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CD69 is highly expressed by lymphocytes at mucosal surfaces. We aimed to investigate the role of CD69 in mucosal immune responses. The expression of CD69 by CD4 T cells isolated from the spleen, mesenteric lymph nodes, small intestinal lamina propria, and colonic lamina propria was determined in specific pathogen-free B6 and TCR transgenic animals, as well as in germ-free B6 mice. Transfer colitis was induced by transplanting RAG−/− mice with B6 or CD69−/−CD45RBhigh CD4 T cells. CD69 expression by CD4 T cells is induced by the intestinal microflora, oral delivery of specific Ag, and type I IFN (IFN-I) signals. CD4 T cells from CD69−/− animals produce higher amounts of the proinflammatory cytokines IFN-γ, TNF-α, and IL-21, whereas the production of TGF-β1 is decreased. CD69-deficient CD4 T cells showed reduced potential to differentiate into Foxp3+ regulatory T cells in vivo and in vitro. The transfer of CD69−/−CD45RBhigh CD4 T cells into RAG−/− hosts induced an accelerated colitis. Oral tolerance was impaired in CD69−/− and IFN-I receptor 1-deficient mice when compared with B6 and OT-II × RAG−/− animals. Polyinosinic-polycytidylic acid treatment of RAG−/− mice transplanted with B6 but not CD69−/− or IFN-I receptor 1-deficient CD45RBhigh CD4 T cells attenuated transfer colitis. CD69 deficiency led to the increased production of proinflammatory cytokines, reduced Foxp3+ regulatory T cell induction, impaired oral tolerance, and more severe colitis. Hence, the activation Ag CD69 plays an important role in regulating mucosal immune responses.

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become Foxp3+ regulatory T (Treg) cells in vivo and in vitro. Transfer of CD69-deficient T cells into RAG−/− hosts induced a severe colitis. CD69−/− cells showed impaired IFN-β1 induction by polyinosinic-polycytidylic acid [poly (I:C)], and beneficial effects of poly (I:C) treatment could not be observed in RAG−/− hosts after transfer of CD69−/− or IFNAR−/− CD4 T cells.

**Materials and Methods**

**Mice**

Inbred C57BL/6J (B6) mice, RAG−/− (RAGtm1Mom) mice, CD69−/− mice (17, 19), IFNAR−/− mice (34), and transgenic OT-II, OT-II × RAG−/− (35), and OT-II × CD69−/− mice were bred and kept under specific pathogen-free (SPF) conditions in the animal facility of Ulm University (Ulm, Germany). Germ-free (GF) B6 mice were screened weekly for viral, bacterial, and fungal contamination. Female and male mice were used at 6–12 wk of age. All animal experiments were performed according to the guidelines of the local Animal Use and Care Committee and the National Animal Welfare Law.

**Depletion of murine intestinal microbiota by antibiotic treatment**

Mice were treated with ampicillin (catalog no. A6140-5G; Sigma-Aldrich, Steinheim, Germany) 1 g/l added in water flasks and an antibiotic mixture consisting of 5 mg/ml vancomycin (catalog no. 861987-1G; Sigma-Aldrich), 10 mg/ml neomycin (catalog no. N6386-5G; Sigma-Aldrich), and 29 mg/ml polymyxin (catalog no. M3761-5G; Sigma-Aldrich) diluted in distilled H2O. Gavage volume of 10 μl/g body weight was delivered intragastroscopically every 12 h. Fresh antibiotic mixture was displayed at every feeding point, and ampicillin in fresh water was renewed every seventh day. Mice subjected to this protocol for 17 d displayed the properties of GF mice as previously reported (36), and CD69 expression by spleen, mesenteric lymph nodes (MLN), small intestinal LP (siLP), and colonic LP (cLP) CD4 T cells was analyzed by flow cytometry.

**CD45RBhigh CD4 T cell transfer colitis**

Total spleen cells of B6, CD69−/−, or IFNAR−/− mice were isolated and stained for CD3, CD4, and CD45RB with PE-conjugated mAb binding CD4 (catalog no. 145-2C11; eBioscience, Frankfurt, Germany), FITC-conjugated mAb binding CD4 GK1.5 (catalog no. 11-0041-86; eBioscience), and anti-ICOS (catalog no. 552145; BD Biosciences, Heidelberg, Germany) followed by the second-step reagent Streptavidin-Peridinin Chlorophyll-a Protein-Cy5.5 (catalog no. 45-4317-80; eBioscience). CD45RBhigh CD4 T cells from the CD3+ CD4+ population were enriched using the FACSARia system (BD Biosciences) to a purity >95%. Purified CD45RBhigh CD4 T cells were injected i.p. (3 × 10^5 cells/mouse) into RAG−/− mice. In some experiments, hosts were i.p. treated twice a week with 20 μg poly (I:C) (catalog no. 14-0729-01, AMSHERM, Freiburg, Germany). The weight of transplanted mice and their clinical condition were monitored twice weekly. Tissue samples for histopathological examination were taken from the large intestine, fixed in neutral-buffered formalin, embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with H&E. Histology of the large intestine was categorized as normal (score 0); mild colitis (score 1), with few inflammatory cells in the cLP, stroma edema, and a slight reduction of goblet cells; moderate colitis (score 2), with an intense inflammatory infiltration of the LP, hyperplasia of crypts, and a marked reduction of goblet cells; or severe colitis (score 3), with a spillover of leukocytes beyond the mucosa into deeper layers of the colonic wall, complete loss of goblet cells, distortion of the mucosal architecture, erosions or ulcerations, and crypt abscesses as previously published (9, 35).

**Tolerance induction by intragastric Ag delivery**

Mice received intragastrically 1 mg OVA protein (catalog no. A5253, grade II; Sigma-Aldrich) dissolved in 100 μl PBS daily five times as previously reported (37). Control mice received PBS only. Seven days after the last treatment, mice were immunized s.c. in the base of the tail with 50 μl/mouse OVA protein and 50 μl/mouse oligodeoxynucleotide (ODN) 1826 (Thermo Fisher Scientific, Ulm, Germany) emulsified in 50 μl/mouse IFA (catalog no. 552075, Sigma-Aldrich) containing 50 μl/mouse PBS. One week later, spleen cells were isolated from the treated mice and restimulated with OVA233_239 peptide ISQAVHAAHAEINEAGR (ISQAV) (catalog no. OR266830; Thermo Fisher Scientific). After 72 h, supernatants were collected, and IFN-γ concentration was determined by ELISA. Some mice were challenged by s.c. injection of 50 μg OVA protein in 12.5 μl PBS into the right ear pinna 1 wk after immunization. As a control, 12.5 μl only PBS was injected into the left ear pinna of the same mice. Ear swelling was measured before injection and daily for 3 d after injection. OVA-specific ear swelling was calculated as the following: (right ear thickness – left ear thickness)x100 – (right ear thickness – left ear thickness)x100, as 48 h after injection was the peak of swelling.

**CD4 T cell isolation**

CD4 T cells were isolated from the cLP, siLP, spleen, and MLN of B6, CD69−/−, IFNAR−/−, RAG−/−, OT-II, OT-II × RAG−/−, and OT-II × CD69−/− animals.

**Isolation of spleen and MLN cells.** Single-cell suspensions were aseptically prepared from spleen and MLN, washed, and resuspended in PBS supplemented with 1% FCS.

**Isolation of siLP and cLP cells.** Segments of the small intestine or colon were washed with PBS to remove debris and mucus. The epithelium was removed by incubation at 37°C for 10–15 min under gentle shaking with 1 mM DTT (and 1 mM EDTA for colon tissue) in 25 ml PBS supplemented with 1% FCS. The remaining tissue was washed in PBS to remove residual epithelial cells, and the supernatants were discarded. Intestinal tissues were cut into 2 × 2-mm pieces and digested by incubation with 0.25 mg/ml collagenase type VIII from *Clostridium histolyticum* (catalog no. C-2139; Sigma-Aldrich) for 30–45 min at 37°C in RPMI under shaking. Supernatants were collected from which LP lymphocytes were pelleted. LP lymphocytes were resuspended in RPMI medium containing 35% Percoll (density 1.124 g/ml; catalog no. L-6145; Biochrom, Berlin, Germany). This cell suspension was overlaid onto 70% Percoll and centrifuged for 20 min at 750 × g. Viable cells at the 35/70% Percoll interface were collected and washed twice.

**mAbs**

The following reagents and mAbs from eBioscience were used: FITC-conjugated mAb binding CD4 GK1.5 (catalog no. 11-0041-86), allophycocyanin-conjugated mAb binding CD4 GK1.5 (catalog no. 17-0041-83), PE-conjugated mAb binding CD69 H1.2F3 (catalog no. 12-0691-82), IL-21R mAb (catalog no. 12-2191), biotinylated mAb binding CD25 PC10.5 (catalog no. 13-0251-81), and CD69 H1.2F3 (catalog no. 13-0091-81). From BD Biosciences, the following biotinylated mAbs were used: anti-CD103 M290 (catalog no. 557493), anti-CD122 TM-β1 (catalog no. 559884), anti–IFN-γR GR20 (catalog no. 550482), and anti–ICOS 7E.17G9 (catalog no. 552145) as well as PE-conjugated anti–IL-10R b1.3a (catalog no. 559914). The biotinylated mAbs binding TGF-βRII (catalog no. BA532) and APC-conjugated anti-LAP (TGF-β1) 2745 (catalog no. FAB2463A) were purchased from R&D Systems. As a second-step reagent, PerCP-Cy5.5–conjugated streptavidin (catalog no. 45-4317-80; eBioscience) was used.

**Intracellular cytokine staining**

Cells were isolated, washed with PBS supplemented with 0.3% BSA and 0.1% sodium azide, and stained extracellular with FITC-conjugated anti-CD4 mAb binding CD4 GK1.5 (catalog no. 11-0041-86; eBioscience) as described above. Surface-stained cells were fixed with 4% paraformaldehyde/hyde for 15 min in the dark, then pelleted and resuspended in 200 μl Fixation/Permeabilization buffer (catalog no. 00-5123-43; eBioscience). After overnight incubation at 4°C in the dark, permeabilized cells were washed two times with 1× Permeabilization buffer (catalog no. 00-8333-56; eBioscience) and incubated for 15 min, 4°C, with mAb 2A4 directed against the FcγRIII/II CD16/CD32 (0.5 μg/ml Ab/10^5 cells) for preventing nonspecific binding of the Abs. Cells were pelleted and, after discarding the supernatant, incubated for 30 min at 4°C in the dark with 0.5 ng/ml cells of the following PE-conjugated Abs: anti-Foxp3-FITC-16s (catalog no. 12-5737-80; eBioscience), anti–T-bet-eBio410 (catalog no. 12-5825-80; eBioscience), anti–GATA-3-TRW1 (catalog no. 12-9966-42; eBioscience), and anti–RORγT AFKJS-9 (catalog no. 12-0988-82; eBioscience). PE-conjugated Abs were diluted in 1× Permeabilization buffer. Stained cells were washed once with 1× Permeabilization buffer, once with PBS supplemented with 0.3% BSA and 0.1% sodium azide, resuspended in PBS supplemented with 0.3% BSA and 0.1% sodium azide, and analyzed on a FACSCalibur (BD Biosciences).

**Flow cytometry analyses**

Cells were washed twice in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. Nonspecific binding of Abs to FcRs was blocked by...
preincubation of cells with mAb 2.4G2 directed against the FcγRII/II CD16/CD32 (0.5 ng/ml) and then washed with 0.5 ng/ml CD69− mAb for 20 min at 4°C and washed again twice. In most experiments, cells were subsequently incubated with a second-step reagent for 20 min at 4°C. Four-color flow cytometry (FCM) analyses were performed using an FACSCalibur (BD Biosciences). The forward–angle light scatter was used as an additional parameter to facilitate the exclusion of dead cells and aggregated cell clumps. Data were analyzed using FCS Express V3 software.

**Stimulation of CD4 T cells with anti-CD3/CD28 beads, CD69 activation, or poly IC**

Total spleen, siLp, or cpLp cells of B6 or CD69−/− mice were cultured in 200 μl medium containing RPMI, 10% FCS, 1% Penicillin/Streptomyicin (catalog no. P11-010; PAA Laboratories, Pasching, Austria) with addition of anti-CD3/anti-CD28 dynabeads (catalog no. 11452D; Invitrogen, Karlsruhe, Germany) at a cell/bead ratio of 10:1. After 24 h at 37°C, 5% CO2, the concentration of IFN-γ, TNF-α, and TGF-B1 was measured in the supernatants by ELISA. CD4+ T cells were enriched via a MACS isolation kit (catalog no. 130-090-860; Miltenyi Biotec, Bergisch Gladbach, Germany). From total spleen cells of B6 or CD69−/− mice and cultured in 500 μl medium containing RPMI, 10% FCS, and 1% Penicillin/Streptomyicin in the supernatants by ELISA. CD4+ T cells were enriched via a MACS isolation kit (catalog no. 130-090-860; Miltenyi Biotec, Bergisch Gladbach, Germany). From total spleen cells of B6 or CD69−/− mice and cultured in 500 μl medium containing RPMI, 10% FCS, and 1% Penicillin/Streptomyicin with addition of 2 μg/ml RNA isolated from tissue or 200 ng RNA isolated from CD4+ T cells was reverse transcribed with SuperScript II Reverse Transcriptase (catalog no. 18064-014; Invitrogen) using random primers (catalog no. 48190-011; Invitrogen) according to the manufacturer’s instructions. SYBR Green qPCR Master mix (catalog no. PA-012-12; SABiosciences, Qiagen) was used for amplification and detection. Real-time PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and the following conditions: 50°C for 2 min, repeat 1; 95°C for 10 min, repeat 1; 95°C for 15 s, 60°C for 1 min, repeat 40; 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s, 95°C for 1 min, repeat 15. β-actin PCR signals were used to equalize cDNA amounts between preparations. The following primers were used: β-actin (catalog no. PPM02945A; SABiosciences, Qiagen); IFN-γ (catalog no. PPM03121A; SABiosciences); IL-12 (catalog no. PPM03761E; SABiosciences); IFN-β (catalog no. PPM03594B; SABiosciences); TLR3 (catalog no. PPM04216B; SABiosciences); TGF-β1 (forward) 5′-GTA CAG CAA GGT CTT TGC CCT CTT-3′; and TGF-β1 (reverse) 5′-TAG TAG AGC ATG GCC AGT GGC-3′ (Thermo Scientific). Expected product length was: for β-actin, 154 bp; for IFN-γ, 95 bp; for IL-12, 189 bp; for IFN-β, 52 bp; for TLR3, 184 bp; and for TGF-β1, 102 bp. In some experiments, RT-PCR results were performed in five repeats.

**Cytokine detection by quantitative RT-PCR**

RNA was prepared from frozen colon tissue or pelleted CD4+ T cells using the RNAeasy mini kit (catalog no. 74904; Qiagen, Hilden, Germany). Contaminating genomic DNA was eliminated from samples by treatment with RNase-free DNase I (catalog no. 1010395; Qiagen). The total RNAs were amplified and purified using the RNeasy kit (catalog no. 74104; Qiagen). A total of 2 μg RNA was reverse transcribed with SuperScript II Reverse Transcriptase (catalog no. 18064-014; Invitrogen) using random primers (catalog no. 48190-011; Invitrogen) according to the manufacturer’s instructions. SYBR Green qPCR Master mix (catalog no. PA-012-12; SABiosciences, Qiagen) was used for amplification and detection. Real-time PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and the following conditions: 50°C for 2 min, repeat 1; 95°C for 10 min, repeat 1; 95°C for 15 s, 60°C for 1 min, repeat 40; 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s, 95°C for 1 min, repeat 15. β-actin PCR signals were used to equalize cDNA amounts between preparations. The following primers were used: β-actin (catalog no. PPM02945A; SABiosciences, Qiagen); IFN-γ (catalog no. PPM03121A; SABiosciences); IL-12 (catalog no. PPM03761E; SABiosciences); IFN-β (catalog no. PPM03594B; SABiosciences); TLR3 (catalog no. PPM04216B; SABiosciences); TGF-β1 (forward) 5′-GTA CAG CAA GGT CTT TGC CCT CTT-3′; and TGF-β1 (reverse) 5′-TAG TAG AGC ATG GCC AGT GGC-3′ (Thermo Scientific). Expected product length was: for β-actin, 154 bp; for IFN-γ, 95 bp; for IL-12, 189 bp; for IFN-β, 52 bp; for TLR3, 184 bp; and for TGF-β1, 102 bp. In some experiments, RT-PCR results were performed in five repeats.
products were visualized by agarose gel electrophoresis. A 10-cm length 2% agarose (catalog no. A9539-500G; Sigma-Aldrich) gel was used. Electrophoresis was performed in 1× TAE buffer, 8 V/cm, 45 min. Gene Ruler Plus (catalog no. SM1332; Fermentas, St. Leon-Rot, Germany) was used to identify the band length. Samples were visualized by adding ethidium bromide 0.07% solution (catalog no. A2273, 0015; AppliChem, Darmstadt, Germany) in gel and using the GeneGenius System (Syngene, Frankfurt, Germany) after electrophoresis. Images were obtained by GeneSnap image acquisition software.

**Cytokine detection by ELISA**

Cytokines in supernatants and blood serums were detected by a conventional double-sandwich ELISA. The following mAbs (from BD Biosciences) were used for detection and capture: mAb R4-6A2 (catalog no.

![Image](http://www.jimmunol.org/)

**FIGURE 2.** CD69 surface expression is induced by oral Ag challenge in TCR transgenic mice. B6 (A) and OT-II × RAG<sup>−/−</sup> (B) mice were fed for 2 d with 1 mg OVA protein dissolved in PBS. The expression of the activation Ags CD69 and CD25 by CD4 T cells from spleen (S), MLN, siLP, and cLP was analyzed by multicolor FCM. Data from an individual representative mouse (out of four mice analyzed) are shown. Numbers indicate the percentage of CD4 T cells that express the activation Ags CD69 or CD25.

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551216) and biotinylated mAb XMG1.2 (catalog no. 554410) for IFN-γ, mAb TC11-18H10 (catalog no. 550568), and biotinylated mAb TC11-8H4.1 (catalog no. 550567) for IL-17. TGF-β1 concentration was measured using the human/mouse TGF-β1 ELISA Ready-SET-Go Kit (catalog no. 88-7344; eBioscience). Extinction was measured at 405/490 nm on a TECAN microplate-ELISA reader using EasyWin software (both from Tecan, Wetzlar, Germany).

Statistics
A one-way ANOVA test (for nonparametric data) and a t test for two unequal variances were used. A p value <0.05 was considered statistically significant.

Results
Commensal-dependent upregulation of CD69 surface expression by LP CD4 T cells
We fed 1 mg OVA (in 10 μl PBS) to SPF B6 and (age- and sex-matched) OT-II × RAG−/− mice twice (on consecutive days) by oral gavage. Control animals were gavaged with PBS only. Tested at 24 h after the feeding, SPF B6 mice challenged with OVA showed no change in CD69 and CD25 surface expression on CD4 T cells recently activated in the intestinal LP by specific Ag. Having demonstrated the high CD69 expression by CD4 T cells at the intestinal LP, spleen, and MLN (Fig. 2A), in contrast, oral challenge of SPF OT-II × RAG−/− mice with OVA (using the same protocol) induced CD69 surface expression by CD4 T cells from the siLP, spleen, and MLN, but not cLP, where the CD4 T cells were expressing CD69 even before the OVA gavage (Fig. 2B). Following oral OVA challenge, 40–50% of all CD4 T cells from the intestinal LP and spleen of challenged mice expressed CD69 on the surface (Fig. 2B). Convincing induction of CD69 expression by CD4 T cells was seen in the MLN of OVA-gavaged OT-II × RAG−/− animals, in which a clear population of ∼20% of all CD4 T cells was detected to be CD69+ (Fig. 2B). Interestingly, these specifically activated CD4 T cells did not express the activation marker CD25 that renders them responsive to IL-2 and allows their clonal expansion and survival after specific challenge (Fig. 2B). Hence, CD4 T cells respond to specific priming by an oral Ag challenge by expressing a CD69+CD25− surface phenotype.

We compared the phenotype of CD69+ versus CD69− CD4 T cells from the siLP of OT-II × RAG−/− mice recently challenged orally by OVA in intracellular and surface FCM analyses. As expected, most intestinal LP CD69− CD4 T cells are naive and do not express the master regulators T-bet, GATA-3, or RORγt (required for Th1, Th2, or Th17 differentiation, respectively (43–45)). Almost 20% of the intestinal LP CD69+ CD4 T cells were Foxp3+ Treg cells. A fraction of (≈20%) of the intestinal LP CD69+ CD4 T cells expressed the IFN-γR (binding IFN-γ), CD122 (binding IL-2/IL-15), TGF-βRII (binding TGF-β), the TGF-β–regulated integrin CD103, and IL-21R1. A fraction (≈5%) of the activated intestinal LP CD69+ CD4 T cells expressed LAP associated with TGF-β1. The cytokine receptor IL-10R and the costimulatory molecule ICOS were not expressed by LP CD69+ CD4 T cells. In contrast, none of the transcription factors—T-bet, GATA-3, or RORγt—or the cytokine receptors—IFN-γR, CD122, TGF-βRII, IL-21R1, IL-10R, the integrin CD103, or the costimulatory molecule ICOS—were detected in activated intestinal LP CD69+ CD4 T cells (Fig. 2C). A fraction (∼40%) of the activated intestinal LP CD69+ CD4 T cells expressed LAP/TGF-β1. Hence, CD69+ CD25/CD122+ CD4 T cells recently activated in the intestinal LP by specific Ag challenge are not committed to the development of a proinflammatory phenotype and are largely unresponsive to key cytokines. Activated CD69+CD25/CD122+ LP CD4 T cells expressed LAP/TGF-β1, indicating that these cells could serve as regulatory cells.

CD69 negatively regulates the production of proinflammatory cytokines
Having demonstrated the high CD69 expression by CD4 T cells at mucosal surfaces, we analyzed by microarray experiment the CD69-dependent gene expression by comparing CD4 T cells from CD69-deficient animals, CD69-activated CD4 T cells and CD4 T cells from B6 animals. When CD4 T cells from B6 animals were compared with those from CD69−/− animals, 2472 genes were differentially regulated. The comparison of B6 CD4 T cells with CD69-activated CD4 T cells yielded 2641 differentially regulated genes. The comparison of CD4 T cells from CD69−/− animals with CD69-activated CD4 T cells yielded 3259 differentially

Table I. Expression of selected genes differentially expressed in B6, CD69−/−, and CD69-activated B6 CD4 T cells analyzed by microarray

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>Fold Change (log2) B6 versus CD69−/−</th>
<th>Fold Change (log2) B6 versus CD69 Activated</th>
<th>Fold Change (log2) CD69 Activated versus CD69−/−</th>
<th>FDR B6 versus CD69 Activated</th>
<th>FDR CD69 Activated versus CD69−/−</th>
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<tr>
<td>Il21</td>
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<tr>
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<td>TGF-β regulated gene 3</td>
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<td>−1.07</td>
<td>1.10</td>
<td>0.909</td>
<td>2.72e-05</td>
</tr>
</tbody>
</table>

FDR <0.05 was considered statistically significant.
FIGURE 3. CD69 negatively regulates the production of proinflammatory cytokines. For CD69 ligation, CD4 T cells from spleen of B6 or CD69$^{-/-}$ animals were activated with anti-CD3/anti-CD28 dynabeads followed by anti-CD69 and Fc fragments for cross-linking. RNA from the cell lysates was reverse transcribed to cDNA, and relative expression of TGF-$\beta_1$ (A), IFN-$\gamma$ (B), and IL-21 (C) to $\beta$-actin was analyzed by real-time PCR. TGF-$\beta_1$ (D) and IFN-$\gamma$ (E) concentrations in the supernatants were measured by ELISA. Data are representative of two individual experiments. *p, 0.05 was considered statistically significant.

B6 CD4 T cells
CD69$^{-/-}$ CD4 T cells
$\alpha$CD3/CD28 microbeads
Iso control antibody
$\alpha$CD69 antibody
Cross-linking antibody

B6 CD4 T cells
CD69$^{-/-}$ CD4 T cells
$\alpha$CD3/CD28 microbeads
Iso control antibody
$\alpha$CD69 antibody
Cross-linking antibody

B6 CD4 T cells
CD69$^{-/-}$ CD4 T cells
$\alpha$CD3/CD28 microbeads
Iso control antibody
$\alpha$CD69 antibody
Cross-linking antibody

B6 CD4 T cells
CD69$^{-/-}$ CD4 T cells
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B6 CD4 T cells
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B6 CD4 T cells
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Iso control antibody
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B6 CD4 T cells
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Cross-linking antibody

B6 CD4 T cells
CD69$^{-/-}$ CD4 T cells
$\alpha$CD3/CD28 microbeads
Iso control antibody
$\alpha$CD69 antibody
Cross-linking antibody

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regulated genes. IL-21, TNF-α, and IFN-γ were downregulated, and the TGF-β3 was upregulated in CD69-activated CD4 T cells (Table I). The microarray data sets have been uploaded in the Gene Expression Omnibus (accession number GSE27706).

To map the CD69-dependent pathways, we checked for enrichment of our microarray data sets in the gene ontology category and Kyoto Encyclopedia of Genes and Genomes pathways. These pathways included cytokine–cytokine receptor interaction, chemokine signaling pathway, TLR signaling pathway, nucleotide-binding oligomerization domain-like receptor signaling pathway, and the TGF-β signaling pathway (p value from hypergeometric test [rawP] was 7.24e-05, and p value adjusted by the multiple test adjustment [adjP] was 0.0004, respectively) (data not shown).

Spleen CD4 T cells from B6 or CD69−/− mice were then isolated and stimulated with anti-CD3/anti-CD28 Ab-coated beads in the presence or absence of a cross-linked anti-CD69 Ab. CD69 ligation enhanced TGF-β1 transcript levels expressed by B6 CD4 T cells (measured by quantitative RT-PCR [qRT-PCR]) but decreased IFN-γ and IL-21 transcript levels (Fig. 3 A–C). Activation of CD69 also increased the amount of TGF-β1 released into supernatants by stimulated B6 CD4 T cells but decreased IFN-γ secretion (Fig. 3D, 3E). No differences in IL-2 and IL-17A transcript levels or secreted protein by CD4 T cells with ligated or unligated CD69 were detectable (data not shown).

Furthermore, (CD3/CD28 ligation) stimulated cells from the spleen, siLP, and cLP of CD69−/− B6 mice produced significantly increased IFN-γ and TNF-α but decreased TGF-β1 levels when compared with cells from (age- and sex-matched) B6 mice (Fig. 3F–H). IL-21 transcript level was increased in the small intestine and colon tissue of nontreated CD69−/− mice compared with B6 mice (Fig. 3J). CD69 deficiency hence results in the reduction of induced TGF-β1 expression by CD4 T cells that is associated with enhanced IFN-γ, TNF-α, and IL-21 production.

Reduced numbers of Foxp3+ CD4 Treg cells in CD69−/− animals

Because CD69 influences the secretion of cytokines, we then analyzed the numbers of Foxp3+ CD4 Treg cells in CD69−/−.
animals. The fraction of Foxp3+ CD4 Treg cells was reduced in the MLN, siLP, and cLP CD4 T cell population from nontreated OT-II mice when compared with B6 mice (Fig. 4A). In the absence of TCR signaling in nontreated OT-II animals, reduced LAP/TGF-β1 expression was observed in isolates from the siLP of OT-II × CD69−/− mice as compared with OT-II mice (Supplemental Fig. 1). Feeding with OVA protein induced significantly higher fraction and cell number of Foxp3+ Treg cells among spleen, MLN, siLP, and cLP CD4 T cells of OT-II mice compared with OT-II × CD69−/− mice (Fig. 4B, 4C). This effect could be due to reduced TGF-β1 level in the absence of CD69, and we tested if exogenous addition of TGF-β1 can restore the ability of CD69−/− CD4 T cells to become Foxp3+ Treg. Naive CD4+CD25− B6 or CD69−/− cells were cultured in Treg-polarizing conditions, with B6 CD11c+ DC and exogenous IL-2 and TGF-β1 for 5 d. Although a significant fraction of CD69−/− CD4 T cells induced Foxp3 expression (~45%), this was still significantly reduced as compared with the fraction of B6 CD4 T cells expressing Foxp3 (~55%) (Fig. 4D, 4E). These data are showing that other factors that reduced TGF-β1 production are influencing reduced Foxp3+ Treg cell induction in the absence of CD69.

CD69-deficient CD4 T cells induce a severe colitis correlating with increased IFN-γ, IL-17A, and TNF-α serum levels

Because CD69 deficiency in transgenic animals is associated with reduced numbers of Foxp3+ Treg cells and an increased secretion of IFN-γ and TNF-α by CD69−/− CD4 T cells, CD69−/− animals were monitored for >6 mo for the occurrence of clinical and histopathological signs of a spontaneous colitis. However, clinical and histopathological signs for the development of a spontaneous colitis could not be observed in our animal facility (data not shown). We then transferred B6 or CD69−/− CD45RBhigh CD4 T cells into RAG−/− hosts to test if CD69 has an influence on the severity of CD4 T cell-induced transfer colitis. We monitored the clinical course, percent loss of body weight, and histological severity of the progressive and lethal colitis. Transfer of CD45RBhigh CD4 T cells from CD69−/− donors induced a colitis that was more severe than the colitis induced by transfer of an equal number of CD45RBhigh CD4 T cells from B6 donors. This was evident by accelerated body weight loss (Fig. 5A), more severe histopathology (Fig. 5B–E), and early lethality. The transfer of CD69−/−/CD45RBhigh CD4 T cells into RAG−/− hosts induced a higher rise in IL-17A, IFN-γ, and TNF-α serum levels when compared with transfers of B6 CD45RBhigh CD4 T cells (Fig. 5F–H). These data

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FIGURE 5. CD69-deficient CD4 T cells induce a severe colitis correlating with increased IFN-γ, IL-17A, and TNF-α serum levels. A, RAG−/− mice were i.p. transplanted with B6 or CD69−/− CD45RBhigh CD4 T cells. Mean ± SEM loss of body weight (%) of nine mice per group is shown for control, nontreated, and cell-transplanted RAG−/− mice. The p values were calculated with a nonparametric Student t test; p < 0.05 was considered statistically significant. B, Histopathological scores of colon sections taken from control or RAG−/− mice transplanted with the indicated CD45RBhigh CD4 T cells. Each experimental group consisted of nine mice. Mean ± SEM loss of body weight (%) of nine mice per group is shown for control, nontreated, and cell-transplanted RAG−/− mice. The p values were calculated with a nonparametric Student t test; p < 0.05 was considered statistically significant. Large intestinal tissue samples were taken from nontransplanted RAG−/− (C), RAG−/− hosts transplanted with B6 CD45RBhigh CD4 T cells (D), and hosts transplanted with CD69−/− CD45RBhigh CD4 T cells (E), embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with H&E. Representative images from one individual mouse per group (from nine mice per group analyzed) are shown (original magnification ×20). IFN-γ (F), IL-17A (G), and TNF-α (H) concentrations were detected in sera of nontransplanted RAG−/− and mice transplanted with B6 or CD69−/− CD45RBhigh CD4 T cells by ELISA. Each experimental group consisted of nine mice. The p values were calculated with the nonparametric Student t test; p < 0.05 was considered statistically significant.
point to a critical role of CD69 in regulating mucosal immune responses in inflammatory conditions.

**Poly (I:C) induces CD69 surface expression in an IFN-I–dependent manner**

IFN-IIs induce and upregulate CD69 surface expression in many cell types (16), which is confirmed by our data. Injection of 20 μg of the IFN-I inducer poly (I:C) induced CD69 surface expression by CD4 T cells in B6 and OT-II × RAG-2/− animals. After injection of 200 μg poly (I:C), most CD4 T cells (from the spleen, MLN, and intestinal LP) expressed CD69 in B6 or OT-II × RAG-2/− mice but not in (IFN-I–unresponsive) IFNAR-1/− mice within 24 h (Fig. 6). These data indicated that poly (I:C) is a strong inducer of CD69 on CD4 T cells in an IFN-I–dependent manner.

**Impaired oral tolerance in CD69 and IFNAR-deficient animals**

To examine the role of CD69 and IFN-I signals in mucosal CD4 T cells analyzed by multicolor FCM. Four mice per group were analyzed, and data from one representative individual mouse are shown. Filled curves represent the corresponding negative control. Numbers indicate the percentage of CD69+ CD4 T cells.

**CD69 is involved in IFN-I induction after poly (I:C) stimulation**

In RAG-2/− animals reconstituted with B6, CD69−/−, or IFNAR-1/− CD4 T cells, the observed protective effects on colitis development could be a direct effect of poly (I:C) on CD4 T cells. Alternatively, poly (I:C) acts on non-CD4 T cells (i.e., myeloid cells) in reconstituted RAG-2/− animals that elicits protective effects on CD4 T cells in animals that elicits protective effects on CD4 T cells. Hence, poly (I:C)-derived protective signals to CD4 T cells are depending on CD69 and IFN-I.
and B6 myeloid cells. When CD69−/− CD4 T cells were cultured with myeloid cells from B6 animals, reduced IFN-β responses were observed (Fig. 9C). Interactions of CD69 with a yet-unknown ligand (potentially) expressed by myeloid cells may be involved in the observed effects.

Discussion

The data presented in our study indicated that the activation Ag CD69 plays a role in regulating mucosal immune responses in the intestine. CD69 expression is upregulated on intestinal CD4 T cells by the commensal microflora, recognition of specific Ag, or innate stimuli [i.e., poly (I:C) injections]. The upregulation of CD69 surface expression by the commensal microflora may hence require innate and/or TCR-specific stimuli. CD69+ CD4 T cells are characterized by LAP/TGF-β1 expression. We confirmed that CD69 activation is associated with an increased TGF-β1 expression and that the lack of this molecule in CD69−/− animals is associated with CD69−/− CD4 T cells producing proinflammatory cytokines and reduced TGF-β1 (18). The CD4 T cells from spleen, siLP, and cLP showed differential expression of IFN-γ, TNF-α, and TGF-β1 (Fig. 3F–I), but the highest CD69 surface expression was determined on CD4 T cells isolated from intestinal tissues (Fig. 1A), indicating the most important role of this molecule in the mucosal immune system of the gut. Furthermore, CD69 affected the peripheral Foxp3+ Treg cell pool, as demonstrated in vivo both in steady-state conditions and after oral Ag challenge of OT-II CD69−/− animals. TGF-β1 is known to promote Foxp3 Treg cell generation (49), and we speculated that reduction in Foxp3+ Treg cell fraction in CD69−/− mice was in part due to lack of CD69-induced TGF-β1 production in these animals. Still, exogenous addition of TGF-β1 could not restore the normal potential of naive CD69−/− CD4 T cells to become Foxp3+ Treg cells in vitro. We speculate that CD69 intracellular signaling may interfere with Foxp3 expression (50) or that other cytokines, such as IL-10, are involved in our observed findings (51). Although CD69−/− animals did not develop a spontaneous colitis in our animal facility, the reconstitution of RAG2−/− hosts with CD69−/−CD45RBhigh CD4 T cells was associated with the accelerated transfer colitis as compared with RAG2−/− hosts transplanted with CD45RBhigh CD4 T cells from syngeneic B6 donor mice. In addition, CD69−/− animals are impaired in fully establishing oral tolerance. The increased expression of IFN-γ and TNF-α, reduced expression of TGF-β1, and reduction in the Foxp3+ Treg cell fraction in CD69−/− mice could contribute to the accelerated transfer colitis develop-
FIGURE 8. Poly (I:C) attenuates transfer colitis in RAG\(^{-/-}\) hosts transplanted with B6 but not with CD69\(^{-/-}\) and IFNAR\(^{-/-}\)-CD45RB\(^{high}\) CD4 T cells. RAG\(^{-/-}\) mice were i.p. transplanted with B6 (A), CD69\(^{-/-}\) (B), or IFNAR\(^{-/-}\)CD45RB\(^{high}\) (C) CD4 T cells. Poly (I:C) (20 μg/mouse) was i.p. injected twice a week. Mean ± SEM loss of body weight (%) of seven mice per group is shown. 
p values were calculated with a nonparametric Student t test; \(p < 0.05\) was considered statistically significant. D. Histopathological scores of colon sections taken from RAG\(^{-/-}\) mice transplanted with the indicated CD4 T cells and treated or not with poly (I:C) (20 μg/mouse) twice a week. Each experimental group consisted of seven mice. In the nonparametric Student t test, \(p < 0.05\) was considered statistically significant. E. RNA was isolated from frozen colon tissue samples of RAG\(^{-/-}\) hosts transplanted with B6, CD69\(^{-/-}\), or IFNAR\(^{-/-}\)CD45RB\(^{high}\) CD4 T cells treated or not with poly (I:C) and reverse transcribed to cDNA. Relative expression of IFN-γ gene was measured by qRT-PCR and presented as mean ± SEM of seven mice per group. \(p\) values were calculated with a nonparametric Student t test; \(p < 0.05\) was considered statistically significant.

FIGURE 9. CD69 is involved in IFN-I production after poly (I:C) stimulation. A. RNA was isolated from total spleen cells of B6, CD69\(^{-/-}\), or IFNAR\(^{-/-}\) and reverse transcribed to cDNA. Relative expression of TLR-3 was measured by qRT-PCR, and the mean ± SEMs of five samples per group are shown. B. Eight microliters of qRT-PCR product was loaded on 2% agarose gel, electrophoresis was performed, and picture obtained using GeneSnap acquisition software. C. Total spleen cells from B6, CD69\(^{-/-}\), or IFNAR\(^{-/-}\) were cultured with or without addition of 200 μg/sample of poly (I:C) for 48 h. Total RNA was isolated from cell lysates and reverse transcribed to cDNA. IFN-β1 expression was measured by qRT-PCR, and mean ± SEM of five samples from one mouse per group is presented. D, CD4\(^{+}\) and CD4\(^{+}\) spleen cells of the B6 or CD69\(^{-/-}\) were separated via MACS columns. B6 CD4\(^{+}\) cells were mixed with CD69\(^{-/-}\) CD4\(^{+}\) cells and CD69\(^{-/-}\)CD4\(^{+}\) T cells with B6 CD4\(^{+}\) and treated or not in vitro with 200 μg of poly (I:C). Forty-eight hours later, total RNA was obtained from the cell lysates and reverse transcribed to cDNA for qRT-PCR measuring of IFN-β1 expression. Mean ± SEMs of five samples from one mouse per group are shown. All of these experiments were performed twice, and the data from the representative one are presented. \(p\) values were calculated with a nonparametric Student t test; \(p < 0.05\) was considered statistically significant.
development of ulcerative colitis (30), has been suggested to contribute to the protective effects of IFN-I in colitis models (33).

The treatment of dextran sulfate sodium colitis with s.c. injections of poly (I:C) (20 μg/mouse) attenuates the colitis in an IFN-I–dependent manner (47). The protective effects of poly (I:C) on colitis models are dose dependent because injections of high amounts of poly (I:C) can induce mucosal damage (55). IFN-I responses after poly (I:C) injection are modulated by CD69.

Poly (I:C) injections did not affect the course of transfer colitis after transfer of CD69+/− or IFNAR−/−CD45RBhigh CD4 T cells. IFNAR−/−CD4 T cells are unable to respond to IFN-I, showing that poly (I:C) protects from transfer colitis in an IFN-I–dependent manner.

Because CD69 suppresses spingosine 1-phosphate receptor 1 expression and is hence involved in the regulation of lymphocyte egress (16), the expression of CD69 by T cells may be involved in the arrest of T cells at mucosal sites. In parallel, our data indicated that CD69 is involved in the regulation of IL-21, IFN-γ, TNF-α, and TGF-β1 expression by CD4 T cells. CD69 may hence regulate T cell migration and production of IL-21, IFN-γ, TNF-α, and TGF-β1 by CD4 T cells. The upregulation of the activation Ag CD69 by the commensal flora is not only an indicator of lymphocyte activation but also has significant functional relevance. CD69 may help to control the potential harmful impact of the intestinal microflora to the host by controlling lymphocyte migration and regulating the expression of proinflammatory cytokines and TGF-β1 in CD4 T cells, but further investigations are needed to address the role of CD69 in detail. The activation of CD69 by specific ligands could be a novel option for the treatment of intestinal inflammation.

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