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CD69+CD4+CD25− T Cells, a New Subset of Regulatory T Cells, Suppress T Cell Proliferation through Membrane-Bound TGF-β1

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The underlying mechanisms of tumor-induced immune suppression need to be fully understood. Regulatory T (Treg) cells have been shown to play an important role in tumor immune escape. Until now, many subsets of Treg cells have been described that can suppress T cell response via different mechanisms. CD69 is generally regarded as one of the activating markers; however, recent studies show that CD69 may exert regulatory function in the immune response. In this study, we have identified tumor-induced CD69+CD4+CD25− T cells as a new subset of CD4+ Treg cells. CD69+CD4+CD25− T cells increase dramatically along tumor progression, with up to 40% of CD4+ T cells in the advanced tumor-bearing mice. Distinct from the previously described CD4+ Treg cell subsets, CD69+CD4+CD25− T cells express high CD122, but they do not express Foxp3 and secrete IL-10, TGF-β1, IL-2, and IFN-γ. CD69+CD4+CD25− T cells are hyperresponsive and can suppress CD4+ T cell proliferation in a cell-cell contact manner. Interestingly, the fixed CD69+CD4+CD25− T cells still have suppressive activity, and neutralizing Abs against TGF-β1 can block their suppressive activity. We found that CD69+CD4+CD25− T cells express membrane-bound TGF-β1, which mediates suppression of T cell proliferation. Furthermore, engagement of CD69 maintains high expression of membrane-bound TGF-β1 on CD69+CD4+CD25− T cells via ERK activation. Our results demonstrate that CD69+CD4+CD25− T cells act as a new subset of regulatory CD4+ T cells, with distinct characteristics of negative expression of Foxp3, no secretion of IL-10, but high expression of CD122 and membrane-bound TGF-β1. Our data contribute to the better understanding of mechanisms for tumor immune escape. The Journal of Immunology, 2009, 182: 111–120.
of TGF-β in inflamed joints, suggesting that CD69 may also exert a regulatory function (20).

During analysis of T cell subsets in tumor-bearing models, including liver cancer-, lung cancer-, and melanoma-bearing mice, we observed that the number of splenic CD69+CD4+ T cells increased dramatically along with tumor progression. Interestingly, we prove that CD69+CD4+ T cells are CD25-negative and that they do not express Foxp3 and secrete IL-10, TGF-β1, IL-2, and IFN-γ. Importantly, CD69+CD4+CD25− T cells can suppress T cell proliferation via membrane-bound TGF-β1, and CD69 engagement maintains expression of membrane-bound TGF-β1 via ERK activation. Our results demonstrate that CD69+CD4+CD25− T cells are a new subset of regulatory CD4+ T cells involved in tumor-induced immunosuppression. Furthermore, we provide novel evidence for the regulatory function of CD69.

Materials and Methods

**Mice and cell lines**

C57BL/6j mice and BALB/c mice were obtained from Joint Ventures. Sipper BK Experimental Animal and used at the age of 6–8 wk. OVA233-339-specific TCR-transgenic mice (DO11.10) were obtained from The Jackson Laboratory and were bred in specific pathogen-free conditions. All experimental manipulations were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Second Military Medical University, Shanghai, China. The murine liver carcinoma cell line (Hepa), Lewis lung carcinoma cell line (3LL), and melanoma cell line (B16) were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium (PAA Laboratories) supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% (v/v) heat-inactivated FCS (PAA Laboratories).

**Reagents**

Recombinant mouse TGF-β1 (rTGF-β1) and anti-TGF-β1 Ab (1D11) and its isotype control mouse IgG1 mAb (clone 11711) were obtained from R&D Systems. The Abs for flow cytometry, including allophycocyanin-labeled Abs against CD25 (PC61), FITC-labeled Abs against CD69 (H1.2F3) and biotin, PE-labeled Ab against CD122 (TM-β1), PerCP-labeled Abs against CD4 (L3T4), fluorescence-conjugated anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), anti-IL-10 (JES5-16E3), anti-TNF-α (MP6-XT22), anti-IFN-γ (XMG1.2) or anti-ERK1/2 (P202/Py204, 20A) mAbs or isotype-matched Ig controls. For intracellular staining of ERK1/2, mixed leukocyte reactions, and analysis of intracellular cytokine expression and ERK activation by FACS, cells were labeled with CFSE (Sigma-Aldrich), and then the target cells were sorted by flow cytometry and then used as maDCs.

**Flow cytometry**

Before staining with fluorescent Abs, all cells were incubated for 15 min at 4°C with Ab to CD16/CD32 at a concentration of 1 mg/10 ml for blockade of Fc receptors. Fluorescent Abs and the respective isotype controls were then added at a concentration of 1 mg/10 ml to 100 ml and cells were incubated for a further 30 min at 4°C. The cells were washed once with ice-cold PBS (pH 7.2) containing 0.1% NaN3 and 0.5% BSA and resuspended in 200 μl PBS. Cell phenotype was analyzed by flow cytometry with a FACS LSRII (BD Biosciences), and data were analyzed with FACSDiva software (24). Membrane-bound TGF-β1 expression was determined by mean fluorescence intensity (MFI) on CD69+CD4+CD25− T cells and CD69+CD4+CD25+ T cells.

**Detection of cytokine secretion by ELISA**

For assay of cytokine secretion, cell supernatants were collected and the cytokine concentrations were determined by ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Analysis of intracellular cytokine expression and ERK activation by FACS**

For intracellular staining, the cells were stained with the Abs against cell-surface Ag and then fixed and permeabilized (IC fixation/permeabilization buffer; eBioscience) for 20 min. The cells were washed with permeabilization buffer and labeled with cytokine-specific fluorescence-conjugated anti-IL-2, anti-IL-4, anti-IL-10, anti-TNF-α, anti-TGF-β1, or anti-IFN-γ mAbs or isotype-matched Ig controls. For cellular staining of ERK1/2 (P202/Py204), the cells were fixed with prewarmed the BD Cytofix buffer, permeabilized with BD Phosflow Perm buffer (BD Biosciences), and then labeled with Alexa Fluor 647-conjugated ERK1/2 (P202/Py204). Flow cytometry was done with a FACS LSRII and data were analyzed with FACSDiva software. ERK1/2 (P202/Py204) expression was determined by MFI on CD69+CD4+CD25− and CD69+CD4+CD25+ T cells.

**Mixed leucocyte reactions**

CD69+CD4−CD25− or CD4+CD69+ T cells (5 × 105/ml) were purified from splenocytes of tumor (Hepa)-bearing mice (2 wk after tumor inoculation) and cultured with allogeneic maDCs for 5 days at the ratio of 10:1 (T cells/maDCs). Then the cells were harvested, double stained with anti-CD4-FITC and 7-aminoactinomycin D (7-AAD) reconstituted in 200 μl PBS, and cellular data were acquired for 70 s with a flow cytometer (1 × 106 PE-labeled beads 3 mm in diameter were added to each well as an internal control before Ab labeling). The numbers of CD4+ 7-AAD-negative live cells and control bead events acquired were analyzed, and the total cells in each well were calculated according to the formula: total no. = (no. live CD4+ no. beads) × 105. In a variation of this assay, MLR (T cell/maDC coculture) was performed using CD69+CD4+CD25− or CD4+CD69+ T cells that were labeled with CFSE (Sigma-Aldrich), and then the degree of cell division was gauged from the serial diminution of the CFSE label, as determined by flow cytometry.

**Isolation and purification of CD69+CD4+CD25− T cells and CD69+CD4+CD25+ T cells**

Single-cell suspensions of splenocytes were prepared from tumor (Hepa)-bearing mice 2 wk after tumor inoculation. For isolation of CD69+CD4+CD25− T cells and CD69+CD4+CD25+ T cells, the cells were stained with allophtocyanin-labeled anti-CD25 mAb, FITC-labeled anti-CD69 mAb, and PerCP-labeled anti-CD4 mAb, and then the target cells were sorted by a MoFlo high-speed cell sorter (Dako). The purity of each population was confirmed by FACS to be >97%.

**Preparation of mature dendritic cells (maDCs) from mouse bone marrow**

DCs were prepared from bone marrow progenitors by culturing in 10 ng/ml recombinant mouse GM-CSF and 1 ng/ml of recombinant mouse IL-4, IL-7, IL-15, and Flt3L as described previously (23). Nonadherent cells were gently washed out on day 3 of culture, and the remaining loosely adherent clusters were cultured for a further 4–5 days in the presence of 10 ng/ml LPS (Sigma-Alrich). On day 8, cells were positively selected from floating cells with CD11c magnetic microbeads (Miltenyi Biotec). CD11c+CD11b+CD86+ cells were sorted by flow cytometry and then used as maDCs.

**Isolation of mononuclear cells (MNC) from tumor tissue**

Tumors, obtained from tumor-bearing mice at different stages, were digested with collagenase IV (Sigma-Aldrich) for 3 h, and then the single-cell suspension was passed through a 40-μm cell strainer (BD Falcon). MNC populations were purified by centrifugation through a Percoll gradient. Cells were collected, washed in PBS, and resuspended in 40% Percoll (Sigma-Aldrich) in complete RPMI 1640 medium. The cell suspension was gently overlayed onto 70% Percoll and centrifuged for 20 min at 750 × g. MNC were collected from the interface. Cells were washed twice in PBS and resuspended in complete RPMI 1640 medium.

**Preparation of orthotopic tumor-bearing models**

An orthotopic hepatic tumor model was established by subcapsular intrahepatic injection of Hepa cells (1 × 107/50 μl per mouse) into the left liver lobe of mice (21). An orthotopic lung cancer model was prepared by intrapulmonary injection with 3LL cells (1 × 105/50 μl per mouse) as described previously (22). The murine melanoma-bearing mouse model was established by s.c. injection of B16 cells (5 × 105/50 μl per mouse). Tumors were measured every 7 days using Vernier calipers, and volume was calculated as (length × width2) × 0.52.
Assay for Ag-specific T cell proliferation in vitro

Splenic CD4 T cells from (DO11.10 × C57BL/6)F1 hybrid mice were positively selected by magnetic-activated cell sorting for use as Ag-specific responders, labeled with CFSE or not, and then cocultured for 5 days with live maDCs, together with or without CD69+CD4+CD25+ T cells, in the presence of OVA323–339 at a ratio of 10:1 (T cells/DCs) (1 × 10⁶ T cells in 200 µl/well). The ratios of maDCs and CD69+CD4+CD25+ T cells were 1:1. For blocking assay, anti-TGF-β1 mAb was added to the coculture system, with the concentration up to 10 µg/ml. To investigate whether soluble factors or cell-cell contact was responsible for the suppressive effect of CD69+CD4+CD25+ T cells, CD4+CD69+CD25+ T cells were fixed with 1% glutaraldehyde and then added to the coculture system, or 0.4-µm transwells were used to separate CD69+CD4+CD25+ T cells from maDCs/T cells in the coculture system. Five days later, the cells were stained with anti-CD4-FITC, PE-labeled clonotypic mAb (KJ1-26; BD Pharmingen), and 7-AAD resuspended in 200 µl PBS, and cellular data were acquired for 70 s with a flow cytometer. The number of CD4+KJ1-26+7-AAD− live cells was analyzed as above.

FIGURE 1. Increase of CD69+CD4+ T cells in spleen of tumor-bearing mice. A. The splenocytes of orthotopic liver cancer (Hepa)-, Lewis lung carcinoma (3LL)-, and melanoma (B16)-bearing mice at different stages (7, 14, and 21 days after tumor inoculation) were stained with CD3-PE, CD4-PerCP, and CD69-FITC. CD4+ T cells were gated in the FACS analysis. The dot plots represent one of four independent experiments with similar results. B. The MNC of tumor mass and tumor draining lymph nodes isolated from the above orthotopic liver cancer-, Lewis lung carcinoma-, and melanoma-bearing mice (2 wk after tumor inoculation) were stained with CD3-PE, CD4-PerCP, and CD69-FITC. CD4+ T cells were gated in the FACS analysis. The dot plots represent one of three independent experiments with similar results.
Distinct phenotype and cytokine profile of tumor-induced \( CD69^+ \) \( CD4^+ \) \( CD25^- \) T cells

OVA\(_{23-33}\)-specific TCR-transgenic splenic CD4 T cells (5 \( \times \) 10\(^6\)) from F1, hybrid mice were injected i.p. into each F1 (BALB/c \( \times \) C57BL/6j) hybrid mouse. After 24 h, 5 \( \times \) 10\(^6\) OVA\(_{23-33}\)-pulsed maDCs, together with or without the same number of CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells, were transferred i.p. into the mice. After 5 days, single-cell suspensions of mesenteric lymph nodes (MLN) and spleen were prepared, and the cells were stained with anti-CD4-FITC, PE-conjugated KJ1-26 and 7-AAD, and then analyzed by flow cytometry. The ratio of KJ1-26\(^+\) cells to CD4 cells was calculated.

Statistical analysis

Data were analyzed for statistical significance using Student’s \( t \) test. Survival estimates and median survivals were determined using the method of Kaplan and Meier. Statistical significance was determined as \( p \) values <0.05.

Results

Significant increase of CD69\(^+\) CD4\(^+\) T cells in the spleen and MLN of tumor-bearing mice

Splenic CD4\(^+\) T cells were purified from tumor-bearing mice 7, 14, or 21 days after tumor cell inoculation and analyzed for components of subpopulations. As shown in Fig. 1A, the percentage of CD69\(^+\) CD4\(^+\) T cells in splenic CD4\(^+\) T cells from orthotopic liver cancer-bearing mice increased significantly along with tumor progression, and increased to 40–50% among the total splenic CD4\(^+\) T cells of liver cancer-bearing mice in the advanced stage. In normal mice, the percentage of CD69\(^+\) CD4\(^+\) T cells in splenic CD4\(^+\) T cells was <6%. Similar results of the dramatic increase of CD69\(^+\) CD4\(^+\) T cells in splenic CD4\(^+\) T cells were also observed in the lung cancer- and melanoma-bearing mice. Additionally, the dramatic increase of CD69\(^+\) CD4\(^+\) T cells in CD4\(^+\) T cells from tumor tissue and tumor draining lymph nodes was also observed in all three kinds of tumor-bearing mice (Fig. 1B). The data suggest that CD69\(^+\) CD4\(^+\) T cells can be induced and expanded systemically in tumor-bearing mice.

Distinct phenotype and cytokine profile of tumor-induced CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells

Next, we wanted to know the characteristics of this population of CD69\(^+\) CD4\(^+\) T cells induced and expanded by tumor. We sorted CD69\(^+\) CD4\(^+\) T cells and CD69\(^+\) CD4\(^+\) T cells from splenic CD4\(^+\) T cells of orthotopic liver cancer-bearing mice and then analyzed their expression of CD25, CD27, CD44, CD62L, CD122, and CD127. We found that there was no obvious difference in the expression of CD27, CD44, CD46L, and CD127 between the two populations (data not shown). Interestingly, CD69\(^+\) CD4\(^+\) T cells minimally expressed CD25, one important marker of conventional regulatory CD4\(^+\) T cells; however, ~20% CD69\(^+\) CD4\(^+\) T cells expressed CD25. In contrast, CD69\(^+\) CD4\(^+\) T cells expressed higher CD122 than did CD4\(^+\) CD69\(^-\) T cells (Fig. 2A). Thus, we designated the new subpopulation of tumor-induced CD4\(^+\) T cells as CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells. Then, we detected Foxp3 expression of CD4\(^+\) CD69\(^+\) CD25\(^-\) T cells with intracellular staining. As shown in Fig. 2B, CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells did not express Foxp3, which could distinguish the phenotype of CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells from that of conventional CD4\(^+\) CD25\(^+\) Foxp3\(^+\) Treg cells.

Since CD69 is generally considered as the earliest activation cell marker of T cells, we further tested whether CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells were the activated T cells. Splenic CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells were sorted from tumor-bearing mice and then detected for their secretion of IL-2, IFN-\(\gamma\), IL-10, IL-17, and TGF-\(\beta\)1 in response to stimulation with 25 ng/ml PMA and 1 \( \mu \)g/ml ionomycin. Unexpectedly, the above cytokines were not detected in the supernatants of CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells stimulated with or without PMA/ionomycin (Fig. 2C). Therefore, the CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells are not the activated CD4\(^+\) T cells. Taken together, the data indicate that CD69\(^+\) CD4\(^+\) CD25\(^-\) T cells do not express CD25.
and Foxp3, and they do not secrete the cytokines we detected. Therefore, tumor-induced CD69\(^+\)CD4\(^+\)CD25\(^+\) T cells are a new subset of CD4\(^+\) T cells with distinct phenotype and cytokine profile.

**CD69\(^+\)CD4\(^+\)CD25\(^+\) T cells suppress CD4 T cell proliferation in vitro**

Considering that CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells are not the activated T cells but could be induced and expanded significantly in the tumor-bearing mice, we wondered whether the tumor-induced CD69\(^+\)CD4\(^+\)CD25\(^+\) T cells may be involved in the tumor immune escape by acting as Treg cells to suppress CD4\(^+\) T cell response. First, we observed the proliferation of CD4\(^+\)CD25\(^-\) T cells in response to the stimulation with allogeneic mature DCs by measuring cell number and cell division. The proliferation of CD69\(^+\)CD4\(^+\)CD25\(^+\) T cells was lower as compared with that of CD69\(^+\)CD4\(^+\) T cells (\(p < 0.001\) vs CD69\(^+\)CD4\(^+\) T cells, Fig. 3, A and B), indicating that CD69\(^+\)CD4\(^+\)CD25\(^+\) T cells are hyporesponsive, having one of the characteristics of Treg cells. To investigate whether CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells could suppress an Ag-specific CD4\(^+\) T cell proliferation, we cocultured the CD69\(^+\)CD4\(^+\)CD25\(^+\) T cells with TCR-transgenic CD4\(^+\) T cells (DO11.10 T cells) and OVA\(_{323-339}\) peptide-pulsed maDCs. After coculture for 5 days, we counted the viable DO11.10 T cells by flow cytometry and found that CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells could markedly suppress maDC-initiated Ag-specific CD4\(^+\) T cell proliferation, whereas CD69\(^-\) CD4\(^+\) T cells could not (Fig. 3C). Furthermore, CFSE-labeling experiments confirmed the suppression of CD4 T cell proliferation by CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells (Fig. 3D).

**CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells suppress CD4 T cell proliferation in vivo**

To confirm the suppressive effect of CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells on CD4\(^+\) T cell proliferation in vivo, we adoptively transferred the OVA peptide-loaded maDCs, together with CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells or CD69\(^-\) CD4\(^+\) T cells, into the mice preinjected with DO11.10 T cells. Consistent with the above observations in vitro, the transferred CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells significantly reduced the frequency of DO11.10 (KJ1-26\(^+\)) T cells in spleen and MLN, whereas CD69\(^-\) CD4\(^+\) T cells could not (Fig. 4). Taken together, although CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells do not express Foxp3 and IL-10, CD69\(^+\)CD4\(^+\)CD25\(^-\) T cells are hyporesponsive and can
suppress CD4+ T cell proliferation both in vitro and in vivo, demonstrating that CD69+CD4+CD25− T cells are a new subset of Treg cells.

On the basis of the above data showing that CD69+CD4+CD25− T cells suppress CD4+ T cell proliferation both in vitro and in vivo, we are wondering whether CD69+CD4+CD25− T cells can promote tumor growth in vivo. As shown in Fig. 5, adoptive transfer of CD69+CD4+CD25− T cells could increase tumor growth and shorten the survival of liver cancer (Hepa)-bearing mice significantly (p < 0.05). We also observed the similar tumor-promoting effect of the adoptive transfer of CD69+CD4+CD25− T cells in B16 melanoma or 3LL lung cancer-bearing mice (data not shown). These observations further confirm the immunosuppressive function of CD69+CD4+CD25− T cells.

FIGURE 4. CD69+CD4+CD25− T cells inhibit CD4 T cell proliferation in vivo. CD69+CD4+CD25− and CD69+CD4+ splenic T cells were purified from tumor-bearing mice (2 wk after tumor inoculation). OVA-pulsed maDCs and DO11.10 T cells were transferred, together or without CD69+CD4+CD25− T cells, into recipient mice. Five days later, the lymphocytes in spleen and MLN were stained with CD4-FITC, KJ1-26-PE, and 7-AAD for analysis by flow cytometry. A. The dot plots represent one of four independent experiments. The proportions of KJ1-26+ in total CD4+ T cells from spleen (B) and MLN (C) are shown. Data are means ± SD of triplicate experiments. *, p < 0.05; ***, p < 0.001.

CD69+CD4+CD25− T cells suppress CD4 T cell proliferation in a cell-cell contact manner

We went further to analyze the underlying mechanisms for the regulatory function of CD69+CD4+CD25− T cells. To identify whether soluble factor(s) derived from CD69+CD4+CD25− T cells or cell-cell contact is required for this process, we incubated CD69+CD4+CD25− T cells and OVA peptide/maDCs/DO11.10 T cells in the transwell system (0.4 μM), or incubated the fixed CD69+CD4+CD25− T cells with OVA peptide/maDCs/DO11.10 T cells. In the transwell system, CD69+CD4+CD25− T cells lost their suppressive capacity, whereas the fixed CD69+CD4+CD25− T cells still exerted their suppressive effect on the DC-initiated Ag-specific T cell proliferation (Fig. 6A). The data suggest that the suppression of CD4+ T cell proliferation by CD69+CD4+CD25− T cells is dependent on cell-cell contact.

Membrane-bound TGF-β1 on CD69+CD4+CD25− T cells is responsible for their immunosuppressive activity

Subsequently, we looked for which membrane molecule(s) mediated the suppressive activity of CD69+CD4+CD25− T cells. As inspired by the previous studies showing that membrane-bound TGF-β1 is a critical regulator for T cell proliferation (25, 26), we wondered whether CD69+CD4+CD25− T cells could express membrane-bound TGF-β1 and, if so, whether membrane-bound TGF-β1 could mediate the suppressive activity of CD69+CD4+CD25− T cells. As shown in Fig. 6, B and C, most CD69+CD4+CD25− T cells expressed membrane-bound TGF-β1, and CD69+CD4+CD25− T cells almost did not express membrane-bound TGF-β1.
Although neutralization of TGF-β1 in the coculture system could restore the DC-initiated Ag-specific CD4 T cell proliferation in the presence of CD69+CD4+CD25− T cells. Interestingly, supplement of exogenous recombinant TGF-β1 into the OVA peptide/maDCs/DO11.10 T cells coculture system could not suppress DC-initiated Ag-specific T cell proliferation (Fig. 6A). Therefore, our results demonstrate that CD69+CD4+CD25− T cells can suppress CD4+ T cell proliferation through their membrane-bound TGF-β1.

**Engagement of CD69 maintains expression of membrane-bound TGF-β1 on CD69+CD4+CD25− T cells via ERK activation**

Finally, we investigated the mechanisms by which CD69+CD4+CD25− T cells expressed membrane-bound TGF-β1. One of the unique characteristics of this population is CD69+. It was reported that TGF-β1 production was reduced in CD69-deficient mice (20). Also, CD69 engagement has been shown to activate ERK (27), and ERK activation has been shown to be involved in the expression of cytokines. Thus, we first observed the ERK activation in the CD69+CD4+CD25− T cells. To exclude the possibility of ERK activation by the sorting process with CD69 mAb, we freshly purified splenic CD4+ T cells and then analyzed expression of p-ERK in gated CD69+CD25− T cells. As shown in Fig. 7, more ERK activation was observed in the freshly isolated CD69+CD4+CD25− T cells, indicating that CD69 ligand(s), present in vivo, could activate ERK in the CD69+CD4+CD25− T cells. Because no definite ligand(s) of CD69 has been identified until now, we engaged the CD69+CD4+CD25− T cells with the agonistic Ab against CD69 (H1.2F3), the mimic of the CD69 ligand in vitro. As shown in Fig.

8, engagement of CD69 could prolong ERK activation in the CD69+CD4+CD25− T cells and, accordingly, maintain high expression of membrane-bound TGF-β1 on CD69+CD4+CD25− T cells. In contrast, ERK inhibitor could reduce the expression of membrane-bound TGF-β1 on CD69+CD4+CD25− T cells engaged with CD69. The data suggest that CD69 engagement can maintain high expression.

**FIGURE 5.** Adoptive transfer of CD69+CD4+CD25− T cells promotes tumor growth in vivo. CD69+CD4+CD25− or CD69+CD4+ splenic T cells (3 × 10⁶/mouse), purified from tumor-bearing mice (2 wk after tumor inoculation), were adoptively transferred into tumor (Hepa)-bearing mice on days 1, 6, 13, and 20. The tumor size was measured every 7 days, and the surviving mice were counted every 5 days. Tumor volumes were measured and expressed in cubic centimeters. Each datum point represents the mean tumor volume ± SEM of six mice (A). The number of survival mice is indicated as a percentage of total mice inoculated (n = 7 mice/group, B). Data are representative of three independent experiments with similar results. Survival estimates and median survivals were determined using the method of Kaplan and Meier. **, p < 0.05.

**FIGURE 6.** CD69+CD4+CD25− T cells express membrane-bound TGF-β1, which mediates suppression of CD4 T cell proliferation. A, DO11.10 T cells were cocultured with maDCs in the presence of OVA323–329, together with or without CD69+CD4+CD25− T cells, for 5 days. In some experiments, 0.4-μm Transwells were used to separate maDCs/T cells from CD69+CD4+CD25− T cells in the coculture system, or CD69+CD4+CD25− T cells fixed by 1% glutaraldehyde were used, or neutralizing Ab against mouse TGF-β1 or recombinant mouse TGF-β1 (10 ng/ml) was added to the coculture system. Transwell indicates coculture system of CD69+CD4+CD25− T cells and maDCs/DO11.10 T cells in Transwells; fixation, coculture system of maDCs/DO11.10 T cells and CD4+CD69+CD25− T cells that were fixed before being added to coculture system; and isotype, isotype control for TGF-β1 Ab. The concentration of Ab used for blocking was up to 10 μg/ml. Data are means ± SD of triplicate experiments. ***, p < 0.001. B and C, Membrane-bound TGF-β1 was expressed on CD69+CD4+CD25− T cells. The percentage of membrane-bound TGF-β1-expressing CD69+CD4+CD25− T cells (B) and MFI of TGF-β1 expression on CD69+CD4+CD25− T cells (C) were assayed by FACS. CD69+CD4+CD25− T cells were gated in the FACS analysis. The dot plots represent one of four independent experiments. For MFI, data are means ± SD of three experiments.
of membrane-bound TGF-β1 on CD69⁺CD4⁺CD25⁻ T cells by activating ERK.

Discussion

Among the different subsets of Treg cells, CD4⁺CD25⁺Foxp3⁺ Treg cells have been extensively studied for their important roles in the tumor immune escape, allograft tolerance, and also in the pathogenesis of autoimmune diseases and even the chronic viral infection (1–4). CD4⁺CD25⁺Foxp3⁺ Treg cells comprise two main groups: those that are naturally arising in thymus, and those that are peripherally induced. The suppressive mechanism of this population has been shown to be due to the inhibition of the IL-2 receptor α-chain in target T cells, which is primarily induced by CTLA-4 and membrane-bound TGF-β1 (5). Tr1 Treg cells are characterized by constitutive expression of the immunosuppressive cytokine IL-10 and are induced in the periphery by regulatory cytokines (6, 28, 29). Th3 Treg cells are characterized by preferential expression of TGF-β but low expression of CD25. In mice, Th3 Treg cells seem to be important for the induction and maintenance of oral tolerance (7, 30). Additionally, other subsets of Treg cells identified by different markers may also mediate negative regulation of immune response. For example, a subset of human CD8⁺ lymphocyte activation gene-3 (LAG-3)⁺CD25⁺Foxp3⁺ Treg cells can inhibit T cell proliferation via secretion of CCL4 (31). There are subsets of HLA-G-expressing but CD25⁻ and Foxp3-negative CD4⁺ and CD8⁺ T cells in the peripheral blood, and the subset of T cells can suppress T cell proliferation via HLA-G (32). GRAIL (gene related to anergy in lymphocytes) is expressed in one population of CD25⁺ Foxp3⁺ Treg cells, and the expression of GRAIL confers the regulatory function of these Treg cells (33). These data suggest that different Treg cells may exert their immunosuppressive function through different mechanisms.

In addition to the previously known CD4⁺CD25⁺ Treg cells, CD4⁺CD25⁻ T cells have been also demonstrated to exert immunosuppressive function in several experimental models (34–36). These CD4⁺CD25⁻ T cells contribute to the maintenance of immune homeostasis and tolerance, and they also attenuate the pathogenesis of autoimmune diseases. The mechanistic studies show that CD4⁺CD25⁻ T cells express Foxp3, IL-10, and IFN-γ; furthermore, CD4⁺CD25⁻ T cells suppress T cell response through IL-10 and IFN-γ (15). In contrast, CD69⁺CD4⁺CD25⁻ T cells, the new subset of CD4⁺ Treg cells we identified in this study, do not express Foxp3 and CD25, and they do not secrete IL-10, TGF-β1, IL-2, and IFN-γ. It is well known that TGF-β1 produced by Treg cells can exert its inhibitory effects in soluble or membrane-bound manner. However, addition of recombinant TGF-β1 did not suppress DC-initiated Ag-specific T cell proliferation in our system, whereas blockade of TGF-β1 reversed the suppression of T cell proliferation by CD69⁺CD4⁺CD25⁺ T cells, indicating that this population of Treg cells can suppress T cell proliferation through membrane-bound TGF-β1. All of these data make this population distinct from the previously described CD4⁺ Treg cell subsets.

In comparison with the CD69⁺CD4⁺ T cells, CD69⁺CD4⁺CD25⁻ T cells were characterized by the increased percentage of CD122. CD122 has been found to be highly expressed on one population of CD8⁺ Treg cells, and the CD122⁺CD8⁺ T cells can suppress T cell proliferation and IFN-γ production through IL-10 (37–39). Until now, there was no report about the high expression of CD122 on CD4⁺ regulatory T cells. The biological significance of high expression of CD122 on the regulatory CD69⁺CD4⁺CD25⁻ T cells for suppressive function needs to be investigated.

CD69 belongs to the C-lectin type superfamily, and its gene maps in the NK gene complex. Early studies have shown that CD69, generally accepted as an activating marker of T cells, is persistently expressed in chronic inflammation. Engagement of CD69 can activate NK and T cells, resulting in increased cytotoxic
activity and proinflammatory cytokine production (17). Owing to the absence of a known ligand, the biological function of CD69 has not been extensively studied. In recent years, CD69 has been proposed as the negative regulator for the T cell response because CD69-deficient mice develop more severe T cell-mediated autoimmune diseases (20), and, also, CD69 deficiency could enhance the induction of antitumor responses (40). Additionally, CD69 engagement can induce apoptosis in monocytes and eosinophils and also trigger the inhibitory signal for IL-1 receptor- or CD3-mediated T cell proliferation (41–43). One subset of CD4+CD69+ T cells was detected in peripheral lymphoid tissues of a murine lupus model, and these CD4+CD69+ T cells are anergic, with impaired ability to produce proinflammatory cytokines. Considering that CXCR4/CXCL12 play an important role in the promotion of tumor growth and metastasis, Wald et al. analyzed the CXCR4 expression of the tumor-infiltrating lymphocytes isolated from lung adenocarcinoma, and they demonstrated that the percentage of CD4+CD69+CXCR4+ T cells increased in the tumor. Thirty percent of these CD4+CD69+CXCR4+ T cells expressed high CD25 and Foxp3; however, there was no functional study of these T cells as suppressor of T cell proliferation, and the biological significance of CD69 for the function of these T cells was not described (44).

Our experiments showed that CD69 engagement can activate ERK in the CD69+CD4+CD25− T cells, leading to maintenance of high expression of membrane-bound TGF-β1. CD69 is first suggested as an immunoregulatory receptor with a close relationship with TGF-β synthesis. Supporting this hypothesis, CD69 deficiency enhanced antitumor responses in MHC class I−/− tumor models through high production of chemokines, diminished production of TGF-β, and decreased lymphocyte apoptosis. CD69 deficiency leads to diminished TGF-β levels that contribute to an enhanced immune response, resulting in the increased inflammation in the CIA model. These experiments link CD69 signaling to TGF-β production directly. However, we did not find that the CD69 engagement can promote CD69+CD4+CD25− T cells to secrete soluble TGF-β. Additional experiments need to be performed to study the mechanisms by which this subset of CD69+CD4+CD25− T cells is induced and expanded in tumor-bearing host, especially how CD69 can be induced by tumor to be selectively expressed on these T cells. Also, whether CD69+CD4+CD25− T cells can express other inhibitory molecules that mediate suppression of T cells should be investigated in the future. Additionally, whether and how CD69+CD4+CD25− T cells can recognize tumor cells and promote tumor growth and metastasis directly, as well as how CD69+CD4+CD25− T cells are initially activated in vivo, need to be investigated in the future.

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Disclosures
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