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CD69 targeting enhances anti-Vaccinia virus immunity

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Abstract

CD69 is highly expressed on the leukocyte surface upon viral infection, and its regulatory role in the vaccinia virus (VACV) immune response has been recently demonstrated using CD69^{-/-} mice. Here, we show augmented control of VACV infection using the anti-human CD69 monoclonal antibody (mAb) 2.8 as a therapeutic treatment for mice expressing human CD69. This control was related to increased natural killer (NK) cell reactivity and increased numbers of cytokine-producing T and NK cells in the periphery. Moreover, similarly increased immunity and protection against VACV were reproduced over both long and short periods in anti-mouse CD69 mAb 2.2-treated immunocompetent wild-type (WT) mice and immunodeficient Rag2^{-/-} CD69^{+/+} mice. This result was not due to synergy between infection and anti-CD69 treatment since, in the absence of infection, anti-human CD69 targeting induced immune activation, which was characterized by mobilization, proliferation, and enhanced survival of immune cells as well as marked production of several innate proinflammatory cytokines by immune cells. Additionally, we showed that the rapid leukocyte effect induced by anti-CD69 mAb treatment was dependent on mTOR signaling. These properties suggest the potential of CD69-targeted therapy as an antiviral adjuvant to prevent derived infections.

21 **Importance**

22 In this study, we demonstrate the influence of human and mouse anti-CD69 therapies on
23 the immune response to VACV infection. We report that targeting CD69 increases the
24 leukocyte numbers in the secondary lymphoid organs during infection and improves the
25 capacity to clear the viral infection. Targeting CD69 increases the numbers of gamma
26 interferon (IFN γ)- and tumor necrosis factor alpha (TNF α)-producing NK and T cells.
27 In mice expressing human CD69, treatment with an anti-CD69 mAb produces increases
28 in cytokine production, survival and proliferation mediated in part by mTOR signaling.
29 These results, together with the fact that we have mainly worked with a human-CD69
30 transgenic model, reveal CD69 as a treatment target to enhance vaccine protectiveness.

Introduction

The use of vaccinia virus (VACV) had an essential role in the eradication of smallpox, and since then, VACV has been used as a model to study virus-host interactions. It is a complex, enveloped, linear, double-stranded DNA virus with many genes dedicated to the evasion of the innate immune system and the action of interferons. VACV infection clearance is first controlled by the innate immune response mostly through NK cells due to the production of cytokines such as type I and II IFNs and TNF α (1). However, ultimately, if VACV infection is not resolved, a powerful adaptive immune response essential for the proper elimination of VACV infection is activated by specific CD8⁺ and CD4⁺ T cells (2). Currently, in many clinical trials, vaccinia virus is being used to construct directed targets for vaccine candidates against various diseases, such as HIV/AIDS (3), hepatitis (4), influenza (5), malaria (6), tuberculosis (7), and even oncogenic diseases of both human and domestic animals (8).

CD69 is a leukocyte receptor constitutively expressed on the surface of subsets of thymocytes, T cells, NK cells, plasmacytoid dendritic cells and progenitor cells (HSPC) (9-12). This receptor is known to be one of the earliest activation markers since its expression is promptly induced or further upregulated on all studied leukocyte subtypes upon activation (10). CD69 is highly expressed in leukocytes during infections and at inflammatory locations in autoimmune and allergic diseases. The *in vivo* function of CD69 as a regulator of the immune response has been revealed by the study of CD69-deficient mice using different murine inflammatory models, including tumor immunity, infection and autoimmune disease models (13-20). The effects of treatment with the anti-mouse CD69 2.2 nondepleting antibody (anti-mCD69-2.2) partially resemble the CD69-deficient phenotypes in tumor, arthritis, and contact hypersensitivity models (17, 21, 22). Roles for CD69 in leukocyte retention in the thymus and secondary lymphoid organs have been associated with the cis interaction between CD69 and Sphingosine-1-Phosphate Receptor 1 (S1P₁), which downregulates S1P₁ surface expression and inhibits lymphocyte egress from the thymus and peripheral lymphoid organs (23). In agreement with the role of CD69 in leukocyte retention in the bone marrow (BM), our recent published study showed that CD69 deficiency or targeting with nondepleting anti-CD69 mAbs promotes the egress of hematopoietic precursor cells from the BM (11). Moreover, anti-CD69 mAb treatment induces the expansion of HSPC dependent on mTOR signaling (11). mTOR, which is of chief importance in cell metabolism,

proliferation and survival, can exist in two distinct complexes: mTORC1, whose downstream targets are p70S6K and 4E-BP1, and mTORC2, whose activity is monitored by the downstream phosphorylation of Akt at Serine 473 (24). Previous studies on the role of CD69 in the immune response to VACV infection, showed the importance of CD69 in the formation of virus specific and protective skin resident memory T cells [Mackay, 2013 #388][Khan, 2016 #387]. Also, in our preceding study, the CD69 deficiency resulted in increased early NK cell-dependent control of the infection (18). These results are in accordance with those obtained investigating in vivo infections with deletion mutant virus, where inactivation of N1L VACV gene resulted in an enhanced NK response and reduced numbers of CD69+ leukocytes. In the present study, we analyzed the effect of targeting CD69 in the VACV infection model. Anti-CD69 therapy resulted in increased control of VACV infection, and this control was associated with increased numbers of IFN γ - and TNF α -producing NK cells and CD4⁺ and CD8⁺ T cells reacting in a noncognate fashion. In addition, the percentages of these cytokine-producing cells were increased, indicating that targeting CD69 increases the reactivity of these effector cells. We showed that the increase in leukocyte numbers in the peripheral organs induced by targeting CD69 was mediated by S1P receptor-dependent mTOR signaling. In agreement with the roles of mTOR in proliferation and cell survival, we found that these parameters increased upon anti-CD69 therapy, which contributed to the increased leukocyte counts observed in the long term. Moreover, we observed a sharp induction of the expression of cytokines, many of which are important in innate immune responses.

RESULTS

Anti-CD69 mAb treatment enhances protection against VACV infection.

First, we tested whether anti-CD69 pretreatment could enhance the control of VACV infection. To do this we used a human-CD69 transgenic mouse model that carries a BAC containing the human CD69 gene locus on the mouse CD69^{-/-} background (huCD69). The mice were treated with two doses of 200 µg of anti-human CD69 mAb 2.8 (anti-huCD69-2.8) separated by one week and i.p infected with 1x10⁷ plaque-forming units (pfu) of VACV 5 days after the last mAb dose. The mice were analyzed for ovarian viral counts and peripheral leukocyte numbers seven days after infection, which is the peak of the T cell primary response (Fig. 1A). The treated mice controlled VACV infection more efficiently than control mice (Fig. 1B). A decrease and increase in the total leukocyte counts in the BM and spleen, respectively, were observed, as well as small increases in the lymph node and blood leukocyte counts (Fig. 1C). The numbers of CD8⁺ T cells, macrophages and eosinophils were slightly but significantly increased (Fig. 1D). The treatment also induced an increase in the number of CD4⁺ T cells producing IFN γ and a tendency toward higher numbers of CD4⁺ T cells producing TNF α and CD8⁺ T cells producing IFN γ (Fig. 1E-F). Thus, treatment with the anti-huCD69-2.8mAb could affect the primary adaptive response to VACV infection by increasing the number of activated T cells in the periphery. Similarly, WT mice treated with the anti-mCD69-2.2 mAb using the same schedule more efficiently limited infection than control-treated mice and had strong increases in leukocyte numbers in the spleen, lymph nodes and blood, and the numbers of the main splenic lymphocyte subsets, dendritic cells (DCs) and TNF α - and IFN γ -producing CD4⁺ T cells were augmented (data not shown).

To assess the role of CD69 treatment in a more natural experimental model we used a low-dose VACV infection and study the weight of mice intranasal infected with 10⁴ pfu of VACV overtime. Non treated mice showed a reduced weight loss relative to their anti-CD69 counterparts (Figure 2). The differences were already significant 1 day after infection, maintained overtime and augmented after seven days which suggested that anti-CD69 treatment act along the immune response. In summary, targeting human and mouse CD69 resulted in an increased ability to control VACV infection and increased the accumulation of leukocytes, including effector lymphocytes, in peripheral sites.

Targeting CD69 enhances the innate immune response to VACV infection

To study whether the enhanced antiviral control was in part due to a more efficient early innate response, we performed the same treatment regimen in huCD69 mice and analyzed viral titers and leukocyte subsets two days after VACV infection (Fig. 3A), at which time point the adaptive response to VACV infection had not yet developed. At this early time point, anti-huCD69-2.8 mAb treated mice already showed slightly lower ovarian viral titers (Fig. 3B) and this increased control is already detected in the spleen after 6 hours of infection (Fig. 3G-H). The numbers of total blood and splenic leukocytes (Fig. 3C) and all splenic subtypes analyzed in the mAb-treated mice were increased compared to those in control mice (Fig. 3D). Moreover, the treatment induced marked increases in the numbers of TNF α -producing NK, CD8 $^{+}$ T and CD4 $^{+}$ T cells and IFN γ -producing NK and CD4 $^{+}$ T cells and a tendency toward higher numbers of IFN γ -producing CD8 $^{+}$ T cells (Fig. 3E,F). Very similar results were obtained when WT mice were treated with only one injection of anti-mCD69-2.2 administered 24 hours before VACV infection and analyzed at 2 days postinfection (Fig. 4A-H). Also, there was a significant differences in viral titers when the treatment with anti-mCD69-2.2 was administered 24 h after VACV exposure.

This early time point postinfection at which the antibody pretreatment affects viral titers suggests a contribution of the innate immune system. To assess the importance of the innate immune response against VACV infection in the absence of T and B cells, we infected Rag2 $^{-/-}$ mice pretreated with anti-mCD69-2.2 mAb with 1×10^6 pfu of VACV and analyzed them two days after infection (Fig. 5A). As in the treated WT and huCD69 mice, the anti-CD69-treated Rag2 $^{-/-}$ mice showed lower ovarian viral titers than control mice (Fig. 5B) and decreased and increased leukocyte numbers in the bone marrow and spleen, respectively (Fig. 5C). Consistently, in the spleen, targeting CD69 induced increases in NK cell, monocyte and neutrophil counts (Fig. 5D). Moreover, we found that the antibody treatment increased the percentages (Fig. 5E) and numbers (Fig. 5F) of NK cells producing IFN γ - and TNF α - and undergoing degranulation (CD107+).

Altogether, these results show that anti-CD69 pretreatment increases the innate immune control of viral infection accompanied by increases in peripheral innate leukocyte numbers and cytokine-producing T and NK cell numbers. Unlike CD69 deficiency, however, targeting CD69 increases the percentages of cytokine-producing T and NK

cells and degranulating NK cells, suggesting that targeting CD69 augments T and NK cell reactivity.

In the absence of infection, targeting CD69 promotes the proliferation and survival of leukocytes.

Next, we studied whether the differences observed in the infection setting are due to the effects of the mAb treatment on components of the immune system that will later respond to the infection. Thus, we were interested in analyzing the effects of the anti-CD69 mAb treatment schedule in the absence of infection. To study this possibility, we injected huCD69 mice with two doses of 200µg of anti-huCD69 2.8 separated by one week and analyzed the BM and splenic numbers of leukocyte subtypes 5 days after the second dose. Total leukocyte counts were decreased in the BM and increased in the secondary lymphoid organs of the treated mice (Fig. 6A). The numbers of NK cells, T cells and B cells were augmented in the spleen (Fig. 6B). Then, we studied whether mechanisms such as increased leukocyte proliferation and/or survival might contribute to the increased peripheral cellularity observed in the longer term. Thus, we tested the effect of anti-huCD69 2.8 treatment on leukocyte proliferation. HuCD69 mice were administered the anti-huCD69 2.8 mAb, and after 24 hours, they were injected with bromodeoxyuridine (BrdU) and analyzed 3 hours later. BrdU incorporation was increased in most of the major leukocyte lineages in the BM and spleen (Fig. 7A-B). Of note, after only a 3-hour pulse, the BrdU⁺ myeloid cells likely consisted mainly of differentiated myeloid cells, which have been demonstrated to be able to proliferate *in vivo* (25-28).

For cell survival analysis, since dead cells are quickly phagocytized *in vivo*, we performed *ex vivo* cultures of splenocytes from anti-human CD69 2.8-treated mice and analyzed the spontaneous cell death by propidium iodide (PI) staining at different times of culture. Significant differences were found at early time points, and these differences increased over time (Fig. 7C). Altogether, these data suggest that anti-CD69 mAb treatment may achieve greater peripheral cellularity through increased survival and proliferation of mature leukocytes and that this state can be maintained by repeated anti-CD69 mAb administration.

Targeting CD69 promotes mitogenic and proinflammatory cytokine production

We previously described that the anti-mCD69-2.2 mAb induces T cell proliferation through IL-2 production by plasmacytoid DCs and interleukin-2 receptor alpha (IL-2Ra) expression upregulation in memory T cells (9). Thus, we tested whether anti-human CD69 2.8 treatment could also upregulate IL-2 and IL-2Ra expression. As expected, we found increased intracellular IL-2 and surface IL-2Ra levels in cells from the bone marrow, spleen and blood 24 h after anti-huCD69 2.8 treatment (Fig. 8A). These increases may contribute to the higher T and NK cell proliferation observed in anti-CD69-treated mice.

Previous studies performed in our laboratory showed decreased transforming growth factor beta (TGF- β) and increased proinflammatory cytokine expression in CD69^{-/-} mice, which was related to increased splenic cellularity and decreased spontaneous cell death. We wanted to test whether this could also be achieved by targeting CD69 with a mAb. Thus, we assessed the expression of TGF- β and several proinflammatory cytokines (IL-1 α and β ; LT α and β ; IL-17 α , β and f; IL-21; IFN γ ; and TNF α) and chemokines (CCL2 and CCL12) 24 h after anti-huCD69 2.8 treatment by real-time PCR (RT-PCR). We found a decrease in TGF- β expression in the spleen of treated mice (Fig. 8B). Instead, the splenic messenger RNA (mRNA) levels of IL-1 α and β , LT α and β , IL-17 β and f, IL-21, IFN γ , TNF α , CCL2 and CCL12 were increased (Fig. 8D-E). We also measured the expression of IL-1 α and β , IL-21, IL-17 f, IFN γ , IL-3 and IL-6 at the protein level in supernatants of huCD69 splenocytes 1 day after anti-huCD69 mAb 2.8 treatment with a Luminex assay. The results showed highly significant increases in IL-1 α , IL-1 β , IFN γ and IL-6 production (Fig. 8G) in the anti-CD69 mAb 2.8-treated mice. In summary, targeting CD69 rapidly induces a promitogenic and proinflammatory cytokine profile.

Targeting CD69 induces leukocyte accumulation in the spleen through mTOR signaling.

We reported that anti-CD69 mAb-induced BM mobilization is dependent on S1P receptor function since this migration is inhibited by S1P receptor desensitization with fingolimod (FTY720) treatment (11).

Several reports have shown that S1P mediates BM mobilization through mTOR (29-31). We showed that mTOR inhibition in huCD69 mice by rapamycin pretreatment blocked the increase in splenic cellularity and partially inhibited the decrease in whole BM cell numbers induced by the anti-hCD69 mAb 2.8 at 24 h posttreatment (Fig. 9A), suggesting that mTOR also mediates the accumulation of leukocytes in the spleen induced by targeting CD69. Consistently, anti-CD69 mAb 2.8 treatment induced the phosphorylation of the mTOR target p70S6K, and this effect was inhibited in the spleen by rapamycin treatment (Fig. 9B). Moreover, FTY720 treatment also inhibited the phosphorylation of p70S6K in the spleen (Fig. 9C), suggesting that targeting CD69 induces mTOR signaling through S1P receptors. Altogether, the presented data suggest that the effect on leukocyte accumulation in the spleen induced by targeting CD69 is mediated by S1P receptor signaling through the mTORC1 cascade.

DISCUSSION

In the present study, we show that anti-CD69 mAb pretreatment enhances innate immune protection against VACV infection related to enhanced numbers of peripheral cellular effectors, which can be explained by increased BM cell output and leukocyte proliferation and survival. In previous reports, targeting CD69 resulted in outcomes similar to those seen with CD69 deficiency in several murine models of cancer and autoimmune disease (19, 21, 22). Similar outcomes are also seen in the VACV infection model since, like targeting CD69, CD69 deficiency also enhances the host innate immune resistance to VACV infection accompanied by increases in peripheral cellularity and the numbers of T and NK cells producing IFN γ and TNF α and degranulating NK cells (18). The similarities in cellularity and immune control in early VACV infection between targeting CD69 and CD69 deficiency could be due to targeting CD69 simulating the avoidance of the cis interaction between CD69 and S1P1 and the downregulation of S1P1 expression, which hinders BM cell egress, seen in CD69 deficiency. However, several data points indicate that the mechanisms underlying the effects of targeting CD69 and CD69 deficiency may differ to some extent.

On the one hand, although both settings show increases in the numbers of cytokine-producing T and NK cells, only targeting CD69 shows an increase in the percentages of these cells. For NK cells, this finding suggests that, unlike CD69 deficiency, targeting CD69 enhances NK cell reactivity, probably through cytokine-mediated NK cell priming. The T cells producing IFN γ and TNF α upon restimulation at two days postinfection are likely T cells activated in a noncognate fashion by cytokines. This result may support the conclusion that targeting CD69 but not CD69 deficiency induces proinflammatory cytokine production *per se*.

At seven days postinfection, the detected IFN γ ⁺ and TNF α ⁺ T cells may include VACV-specific T cells. However, we do not expect that specific T cells account for the differences since anti-mCD69-2.2 mAb treatment *in vivo* did not affect the frequency of VACV-specific T cells at the peak of the primary immune response to VACV infection (32).

Another difference between CD69 deficiency and targeting CD69 is that while targeting CD69 augments the leukocyte proliferation rate, CD69 deficiency has no effect on that

rate (32, 18). This result also argues against the presence of increased amounts of mitogenic cytokines in CD69 knockout (KO) mice under steady-state conditions, although CD69 KO mice do show proinflammatory cytokine profiles upon the induction of cancer or autoimmune disease (15, 22).

In both CD69-deficient mice and CD69-targeted mice, cell survival is increased (18). This result may be related to the fact that TGF β expression is decreased in almost all disease models performed with both CD69^{-/-} and anti-CD69 mAb-treated mice (15, 19-22, 33, 34). TGF- β is a pleiotropic cytokine, and one of its reported functions is the control of cell survival, including the survival of leukocytes (35). Interestingly, CD69 deficiency in FoxP3 Tregs leads to decreased TGF β production, and both CD69 deficiency and targeting CD69 in these cells hinder their suppressive function (33).

Another observation indicates that the situations in CD69-targeted and CD69-deficient mice differ; in CD69-targeted mice, there is massive egress of cells with undetectable CD69 surface levels, such as neutrophils, which suggests the contribution of a cell extrinsic mechanism, putatively mediated by soluble mediators. One of these soluble mediators is likely to be S1P acting through mTOR signaling since S1P receptor desensitization with FTY720 inhibits anti-CD69 treatment-induced egress from the BM (11) and mTOR signaling, and mTOR inhibition with rapamycin partly inhibits BM mobilization. Other possible soluble mediators are cytokines and chemokines. In the present work, we observed enhanced expression of several innate cytokines and chemokines. Some of these cytokines, i.e., IL-1, IL-2 and IFN γ , activate mTOR through PI3K and phospho-Akt T308. Growth factors can also activate mTOR through ERK1/2 signaling (36). Instead, S1P activates mTORC1 through the E3-ubiquitin ligase PAM in a manner independent of PI3K, Akt and ERK1/2 (37). The fact that we did not detect any phosphorylation of Akt T308 or ERK1/2 may suggest that, at the time of analysis, targeting CD69 had induced mTORC1 activation mainly through S1P receptors rather than through cytokines or growth factors.

VACV employs numerous immune evasion mechanisms to decoy, inhibit the production, and block the signaling of type I and II IFNs, TNF α , IL-1 and IL-18, which amplify the innate immune response, influence the adaptive response to the virus and, in the case of type I and II IFNs and TNF α , have direct antiviral activity (38). Thus, the observed augmented expression of IFN γ , TNF α and IL-1 as well as the possible

290 augmentation of the expression of other cytokines may counteract these immune
291 evasion mechanisms of the virus. Instead, an enhanced proinflammatory cytokine
292 response is detrimental in the cases of other pathogens.

293 The data presented here showing that targeting CD69 enhances the early control of
294 pathogens encourage future testing of anti-CD69 mAbs as agents for enhancing the
295 immune responses in vaccine-mediated protection and against infection in leukopenia
296 treatment.

Materials and methods

Mice and *in vivo* protocols

All mice used were males or females between 6 and 12 weeks of age that were bred and housed under specific pathogen-free conditions in the *Instituto de Salud Carlos III* (ISCIII) animal facilities (Madrid). HuCD69 mice were obtained by transgenesis of a human CD69 BAC containing 100 kb of the CD69 gene, which was generated by Bristol Myers and kindly provided by Dr. Robert Graziano. These mice, as carry the entire gene locus expressed human CD69 in a regulated fashion driven by its regulatory regions of the CD69 locus, as previously shown (11). These mice were maintained as hemizygous animals, and had three copies of the human CD69 BAC on the mouse CD69^{-/-} background. The Rag2^{-/-} in Balb/c genetic mice background were used .

Mice treated with FTY720 (Sigma-Aldrich) were injected intraperitoneally (i.p) with two doses of FTY720 (5 mg/kg) separated by 24 hours.

The mice that received rapamycin (Selleckchem) were treated with 480 mg of rapamycin/kg administered i.p for five days.

In proliferation assays, mice were injected intraperitoneally with 1 mg of BrdU and after three hours, splenocytes and BM cells were collected. BrdU staining was performed using a BrdU Flow kit (Beckton Dickinson) and an anti-mouse BrdU antibody (clone B44) according to the manufacturer's instructions.

Antibodies and cell culture

The anti-huCD69-2.8 mAb and anti-mCD69-2.2 mAb both IgG1 isotype, which binds to the human and the mouse CD69 molecule respectively, were generated in our laboratory immunizing CD69-deficient mice with cells that co-expressed (11, 21). Both antibodies, as previously experimentally demonstrated (11, 21), do not activate the complement system or induced ADCC. Because anti-huCD69 mAb 2.8 does not cross-react with mouse CD69 and anti-mCD69-2.2 mAb does not cross-react with human CD69, we used the anti-mouse-CD69 mAb as isotype control of anti-human-CD69 mAb experiments and vice versa. These mAbs were purified by standard methods and tested with CD69^{-/-} BM-derived DC (BMDC) cultures at a concentration of 10 µg/ml, and these cultures were unable to upregulate CD80 or CD86 expression. HuCD69 mice were intravenously treated with one dose of 500 µg of anti-huCD69 2.8 or with two

doses of 200 µg of anti-huCD69 2.8 separated by a week, and 5 days after the second dose, the mice were analyzed or infected as appropriate for the experiment. In some cases, the anti-mCD69-2.2 mAb was used as a control, while at other times, phosphate-buffered saline (PBS) was used as a control. CD69^{+/+} and Rag2^{-/-} mice were treated following a protocol similar to that used for the HuCD69 mice but with the anti-mCD69-2.2 mAb administered i.v. and the anti-huCD69-2.8 mAb or PBS used as a control.

The mAbs were purified from concentrated hybridoma supernatants using protein G columns (GE Healthcare, Piscataway, NJ, USA), further purified by Zeba Spin desalting columns (*Thermo Scientific*) and stored at -80°C.

Cell Isolation

Bone marrow was collected from the two femurs of each mouse. Blood was collected by intracardiac puncture and diluted in 10 ml of 2 mM EDTA PBS, and the numbers shown are the white blood cell counts per 1 ml of blood. The thymus and spleen were disaggregated, and the cells were washed in PBS. Erythrocytes were lysed with an ammonium chloride potassium (ACK) solution in all samples, and leukocytes were labeled and analyzed by flow cytometry.

Abs and flow cytometry

Cells from the bone marrow, spleen, blood, thymus and lymph nodes were incubated with anti-CD16/32 (Fc-block 2.4G2; BD Biosciences, Franklin Lakes, NJ, USA). The following antibodies 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), anti-CD49b (clone DX5), anti-CD69 (clone H1.2F3), anti-CD107a (clone eBio4A3), anti-CD117 (clone 2B8), anti-F480 (clone BM8), anti-GR1 (clone RB6-8C5), anti-IFN-γ (clone XMG 1.2), anti-NKp46 (clone 29A1.4), anti-CD3 (clone 17A2), anti-IL2 (clone JES6-5H4), anti-CD25 (clone 3C7), anti-H-2K^b (clone AF6-88.5) and anti-TNF-α (clone MP6-XT22). Cells were analyzed with a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using BD FACSDiva software (Becton Dickinson), and data were analyzed with FlowJo (TreeStar Inc., Ashland, OR, USA).

To assess the intracellular production of IFN- γ , TNF- α and IL-2, 2×10^6 splenocytes were incubated in the presence of brefeldin A (BFA) (5 μ g/ml) for 4 h at 37°C and washed. Alternatively, cells were restimulated with 10 ng/ml PMA (*Phorbol* 12-myristate 13-acetate) and 1 μ g/ml ionomycin or RPMI medium only in the presence of BFA (5 μ g/ml) for 4 hours at 37°C. Following incubation, the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 12 minutes at room temperature in the dark and permeabilized with 1% saponin (Sigma-Aldrich) in 1x PBS and 3% fetal bovine serum (FBS) for 20 min at 4°C. The cells were acquired with a FACSCanto instrument (Becton Dickinson, Franklin Lakes, NJ, and USA) using BD FACSDiva software (Becton Dickinson), and the data were analyzed with FlowJo (TreeStar Inc., Ashland, OR, USA).

Vaccinia virus

The Western Reserve strain of VACV (provided by Dr. Daniel Lopez) was grown in African green monkey kidney fibroblast cells (CV1 cells, provided by Dr. Daniel Lopez) cultured in D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 5 μ M β -mercaptoethanol. The titer was determined by a plaque assay using CV1 cells, and the viral stock was stored at -80°C in PBS until use. A total of 1×10^6 pfu was injected into Rag2^{-/-} mice, and 1×10^7 pfu was injected intraperitoneally into immunocompetent mice in 0.2 ml of PBS. The viral load was measured by a plaque-forming assay. In brief, female mice were sacrificed at the indicated times, and the ovaries were harvested and stored at -80°C in 0.5 ml of PBS until use. The ovaries from individual mice were first homogenized and subjected to three freeze-thaw cycles. Serial dilutions were plated on confluent CV1 cells. After one day of culture at 37°C, plates were stained with crystal violet, and the plaques were counted.

Luminex assay

Splenocytes were collected from HuCD69 mice 24 hours after treatment with the anti-huCD692.8 mAb or no treatment in 1 ml of RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 5 μ M β -mercaptoethanol. Supernatants were obtained after centrifugation. The serum levels of IFN- γ , IL-1 α , IL-1 β , IL-17f, IL-21, IL-3 and IL-6 were determined by using ProcartaPlex custom assays (Affymetrix) and Luminex.

Gene expression analysis

The expression of inflammatory genes was evaluated with the Mouse RT²*Profiler* PCR Inflammatory Cytokines and Receptors Array (SABiosciences). Ribonucleic acid (RNA) was obtained from the spleen of mice treated with the anti-huCD69-2.8 mAb or untreated mice using the RNeasy Mini Kit, and a First Standard kit (SABiosciences) was used for cDNA synthesis. The RT²*Profiler* array was probed according to the manufacturer's protocol using the Profiler PCR Array System and SYBR Green/Fluorescein qPCR Master Mix (SABiosciences) with an ABI 7500 Fast sequence analyzer (Applied Biosystems). Gene expression was measured with a web-based software package for the PCR Array System (<http://www.superarray.com/pcr/arrayanalysis.php>), which automatically performs all $\Delta\Delta C_t$ -based fold-change calculations from the specific uploaded raw threshold cycle data.

GEN	PRIMER SEQUENCE (5'-3')
IL1-α	acgtcaagcaacgggaagat
	aaggtgctgatctgggttg
IL1-β	aatgaaagacggcacacca
	accgtttttccatcttcttcttg
TNF-α	gcctcttctcattcctgcttg
	ctgatgagaggaggccatt
IL-17F	gcatttctgtcccacgtgaa
	tgggggtctcgagtgatgt
IL-21	cgcctcctgattagacttcgt
	tgctcacagtgtcccctttac
CCL2	caggtccctgtcatgcttct
	gtggggcggttaactgcatct
CCL12	gaatcacaagcagccagtgtc
	ttctccttggggtcagcaca
β-ACTIN	actgtcgagtcgctcca
	tcatccatggcgaaactggtg

For qPCR assays, RNA was treated with RQ1 RNase-Free DNase (Promega) prior to cDNA synthesis from 5 μ g of total RNA with GoScriptTM Reverse Transcriptase (Promega) using a combination of random primers and oligo dT. The cDNA samples were diluted to 50 ng/ μ l. Real-time PCR with SYBR green detection was performed with 1 μ l of cDNA. The primer sets used for qPCR are listed in the table below.

410 Expression was calculated by the formula $2^{-\Delta C_t}$, using β -actin to normalize sample
411 loading.

Cell death assay

Splenocytes from noninfected mice were cultured in 24-well plates (1×10^6 cells/ml), and cell death was assayed at different times during the culture by staining with PI, followed by flow cytometric analysis.

Biochemical Analysis

Collected cells from the BM and spleen were lysed using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin and leupeptin). Extracts (30 μ g of protein) were examined by protein immunoblot analysis with antibodies against phospho-(Thr389)-p70S6K, p70S6K, phospho-(Ser240/244)-S6, S6, phospho-(Ser2448)-mTOR, and mTOR, all of which were obtained from Cell Signaling, and antibodies against GAPDH (Santa Cruz Biotechnology Inc.) or vinculin (Sigma).

Ethics Statement

All procedures involving animals and their care were approved by the *ISCIII* Ethics Committees (OEBA M-05-2015) and the Comunidad de Madrid (PROEX 156/15) and followed the current Spanish legislation (Real Decreto 53/2013), which are in compliance with European laws (Directive 2010/63/EU).

Statistical analysis

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

Author contributions

LN, JR and ML performed the experiments. LN, GS and PL designed the experiments. AA provided technical support. LN, EAP, GS and DL discussed the data. LN, EAP and PL wrote the manuscript. PL was responsible for the project design and management.

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442 **Conflict of interests**

443 No competing interests declared.

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Figure legends

Figure 1. HuCD69 mice treated with the mAb anti-huCD69-2.8 exhibited a higher antiviral response seven days after vaccinia virus infection than control-treated mice. A, Mice were treated with PBS or 200 µg of anti-huCD69-2.8 with two doses separated by one week. Five days after the second treatment, the mice were infected with 1×10^7 pfu administered i.p, and 7 days after infection, the mice were analyzed. B, Viral titers in the ovaries were measured. C, Absolute cell numbers in the spleen, bone marrow, lymph nodes and blood were measured. D, The numbers of different lymphoid and myeloid cell populations in the spleen were measured. E-F, The numbers of IFN γ and TNF α -producing cells in the spleen were measured. Two independent experiments with n=8 mice per group were pooled. Data were analyzed by an unpaired t test, where $*=p \leq 0.05$, $**=p \leq 0.01$, $***=p \leq 0.001$ and $****=p \leq 0.0001$

Figure 2. HuCD69 mice treated with mAb anti-huCD69 2.8 are more resistant to VACV infection than non-treated mice. A, Mice were treated with PBS or 500ug of anti-huCD69 2.8 and one day after were infected intranasally with 1×10^5 pfu of VACV-WR. One week after infection, mice received a second dose of treatment. Weight loss was evaluated over 9 days. Data shown are representative of one experiment with n=5-6 mice per group. Data were analyzed by unpaired t test, where $*=p \leq 0.05$, $**=p \leq 0.01$, $***=p \leq 0.001$, and $****=p \leq 0.0001$.

Figure 3. Early anti-VACV response upon targeting CD69 with the anti-huCD69- 2.8 mAb promoted better viral clearance and a concomitant accumulation of leukocytes two days after infection. A, Mice were treated with 2 doses of 200 µg of anti-huCD69-2.8 or PBS administered i.v with the doses separated by 1 week, and 5 days after the second dose, the mice were infected with VACV and analyzed 2 days after infection. B, Viral titers in the ovaries were measured. C, Absolute cell numbers in the spleen, bone marrow, lymph nodes, thymus and blood were measured. D, The numbers of leukocyte subpopulations in the spleen were measured. E-F, The numbers of IFN γ - and TNF α -producing cells in the spleen were measured. G, Mice were treated with 1 dose of 500 µg of anti-huCD69-2.8 or PBS administered i.v and 24 hours after treatment, the mice were infected with VACV i.v and viral titers were analyzed 6 hours after infection. Viral titers in spleen were measured. B-F, Two independent experiments with n=7 mice per group were pooled. G, One experiment with n=4-5 mice per group. Data were analyzed by an unpaired t test, where $*=p \leq 0.05$, $**=p \leq 0.01$, $***=p \leq 0.001$ and $****=p \leq 0.0001$.

Figure 4. Immunocompetent mice treated with the anti-mCD69-2.2 mAb exhibited better viral clearance. A, Mice were treated with 1 dose of 500 µg of anti-mCD69- 2.2 mAb or PBS administered i.v 24 hours before infection with VACV and analyzed 2 days after infection. B,

Viral titers in the ovaries were measured. C, Absolute cell numbers in the spleen, bone marrow, lymph nodes, thymus and blood were measured. D, The numbers of leukocyte subpopulations in the spleen were measured. E-F, The percentages and G-H, numbers of IFN γ - and TNF α -producing cells in the spleen were measured. I, Mice were infected with 10⁷ pfu of VACV and one day after were treated with 500 μ g of anti-mCD69- 2.2 mAb or PBS administered i.v 24 hours. Viral titers were analyzed 2 days after infection. J, Viral titers were measured in ovaries. B-H, Two independent experiments with n $\geq$ 7 mice per group were pooled. J, One experiment with n=4 mice per group. Data were analyzed by an unpaired t test, where *=p $\leq$ 0.05, **=p $\leq$ 0.01, ***=p $\leq$ 0.001, and ****=p $\leq$ 0.0001.

Figure 5. mAb anti-mCD69 2.2 treatment in Rag2^{-/-} CD69^{+/+} mice induced an increased accumulation of leukocytes and promoted NK cell activity. A, Mice were treated with anti-mCD69-2.2 or PBS with two doses separated by one week; 5 days after the second treatment, the mice were infected with 1x10⁶ pfu administered i.p, and two days after infection, the mice were sacrificed. B, Viral titers were measured. C, Absolute cell numbers in the spleen, thymus, bone marrow and blood were compared between anti-mCD69-2.2-treated mice and untreated mice. D-E, Lymphoid and myeloid subpopulation cell numbers were analyzed in the D, bone marrow and E, spleen. F, and G, The intracellular production of IFN- γ and TNF α and surface expression of CD107a in NK cells were measured. B, One experiment representative of two independent experiments is shown. C-H, Three independent experiments with n $\geq$ 8 mice per group were pooled. Data were analyzed by an unpaired t test, where *=p $\leq$ 0.05, **=p $\leq$ 0.01, ***=p $\leq$ 0.001 and ****=p $\leq$ 0.0001.

Figure 6. HuCD69 mice treated with two doses of an anti-human CD69 antibody showed similar effects on the cell numbers of the bone marrow and spleen compared to mice given a one-dose treatment. A and B, Mice were treated with two doses of 200 μ g of anti-huCD69-2.8 separated by one week, and five days after the second treatment, the mice were analyzed. A, Total cell number. B, Numbers of lymphoid and myeloid subpopulation cells in the spleen. Two independent experiments with n $\geq$ 8 mice per group were pooled. Data were analyzed by an unpaired t test, where *=p $\leq$ 0.05, **=p $\leq$ 0.01, ***=p $\leq$ 0.001 and ****=p $\leq$ 0.0001.

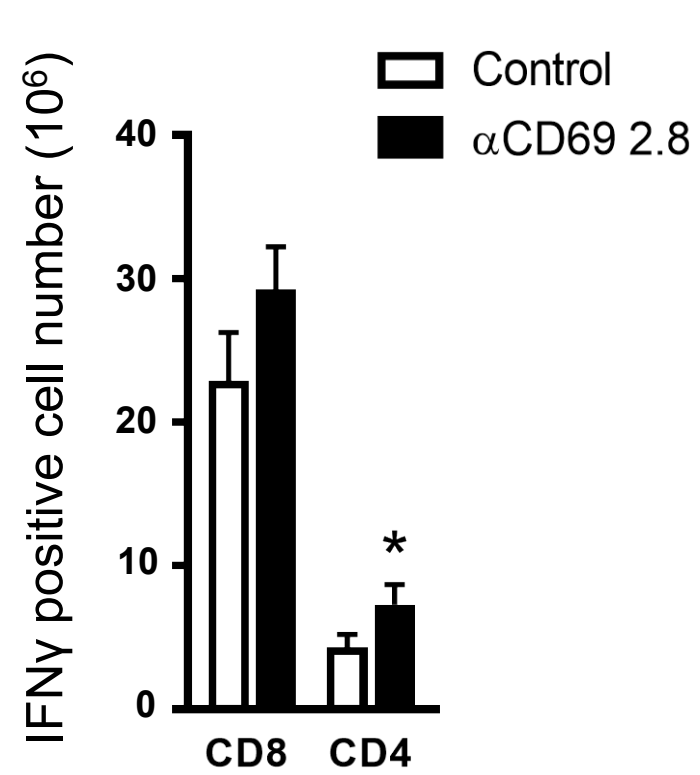
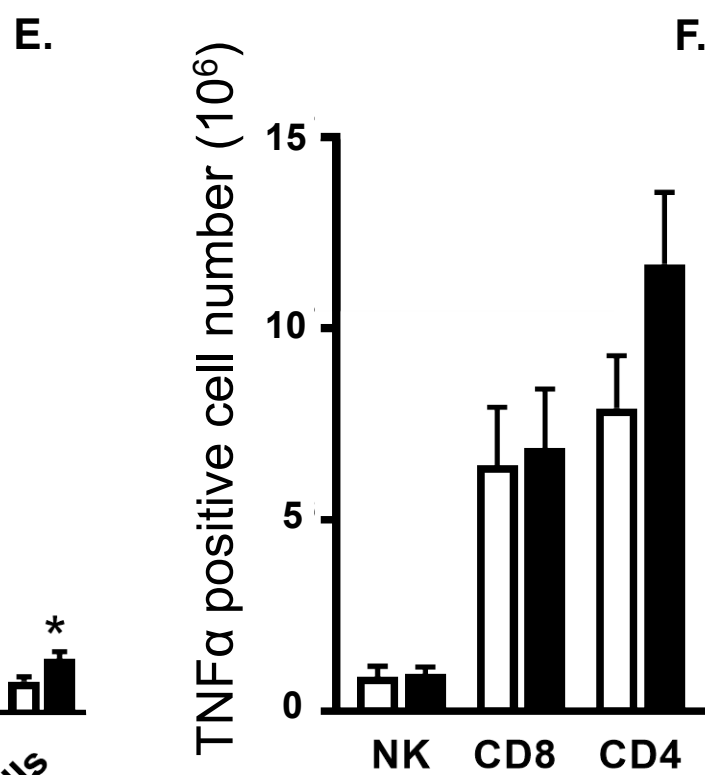
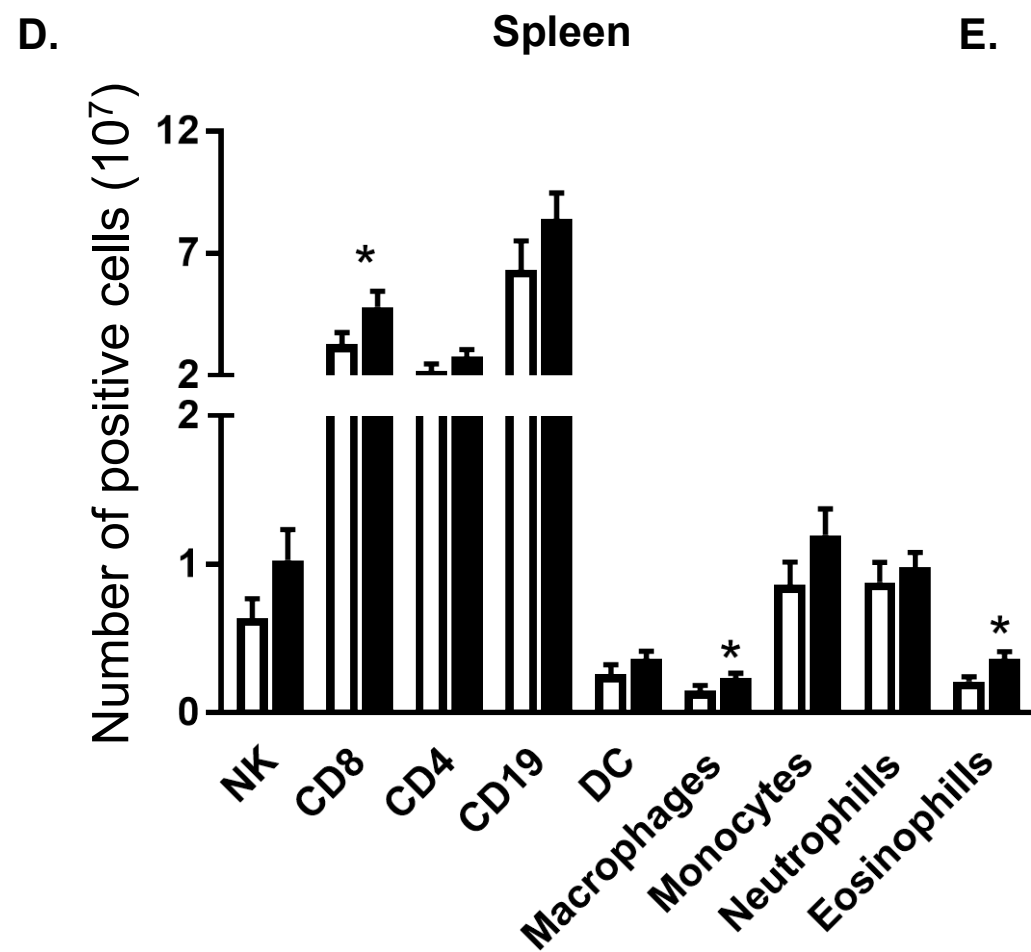
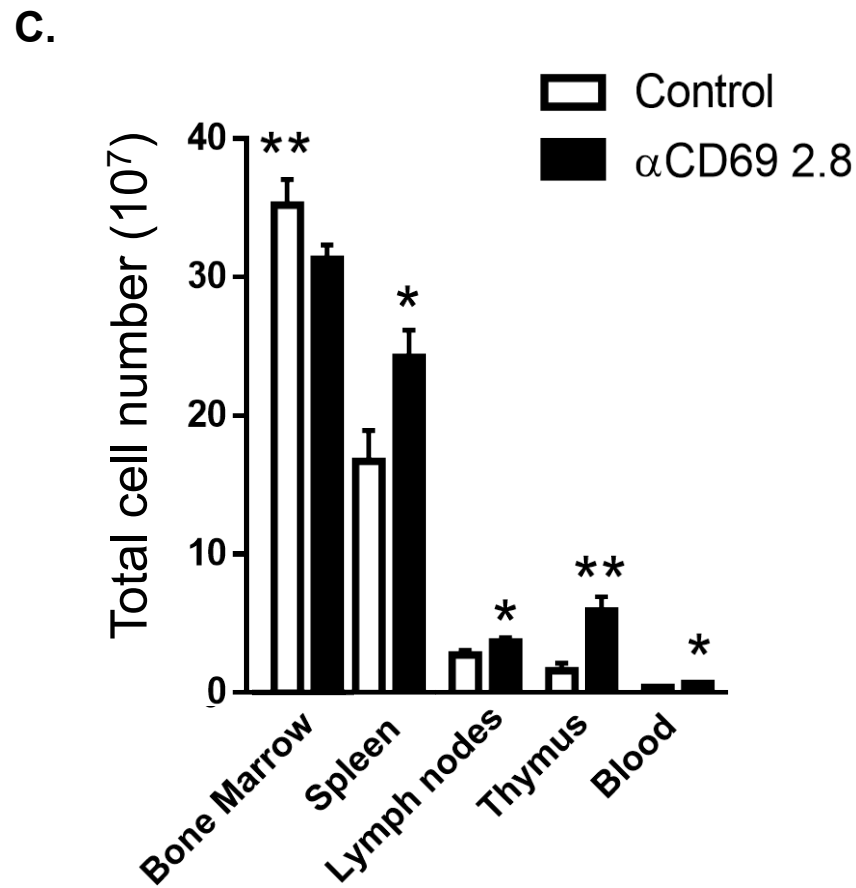
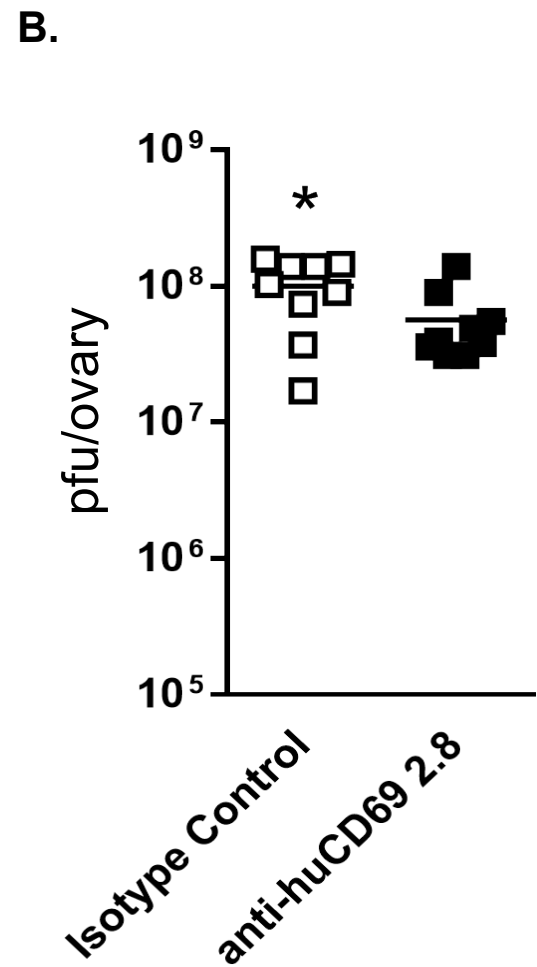
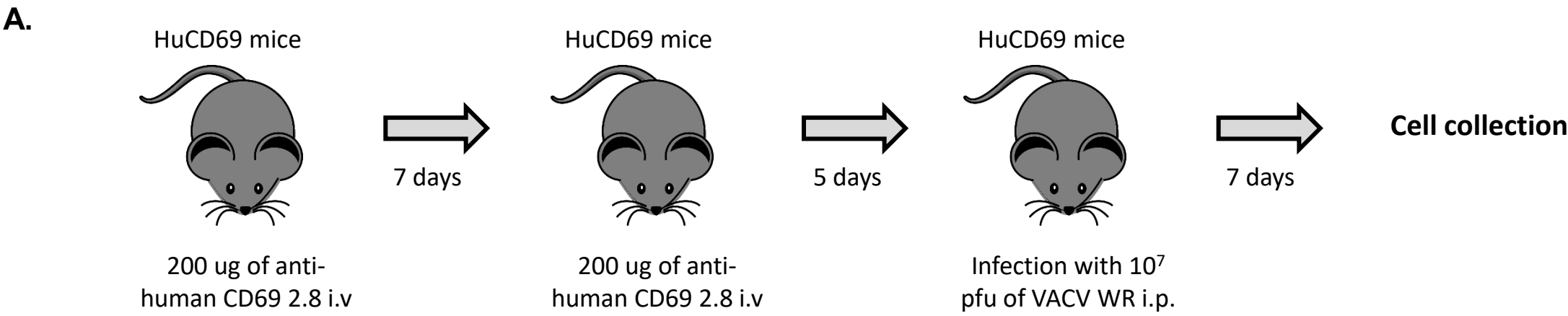
Figure 7. Treatment with anti-human-CD69-2.8 antibody increased proliferation and survival in the spleen. A-B, Mice were treated or not treated with 500 μ g of anti-huCD69-2.8 24 hours before a BrdU injection. The mice received 1 mg of BrdU intraperitoneally, and, three hours later, were sacrificed. BrdU incorporation was assessed in the lymphoid and myeloid subpopulations of the BM (A) and spleen (B) by flow cytometry. C. Survival was measured by PI staining of unfractionated splenocytes from uninfected HuCD69 mice treated with the anti-

huCD69-2.8 mAb or an isotype control. A-B, Two independent experiments with $n \geq 8$ mice per group were pooled. C, One independent experiment representative of two experiments is shown. Data were analyzed by an unpaired t test, where $*=p \leq 0.05$, $**=p \leq 0.01$, $***=p \leq 0.001$ and $****=p \leq 0.0001$.

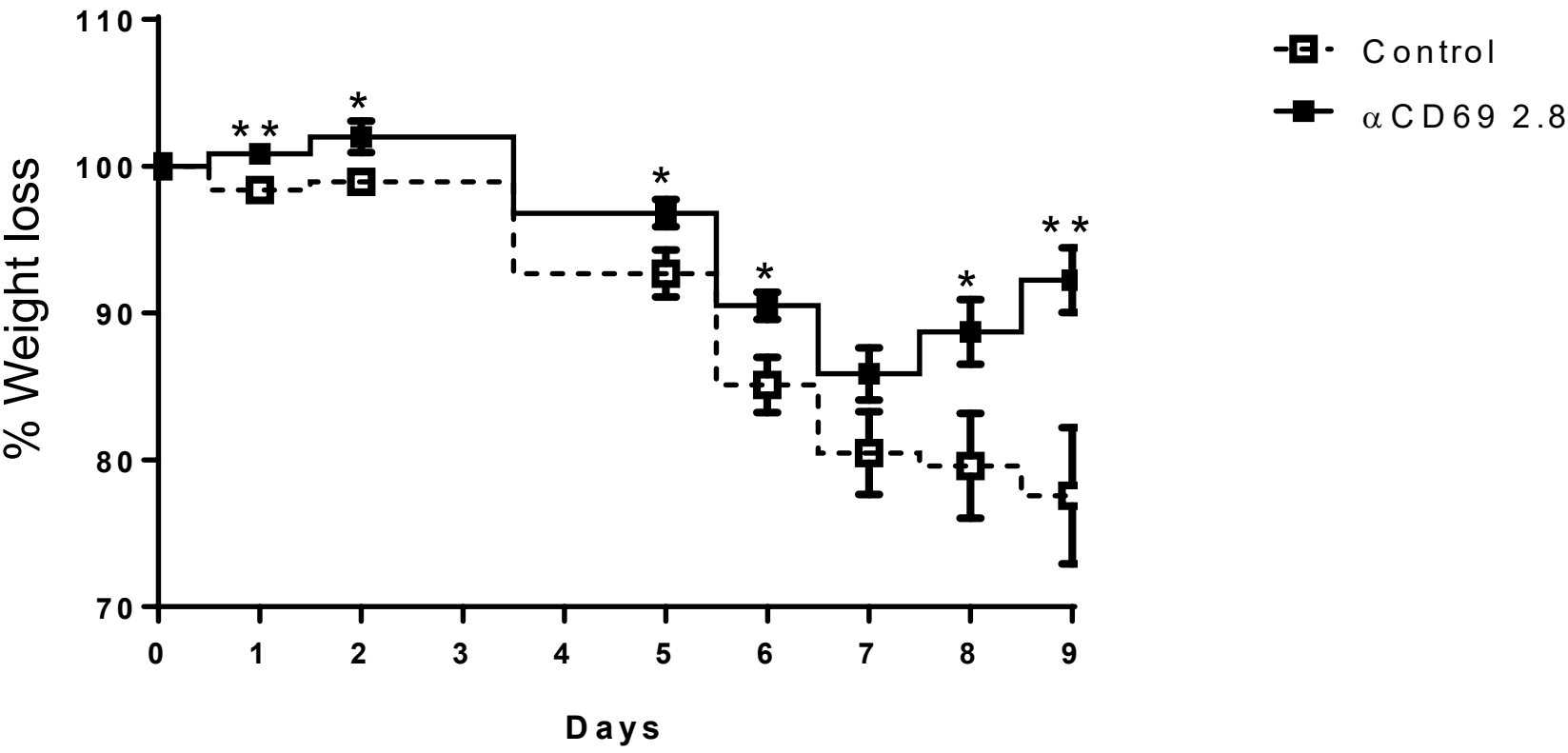
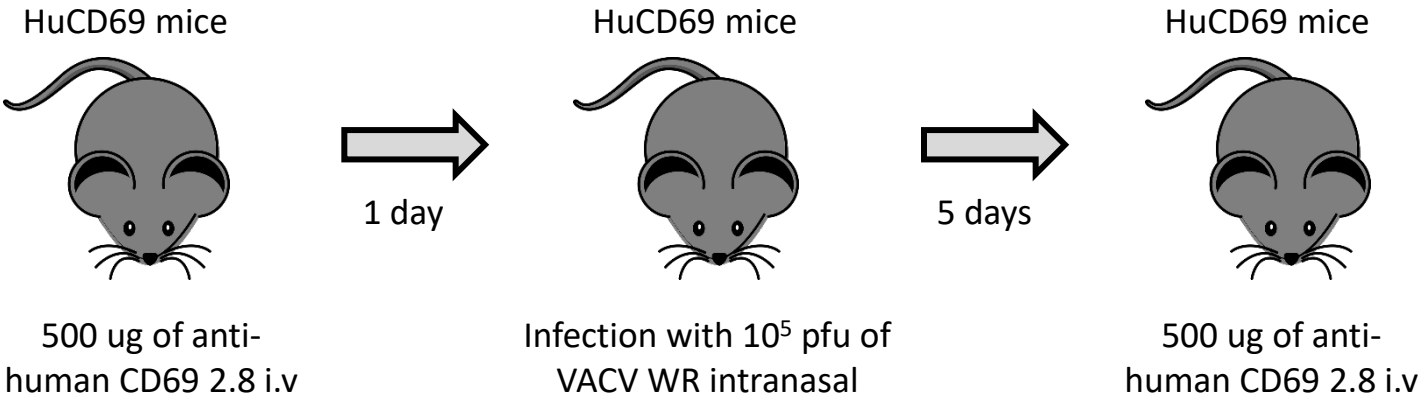
Figure 8. Targeting human CD69 promoted an increase in the levels of some proinflammatory cytokines. A-C, Mice were treated or not treated with 500 μ g of anti huCD69-2.8 24 hours before analysis. A, IL-2 and CD25 expression were measured in the bone Marrow, spleen and blood. B, The relative expression of TGF- β was compared between treated and control mice. C, The expression of certain cytokines in supernatants of splenocytes was measured by a Luminex assay. D-G, Splenocytes were collected from mice treated with 500 μ g of anti-huCD69-2.8 for 24 hours or left untreated. D-E, Proinflammatory cytokines and their receptors were assayed through an array. D, data for IL-17a, IL-17b, IL-17f, IL-1- α , IL-1 β , LTa, LTb and IFN γ are shown. E, Data for CCL2 and CCL12 are shown. Data are represented in a graph as the fold-induction relative to the β -actin level. F, qPCR analysis of IL-17f, IL-1- α , IL-1 β , TNF α and IL-21 is shown. G, qPCR analysis of CCL2 and CCL12 is shown.

A, Three experiments with $n \geq 10$ mice per group were pooled. B, One experiment is shown. C, Four independent experiments with $n \geq 12$ mice per group were pooled. D-G, One experiment is shown with samples pooled of two or three experiments. Data were analyzed by an unpaired t test, where $*=p \leq 0.05$, $**=p \leq 0.01$, $***=p \leq 0.001$ and $****=p \leq 0.0001$.

Figure 9. Targeting human CD69 induced increased splenic cellularity mediated by mTOR. A-C, Mice were treated with 500 μ g of anti-huCD69-2.8 administered i.v 24 hours before analysis, 480 mg of rapamycin/kg administered i.p for five days or both, as indicated. A, The total cell numbers in the spleen (left) and bone marrow (right) are shown. B, p70, S6K and S6 phosphorylation was assessed by immunoblotting splenocyte extracts from the indicated mice. C, Mice were treated with two doses of FTY720 (5 mg/kg) administered i.p and separated by 24 hours; 6 hours after the first dose of FTY720, the mice were treated with 500 μ g of anti-huCD69-2.8 administered i.v. as appropriate, and 24 hours after anti-huCD69-2.8 injection, the mice were analyzed. mTOR and S6 phosphorylation was assessed by immunoblotting splenocyte extracts. A, Two independent experiments were performed. B-C, One experiment was performed. Data were analyzed by an unpaired t test, where $*=p \leq 0.05$, $**=p \leq 0.01$, $***=p \leq 0.001$ and $****=p \leq 0.0001$.

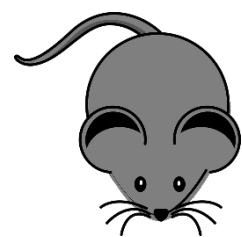


A.



A.

HuCD69 mice

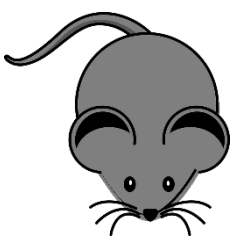


200 ug of anti-human CD69 2.8 i.v



7 days

HuCD69 mice

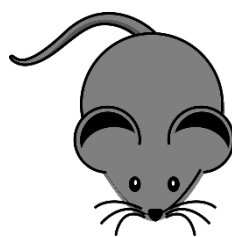


200 ug of anti-human CD69 2.8 i.v



5 days

HuCD69 mice



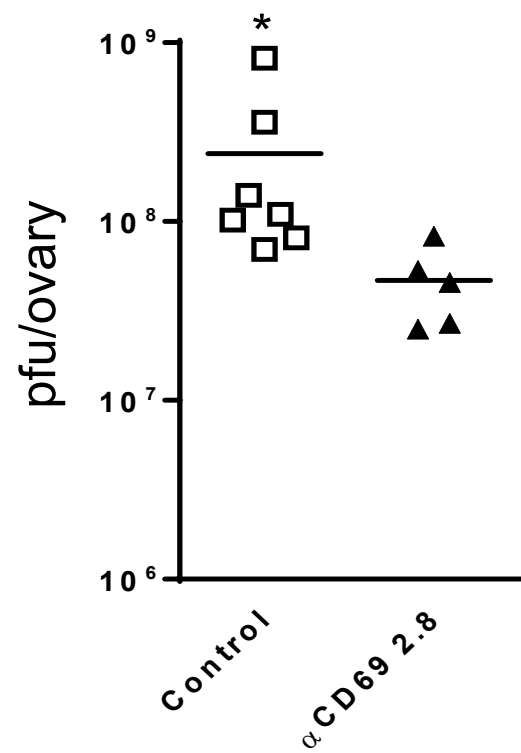
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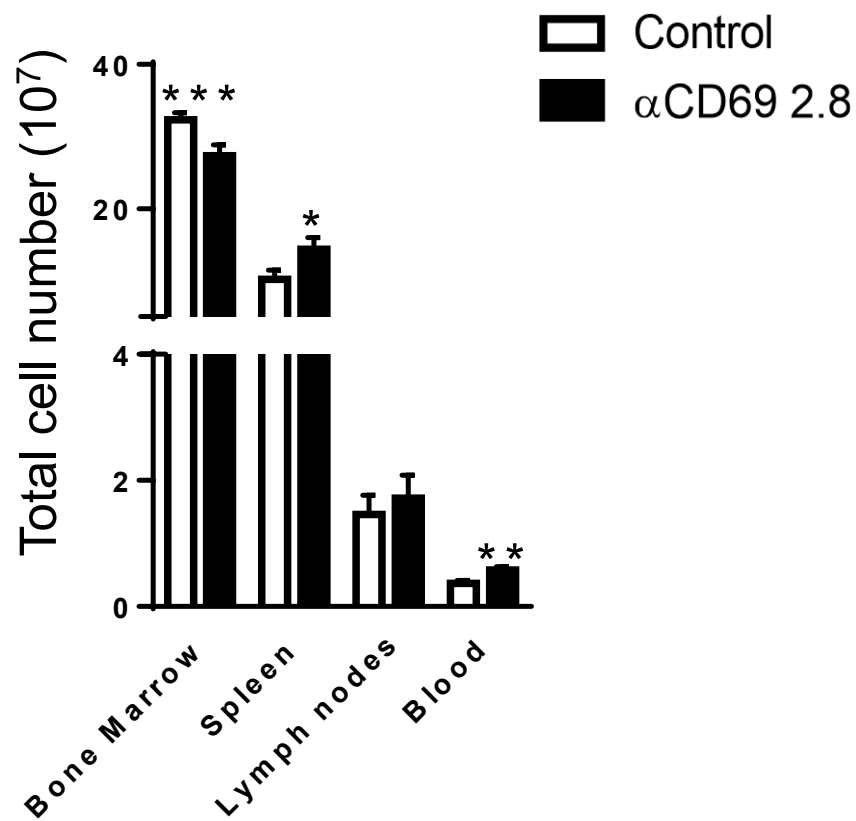
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Cell collection

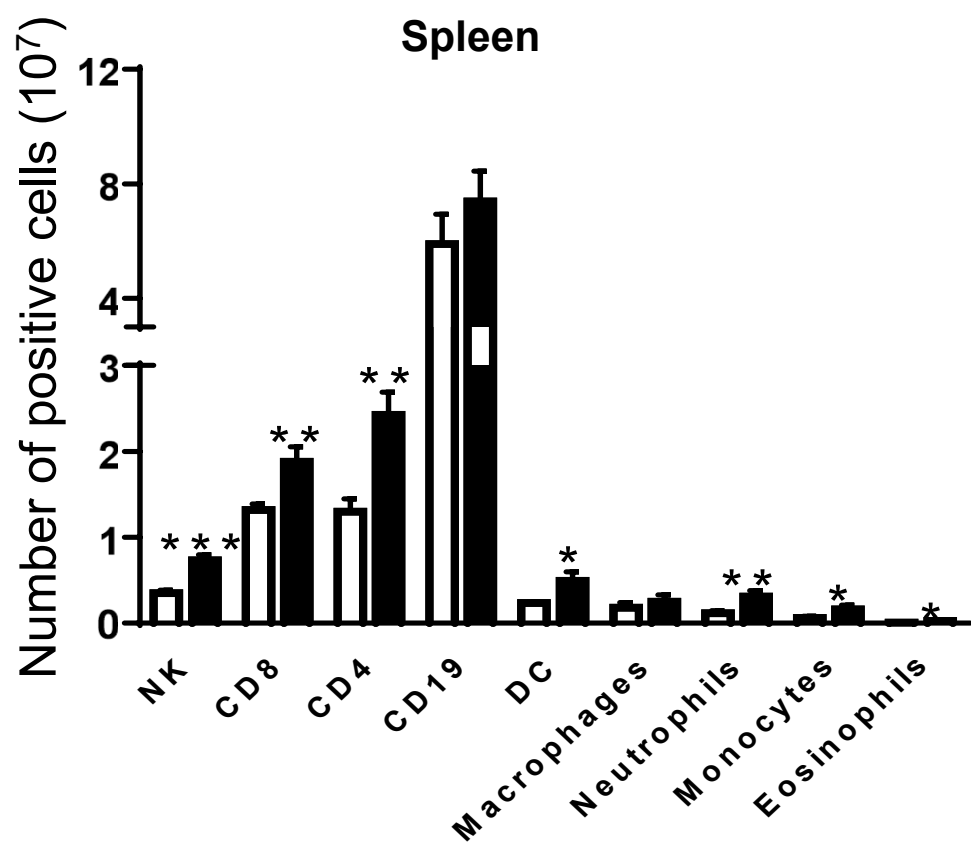
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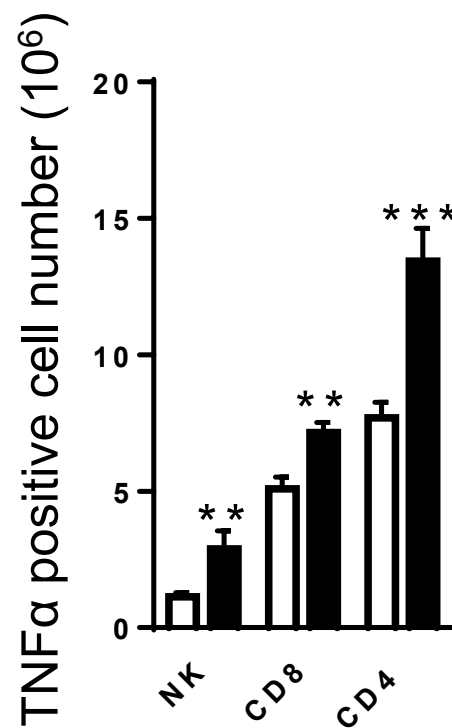
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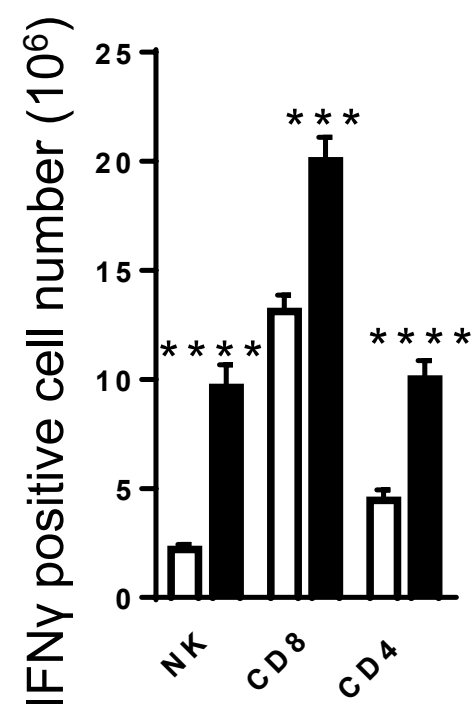
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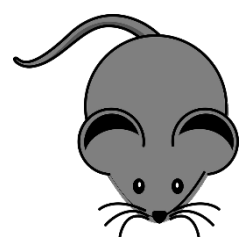


F.



G.

HuCD69 mice

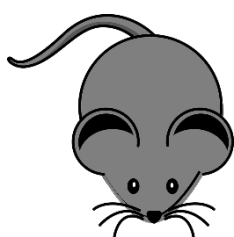


500 ug of anti-human CD69 2.8 i.v



1 days

HuCD69 mice

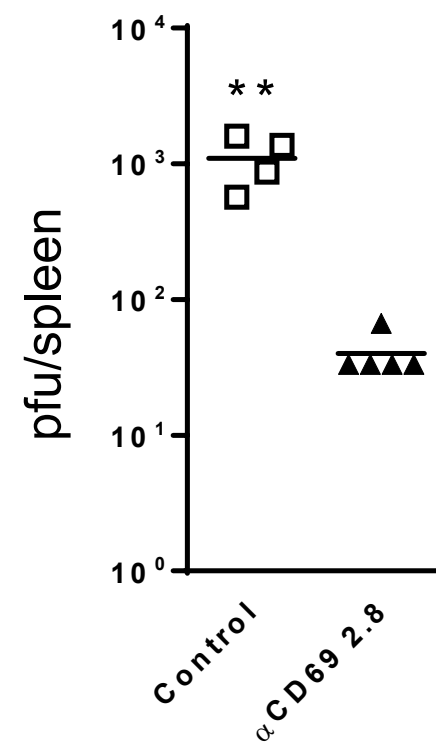


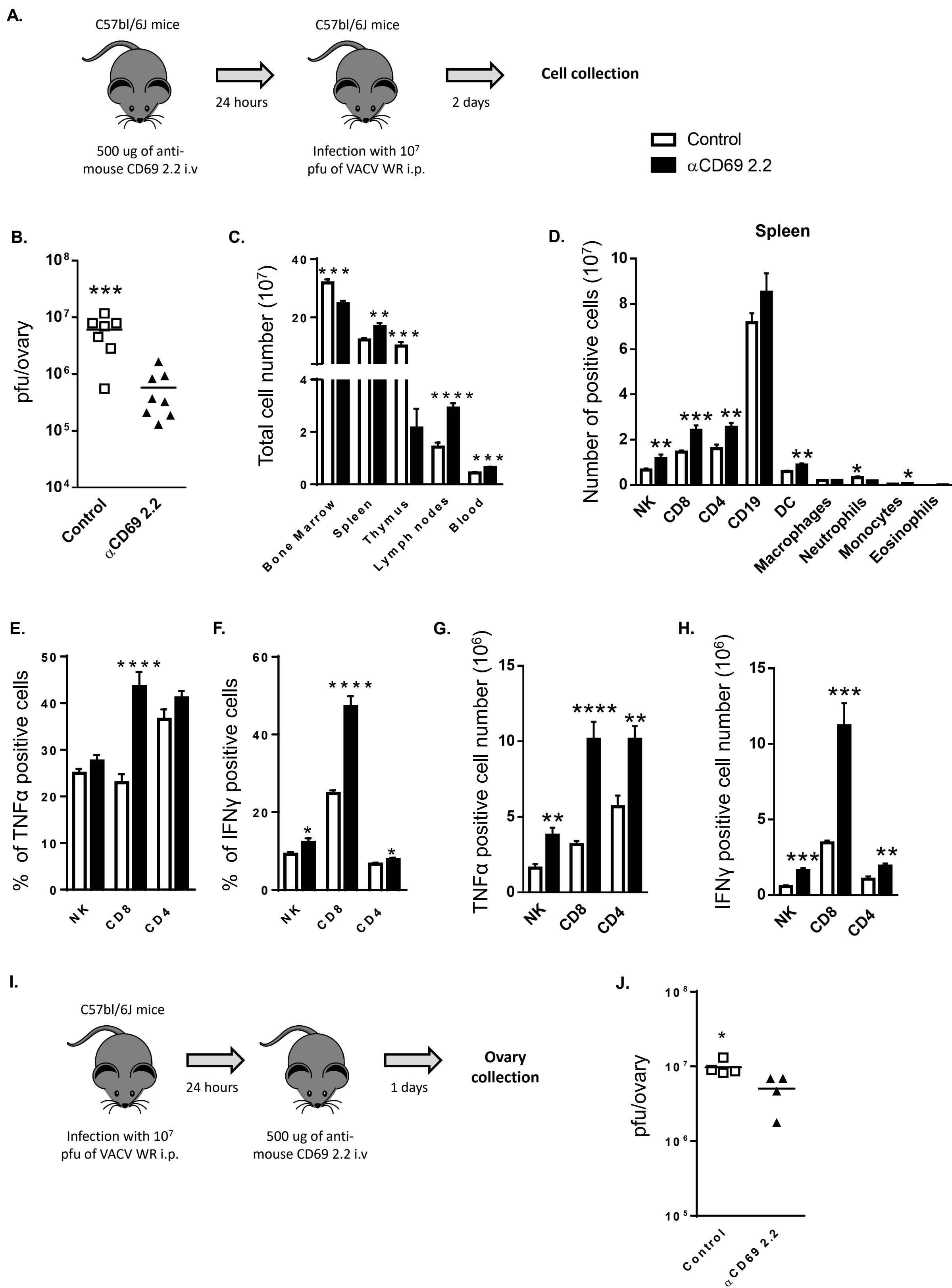
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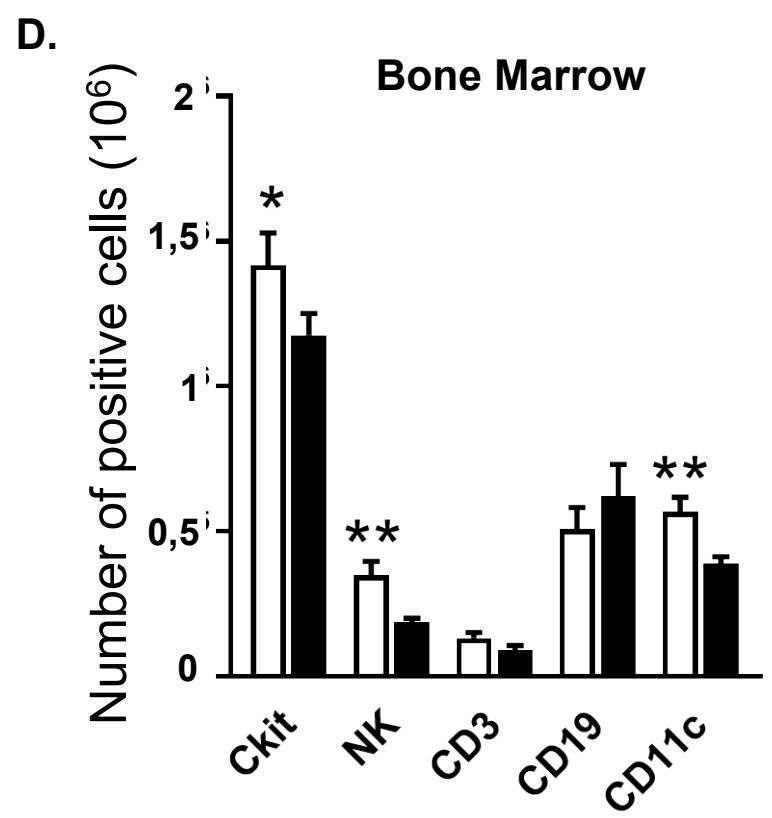
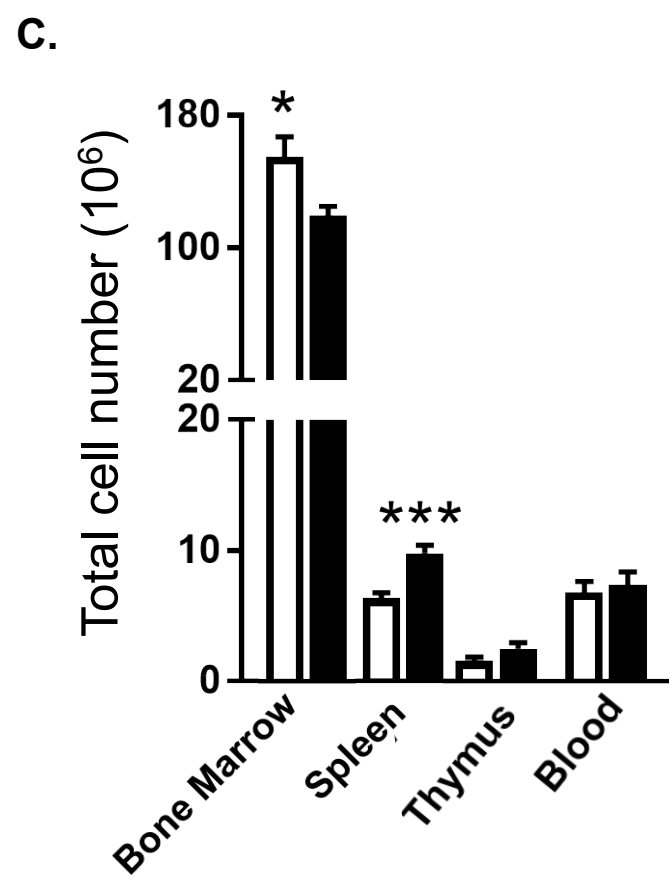
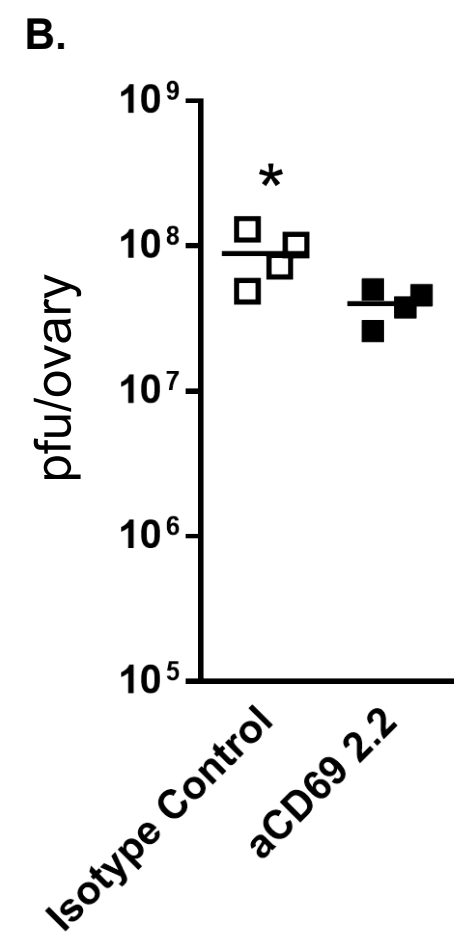
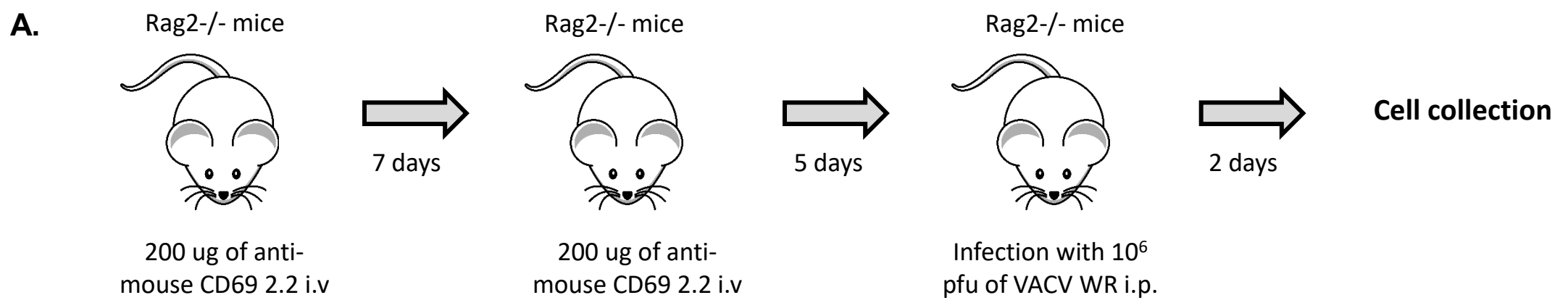


6 hours

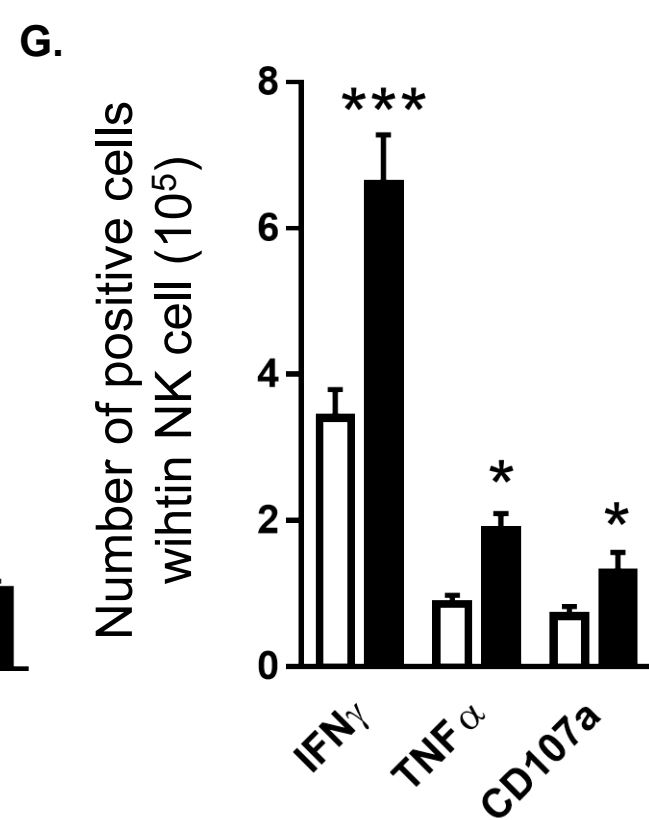
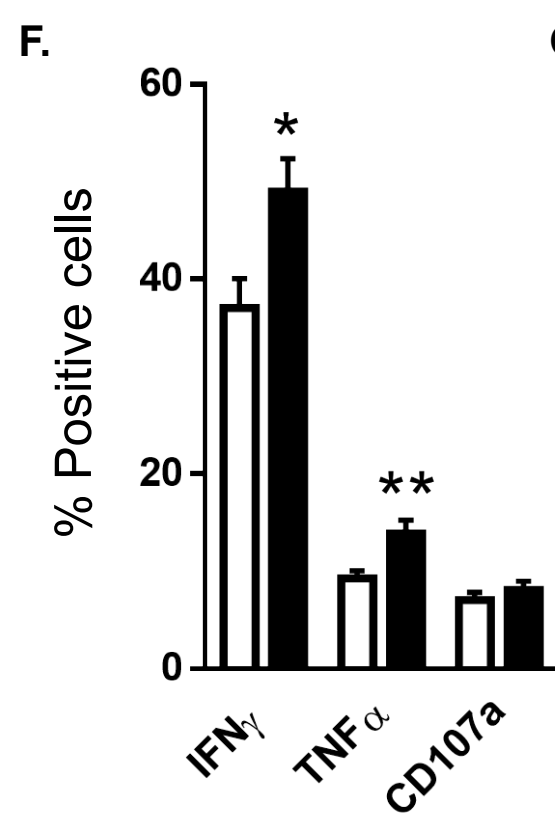
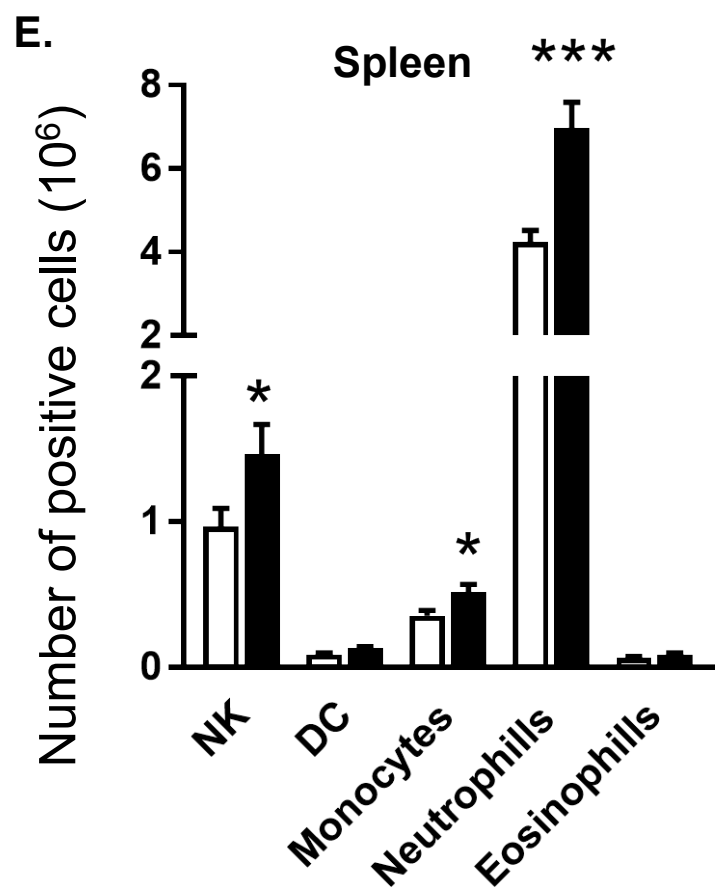
Spleen collection



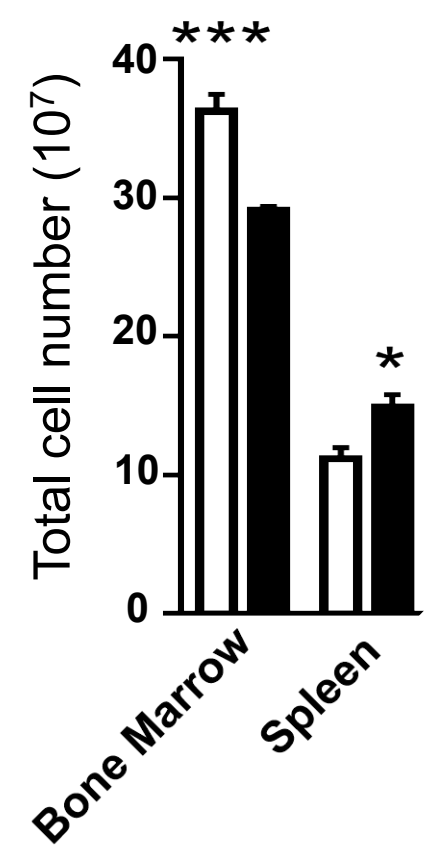




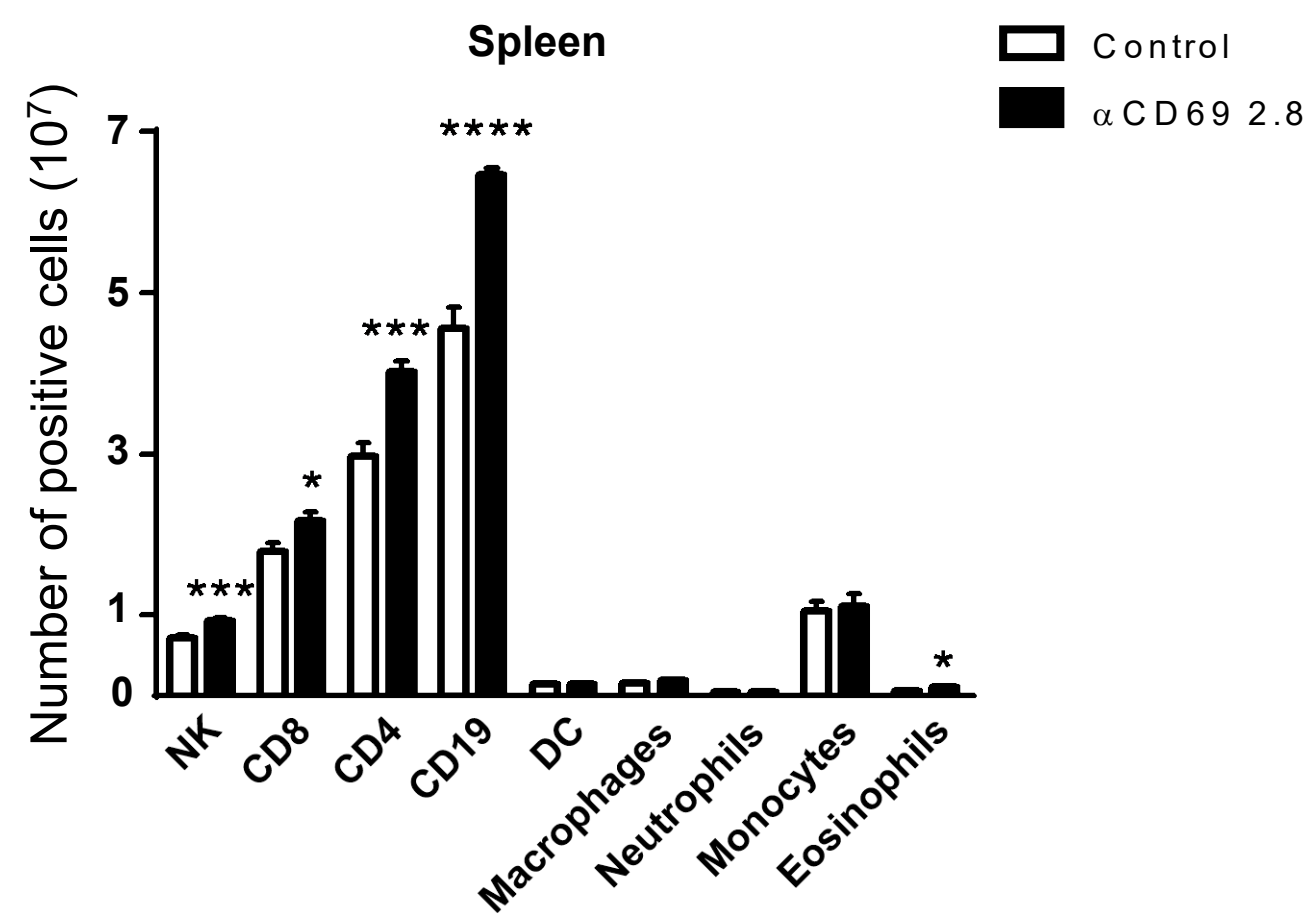
Legend:
 Control
 α CD69 2.2



A.



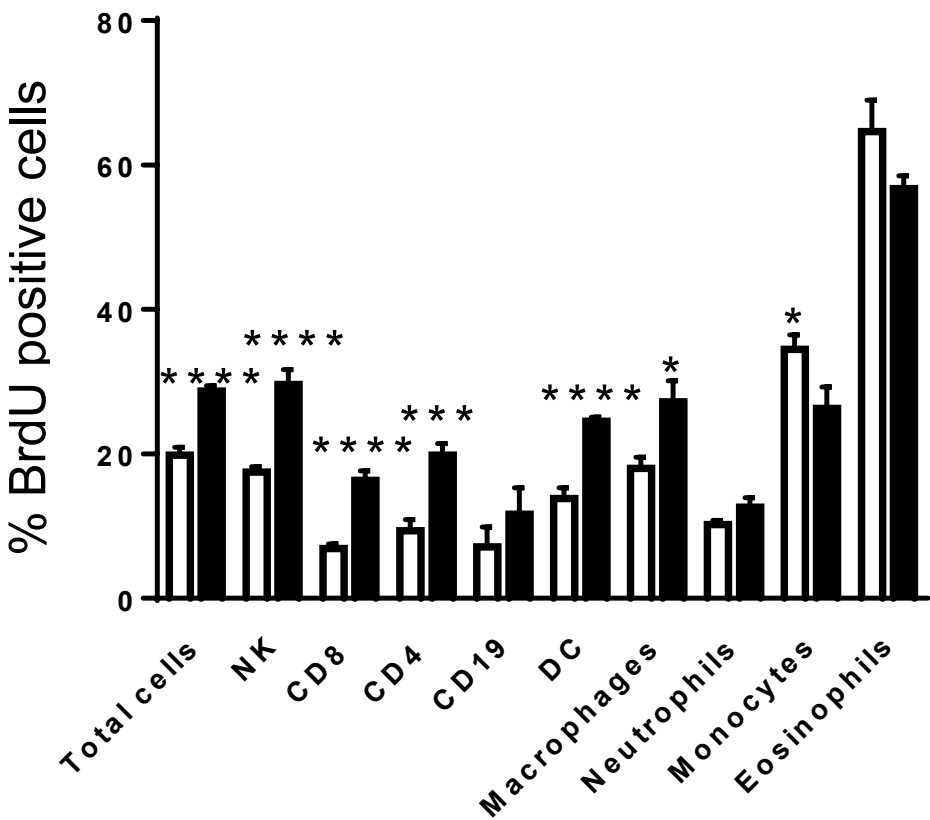
B.



Control
 α CD69 2.8

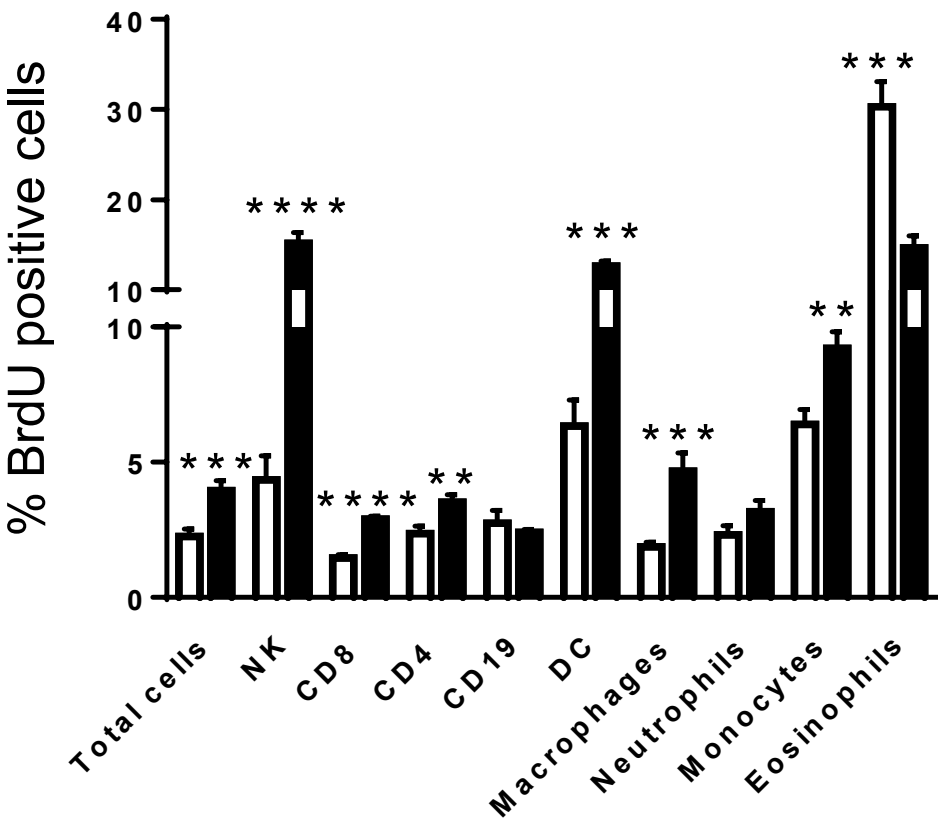
A.

Bone Marrow



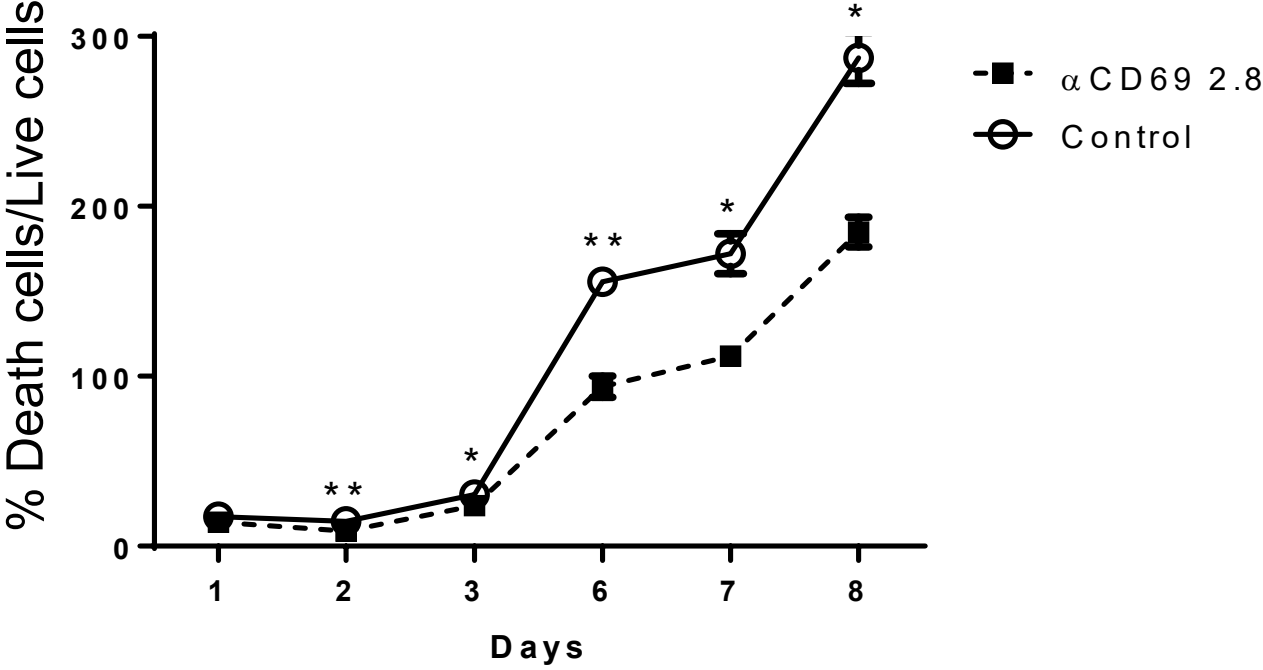
B.

Spleen

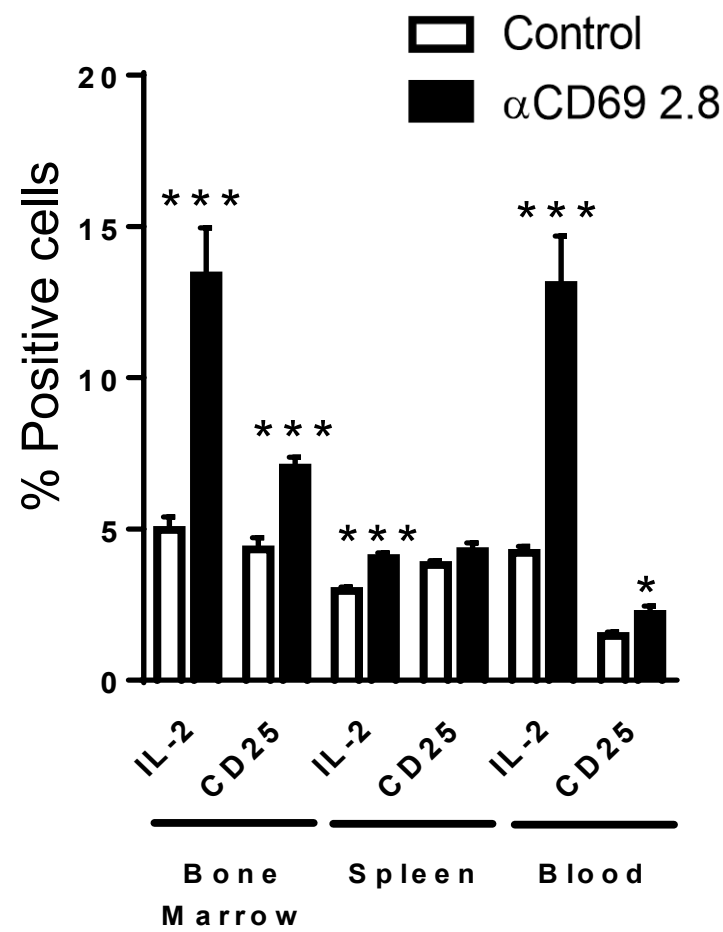


C.

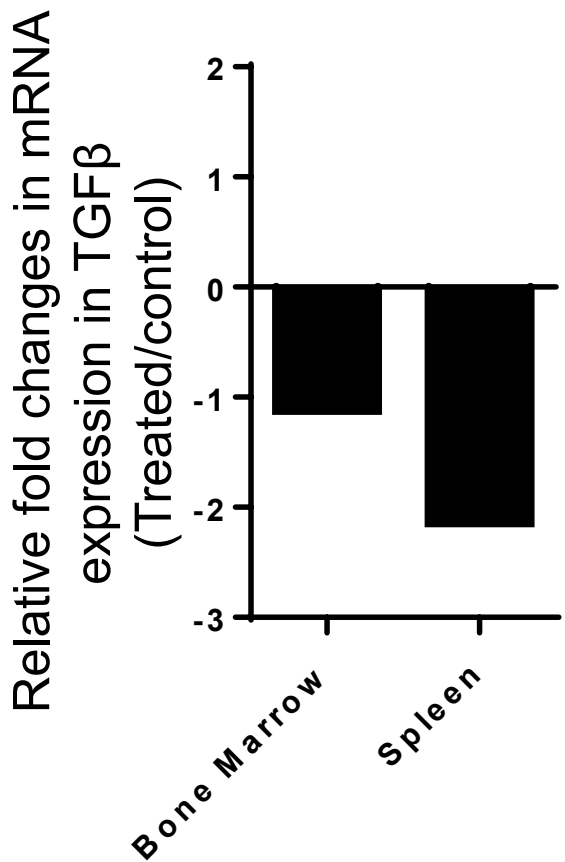
Spleen



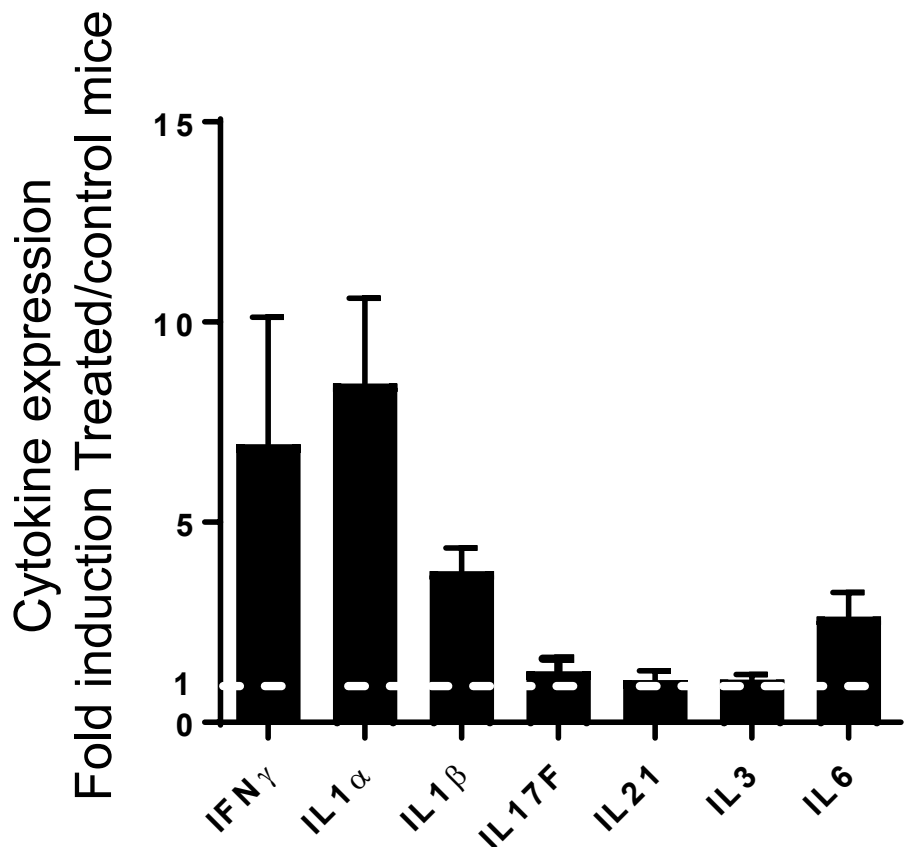
A.



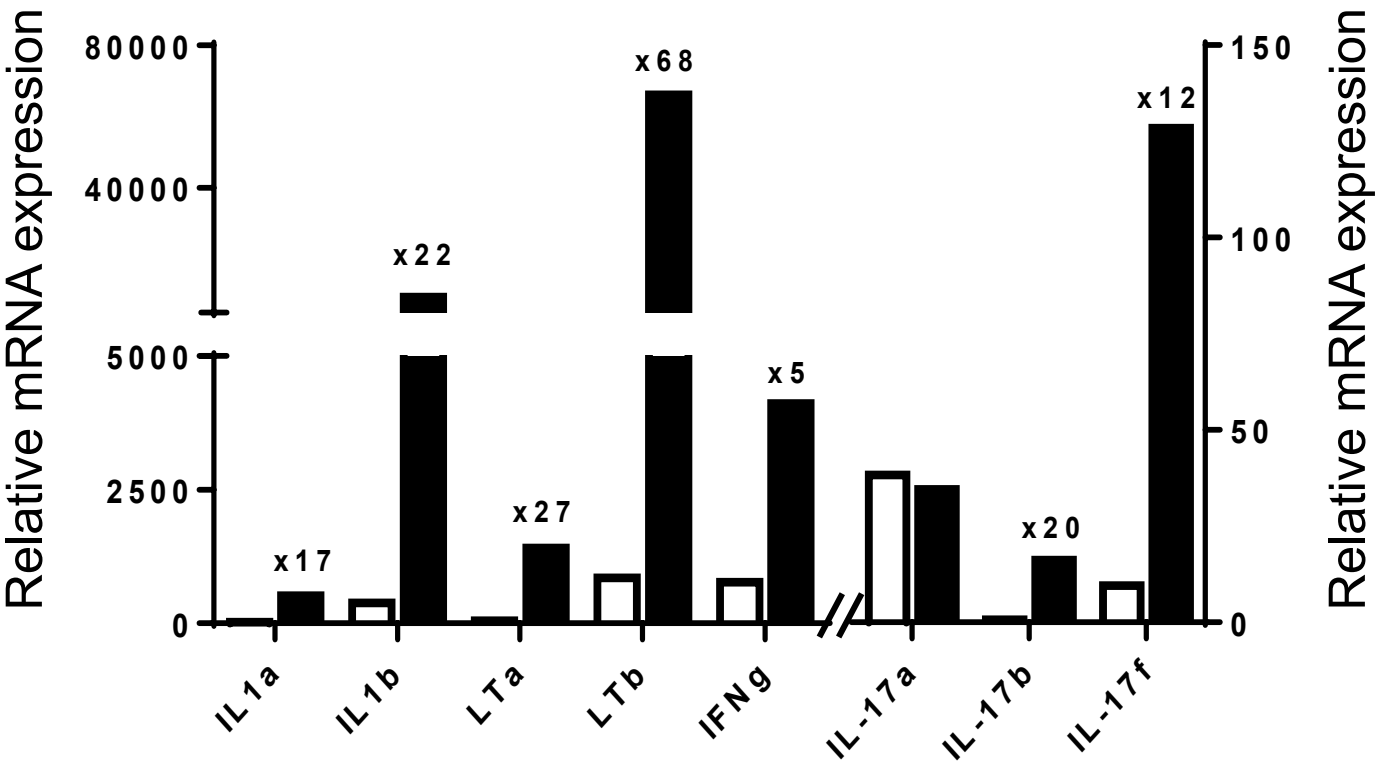
B.



C.



D.

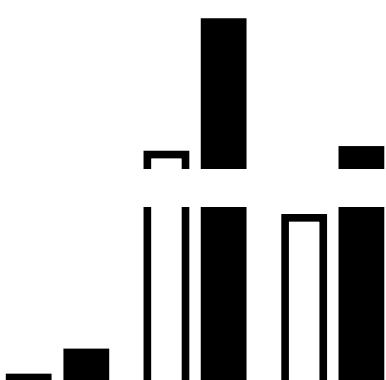


E.

Relative mRNA expression

F.

mRNA expression (relative to βACT x10⁻⁶)

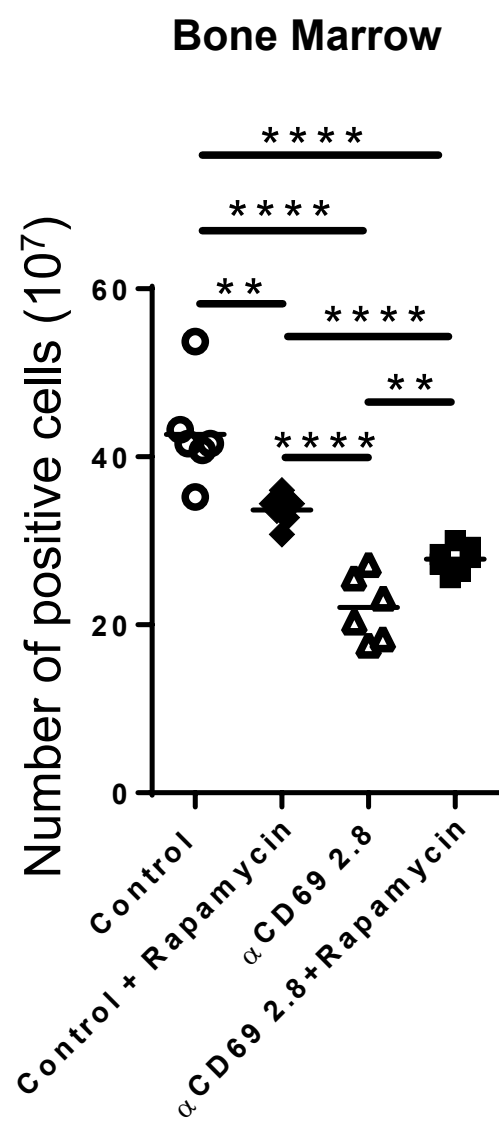
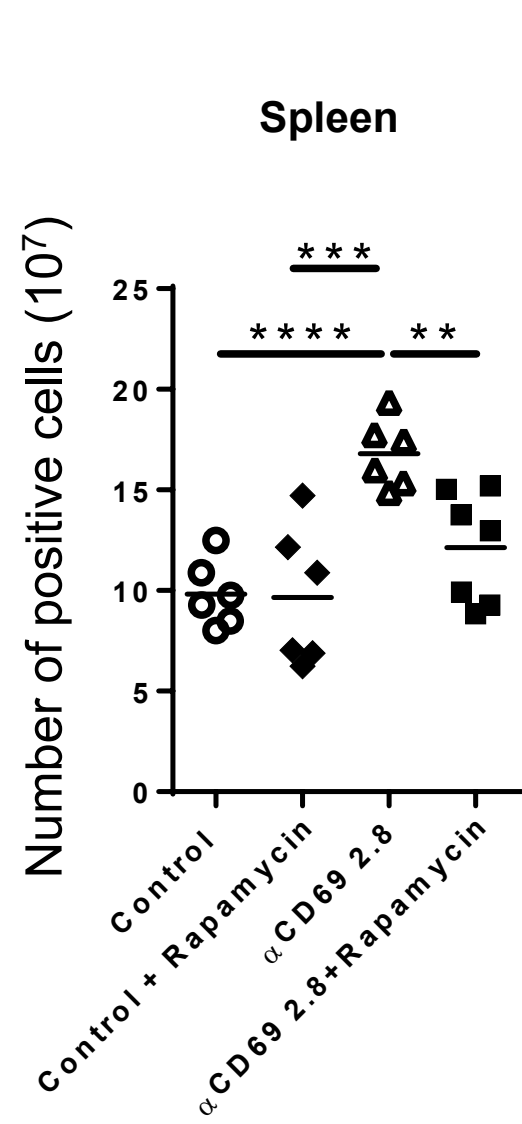


G.

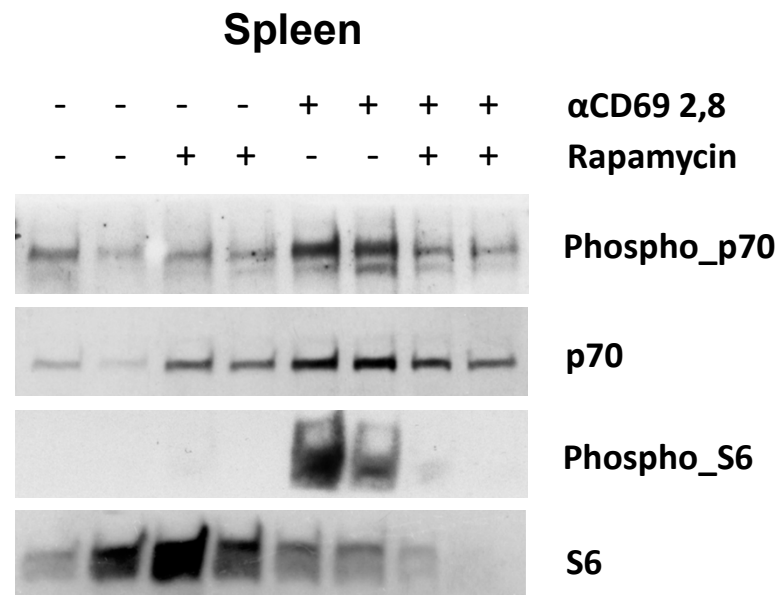
mRNA expression (relative to βACT x10⁻⁴)

Control
αCD69 2.8

A.



B.



C.

