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The Leukocyte Activation Receptor CD69 Controls T Cell Differentiation through Its Interaction with Galectin-1

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CD69 is involved in immune cell homeostasis, regulating the T cell-mediated immune response through the control of Th17 cell differentiation. However, natural ligands for CD69 have not yet been described. Using recombinant fusion proteins containing the extracellular domain of CD69, we have detected the presence of a ligand(s) for CD69 on human dendritic cells (DCs). Pull-down followed by mass spectrometry analyses of CD69-binding moieties on DCs identified galectin-1 as a CD69 counterreceptor. Surface plasmon resonance and anti-CD69 blocking analyses demonstrated a direct and specific interaction between CD69 and galectin-1 that was carbohydrate dependent. Functional assays with both human and mouse T cells demonstrated the role of CD69 in the negative effect of galectin-1 on Th17 differentiation. Our findings identify CD69 and galectin-1 to be a novel regulatory receptor-ligand pair that modulates Th17 effector cell differentiation and function.

CD69, a C-type lectin, is a member of the natural killer (NK) receptor family and is induced early following activation of leukocytes (1). The physiological role of this receptor, which is persistently expressed by infiltrating leukocytes in different chronic inflammatory diseases, has been studied in CD69-deficient mice in multiple different models of chronic inflammation (2–5). Thus, we have previously described that CD69$^{-/-}$ mice develop an exacerbated form of collagen-induced arthritis (CIA) (3), a Th1 and Th17 cell-mediated autoimmune condition. Moreover, in an experimental model of autoimmune myocarditis (EAM), CD69 negatively regulates cardiac inflammation through the regulation of heart-specific Th17 responses (4). In this regard, we have detected that CD69 modulates the *in vitro* differentiation of T cells toward the Th17 lineage through the activation of the Jak3/Stat5 inhibitory pathway (5). On the other hand, CD69 negatively regulates the chemotactic responses of effector lymphocytes and dendritic cells (DCs) to sphingosine 1 phosphate (S1P); CD69 can associate with S1P1 in the cell membrane and induce a conformation of S1P1 that favors its internalization and degradation (6–8). It is clear that the identification of cellular ligands for CD69 is a critical next step to better understand the physiological role of this receptor.

Galectins are characterized by a common structural fold and a conserved carbohydrate recognition domain (CRD) with a high affinity for beta-galactosides (9). Despite being soluble proteins, galectins are also expressed on the cell surface due to their association with membrane glycoproteins. Thus, galectin-1 (Gal-1) is expressed by most activated but not resting T and B cells, and it is significantly upregulated in activated macrophages and T regulatory lymphocytes (10). In addition, tolerogenic DCs show a high expression of Gal-1 (11), which is rapidly downregulated in response to maturation signals. Furthermore, Gal-1-deficient DCs show a greater immunogenic potential and an impaired ability to halt the inflammatory phenomenon in a model of experimental autoimmune encephalomyelitis (11). Altogether this evidence suggests that Gal-1 expressed on DCs could act as a negative regulator of T cell differentiation. The beneficial effect of Gal-1 administration in experimental models of T cell-mediated autoimmune disorders (12, 13) and graft-versus-host disease (14) indicates that this galectin may be critical for T cell homeostasis and peripheral tolerance. Gal-1-deficient (Lgals1$^{-/-}$) mice show augmented Th1 and Th17 responses and are considerably more susceptible to immune-mediated fetal rejection and autoimmune diseases than their wild-type (WT) counterparts (11, 15, 16). Accordingly, Th1 and Th17 lymphocytes express the cell surface glycans critical for Gal-1-induced cell death (15).

Here, we demonstrate for the first time the presence of cell membrane ligands for CD69 on human monocyte-derived DCs. Mass spectrometry, surface plasmon resonance (SPR), and other binding assays show that Gal-1 interacts specifically and directly with CD69. The treatment with recombinant Gal-1 suppressed human Th17 cell differentiation through its interaction with CD69 expressed by activated T cells. Thus, our data indicate that the expression of CD69 by activated T lymphocytes triggers an anti-inflammatory mechanism mediated by Gal-1, which regulates the immune response and prevents pathogenic Th17 responses.

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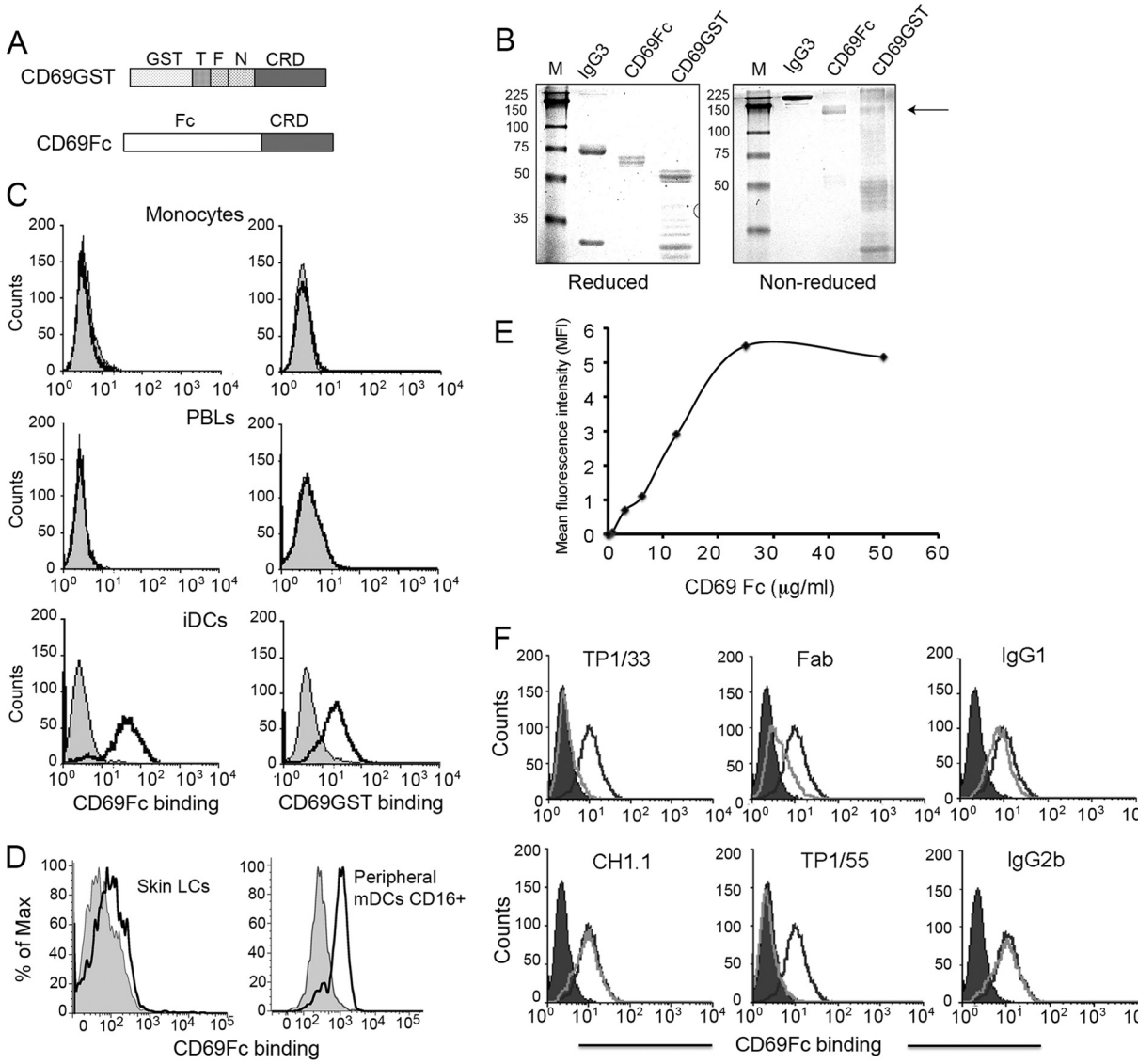

FIG 1 Recombinant CD69 proteins bind to immature DCs. (A) A CD69-GST fusion protein has the extracellular domain of CD69, the carbohydrate recognition domain (CRD) and a short neck (N) fused to the Flag (F) tag, a thrombin cleavage site (T), and glutathione *S*-transferase (GST). The CD69-Fc recombinant protein has the CRD of CD69 fused to the human immunoglobulin G3 Fc tail. (B) Electrophoresis gels under reduced and nonreduced conditions. Coomassie staining for detection of recombinant CD69 proteins after their purification is shown. Arrow, dimeric proteins; lanes M, molecular mass markers (kDa) indicated on the left. (C) Representative binding of recombinant CD69 proteins to human monocytes, PBLs, and iDCs from 5 independent experiments is shown. Analysis was on a FACSCalibur flow cytometer. Filled histograms, human IgG3 or GST; empty histograms, CD69-Fc or CD69-GST. (D) CD69-Fc binding to human skin LCs and peripheral CD16$^+$ myeloid DCs. CD69-Fc binding was evaluated on isolated LCs from skin samples and purified peripheral mDCs, as indicated in Materials and Methods. Cells were analyzed on a FACSCanto flow cytometer. Data correspond to those from 1 out of 3 experiments that were done. (E) Dose response of CD69-Fc binding to iDCs. Binding is represented as the mean fluorescence intensity (MFI) and was analyzed as described for panel C. Mean fluorescence intensity values are represented on a linear scale. (F) Inhibition of CD69-Fc binding by anti-CD69 MAbs. CD69-Fc was preincubated with anti-CD69 MAbs directed against epitope E1 (TP1/33, TP1/55), epitope E2, epitope E3 (Fab and CH1.1), or isotype-matched controls (IgG1 and IgG2b) and then added to DCs. Black line, CD69-Fc; gray line, CD69-Fc preincubated with the indicated MAbs; filled histogram, control human IgG3. Histograms are representative of those from at least 5 independent experiments.

MATERIALS AND METHODS

Cells and reagents. The study was approved by the institutional review board and the independent ethics committee of the Hospital Universitario de la Princesa and conformed to the Declaration of Helsinki principles. Human monocyte-derived DCs were obtained as described previously (17). At day 6, maturation of DCs was induced by lipopolysaccharide (LPS; 10 ng/ml; Sigma Chemical Co., St. Louis, MO). Mouse bone marrow-derived DCs were generated as described previously (18). Langerhans cells (LCs) were isolated from a skin sample of a healthy subject. Skin cell suspensions were obtained after epidermis and dermis separation with trypsin. The epidermis was then cultured overnight; nonadherent cells that migrated out the tissue into the medium were collected. Human peripheral myeloid DCs (mDCs) were purified from peripheral blood mononuclear cells by cell sorting according to next staining: lineage negative (CD3$^-$, CD14$^-$, CD19$^-$, CD20$^-$, and CD56$^-$), HLA-DR$^+$, CD11c$^+$, and CD123$^-$. Murine monoclonal antibodies (MAbs) specific for human CD69 (TP1/8, TP1/33, TP1/55, TP1/22, and CH1.1) and Fab were generated and charac-

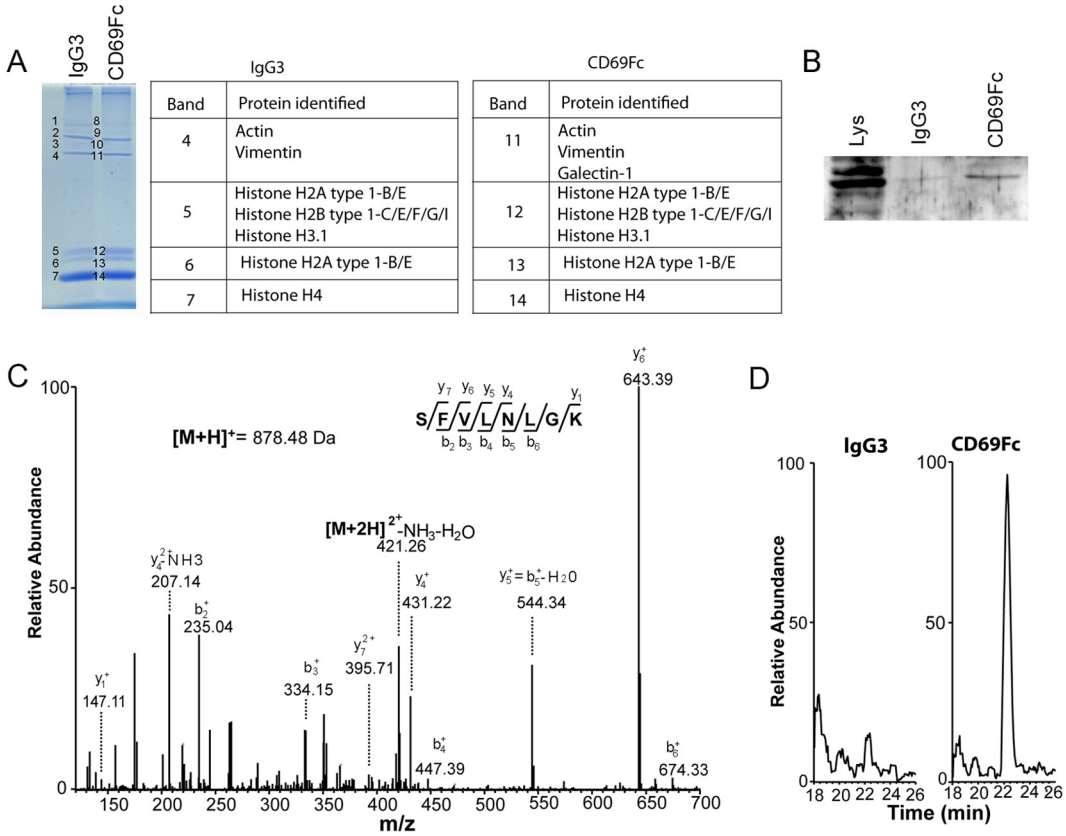

FIG 2 Identification of galectin-1 as a binding protein of CD69. (A) Identification of binding proteins in pulldown experiments performed with iDCs incubated with either IgG3 (control) or CD69-Fc. CD69-Fc and IgG3 were coupled to a photoactivatable biotin-labeled moiety and then incubated with iDCs for 1 h. Upon UV photoactivation, iDCs were lysed and incubated with streptavidin-Dynabeads. Pulldown samples were resolved on a 12% polyacrylamide gel, and the gel was stained with Coomassie blue. The indicated protein bands were sliced and subjected to in-gel trypsin digestion, and the resulting peptides were analyzed by liquid chromatography-MS/MS using an LTQ-Orbitrap XL mass spectrometer. The proteins identified in the bands numbered in the gel are listed in the tables on the right. (B) Western blot of the pulldown samples from CD69-Fc and IgG3 revealed with anti-Gal-1. CD69-Fc and IgG3 were coupled to a photoactivatable biotin-labeled moiety and then incubated with iDCs, as described for panel A. Pulldown samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The results of 1 of 3 independent experiments are shown. (C, D) Confirmation of the specific presence of Gal-1 in CD69-Fc pulldown. The peptide pool from the band was subjected to selected MS/MS ion monitoring to unequivocally confirm the presence of the Gal-1 peptide from its MS/MS spectrum (C) and to quantify the abundance of this peptide in the pulldowns from IgG3 and CD69-Fc, revealing that it was present only in the latter (D).

terized as described previously (19) (20). Recombinant Gal-1, Gal-3, and Gal-7 proteins were produced as described previously (21). The source of Gal-1 is important for protein-protein binding experiments, likely due to the presence of reducing agents during the purification procedure; thus, we avoided the use of commercial galectins.

Mice. CD69-deficient mice were generated in the C57BL/6J genetic background as described previously (5). C57BL/6-Tg (TcraTcrb) 425Cbn/J OTII mice expressing a T-cell receptor (TCR) specific for the peptide from amino acids 323 to 339 of ovalbumin in the context of I-A^b were purchased from The Jackson Laboratory, Bar Harbor, ME (stock number 004194). OTII mice were backcrossed with CD69-deficient mice in the C57BL/6 background (OTII knockout mice). Experimental procedures were approved by the CNIC Animal Welfare and Ethics Committee and conducted in accordance with institutional guidelines for the care and experimental use of animals that comply with Spanish and European Union guidelines.

Generation of CD69 recombinant proteins. CD69–Flag–glutathione *S*-transferase (GST) was generated by PCR using full-length CD69 cDNA as the template, and the Flag tag was engineered for attachment to the 5′ end of the entire extracellular domain of human CD69. The PCR product was cloned into the baculovirus transfer vector pAcSecG2T (BD Pharmingen, CA) in frame with the GST open reading frame (ORF). CD69-Fc and CD94-Fc were constructed by fusing the C-type lectin domain (CTLD) of the respective human molecules with the Fc region of human IgG3, and the fusion was cloned into the baculovirus transfer vector pAcGP67A. These three recombinant proteins were purified from insect cell supernatants using a GSTrap FF column for CD69-Flag-GST and a protein G HP column (Amersham, NJ) for CD69-Fc and CD94-Fc.

Flow cytometry and binding assays. For analyses of CD69 binding, cells were first incubated with serum to block Fc receptors and then with CD69-Fc for 1 h at 4°C (10 μg/ml), followed by staining with a secondary fluorescein isothiocyanate- or phycoerythrin-labeled anti-human Fc antibody (Jackson ImmunoResearch, CA). CD69-Flag-GST binding was detected using an anti-Flag–biotin conjugate plus streptavidin R-phycoerythrin (Molecular Probes, OR). For blocking binding assays, recombinant CD69 (10 μg/ml) was preincubated or not with anti-CD69 MAbs (20 μg/ml) or recombinant galectins for 30 min at room temperature before incubation with DCs. To detect the binding of Gal-1 to mouse T cells, lymphocytes were incubated for 30 min at 4°C with biotinylated Gal-1 and then with streptavidin-Alexa Fluor 647 (Molecular Probes). Where indicated, lymphocytes were preincubated with lactose (Lac) for 30 min at 4°C. Cells were analyzed with a FACSCalibur or with a FACSCanto flow cytometer, as indicated below.

Enzyme-linked immunosorbent assay (ELISA). High-binding 96-well plates were coated for 1 h at 37°C with human recombinant Gal-1, Gal-3, or Gal-7 (10 μg/ml), followed by a blocking step of 1 h at 37°C with

2% (wt/vol) bovine serum albumin, and then incubated for 1 h at 37°C with CD69-Fc or IgG3 (2 μg/ml). After extensive washing, biotinylated anti-human Fc was added for 1 h at 37°C. Binding was detected with peroxidase-labeled streptavidin.

Cellular adhesion assays. Jurkat cells and Jurkat cells expressing CD69 (J1; JKCD69) were loaded with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) and seeded at a density of 0.2×10^5 cells per well in 96-well plates precoated with recombinant Gal-1, Gal-3, or Gal-7. Cells were incubated for the indicated times at 37°C, and then, nonadherent cells were removed by gentle washing. The number of adhered cells was quantified on a Fluorstar spectrofluorimeter.

Polarization of human and mouse Th17 cells. For human Th17 polarization, naive $CD4^+$ T cells were purified to a purity of >96% by immunomagnetic depletion with a human naive $CD4^+$ T cell isolation kit II (Miltenyi Biotec, CA). Human $CD4^+$ T cells (1×10^6) were polarized as described previously (22). Where indicated, plates were coated with recombinant Gal-1 (10 μg/ml), and anti-CD69 (TP1/55) or an isotype control (10 μg/ml) was added to the cultures. After 10 days, the percentage of interferon (IFN)- and interleukin-17 (IL-17)-producing cells was analyzed by intracellular staining. Mouse naive $CD4^+$ T cells were isolated from spleen and lymph nodes by negative selection using an autoMACSPro separator (Miltenyi Biotec) according to the manufacturer's instructions. For polyclonal activation, naive $CD4^+$ T cells (0.2×10^6 cells/well) were stimulated with plate-bound anti-CD3 (5 mg/ml) plus CD28 (2 mg/ml) MAbs for 72 h. For Th17 differentiation, $CD4^+$ T cells were also cultured for 5 days with anti-CD3 plus CD28 MAbs in the presence of a cytokine and antibody combination appropriate for polarization: recombinant murine IL-6 (rmIL-6; 20 ng/ml), recombinant murine IL-23 (20 ng/ml), recombinant human transforming growth factor β1 (10 ng/ml), anti-IFN-γ (10 mg/ml), and anti-IL-4 (10 mg/ml). Where indicated, plates were coated with recombinant Gal-1 (10 μg/ml). For analysis of IL-17 production, activated $CD4^+$ T cells or fully differentiated Th17 cells were restimulated with 50 ng/ml phorbol myristate acetate (PMA) and 750 ng/ml ionomycin for 6 h. Cytokine production was determined using a BD cytometric bead array for IL-17, followed by flow cytometry analysis on a BD FACSCanto II cytometer or intracellular staining. All cytokines and antibodies were purchased from R&D Systems, MN.

Pulldown and biochemical assays. For the pulldown assay, CD69-Fc and IgG3 were labeled with sulfo-SBED, a cross-linker reagent containing a photoactivatable biotin residue (Pierce, IL). Human dendritic cells were incubated with the biotin-labeled proteins for 1 h, and after UV photoactivation, cells were lysed in 0.5% NP-40 containing phosphate and protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). Pulldown was performed using streptavidin-Dynabeads for 3 h at 4°C. After 5 washes with lysis buffer, the beads were resuspended in Laemmli buffer and resolved by SDS-PAGE. After staining with Coomassie blue, gel samples were digested in gel with trypsin, and the resulting peptides were identified by mass spectrometry (MS) as described previously (23). Selected tandem MS (MS/MS) ion monitoring was performed as described previously (24). Primary antibodies for immunoblotting were goat polyclonal anti-Gal-1, anti-Gal-3, and anti-Gal-9 (R&D Systems, MN) and monoclonal anti-Gal-1 (Vector Laboratories, CA).

Surface plasmon resonance. CD69-Fc or CD94-Fc was covalently immobilized to functionalized carboxymethylated dextran on a sensor chip. The sensor chip surface was activated with a 4:1 molar ratio of 1-ethyl-3-(3-dimethylpropyl)-carboiimide and *N*-hydroxysuccinimide in water. Proteins were injected at ~75 μg/ml in sodium acetate (pH 4.0) until the appropriate immobilization level was reached. Remaining active groups were neutralized with ethanolamine. Solutions of Gal-1 or Gal-3 (purified as described previously [21]) at the indicated concentrations in HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% [vol/vol] surfactant P20) buffer were injected for 3 min over CD69-Fc and CD94-Fc flow cells and allowed to dissociate for at least 3 min before regeneration with lactose (50 mM). The flow cell with immobilized CD94-Fc was used as a reference surface, and double referencing was performed. Kinetic parameters (k_a and the dissociation constant [K_d]) and equilibrium dissociation constants (K_Ds) were determined by nonlinear fitting of the sensorgrams to a 1:1 interaction model (Langmuir fitting) using BIAevaluation software (version 4.0.1).

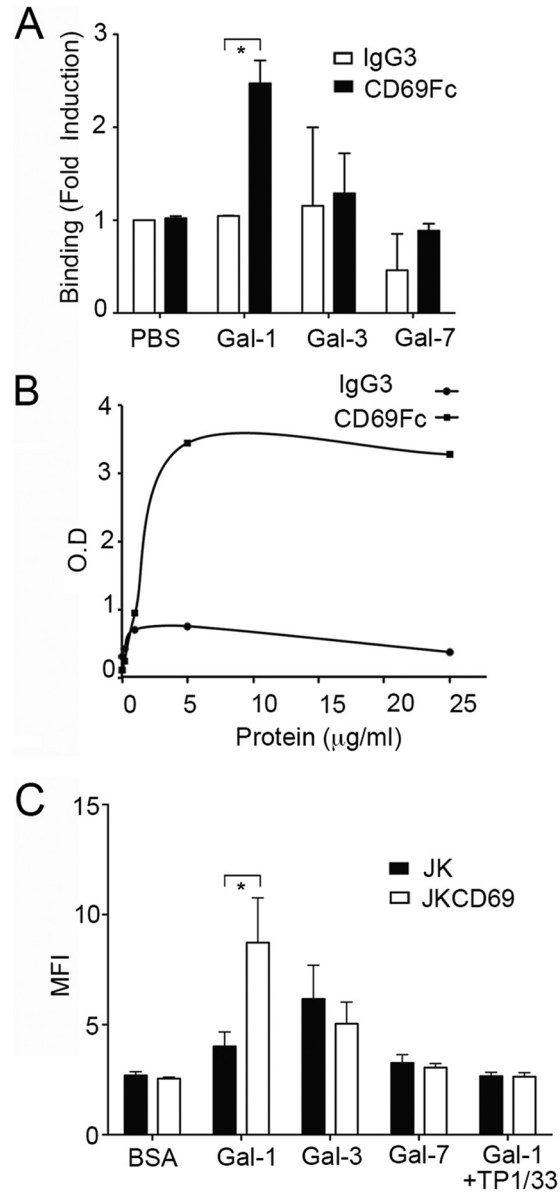

FIG 3 Specific association of CD69 with galectin-1. (A) Human recombinant galectins (10 μg/ml) were precoated on 96-well plates, and then CD69-Fc or IgG3 (10 μg/ml) was added. The interaction of CD69-Fc or IgG3 was assessed by ELISA. Bars correspond to the means ± SDs of three experiments. *, $P = 0.007$ (*t* test) for CD69-Fc versus IgG3. (B) Increasing doses of recombinant Gal-1 were coated on a plate, and the interaction of CD69-Fc or control IgG3 was detected, as described for panel A. O.D, optical density. (C) Results of assays for adhesion of Jurkat cells (JK) or CD69-expressing Jurkat cells (JKCD69) to recombinant galectins. Jurkat cells were loaded with CFSE and seeded onto plates precoated with recombinant Gal-1, Gal-3, or Gal-7. Where indicated, anti-CD69 MAb TP1/33 was incubated with cells before its addition to plates coated with galectins. The graph presents the mean ± SD of 3 independent experiments. BSA, bovine serum albumin. *, $P = 0.007$ by one-way analysis of variance and the Bonferroni test.

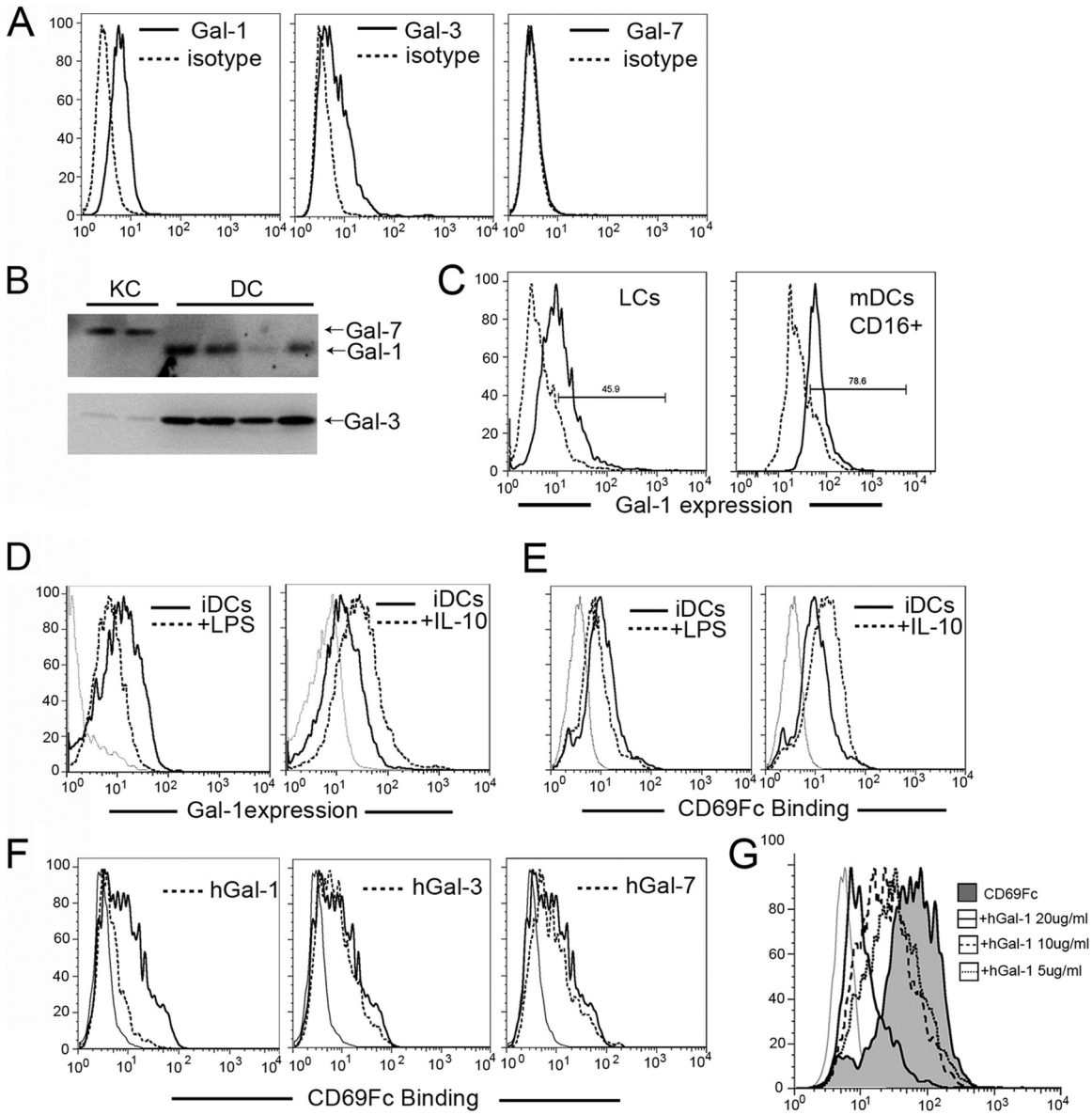

FIG 4 Binding of CD69-Fc to Gal-1 expressed on human DCs. (A) Surface expression of Gal-1, Gal-3, and Gal-7 in iDCs analyzed by flow cytometry. (B) Expression of Gal-1, Gal-3, and Gal-7 in protein lysates from iDCs analyzed by immunoblotting. Total lysates of human keratinocytes (KC) were included as controls. (C) Gal-1 expression on human skin Langerhans cells and peripheral CD16$^+$ myeloid DCs. LCs were isolated from a skin sample of a healthy subject. Peripheral mDCs were purified by cell sorting. After purification, mDCs were tested for CD69-Fc binding. Binding of CD69 to mDCs is shown. Continuous line, Gal-1 expression; dotted line, Alexa Fluor 647–donkey anti-goat signal. (D) Gal-1 expression in iDCs stimulated with LPS or IL-10. Dotted line, isotype control. (E) Representative binding of CD69-Fc to DCs exposed to IL-10 and LPS. Dotted lines, IgG3 binding. The data in panels A to E are from 1 out of 3 independent experiments. (F) CD69-Fc binding to iDCs is prevented by preincubation with recombinant human Gal-1 (hGal-1). CD69-Fc was preincubated with 20 μg/ml human Gal-1, human Gal-3 (hGal-3), or human Gal-7 (hGal-7) before its addition to iDCs. Histograms are representative of those from 4 independent experiments. Thick lines, CD69-Fc; thin lines, IgG3. (G) Inhibition of CD69-Fc binding by increasing doses of human Gal-1. Thick lines, CD69-Fc; thin lines, IgG3. Data from 1 out of 2 experiments are shown. *y* axis values are percentages of maximum.

RESULTS

We generated two different recombinant proteins containing the extracellular domain of human CD69 (CD69-GST and CD69-Fc; Fig. 1A). Both recombinant proteins resolved as dimers by gel electrophoresis under reducing and nonreducing conditions (Fig. 1B). CD69 chimeric proteins were able to bind to human immature monocyte-derived DCs (iDCs) as well as primary Langerhans cells (LCs) and human peripheral myeloid DCs, whereas no binding to other leukocyte cell types, such as monocytes or peripheral blood lymphocytes, was detected (Fig. 1C and D). Furthermore, blast T cells showed slight binding of CD69, while a binding signal was not detected in activated B lymphocytes or a panel of human cell lines (data not shown). The binding of CD69-Fc and CD69-GST to iDCs was dose dependent (Fig. 1E and data not shown), and the binding of recombinant proteins was specifically inhibited by anti-human CD69 MAbs (Fig. 1F). In these assays, only MAbs directed to epitope E1 were able to inhibit the binding of CD69-Fc to iDCs (Fig. 1F), whereas anti-CD69 MAbs that recognize other

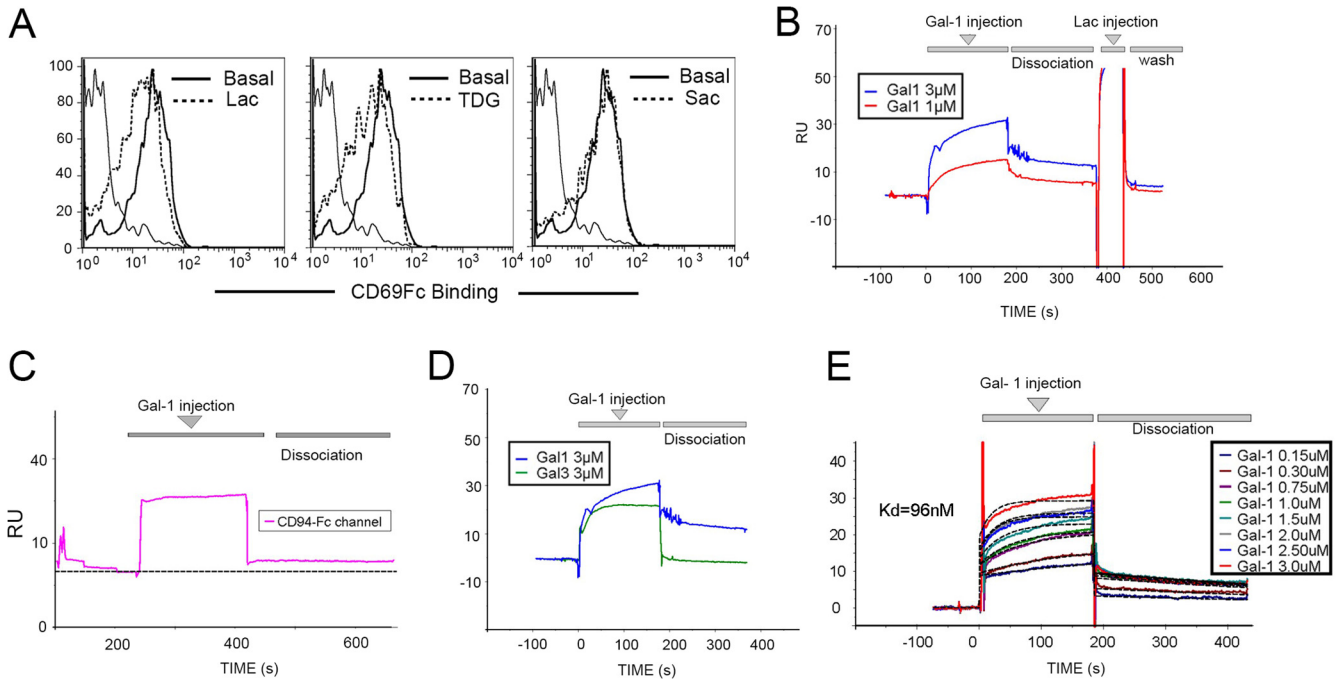

FIG 5 Direct and carbohydrate-dependent interaction on CD69 to Gal-1. (A) CD69-Fc binding to iDCs is dependent on carbohydrate recognition. Binding assays were performed in the presence or absence of 50 mM lactose (Lac), 2 mM thiodigalactoside (TDG), or 50 mM saccharose (Sac). Dotted lines, carbohydrates; thick lines, CD69-Fc; thin lines, IgG3. The results of 1 out of 3 experiments are shown. *y* axis values are percentages of maximum. (B to E) SPR analysis of the interaction between CD69 and galectin-1. Differential surface plasmon resonance was performed over a surface coated with either CD69-Fc or CD94-Fc, and the sensorgrams show the normalized response (in relative units [RU]) to CD69-Fc/CD94-Fc. (B) Two different concentrations of Gal-1 (3.0 and 1.0 μM) were injected for 3 min over CD69-Fc and CD94-Fc flow cells, and after a 3-min dissociation, 50 mM lactose (1 min) was injected. (C) Control SPR analysis of the lack of interaction of CD94-Fc and Gal-1. Differential surface plasmon resonance was performed over a surface coated with CD94-Fc. (D) Gal-1 and Gal-3 (3.0 μM) were injected through both flow cells, as described for panel B. (E) Kinetic experiment. Increasing concentrations of Gal-1 (0.15 to 3.0 μM) were injected through both flow cells, and the kinetic constant was obtained from mathematical fitting.

epitopes (E2 and E3) or isotype control MAbs did not inhibit or only partially blocked the binding of CD69 (Fig. 1F).

To identify the putative cellular ligands of CD69 expressed by DCs, assays in which photoactivatable biotin-labeled CD69-Fc was allowed to bind to iDCs under cross-linking conditions were performed. CD69-Fc and IgG3 (as a control) pulldown samples were resolved by polyacrylamide gel electrophoresis, and proteins present in the Coomassie-stained bands were identified by mass spectrometry; Gal-1 was identified in one of the bands from CD69-Fc but not in the band from the control (Fig. 2A). Western blotting from CD69-Fc pulldown corroborated the specific binding to Gal-1 (Fig. 2B). The specific interaction of Gal-1 with CD69-Fc was confirmed by performing selected MS/MS ion monitoring of a Gal-1-specific peptide (Fig. 2C and D).

The binding of CD69-Fc to different human recombinant galectins was tested by ELISA. CD69-Fc interacted with Gal-1, whereas no binding to Gal-7 or Gal-3 was observed (Fig. 3A and B). Jurkat cells expressing CD69 (JKCD69) also specifically interacted with Gal-1, mediating their adhesion to plastic surfaces coated with Gal-1, which was blocked with anti-CD69 MAb (Fig. 3C).

Galectin expression was analyzed in human DCs using flow cytometry and Western blot assays (Fig. 4A and B). Expression of Gal-1 and Gal-3 but not Gal-7 was observed on the surface of iDCs (Fig. 4A). Gal-1 was also expressed in primary LCs and peripheral myeloid DCs (Fig. 4C) Interestingly, LPS treatment decreased the expression of Gal-1 on iDCs, whereas the cytokine IL-10 exerted the opposite effect (Fig. 4D). Accordingly, LPS stimulation reduced the binding of CD69-Fc, while preincubation with IL-10 enhanced it (Fig. 4E). The binding of CD69-Fc to iDCs was prevented by the addition of soluble recombinant Gal-1 but not Gal-3 or Gal-7 (Fig. 4F and G).

The results of assays of the binding of CD69-Fc to iDCs in the presence of lactose or thiodigalactoside (TDG) (Fig. 5A) suggested that the interaction with Gal-1 is influenced by carbohydrates. Surface plasmon resonance (SPR) analysis using a Biacore biosensor demonstrated a direct interaction between CD69 and Gal-1 (Fig. 5B). The presence of lactose (50 mM) inhibited the binding of Gal-1 to CD69-Fc (Fig. 5B), indicating that carbohydrate moieties contribute to this interaction. Gal-1 did not bind C-type lectin CD94-Fc (Fig. 5C), and Gal-3 did not interact with immobilized CD69-Fc, confirming the binding specificity (Fig. 5D). Kinetic experiments showed that the response with CD69-Fc immobilized on the sensor chip was augmented with increasing concentrations of human Gal-1, and the data could be fitted to a 1:1 binding model with a K_d of 96 nM (Fig. 5E).

We then explored the possible functional consequences of the CD69–Gal-1 association. As shown in Fig. 6A and B, recombinant Gal-1 was able to inhibit IFN-γ- and IL-17-producing cells in a Th17 polarization culture, a phenomenon that was blocked with the anti-CD69 MAb TP1/55. Accordingly, the inhibitory effect of Gal-1 on the mRNA levels of RORC2, a key Th17 transcription factor, was also reverted by the anti-CD69 MAb TP1/55 (Fig. 6C). In contrast, an isotype-matched antibody did not affect Th17 cell

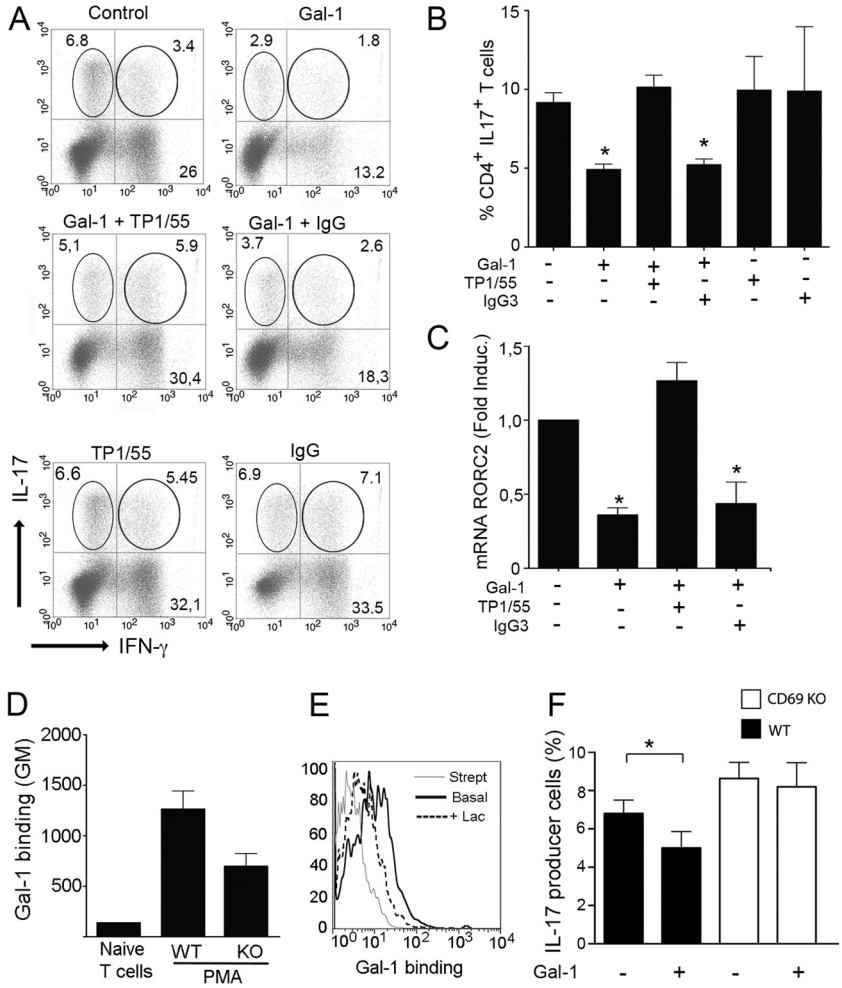

FIG 6 Human Th17 cell differentiation is regulated by the Gal-1–CD69 association. (A) Analysis of intracellular IFN-γ and IL-17 expression by flow cytometry. Human naive $CD4^+$ T cells were differentiated toward effector Th17 cells in the presence of the indicated stimuli. After 10 days, the percentages of IFN-γ- and IL-17-producing cells were analyzed. (B) The bar charts represent the means ± SDs of 4 independent experiments of total $CD4^+$ IL-17-positive T cells obtained with the different stimuli. (C) Reverse transcription-PCR analysis of the transcription factor RORC2 in differentiated Th17 cells. Induc., induction. *, $P = 0.04$ by one-way analysis of variance and the Bonferroni test. (D) $CD4^+$ T cells isolated from LNs of CD69 OTII knockout (KO) or OTII WT mice were stimulated with PMA and incubated with biotinylated Gal-1 for 30 min at 4°C. After staining with streptavidin-Alexa Fluor 647, cells were analyzed by flow cytometry. Bars correspond to means ± SDs of Gal-1 binding, represented as the geometric mean (GM) fluorescence intensity ($n = 3$). (E) Gal-1 binding to activated lymphocytes is inhibited by Lac. LN lymphocytes were preincubated with Lac for 30 min at 4°C, and Gal-1 binding was analyzed. Strept, streptavidin. (F) Naive $CD4^+$ T cells (0.2×10^6 cell/well) were cultured with anti-CD3 (5 mg/ml) plus anti-CD28 (2 mg/ml) MAbs in the presence of a cytokine and antibody combination appropriate for Th17 polarization. Where indicated, recombinant Gal-1 (10 μg/ml) was included. The percentage of cells producing IL-17 was determined by intracellular staining.

differentiation in either the absence or the presence of Gal-1. In addition, MAb TP1/55 alone did not exert any effect on the percentage of $CD4^+$ IL-17-positive or IFN-γ-positive T cells. At the low dose employed (10 μg/ml), no significant Gal-1-induced apoptosis or loss of viability was observed (data not shown).

Gal-1 ligands are absent from naive mouse T cells but highly expressed on activated $CD4^+$ T cells in lymph nodes (LNs) draining antigen-sensitive skin (25). To assess the possible association of Gal-1 with CD69 in mouse cells, we performed binding assays with naive and PMA-activated $CD4^+$ T cells from WT and CD69-deficient mice. We found that recombinant Gal-1 did not interact with resting $CD4^+$ T cells but bound to PMA-activated lymphocytes. In addition, activated T cells from $CD69^{-/-}$ mice showed decreased binding of Gal-1 compared with activated WT T cells (Fig. 6D). As expected, Gal-1 binding was inhibited by lactose (Fig. 6E). To further assess the role of the CD69/Gal-1 interaction in mouse IL-17 production, Th differentiation was carried out using CD69-deficient T cells. Recombinant Gal-1 was able to inhibit Th17 differentiation in a Th17 polarization culture of WT $CD4^+$ T cells, while CD69-deficient cells were unresponsive to the Gal-1 effect (Fig. 6F). Thus, Gal-1 binds mouse CD69, and this association modulates the differentiation of mouse Th17 lymphocytes.

DISCUSSION

The leukocyte activation receptor CD69 controls inflammation in several autoimmune processes by limiting the differentiation of Th17 pathogenic cells. However, full elucidation of the functional role of CD69 in immune responses *in vivo* has constantly been hampered by the unknown identity of the CD69 ligand(s). In this

study, we identified Gal-1 to be a ligand for CD69. Transfection assays, blocking antibodies, and pulldown, proteomic, and SPR studies demonstrated that Gal-1 but not other galectins binds to the extracellular domain of CD69. Gal-1 is expressed by DCs, and its expression is upregulated by tolerogenic stimuli correlating with increased binding of the extracellular domain of CD69 to these cells. We have identified a key function for CD69 in the regulation of both mouse and human Th17 effector cells through the interaction with Gal-1.

CD69 belongs to the C-type lectin superfamily and is a member of the NK receptor family (1). The ligands of a number of NK receptors have recently been identified and characterized, including Nkrp1d and Nkrp1f (26, 27), lectin-like transcript 1 (LLT1) (28), and NKp80 (also called KLRF1) (29); some of these ligands are lectins (30). However, the ligand(s) for CD69 has remained elusive, although it has been postulated to involve carbohydrate moieties (31, 32). Remarkably, our data demonstrate the association of CD69 with Gal-1. CD69 contains sites for N-glycosylation (33), and conceivably, the interaction of Gal-1 with CD69 may involve carbohydrate recognition. Indeed, our data show that in the presence of lactose, CD69 binding to iDCs is partially inhibited. SPR studies not only confirmed the direct and specific interaction of CD69 with Gal-1 but also demonstrated that this interaction is influenced by carbohydrates. Other additional membrane glycoproteins, besides CD69, have been reported to be binding partners of Gal-1 in T cells, including CD2, CD3, CD4, CD7, CD43, CD45, CD90, and Thy-1 (34–37). Some of these Gal-1 partners have been described to be responsible for apoptosis induction in T cells. Our results show that addition of low concentrations of Gal-1 inhibits Th17 differentiation through CD69 interaction on activated T cells. However, this suppressive effect is not due to Gal-1-induced apoptosis. Thus, besides the induction of apoptosis, nonapoptotic mechanisms might contribute to the immunosuppressive effects of this protein. In accord with this, similar results were observed when low concentrations of Gal-1–human Fc were incubated with human skin-resident memory T cells, which dramatically lowered the numbers of IL-17-producing T cells (25). Depending on the dose of recombinant Gal-1 treatment, the effects could be different (25, 37). In this regard, an irreversible dimeric form of Gal-1 is a potent inducer of apoptosis in T cells (38). Recently, the Gal-1 association with CD45 has been reported to modulate IL-10 (39). However, our data provide an unequivocal explanation for Gal-1 regulation of Th17 differentiation through the interaction with CD69.

We have previously reported that CD69$^{-/-}$ mice develop an exacerbated form of autoimmune disease due to an enhanced inflammatory response associated with Th17 lymphocytes. This regulatory effect of CD69 is mediated, at least in part, through the activation of the Jak3/Stat5 signaling pathway, which inhibits Th17 differentiation (5). Although the putative mechanism that triggers the regulatory effect mediated by CD69 remains to be established, we have previously proposed the existence of a cellular ligand for CD69 on antigen-presenting cells (APCs) at specific times or in specific cell subsets (i.e., tolerogenic DCs) (40). Our results demonstrate that DC-expressed Gal-1 fulfills these criteria as a counterreceptor for CD69 and that this ligand-receptor pair represents a novel regulatory pathway for the control of inflammation and immune-mediated tissue damage mediated largely by Th17 cells. Thus, our data further support the suggestion that CD69 is a therapeutic target in inflammation (40) and reinforces the potential for Gal-1 to be an immunomodulatory agent.

In summary, we have identified for the first time a cellular counterreceptor for CD69 which exerts a relevant functional role, mainly in the differentiation of Th17 lymphocytes. These data significantly contribute to our understanding of the pathophysiological role of CD69 and further confirm the relevance of its immune-regulatory effect.

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H. de la Fuente and A. Cruz-Adalia performed experimental work and data analysis and wrote the manuscript, G. Martinez del Hoyo, D. Cibrian-Vera, and M. Ramirez-Huesca performed experiments with cells from deficient mice, P. Bonay purified Gal-1 and created some of the experimental design, D. Pérez-Hernández and J. Vázquez performed proteomic assays, P. Navarro and R. Gutierrez-Gallego performed the SPR protein interaction experiments, P. Martín designed the experiments and prepared the discussion of the data, and F. Sánchez-Madrid designed experiments, performed experimental work, analyzed the data, and wrote the manuscript.

We declare that we do not have any conflicts of interest.

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