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CD69 Regulates Type I IFN-Induced Tolerogenic Signals to Mucosal CD4 T Cells That Attenuate Their Colitogenic Potential

Katarina Radulovic,* Calin Manta,* Valerio Rossini,* Karlheinz Holzmann,† Hans A. Kestler,*§ Ursula Maria Wegenka,* Toshinori Nakayama,§ and Jan Hendrik Niess*

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 RAG2−/− 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 Rag2−/− 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 × Rag2−/− animals. Polyinosinic-polycytidylic acid treatment of Rag2−/− 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. The Journal of Immunology, 2012, 188: 000–000.

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The microarray data sets presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE27706.

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The online version of this article contains supplemental material.

Abbreviations used in this article: cLP, colonic lamina propria; DC, dendritic cell; DTH, delayed-type hypersensitivity; FCM, flow cytometry; FDR, false discovery rate; GF, germ-free; IFN-I, type I IFN; ISQAV, OVA323–339 peptide ISQA VHAA-HAEINEAGR; LAP, latency-associated peptide; LP, lamina propria; MLN, mesenteric lymph node; ODN, oligodeoxymethylcoidetide; poly (I:C), polyinosinic-polycytidylic acid; qRT-PCR, quantitative RT-PCR; siLP, small intestinal lamina propria; SPP, specific pathogen-free; Treg, regulatory T.

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The intestinal immune system is continuously exposed to large amounts of potential beneficial or harmful Ags derived from constituents of the commensal flora and ingested food Ags (1–3). In healthy individuals, rare inflammatory immune responses against these Ags are observed although T cell reactivity against commensal-derived Ags have been described (4). These Ags induce an Ag-specific state of systemic immunologic unresponsiveness, a phenomenon called oral tolerance (5). Breakdown of oral tolerance is associated with severe immunologic unresponsiveness, a phenomenon called oral tolerance (5).

T cells play an important role in regulating mucosal immune responses. The Journal of Immunology, 2012, 188: 000–000.
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-γ 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 B (catalog no. M3761-5G; Sigma-Aldrich) diluted in distilled H2O. Gavage volume of 10 μl/g body weight was delivered intragastrically 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 analysed after 18 d of treatment.

### 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 CD3ε 145-2C11 (catalog no. 12-0031-83; eBioscience, Frankfurt, Germany), FITC-conjugated mAb binding CD4 GK1.5 (catalog no. 11-0041-86; eBioscience), and biotinylated mAb binding CD45RB 16A (catalog no. OR266830; Thermo Fisher Scientific). After 72 h, supernatants from 2 CD69 AND TYPE I IFN IN COLITIS

### Intraacellular 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) and PE-conjugated mAb binding CD4 GK1.5 (catalog no. 17-0041-83; PE-conjugated mAb binding CD69 H1.2F3 (catalog no. 12-0061-82), IL-21R eBio4A9 (catalog no. 12-1219), biotinylated mAb binding CD25 PC01.5 (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. 559984), anti–IFN-γ GR20 (catalog no. 550482), and anti–ICOS 7E.17G9 (catalog no. 552145) as well as PE-conjugated anti–IL-10R1 B1.3a (catalog no. 559914). The biotinylated mAbs binding TGF-βRII (catalog no. BA532) and APC-conjugated anti–LAP (TGF-β1) 27232 (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.

### 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 incubation with 5 μg/ml of F(ab′)2 goat anti-rat-Ig (Southern Biotechnology Associates).
preincubation of cells with mAb 2.4G2 directed against the FcγRIIB/II CD16/CD32 (0.5 ng/mL 10^9 cells). Cells were washed and incubated with 0.5 ng/10^9 cells relevant 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 narrow-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 cLP cells of B6 or CD69^{-/-} mice were cultured in 200 μl medium containing RPMI, 10% FCS, 1% Penicillin/Streptomycin with CD11c MicroBeads, catalog no. 130-052-001; Miltenyi Biotec). T cells and DC were mixed in the ratio 2:1 (8 x 10^6 T cells:4 x 10^7 DC) and cultured together with T cell activating anti-CD3/anti-CD28 dynabeads (T cell/bead ratio 10:1) in 200 μl final/well medium RPMI, 10% FBS, and 1% Penicillin/Streptomycin to with or without addition of 200 μg poly (I:C) per well. In one experiment, 1 x 10^6 enriched B6 CD4^+ T cells from spleen were mixed with 9 x 10^5 CD4^+ CD69^{-/-} spleen cells or 1 x 10^6 CD4^+ T cells from spleen of CD69^{-/-} mice and cultured in 500 μl medium containing RPMI, 10% FCS, and 1% Penicillin/Streptomycin with anti-CD3/anti-CD28 dynabeads at a cell/bead ratio of 10:1. After overnight incubation, anti-CD69 H1.2F3 (catalog no. 13-0691-81; eBioscience) Ab was added (1:200 Ab dilution finally) to cross-link the anti-CD69 Abs. In some experiments, CD4-enriched spleen cells (from B6, CD69^{-/-}, or IFNAR^{-/-} mice) were cultured at 37°C, 5% CO_2, 48 h in 500 μl medium containing RPMI, 10% FBS, and 1% Penicillin/Streptomycin with or without addition of 200 μg poly (I:C) per well. In one experiment, 1 x 10^6 enriched B6 CD4^+ T cells from spleen of B6 mice with 9 x 10^5 CD4^+ CD69^{-/-} spleen cells or 1 x 10^6 CD4^+ T cells from spleen of CD69^{-/-} mice with 9 x 10^5 CD4^+ cells from spleen of B6. These mixed cells were cultured for 48 h in 500 μl medium containing RPMI, 10% FBS, and 1% Penicillin/Streptomycin with or without addition of 200 μg poly (I:C) per well. Assays for all reactions were performed in five repeats.

**In vitro Foxp3^{+} CD4 Treg cell induction**

Naïve CD4^+CD25^{-} T cells were enriched via MACS from a total cell population of B6 or CD69^{-/-} spleen cells. These cells were cocultured with CD11c^{+} dendritic cells (DC) from the spleen of B6 mice (enriched via CD11c MicroBeads, catalog no. 130-052-001; Miltenyi Biotec). T cells and DC were mixed in the ratio 2:1 (8 x 10^6 T cells:4 x 10^7 DC) and cultured together with T cell-activating anti-CD3/anti-CD28 dynabeads (T cell/bead ratio 10:1) in 200 μl final/well medium RPMI, 10% FBS, and 1% Penicillin/Streptomycin to with or without addition of 200 μg poly (I:C) per well. Expected product length was: for β-actin, 154 bp; for IFN-γ, 95 bp; for IL-21, 189 bp; for IFN-β, 52 bp; for TLR3, 184 bp; and for TGF-β1, 102 bp. In some experiments, RT-PCR

**Microarray**

Microarray analyses were performed using 200 ng total RNA as starting material and 5.5 μg ssDNA per hybridization (GeneChip Fluidics Station 450; Affymetrix, Santa Clara, CA). The total RNAs were amplified and labeled following the Whole Transcript Sense Target Labeling Assay (http://www.affymetrix.com). Labeled ssDNA was hybridized to Mouse Gene 1.0 ST Affymetrix GeneChip arrays (Affymetrix). The chips were scanned with an Affymetrix GeneChip Scanner 3000 and subsequent images analyzed using Affymetrix Expression Console Software (Affymetrix). Gene expression was than analyzed using the Affymetrix GeneChip Mouse Gene 1.0 ST Array platform (Affymetrix). Gene expression microarray files (Affymetrix CEL files) were generated using the GCOS software (Affymetrix). Statistical analyses were carried out using R (v. 2.12.1; R-Development-Core-Team). Arrays have been normalized using robust multivariate-array average (38). Expression data were analyzed using Bioconductor oligo package for R (39). Differentially expressed genes were determined by the shrinkage T-statistic (40). Functional enrichment analysis (gene ontology category enrichment and Kyoto Encyclopedia of Genes and Genomes Pathway enrichment) was determined using the hypergeometric test statistic with all genes on the Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix) as a reference set. Multiple comparison results were controlled by maintaining a false discovery rate (FDR) <0.05 (41).

**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). A total of 2 μg 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; SA Biosciences, 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, repeats 40; 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s, repeat 1. β-actin PCR signals were used to equalize cDNA amounts between preparations. The following primers were used: β-actin (catalog no. PPM02045A; SA Biosciences, Qiagen); IFN-γ (catalog no. PPM03121A; SA Biosciences); IL-21 (catalog no. PPM03761E; SA Biosciences); IFN-β (catalog no. PPM03594B; SA Biosciences); TLR3 (catalog no. PPM04216B; SA Biosciences); TGF-β1 (forward) 5′-GTA CAG CAA GGT CCT TGC CCT-3′; and TGF-β1 (reverse) 5′-TAG TAG AGC ATG GCC AGT GCC-3′ (Thermo Scientific). Expected product length was: for β-actin, 154 bp; for IFN-γ, 95 bp; for IL-21, 189 bp; for IFN-β, 52 bp; for TLR3, 184 bp; and for TGF-β1, 102 bp. In some experiments, RT-PCR

**FIGURE 1.** CD69 surface expression is upregulated by the commensal microflora. CD69 expression by CD4 T cells from spleen (S), MLN, siLP, and cLP of SPF B6 and age- and sex-matched OT-II and OT-II × RAG^{-/-} transgenic mice (A) as well as GF B6 and intestinal microflora-depleted OT-II and OT-II × RAG^{-/-} mice (B) was analyzed by multicolor FCM. Filled curves represent the respective negative controls. Numbers indicate the percentage of CD4^+CD69^+ cells. Four individual mice per strain were analyzed, and data from a representative mouse are shown.
products were visualized by agarose gel electrophoresis. A 10-cm length 2% agarose 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, Darmstad, 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. A9539-500G; Sigma-Aldrich)

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**FIGURE 2.** CD69 surface expression is induced by oral Ag challenge in TCR transgenic mice. B6 (A) and OT-II × RAG\(^{-/-}\) (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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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. A9539-500G; Sigma-Aldrich)
port (42). Compared to SPF B6 mice, the CD69+ CD4 T cell mice, in which the fraction of CD69+ cells was reduced, especially population. This was observed in SPF but not syngeneic GF B6 is hence increased substantially in the intestinal LP CD4 T cell expression by CD4 T cells is induced by oral Ag challenge (Fig. 1 A). The fraction of CD69+ cells is hence increased substantially in the intestinal LP CD4 T cell population. This was observed in SPF but not syngeneic GF B6 mice, in which the fraction of CD69+ cells was reduced, especially in the intestinal LP CD4 T cell population (Fig. 1 B). The fraction of CD69+ CD4 T cells in spleen, MLN, and particularly the intestinal LP was also reduced in SPF OT-II mice, although a substantial fraction of the transgenic TCR-expressing CD4 T cells did express CD69 in these mice (Fig. 1A), confirming a previous report (42). Compared to SPF B6 mice, the CD69+ CD4 T cell fraction in OT-II × RAG−/− mice was reduced in spleen, MLN, and siLP but not in cLP (i.e., a 10-fold reduction in CD69+ CD4 T cell population was seen in spleen, MLN, and siLP) (Fig. 1A). The treatment of OT-II and OT-II × RAG−/− animals with an antibiotic mixture led to the reduction of CD69+ CD4 T cell numbers as compared with SPF OT-II and OT-II × RAG−/− animals (Fig. 1B). These data indicate that the endogenous microflora have a major impact on the CD69 surface expression by CD4 T cells in all tissues tested.

CD69 expression by CD4 T cells is induced by oral Ag challenge

We fed 1 mg OVA (in 100 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 from 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 sLP, 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.

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 determined the fraction of CD4 T cells in spleen, MLN, and intestinal LP of B6 mice that expressed on the surface the activation marker CD69. Approximately 10% of the CD4 T cells from the spleen, 17% of CD4 T cells from the MLN, and 50% of CD4 T cells from the intestinal LP expressed CD69 in B6 mice were raised and kept under SPF conditions (Fig. 1A). The fraction of CD69+ cells is hence increased substantially in the intestinal LP CD4 T cell population. This was observed in SPF but not syngeneic GF B6 mice, in which the fraction of CD69+ cells was reduced, especially in the intestinal LP CD4 T cell population (Fig. 1B). The fraction of CD69+ CD4 T cells in spleen, MLN, and particularly the intestinal LP was also reduced in SPF OT-II mice, although a substantial fraction of the transgenic TCR-expressing CD4 T cells did express CD69 in these mice (Fig. 1A), confirming a previous report (42). Compared to SPF B6 mice, the CD69+ CD4 T cell fraction in OT-II × RAG−/− mice was reduced in spleen, MLN, and siLP but not in cLP (i.e., a 10-fold reduction in CD69+ CD4 T cell population was seen in spleen, MLN, and siLP) (Fig. 1A). The treatment of OT-II and OT-II × RAG−/− animals with an antibiotic mixture led to the reduction of CD69+ CD4 T cell numbers as compared with SPF OT-II and OT-II × RAG−/− animals (Fig. 1B). These data indicate that the endogenous microflora have a major impact on the CD69 surface expression by CD4 T cells in all tissues tested.

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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 (of ~11%) expressed the 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.

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

<table>
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<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−/−</th>
<th>FDR B6 versus CD69 Activated</th>
<th>FDR CD69 Activated versus CD69−/−</th>
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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-β1 (A), IFN-γ (B), and IL-21 (C) to β-actin was analyzed by real-time PCR. TGF-β1 (D) and IFN-γ (E) concentrations in the supernatants were measured by ELISA. Data are representative of two individual experiments. The p values were calculated with a nonparametric Student t test; p < 0.05 was considered statistically significant.

Spleen (S), siLP, and cLP cells of B6 and CD69−/− mice were cultured with anti-CD3/anti-CD28 dynabeads. After 18 h, supernatants were collected and analyzed for IFN-γ (F), TNF-α (G), and TGF-β1 (H) by ELISA. The mean ± SEMs of five mice per group are shown. The p values were calculated with a nonparametric Student t test; p < 0.05 was considered statistically significant. Total RNA isolated from frozen small intestine and colon tissue of B6 and CD69−/− was reverse transcribed to cDNA, and expression of IL-21 (I) was analyzed by RT-PCR. Data are representative of two individual experiments, and mean ± SEM of five mice per group is presented. In the nonparametric Student t test, p < 0.05 was considered statistically significant (*p < 0.05).
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 ligand 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. 3A–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.

**FIGURE 4.** Reduced Foxp3+ Treg cell number in the absence of CD69. A, Cells were isolated from spleen (S), MLN, siLP, and cLP of nontreated B6 and CD69−/− mice and analyzed for intracellular expression of Foxp3 in CD4 T cell population by multicolor FCM. Numbers indicate the percentage of CD4+Foxp3+ cells. Four individual mice were analyzed, and data from a representative mouse are shown. B, TCR-transgenic OT-II and OT-II−/− CD69−/− mice were fed 2 d with 1 mg OVA protein dissolved in PBS. The intracellular expression of Foxp3 by CD4 T cells from spleen (S), MLN, siLP, and cLP was analyzed by multicolor FCM. Numbers indicate the percentage of CD4+Foxp3+ cells. Four individual mice were analyzed, and data from a representative mouse are shown. C, Mean (± SEM) total number of Foxp3+ CD4 T cells in spleen, MLN, siLP, and clp of two times OVA-fed OT-II and OT-II−/− mice are shown. Four mice of each strain were analyzed. In the nonparametric Student *t* test, *p* < 0.05 was considered statistically significant. D, Naive CD4+CD25− T cells were enriched from the spleen of B6 or CD69−/− animals and mixed with B6 CD11c+ DCs in the proportion 2:1. Cells were cultured for 5 d with activating anti-CD3/anti-CD28 microbeads in the presence of exogenous IL-2 and TGF-β1. Cells were pelleted, washed, and intracellular Foxp3 expression was measured with multicolor FCM. As the control, Foxp3 expression was analyzed in the population of naive cells before the culture. Numbers indicate the percentage of Foxp3+CD4+ cells. Experiment was repeated twice with 10 individual samples from one mouse per strain per experiment, and representative dot plots are shown. E, Mean (± SEM) of in vitro-induced Foxp3+CD4+ cell percentage between B6 and CD69−/− cells for 10 samples per group is presented. Experiment is repeated twice, and the representative data are shown. In the nonparametric Student *t* test, *p* < 0.05 was considered statistically significant.
The fraction of Foxp3+ CD4 Treg cells was reduced in the MLN, siLP, and cLP CD4 T cell population from nontreated CD69−/− mice as 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 TFG-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.

**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−/− animals. 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. In the 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–/– 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–/– mice but not in (IFN-I–unresponsive) IFNAR–/– 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 cell responses, the protocol for the induction of oral tolerance was conducted in B6, OT-II mice. Animals were fed with OV A, rested, and immunized with OV A peptide, and the concentration of specifically induced IFN-γ was measured in supernatants by ELISA. OVA feeding prior to immunization significantly reduced IFN-γ production by spleen cells from B6 and OT-II × RAG–/– mice, indicating establishment of oral tolerance (Fig. 7A). Checking of delayed-type hypersensitivity (DTH) response in B6 and OT-II × RAG–/– mice confirmed establishment of tolerance in OVA-fed mice, as these animals showed significantly reduced response to ear challenge with OVA (conducted 7 d after immunization and measured as ear swelling) compared with those fed with PBS (Fig. 7B). These data confirmed a previous report that oral tolerance can be induced in TCR-transgenic animals (46).

We used the same protocol to test if oral tolerance to OVA can be induced in CD69–/– and IFNAR–/– B6 mice. The specific IFN-γ response of spleen cells from CD69–/– and IFNAR–/– B6 mice fed with OVA, rested, and immunized was not impaired compared to the animals that were PBS fed, rested, and immunized (Fig. 7A). Ear challenge with OVA 7 d after immunization revealed that DTH response in OVA-fed mice of these two mice strains was not altered compared to PBS-fed animals (Fig. 7B). Oral tolerance can hence not be induced either in CD69–/– or IFNAR–/– B6 mice, pointing to a critical role of CD69- and IFN-I–mediated signaling in the induction and/or maintenance of oral tolerance.

**Poly (I:C) treatment attenuates colitis after transfer of B6 but not CD69–/– or IFNAR–/–CD45RBhigh CD4 T cells**

It is reported that poly (I:C) injection can diminish dextran sulfate sodium colitis (47). We tested if poly (I:C) injections attenuate transfer colitis. The transfer of CD45RBhigh CD4 T cells in RAG–/– hosts results in progressive body weight loss and histopathological signs of colitis (Fig. 8A, 8D). Poly (I:C) injections diminish the severity of transfer colitis in RAG–/– hosts transplanted with B6 CD45RBhigh CD4 T cells as indicated by significant reduced body weight loss, histopathological signs of colitis, and IFN-γ transcript levels in colon (Fig. 8A, 8D, 8E). Compared to nontreated RAG–/– hosts transplanted with CD69–/–CD45RBhigh CD4 T cells, body weight loss, the severity of histopathological signs of colitis, as well as IFN-γ transcript levels in colon were not reduced in RAG–/– hosts transplanted with CD69–/–CD45RBhigh CD4 T cells and treated with poly (I:C) (Fig. 8B, 8D, 8E). Body weight loss, histopathological signs of colitis, and IFN-γ transcript levels in colon between poly (I:C)-treated and nontreated RAG–/– hosts transplanted with IFNAR–/–CD4 CD45RBhigh T cells were not significantly different (Fig. 8C–E). Hence, poly (I:C)-derived protective signals to CD4 T cells are depending on CD69 and IFN-I.

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

In RAG–/– animals reconstituted with B6, CD69–/–, or IFNAR–/–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–/– animals that elicits protective effects on CD4 T cells in animals with transfer colitis. We first investigated TLR3 expression in B6, CD69–/–, and IFNAR–/– cells by qRT-PCR. A significant difference in TLR3 expression among B6, CD69–/–, and IFNAR–/– animals was not observed (Fig. 9A, 9B). Myeloid cells (plasmacytoid DC, conventional DC, and macrophages) are the main producers of IFN-I after poly (I:C) challenge (48). We analyzed IFN-β1 expression in lymphocytes from B6, CD69–/–, and IFNAR–/– mice stimulated in vitro with poly (I:C). IFN-β1 expression after poly (I:C) stimulation was significantly increased in B6 and IFNAR–/– cells, but not in CD69-deficient cells (Fig. 9B). We then carried out cocultures with B6 CD4 T cells and CD69–/– myeloid cells or with CD69–/– CD4 T cells.

### Table: CD69 Expression

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<tr>
<th>CD4 T cells</th>
<th>S</th>
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<th>siLP</th>
<th>cLP</th>
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**FIGURE 6.** Poly (I:C) induces CD69 surface expression in an IFN-I–dependent manner. Poly (I:C) (20 or 200 μg/mouse) was injected i.p. into B6, OT-II × RAG–/–, and IFNAR–/– mice. Cells were isolated from spleen (S), MLN, siLP, and cLP and surface expression of CD69 by 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.
FIGURE 7. CD69 and IFNAR-deficient animals are impaired in establishing oral tolerance. A, B6, OT-II × RAG−/−, CD69−/−, and IFNAR−/− mice were fed for 5 d with 1 mg OVA protein dissolved in 100 μl PBS or PBS only. After 7 d, mice were immunized s.c. with 50 μg OVA protein and 50 μg ODN emulsified in IFA with PBS. Splenocytes were harvested and restimulated with ISQAV OVA peptide in indicated concentrations on day 19. After 72 h restimulation with ISQAV OVA peptide, IFN-γ was measured in the supernatants by ELISA. B. After feeding of the indicated mice with 1 mg OVA for 5 d, mice were immunized s.c. with 50 μg OVA protein and 50 μg ODN emulsified in IFA with PBS on day 12. Nineteen days after oral OVA feeding, the indicated mice were challenged by injection of 50 μg OVA protein in 12.5 μl PBS into the right ear pinna. As a control, 12.5 μl of PBS was injected into left ear of the same mice. Ear swelling was measured before ear injection and daily for 3 d after injection. Peak reaction was observed after 48 h. DTH response was calculated as: (right ear thickness − left ear thickness) × (right ear thickness − left ear thickness) ×, Data are representative of two individual experiments. In the nonparametric Student t test, p < 0.05 was considered statistically significant.

and B6 myeloid cells. When CD69−/− CD4 T cells were cultured with myeloid cells from B6 animals, reduced IFN-β1 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 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–H), 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 RAG−/− hosts with CD69−/− CD45RBhigh CD4 T cells was associated with the accelerated transfer colitis as compared with RAG−/− 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-
ment after reconstitution of RAG−/− hosts with CD69−/− CD4 T cells. Besides colonization of the host with the commensal flora, CD69 surface expression can be induced by the injection of the IFN-I inducer poly (I:C) (16). Our data confirmed that this effect of poly (I:C) is IFN-I dependent, because IFNAR−/− animals did not show CD69 upregulation after poly (I:C) injection. However, IFNAR−/− mice had a high fraction of CD69-expressing CD4 T cells even before treatment (CD69 expression in intestine of IFNAR−/− mice was comparable with B6 mice), suggesting that stimuli other than IFN-I are inducing CD69 expression in the steady-state conditions. In contrast, CD69−/− CD4 T cells showed an aberrant IFN-I response after poly (I:C) stimuli. Both CD69- and IFNAR-derived signals are necessary for the establishment of oral tolerance. IFN-I has been reported to have protective effects in colitis models and patients with ulcerative colitis (29–33). The ability of IFN-I to stimulate the production of IL-10 by human T cells and monocytes (52), modulate Th1 and Th2 responses (53, 54), and inhibit the production of IL-13, a key cytokine in the

FIGURE 8. Poly (I:C) attenuates transfer colitis in RAG−/− hosts transplanted with B6 but not with CD69−/− and IFNAR−/−CD45RBhigh CD4 T cells. RAG−/− mice were i.p. transplanted with B6 (A), CD69−/− (B), or IFNAR−/−CD45RBhigh (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−/−CD45RBhigh 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. Poly (I:C) injection was 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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Disclosures

The authors have no financial conflicts of interest.

References


**Figure legends to supplemental material**

**FIGURE S1. CD69 induces LAP/TGF-β1 expression without TCR triggering only on siLP CD4 T cells.**

A. LAP/TGF-β1 expression by CD4 T cells from spleen, MLN, siLP and cLP of non-treated OT-II and OT-II x CD69+/− transgenic mice was analyzed by multicolour flow cytometry. Filled curves represent the respective negative controls. Numbers indicate the percentage of CD4+ LAP/TGF-β1+ cells. Five individual mice were analyzed and data from a representative mouse are shown.  

B. Mean (± SEM) total number of LAP/TGF-β1+ CD4 T cells in spleen, MLN, siLP and cLP of OT-II and OT-II x CD69+/− mice are shown. Five mice of each strain were analyzed. In the non-parametric student’s t test p< 0.05 was considered statistically significant.
Figure S1 (related to Figure 3)