Published in final edited form as:
J Autoimmun. 2014 December ; 55: 51–62. doi:10.1016/j.jaut.2014.05.007.

Maintenance of immune tolerance by Foxp3+ regulatory T cells requires CD69 expression

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Abstract

Although FoxP3$^+$ regulatory T cells are key players in the maintenance of immune tolerance and autoimmunity, the lack of specific markers constitute an obstacle to their use for immunotherapy protocols. In this study, we have investigated the role of the C-type lectin receptor CD69 in the suppressor function of Tregs and maintenance of immune tolerance towards harmless inhaled antigens. We identified a novel FoxP3$^+$CD69$^+$ Treg subset capable to maintain immune tolerance and protect to developing inflammation. Although CD69$^+$ and CD69$^-$FoxP3$^+$ Tregs exist in homeostasis, only CD69-expressing Tregs express high levels of CTLA-4, ICOS, CD38 and GITR suppression-associated markers, secrete high amounts of TGFβ αvδ have potent suppressor activity. This activity is regulated by STAT5 and ERK signaling pathways and is impaired by antibody-mediated down-regulation of CD69 expression. Moreover, immunotherapy with FoxP3$^+$CD69$^+$ Tregs restores the homeostasis in *Cd69*$^{-/-}$ mice, that fail to induce tolerance, and is also highly proficient in the prevention of inflammation. The identification of the FoxP3$^+$CD69$^+$ Treg subset paves the way toward the development of new therapeutic strategies to control immune homeostasis and autoimmunity.

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6. Author Contributions

J.R.C. and R.S-D performed most experiments and analyzed the data; O.B. contributed confocal microscopy images, analyzed the data and reviewed the manuscript; E.R.B., S.L. and A.M-M. performed experiments; M.L.T. designed experiments and revised the manuscript; F.S-M. designed experiments and revised the manuscript; and P.M. conceived and coordinated the study, designed experiments, oversaw the data analysis and wrote the paper.

7. Conflict of interest: The authors declare that they have no conflict of interest.

Europe PMC Funders Author Manuscripts

Keywords

Regulatory T cells; Immune tolerance; FoxP3; CD69

1. Introduction

FoxP3$^+$ regulatory T (Treg) cells are a subset of CD4$^+$ T lymphocytes with suppressive activity, key mediator of peripheral tolerance and essential for preventing autoimmune and chronic inflammatory diseases. However, the lack of specific markers and insufficient understanding of Tregs biology constitute the two largest obstacles to develop immunotherapy protocols for the treatment of these diseases [1]. Treg cells inhibit proliferation and function of effector T cells through various mechanisms, including cell-cell contact and the production of anti-inflammatory cytokines such as TGF-β or IL-10. Tregs can develop in the thymus, or from naïve CD4$^+$ T cells in the periphery [2]. After antigen challenge lymph nodes are the sites of differentiation of effector T cells and/or Tregs, which upregulate specific adhesion and chemokine receptors and migrate to the inflamed tissue. This regulatory mechanism of cell migration is critical for a proper balance between the innate and adaptive immune responses in the inflamed tissue [3]. In this regard, $S1P_1$ has been described as an important molecule controlling lymphocyte egress from lymphoid organs and leukocyte receptor CD69 negatively regulates its expression [4].

The early leukocyte activation antigen CD69 is a membrane receptor from the family of type II C-type lectins. CD69 is rapidly induced after cell activation in all bone marrow derived cells except erythrocytes [5, 6]. Expression of CD69 *in vivo* is restricted to positively selected thymocytes and leukocytes undergoing activation, particularly at inflammatory sites. Engagement of CD69 with monoclonal antibodies (mAbs) in the presence of phorbol esters induces a strong Ca^{2+} influx leading to the activation of ERK, induction of IL-2 and IFN-γ genes, and T cell proliferation [7, 8]. However, *in vivo* studies with CD69-deficient mice revealed an unexpected immunoregulatory role [9]. A mouse model of lymphocyte-dependent collagen-induced arthritis (CIA) suggested that CD69 might act as a regulatory molecule by modulating TGF-β levels at the site of inflammation [10]. Since TGF-β participates in the differentiation both of regulatory T cells and of Th17 cells [11, 12], CD69 might regulate the immune response at the stage of T cell differentiation. The balance between Th17 and Treg cells is critical for the regulation of the immune response by determining the net balance of pro- and anti-inflammatory cytokines at the inflammatory foci.

In this report we have analyzed the role of CD69 in the function of FoxP3$^+$ Tregs. We show that around half of the Tregs located in lymphoid organs express CD69 in steady state. CD69$^+$ Tregs express higher surface levels of suppression-associated markers than CD69$^-$ Tregs cells or Tregs from *Cd69*$^{-/-}$ mice, and have enhanced suppressor activity *in vitro* and in a mouse model of lung tolerance to harmless antigens. Our results strongly support a role for CD69 as a critical receptor for controlling Treg-suppressor function in both physiological and pathological immune responses, including autoimmunity and allergy.

2. Materials and Methods

2.1 Animals

CD69-deficient mice were generated as described [13]. C57BL/6 Tg(TcraTcrb)425Cbn/J mice expressing a T-cell receptor specific for peptide 323-339 of OVA in the context of I-Ab (OTII mice) were purchased from the Jackson Laboratory (stock number 004194). OTII mice were backcrossed with CD69-deficient mice in the C57BL/6 background (OTKO mice). For *in vivo* tolerogenic asthma experiments, we used females 10 to −12 weeks old that were either littermates or age-matched offspring in BALB/c or OTII background. Foxp3-RFP reported mice were kindly provided by Dr. R.A. Flavell (Yale University). Mice were kept in SPF conditions at the Animal Facility of CNIC. Experimental procedures were approved by the CNIC Committee for Research Ethics and conducted under Animal Welfare and Health Spanish and European guidelines.

4.2 T-cell isolation

Single-cell suspensions were obtained from spleen or mesenteric lymph nodes and incubated with the following biotinylated antibodies: CD8, CD19, B220, MHCII, CD11c, IgM, DX5 and CD11b, followed by streptavidin Microbeads. $CD4^+$ T cells were negatively selected with auto-MACS Pro (Miltenyi) separator. The negative fraction was incubated with anti-CD25 biotin to obtain Tregs.

2.3 Treg cell suppression assays

Tconvs were cultured with coated a-CD3 antibody (1 μg/mL) plus indicated amounts of soluble a-CD28 or irradiated T cell–depleted splenocytes. For antigen-specific assays, $CD4^+$ T cells from OTII and OTKO mice were cultured in the presence of irradiated APC and OVAp (10 mg/mL). T cell proliferation was measured by $[^3H]$-thymidine incorporation for the last 16h of culture or by CFSE or CellTraceViolet staining of $CD4^+$ Tconv cells.

2.4 Intracellular staining and FACS

Tregs or Tconv cells were treated with 1 μg/ml anti-CD3 (145-2C11) and anti-CD28 (37.51), and crosslinked with anti–Armenian Hamster IgG F(ab')2 (20 μg/mL). Activation was arrested by fixation with 4 % formaldehyde and permeabilized with 90 % methanol and cells were incubated with phospho-Akt, phospho-Erk and phospho-Stat5 from Cell Signaling.

2.5 *In vitro* differentiation of Tregs

OVA-specific TCR transgenic naïve T cells were obtained from OTII and OTKO mice and cultured for 72h with irradiated APCs in the presence of 10μg/ml OVAp, recombinant TGF-β1, and IL-2. *In vitro* differentiation of iTregs from C57BL/6 mice with normal TCR was performed from naïve CD4 T cells from CD69-proficient or deficient mice, co-cultured 120h with WT splenic dendritic cells in the presence of plate-bound CD3 and soluble CD28 plus recombinant TGF-β1 and IL-2.

2.6 Tolerogenic challenge with harmless antigen

To analyze the *in vivo* function of Treg, we used a previously described tolerogenic model[14]. Briefly, at day 0, mice were i.t injected with 800 μg LPS-free OVA (Calbiochem). At day 20, mice received 3 OVA aerosols (10 mg/mL in PBS, generated using a jet nebulizer) of 30 min on 3 consecutive days. In some experiments, 10 days later after the i.t administration of OVA, the mice were immunized with OVA-alum (10 μg OVA adsorbed to 2 mg aluminium hydroxide) and 10 day later, mice were challenged with three daily 30-min OVA aerosols as described before.

2.7 Adoptive transfer experiments

Mice were intraperitoneally injected with 10 μg OVA in 2 mg alum, and 5 days later $CD4^+$ T cells were isolated and expanded in vitro with TGF-β and IL-2 in presence of irradiated APC and OVAp. iTreg cells were intravenously injected (5×10^6 cells) into $Cd69^{+/+}$ or $Cd69^{-/-}$ recipient mice previously intratracheally sensitized with OVA. The recipients were exposed to inhaled OVA (10 mg/mL) for 3 days. For the adoptive transfer experiment with natural Tregs, $CD4^+CD25^+$-$CD69^+$ or $-CD69^-$ Tregs were isolated by FACS sorting from spleens and 2×10^5 cells were i.v. transferred to $Cd69^{+/+}$ or $Cd69^{-/-}$ recipients.

2.8 Whole-mount staining of mouse tracheas

Tracheas were incubated with PBS containing 0.5% Triton X-100, 5% goat serum and primary antibodies [rabbit polyclonal anti-mouse CD31 (Abcam) and rat clone FJK-16s anti-mouse Foxp3 coupled to FITC (eBiosciences)]. Samples were washed with 0.3% Triton X-100 in PBS and stained with goat anti-rabbit Rhodamine Red-X (Molecular Probes) Stained samples were fixed with PFA 4% and mounted with Prolong® Gold antifade reagent (Molecular Probes, Life Technologies). Confocal z-stacks from the inner side of tracheas were obtained using a LSM 700 Laser Scanning Microscope equipped with a LD LCI Plan/Apochromat 25x/0.8 Imm Korr DIC M27 (Zeiss). Images were analyzed with Imaris v.7.3.1 (Bitplane).

2.9 In Vivo FMT 1500 Tomographic Imaging and Analysis

Mice were injected intravenously with 4 nmol of Neutrophil Elastase 680 FAST (PerkinElmer, Inc.). OVA-challenged and control mice were imaged using the FMT 1500 fluorescence tomography *in vivo* imaging system (PerkinElmer, Inc.). The collected fluorescence data were reconstructed by FMT 2500 system software version 1.1 for three-dimensional fluorescence quantification. The total amount of lung fluorescence (in picomols) was calculated relative to internal standards.

2.10 RNA extraction and Micro Fluidic Card analysis

RNA was extracted using Absolutely RNA Nanoprep Kit (Agilent Technologies). 100-200 ng of total RNA was converted into cDNA and loaded in a TaqMan® Array Micro Fluidic Card (Applied Biosystems). Relative gene expression was calculated with qbase software (Biogazelle) using Actb, B2m, Gapdh and Hprt1 as reference targets genes. Heat map images were generated with Gene Cluster 3.0 and Java Treeview software.

2.11 Statistical analysis

P values were calculated with the Student t test, and values below 0.05 were considered significant. Means of the experimental groups were compared by using One-way ANOVA. To account for multiple comparisons, the Tukey or Bonferroni post-test were used to compare selected pairs of means and all pairs of means, respectively. All statistical analyses were carried out with GraphPad Prism v5.

3. Results

3.1 CD69 is constitutively expressed by a subset of thymic and peripheral Tregs in steady state

A subpopulation of about fifty percent of total $CD4^+CD25^+FoxP3^+$ Treg cells in thymus and secondary lymphoid organs express CD69 on their membrane in steady state (Fig. 1A). Further phenotypic analysis of peripheral $CD4^+$ $FoxP3^+$ gated $CD69^+$ and $CD69^-$ Tregs from *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice in homeostasis (Fig. 1B), revealed that the suppression-associated markers CTLA-4, ICOS, CD38 and GITR are expressed at higher levels in $CD69^+$-Tregs in steady state compared to $CD69^-$ Tregs or Tregs from *Cd69*$^{-/-}$ mice (Fig. 1C), suggesting that the phenotypic features of $CD69^+$ Tregs cells are consistent with an effector Treg phenotype. However, expression levels of other relevant proteins for Treg function, such as CD25, CD86 or CD27, are unaffected by CD69 expression (Supplementary Fig. S1). Interestingly, $CD4^+FoxP3^+$ Tregs homeostasis is not altered in peripheral lymphoid organs from *Cd69*$^{-/-}$ mice in comparison with *Cd69*$^{+/+}$ mice (Fig. 1D), indicating that, although Treg development seems not to be affected by the lack of CD69, their function could be dampened.

3.2 The suppressive function of Tregs is dependent on CD69 expression

We next assessed the role of CD69 in the suppressive function of Tregs. Naïve conventional T (Tconv) cells from *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice were co-cultured with $CD4^+CD25^+FoxP3^+$Tregs of *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice and irradiated APCs (iAPCs). Suppressor function of CD69-deficient Tregs was diminished compared with Tregs from *Cd69*$^{+/+}$ mice (Fig. 2A). This effect was confirmed in the presence of CD3/CD28 antibodies suppression assays devoid of APCs (Supplementary Fig. S2). Tregs from *Cd69*$^{-/-}$ mice bearing OTII T-cell receptors (TCR) specific for OVA peptide showed almost no ability to inhibit Tconv proliferation in antigen-specific suppression cultures (Fig. 2B).

To confirm those results, we sorted $CD69^+$ and $CD69^-$ Tregs from *Cd69*$^{+/+}$ mice and compared them with the total $CD4^+CD25^+$ Tregs from *Cd69*$^{-/-}$ mice (referred hereafter as *Cd69*$^{-/-}$Tregs) (see Supplementary Fig. S3A for sorting strategy). All Treg subpopulations have identical levels of $FoxP3^+$ (Supplementary Fig. S3B). $CD69^+$ Tregs showed the highest capacity to inhibit the proliferation of Tconvs, whereas $CD69^-$ and *Cd69*$^{-/-}$ Tregs showed a weaker suppressor function (Fig. 2C). The involvement of CD69 in Treg function was ascertained in *Cd69*$^{+/+}$ Tregs by addition of the anti-CD69 monoclonal antibody (*2.2*), which downregulates CD69 expression [15] and blocks the suppressive function (Fig. 2D). It is worth noting that CD69 expression in untreated $CD69^+$ Treg subpopulation was maintained at high levels *in vitro* after 3 days of co-culture with Tconv cells (Fig. 2, C-D),

indicating that sustained CD69 expression is critical for the suppressive function of Tregs. We also analyzed the ability of Tregs differentiated *in vitro* (iTreg) to induce effector T cells suppression; although Treg differentiation process is not compromised in OTII *Cd69*$^{-/-}$ cells (Fig. 2E) or in *Cd69*$^{-/-}$ cells with normal TCR (Fig. 2G), these cells were unable to properly induce T cell suppression compared to OT-II-*Cd69*$^{+/+}$ iTregs (Fig. 2F) or B6 *Cd69*$^{+/+}$ iTregs (Fig. 2H), respectively. These data indicate that CD69 expression is also required for iTreg suppressive function. To further support these results, we analyzed the expression of CD69 in Tregs from Foxp3-RFP reported mice. As in the WT B6 mice, CD69^{+} and CD69^{-} Tregs maintain the same proportions in lymphoid organs and more importantly, Foxp3-RFP^{+} CD69^{-} Tregs showed almost no ability to suppress Tconv proliferation (Fig. 2I).

3.3 Activation of CD69^{+} Tregs enhances STAT5 phosphorylation and decreases ERK phosphorylation

Activation of STAT5 has been reported to be required for Treg development and suppressive function [16]. To study the molecular mechanism of suppression by FoxP3^{+}CD69^{+} Tregs, we stimulated sorted Tregs and naïve T cells through the TCR and measured the levels of STAT5, ERK and AKT phosphorylation by FACS. Naïve T cells from *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice showed no significant difference in STAT5, ERK or AKT phosphorylation (Fig. 3A). However STAT5 phosphorylation in *Cd69*$^{-/-}$ Tregs was inhibited, whereas phospho-ERK was enhanced at late time points and AKT activation was unaltered (Fig. 3B). These results are consistent with our previous results in Th17 cells, where STAT5 activation is partially inhibited in the absence of CD69 [13]. Addition of IL-2 abolished the differences in STAT5 phosphorylation between *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ Tregs (Fig. 3C), thus indicating that this cytokine restores the activation of the pathway.

To further study the role of ERK activation in the suppressor function of Tregs, Tregs isolated from *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice were treated with the ERK inhibitor U0126. ERK inhibition in Tregs from *Cd69*$^{-/-}$ mice restored their suppressor activity, whereas it had no detectable effect in Tregs from *Cd69*$^{+/+}$ mice (Fig. 3D). Moreover, sorted CD69^{-} Tregs and *Cd69*$^{-/-}$ Tregs partially recovered their suppressor potential after pre-incubation with U0126 (Fig. 3E). To assess whether ERK inhibitors could also restore the suppressor potential of *Cd69*$^{-/-}$ Tregs *in vivo*, mice were given daily i.p. injection with the ERK inhibitor *ci 1040* [17]. *In vivo* inhibition of ERK phosphorylation in *Cd69*$^{-/-}$ mice (Supplementary Fig. S4) restored the suppressor capacity of Tregs isolated from *Cd69*$^{-/-}$ mice to the level detected for Tregs from *Cd69*$^{+/+}$ mice (Fig. 3F).

3.4 CD69^{+} Tregs display specific cytokine, migratory and Treg cell-associated gene expression

We analyzed the secretion of TGF-β1 and IL-2, cytokines involved in the regulation of T cell proliferation. Sorted Tregs were stimulated with anti-CD3/CD28 mAbs. *Cd69*$^{-/-}$ Tregs secreted significantly less TGF-β1 than *Cd69*$^{+/+}$ Tregs (Fig. 4A). Moreover, lower levels of soluble TGF-β1 were found in co-cultures of *Cd69*$^{-/-}$ Tregs and Tconvs (Fig. 4B). IL-2 secretion was low in cultures of *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ Tregs, consistent with the notion that lack of IL-2 secretion is a typical feature of mature and functional Tregs [18] (Fig. 4A).

However we observed increased IL-2 secretion in co-cultures of *Cd69*$^{-/-}$ Tregs and Tconvs (Fig. 4B), which could be a consequence of the higher proliferation rate of Tconvs in this condition (Fig. 2, A and B). Intracellular content of FoxP3 (Fig. 1B, and Supplementary Fig. S3B) as well as *Foxp3* mRNA levels (Fig. 4C) was the same in CD69^{+}, CD69^{-} and *Cd69*$^{-/-}$ Tregs or in iTregs from *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice (Fig. 4D). Thus the functional differences between CD69^{+} and CD69^{-} Tregs are unrelated to FoxP3 expression.

To identify differential gene expression patterns we conducted TaqMan immune function gene expression analysis on sorted CD69^{+}, and CD69^{-} Tregs (Fig. 4, E, F and G, and Supplementary Table S1). CD69^{+} Tregs showed higher expression of cytokine genes involved in T cell differentiation and function such as *Il2*, *Il10*, *Il4* and *Ifnγ* (Fig. 4E). Expression of these transcripts was barely detected in CD69^{-} Tregs. CD69^{+} Tregs express higher levels of *Cxcl10*, *Cxcl11* and *Ccl5* and chemokine receptors *Cxcr3* and *Ccr2*, involved in the migration of Tregs to inflamed tissue [19]. CD69^{-} Tregs express low levels of most of the receptors and chemokines analyzed (Fig. 4F). Analysis of surface receptor gene expression showed that CD69^{+} and CD69-Tregs express similar levels of the membrane antigens analyzed (Supplementary Fig. S5A). CD69^{+} Tregs expressed high levels of *Tbx21* (*T-bet*), which together with *FoxP3* is important for the suppression of immune responses *in vivo* [20] (Supplementary Fig. S5B). Interestingly, expression of *Icos* and *FasL*, which are involved in immune-suppression pathways, was enhanced in CD69^{+} Tregs (Fig. 4G). These data indicate that CD69^{+} Tregs and CD69^{-} Tregs exist as two independent populations with different pattern of gene expression.

3.5 Tregs from *Cd69*$^{-/-}$ mice have reduced capacity to maintain immune tolerance and suppress inflammation *in vivo*

We next investigated the tolerogenic potential of *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ Tregs in a model of sensitization to inhaled harmless antigens. In this model, primary tolerogenic challenge by intratracheal (i.t.) injection of OVA did not lead to sensitization or lung inflammation upon repeated challenge with aerosolized OVA in *CD69*$^{+/+}$ mice. However, analysis of bronchoalveolar lavage (BAL) fluids as previously described [14] showed increased neutrophil and macrophage infiltration in *Cd69*$^{-/-}$ mice (Fig. 5A). Detection of activated neutrophils by tomographic imaging after Neutrophil Elastase probe administration confirmed that only *Cd69*$^{-/-}$ mice developed inflammation under tolerogenic conditions, indicating that the absence of CD69 impairs the maintenance of tolerance in lungs (Fig. 5B). We found higher neutrophil infiltration in Me-LNs from OVA-sensitized *Cd69*$^{-/-}$ mice (Fig. 5C) and this was confirmed by tomographic imaging (Fig. 5D). Moreover, transgenic OTKO mice fail to establish lung tolerance, compared to OTII, after OVA exposure, similar to CD69$^{-/-}$ mice in BALB/c background (Fig. 5E). In a different experiment, after the i.t. tolerogenic challenge, mice were i.p. immunized with OVA, in order to study the induction of tolerance after immunization with the same antigen. After aerosol exposure to OVA mice developed airway inflammation, although the neutrophil infiltration was significantly higher in *Cd69*$^{-/-}$ mice than in *Cd69*$^{+/+}$ mice (Fig. 5F). Neutrophil infiltration in lungs and tracheas assessed by tomographic imaging was higher in OVA-treated *Cd69*$^{-/-}$ mice (Fig. 5G), indicating that CD69 expression is necessary for the maintenance of tolerance in a mouse model of airway inflammation.

The lack of CD69 expression on Tregs diminishes the potential to suppress inflammation in response to inhaled antigens (Fig. 5). To rule out an effect to differences in cell migration capacities of *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ Tregs after OVA challenge, we analyzed the content of CD4^{+}FoxP3^{+} cells in BALS fluid (Fig. 6A) and Me-LNs (Fig. 6B) finding no significant differences in Tregs cell numbers between the two genotypes. Analysis of tracheas by whole mount staining confocal microscopy detected higher numbers of perivascular infiltrating neutrophils in *Cd69*$^{-/-}$ mice (Fig. 6C); however, we did not observe any differences in total numbers of FoxP3^{+} cells migrating to this tissue, indicating that the migration of FoxP3^{+} cells is not affected by CD69 expression (Fig. 6D). Moreover, we analyzed the expression levels of S1P$_1$ receptor, a molecule involved in T-cell migration regulated by CD69 expression on the membrane [4]. We did not find any differences in S1P$_1$ receptor expression levels between *Cd69−/−* and *Cd69*$^{-/-}$ Treg cell subsets (Supplementary Fig. S6).

3.6 *Cd69*$^{-/-}$ Tregs show impaired function in cell therapy protocols

To demonstrate that CD69 expression on Tregs is required to restore lung homeostasis and suppress inflammation in response to inhaled antigens, we immunized *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice with OVA. MS-LNs were collected and cells were *ex-vivo* expanded with TGF-β1 and IL-2 in the presence of iAPCs pre-incubated with OVAp. The derived *ex-vivo* iTreg-enriched cell suspensions (5×10^6) were adoptively transferred into recipient *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice on day 19 of i.t. injection with OVA (see scheme in Fig. 7A). After the treatment with iTregs, all groups of mice were aerosolized with OVA three consecutive days 30 minutes each and i.v. injected with the neutrophil elastase probe 12 hours previous analysis. *In vivo* imaging analysis by quantitative tomography indicated that none of the recipient mice developed features of lung inflammation when challenged with PBS aerosol or transfer of Tregs derived from *Cd69*$^{+/+}$ donor mice. Interestingly, whereas an inflammatory response was detected in the lungs of *Cd69*$^{-/-}$ recipient mice treated with *Cd69*$^{-/-}$ donor Tregs, inflammation was completely abolished in *Cd69*$^{-/-}$ mice receiving immunotherapy with *Cd69*$^{+/+}$ Tregs (Fig. 7B and C). In parallel, a different group of mice was adoptively transferred with 2×10^5 freshly isolated CD25^{+} natural Tregs cells from WT or CD69$^{-/-}$ mice, obtained from spleens by FACS sorting. Percentage of neutrophils in BALS was analyzed 24 hours after the last exposure to PBS or OVA. Confirming the previous adoptive transfer experiment, the tolerance for OVA was restored in CD69$^{-/-}$ mice treated with CD69 expressing natural Tregs (Fig. 7D). To rule out a role in Treg migration, we analyzed CD4^{+} Foxp3^{+} Treg cells in mediastinal lymph nodes and BALS after adoptive transfer experiment using nTregs. Although we find an increase in Treg numbers from BALS after OVA exposure, we do not detect any significant differences between WT and CD69$^{-/-}$ mice transferred with CD69^{+} or CD69^{-} nTreg cells (Supplementary Fig. S7), thus confirming our previous results pointing to a dysfunction in CD69$^{-/-}$ Tregs rather than a defect in their migration.

4. Discussion

Here we identify a unique CD69-constitutive expressing subset of Treg cells in homeostatic and inflammatory conditions with higher suppressor potential, than the classical FoxP3^{+}

Treg cell population as a whole. Consistent with previous evidence that a subset of thymocytes with high expression of CD69 is the precursor of human natural Tregs [21], the peripheral subset of $CD69^+$ Treg differs from $CD69^-$ Tregs in the basal expression levels of naturally occurring Treg markers, such as CD25, CTLA-4, ICOS, CD38 or GITR [22]. More importantly, $FoxP3^+CD69^+$-Tregs are the more effective subset suppressing Tconvs; and downregulation of the CD69 receptor after antibody treatment inhibited their suppressor potential, indicating that functional suppressor Tregs constitutively express CD69.

Our data support the notion that the $CD69^+$ Treg cell subset is the functional suppressive population within the classical $CD4^+CD25^+FoxP3^+$ Tregs. However, different subsets of inducible $CD69^+$ Tregs have also been reported in mice, $CD4^+CD25^-FoxP3^-$ Treg precursors, express CD69 on their membrane upon activation with oral OVA [23], 2,4,6-trinitro-1-chlorobenzene [24] or after migration within lymphoid organs [25] and tumor induction [26], and exert their function mainly through membrane-bound LAP/TGF-β1. Similarly, recent studies in humans have found the $CD69^+CD4^+FoxP3^-LAP^+$ Treg subset in peripheral blood [27], $CD69^+CD71^+$ Tregs after allospecific activation *in vitro* [28], and the diminished suppressive function of Tregs from systemic sclerosis patients, correlating with lower CD69 expression [29].

FoxP3 has been postulated as a master lineage specification factor for Tregs; however, the data are contradictory, with some groups postulating that $FoxP3^+$ T cells are a suppressive population [30] and others describing an incomplete suppressive function in this population [31-33]. Our data reveal that, within the $FoxP3^+$ cells, the $CD69^+$ subset is the most potent with suppressive capacity *in vitro* and *in vivo*. Recent data indicate that CD69 associates with the Jak3/STAT5 pathway, triggering STAT5 phosphorylation [9, 13], which can directly bind the Foxp3 promoter, thus playing a critical role in Treg development [16]. STAT5 phosphorylation, which is critical for Treg thymic differentiation, is diminished in *Cd69*$^{-/-}$ Tregs and lower strength of TCR or costimulatory signals is required to maintain FoxP3 expression on Tregs, through the inhibition of the AKT/mTOR pathway in a TGF-β independent manner [34, 35]. Moreover, inhibition of the ERK MAPK pathway increases the frequency of $FoxP3^+$ Tregs in a mechanism dependent on TGF-β signaling [36]. Here we show that, although the AKT pathway and FoxP3 expression at mRNA or protein levels are not altered in the absence of CD69 expression in Tregs, the ERK MAPK pathway remains phosphorylated only in $CD69^{-/-}$ Treg cells after long term TCR signaling. Since ERK is not active in $CD69^{+/+}$ Tregs, the pre-treatment of Tregs during 2h with ERK inhibitor may be effective only in $CD69^{-/-}$ Tregs with activated ERK, having no effect on $CD69^{+/+}$ Tregs with down-regulated ERK signaling. Therefore, inhibition of the ERK1/2 pathway in *Cd69*$^{-/-}$ Tregs restores their suppressive function *in vitro* and *in vivo* [37]. Interestingly, the secretion of TGF-β by CD69-deficient Tregs after TCR stimulation is significantly inhibited, indicating that CD69 exerts its immuno-regulatory role through the control of the ERK MAPK pathway in a mechanism dependent on TGF-β.

The regulation of the immune response to aeroantigens is critical for maintaining immune tolerance in the lungs and preventing airway inflammatory disease [38]. In a previous work we demonstrated that CD69 expression in effector T cells negatively regulates Th2 and Th17 cell-driven inflammatory responses associated to allergic asthma and contact

dermatitis [39]. However, the role of CD69 in the function of regulatory T cells and the maintenance of lung tolerance has not been before explored. Treg subset is central to the maintenance of mucosal tolerance, and defects in the development or function of these cells lead to exacerbated airway inflammation typical of allergies and asthma [14, 40, 41]. Our studies with a model of lung tolerance show that *Cd69*$^{-/-}$ Treg cells exhibited a diminished potential to suppress OVA-specific inflammatory responses, indicating that Treg function or migration to the lungs was impaired. Adoptive transfer experiments unequivocally demonstrate that only the FoxP3^{+}CD69^{+} Treg subset is able to maintain self tolerance in *Cd69*$^{-/-}$ mice. However, *in vivo* analysis of Treg migration to trachea, alveoli and mediastinal lymph nodes indicates an identical pattern of migration by CD69$^{+/+}$ and *Cd69*$^{-/-}$ Tregs. The rate of T cell migration to the inflamed tissue is also regulated by the S1P/S1P$_1$ pathway, and it has been demonstrated a role of CD69 in the negative regulation of S1P$_1$ expression levels in T cells [4, 42] and Dendritic cells [43]. In contrast to our previous observations in *CD69*$^{-/-}$ Th17 cells [39], we have detected any significant difference in S1P$_1$ expression between *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ Tregs, suggesting that those are not altered by differential expression of S1P$_1$. Moreover, S1P$_1$ is an inhibitor of Treg cell function through AKT-mTOR pathway [44] and we have not found any differences in this signaling pathway by *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ Tregs, indicating that CD69 regulation of Tregs function or migration is independent on their expression of S1P$_1$.

4.1 Conclusions

Several mechanisms of suppression by Tregs have been proposed; however, Tregs need to express CD69 to sustain immune tolerance. Our results thus show that CD69 is a key molecular target for controlling Treg-suppressive function and development of inflammation. The identification of this new subset of CD69^{+}Tregs paves the way toward the development of new strategies of Treg cell isolation for therapeutic purposes towards chronic inflammatory and autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

5. Acknowledgments

The authors thank Dr. R.A. Flavell for kindly provided the Foxp3-RFP reporter mice, S. Bartlett for editorial assistance, R. Strippoli and M.A. del Pozo for kindly providing the Ci1040 ERK inhibitor and M^{a} Carmen Gómez de Frutos for technical help. This work was supported by funding from the Spanish Ministry of Economy and Competitiveness: SAF2011-27330 to P.M., SAF2010-15106 to M.L.T and SAF2011-25834 to F.S-M.; grant INDISNET 01592006 from Comunidad de Madrid and RETICS *Enfermedades Cardiovasculares* from Instituto de Salud Carlos III to P.M and F. S-M; and ERC-2011-AdG294340-GENTRIS to F.S-M. J.R.C. was supported by a CNIC post-doctoral fellowship, R. S-D is funded with a pre-doctoral fellowship from Comunidad de Madrid and E.R.B. and A.M-M. were supported by a FPI pre-doctoral fellowship from the Spanish Ministry of Economy and Competitiveness. The CNIC is supported by the Spanish Ministry of Economy and Competitiveness and the Pro CNIC Foundation.

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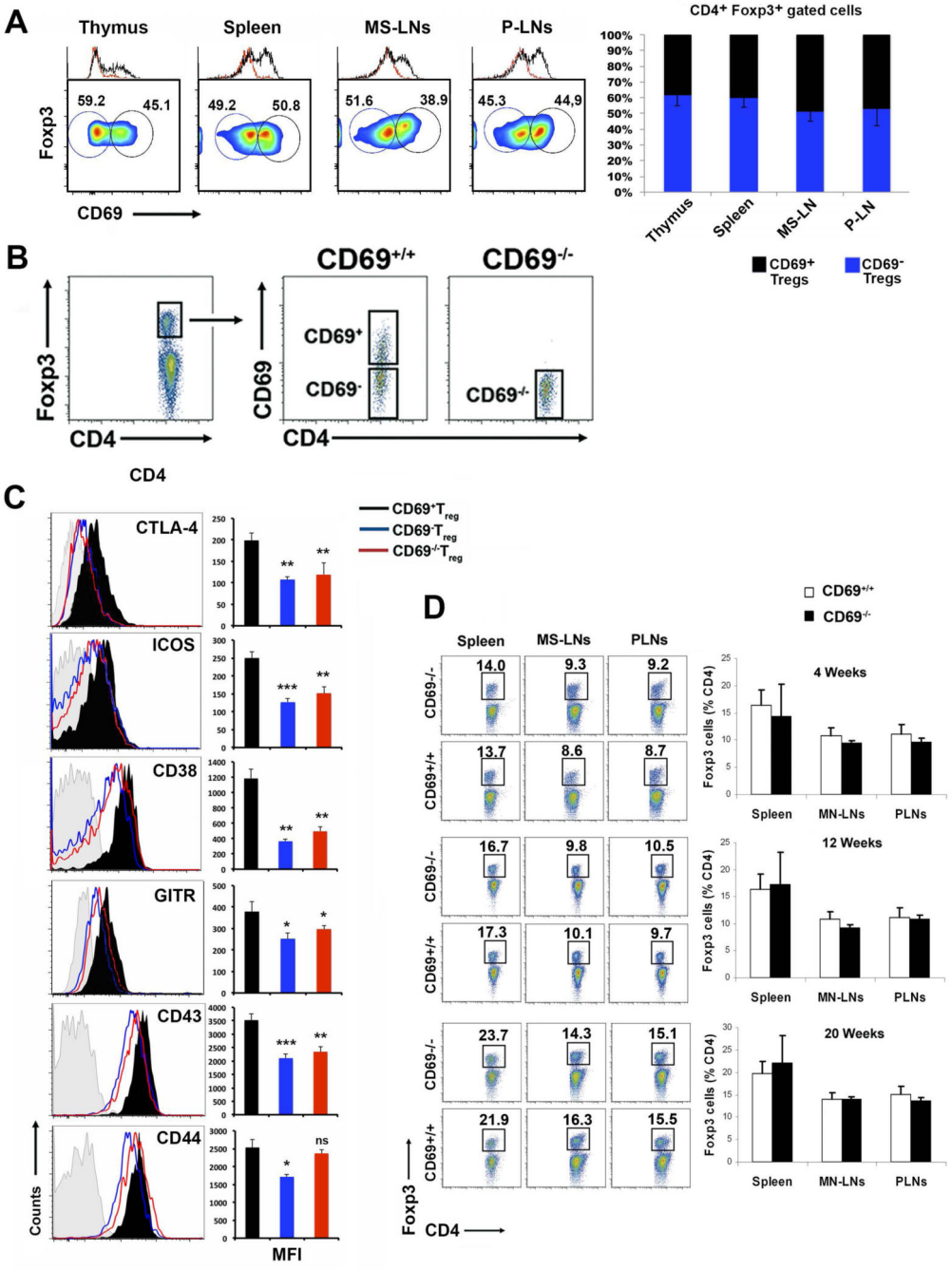

Figure 1. CD69 is constitutively expressed by a subset of peripheral Tregs in steady state

(**A**) Left, density plots show CD69 expression in gated $CD4^+FoxP3^+$ cells in thymus, spleen, MSLNs and PLNs from *Cd69*$^{+/+}$ mice. Histograms represent CD69 expression on $CD4^+FoxP3^+$ cells in *Cd69*$^{+/+}$ (black line) and *Cd69*$^{-/-}$ (red line) mice. Right, the bar chart shows the percentage of $CD69^+$ and $CD69^-$ Tregs within the indicated organs from *Cd69*$^{+/+}$ mice ± S.D. (**B**) FACS analysis of $CD4^+FoxP3^+CD69^+$ or $-CD69^-$ gated spleen cells from *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ mice. (**C**) Cell surface expression of Treg-related markers in $CD69^+$ and $CD69^-$ Tregs gated as in (B). Gray-shaded areas represent antibody isotype control and

bar charts show mean fluorescence intensity (MFI) from $CD69^+$ (solid black), $CD69^-$ (blue) or *Cd69*$^{-/-}$ (red) Tregs. (**D**) *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$mice have comparable proportions of $CD4^+CD25^+FoxP3^+$ Treg cells in secondary lymphoid organs. Left panels, flow cytometry analysis of $CD4^+FoxP3^+$ cells isolated from spleen, mesenteric (MS-LNs) and peripheral (P-LNs) lymph nodes of *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ mice of 4, 12 and 20 weeks of age. Right panels, percentage of $FoxP3^+$ on gated $CD4^+$ T cells. Data are representative of three independents experiments (n = 3 each). Errors bars represent mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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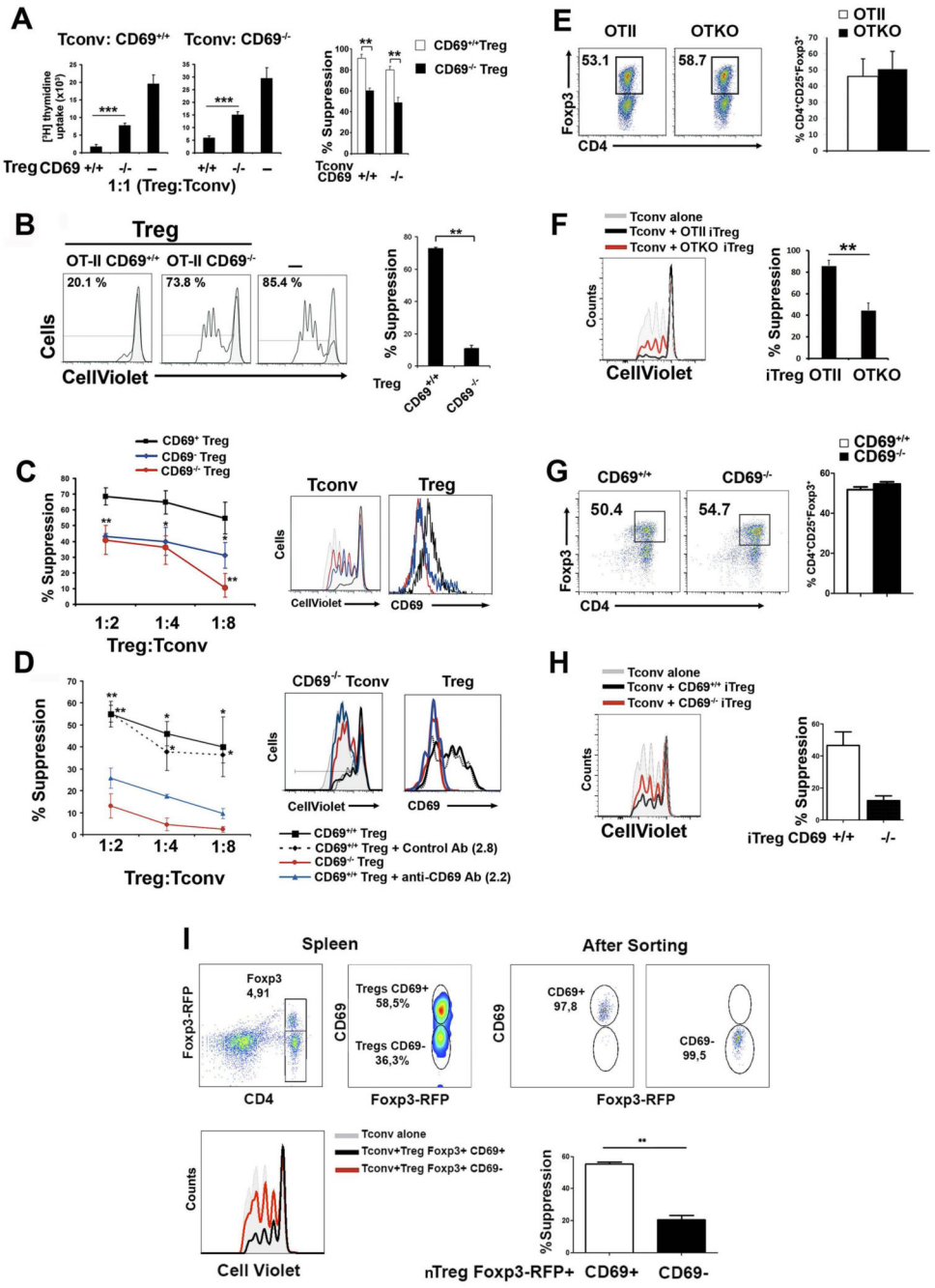

Figure 2. Impaired suppressive function of CD69^{-} and *Cd69*$^{-/-}$ Tregs *in vitro*

(**A**) *In vitro* Treg polyclonal suppression assay of CD4^{+}FoxP3^{+} Treg cells from *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ mice and Tconv cells from *Cd69*$^{+/+}$ (left) and *Cd69*$^{-/-}$ (right) mice with coated anti-CD3 Ab in the presence of iAPCs; left panels show [^{3}H]thymidine uptake, and right panels show percentage of suppression relative to maximum Tconv proliferation in culture without Tregs. (**B**) FACS analysis of OT-II CD69$^{+/+}$ or OT-II CD69$^{-/-}$ Tregs suppression function under antigen-specific conditions (iAPCs and OVAp). CellViolet show proliferation of Tconvs and bar charts percentage of suppression. (**C**) Sorted CD69^{+} and

CD69^{-} Tregs from *Cd69*$^{+/+}$ mice and *Cd69*$^{-/-}$Tregs were tested in suppression assays with coated anti-CD3 and iAPCs. *Left*, Percentage suppression of Tconvs. *Center*, CellViolet shows Tconvs proliferation. *Right*, CD69 expression in Tregs co-cultures. (**D**) *In vitro* suppression function assay of CD69^{+} or CD69^{-} Tregs under polyclonal stimulus with or without anti-CD69 *2.2* or isotype control *2.8* mAbs. *Left*, percentage of suppression. *Center*, Tconv proliferation. *Right*, CD69 expression in gated Tregs. (**E**) FACS analysis of *in vitro* iTreg differentiation cultures of CD4^{+} naïve TCs from OT-II *Cd69*$^{+/+}$ or OT-II *Cd69*$^{-/-}$ mice. Bars show the percentage of CD4^{+}Foxp3^{+} iTregs obtained. (**F**) iTreg suppression assay of Tconvs cultured with OT-II *Cd69*$^{+/+}$ or OT-II *Cd69*$^{-/-}$ iTregs. (**G**) iTreg differentiation cultures with naive CD4^{+} T cells from B6 WT and CD69KO with normal TCR repertoire and cultured together with DCs, anti-CD3/CD28, TGF-β and IL-2 and (**H**) iTreg suppression assay of Tconvs cultured with iTregs WT and CD69$^{-/-}$ from B6 mice. (**I**) FACS analysis of splenic CD4^{+}Foxp3^{+} −CD69^{+} and −CD69^{-} natural Tregs from Foxp3-RFP reporter mice (*up left*). CD4^{+}Foxp3^{+} −CD69^{+} and −CD69^{-} nTregs were sorted (*up right*) and suppression assays of Tconvs were performed. CellViolet show proliferation of Tconvs and bar charts percentage of suppression (*lower panels*). Data are representative of three (A-C) or nine (D) independent experiments (n = 3). Errors bars represent mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001

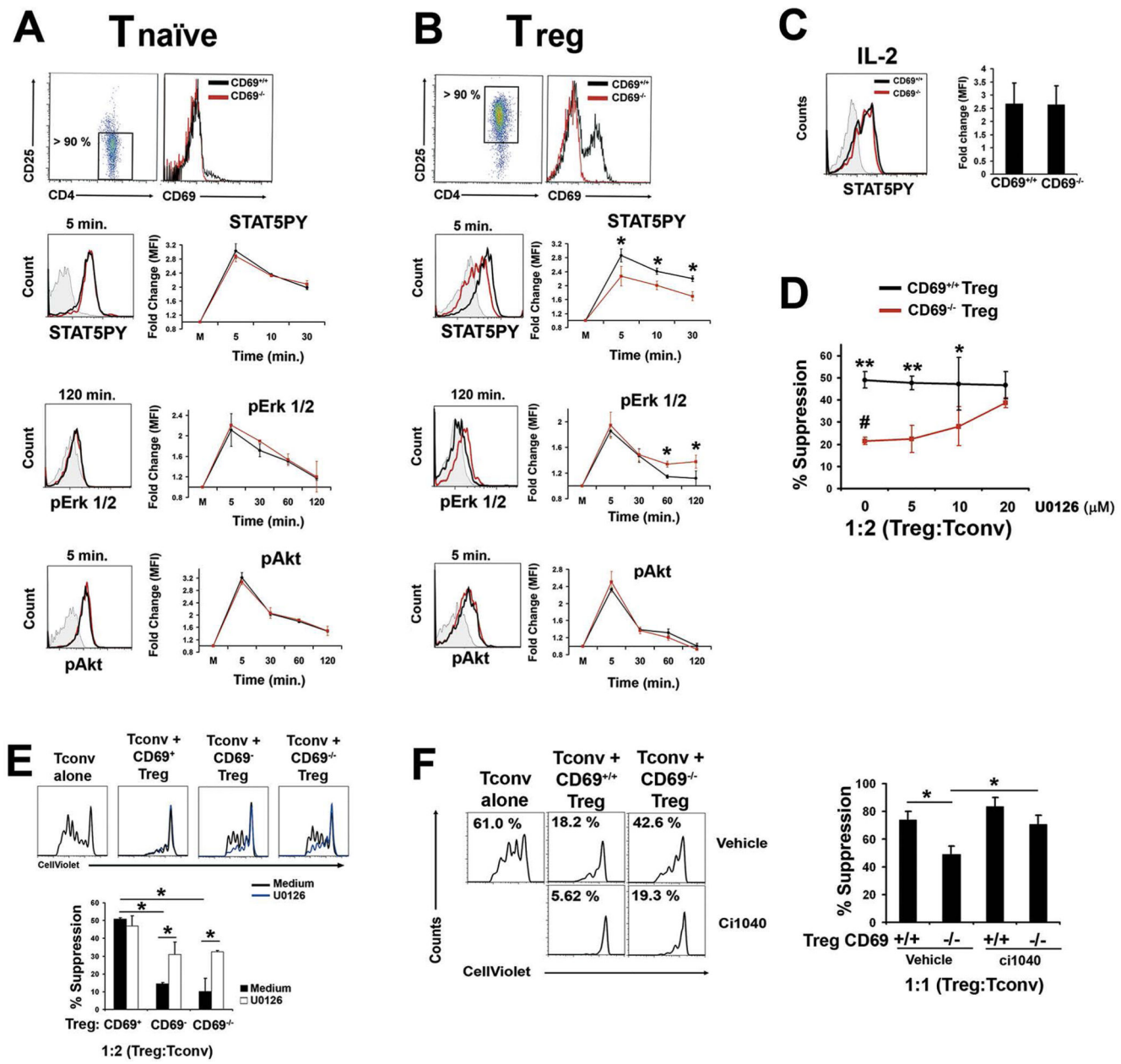

Figure 3. STAT5 and Erk 1/2 activation controls the suppressor potential of Tregs

FACS analysis shows purity and CD69 expression of naïve T cells (**A**) and Tregs (**B**) from *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ mice. Kinetics of STAT5, Erk and Akt phosphorylation after TCR stimulation; grey histograms represent non-stimulated cells and phosphorylation was measured as MFI fold change. Data are representative of three independent experiments. (**C**) STAT5 phosphorylation in Tregs stimulated 30min with IL-2. (**D**) *In vitro* suppression assays after pre-treatment of Tregs with the indicated concentrations of Erk inhibitor U0126 for 2h. * $P < 0.5$, ** $P < 0.01$ (comparison of *Cd69*$^{+/+}$ versus *Cd69*$^{-/-}$ Treg), $^{\#}$ $P < 0.05$ (untreated CD69$^{-/-}$ Tregs versus 20μM U0126 pre-treated CD69$^{-/-}$ Tregs). CellViolet indicate Tconv proliferation. (**E**) *In vitro* pre-treatment of Tregs with 20 μM U0126 partially

restores suppressor ability of sorted CD69^{-} Tregs and *Cd69*$^{-/-}$ Tregs. *Upper histograms*, cell violet analysis of Tconv proliferation. *Lower*, percentage of suppression. (**F**) *In vivo* pre-treatment daily with 100 mg/Kg of Erk inhibitor *ci1040* (100mg/kg) or vehicle. Suppressor ability of derived Tregs was tested *in vitro*. *Left*, percentage of suppression. *Right*, Tconv proliferation in co-culture. Data are representative of at least 3 independent experiments. Errors bars represent mean ± SD. * $P < 0.05$, ** $P < 0.01$.

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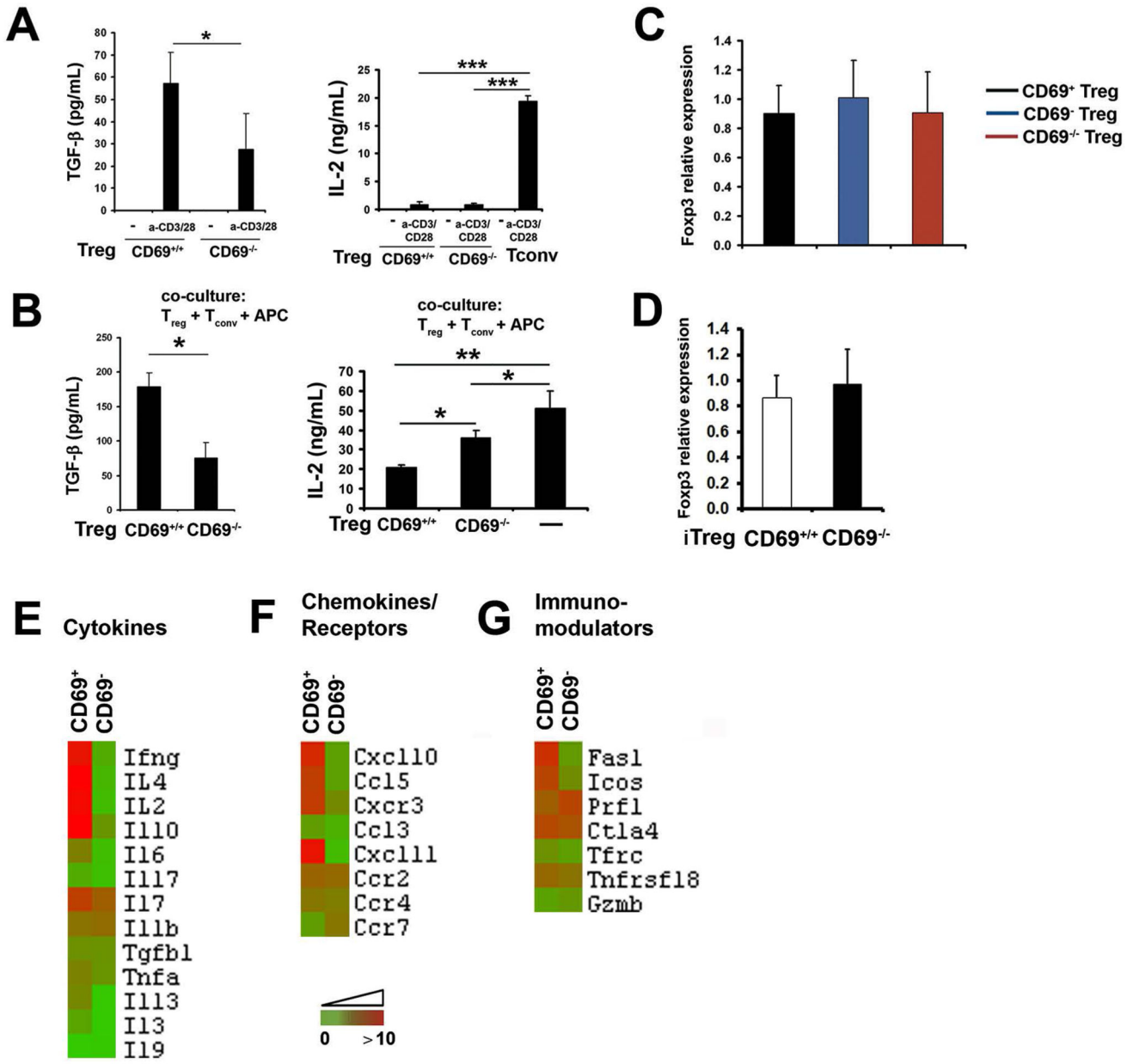

Figure 4. CD69^{+} Tregs secrete high amounts of TGF-β and express high levels of immune-suppression related genes

TGF-β and IL-2 secretion by Tregs after TCR stimulation with anti-CD3/CD28 for 48 h (**A**) or after co-culture with Tconvs and iAPCs (**B**) measured by ELISA. Results are representative of three independent experiments. (**C-D**) Relative *Foxp3* mRNA expression in *ex-vivo* sorted CD69^{+} and CD69^{-} Tregs from *Cd69*$^{+/+}$ mice, and *Cd69*$^{-}$ Tregs from *Cd69*$^{-/-}$ mice (**C**) or *in vitro* differentiated iTregs obtained from OT-II *Cd69*$^{+/+}$ or OT-II *Cd69*$^{-/-}$mice (**D**). (**E-G**) qPCR analysis of immune-related genes in *ex-vivo* sorted CD69^{+} and CD69-Tregs. Heat map represent fold increase to internal standards Data are means from three biological samples. Errors bars represent mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

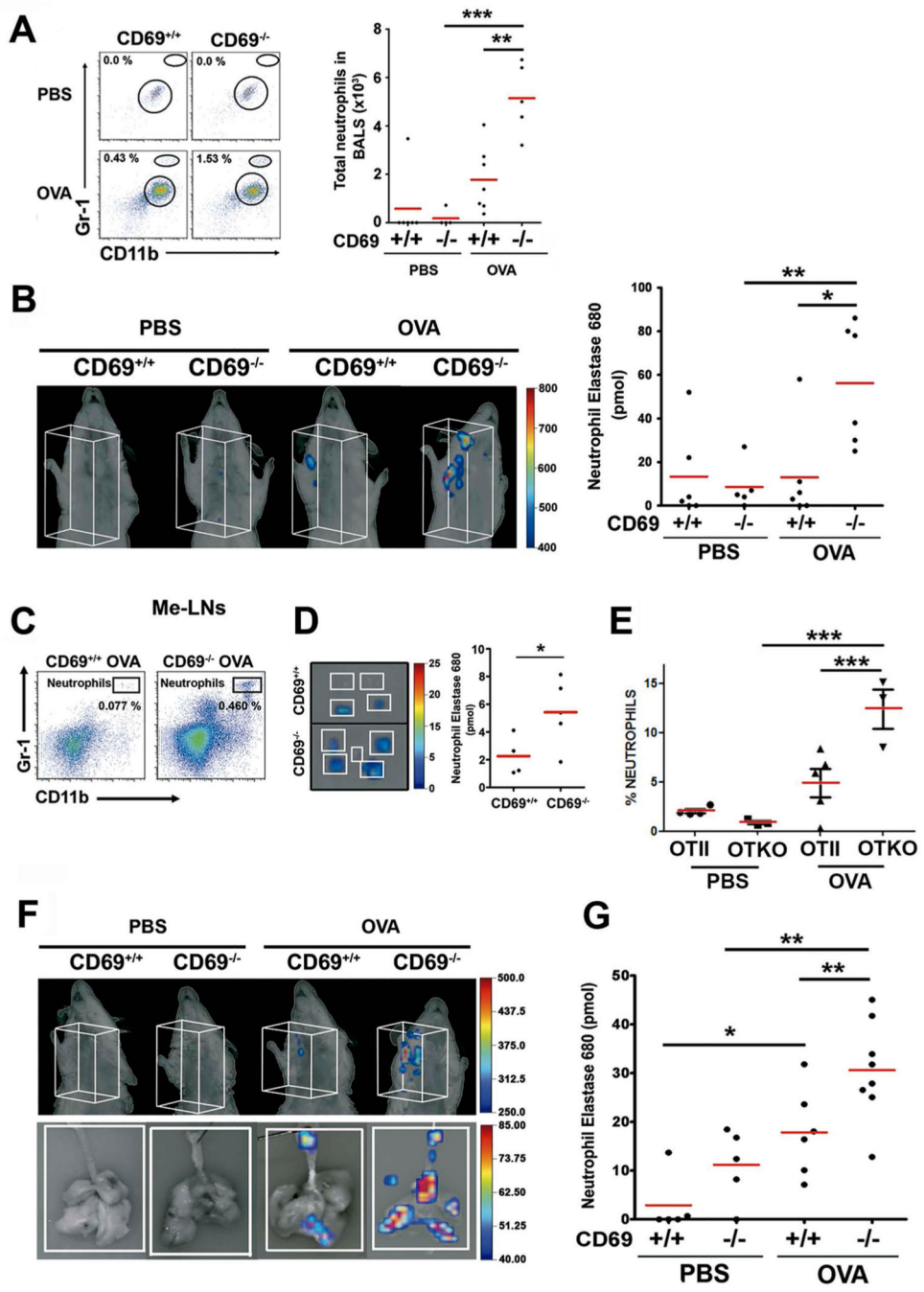

Figure 5. ***Cd69*$^{-/-}$ Tregs show impaired ability to suppress airway inflammation *in vivo* in a model of lung tolerance**

(**A**) Mice were treated with OVA i.t. 20d before PBS or OVA-aerosolized challenge. *Left*, flow cytometry analysis of BALS cells stained with a-CD11b and a-Gr-1, showing the percentage of neutrophils. *Right*, absolute numbers of neutrophils recruited in BALS are shown (**B**) *In vivo* detection of activated neutrophils in airways by three-dimensional tomographic imaging in mice treated as in A (*left*). *Right*, quantification of Neutrophil Elastase 680 probe fluorescence activity. (**C**) FACS analysis of neutrophils in cell

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suspensions from Me-LNs. (**D**) *Left*, *ex vivo* tissue imaging of excised Me-LNs from OVA-challenged *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ mice. *Right*, quantification of Neutrophil Elastase 680 probe fluorescence activity in the individual lymph nodes excised from different mice (white boxes). Red bars represent means. (**E**) OTII and OTKO mice were injected i.t. with endotoxin free OVA previous nebulizations with PBS or OVA and percentage of neutrophils in BALS were analyzed 24 hours after the last exposure to the antigen by FACS. (**F-G**) Mice were treated with OVA i.t. to induce tolerance and then with OVA i.p. before PBS or OVA-aerosolized challenge. Detection of neutrophils by tomographic imaging of airways *in vivo* or in resected lungs and tracheas (*lower panels*). (**G**) Neutrophil Elastase 680 probe fluorescence activity quantification. Data are representative of three (A-B) or two (E) independent experiments (n = 5-7). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

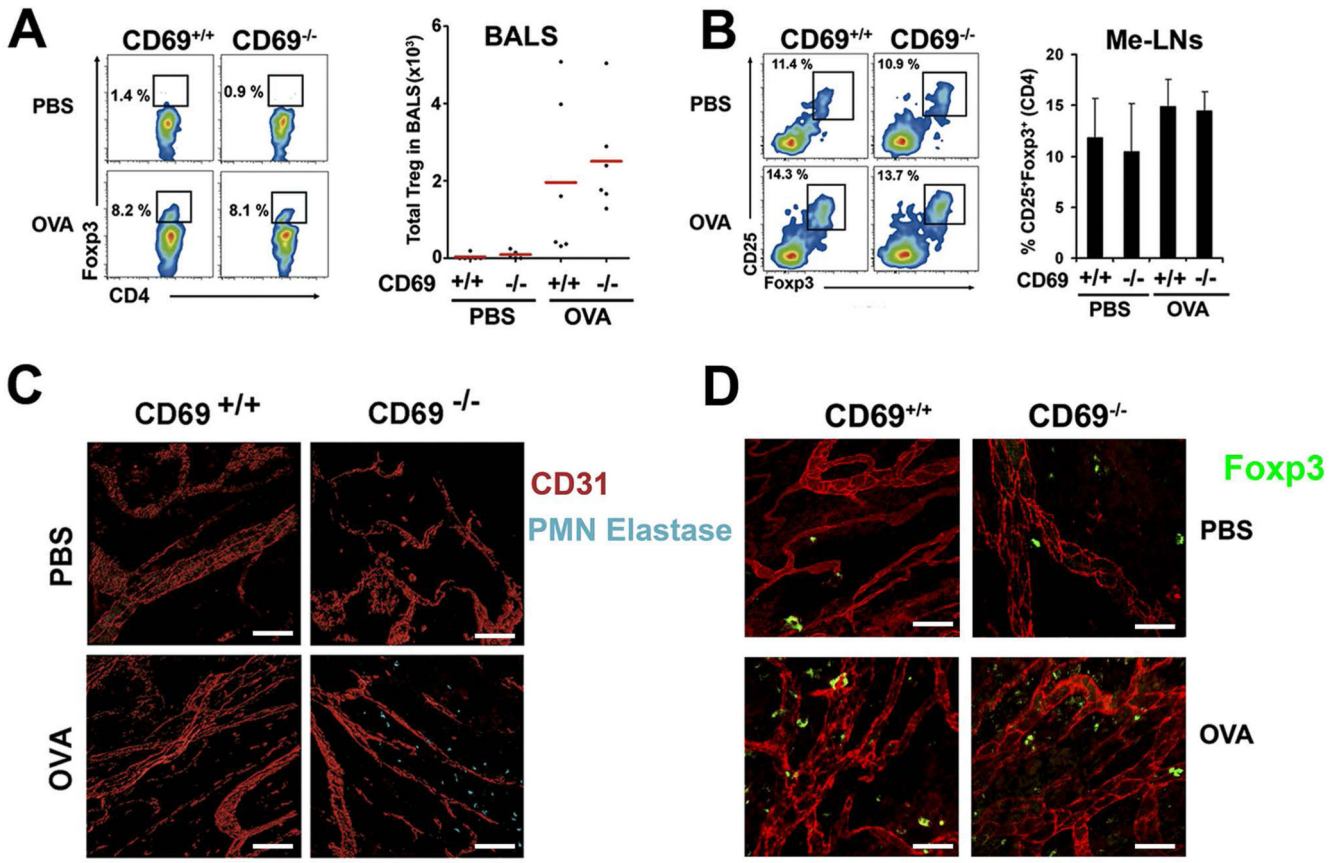

Figure 6. Migratory behavior of FoxP3$^+$ cells to the airways and lymph nodes is not affected in *CD69*$^{-/-}$ mice

(**A**) FACS analysis of CD4$^+$FoxP3$^+$ Tregs in BALS from mice treated with OVA i.t. before PBS or OVA-aerosolized challenge. *Left*, numbers indicate the percentage of cells within squares in dot plots. *Right*, absolute cell numbers of Tregs; red bars indicate means. **(B)** Analysis of Tregs in Me-LNs from *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ mice treated as in (A). (**C**-**D**) Whole-mount staining with CD31 to visualize vasculature (red) from tracheas of mice treated as in A. (**C**) Neutrophil infiltration in the trachea analysed by whole-mount staining as described under methods. Tissues were stained with a rabbit anti-mouse CD31 followed by a goat anti-rabbit Rhodamine Red-X to visualize vasculature (red). In parallel, Neutrophil elastase 680 flourescent probe was detected with a 633 laser line (bright blue). Figure shows the three-dimensional isosurface rendering of confocal image stacks. Bars, 30 μm. (**D**) Whole-mount staining of Foxp3 (Tregs, green) and CD31 (vasculature, red) in tracheas from PBS or OVA-challenged *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ mice. Bars, 20 μm. Data are from three independent experiments (n = 6).

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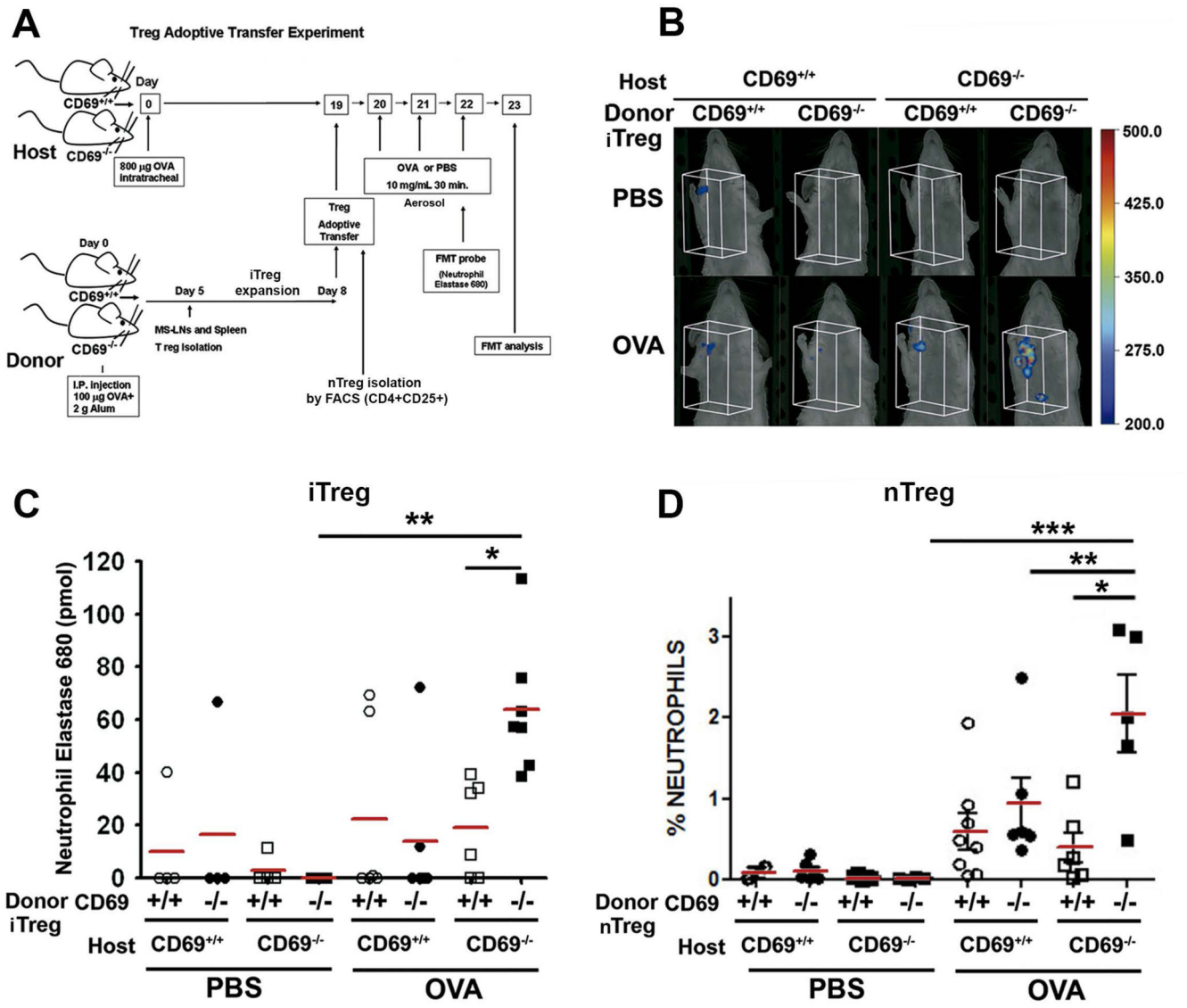

Figure 7. Cell therapy with *Cd69*$^{+/+}$ Tregs restores lung tolerance in *Cd69*$^{-/-}$ mice

(**A**) Scheme depicting adoptive transfer with inducible iTregs or natural nTregs obtained from *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ mice before PBS or OVA-challenge. (**B**) Detection of activated neutrophils in airways by tomographic imaging. Three-dimensional regions represent neutrophil elastase activity in upper airways and lung (white boxes) of PBS or OVA-challenged *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ BALB/c mice treated with *ex vivo* expanded *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ iTregs. Quantification of Neutrophil Elastase 680 probe fluorescence activity after treatment with iTregs (**C**) or nTregs (**D**). Circles and squares indicate *Cd69*$^{+/+}$ and *Cd69*$^{-/-}$ host mice and white and black symbols indicate mice treated with exogenous *Cd69*$^{+/+}$ or *Cd69*$^{-/-}$ Tregs, respectively. Data are representative of two independent experiments (n = 4-7). * P < 0.05, ** P < 0.01, ***P<0.001.