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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 both preventive and 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.

Importance

In this study, we demonstrate the influence of human and mouse anti-CD69 therapies on the immune response to VACV infection. We report that targeting CD69 increases the leukocyte numbers in the secondary lymphoid organs during infection and improves the capacity to clear the viral infection. Targeting CD69 increases the numbers of gamma interferon (IFNγ)- and tumor necrosis factor alpha (TNFα)-producing NK and T cells. In mice expressing human CD69, treatment with an anti-CD69 mAb produces increases in cytokine production, survival and proliferation mediated in part by mTOR signaling. These results, together with the fact that we have mainly worked with a human-CD69 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 TNFα (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. Galectin-1, S100A8/A9 and myosin light chains 9 and 12 have been proposed as CD69 ligands (13-15). T he *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 (16-23). 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 (20, 24, 25). 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 ($S1P_1$), which downregulates $S1P_1$ surface expression and inhibits lymphocyte egress from the thymus and peripheral lymphoid organs (26). 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 (27).

CD69 has been shown to be expressed in lung lymphocytes after intranasal VACV infection proportionally to infection virulence. It is also highly expressed on virus-specific tissue resident memory T cells of virally infected skin (28, 29) and is important for the retention in the skin of this population (28). In our preceding study, CD69 deficiency resulted in increased early NK cell-dependent control of the infection (21). 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 (30).

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 IFNγ- and TNFα-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 $1x10^7$ 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, when the primary adaptive response is already taking place (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 IFNγ and a tendency toward higher numbers of CD4+ T cells producing TNFα and CD8+ T cells producing IFNγ (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. To assess the effect of CD69 treatment in a more natural experimental model we used an intranasal low-dose (10^4 pfu) VACV innoculation and monitored weight overtime. Anti-CD69-treated mice showed a reduced weight loss relative to their non-treated counterparts (Figure G-H). The differences were already significant 1 day after infection, maintained overtime and augmented after seven days, showing that the effects of anti-CD69 treatment are long lasting. Similarly, WT mice treated with the anti-mCD69-2.2 mAb using the same schedule more efficiently limited infection than control-treated mice. Moreover, the WT mice 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 TNFα- and IFNγ-producing CD4+ T cells were augmented (data not shown). 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. 2A), 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. 2B). The numbers of total blood and splenic leukocytes (Fig. 2C) and all splenic subtypes analyzed in the mAb-treated mice were increased compared to those in control mice (Fig. 2D). Moreover, the treatment induced marked increases in the numbers of TNFα-producing NK, CD8+ T and CD4+ T cells and IFNγ-producing NK and CD4+ T cells and a tendency toward higher numbers of IFNγ-producing CD8+ T cells (Fig. 2E-F). In a short-term treatment experiment, this increased control is already detected in the spleen after 6 hours of infection (Fig. 2G).

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. 3A-H). Also, there was a significant difference in ovarian viral titers when the treatment with anti-mCD69-2.2 was administered 24 h after VACV exposure (Fig. 3I-J).

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 $1x10^6$ pfu of VACV and analyzed them two days after infection (Fig. 4A). As in the treated WT and huCD69 mice, the anti-CD69-treated $Rag2^{-/-}$ mice showed lower ovarian viral titers than control mice (Fig. 4B) and decreased and increased leukocyte numbers in the bone marrow and spleen, respectively (Fig. 4C). Consistently, in the spleen, targeting CD69 induced increases in NK cell, monocyte and neutrophil counts (Fig. 4D). Moreover, we found that the antibody treatment increased the percentages (Fig. 4E) and numbers (Fig. 4F) of NK cells producing IFNγ- and TNFα- 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. 5A). The numbers of NK cells, T cells and B cells were augmented in the spleen (Fig. 5B). 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. 6A-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* (31-34).

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. 6C). 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. 7A). 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-1α and β; LTα and β; IL-17α, β and f; IL-21; IFNγ; and TNFα) 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. 7B). Instead, the splenic messenger RNA (mRNA) levels of IL-1α and β, LTα and β, IL-17β and f, IL-21, IFNγ, TNFα, CCL2 and CCL12 were increased (Fig. 7D-G). We also measured the expression of IL-1α and β, IL-21, IL-17 f, IFNγ, 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-1α, IL-1β, IFNγ and IL-6 production (Fig. 7C) 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 (35-37). 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. 8A), 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. 8B). Moreover, FTY720 treatment also inhibited the phosphorylation of p70S6K in the spleen (Fig. 8C), 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. Although they rely on the murine immune system, the use of huCD69 mice allows to show that the interaction of the anti-human CD69-2.8 mAb with the human CD69 molecule is such that, like the treatment of WT mice with anti-mouse-2.2, will also induce innate immune activation and protection in front of viral infection *in vivo*. Human and mouse CD69 have more than 70% of conserved residues. The comparable effects of anti-human and anti-mouse CD69 targeting suggest that conserved motifs allow the human CD69 molecule to act like the mouse CD69 in the context of murine immune system in the CD69-/- background.

In previous reports, targeting CD69 resulted in outcomes similar to those seen with CD69 deficiency in several murine models of cancer and autoimmune disease (22, 24, 25). 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 IFNγ and TNFα and degranulating NK cells (21). 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 IFNγ and TNFα 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

IFNγ+ and TNFα+ 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 splenic VACV-specific T cells 1 week after intraperitoneal VACV innoculation (38). Instead, in a model of local virus infection of the skin, CD69 enhanced the retention of specific resident memory T cells in the infected skin already at week 1 post-infection, but not in the spleen (28). The differences may be attributed to the CD69 function in the retention of memory T cells in tissue and non in lymphoid organs.

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 (21, 38). 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 (18, 25).

In both CD69-deficient mice and CD69-targeted mice, cell survival is increased (21). This result may be related to the fact that TGFβ expression is decreased in almost all disease models performed with both $CD69^{-/-}$ and anti-CD69 mAb-treated mice (18, 22-25, 39, 40). TGF-β is a pleiotropic cytokine, and one of its reported functions is the control of cell survival, including the survival of leukocytes (41). Interestingly, CD69 deficiency in FoxP3 Tregs leads to decreased TGFβ production, and both CD69 deficiency and targeting CD69 in these cells hinder their suppressive function (39).

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 IFNγ, activate mTOR through PI3K and phospho-Akt T308. Growth factors can also activate mTOR through ERK1/2

signaling (42). Instead, S1P activates mTORC1 through the E3-ubiquitin ligase PAM in a manner independent of PI3K, Akt and ERK1/2 (43). 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.

CD69 has been described to negatively regulates Th17 cell differentiation through the immunoregulatory molecule Galectin 1(15) and to promote Treg differentiation thorugh interaction with the complex S100A8/S100A9 (14). The antibody could be disrupting the interactions with these trans ligands and thus inducing immune activation. Moreover, the antibody could be affecting CD69 interaction with its ligands in cis S1P1 and CD69 downregulation of surface S1P1 may be hindered by mAb binding. This, together with that sustained S1P1 surface expression enhances mTOR activation on T cells (44) may contribute to anti-CD69-induced mTOR activation.VACV employs numerous immune evasion mechanisms to decoy, inhibit the production, and block the signaling of type I and II IFNs, TNFα, 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 TNFα, have direct antiviral activity (45). Thus, the observed augmented expression of IFNγ, TNFα and IL-1 as well as the possible augmentation of the expression of other cytokines may counteract these immune evasion mechanisms of the virus. Instead, an enhanced proinflammatory cytokine response is detrimental in the cases of other pathogens.

The data presented here showing that targeting CD69 enhances the early control of pathogens encourage future testing of anti-CD69 mAbs as agents for enhancing the immune responses in vaccine-mediated protection and against infection in leukopenia 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 are in the mouse $CD69^{-/-}$ background and carry three copies of the human CD69 BAC, which were maintained in hemizygosity. Since the human CD69 BAC contains the entire human CD69 gene locus (around 100kb), where CD69 transcription is driven by the original regulatory regions, these mice express human CD69 in a regulated fashion, as previously shown (24). $Rag2^{-/-}$ in Balb/c genetic background and C57BL6 mice were also 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 of the IgG1 isotype, bind to the human and the mouse CD69 molecule respectively. They were generated in our laboratory (24). Neither of them activates the complement system or induces ADCC, as previously experimentally demonstrated (24). Since anti-huCD69-2.8 mAb 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-CD69-2.2 mAb as isotype control of anti-human-CD69 mAb experiments and *vice versa.* 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. The purified mAbs were tested with $CD69^{-/-}$ BM-derived DC (BMDC) cultures at a concentration of 10 μg/ml, and were unable to upregulate CD80 or CD86 expression in these cultures. 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 repeated experiments, we injected control mice with isotype control mAb in the first experiment, and PBS when the experiment was repeated, obtaining comparable results. $CD69^{+/+}$ 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.

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), 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, $2x10^6$ splenocytes 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 medium only in the presence of BFA (5 µg/ml) for 4 hours at 37ºC. 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 µg/mL streptomycin and 5 µM 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 37ºC, 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 µg/mL streptomycin and 5 µM β-mercaptoethanol. Supernatants were obtained after centrifugation. The levels of IFN-γ, IL-1α, IL-1β, 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 RT^2*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 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) 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 ΔΔCt-based fold-change calculations from the specific uploaded raw threshold cycle data.

For qPCR assays, RNA was treated with RQ1 RNase-Free DNase (Promega) prior to cDNA synthesis from 5 µg of total RNA with GoScript™ Reverse Transcriptase (Promega) using a combination of random primers and oligo dT. The cDNA samples were diluted to 50 ng/µl. Real-time PCR with SYBR green detection was performed with 1 µl of cDNA.. Expression was calculated by the formula $2^{-\Delta Ct}$, using β-actin to normalize sample loading. The primer sets used for qPCR are listed below.

IL1–α Forward: acgtcaagcaacgggaagat

IL1–α Reverse: aaggtgctgatctgggttgg

IL1–β Forward: aatgaaagacggcacaccca

IL1–β Reverse: accgtttttccatcttcttctttg

TNF-α Forward:gcctcttctcattcctgcttg

TNF-α Reverse: ctgatgagagggaggccatt

Il-17F Forward:gcatttctgtcccacgtgaa

Il-17F Reverse: tgggggtctcgagtgatgt

IL-21 Forward: cgcctcctgattagacttcgt

IL-21 Reverse: tgctcacagtgcccctttac

CCL2 Forward: caggtccctgtcatgcttct

CCL2 Reverse: gtggggcgttaactgcatct

CCL12 Forward: gaatcacaagcagccagtgtc

CCL12 Reverse: ttctccttggggtcagcaca

β-ACTIN Forward: actgtcgagtcgcgtcca

β-ACTIN Reverse: tcatccatggcgaactggtg

Cell death assay

Splenocytes from noninfected mice were cultured in 24-well plates ($1x10^6$ 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 μg/ml aprotinin and leupeptin). Extracts (30 μg 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

471 determined using an unpaired two-tailed *t*-test (* $p<0.05$, ** $p<0.01$, *** $p<0.005$).
472 $p<0.05$ was considered significant

473 **Author contributions**

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

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479 husbandry.

480 **Conflict of interests**

481 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 several days after vaccinia virus infection than control-treated mice. A-F, 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 $1x10^7$ 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 IFNγ and TNFα-producing cells in the spleen were measured. Two independent experiments were pooled, with a total of n=8 mice per group. G,H. Mice were treated with PBS or 500ug of anti-huCD69 2.8 and one day after were infected intranasally with $1x10^5$ 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≤0.05, **=p≤0.01, ***=p≤0.001, and ****=p≤0.0001.

Figure 2. 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 IFNγ- and TNFα-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 were pooled, with a total of n=7 mice per group. G, One experiment with n=4-5 mice per group. Data were analyzed by an unpaired t test, where *=p≤0.05, **=p≤0.01, ***=p≤0.001 and ****=p≤0.0001.

Figure 3. 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 IFNγ- and TNFα-producing cells in the spleen were measured. I, Mice were infected with 10^7 pfu of VACV and one day after were treated with 500 µg 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 were pooled, with a total of n≥7 mice per group. J, One experiment with n=4 mice per group. Data were analyzed by an unpaired t test, where *=p≤0.05, **=p≤0.01, ***=p≤0.001, and ****=p≤0.0001.

Figure 4. 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 $1x10^6$ 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 TNFα 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 were pooled, with a total of n≥8 mice per group. Data were analyzed by an unpaired t test, where *=p≤0.05, **=p≤0.01, ***=p≤0.001 and ****=p≤0.0001.

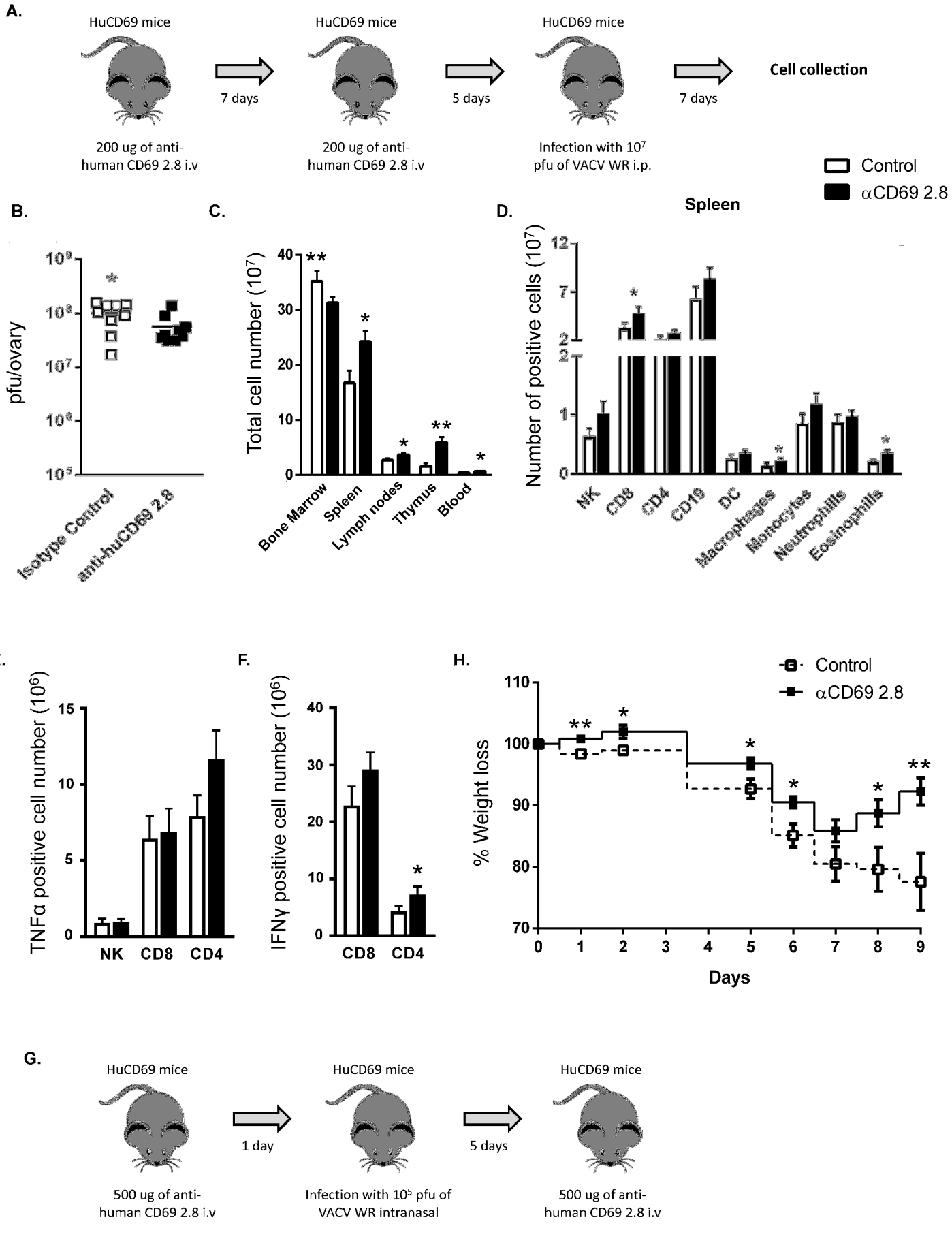
Figure 5. HuCD69 mice treated with two doses of an anti-human CD69 antibody showed similar effects on the cell numbers of the bone marrow and spleen compared to mice given a one-dose treatment. A and B, Mice were treated with two doses of 200 µg of anti-huCD69-2.8 separated by one week, and five days 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 were pooled, with a total of n≥8 mice per group. Data were analyzed by an unpaired t test, where *=p≤0.05, **=p≤0.01, ***=p≤0.001 and ****=p≤0.0001.

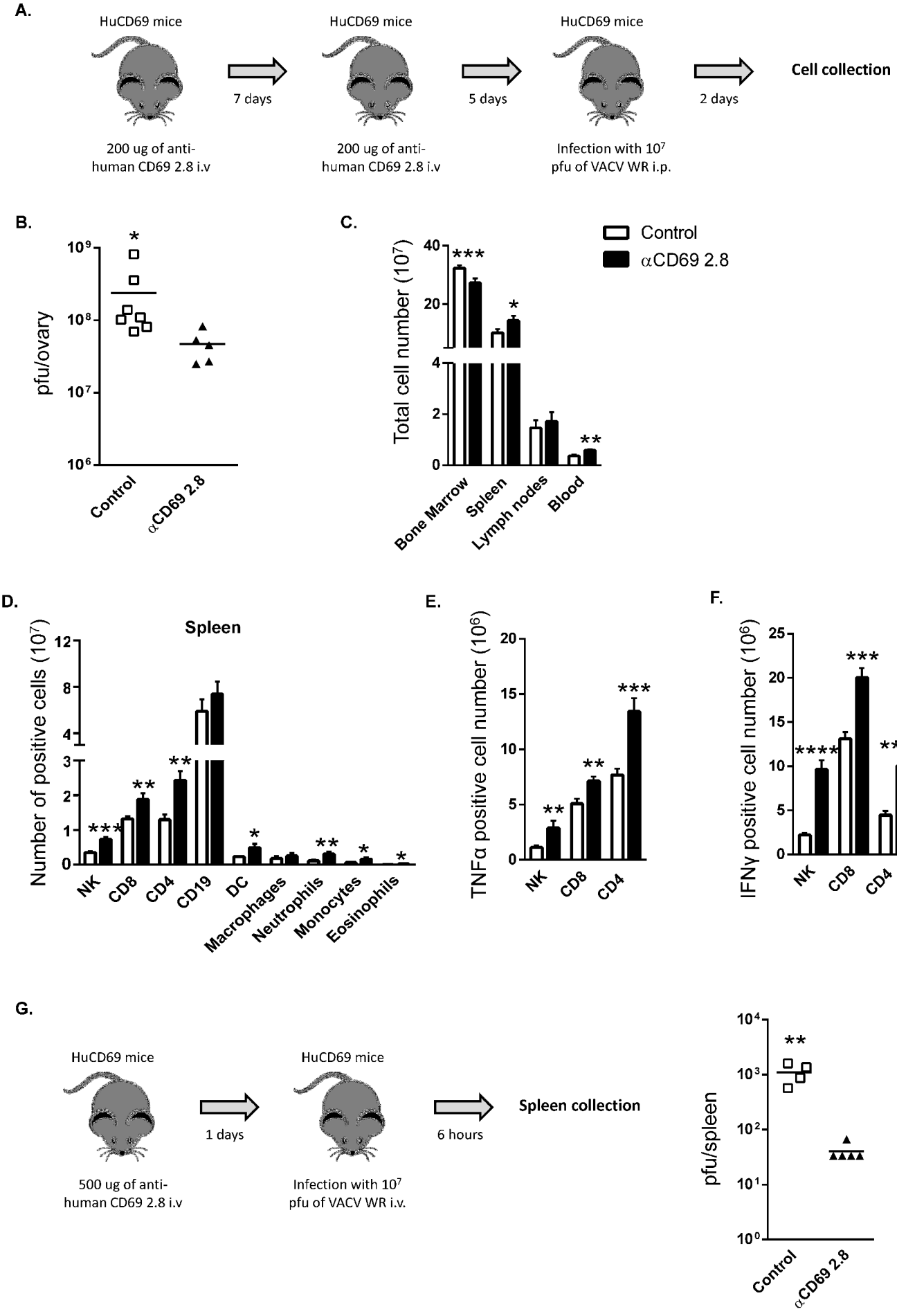
Figure 6. Treatment with anti-human-CD69-2.8 antibody increased proliferation and survival in the spleen. A-B, Mice were treated or not treated with 500 µg of anti-huCD69-2.8 24 hours before a BrdU injection. The mice received 1 mg of BrdU intraperitoneally, and, three hours later, were sacrificed. BrdU 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 were pooled, with a total of n≥8 mice per group. C, One independent experiment representative of two

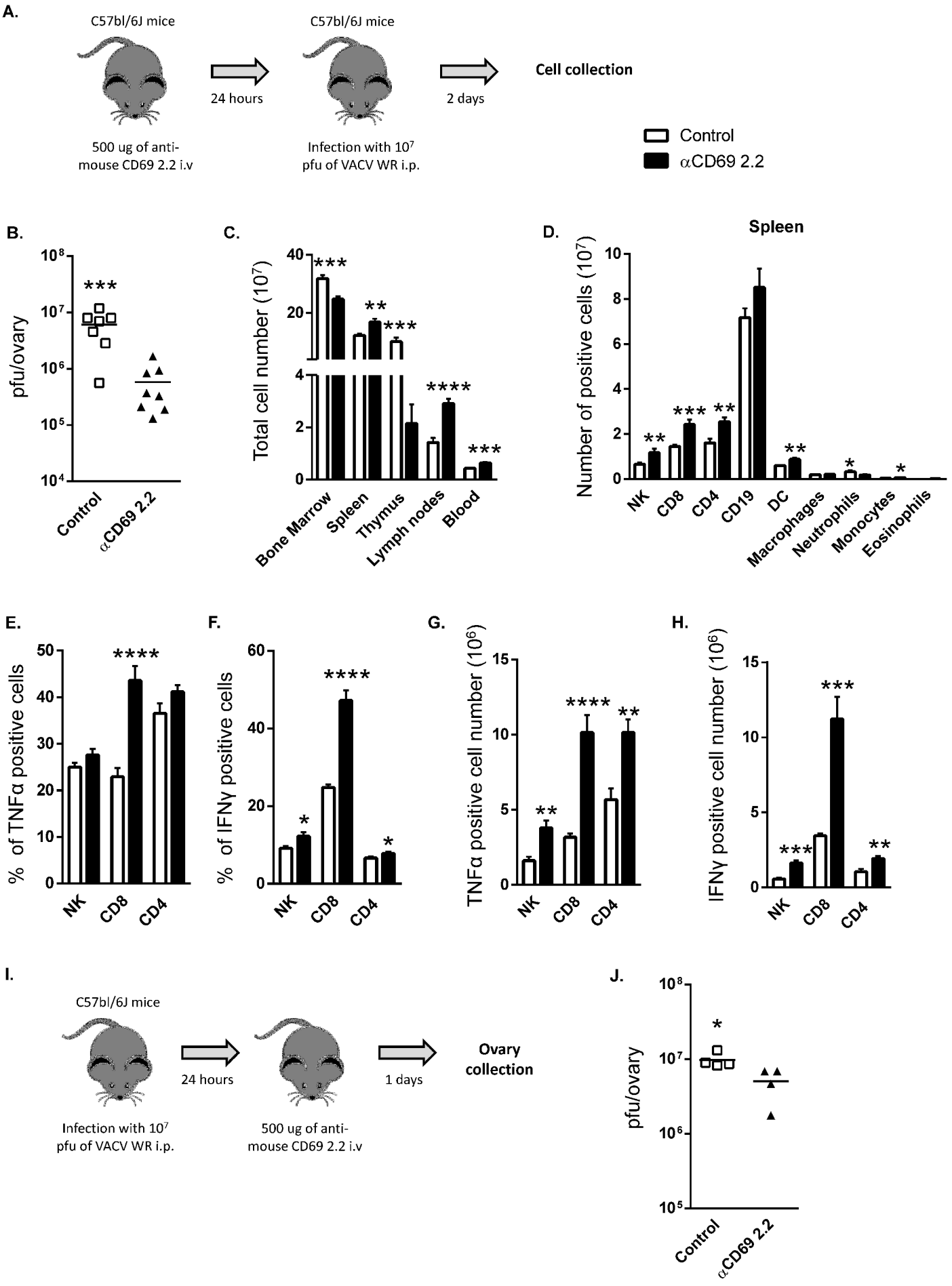
experiments is shown. Data were analyzed by an unpaired t test, where *=p≤0.05, **=p≤0.01, ***=p≤0.001 and ****=p≤0.0001.

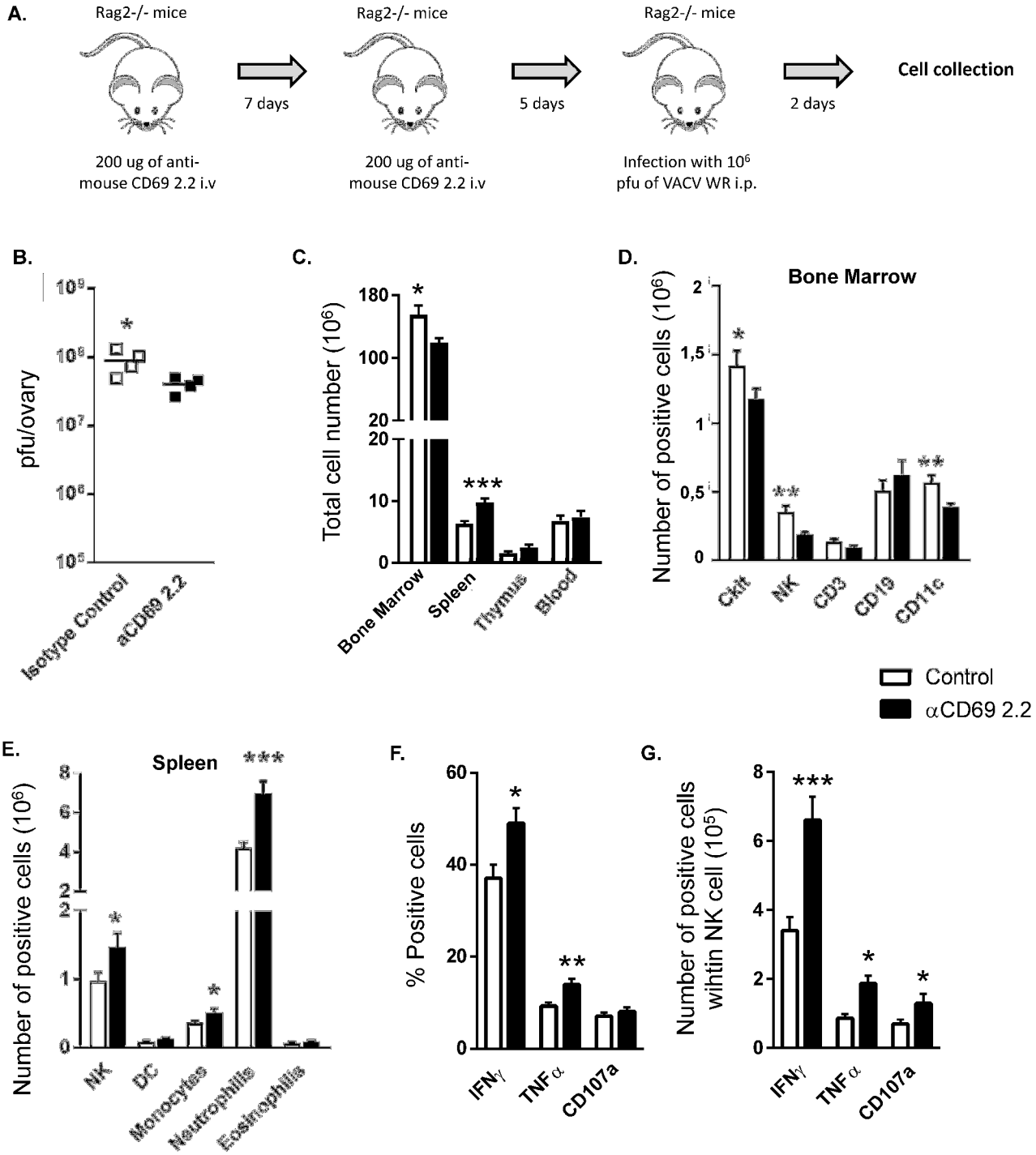
Figure 7. Targeting human CD69 promoted an increase in the levels of some proinflammatory cytokines. A-C, Mice were treated or not treated with 500 µg of anti huCD69-2.8 24 hours before analysis. A, IL-2 and CD25 expression were measured in the bone Marrow, spleen and blood by flow cytometry. B, The relative mRNA expression of TGF-β was compared between treated and control mice. C, The protein levels of certain cytokines in supernatants of splenocytes was measured by Luminex assay. D-G, Splenocytes were collected from mice treated with 500 µg of anti-huCD69-2.8 for 24 hours or left untreated. D-E, mRNA of proinflammatory cytokines and their receptors were assayed through an array. D, data for IL-17a, IL-17b, IL-17f, IL1-α, IL-1β, LTa, LTb and IFNγ 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, IL1- α, IL-1β, TNFα and IL-21 is shown. G, qPCR analysis of CCL2 and CCL12 is shown.
A, Three experiments were pooled, with a total of n≥10 mice per group. B, One experiment is shown. C, Four independent experiments were pooled, with a total of n≥12 mice per group. D-G, One experiment is shown with samples pooled of two or three experiments. Data were analyzed by an unpaired t test, where *=p≤0.05, **=p≤0.01, ***=p≤0.001 and ****=p≤0.0001.

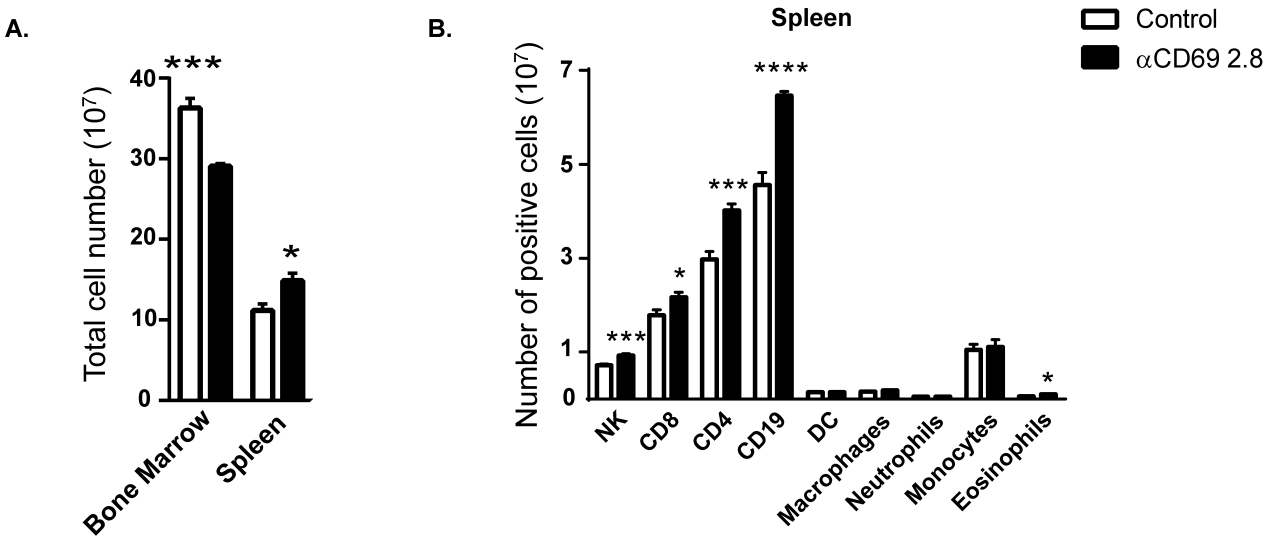
Figure 8. Targeting human CD69 induced increased splenic cellularity mediated by mTOR. A-C, Mice were treated with 500 µg of anti-huCD69-2.8 administered i.v 24 hours before analysis, 480 mg of rapamycin/kg administered i.p for five days or both, as indicated. A, The total cell numbers in the spleen (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 µg 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≤0.05, **=p≤0.01, ***=p≤0.001 and ****=p≤0.0001.

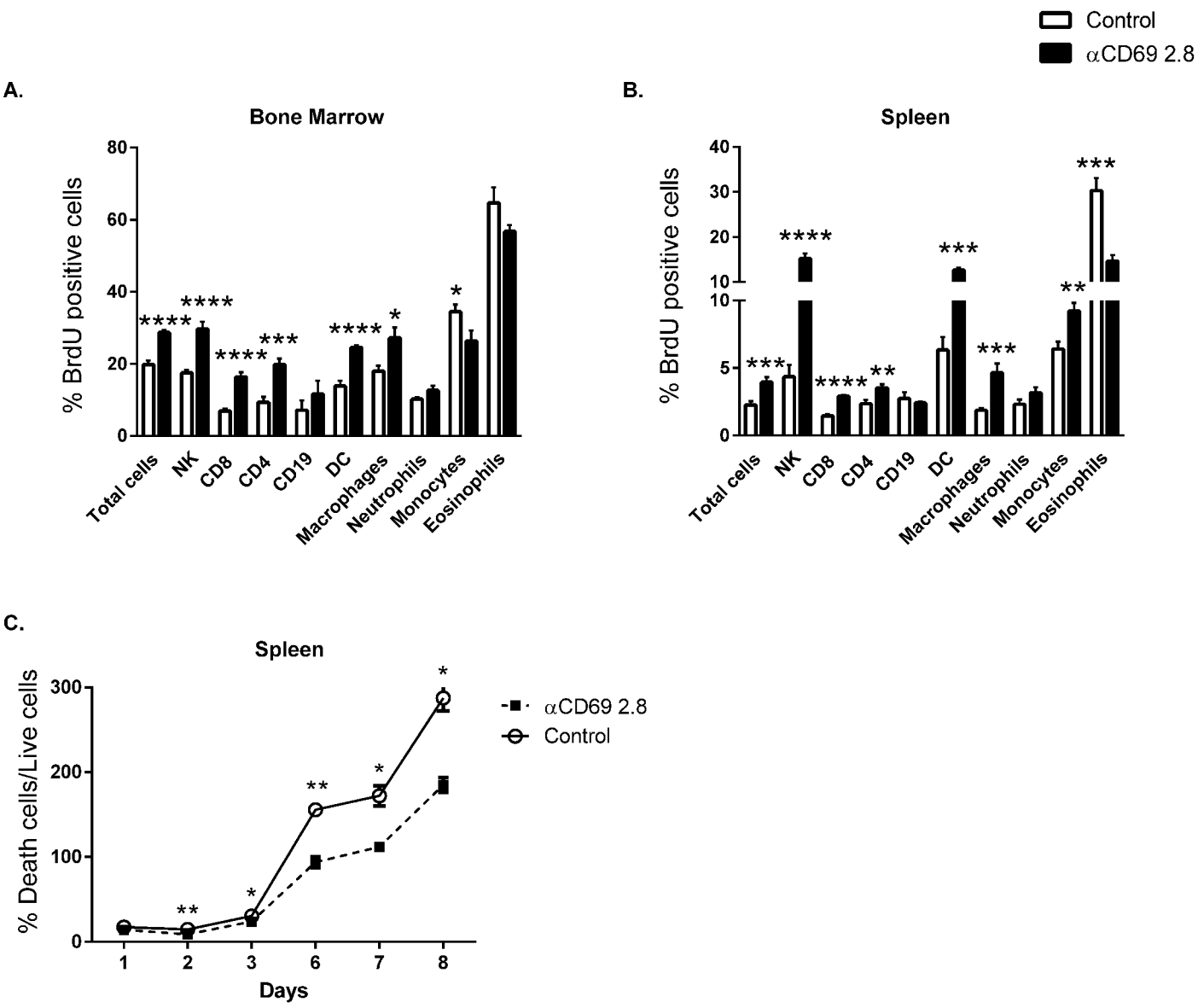

A.
HuCD69 mice
7 days
HuCD69 mice
5 days
HuCD69 mice
7 days
Cell collection
200 ug of anti-human CD69 2.8 i.v
200 ug of anti-human CD69 2.8 i.v
Infection with 10^7 pfu of VACV WR i.p.
Control
αCD69 2.8
B.
pfu/ovary
Isotype Control
anti-huCD69 2.8
C.
Total cell number (10^7)
Bone Marrow
Spleen
Lymph nodes
Thymus
Blood
D.
Spleen
Number of positive cells (10^7)
NK
CD8
CD4
CD19
DC
Macrophages
Monocytes
Neutrophils
Eosinophils
E.
TNFα positive cell number (10^6)
NK
CD8
CD4
F.
IFNγ positive cell number (10^6)
CD8
CD4
H.
Control
αCD69 2.8
% Weight loss
Days
G.
HuCD69 mice
1 day
HuCD69 mice
5 days
HuCD69 mice
500 ug of anti-human CD69 2.8 i.v
Infection with 10^5 pfu of VACV WR intranasal
500 ug of anti-human CD69 2.8 i.v

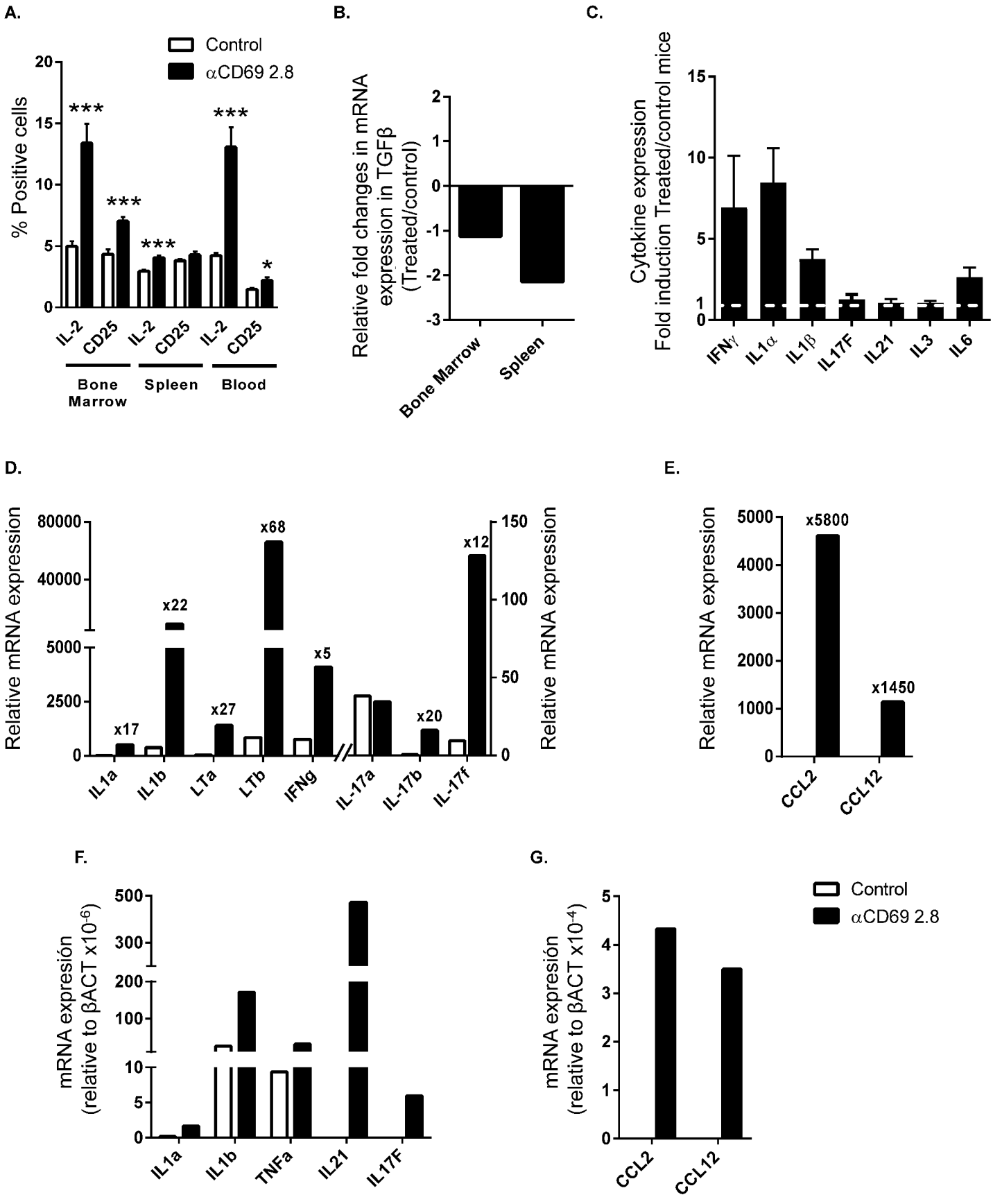

A.
HuCD69 mice
7 days
HuCD69 mice
5 days
HuCD69 mice
2 days
Cell collection
200 ug of anti-human CD69 2.8 i.v
200 ug of anti-human CD69 2.8 i.v
Infection with 10^7 pfu of VACV WR i.p.
B.
pfu/ovary
Control
αCD69 2.8
C.
Control
αCD69 2.8
Total cell number (10^7)
Bone Marrow
Spleen
Lymph nodes
Blood
D.
Spleen
Number of positive cells (10^7)
NK
CD8
CD4
CD19
DC
Macrophages
Neutrophils
Monocytes
Eosinophils
E.
TNFα positive cell number (10^6)
NK
CD8
CD4
F.
IFNγ positive cell number (10^6)
NK
CD8
CD4
G.
HuCD69 mice
1 days
HuCD69 mice
6 hours
Spleen collection
500 ug of anti-human CD69 2.8 i.v
Infection with 10^7 pfu of VACV WR i.v.
pfu/spleen
Control
αCD69 2.8


A.
C57bl/6J mice
24 hours
C57bl/6J mice
2 days
Cell collection
500 ug of anti-mouse CD69 2.2 i.v
Infection with 10^7 pfu of VACV WR i.p.
Control
αCD69 2.2
B.
pfu/ovary
Control
αCD69 2.2
C.
Total cell number (10^7)
Bone Marrow
Spleen
Thymus
Lymph nodes
Blood
D.
Spleen
Number of positive cells (10^7)
NK
CD8
CD4
CD19
DC
Macrophages
Neutrophils
Monocytes
Eosinophils
E.
% of TNFα positive cells
F.
% of IFNγ positive cells
G.
TNFα positive cell number (10^6)
H.
IFNγ positive cell number (10^6)
I.
C57bl/6J mice
24 hours
1 days
Ovary collection
Infection with 10^7 pfu of VACV WR i.p.
500 ug of anti-mouse CD69 2.2 i.v
J.
pfu/ovary
Control
αCD69 2.2

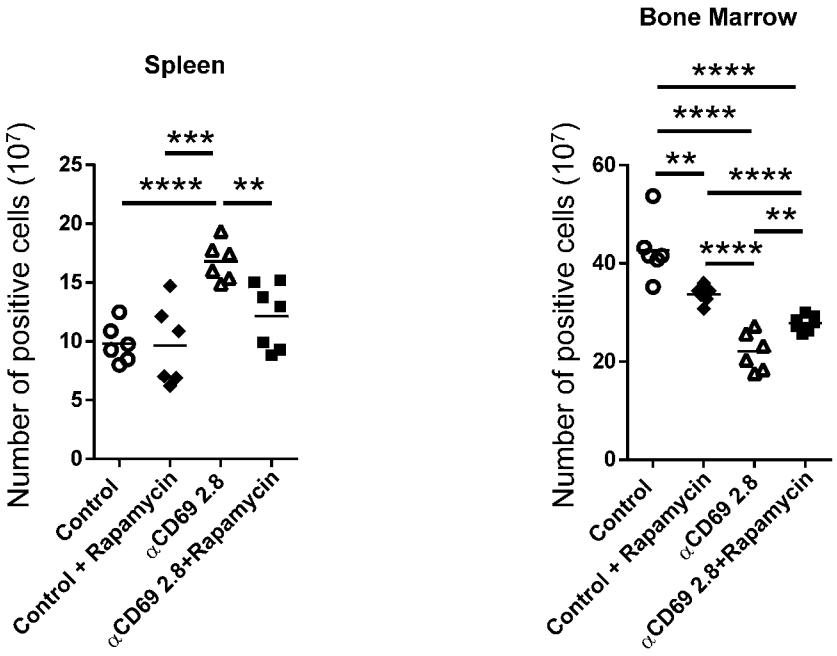

A.
Rag2-/- mice
7 days
Rag2-/- mice
5 days
Rag2-/- mice
2 days
Cell collection
200 ug of anti-mouse CD69 2.2 i.v
200 ug of anti-mouse CD69 2.2 i.v
Infection with 10^6 pfu of VACV WR i.p.
B.
pfu/ovary
Isotype Control
aCD69 2.2
C.
Total cell number (10^6)
Bone Marrow
Spleen
Thymus
Blood
D.
Bone Marrow
Number of positive cells (10^6)
Ckit
NK
CD3
CD19
CD11c
Control
αCD69 2.2
E.
Spleen
Number of positive cells (10^6)
NK
DC
Monocytes
Neutrophils
Eosinophils
F.
% Positive cells
IFNγ
TNFα
CD107a
G.
Number of positive cells wihtin NK cell (10^5)
IFNγ
TNFα
CD107a


A.
B.
Spleen
Control
αCD69 2.8
Total cell number (10^7)
Number of positive cells (10^7)
***
*
****
Bone Marrow
Spleen
NK
CD8
CD4
CD19
DC
Macrophages
Neutrophils
Monocytes
Eosinophils

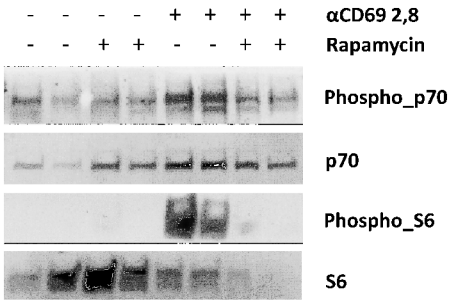

Control
αCD69 2.8
A.
Bone Marrow
% BrdU positive cells
Total cells
NK
CD8
CD4
CD19
DC
Macrophages
Neutrophils
Monocytes
Eosinophils
B.
Spleen
% BrdU positive cells
C.
Spleen
% Death cells/Live cells
Days
αCD69 2.8
Control


A.
Control
αCD69 2.8
% Positive cells
20
15
10
5
0
***
***
***
***
*
IL-2
CD25
IL-2
CD25
IL-2
CD25
Bone Marrow
Spleen
Blood
B.
Relative fold changes in mRNA expression in TGFβ (Treated/control)
2
1
0
-1
-2
-3
Bone Marrow
Spleen
C.
Cytokine expression Fold induction Treated/control mice
15
10
5
1
0
IFNγ
IL1α
IL1β
IL17F
IL21
IL3
IL6
D.
Relative mRNA expression
80000
40000
5000
2500
0
x17
x22
x27
x68
x5
x20
x12
150
100
50
0
IL1a
IL1b
LTa
LTb
IFNg
IL-17a
IL-17b
IL-17f
E.
Relative mRNA expression
5000
4000
3000
2000
1000
0
x5800
x1450
CCL2
CCL12
F.
mRNA expresión (relative to βACT x10-6)
500
300
200
100
10
5
0
IL1a
IL1b
TNFa
IL21
IL17F
G.
Control
αCD69 2.8
mRNA expresión (relative to βACT x10-4)
5
4
3
2
1
0
CCL2
CCL12

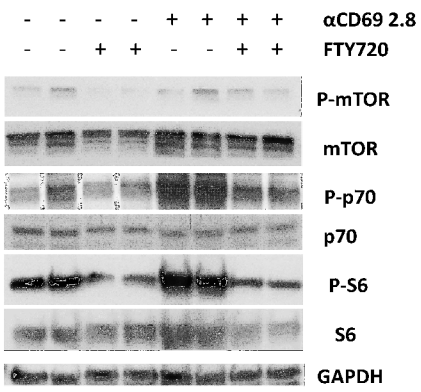
A.


Spleen
Number of positive cells (10^7)
25
20
15
10
5
0
***
****
**
Control
Control + Rapamycin
αCD69 2.8
αCD69 2.8+Rapamycin
Bone Marrow
Number of positive cells (10^7)
60
40
20
0
****
****
**
****
****
**
Control
Control + Rapamycin
αCD69 2.8
αCD69 2.8+Rapamycin

B.


Spleen
- - - - + + + + αCD69 2,8
- - + + - - + + Rapamycin
Phospho_p70
p70
Phospho_S6
S6

C.


Spleen
- - - - + + + + αCD69 2.8
- - + + - - + + FTY720
P-mTOR
mTOR
P-p70
p70
P-S6
S6
GAPDH