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# Differential effect of CD69 targeting on bystander and antigen-specific T cell proliferation

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## ABSTRACT

**In spite of an initially proposed role as a costimulatory molecule for CD69, in vivo studies showed it as a regulator of immune responses and lymphocyte egress. We found constitutive CD69 expression by T cell subsets and pDC. We examined a possible effect of CD69 on T cell proliferation using transfer models and in vitro assays. In mice locally expressing or receiving antigen, anti-CD692.2 treatment did not affect the proliferation of antigen-specific transgenic T cells in ADLN, although we observed the presence of proliferated T cells in non-ADLN and spleen. This was not affected by FTY720 treatment and thus, not contributed by increased egress of proliferated lymphocytes from ADLN. In the absence of antigen, anti-CD69 2.2 treatment induced bystander proliferation of transferred memory phenotype T cells. This proliferation was mediated by IL-2, as it was inhibited by anti-IL-2 or anti-CD25 antibodies in vitro and by anti-CD25 antibodies in vivo. It was also dependent on CD69 expression by donor T cells and recipient cells. CD69 targeting on T cells enhanced IL-2-mediated proliferation and CD25 expression. However, it did not lead to increased early IL-2 production by T cells. No T cell subset was found to be specifically required in the recipient. Instead, CD69 targeting on pDC induced their expression of IL-2 and CD25, and pDC depletion showed that this subset was involved in the proliferation induction. These results indicate that CD69 targeting induces bystander T cell proliferation through pDC IL-2 production and T cell sensitization to IL-2 without affecting antigen-driven T cell proliferation.** ***J. Leukoc. Biol.* 92: 145–158; 2012.**

## Introduction

CD69 is a type II C-type lectin, rapidly up-regulated on all leukocytes in response to innate or adaptive stimuli [1]. On T cells, it is promptly up-regulated by TCR engagement [2] and by cytokines [3], mainly by IFN-$\alpha\beta$ [4]. Certain C-type lectins are up-regulated upon activation on T cells and have been implicated in costimulation [5, 6]. Early in vitro studies pointed to the same function for CD69, as addition of anti-CD69 mAb increased activated T cell proliferation through increased IL-2 and IL-2R expression [2, 7]. However, $CD69^{-/-}$ mice had increased anti-tumor response to NK-sensitive tumors [8], [9] and increased severity of collagen II-induced arthritis [10] [11], allergic asthma, skin contact hypersensitivity [12], and autoimmune myocarditis [13]. In the tumor, arthritis, and contact hypersensitivity models, the treatment with the anti-CD69 mAb 2.2 mimicked the $CD69^{-/-}$ phenotype [9, 11, 12]. $CD69^{-/-}$ mice showed deficient control of *Listeria monocytogenes* infection at early time-points, associated with increased IFN-$\gamma$ production and dependent on the presence of lymphocytes, as $RAG2^{-/-}$ $CD69^{-/-}$ mice did not have increased bacterial burden compared with $RAG2^{-/-}$ $CD69^{+/+}$ mice [14]. In addition, CD69 is implicated in retention of activated lymphocytes in lymphoid organs through interaction with and negative regulation of surface S1P1 [15].

Antigen-specific T cell activation depends on MHC-antigen-TCR interactions, but costimulatory molecules and cytokines contribute to determine the extent of the initial proliferative response and the function acquired by the primed T cell [16].

Abbreviations: 7AAD=7-amino-actinomycin D, $\gamma$c=$\gamma$-chain, ADLN=antigen draining LN, BFA=Brefeldin A, BM=bone marrow, BMDC=bone marrow-derived DC, CD40L/CD62L=CD40/CD62 ligand, cDC=conventional DC, DLN=draining LN, Flt3l=Fms-related tyrosine kinase 3 ligand, FoxP3=forkhead box P3, ISCIII=Instituto de Salud Carlos III, MFI=mean fluorescence intensity, mOVA=membrane-bound OVA, pDC=plasmacytoid DC, PDL-1=programmed death ligand 1, RIP=rat insulin promoter, rm=recombinant mouse, RMMA-1=rat mAb against mouse IFN-$\alpha$, S1P1=sphingosine-1-phosphate receptor 1

*The online version of this paper, found at www.jleukbio.org, includes supplemental information.*

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Nevertheless, T cells, especially of the memory-phenotype subset, can also undergo antigen-unspecific bystander activation, which involves proliferation, cytokine secretion, and cytotoxicity [17]. This is a cytokine-driven proliferation, and it has been described to be induced by agents activating innate immune cells, such as TLR ligands [18, 19] and anti-CD40L targeting [20]. Activated DCs secrete IFN-$\alpha\beta$, IL-12, and IL-18, which activate NK cells to produce IFN-$\gamma$. IFN-$\alpha\beta$ and IFN-$\gamma$ induce secretion of IL-15, which has a direct mitogenic effect on T cells in vitro and in vivo [21–23]. Other mitogenic cytokines, also sharing the common $\gamma$-c receptor, such as IL-2 and IL-4, have also been described to directly induce bystander T cell proliferation in vivo [24–26].

We studied a possible effect of CD69 targeting on the extent of T cell priming by analyzing the initial antigen-specific T cell proliferation in in vivo transgenic T cell transfer models. Surprisingly, we did not find any alteration of this parameter in ADLNs. Instead, we found that the anti-CD69 2.2 treatment induced bystander proliferation of memory phenotype T cells and that this was mediated by pDC and IL-2.

## MATERIALS AND METHODS

### Mice

Balb/c, DO10.11 RAG2$^{-/-}$ Balb/c, C57BL/6, and OT-I C57BL/6 mice, all CD69$^{+/+}$ and CD69$^{-/-}$, and CIITA$^{-/-}$ Balb/c, RAG1$^{-/-}$ C57BL/6, OT-I RAG1$^{-/-}$ C57BL/6, and RIP-mOVA C57BL/6 mice were bred and housed under specific, pathogen-free conditions in the animal facilities of the Parc Cientific de Barcelona (Spain), ISCIII (Madrid, Spain), The Walter and Eliza Hall Institute (Melbourne, Australia), and Dana-Farber Cancer Institute (Boston, MA, USA). CD69$^{-/-}$ mice had been backcrossed on the C57BL/6 and the Balb/c backgrounds at least nine times [27]. All procedures involving animals and their care were approved by the University of Barcelona and ISCIII Ethics Committees and were conducted according to institutional guidelines in compliance with local (Generalitat de Catalunya Decree 214/1997, DOGC 2450) and international (Guide for the Care and Use of Laboratory Animals, NIH 85-23, 1985) laws and policies. Unless otherwise stated, C57BL/6 mice were used.

### Antibodies and immunological reagents

The anti-CD69 mAb 2.2 (IgG1 isotype) was generated in our laboratory [9] by the fusion of NS-1 myeloma cells with spleen cells from a CD69$^{-/-}$ mouse, previously immunized three times with mouse 300–19 pre-B cells. The antibody was purified from concentrated hybridoma supernatants using a protein G column (GE Healthcare, Piscataway, NJ, USA), dialyzed extensively against PBS, further purified by HPLC using a Superdex 200 column (GE Healthcare), and stored at −80°C. The IgG1 isotype control antibody was produced and purified likewise. The resulting antibody preparations were tested on CD69$^{-/-}$ BMDC cultures at 10 $\mu$g/ml and were unable to up-regulate CD80 and CD86 expression levels on these cells. The anti-CD25 PC61.5 was a kind gift from Dr. J. C. Ochando. Functional-grade anti-IL-2 (JES6-1A12) and anti-IL-15 (AIO.3) were purchased from eBioscience (San Diego, CA, USA). The rmIL-2 and human rIL-15 were from ImmunoTools (Friesoythe, Germany). The supernatant of the rmIL-7-transfected 3T3 cell line [28] was a kind gift from Dr. B. de Andrés.

### Flow cytometry

LN and spleen cells were incubated with anti-CD16/32 (Fc-block 2.4G2; BD Biosciences, Franklin Lakes, NJ, USA), and 7AAD (BD PharMingen, San Diego, CA, USA) or PI (Molecular Probes, Eugene, OR, USA) was added to exclude dead cells. The following antibodies against mouse surface antigens were used: anti-CD4 (GK1.5), -CD8 (YTS 169.4), -CD11c (N418), -B220/CD45RA (14.8), -CD69 (H1.2F3), and -Bst2 (120G8), all in-house-produced; anti-CD11b (M1/70.15), -TCR-$\gamma\delta$ (GL-2), -CD19 (PeCa1), and -CD62L (MEL-14) from ImmunoTools; anti-CD3 (145-2C11), -CD4 (GK1.5), -CD8 (53-6.7), -CD11c (N418), -CD19 (1D3), -CD25 (PC61.5), -CD44 (1M7), -B220/CD45RA (RA3-6B2), -CD69 (H1.2F3), -V$\alpha$2 TCR (B20.1), and -DO10.11 TCR (KJ1), from eBioscience; and anti-Ly6C/G (Gr1) and -CD49b (DX5), from BD PharMingen. Anti-SiglecH mAb (440c; Hycult Biotech, Uden, The Netherlands) was a kind gift of Dr. J. Cano Ochando. Anti-CD127 (A7R34; eBioscience) was a kind gift of Dr. B. de Andres. For intracellular IFN-$\alpha$ staining, spleen cells were cultured in the presence of 3 $\mu$g/ml BFA (Sigma-Aldrich, St. Louis, MO, USA) for 4 h. For intracellular IL-2 staining, cells were stimulated with 10 ng/ml PMA and 1 $\mu$g/ml ionomycin (both from Sigma-Aldrich) in the presence of 3 $\mu$g/ml BFA for 4h. In both cases, cells were then surface-labeled, fixed, and permeabilized with Cytofix/Cytoperm (Becton Dickinson, Franklin Lakes, NJ, USA), stained with anti-IFN-$\alpha$ (RMMA-1; PBL InterferonSource, Piscataway, NJ, USA) or anti-IL-2 (JES6-5H4; eBioscience), and analyzed. For FoxP3 intranuclear staining, the FoxP3 staining buffer set and anti-FoxP3 (FJK-165), all from eBioscience, were used. Cells were analyzed on FACSCalibur or FACSCanto (Becton Dickinson) using CELLQuest or BD FACSDiva software (Becton Dickinson), and data were analyzed with FlowJo (Tree Star, Ashland, OR, USA). In the experiments where the MFI is represented, this parameter corresponds to the geometric mean.

### Adoptive transfer experiments

Total LN and spleen OT-I, OT-I RAG1$^{-/-}$, or DO10.11 RAG2$^{-/-}$ cells were collected, and where indicated, T cells were negatively purified using MACS columns and MACS streptavidin microbeads (Miltenyi Biotec, Auburn, CA, USA); anti-CD11c (HL3), -CD11b (M1/70), -Gr1 (RB6-8C5), and -CD49b (DX5), all from BD PharMingen; anti-B220 (RA3-6B2, eBioscience) and -CD4 (for OT-I CD8$^+$ T cell isolation, L3T4; Southern Biotech, Birmingham, AL, USA); or anti-CD8$\alpha$ (for DO10.11 CD4$^+$ T cells isolation, eBioH35-17.2; eBioscience) biotinylated antibodies. Alternatively, CD4 and CD8$\alpha$ T cell isolation kits (Miltenyi Biotec) were used. Purified cells were 90–95% T cells, and T cells were stained with 5–8 $\mu$M CFSE (Molecular Probes) and were injected i.v. into C57BL/6 or Balb/c recipients, respectively. Where indicated, 1 $\mu$g OVA (Sigma-Aldrich) and/or 5 $\mu$g LPS (Sigma-Aldrich) were injected s.c. into a posterior footpad. In experiments with FTY720 (Cayman Chemicals, Ann Arbor, MI, USA) treatment, this compound was administered at 1 mg/kg i.p., 14 h after cell transfer and 48 h before sample collection. B16-OVA-bearing mice had received $2 \times 10^6$ B16-OVA cells s.c., 10 days before. In all of the transfers, after the indicated times, LN and spleen samples were stained with anti-V$\alpha$2 and anti-CD8$\alpha$ mAb for OT-I T cell transfer or KJ1 and anti-CD4 mAb for DO10.11 T cell transfer. Proliferation was measured by assessing CFSE dilution in the indicated cell populations.

### In vivo pDC depletion and CD25 blockade

For pDC depletion, 1 mg in-house-produced anti-Bst2 120G8 antibody was administered i.p. on Days −1 and + 1, with respect to anti-CD69 2.2 treatment and adoptive transfer. For CD25 blocking experiments, 300 $\mu$g of the anti-CD25blocking mAb PC61.5 [27] was injected i.v. on Days −1 and + 1. LN and spleen samples were taken on Day 3

### pDC obtention and purification

For BM-pDCs, BMs were obtained from C57BL/6 mice, lysed, and cultured at $1.5 \times 10^6$/ml in 24-well plates in complete medium (RPMI-1640 medium, supplemented with 10% FCS, 50 $\mu$M 2-ME, 2 mM L-glutamine, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin), supplemented with in-house- produced Flt3l for 9 days. After that time, floating cells were collected and stained with home-produced anti-CD11c, -CD45RA, and -Bst2. CD11c$^{mid}$, CD45R$^+$ Bst2$^+$ were purified as pDCs with a FACSAria sorter (Becton Dickinson). pDCs were cultured at $10^5$ cells/well in the presence of GM-CSF and various doses of phosphorothioated CpG oligodeoxynucle-

otide 1668 (GeneWorks, Hindmarsh, Australia) and with 10 μg/ml anti-CD69 2.2 or its isotype control for 24 h. After that time, the cultures were centrifuged and supernatants collected.

To obtain endogenous pDC, spleens and LNs were snipped, and a cell suspension was made. Cells were prepurified using anti-mouse CD11c microbeads and MACS columns. The positive fraction was then stained with anti-CD3 and -CD19, 7AAD (all in a dump channel), and anti-CD11c and -CD45RA. CD3/19/7AAD$^-$ CD11c$^{mid}$ CD45R$^+$ were purified as pDCs with a FACSAria sorter (Becton Dickinson). pDCs were cultured at $2 \times 10^4$ cells/well in U-bottom, 96-well plates in complete media, supplemented with 20 ng/ml GM-CSF (ImmunoTools) and with 10 μg/ml anti-CD69 2.2 or its isotype control for 2 days. After that time, the cultures were centrifuged and supernatants collected.

### In vitro cultures

Total LN and spleen cells of Balb/c, OT-I, or OT-I RAG1$^{-/-}$ were isolated. When proliferation was to be measured, cells were stained with 2–5 μM CFSE. Cells were cutured at 1–5 $\times$ $10^6$ cells/ml in media, supplemented or not with 1 μg/ml LPS, as indicated, and the anti-CD69 2.2 or isotype control antibodies were added at 10 μg/ml. Where indicated, anti-IL-2 (JES6-1A12), anti-CD25 (PC61.5), or the corresponding isotype control antibodies were added at 5 μg/ml and 10 μg/ml, respectively. For experiments measuring T cell proliferation in response to IL-2, LN and spleen OT-I RAG$^{-/-}$ CD8$^+$ T cells were negatively purified using MACS columns and MACS streptavidin microbeads (Miltenyi Biotec); anti-CD11c (HL3), -CD11b (M1/70), -Gr1 (RB6-8C5), and -CD49b (DX5), all from BD PharMingen; and anti-B220 (RA3-6B2, eBioscience) and -CD4 (L3T4, Southern Biotech) biotinylated antibodies and were CFSE-stained and cultured with the indicated rmIL-2 doses. After the indicated days of culture, cells were stained and analyzed by flow cytometry. Callibrite beads (Becton Dickinson; $2.5 \times 10^4$) were added to each sample before acquisition to normalize cell counts. For CFSE-stained cultures, proliferation was measured by assessing CFSE dilution in the indicated cell populations.

### ELISA

Supernatants of BM-pDCs were incubated in rat anti-mouse IFN-α mAb (RMMA-1; PBL Biomedical Laboratories, New Brunswick, NJ, USA)-coated plates, followed by rabbit anti-mouse IFN-α polyclonal antibodies (PBL Biomedical Laboratories) and HRP-conjugated donkey anti-rabbit IgG for detection with the substrate ABTS. rIFN-α (PBL Biomedical Laboratories) was used to generate a standard curve. IL-2, in the supernatants of purified pDCs, was quantified by ELISA (eBioscience), following the manufacturer's instructions.

### Statistical analysis

Data were plotted and statistically analyzed using Prism software (GraphPad Software, La Jolla, CA, USA). When homogeneity of variances between the groups to be compared could be demonstrated, a two-tailed, unpaired *t* test was used, with the *P* values indicated: $*P < 0.05$; $**P < 0.01$; and $***P < 0.005$.

## RESULTS

### CD69 expression at steady-state

To assess CD69 expression levels at steady-state in the different LN and spleen cell subtypes, we stained LN and spleen samples from CD69$^{+/+}$ and CD69$^{-/-}$ mice for different cell subsets. These characterizations showed constitutive CD69 expression in LN and spleen pDCs. cDCs showed a certain level of CD69 expression in the LN, but not in the spleen (**Fig. 1**), which could reflect the presence of mature migratory DCs in the LN rather than in the spleen [29]. In addition, CD69 was found to be constitutively expressed by LN NK cells and subsets of spleen NK and NKT cells, as well as by important subsets of CD4$^+$ T cells, CD8$^+$ T cells, and γδ T cells, being higher on CD4$^+$ T cells, and by a very small subset of B cells (Fig. 1). Relevant differences were found in the frequency of CD69$^+$ cells, which are markedly increased in CD44$^{high}$ CD8$^+$ and CD4$^+$ T cells and in FoxP3$^+$ CD4$^+$ T cells (Supplemental Fig. 1). Granulocytes express CD69 at low levels, whereas macrophages seem not to express it. Thus, most cell subtypes show a certain expression of CD69 at steady-state, but this is especially remarkable for pDCs and NK, NKT, and T cells.

### In vivo CD69 targeting does not affect antigen-specific T cell proliferation in ADLN but induces the presence of proliferated T cells in distant LN

To study the effect of CD69 targeting on T cell priming in vivo, we performed OT-I transfers and anti-CD69 2.2 treatment in recipients locally receiving or expressing OVA antigen. In a model where mice received a s.c. injection of OVA plus LPS in a posterior hind footpad (**Fig. 2A**), the anti-CD69 treatment did not affect the percentage of proliferated OT-I cells found in the popliteal ADLN. However, proliferated OT-I cells were found in the spleen and in distant LNs in the mice treated with anti-CD69 2.2 but not in untreated mice. We performed the same analysis in two other experimental settings, which exclude the possibility that endotoxin contamination of the OVA antigen could be affecting anti-CD69 2.2 effects: in the first one, OVA was produced by s.c. B16-OVA tumors, and in the second one, it was a pancreatic and renal antigen in the RIP-mOVA model [30] (Fig. 2B and C). In neither of these settings did anti-CD69 2.2 treatment affect the extent of OT-I proliferation in the ADLN (axilary and pancreatic or renal, respectively), but it did induce OT-I proliferated cells appearing in nonantigen draining lymphoid organs in both models (popliteal and inguinal LNs, respectively). It could be reasoned that the high antigen content in DLNs would mask anti-CD69 effects on T cell proliferation, which would be detected only when antigen presence is limited, as would occur in non-ADLN. To investigate this point, we injected decreasing, graded doses of OVA in the footpad, but still, no substantial effect of the antibody treatment was observed in the proliferation of T cells at nonsaturating OVA doses in DLNs (Fig. 2D).

### Anti-CD69 2.2 in vivo treatment does not induce premature T cell egress

CD69 has been implicated in lymphocyte retention within lymphoid organs through the interaction with S1P1, and CD69$^{-/-}$ lymphocytes have been previously seen to be poorly retained into lymphoid organs [15]. It is therfore conceivable that an increased egress of proliferated T cell from the ADLN could account for the presence of proliferated cells in nonantigen draining sites in antibody-treated mice. To test for this possibility, we repeated the OT-I T cell transfers into anti-CD69 2.2-treated or untreated recipients receiving OVA plus LPS into the footpad and treating them or not with the egress

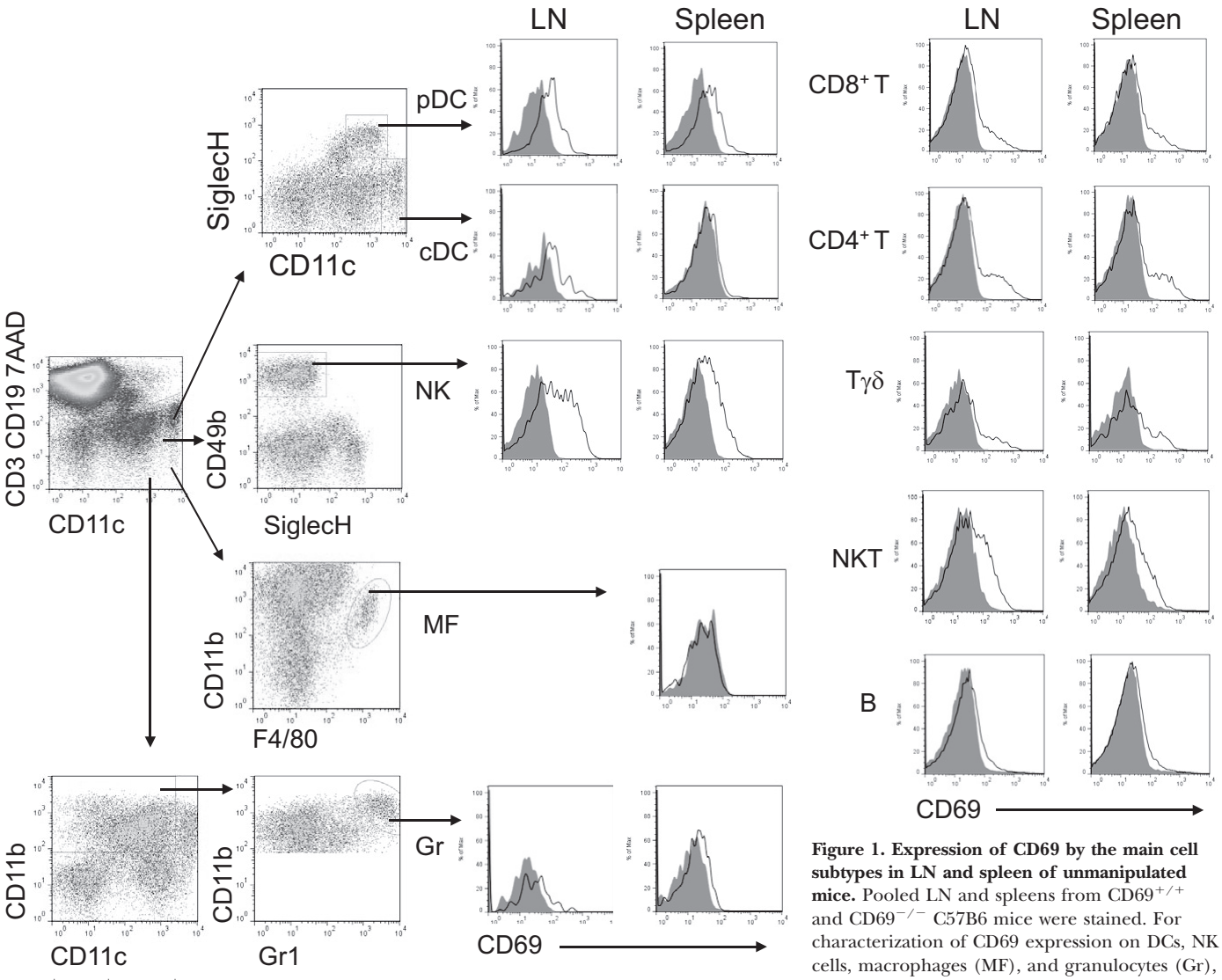


**Figure 1. Expression of CD69 by the main cell subtypes in LN and spleen of unmanipulated mice.** Pooled LN and spleens from $CD69^{+/+}$ and $CD69^{-/-}$ C57B6 mice were stained. For characterization of CD69 expression on DCs, NK cells, macrophages (MF), and granulocytes (Gr), $CD19^+$ $CD3^+$ $7AAD^+$ cells were excluded, and the gatings were performed as depicted. The dot plots are representative of spleen samples. For characterization of CD69 expression on $CD4^+$ T cells ($CD3^+$ $CD4^+$), $CD8^+$ T cells ($CD3^+$ $CD8^+$), Tγδ cells (TCRγδ$^+$), NKT cells ($CD3^+$ $CD49b^+$), and B cells ($CD19^+$, $B220^+$), only $7AAD^+$ cells were excluded. On the right, overlays of $CD69^{+/+}$ (solid line) and $CD69^{-/-}$ (gray-filled) LN and spleen samples gated on the indicated populations. Results are representative of two to four characterizations with similar results.

inhibitor FTY720 [31] 14 h after transfer. In vivo, FTY720 acts as an inhibitor of S1P1 function, blocking lymphocyte egress from lymphoid organs. It acts downstream of CD69, and so, CD69 targeting is not expected to revert the egress blockade, but FTY720 could inhibit a premature egress caused by a putative targeting-mediated blockade of CD69-mediated retention. FTY720 treatment was effective, as demonstrated by the fact that blood leukocyte numbers were reduced by three times (**Fig. 3B**) and by the increased retention in the ADLN (popliteal LN). Nevertheless, it did not inhibit the increased percentage of proliferated OT-I T cells in non-ADLN upon anti-CD69 2.2 treatment (Fig. 3A). This result suggests that CD69 targeting with anti-CD69 2.2 does not induce lymphocyte egress from LNs by interfering with the known CD69 role in T cell retention.

## Anti-CD69 2.2 in vivo treatment induces bystander proliferation of $CD8^+$ and $CD4^+$ T cells

Reasoning that bystander proliferation could explain the CD69 targeting-induced proliferating cells in non-ADLN of antigen-expressing mice, we tested whether the anti-CD69 2.2 was able to induce transferred OT-I T cell proliferation in an OVA antigen-independent manner. For this, we performed OT-I transfer and anti-CD69 2.2 treatment in mice that did not express or receive OVA. In this setting, we could indeed see that 3 days after anti-CD69 2.2 treatment, the anti-CD69 2.2 alone had induced bystander proliferation of the transferred cells in pooled LNs and spleen (**Fig. 4A** and **B**) and that the extent of this proliferation was similar to the one observed in non-ADLN in antigen-bearing mice in the absence of LPS. In experiments in which anti-CD69 2.2 treatments were performed

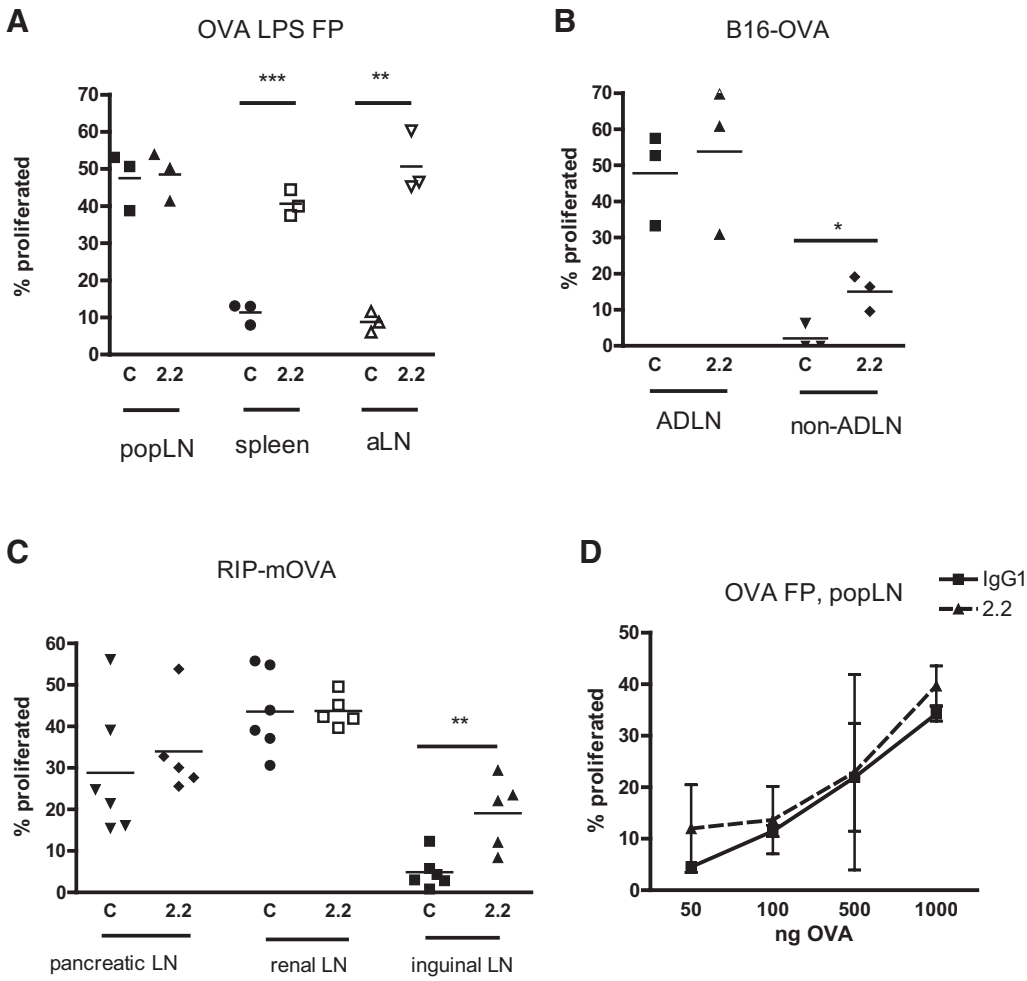


**Figure 2. Anti-CD69 2.2 targeting on T cells does not affect antigen-specific T cell proliferation in ADLN but induces the presence of proliferated T cells in distant LNs.** OT-I CD8$^+$ T cells were transferred into recipients, which were treated or not [control (C)] with anti-CD69 2.2 and received/expressed OVA in various conditions. Proliferation of CD8$^+$ V$\alpha$2$^+$-transferred cells was measured 2 days post-transfer in ADLN, non-ADLN, and spleen. (A) Recipients received 1 $\mu$g OVA and 5 $\mu$g LPS s.c. in a posterior hind footpad (FP); ADLN and non-ADLN analyzed were the popliteal and axillary LNs (popLN and aLN), respectively. (B) Recipients bearing s.c. B16-OVA tumors in the midventral area; ADLN and non-ADLN were axillary and popliteal LNs, respectively. (C) RIP-mOVA expressing OVA in the pancreas and kidney; pancreatic and renal LNs were collected as ADLN and the inguinal as non-ADLN. (D) Recipients received the indicated OVA doses in a posterior hind footpad; popliteal LNs were analyzed. Bars represent SD; $n$ = 1–2 mice/group. Results are representative of two (A–C) or one (D) experiment(s) with similar results.

in parallel in mice which were injected or not with OVA in the footpad, we could see that the percentage of the proliferated OT-I CD8$^+$ T cells in the nonantigen DLN (axillary) was similar in both groups of mice (17.25±0.41 and 14.33±5.2 for antigen-injected and nonantigen-injected mice, respectively). These observations support bystander proliferation as the major responsible for the observed CD69 targeting-influenced presence of proliferated T cells in nonantigen draining lymphoid organs in experiments with antigen-bearing mice. When anti-CD69 treatment was performed in recipients receiving a s.c. LPS injection in the footpad without antigen, we could see that CD69 targeting was enhancing the LPS-induced bystander proliferation, both at DLNs and non-DLNs (Supplemental Fig. 2). The capacity to induce bystander proliferation was not exclusive of anti-CD69 2.2 mAb, as treatment with another anti-CD69 mAb of the same IgG1 isotype (2.3-IgG1-modified) also induced similar CD8$^+$ T cell proliferation (data not shown). The bystander proliferation observed did lead to expansion of the population, as seen by the increase in the transferred cell numbers (Fig. 4C). Most of the proliferating CD8$^+$ T cells were CD44$^{high}$, IL-7R$\alpha^+$ and CD62L$^+$, belonging, thus, to the central memory phenotype subset. They were also mainly Ly6C$^-$ (Fig. 4A). Similarly, the antibody treatment also induced proliferation of transferred DO11.10 CD4$^+$ T cells in the LN and spleen, and the proliferated CD4$^+$ T cells were also mainly of memory phenotype (CD44$^{high}$; Fig. 4E and F). The anti-CD69 2.2 mAb was also able to induce T cell proliferation in in vitro cultures of whole LNs and spleen cells. In the case of CD8$^+$ T cells, this antibody-induced proliferation happened mainly when the culture was supplemented with LPS (Fig. 4D), whereas for CD4$^+$ T cells, the induction occurred in the absence of LPS (Fig. 4G) but was barely detectable with LPS. In conclusion, in vivo CD69 targeting induces bystander proliferation mainly of central memory phenotype CD8$^+$ and memory phenotype CD4$^+$ T cells, and this effect can be reproduced in vitro.

## Anti-CD69 2.2 induces bystander T cell proliferation acting on recipient cells and donor T cells

To determine whether the antibody acts directly on the transferred T cells or secondarily to CD69 targeting of host cells, we performed adoptive transfers of CD69$^{+/+}$ or CD69$^{-/-}$ OT-I cells into CD69$^{+/+}$ or CD69$^{-/-}$ C57B/6 recipients, which were treated or not with anti-CD69 2.2. The different combinations showed that the antibody treatment induced bystander proliferation only when recipient and donor mice were CD69$^{+/+}$ (**Fig. 5A** and **B**). The same observation was made when CD69$^{+/+}$ or CD69$^{-/-}$ DO10.11 cells were trans-

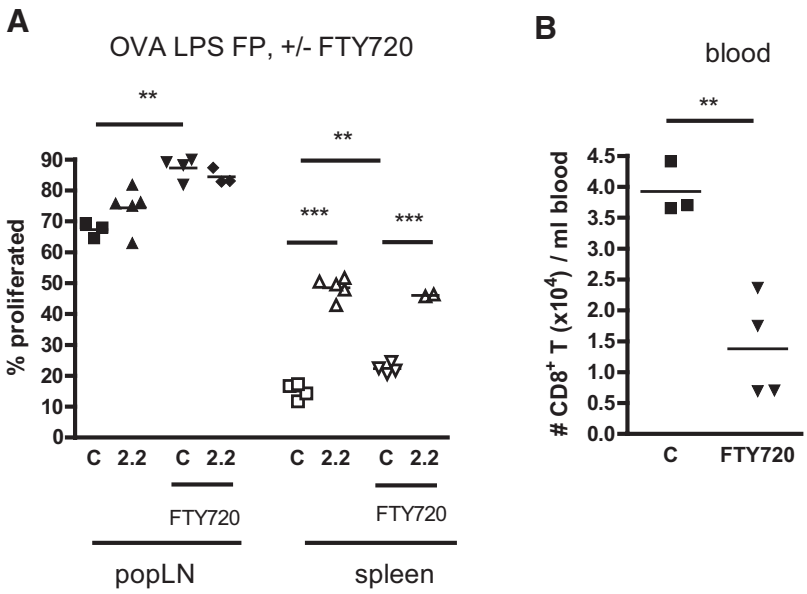


**Figure 3. FTY720 treatment does not avoid the anti-CD69 2.2-induced presence of proliferated OT-I T cells at non-antigen draining sites.** C57BL/6 mice were i.v.-injected or not with 200 $\mu$g anti-CD69 2.2 and transferred with purified OT-I CD8$^+$ T cells. At the same time, they were s.c.-injected with 1 $\mu$g OVA and 5 $\mu$g LPS. After 14 h, they were i.p.-injected with FTY720 (1 mg/kg). Forty-two hours after the OT-I transfer, proliferation was assessed within LN and spleen V$\alpha$2$^+$ CD8$^+$ T cells (A), and blood CD8$^+$ T cells numbers were determined (B). Results are representative of two experiments with similar results.

ferred into CD69$^{+/+}$ or CD69$^{-/-}$ Balb/c recipients (Fig. 5C and D). Therefore, anti-CD69 2.2 induces bystander T cell proliferation, acting on recipient cells and donor T cells. In these experiments, in untreated control mice, we could also observe that CD69 deficiency in donor T cells or recipient cells does not per se induce bystander proliferation. Therefore, CD69 targeting, but not CD69 deficiency, prompts to antigen-independent T cell proliferation.

## CD4$^+$ T, CD8$^+$ T, NKT, T$\gamma\delta$, and B cells are not specifically required for the anti-CD69 2.2-induced proliferation

To test whether CD4$^+$ T, NKT, T$\gamma\delta$, and B cells were necessary antibody-target recipient cells for anti-CD69 mAb to cause CD8$^+$ T cell proliferation, we transferred OT-I RAG1$^{-/-}$ T cells into RAG1$^{-/-}$ mice. In these lymphopenic recipients, transferred T cells undergo homeostatic proliferation. Anti-CD69 treatment increased the extent of homeostatic proliferation, indicating that T CD4$^+$ T and NKT, T$\gamma\delta$, and B cells are dispensable for its effects (**Fig. 6A** and **B**). However, it could be possible that the increased cytokine levels available in these mice over-ride the need for antibody targeting on the recipient cells. To rule this out, we transferred the same OT-I RAG1$^{-/-}$ T cells into OT-I RAG1$^{-/-}$ recipients, in which these transferred cells do not undergo homeostatic proliferation but also lack the mentioned lymphocyte subsets. In these recipients, the antibody treatment still induced bystander proliferation, confirming that these cell types are dispensable (Fig. 6C). To test for the need of CD8$^+$ T cells for CD4$^+$ T cell proliferation, we used a similar approach, by transferring DO10.11 RAG2$^{-/-}$ cells into DO10.11 RAG2$^{-/-}$ mice. In these recipients, the antibody also induced proliferation, indicating that neither CD8$^+$ T cells nor T$\gamma\delta$, B, or NKT cells are needed for the antibody effect on CD4$^+$ T cell proliferation (Fig. 6D). Thus, although their possible contribution cannot be ruled out, CD4$^+$ T, CD8$^+$ T, NKT, T$\gamma\delta$, or B cells are not specifically required for the anti-CD69 2.2-induced proliferation.

## Anti-CD69 2.2-induced T cell bystander proliferation is mediated by IL-2

Bystander proliferation is mediated by mitogenic cytokines whose receptors share the $\gamma$c subunit, such as IL-15 and IL-2. PDL-1 has been described to be up-regulated on T cells in response to the common $\gamma$c cytokines [32], and indeed, its expression level on CD8$^+$ T cells of anti-CD69 2.2-treated mice was increased (**Fig. 7A**). The fact that anti-CD69 2.2 treatment up-regulated CD25 (IL-2R$\alpha$) expression, detected at 3 days on the LN CD8$^+$ T cell (Fig. 7B), suggested that IL-2 could be involved. We then assessed its implication using in vitro cultures. To restrain the possible cell subtypes coming into play and to avoid possible masking effects of other cell subtypes in the induction of this antigen-unspecific proliferation, we chose to use cultures of whole OT-I RAG1$^{-/-}$ LN and spleen cells, supplemented with LPS. In these cultures, the addition of anti-CD69 2.2 mAb induced proliferation and CD25 expression of CD8$^+$ T cells (Fig. 7C and D) but not when purified OT-I RAG1$^{-/-}$ CD8$^+$ T cells were cultured alone (data not shown). The proliferation was inhibited by the addition of anti-CD25 or anti-IL-2 blocking antibodies or both to about the same extent (Fig. 7C), but not by anti-IL-15 blocking mAb (Supplemental Fig. 3D). The addition of anti-IL-2 almost completely inhibited anti-CD69-mediated CD25 expression on OT-1 cells (Fig. 7D), suggesting that the anti-CD69 2.2-induced CD25 up-regulation is dependent on IL-2. We then tested the implication of IL-2 in vivo by blocking CD25 function. For this, we used an in vivo setting equivalent to the one used in vitro, transferring OT-I RAG1$^{-/-}$ cells into OT-I RAG1$^{-/-}$ recipients. In these transfers, we could observe that CD25 blockade by administration of anti-CD25 mAb to recipient mice partly inhibited the anti-CD69 2.2 mAb-induced proliferation (Fig. 7E), thus further suggesting the involvement of IL-2 in the

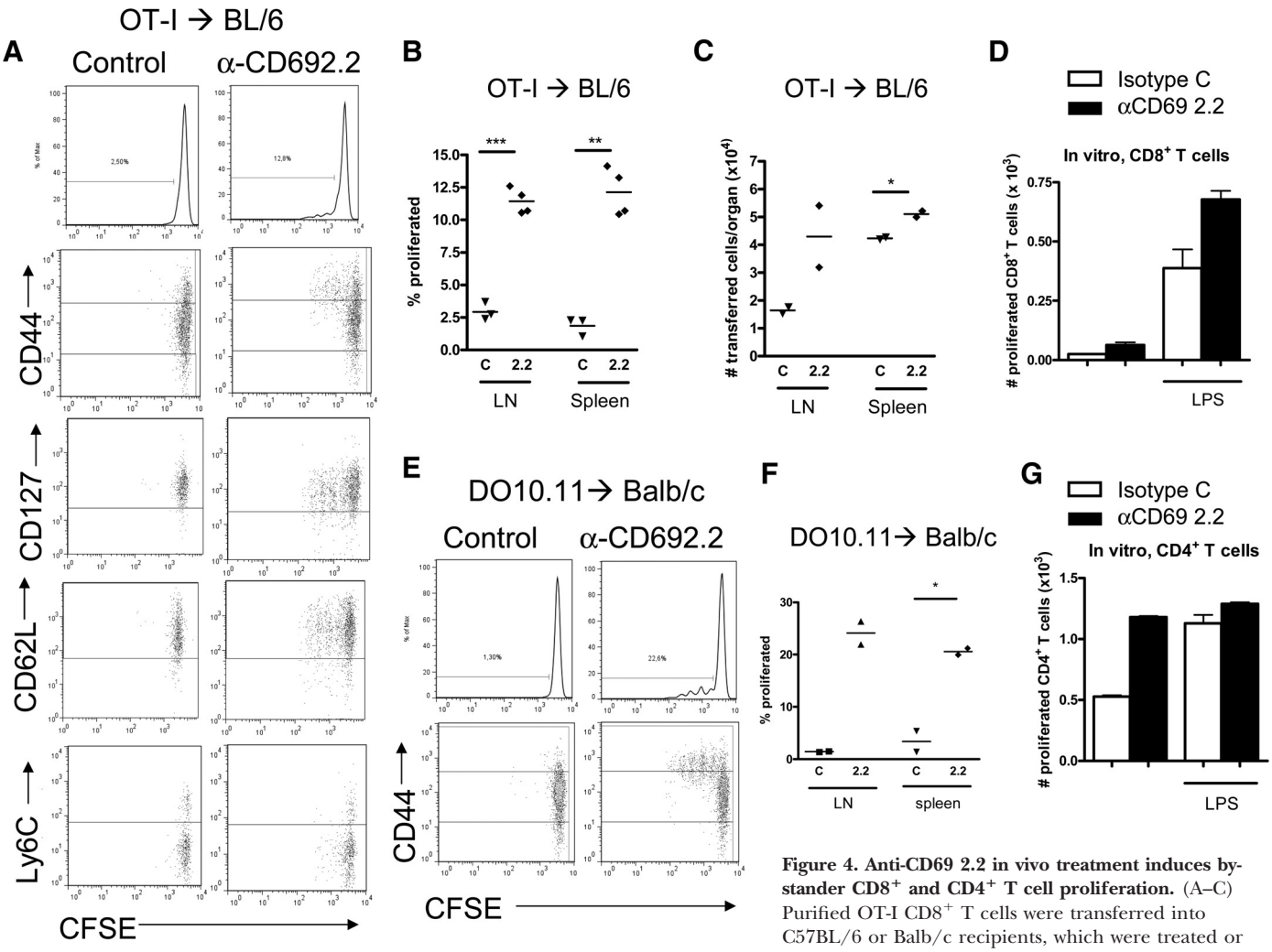


**Figure 4. Anti-CD69 2.2 in vivo treatment induces bystander CD8$^+$ and CD4$^+$ T cell proliferation.** (A–C) Purified OT-I CD8$^+$ T cells were transferred into C57BL/6 or Balb/c recipients, which were treated or not with 100 $\mu$g anti-CD69 2.2. After 3 days, pooled LN and spleen were analyzed. (A) Representative histograms and dot plots of untreated (left) or anti-CD69 2.2-treated mice (right) LN samples, gated on transferred T cells and showing CFSE dilution in relation to CD44, CD127, CD62L, and Ly6C expression levels. Graphs showing percentage of proliferated cells within transferred T cells (B) and numbers of transferred cells/organ (C). (D) Balb/c whole LNs and spleen cells were stained with 2 $\mu$M CFSE and cultured at $5 \times 10^6$ cells/ml in 24-well plates in media, supplemented or not with LPS and with anti-CD69 2.2 or isotype control for 4 days. Graph showing the number of proliferated cells gated on 7AAD$^-$ CD3$^+$ CD8$^+$ cells. (E and F) Purified DO11.10 CD4$^+$ T cells were transferred into Balb/c recipients, which were treated or not with 100 $\mu$g anti-CD69 2.2. After 3 days, pooled LN and spleen were analyzed. (D) Representative histograms and dot plots of untreated (left) or anti-CD69 2.2-treated mice (right) LN samples, gated on transferred T cells and showing CFSE dilution in relation to CD44 expression levels. (E) Graph showing the percentage of proliferated cells within transferred CD4$^+$ T cells. (G) As in D but gated on 7AAD$^-$ CD3$^+$ CD4$^+$ cells. Results are representative of two (A and C), three (E, D, and G), six (F), or at least 10 (B) similar experiments with similar results.

proliferative effect. We tested this involvement by analyzing the possibility that T cells were the actual IL-2-producing cells by means of in vitro cultures of whole LN and spleen cells. We did find increased IL-2 production in anti-CD69 2.2-treated cultures, but that was not until Day 4 of culture. At earlier time-points, IL-2 production was even slightly decreased. Instead, T cell numbers were noticeably increased as soon as Day 2. This observation suggested that increased IL-2 production by T cells is a consequence, rather than a mediator, of anti-CD69-induced proliferation. More importantly, the number of CD25$^+$ T cells drastically increased with anti-CD69 treatment from Day 1, reaching a tenfold difference by Day 4 (Fig. 7H), strongly suggesting that induced proliferation is a consequence of increased T cell sensitivity to available IL-2, as a result of induced CD25 expression. This explanation was further corroborated by in vitro cultures of OT-I CD8$^+$ T cells, incubated alone with different doses of IL-2, in which the presence of anti-CD69 enhanced T cell proliferation to suboptimal IL-2 doses (Fig. 7I), directly correlating with increased CD25 expression (Fig. 7J). Altogether, these results indicate that CD69 targeting-induced bystander T cell proliferation is mediated, at least in part, by IL-2 and CD25.

The participation of other mitogenic cytokines was also assesed in OT-I cultures, by adding increased doses of IL-15 and IL.7. As shown in Supplemental Fig. 3, total CD8$^+$ T cell numbers did not differentially increase upon anti-CD69 treatment

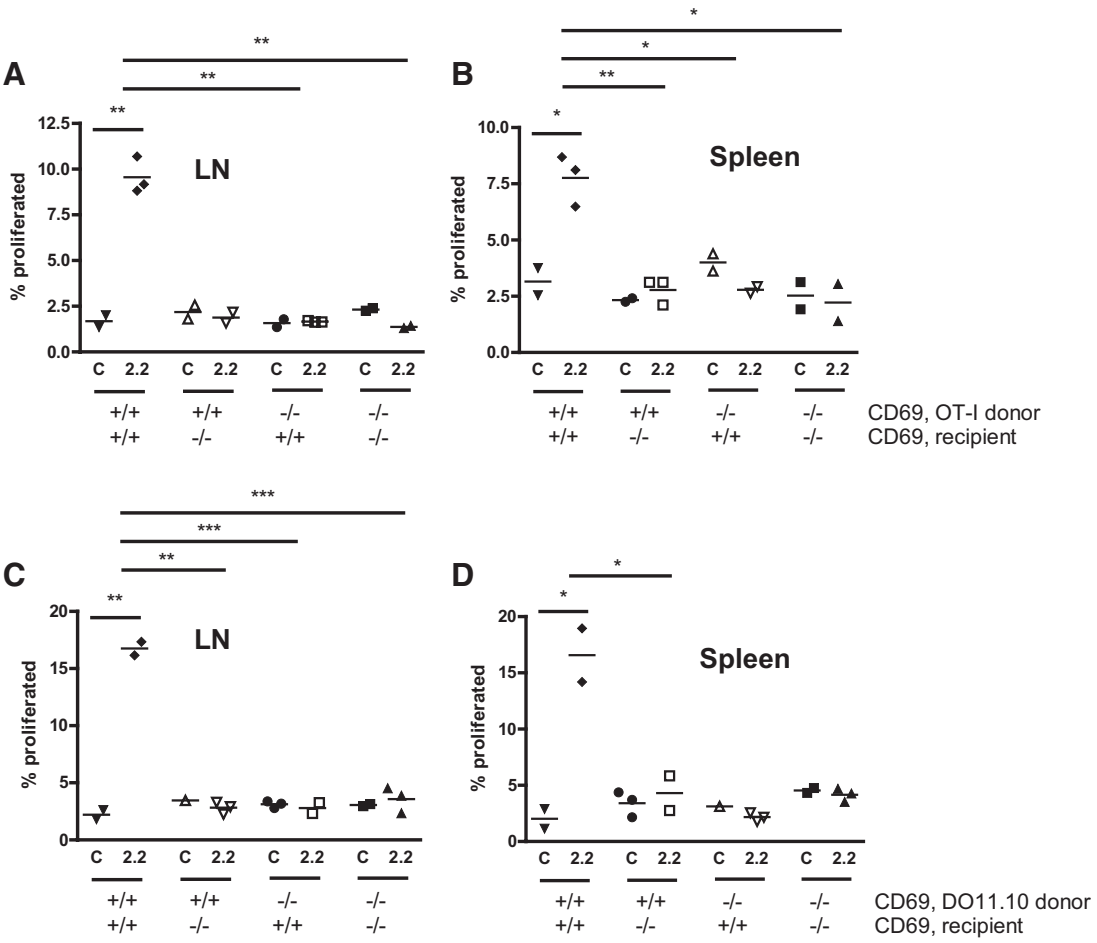


**Figure 5. Anti-CD69 2.2 induces bystander proliferation acting on recipient cells and transferred T cells.** $CD69^{+/+}$ or $CD69^{-/-}$ purified OT-I $CD8^+$ T cells (A and B) or DO11.10 $CD4^+$ T cells (C and D) were transferred into C57BL/6 or Balb/c recipients, respectively; also, $CD69^{+/+}$ or $CD69^{-/-}$, which were treated or not with 100 $\mu$g anti-CD69 2.2. After 3 days, pooled LNs (A and C) and spleens (B and D) were analyzed for the percentage of proliferated cells. Results are representative of two (A and B) and three (C and D) similar experiments with similar results.

at any dose of IL-15 or IL-7, as opposed to the increased numbers found in the presence of IL-2.

These data rule out IL-15 and IL-7 as mediators of anti-CD69 2.2-induced T cell proliferation and establish IL-2 as the active cytokine, mainly through the increased expression of CD25.

### Anti-CD69 2.2-induced T cell bystander proliferation is mediated by pDC

The effect of the antibody increasing the sensitivity to IL-2 could account for the need for CD69 targeting on transferred T cells for the induction of their proliferation, but it could not explain the need for CD69 expression by host cells. Apart from T cells, another cell subtype described to be able to produce IL-2 is the DC [42]. Considering the constitutive CD69 expression by LN and spleen pDCs, we envisaged the possibility that they could be targeted by the antibody and induced to produce IL-2. Indeed, we found increased IL-2 secretion in supernatants of purified LN and spleen pDCs cultured with anti-CD69 2.2 (**Fig. 8A**). Moreover, anti-CD69 2.2 addition to whole OT-I $RAG1^{-/-}$ cell cultures increased CD25 expression by pDCs (Fig. 8B and C), which could participate in IL-2 transpresentation. pDCs are known to be important producers of IFN-$\alpha\beta$, and this cytokine has been described to be one of the main mediators of bystander proliferation. Thus, we aimed to asses a possible effect on the IFN-$\alpha\beta$ production of pDCs, which could contribute to anti-CD69 2.2-induced T cell proliferation. However, anti-CD69 2.2 did not induce IFN-$\alpha\beta$ production by pDCs in vitro nor in vivo (Fig. 8D and E), and it did not increase the expression of Ly6C on $CD8^+$ T cells (Fig. 8F), (as detected by Gr1 staining, which stains for Ly6C and Ly6G, but only Ly6C is expressed on T cells [33]), which on these cells, is up-regulated specifically in response to IFN-$\alpha\beta$ [34].

The putative participation of pDCs in the induction of proliferation was tested in transfer experiments, in which recipient mice were depleted of pDCs with the anti-Bst2 mAb 120G8 [35, 36]. In OT-I- and DO11.10-transferred models treated with anti-CD69 2.2, pDC depletion decreased the induced T cell proliferation in LN and spleen, compared with nondepleted recipients (Fig. 8G and H). The percentage of pDCs ($SiglecH^+$ $CD11c^{int}$) was lowered by 45% in LNs and 60% in spleen, but numbers and percentages of cell types able to express certain Bst2 levels upon stimulation, such as $CD8^+$ and $CD4^+$ T cells and cDCs, were unaltered. Thus, the depletion appeared to be specific for pDCs and indicated that pDCs are implicated in the anti-CD69 2.2-induced bystander proliferation.

## DISCUSSION

There are substantial data about the CD69 role in T cell polarization in vivo [12, 13], but its role in T cell proliferation has

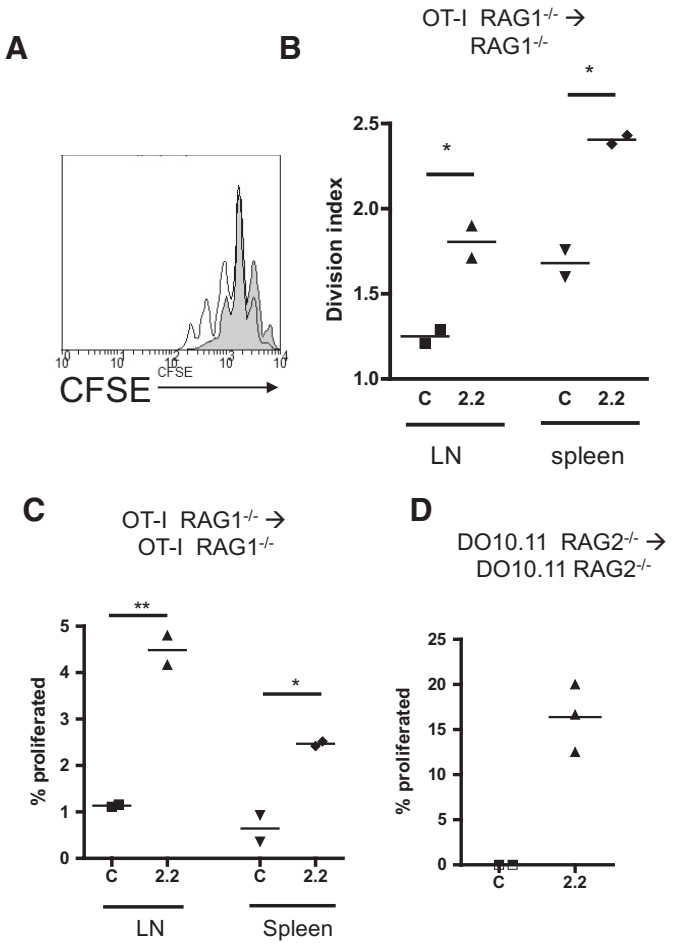


**Figure 6. CD4$^+$, CD8$^+$ T cells and NKT and T$\gamma\delta$ cells are not specifically required for the anti-CD69 2.2-induced proliferation.** (A and B) OT-I CD8$^+$ T cells were transferred into RAG1$^{-/-}$ mice, which were treated or not with 100 $\mu$g anti-CD69 2.2. After 3days, LNs were collected, pooled, and analyzed for the proliferation of transferred cells. (A) Overlay of two representative CFSE histograms of transferred OT-I T cells from untreated (gray-filled) and anti-CD69 2.2-treated (black line) mice. (B) Graph representing the division index of transferred OT-I T cells. The experiment is representative of two independent experiments with similar results. (C) OT-I RAG1$^{-/-}$ cells were transferred into OT-I RAG1$^{-/-}$ recipients, which were treated or not with 100 $\mu$g anti-CD69 2.2, and proliferation was assessed on Day 3 in pooled LN and spleen samples. Data is representative of three experiments. (D) DO10.11 RAG2$^{-/-}$ cells were transferred into DO10.11 RAG2$^{-/-}$, which were treated or not with 100 $\mu$g anti-CD69 2.2. Percentage of proliferated transferred cells in LN samples after 3 days.

not been demonstrated in vivo yet. In this work, we intended to analyze the effect of CD69 targeting on T cell proliferation. First, we did an extensive analyisis of the pattern of CD69 expression in the different LN and spleen immue cell populations at steady-state conditions and, remarkably, found that CD69 is expressed by all LNs and spleen pDCs, as well as all LN NK cells. Other immune cells expressed CD69 in various degrees and affecting different percentages of subpopulations, ranging from prominent expression by a large percentage of spleen NK cells and both spleen and LN NKT cells to minimal or no expression by B cells or macrophages. Expression in T cells was limited to a small percentage of cells but at a relative high level and was higher in memory phenotype T cells and regulatory T cell subsets.

Because of the relevant influence of treatment with anti-CD69 2.2 in different tumor, allergy, and autoimmunity animal models [9, 11, 12], we studied the effect of CD69 targeting in antigen-specific T cell proliferation in vivo using adoptive transfer experiments. In this setting, we did not find any influence of anti-CD69 administration in the antigen-specific T cells (OT-I CD8$^+$) localized in antigen draining lymphoid organs, independently of the administration route of the antigen, either s.c. injection of soluble antigen, s.c. cell-associated antigen, or antigen expression in specific organs. Instead, the treatment with anti-CD69 2.2 induced bystander T cell proliferation, mainly of CD44$^{hi}$ memory phenotype T cells, acting on donor T cells and on recipient cells. No specific subtype of T cells (CD4$^+$ T, CD8$^+$ T, NKT, or T$\gamma\delta$ cells) was found to be necessary for the antibody effect on the transferred T cells. In vitro, the anti-CD69 2.2 mAb induced proliferation, as well as CD25 expression, of CD8$^+$ T cells, and both were inhibited by anti-IL-2 or anti-CD25 blocking antibodies. Treatment with anti-CD25 blocking antibody also reduced anti-CD69 2.2-induced proliferation in vivo. The antibody increased T cell sensitivity to IL-2, but it did not increase early T cell IL-2 secretion. Instead, it induced early CD25 and IL-2 expression by pDCs. In vivo depletion of this cell subtype showed it as an important mediator of proliferation induction.

Anti-CD69 treatment greatly increased the percentage of proliferated T cells found in non-ADLN in the three antigen-delivering conditions tested. This remarkable effect could be explained by induction of egress of activated T cells from the local draining lymphoid organs or by induction of proliferation, independently of antigen. CD69 has been implicated in inhibition of T cell egress from the lymphoid organs through its interaction and down-modulation of the egress mediator S1P1 [15]. Thus, these proliferated T cells could be cells that had been activated and proliferated in the ADLN but that as a result of poor retention, had prematurely egressed from those LNs, returned to blood circulation, and entered other lymphoid organs. Testing this possibility by FTY720 treatment, an inhibitor of T cell egress acting downstream of CD69, we found that the percentage of proliferated T cells in non-ADLN was not decreased, indicating that this phenomenon was attributable to an effect on a process other than T cell egress. It may be possible that anti-CD692.2 was increasing the proliferative response to low, soluble antigen doses, which had reached distant lymphoid organs. However, when we injected lower antigen doses to mimick antigen limitation, anti-CD69 failed to increase the proliferation to lower, unsaturated antigen doses. Moreover, in the case of the RIP-mOVA model, OVA is expressed as a membrane-bound molecule, and it is not expected to diffuse to distant sites, but we still found anti-CD69 proliferation induction in distant LNs. Furthermore, the anti-CD69-induced proliferation levels observed in non-ADLN in antigen-injected mice (in the absence of further stimulating agents, such as LPS) were similar to the ones observed in LNs of OVA-uninjected mice (Fig. 2B and C vs. Fig. 4B), a result corroborated when these analyses were performed in parallel

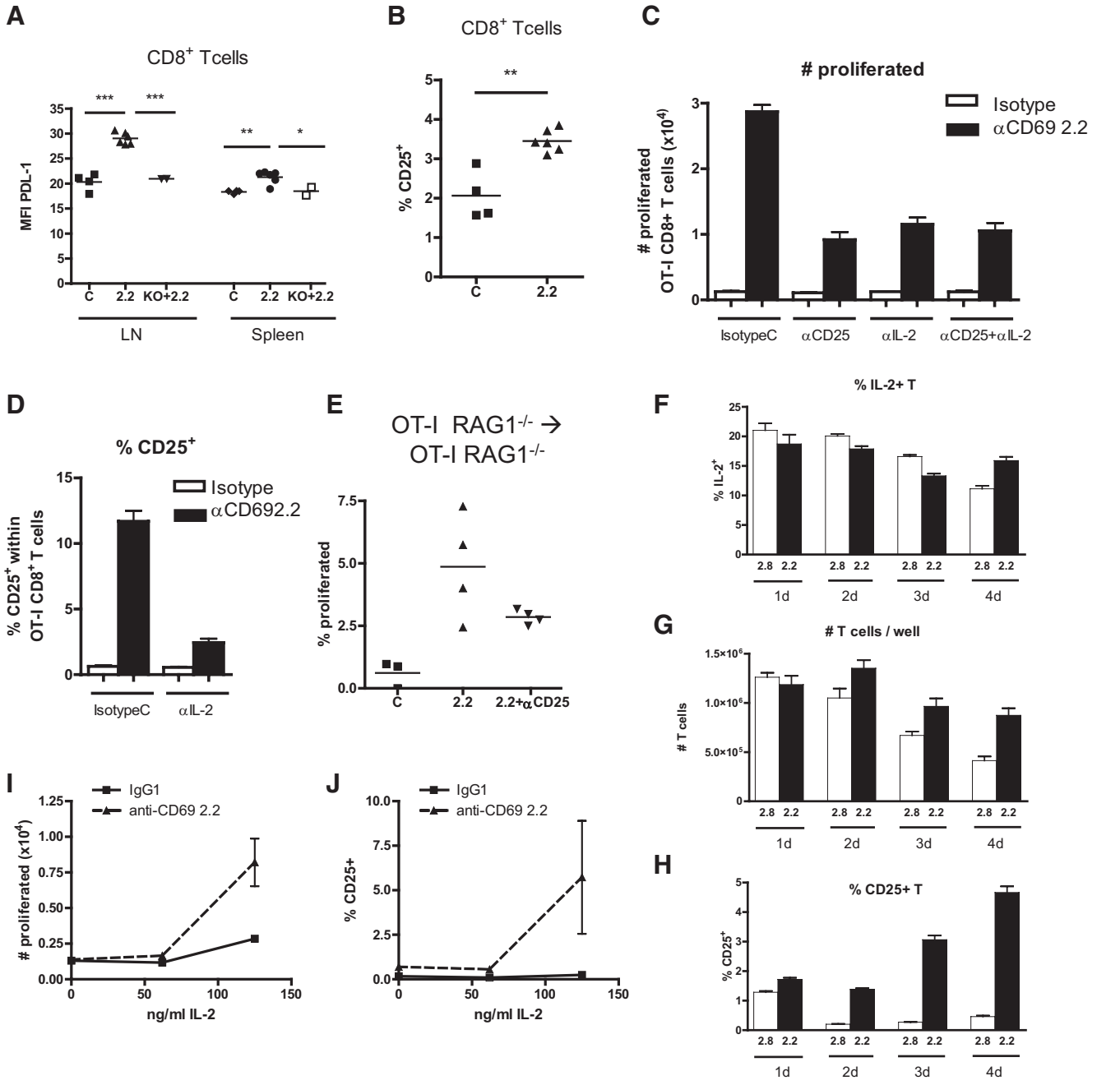


**Figure 7. IL-2 mediates anti-CD69 2.2-induced proliferation.** (A and B) CD69$^{+/+}$ or CD69$^{-/-}$ mice were treated or not with 100 $\mu$g anti-CD69 2.2. After 3days, LN and spleen CD8$^+$ T cells were analyzed for PDL-1. (A) Graph showing the MFI of PDL-1 expression on CD8$^+$ T cells. Results are representative of two independent experiments with similar results. KO, CD69 Knockout. (B) LN CD8$^+$ T cells from 3 days anti-CD69 2.2-treated or untreated mice were analyzed for the percentage of CD25$^+$ cells. Results are representative of four experiments with similar results. (C and D) Whole LNs and spleen cells from OT-I RAG1$^{-/-}$ mice were CFSE-labeled and cultured with 10 $\mu$g/ml anti-CD69 2.2 or IgG1 isotype control and 10 $\mu$g/ml anti-CD25 or 5 $\mu$g/ml anti-IL-2, as indicated, in complete media, supplemented with 1 $\mu$g/ml LPS. The number of proliferated cells (C) and the percentage of CD25$^+$ cells (D) were assessed after 3 days. Results are representative of three (C) and two (D) experiements with similar results. (E) OT-I RAG1$^{-/-}$ cells were transferred into OT-I RAG1$^{-/-}$ recipients, which had been injected or not with 300 $\mu$g anti-CD25 mAb on Days −1 and + 1 and were treated with 100 $\mu$g anti-CD69 2.2. Proliferation was assessed on Day 3 in pooled LNs. Pool of two independent experiments. (F–H) Whole LN and spleen OT-I RAG1$^{-/-}$ cells were cultured in LPS-supplemented media for the indicated times. After that, they were surface-stained for CD3 and stained further for intracytoplasmic IL-2 (F) or costained with CD25 (G and H) and analyzed. Graphs represent the percentage of IL-2$^+$ withinT cells (F), the number of live T cells/well (G), and the percentage of CD25$^+$ within T cells (H). (F–H) Pool of two experiments, from three to six wells/experiment. (I and J) Purified OT-I RAG1$^{-/-}$ CD8$^+$ T cells were CFSE-labeled and cultured with the indicated rmIL-2 doses and with 10 $\mu$g/ml anti-CD69 2.2 or IgG1 isotype control. The number of proliferated cells (D) and the percentage of CD25$^+$ cells (E) were assessed after 3 days. Bars represent SEM.

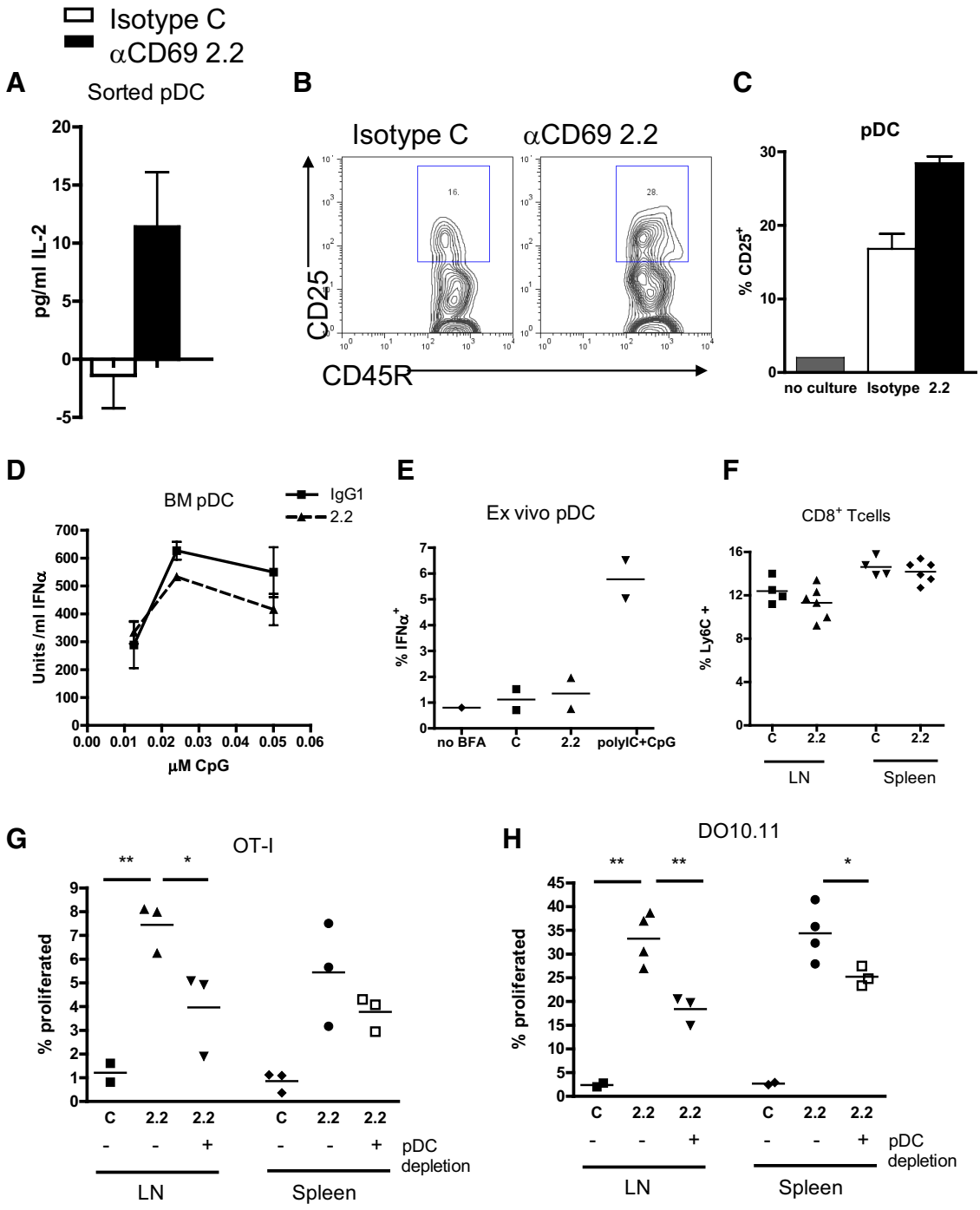


**Figure 8. pDCs express IL-2 and CD25 upon CD69 targeting and contribute to anti-CD69 2.2-induced proliferation, although not through IFN-α production.** (A) Balb/c pDCs were purified and cultured with 10 μg/ml anti-CD69 2.2 or IgG1 isotype control mAb in duplicates for 2 days. Supernatants were tested for IL-2 by ELISA. Pool of three independent experiments. (B and C) Whole LN and spleen OT-I RAG1$^{-/-}$ cells were cultured in LPS-supplemented media for 14 h in triplicate. Representative plots and graph showing percentage of CD25$^+$ cells gated on pDC (CD3$^-$ CD11c$^+$ B220$^+$). Representative of two experiments. (D) Flt3l BMDCs were sorted, and pDCs (CD11c$^+$ CD45RA$^+$ Bst2$^+$) were cultured at $10^5$ cells/well with the indicated concentrations of CpG and with 10 μg/ml anti-CD69 2.2 or IgG1 isotype control mAb in duplicate. After 24 h, supernatants were collected and tested for IFN-α by ELISA. Bars represent SD. Experiments are representative of two with similar results. (E) C57BL/6 mice were left untreated or were injected with 100 μg anti-CD69 2.2 or 13 μg CpG plus 200 μg polyinosinic:policytidylic acid (polyIC). After 5 h, spleen were harvested and spleen cells were incubated with BFA for 3 h and stained for intracytoplasmic IFN-α. Graph showing percentage of IFN-α$^+$ pDC (gated as CD19$^-$ CD3$^-$ CD11c$^{int}$ SiglecH$^+$). Experiments are representative of two with similar results. (F) C57BL/6 mice were treated or not with anti-CD69 2.2. After 3 days, pooled LN and spleen were harvested, and the percentage of Ly6C$^+$ within CD8$^+$ T cells was analyzed. Results are representative of three similar experiments. (G) OT-I cells were transferred into pDC-depleted recipients, receiving or not 100 μg anti-CD69 2.2. Proliferation was analyzed on Day 3 in pooled LN and spleen samples. (H) DO10.11 CD4$^+$ T cells were transferred into pDC-depleted Balb/c recipients, which were treated or not with 100 μg anti-CD69 2.2, and proliferation was analyzed 3 days after.

in the same experiment. Therefore, the proliferated cells in lymphoid organs distant from the antigen inoculation site in the anti-CD69 2.2-treated mice are consistent with an increased bystander T cell proliferation induced by this antibody.

The treatment with anti-CD69 2.2 induced bystander T cell proliferation, mainly of $CD44^{hi}$ memory phenotype T cells, acting on donor T cells and recipient cells. The observation that unlike CD69 targeting, CD69 deficiency does not lead to T cell bystander proliferation suggests that the antibody is not simply acting by blocking CD69. Instead, the antibody might be acting as a ligand mimick and induce signaling cascades from CD69. We envisage the possibility that in physiological conditions, a putative endogenous ligand of CD69 may be up-regulated in a situation of immune activation and may induce CD69-mediated bystander proliferation. This ligand may not be expressed at steady-state, as $CD69^{+/+}$ T cells do not proliferate any more than $CD69^{-/-}$ T cells do when transferred into antibody-untreated mice, suggesting that the $CD69^{+/+}$ T cells are not encountering any ligand.

Bystander proliferation is ultimately mediated by common γc receptor mitogenic cytokines. Concordantly, anti-CD69 2.2 treatment augmented PDL-1 expression on $CD8^+$ T cells, which has been documented to occur in response to γc receptor cytokines [32]. We found that anti-CD69 2.2 treatment induced the expression of CD25 on T cells, strongly suggesting that the mitogenic cytokine was IL-2. The implication of this cytokine was demonstrated by the fact that the induced proliferation is inhibited by anti-IL-2 or anti-CD25 blocking mAb in vitro and even by anti-CD25 mAb in vivo. The role of IL-2 is consistent with the observation that CD69 targeting did not induce bystander proliferation of BDC2.5 NOD $CD4^+$ T cells in non-ADLN of NOD recipients (data not shown), as the NOD strain has impaired IL-2 production [37]. The contribution of other mitogenic cytokines, IL-15 and IL-7, was ruled out, as the antibody did not affect the proliferation mediated by these cytokines in vitro, whereas it did enhance the proliferative response induced by IL-2, and as anti-IL-15 blocking mAb did not inhibit anti-CD69 2.2-induced T cell proliferation. Another mitogenic cytokine, IL-4, is not likely to be implicated, as unlike IL-4-induced proliferating $CD8^+$ T cells, the ones induced to proliferate by CD69 targeting are $Ly6C^-$ [26]. All of these results suggest that anti-CD69 treatment induces bystander T cell proliferation in $CD4^+$ and $CD8^+$ T subsets, at least in part by making them more susceptible to IL-2 by inducing the expression of a high-affinity IL-2Rα chain.

To try to unravel completely the mechanism behind this bystander induction, we demonstrated the need for CD69 expression by recipient cells and donor T cells, suggesting that CD69 targeting on recipient cells induces, directly or indirectly, the secretion of mitogenic cytokine(s), being or including IL-2, but that its secreted levels are unable to induce bystander proliferation of transferred T cells per se and that CD69 targeting on T cells renders them sensitive to otherwise submitogenic levels of that cytokine. Indeed, addition of anti-CD69 2.2 to in vitro cultures of $CD8^+$ T cells induced proliferation in the presence of submitogenic IL-2 doses. This effect likely occurs through the observed parallel CD69 targeting-induced up-regulation of the high-affinity cytokine receptor on T cells. However, the anti-CD69 2.2 mAb alone could not up-regulate CD25 expression on purified T cells, and in LPS-supplemented whole LN and spleen cell cultures, anti-CD69 2.2-up-regulated CD25 expression was inhibited by anti-IL-2. These results indicate that anti-CD69 up-regulation of CD25 expression requires the concomitant presence of IL-2. IL-2R signals through the Jak3/Stat5 signaling pathway [38–40]. As CD69 has also been shown to interact with Jak3/Stat5 [41], it is possible that CD69 targeting on T cells initiates signaling events, which potentiate the signals from the IL-2R, thus inducing the expression of CD25, which renders T cells more responsible to available IL-2.

These results are reminiscent of initial in vitro data showing that CD69 targeting had a costimulatory effect enhancing proliferation of PMA or anti-CD3-preactivated T cells through IL-2 and IL-2Rα up-regulation [2, 7]. The fact that the antibody treatment also induced proliferation in transfers of OTI $RAG1^{-/-}$ cells into OTI-$RAG1^{-/-}$ mice and DO10.11 $RAG2^{-/-}$ cells into DO10.11 $RAG2^{-/-}$ ruled out the specific need for $CD4^+$ T and $CD8^+$ T cells, respectively (apart from that of NKT, Tγδ, and B cells). We show that CD69 targeting enhanced IL-2 production by T cells in vitro but only minimally and at late time-points when the proliferation is already ongoing. This strongly suggests that they are not the host cells inducing IL-2-dependent proliferation. Instead, pDCs have been described previously to be able to produce IL-2 upon activation [42], suggesting that they may be the recipient cells, which would produce IL-2 to enhance the increased T cell responsiveness upon anti-CD69-induced CD25 expression. In fact, we show that isolated pDCs were induced to secrete IL-2 by the anti-CD69 2.2 mAb in vitro. Moreover, they also up-regulated CD25 expression, which has also been described to be up-regulated on these cells upon activation [43]. CD25 expression by DCs has been described recently to participate in IL-2 transpresentation from DCs to T cells [44]. Thus, it is possible that this higher CD25 surface expression by pDCs contributes to increased efficiency of IL-2 recognition by T cells. A proof of the participation of pDCs in the bystander proliferation is shown by depleting pDCs in the recipient mice, which inhibits the anti-CD69 effect in the OT-I and DO10.11 transfer systems to an extent directly correlated with the degree of achieved depletion. It is possible that the pDC role on observed proliferation may be mediated by IFN-αβ, as these cytokines have been described as important mediators of bystander proliferation, and they are produced mainly by pDCs. Notwhistanding, we found no effect of CD69 targeting in vivo on their IFN-αβ production, suggesting that its participation may be more dependent on the production of IL-2. We found that the anti-CD69 2.2-induced $CD8^+$ T cell proliferative response in vitro was enhanced by LPS, which is the ligand of TLR4. As mouse pDCs have been shown to express certain levels of TLR4 [45, 46], it is possible that the addition of LPS enhances anti-CD69 2.2-induced IL-2 production of pDCs, which would increase the $CD8^+$ T cell proliferative response. Instead, the anti-CD69 2.2-induced $CD4^+$ T cell proliferative response did not require LPS. This could be a result of a higher sensitivity of $CD4^+$ T cells to low amounts of IL-2 because of their higher CD25 ex-

pression, as shown in the much higher degree of proliferated cells in the $CD4^+$ T transfer model of DO10.11 compared with the $CD8^+$ T OT-I model.

Although homeostatic proliferation may be mediated by IL-2 [47], it is normally described to be driven by the increased availability of MHC self-ligands and IL-7. Notwhistanding, when we tested whether anti-CD69 2.2 targeting could also enhance IL-7 signals, we found that IL-7-induced T cell survival was unaltered by anti-CD69 2.2 mAb addition in vitro, suggesting that IL-7 is not mediating the observed proliferation.

In summary, this work shows that CD69 targeting induces bystander T cell proliferation through the IL-2 sensitization of T cells as a result of induction of CD25 expression and IL-2 production by pDCs. The anti-CD69 2.2 effect on homeostatic T cell proliferation has potential interest in clinical applications of T cell number restoration in lymphopenic conditions, such as after BM transplantation.

## AUTHORSHIP

E.A-P.: experiment performance, experimental design, study conception, and critical reading and writing; J.V-R.: experiment performance; J-G.Z.: experiment performance and provision of infrastructure; A.R.C.: experiment performance and critical reading and writing; S.J.T. and J.A.V.: experimental design, provision of infrastructure and material, and critical reading and writing; and P.L.: experimental design, provision of infrastructure and material, study conception, and writing.

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KEY WORDS:
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