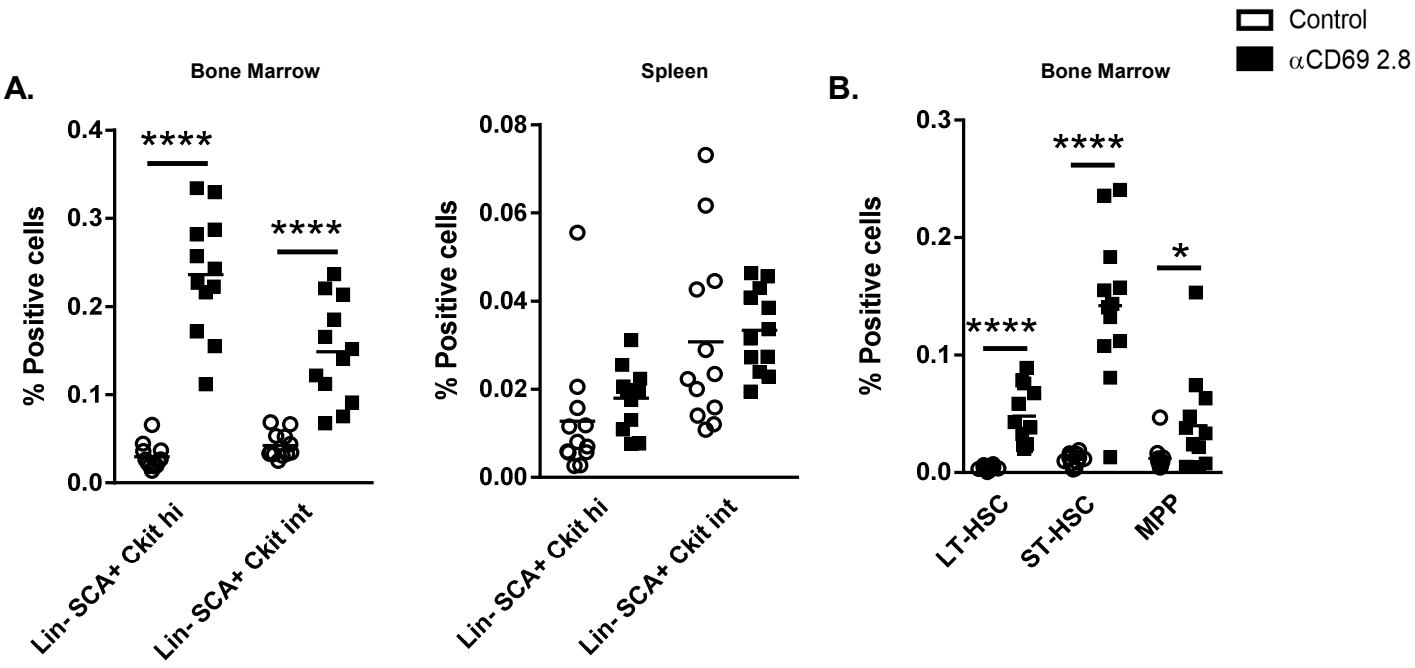
SUPPLEMENTARY CHAPTER II:



Supplementary Figure 1. Targeting of CD69 in HuCD69 mice induced changes in bone marrow and spleen leukocyte distribution. Mice were treated with 500ug of anti-huCD69 2.8 and were analyzed 1 day after treatment. A, Percentages in of lymphoid and myeloid subpopulation cell in A, Bone Marrow and B, spleen. A-B, Pool of four experiments.

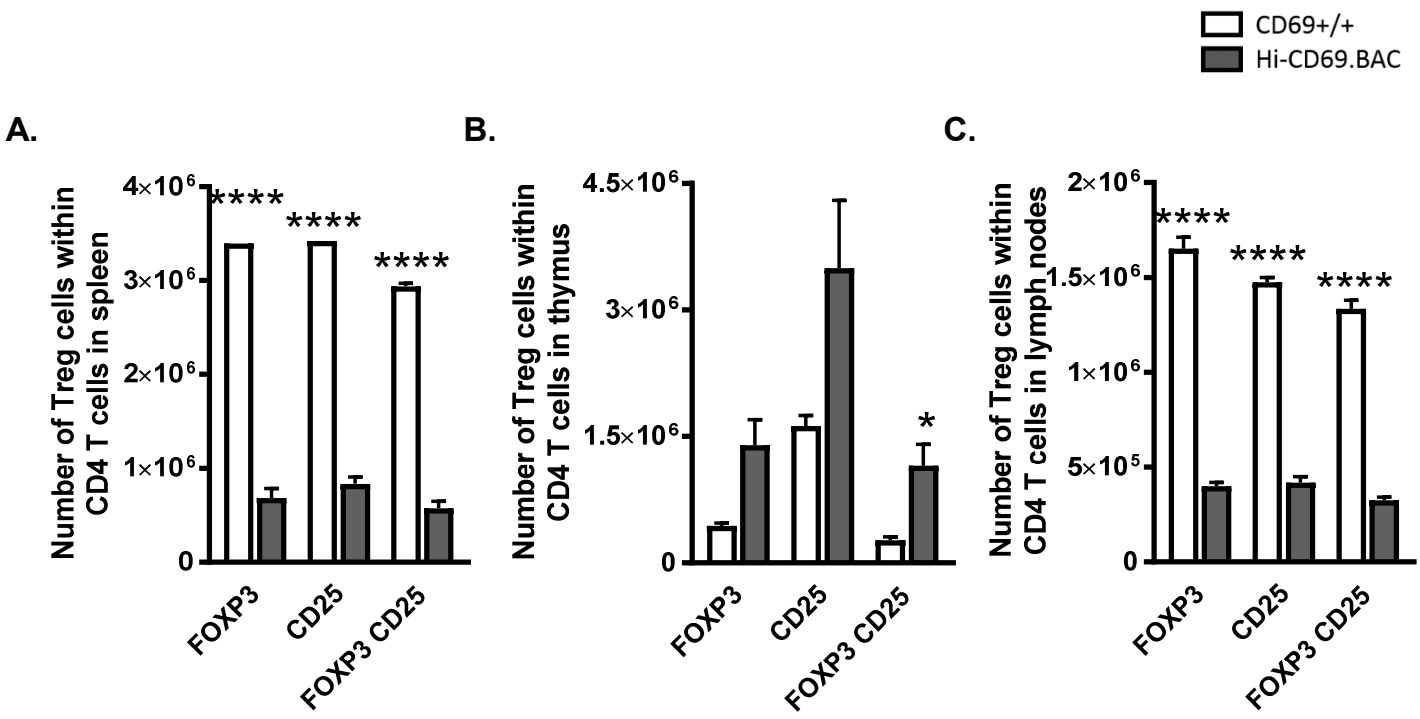


Supplementary Figure 2. Targeting of CD69 in HuCD69 mice induced egress of immature B cells from bone marrow. Mice were treated with 500ug of anti-huCD69 2.8 and were sacrificed 1 day after treatment. A-B, Percentages of B cell subpopulations were measured in Bone Marrow in A and in spleen in B, by flow cytrometry. Pool of three experiments.



Supplementary Figure 3. The treatment with anti-human CD69 increase HSC cells in Bone Marrow. A-B, Mice were treated with anti-human CD69 2.8 24 hours before analyzing. Cells from Bone Marrow and Spleen were analyzed by flow cytometry. A and B, Lin- cells were stained by SCA+ and C-kit hi or C-kit int. Percentages in A, of KSL and CLP cells were measured in (left) Bone Marrow and (right) spleen. B, Cells CD34 and FLT3 were gated in Sca+, C-kit hi cells and numbers of LT-HSC, ST-HSC and MPP are shown in Bone Marrow. A-B, Pool of three experiments.

SUPPLEMENTARY CHAPTER IV:



Supplementary Figure 1. Overexpression of CD69 also retains regulatory T cells in thymus after 7 days of VACV unfection. Mice were infected with $1x10^7$ pfu i.p and 7 days after infection, mice were sacrificed. Number of Tregs were analyzed by flow cytometry according surface CD25 expression and Intranuclear FOXP3 expression in A, spleen, B, Thymus and C, Lymph nodes.