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Both Regulatory T Cells and Antitumor Effector T Cells Are Primed in the Same Draining Lymph Nodes during Tumor Progression[1]

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The peripheral tolerance mechanism prevents effective antitumor immunity, even though tumor cells possess recognizable tumor-associated Ags. Recently, it has been elucidated that regulatory T cells (Treg) play a critical role in maintaining not only self-tolerance, but also tolerance of tumor cells. However, because the Treg that maintain self-tolerance arise naturally in the thymus and are thought to be anergic in peripheral, it is still unclear where and when Treg for tumor cells are generated. In this study we analyze tumor-draining lymph nodes (LNs) and demonstrate that both antitumor effector T cells and Treg capable of abrogating the antitumor reactivity of the effector T cells are primed in the same LNs during tumor progression. The regulatory activity generated in tumor-draining LNs exclusively belonged to the $CD4^+$ T cell subpopulation that expresses both CD25 and a high level of CD62L. Forkhead/winged helix transcription factor gene expression was detected only in the $CD62L^{high}CD4^+CD25^+$ T cells. $CD62L^{high}CD4^+CD25^+$ Treg and $CD62L^{low}CD4^+CD25^+$ T cells, which possess effector T cell functions, had comparable expression of LFA-1, VLA-4, CTLA-4, lymphocyte activation gene-3, and glucocorticoid-induced TNFR. Thus, only CD62L expression could distinguish regulatory $CD4^+CD25^+$ cells from effector $CD4^+CD25^+$ cells in draining LNs as a surface marker. The Treg generated in tumor-draining LNs possess the same functional properties as the Treg that arise naturally in the thymus but recognize tumor-associated Ag. $CD62L^{high}CD4^+CD25^+$ Treg contained a subpopulation that expressed CD86. Blocking experiments revealed that ligation of CTLA-4 on effector T cells by CD86 on Treg plays a pivotal role in regulating $CD4^+$ effector T cells. *The Journal of Immunology,* **2005, 175: 5058–5066.**

The purpose of the immune system is to discriminate and eliminate invading nonself. To do this, it possesses not only an effector system to eliminate nonself, but also a regulatory system that abrogates the attack of effector cells against self-somatic cells. Thus, the balance between immunity and tolerance determines the outcome of an immune reaction. Although tumor cells have Ags altered by mutation, a lack of danger signals and antigenic similarity to self-somatic cells, from which tumor cells are derived, engage the peripheral tolerance mechanism (1, 2). This tolerance makes it difficult to obtain effective antitumor immunity.

Recent studies revealed $CD4^+$ T cells that constitutively express CD25 to play a critical role in maintaining peripheral tolerance during infection, transplantation, autoimmunity, and tumor immunity (2–7). $CD4^+CD25^+$ regulatory T cells (Treg)[4] that arise naturally in the thymus to maintain self-tolerance are considered anergic in peripheral (8). However, it has been demonstrated that Treg proliferate in peripheral tissues in response to antigenic stimulation and can be converted from naive $CD4^+$ T cells (9, 10). Thus, it is still unclear where and when Treg are generated for tumor cells.

APCs that acquire Ags migrate into secondary lymphoid organs, where the Ag information is converted to adaptive immune responses. Although $CD4^+CD25^+$ is the best surrogate marker, it is difficult to distinguish Treg based on CD25 expression, especially in lymph nodes (LNs) where T cell priming is going on, because the expression of CD25 is also up-regulated on effector T cells upon TCR engagement before clonal expansion. This problem makes it impossible to analyze how regulatory T cells are primed in LNs. We reported that effector T cells, which are capable of mediating antitumor reactivity, are primed in LNs draining growing tumors and that these T cells exclusively belong to the $CD62L^{low}$ subpopulation (11). Additional CD40 stimulation as help signals for APC resulted in increased numbers of $CD62L^{low}$ T cells in draining LNs (12, 13). $CD62L^{high}$ T cells have been considered naive cells that have never been encountered by cognate Ag. However, our findings suggested that $CD62L^{high}$ tumor-draining LN T cells contained a regulatory subpopulation, because the elimination of $CD62L^{high}$ cells promotes the generation of highly potent antitumor T cells upon stimulation with CD3 (14).

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[4] Abbreviations used in this paper: Treg, regulatory T cell; CM, complete medium; DC, dendritic cell; Foxp3, forkhead/winged helix transcription factor gene; GITR, glucocorticoid-induced TNFR; LAG3, lymphocyte activation gene-3; LN, lymph node; m, murine.

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Recent studies have demonstrated that $CD62L^{high}$ $CD4^+CD25^+$ T cells possess superior suppressive activity (15–18).

In this study we demonstrate that the expression of CD62L distinguishes regulatory $CD4^+CD25^+$ cells from effector $CD4^+CD25^+$ T cells and that both antitumor effector T cells and regulatory T cells, which are capable of abrogating the therapeutic efficacy of the antitumor effector T cells in vivo, are primed in the same tumor-draining LNs with different kinetics. CTLA-4 ligation by CD86 exclusively expressed on regulatory $CD62L^{high}CD4^+$ $CD25^+$ LN T cells plays a pivotal role in regulating effector $CD4^+$ T cell functions via direct T-T interaction.

Materials and Methods

Mice

Female C57BL/6J (B6) mice were purchased from CLEA Laboratory. They were maintained in a specific pathogen-free environment and used for experiments at the age of 8–10 wk. All animal experiments were conducted with the permission of the Niigata University ethics committee for animal experiments.

Tumors

MCA 205 is a fibrosarcoma of B6 origin induced by i.m. injection of 3-methylcholanthrene (19). Single-cell suspensions were prepared from solid tumors by enzymatic digestion as described previously (20). An MCA 205 tumor cell line was established and maintained in vitro.

mAbs and flow cytometry

Hybridomas producing mAbs against murine CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2), CD3 (2C11), and murine CD62L (MEL14) were obtained from American Type Culture Collection. Anti-CD4 mAb, anti-CD8 mAb, and anti-CD62L mAb were produced as ascites fluid from sublethally irradiated (500 cGy) DBA/2 mice. PE-conjugated anti-CD80 (16-10A), anti-CD86 (GL1), anti-CD62L (MEL14), anti-CTLA-4 (UC10-4F10-11), anti-lymphocyte activation gene-3 (anti-LAG3; C9B7W), anti-CD8 (2.43), and anti-CD25 (PC61) mAbs and FITC-conjugated anti-Thy1.2 (30-H12), and anti-CD4 (GK1.5) mAbs were purchased from BD Pharmingen. PE-conjugated anti-glucocorticoid-induced TNFR (anti-GITR; 108619) mAb was purchased from R&D Systems. Analyses of cell surface phenotypes were conducted by direct immunofluorescent staining of $0.5–1 \times 10^6$ cells with conjugated mAbs. In each sample, 10,000 cells were analyzed using a FACScan flow microfluorometer (BD Biosciences). PE-conjugated subclass-matched Abs used as isotype controls were also purchased from BD Pharmingen.

Fractionation of T cells

T cells in the LN cell suspension were concentrated by passing through nylon wool columns (Wako Pure Chemical Industries). To yield highly purified (>90%) cells with down-regulated CD62L expression ($CD62L^{low}$), LN T cells were further isolated by a panning technique using T-25 flasks precoated with goat anti-rat Ig Ab (Jackson ImmunoResearch Laboratories)/anti-CD62L mAb (MEL14) and sheep anti-rat-Ig Ab/anti-CD62L mAb-coated Dynabeads M-450 (Dynal Biotech). T cells with high CD62L expression ($CD62L^{high}$) were obtained as cells attached to flasks coated with goat anti-rat Ig Ab/anti-CD62L mAb. In some experiments cells were also separated into $CD4^+$ and $CD8^+$ cells by depletion using magnetic beads as described previously (14). For in vitro experiments, highly purified $CD4^+$ cells were obtained using anti-CD4 mAb-coated Dynabeads and Detachabeads (Dynal Biotech) according to the manufacturer's instructions. $CD25^+$ cells were isolated using PE-conjugated anti-CD25 mAb and anti-PE microbeads (Miltenyi Biotec) according to the manufacturer's directions. Cell purity was >90%.

Bone marrow-derived dendritic cells (DCs)

DCs were generated from bone marrow cells as described previously. In brief, bone marrow cells obtained from femurs and tibias of naive mice were placed in T-75 flasks for 2 h at 37°C in complete medium (CM) containing 10 ng/ml rmGM-CSF (a gift from KIRIN). Nonadherent cells were collected by aspirating the medium and were transferred into fresh flasks. On day 6, nonadherent cells were harvested by gentle pipetting. CM consists of RPMI 1640 medium supplemented with 10% heat-inactivated LPS-qualified FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (all from Invitrogen Life Technologies), and 5×10^{-5} M 2-ME (Sigma-Aldrich).

Tumor-draining LN cells

B6 mice were inoculated s.c. with 2×10^6 MCA 205 tumor cells in both flanks. Inguinal LN draining tumors were harvested. Single-cell suspensions were prepared mechanically as described previously (20).

Adoptive immunotherapy

B6 mice were injected s.c. with 1.5×10^6 MCA 205 tumor cells in 100 μl of HBSS to establish s.c. tumors. Three days after inoculation, mice were sublethally irradiated (500 cGy) and then infused i.v. with T cells isolated from tumor-draining LNs. Perpendicular diameters of s.c. tumors were measured with calipers. The significance of differences in the diameters between groups was analyzed by Student t test. A value of $p < 0.05$ was considered significant.

Cytokine ELISA

T cells were stimulated with immobilized anti-CD3 mAb or tumor Ag-pulsed bone marrow-derived DCs in CM. Supernatants were harvested and assayed for mouse IFN-γ content by a quantitative sandwich enzyme immunoassay using a mouse IFN-γ ELISA kit (Genzyme) according to the manufacturer's instructions.

RT-PCR

Total RNA was isolated from T cells using Isogen (Nippon Gene) and used for cDNA synthesis. The cDNAs were used as templates for PCR (94°C for 2 min, 58°C for 30 s, and 72°C for 1.5 min), and 35 cycles were performed using primers specific for forkhead/winged helix transcription factor gene (*foxp3*; forward, 5′-GGCCCTTCTCCAGGACAGA-3′; 5′-GCTGATCAT GGCTGGGTTGT-3′). To ensure the quality of the product, RT-PCR was also performed using primers specific for β_2-microglobulin.

Proliferation assay

T cells isolated from tumor-draining LNs were stimulated with immobilized anti-CD3 mAb for 48 h in 2 ml of CM on 24-well plates at 2×10^6/ml. $CD62L^{low}$ T cells were labeled with 5 μM CFSE (Molecular Probes) in HBSS at 37°C for 15 min and washed twice before CD3 stimulation. The ratio of $CD62L^{low}$ T cells to $CD62L^{high}$ $CD4^+CD25^+$ T cells was 2:1. After a 48-h stimulation, cells were counted and washed twice with HBSS. Then, T cells were cultured in CM supplemented with 10 U/ml human rIL-2 (gift from Shionogi) at 1×10^5/ml. Three wells were analyzed for each condition.

Results

$CD62L^{high}$ T cells derived from tumor-draining LNs, but not from naive spleens, abrogated antitumor reactivity of $CD62L^{low}$ LN T cells

It was believed that $CD62L^{high}$ T cells are naive T cells; however, we reported that the elimination of $CD62L^{high}$ T cells promotes the generation of highly potent antitumor $CD4^+$ T cells upon stimulation with CD3. To determine $CD62L^{high}$ LN T cells possess activity to abolish the antitumor reactivity of effector T cells primed in tumor-draining LNs, mice with established s.c. tumors were infused with 2×10^6 $CD62L^{low}$ LN T cells in the presence or the absence of 10×10^6 $CD62L^{high}$ T cells. T cells were isolated from LNs draining growing MCA 205 tumors for 12 days or from spleens of naive mice. The ratio of $CD62L^{low}$ to $CD62L^{high}$ T cells was approximately the same as that in LNs, because 15–25% of all T cells were $CD62L^{low}$ in 12-day tumor-draining LNs. As shown in Fig. 1*b*, 2×10^6 $CD62L^{low}$ T cells alone successfully mediated the antitumor efficacy to regress s.c. tumor growth. In contrast, the s.c. tumor growth curve of the mice infused with 2×10^6 $CD62L^{low}$ T cells in the presence of 10×10^6 $CD62L^{high}$ T cells derived from tumor-draining LNs was identical with that of the no treatment group. $CD62L^{high}$ T cells derived from naive splenocytes did not affect the antitumor reactivity of $CD62L^{low}$ tumor-draining LN T cells. Thus, $CD62L^{high}$ T cells of tumor-draining LNs contain a subpopulation that is capable of abrogating the antitumor reactivity of effector T cells primed in the same tumor-draining LNs.

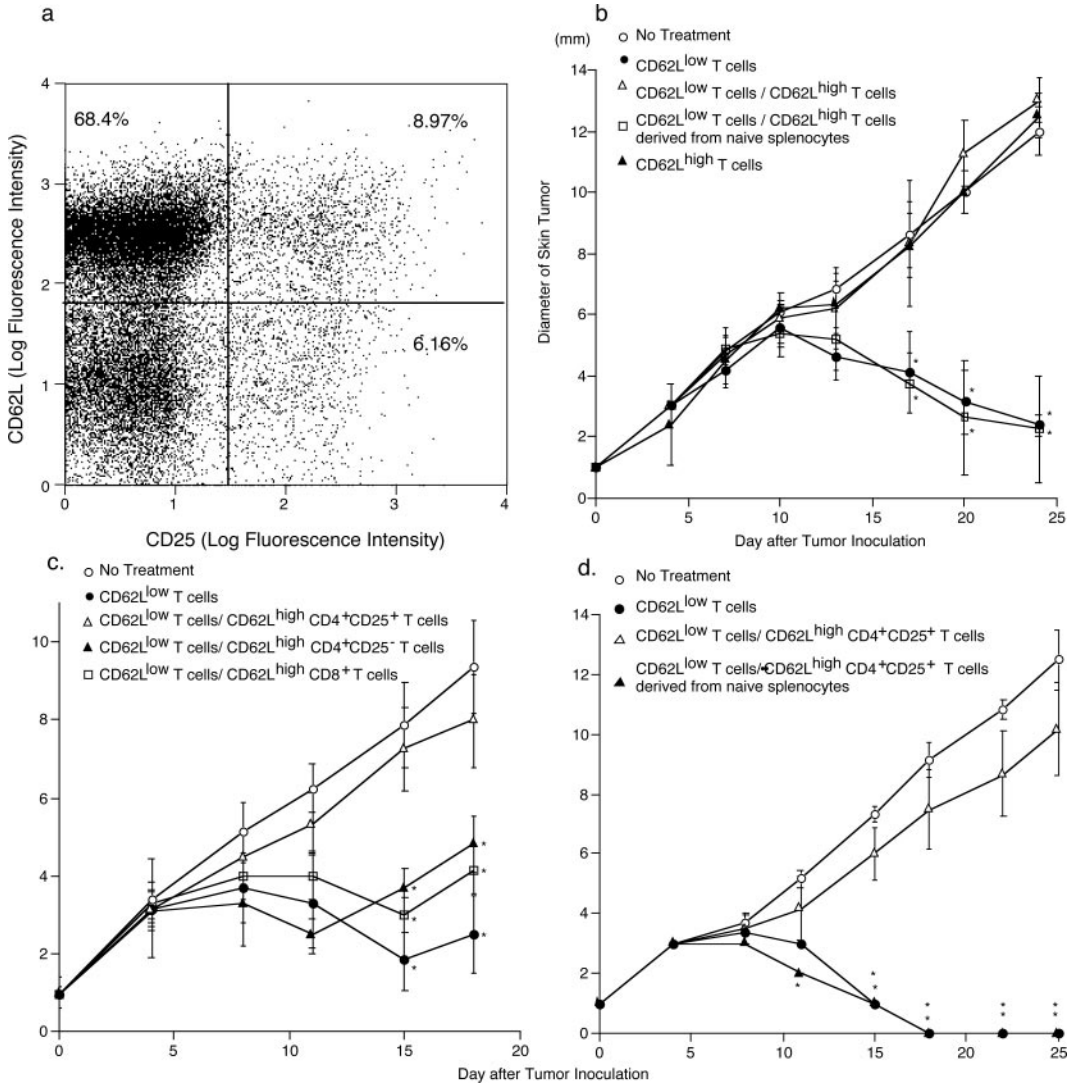

FIGURE 1. *a*, CD62L and CD25 expression of CD4$^+$ T cells in 12-day tumor-draining LNs. LN cells were stained with FITC-conjugated anti-CD4 mAb, PE-conjugated anti-CD62L mAb, and PerCP-conjugated anti-CD25 mAb. Gated CD4$^+$ cells were analyzed with a microfluorometer. *b–d*, Subcutaneous tumor growth of mice infused with CD62L^{low} T cells in the presence or the absence of CD62L^{high} T cells. Two million MCA 205 tumor cells were injected s.c. along the midline of the abdomen to establish s.c. tumors. Three days later, mice were adoptively infused i.v. with 2×10^6 CD62L^{low} T cells alone or with 10×10^6 CD62L^{high} T cells after sublethal whole body irradiation (500 cGy; *b*). CD62L^{low} T cells were isolated from LNs draining growing MCA 205 s.c. tumors for 12 days. CD62L^{high} T cells were isolated from tumor-draining LNs or naive splenocytes. *c* and *d*, CD62L^{high} T cells were further fractionated according to CD4, CD8, and CD25 expression using magnetic beads. One million fractionated CD62L^{high} T cells were infused into mice bearing established 3-day s.c. tumors with 2×10^6 (*c*) or 4×10^6 (*d*) CD62L^{low} LN T cells. Diameters of s.c. tumors were measured twice weekly with calipers, and size was recorded as the average of two perpendicular diameters. Statistical analyses were performed with Student's *t* test. *, $p < 0.01$ compared with the no treatment group. Each group contained five mice.

CD4$^+$CD25$^+$ subpopulation of CD62L^{high} tumor-draining LN T cells mediated regulatory functions

Because CD4$^+$CD25$^+$ is the best surrogate marker for regulatory T cells identified to date, CD4$^+$CD25$^+$, CD4$^+$CD25$^-$, or CD8$^+$ CD62L^{high} T cells isolated from tumor-draining LNs were infused with CD62L^{low} LN T cells into mice bearing established s.c. tumors to determine which subpopulation of CD62L^{high} T cells mediates regulatory functions. In Fig. 1*c*, the antitumor efficacy of 2×10^6 CD62L^{low} T cells was not enough to cure the s.c. tumor, which, after starting to regress, eventually grew in the mice. Neither CD62L^{high}CD4$^+$CD25$^-$ nor CD62L^{high}CD8$^+$ T cells showed any additive antitumor or regulatory activity. The s.c. tumor growth curves showed no significant differences from the curve for the mice who received CD62L^{low} T cells alone. In contrast, CD62L^{high}CD4$^+$CD25$^+$ T cells abolished the antitumor efficacy of effector T cells, resulting in a growth curve identical with that of the no treatment group. Thus, the CD4$^+$CD25$^+$ subpopulation of CD62L^{high} LN T cells is probably made up of Treg cells, whereas CD62L^{high}CD4$^+$CD25$^-$ and CD8$^+$ cells are functionally irrelevant naive T cells. Moreover, Fig. 1*d* shows that 1×10^6 CD62L^{high}CD4$^+$CD25$^+$ tumor-draining LN T cells were capable of abrogating the antitumor reactivity of 4×10^6 CD62L^{low} LN T cells, which was sufficient to cure established 3-day s.c. tumors. In contrast, the same number of CD62L^{high}CD4$^+$CD25$^+$ T cells derived from naive spleens, which are considered resident Treg (21), did not influence the therapeutic efficacy of antitumor effector T cells in vivo.

In 12-day MCA 205 s.c. tumor-draining LNs, ~20% of T cells are CD62L^{low}, and 5–7% are CD62L^{high}CD4$^+$CD25$^+$ (Fig. 1*a*). The ratio of CD62L^{low} to CD62L^{high} CD4$^+$CD25$^+$ T cells is

3–4:1. Thus, it seems that Treg cells sufficient to abolish the antitumor reactivity of primed effector T cells are generated in tumor-bearing hosts.

$CD62L^{high}CD4^+CD25^+$ inhibited IFN-γ production by either $CD4^+$ or $CD8^+$ $CD62L^{low}$ T cells via direct T-T interaction

To test whether $CD62L^{high}CD4^+CD25^+$ T cells generated in tumor-draining LNs influence cytokine production, we measured the amount of IFN-γ produced by 1×10^5 $CD62L^{low}$ T cells in the presence or the absence of 5×10^4 $CD62L^{high}CD4^+CD25^+$ T cells in 200 μl of CM on 96-well plates. As shown in Fig. 2*a*, $CD62L^{high}CD4^+CD25^+$ T cells in tumor-draining LNs abolished the Ag-specific production of IFN-γ by tumor-draining LN effector T cells stimulated with 5×10^4 tumor Ag-loaded DCs. As shown in Fig. 2*b*, $CD62L^{high}CD4^+CD25^+$ T cells derived from naive splenocytes did not affect the production of IFN-γ stimulated by tumor-associated Ag, although they inhibited IFN-γ production in the presence of nonspecific stimulation with immobilized anti-CD3 mAb (data not shown).

Next, we examined whether this suppression of cytokine production can be reproduced without APC and tested whether it is cell-cell contact dependent, because the Treg naturally arise in the thymus to maintain self-tolerance. One million $CD62L^{low}$ LN T cells on the bottom of 24-well plates were cocultured with 0.5×10^6 $CD62L^{high}CD4^+CD25^+$ T cells on either 0.4-μm pore size Transwell inserts (Costar) or the bottom of plates in 0.5 ml of CM. Both 24-well plates and Transwell inserts were coated with anti-CD3 mAb. As shown in Fig. 2, *c* and *d*, $CD62L^{high}CD4^+CD25^+$ Treg cells abrogated IFN-γ production by either $CD8^+$ or $CD4^+$ effector T cells in the absence of APC upon stimulation with CD3, and the suppression was dependent on cell-cell contact.

$CD62L^{high}CD4^+CD25^+$ T cells abrogated proliferation of both $CD4^+$ and $CD8^+$ $CD62L^{low}$ T cells

To elucidate whether $CD62L^{high}CD4^+CD25^+$ T cells generated in tumor-draining LNs inhibit cell proliferation, a T cell proliferation assay was performed as described in *Materials and Methods*. CFSE-labeled $CD62L^{low}$ T cells stimulated with immobilized anti-CD3 mAb increased the total number of cells by 7-fold during a 3-day culture period accompanied by a reduction in the intensity of CFSE (Fig. 3, *a* and *b*). $CD62L^{high}CD4^+CD25^-$ T cells did not affect $CD62L^{low}$ T cell proliferation, because the total number of cells increased and the reduction in intensity of CFSE intensity during the 3-day culture was identical with that of $CD62L^{low}$ T cells alone. In contrast, $CD62L^{low}$ T cells stimulated in the presence of $CD62L^{high}CD4^+CD25^+$ T cells did not proliferate at all. CFSE intensity did not change during the 3-day culture. Fig. 3*c* demonstrates the relative number of $CD8^+$ or $CD4^+$ cells according to phenotypic analysis. Thus, $CD62L^{high}CD4^+CD25^+$ T cells generated in tumor-draining LNs have the ability to abrogate the proliferation of both $CD4^+$ and $CD8^+$ T cells.

$CD62L^{low}CD4^+CD25^+$ LN T cells had effector, but not regulatory, functions

To examine the properties of $CD62L^{low}CD4^+CD25^+$ T cells, which comprise 20–30% of the $CD62L^{low}$ T cell population in 12-day tumor-draining LNs, we tested whether $CD62L^{low}$ $CD4^+CD25^+$ LN T cells affect IFN-γ production by $CD62L^{low}CD4^+CD25^-$ T

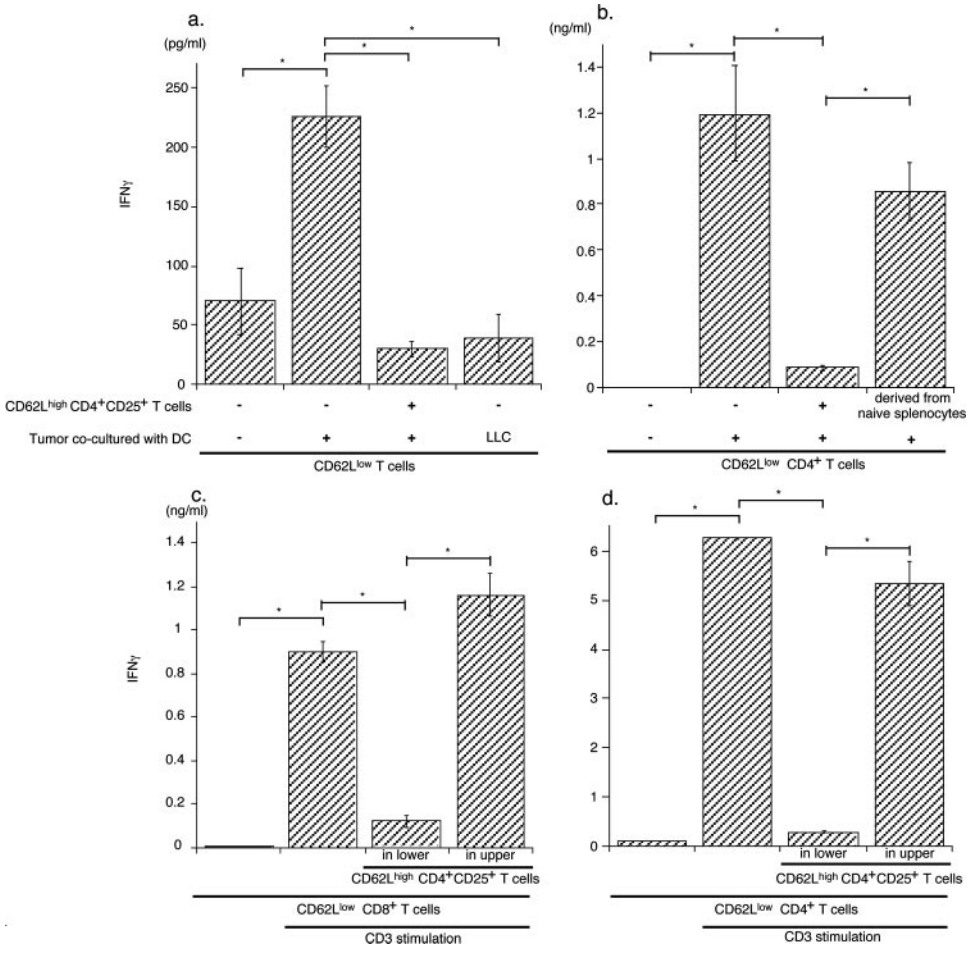

FIGURE 2. Measurement by ELISA of IFN-γ secreted in the medium. $CD62L^{low}$ T cells 1×10^5 were cocultured with 0.5×10^5 DCs in the presence or the absence of 0.5×10^5 $CD62L^{high}CD4^+CD25^+$ T cells for 72 h in 200 μl of CM in 96-well plates (*a* and *b*). Before coculture with T cells, DCs were incubated with 5000 cGy-irradiated MCA 205 or LLC tumor cells overnight. The amount of IFN-γ produced by 1×10^6 $CD62L^{low}CD8^+$ or $CD4^+$ T cells cultured with 0.5×10^6 $CD62L^{high}CD4^+CD25^+$ T cells upon CD3 stimulation is shown in *c* and *d*. $CD62L^{low}$ T cells on the bottom of 24-well plates were cultured with $CD62L^{high}CD4^+CD25^+$ T cells either on 0.4-μm pore size Transwell inserts or on the bottom. Both the Transwell inserts and 24-well plates were coated with anti-CD3 mAb. T cells were isolated from LNs draining growing MCA 205 s.c. tumors for 12 days. Three wells were analyzed for each condition. ELISA was performed in duplicate. Statistical analyses were performed by Student's *t* test. *, $p < 0.01$.

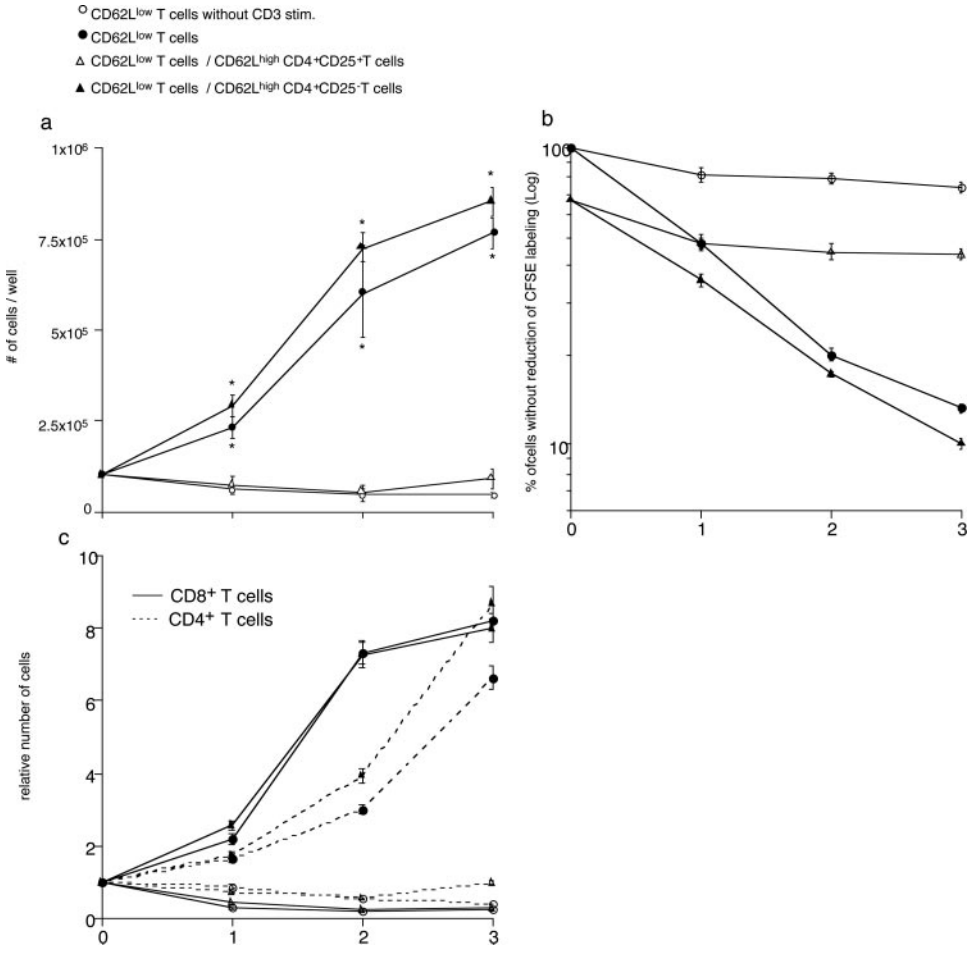

FIGURE 3. T cell proliferation was evaluated in the presence of 10 U/ml IL-2 after stimulation with CD3. CD62L^{low} T cells isolated from tumor-draining LNs were labeled with CFSE and cultured alone or with CD62L^{high}CD4$^+$ LN T cells at 2×10^6/ml in 2 ml of CM on 24-well plates coated with anti-CD3 mAb for 48 h. The ratio of CD62L^{low} to CD62L^{high} cells was 2:1. After CD3 stimulation, T cells were harvested and counted, then recultured at 0.1×10^6/ml in 2 ml of CM supplemented with 10 U/ml IL-2 on new 24-well plates. T cells were counted and analyzed with a microfluorometer every 24 h. *a*, Total number of cells per well. *b*, Percentage of T cells that still possess high levels of CFSE labeling and represent cells without division. *c*, Relative number of CD8$^+$ or CD4$^+$ T cells. Three wells were analyzed for each condition. $*$, $p < 0.01$ compared with the no stimulation group.

cells. CD62L^{high}CD4$^+$CD25$^+$ T cells completely inhibited the production of IFN-γ by CD62L^{low}CD4$^+$CD25$^-$ T cells (Fig. 4), whereas the addition of CD62L^{low}CD4$^+$CD25$^+$ T cells increased production. Moreover, CD62L^{low}CD4$^+$CD25$^+$ T cells alone produced the same amount of IFN-γ as CD62L^{low}CD4$^+$CD25$^-$ T cells. In contrast, CD62L^{high}CD4$^+$CD25$^+$ T cells produced no IFN-γ. Thus, it is likely that CD62L^{low}CD4$^+$CD25$^+$ cells possess effector T cell function, but not regulatory activity.

Foxp3 *mRNA expression was specific to CD62L^{high}CD4$^+$CD25$^+$ T cells*

It has been reported that mutation of Foxp3 is responsible for immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance, a syndrome of systemic autoimmunity in humans (22, 23). It is now believed that Foxp3 is a master switch of regulatory functions (9, 24, 25). Thus, we tested whether fractionated T cells derived from tumor-draining LNs express mRNA for Foxp3. As depicted in Fig. 5*A*, only CD62L^{high}CD4$^+$CD25$^+$ T cells expressed *foxp3* mRNA.

Functionally distinct CD62L^{high} and CD62L^{low} CD4$^+$CD25$^+$ T cells expressed comparable levels of GITR, CTLA-4, LAG3, VLA-4, and LFA-1

Next, we analyzed the phenotype of fractionated T cells derived from tumor-draining LNs. Because it was demonstrated that CD4$^+$CD25$^+$ Treg cells express GITR, CTLA-4, and LAG3 (26–29), we tested for these molecules and adhesion molecules that were important for T cell migration. Although CD62L^{high}CD4$^+$CD25$^+$ regulatory LN T cells have an up-regulated expression of

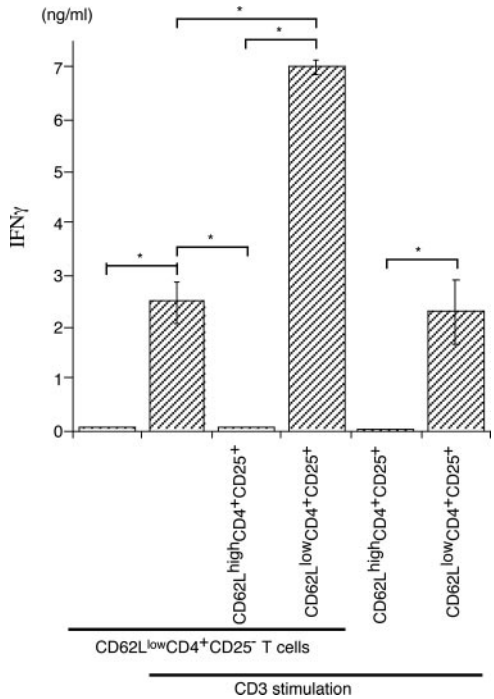

FIGURE 4. Measurement by ELISA of IFN-γ secreted in the medium. CD62L^{low}CD4$^+$CD25$^-$ T cells alone (1×10^5) or with 0.5×10^5 T cells fractionated according to the expression of CD62L and CD25 were stimulated with immobilized anti-CD3 mAb in 200 μl of CM on 96-well plates for 48 h. $*$, $p < 0.01$.

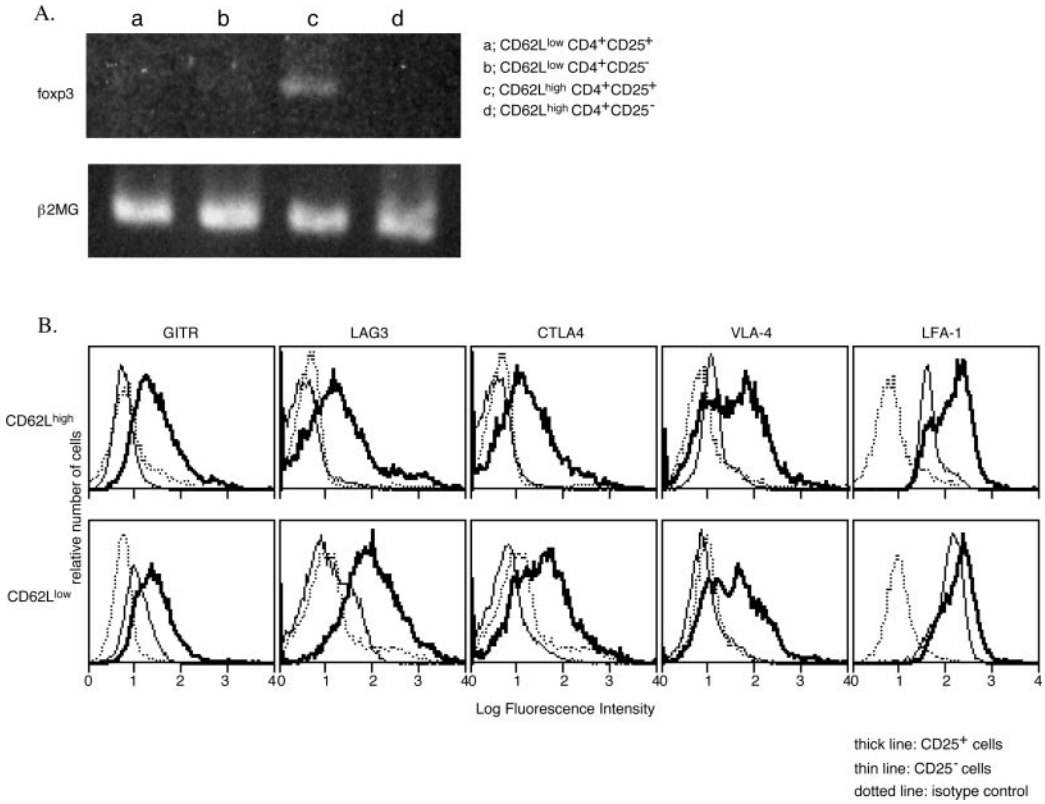

FIGURE 5. *A*, *Foxp3* mRNA expression in isolated $CD62L^{low}CD4^+CD25^+$, $CD62L^{low}CD4^+CD25^-$, $CD62L^{high}CD4^+CD25^+$, or $CD62L^{high}CD4^+CD25^-$ T cells derived from LNs draining MCA 205 s.c. tumors for 12 days. Total RNA was isolated from T cells and analyzed by RT-PCR for *foxp3*. β_2-Microglobulin gene expression is shown to confirm that equal amounts of RNA were used in each RT-PCR. Results shown are representative of three separate experiments. *B*, GITR, LAG3, CTLA-4, VLA-4, and LFA-1 expression on isolated T cells derived from MCA 205 tumor-draining LNs. Immediately after fractionation, T cells were double stained with PE-labeled anti-CD25 and FITC-conjugated anti-GITR, anti-LAG3, anti-CTLA-4, anti-VLA-4, anti-LFA-1, or isotype control Ab. Either $CD25^+$ or $CD25^-$ cells were gated for analyses. A total of 10^6 cells were analyzed for each sample. Each frame consists of 10,000 cells. Dotted lines indicate the isotype control.

GITR, CTLA-4, and LAG3, it is difficult to distinguish $CD62L^{high}CD4^+CD25^+$ T cells from $CD62L^{low}CD4^+CD25^+$ T cells, which possess effector T cell properties, from these molecules (Fig. 5*B*). Furthermore, $CD62L^{high}CD4^+CD25^+$ Treg cells and $CD62L^{low}CD4^+CD25^+$ effector T cells had a comparable upregulated expression of VLA-4 and LFA-1. In contrast, $CD62L^{high}CD4^+CD25^-$ T cells possessed the naive cell phenotype, such as no VLA-4, GITR, or CTLA-4, and a low level of LFA-1.

Different kinetics of $CD62L^{high}CD4^+CD25^+$ and $CD62L^{low}CD4^+$ T cell priming in LNs draining growing s.c. tumors

To address the priming of $CD62L^{high}CD4^+CD25^+$ and $CD62L^{low}CD4^+$ T cells in LNs draining growing MCA 205 s.c. tumors, we examined the number and phenotype of LN cells. Kinetic analysis revealed that the proportion of both $CD62L^{low}CD4^+CD25^+$ and $CD4^+CD25^-$ T cells started to increase 7 days after s.c. tumor inoculation and peaked on the 11th day (Fig. 6). The percentage of $CD62L^{low}$ T cells rapidly decreased, reaching the starting level by the 14th day. Although the proportion of $CD62L^{high}CD4^+CD25^+$ T cells started to increase 7 days after s.c. tumor inoculation, like that of $CD62L^{low}$ cells, it kept increasing until the total number of LN cells started to decrease. The increase in $CD62L^{high}CD4^+CD25^+$ T cells was not caused by a nonspecific accumulation of $CD62L^{high}$ cells, because the proportion of $CD62L^{high}CD4^+CD25^-$ naive T cells decreased in tumor-draining LNs. Hence, it is likely that $CD62L^{high}CD4^+CD25^+$ Treg cells underwent clonal expansion in tumor-draining LNs during tumor progression.

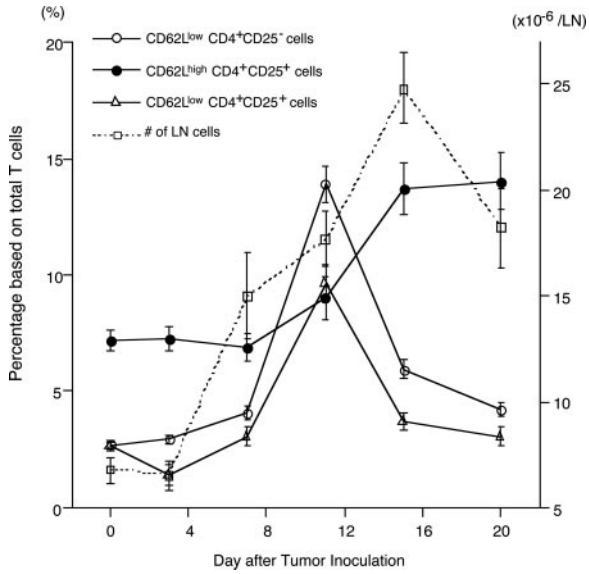

FIGURE 6. Kinetics of cellularity and the ratio of the indicated T cell subpopulations based on total T cells in LNs draining growing MCA 205 s.c. tumors. MCA 205 tumor cells (1.5×10^6) were inoculated s.c. into both flanks of mice. Inguinal LNs were harvested from three mice serially 0, 3, 7, 11, 15, and 20 days after s.c. injection and analyzed.

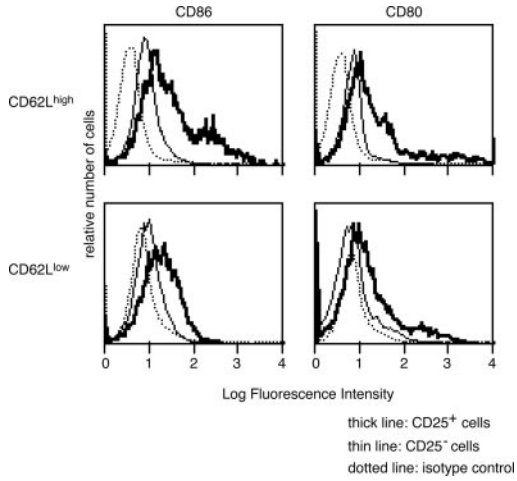

FIGURE 7. T cells isolated from 12-day tumor-draining LNs were analyzed for the expression of CD80 or CD86. CD4$^+$ T cells positively selected with magnetic beads were three-color-stained with FITC-labeled anti-CD80 mAb or FITC-labeled anti-CD86 mAb in the presence of PE-labeled anti-CD62L mAb and PerCP-labeled anti-CD25 mAb. Gated CD62L^{high}CD4$^+$CD25$^+$, CD62L^{high}CD4$^+$CD25$^-$,CD62L^{low}CD4$^+$CD25$^+$, or CD62L^{low}CD4$^+$CD25$^-$ T cells were analyzed for CD80 or CD86 expression. A total of 10^7 cells were analyzed for each sample.

CD62L^{high}CD4$^+$CD25$^+$ regulatory T cells suppress effector CD4$^+$ T cell functions via CD86/CTLA-4 T-T interactions

It has been reported that an inhibitory Ab against CTLA-4 could abrogate the induction of suppression by CD4$^+$CD25$^+$ Treg, and CTLA-4 expressed on Treg was thought to be important for this phenomenon (27). However, the mechanism by which Treg deliver a regulatory signal to effector T cells is still unclear. We examined the expression of CD80 and CD86 on fractionated tumor-draining LN T cells. Unexpectedly, only the CD62L^{high}CD4$^+$CD25$^+$ T cells had a subpopulation that expressed CD86 (Fig. 7). CD62L^{low}CD4$^+$ or CD62L^{high}CD4$^+$CD25$^-$ T cells did not express a detectable level of CD86 even after stimulation with CD3 (data not shown). Next, we tested whether CD86 on regulatory T cells participates in the suppression of T cell functions. CD62L^{low} T cells and CD62L^{high}CD4$^+$CD25$^+$ Treg cells were stimulated with immobilized anti-CD3 mAb in the presence or the absence of an inhibitory mAb against CD80, CD86, or CTLA-4. Anti-CTLA-4 as well as anti-CD86 inhibitory mAb prevented the suppression of CD62L^{low}CD4$^+$ T cells induced by CD62L^{high}CD4$^+$CD25$^+$ Treg cells, whereas anti-CD80 inhibitory mAb or isotype-matched irrelevant Abs did not have any influence (Fig. 8*a*). However, blockade of CTLA-4 or CD86 could not reverse the suppression of CD62L^{low}CD8$^+$ LN T cells (Fig. 8*b*).

Discussion

In this study we demonstrated that both effector T cells and Treg cells are primed in the same LNs that drain growing MCA 205 s.c. tumors. The antitumor effector T cells in tumor-draining LNs belong to a subpopulation that down-regulated CD62L expression, as we previously demonstrated (11, 14). In contrast, the CD62L^{high}CD4$^+$CD25$^+$ subpopulation in tumor-draining LNs abrogate the in vivo antitumor therapeutic efficacy of CD62L^{low} antitumor LN T cells (Fig. 1). Kinetic analyses indicate that CD62L^{high}CD4$^+$CD25$^+$ Treg proliferated in tumor-draining LNs during tumor progression (Fig. 6). Data obtained in vitro reveal that the Treg generated in tumor-draining LNs to abrogate antitumor reactivity possess the same functional properties and level of *foxp3* expression as the Treg that naturally arise in the thymus to maintain self-tolerance (Figs. 2–5).

CD62L^{high}CD4$^+$CD25$^+$ T cells derived from spleens of naive mice could not abrogate antitumor reactivity in vivo (Fig. 1). Moreover, Treg cells from naive mice did not inhibit the production of IFN-γ by antitumor effector T cells upon Ag stimulation by DCs acquired from apoptotic tumor cells (Fig. 2*b*), even though they had comparable suppressive activity as the Treg cells generated in tumor-draining LNs upon nonspecific CD3 stimulation.

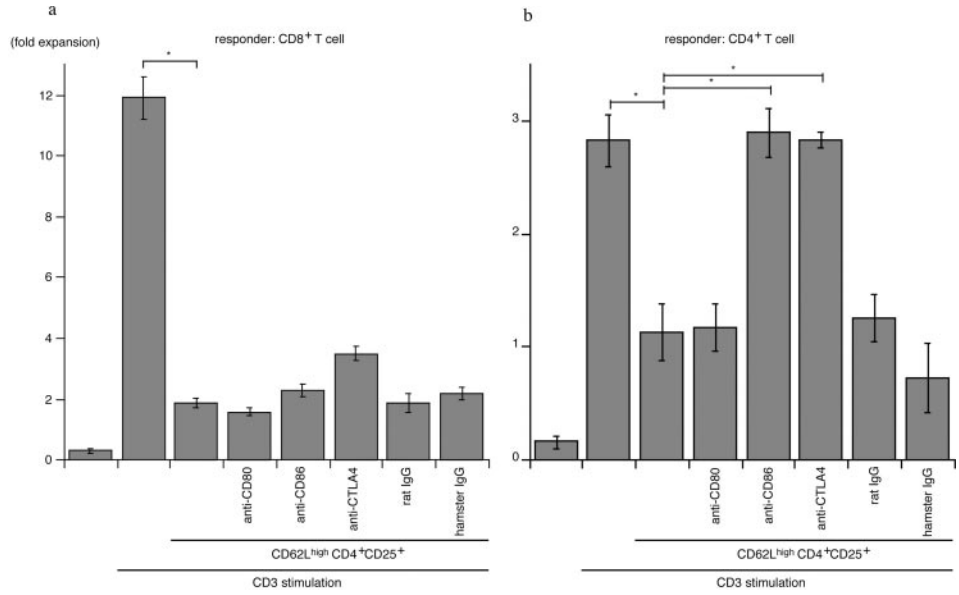

FIGURE 8. Proliferation assay of T cells in the presence or the absence of a blockade between CD86 and CTLA-4. CD62L^{low}CD4$^+$ or CD8$^+$ T cells (1×10^5) were cocultured with 5×10^4 CD62L^{high}CD4$^+$CD25$^+$ T cells in 200 μl of CM on 96-well plates with immobilized anti-CD3 mAb for 48 h. T cells were isolated from LNs draining MCA 205 s.c. tumors for 12 days. To inhibit the interaction between CD86 and CTLA-4, anti-CD86 mAb or anti-CTLA-4 mAb was added to the medium at 10 μg/ml during the 48 h of stimulation. T cells were recultured in 1 ml of CM supplemented with 10 U/ml IL-2 on 24-well plates at 1×10^5/ml. As a control, anti-CD80 mAb, rat IgG, or hamster IgG was added at the same concentration. Three wells were examined for each condition, and cells were enumerated every 24 h. The *y*-axis indicates the fold increase in number on day 2. $*$, $p < 0.01$.

Hence, it is likely that Treg are required to receive TCR/CD3 signaling to interfere with the function of effector T cells and that the Treg generated in tumor-draining LNs recognize tumor-associated Ag.

Although it has been demonstrated that $CD62L^{high}CD4^+CD25^+$ T cells possess superior regulatory activity in several systems, $CD62L^{low}CD4^+CD25^+$ T cells were still considered Treg cells (15, 16, 18). However, the differences in *foxp3* expression and priming kinetics suggest that the $CD62L^{low}CD4^+CD25^+$ T cell subpopulation in tumor-draining LNs is distinct from $CD62L^{high}CD4^+CD25^+$ T cells (Figs. 5*A* and 6). $CD62L^{low}CD4^+CD25^+$ T cells produced even more IFN-γ than $CD62L^{low}CD4^+CD25^-$ T cells upon CD3 stimulation (Fig. 4). Furthermore, $CD62L^{low}CD4^+CD25^+$ LN T cells mediated antitumor efficacy in vivo (Fig. 1). These results indicated that $CD62L^{low}CD4^+CD25^+$ T cells are effector T cells that express CD25 because of recent TCR stimulation before clonal expansion in LNs.

Little is known about the trafficking of Treg cells; however, our study shows that Treg cells generated in tumor-draining LNs have an up-regulated expression of CD62L, VLA-4, and LFA-1 (Figs. 1 and 5*B*). CD62L recognizes specific ligands on high endothelial venules and is considered the homing receptor for secondary lymphoid tissues (30). In contrast, VLA-4 and LFA-1 are thought to play a central role in T cell trafficking to inflammatory sites by recognizing VCAM-1 and ICAM-1 on endothelial cells. Thus, it is likely that $CD62L^{high}CD4^+CD25^+$ Treg cells can follow either $CD62L^{high}$ naive T cells to suppress priming in secondary lymphoid organs or activated $CD62L^{low}$ effector T cells that express VLA-4 and LFA-1 to suppress immune reactions in the effector phase.

$CD62L^{high}CD4^+CD25^+$ Treg cells suppressed either $CD4^+$ or $CD8^+$ effector T cell functions, including cytokine production and cell proliferation, in the absence of APC upon stimulation with CD3 in a cell-cell contact-dependent manner (Fig. 2. *c* and *d*, and Fig. 3). Hence, the regulatory signals sent to either $CD4^+$ or $CD8^+$ effector T cells were received directly from $CD62L^{high}CD4^+CD25^+$ Treg cells and were not mediated by APC. It is postulated that ligation of CD80 and/or CD86 expressed on effector T cells by CTLA-4 on Treg cells causes outside-in signaling and results in suppression (31), although it is well documented that $p56^{lck}$-induced tyrosine phosphorylation, which is the major signal pathway of the TCR/CD3 complex, can be reversed by CTLA-4 ligation (32). We found that the $CD62L^{high}CD4^+CD25^+$ Treg cells contained a subpopulation that expressed CD86 in tumor-draining LNs (Fig. 7). Effector $CD62L^{low}CD4^+$ or naive $CD62L^{high}CD4^+CD25^-$ T cells derived from tumor-draining LNs did not express CD86 or CD80 even after 48-h stimulation with immobilized anti-CD3 mAb (data not shown). The inhibitory mAb against CTLA-4 or CD86, but not CD80, completely reversed the inhibitory effect on the proliferation of $CD4^+$ effector LN T cells by $CD62L^{high}CD4^+CD25^+$ T cells (Fig. 8). In contrast, the same treatment could not reverse the suppression of $CD8^+$ effector LN T cells. $CD62L^{low}CD4^+$ T cells, but not $CD8^+$ T cells, isolated from tumor-draining LNs expressed CTLA-4 (Fig. 5*B*). Our data suggested that CD86 exclusively expressed on Treg cells plays a pivotal role in regulating $CD4^+$ effector T cells by interacting with CTLA-4 on effector T cells. This is a novel mechanism by which Treg cells suppress effector T cell function. This finding is compatible with recent reports demonstrating that $CD4^+CD25^+$ T cells derived from CTLA-4-deficient mice mediated suppression (33) and that T cells transfected with cDNA encoding CD86 suppressed graft-vs-host disease (34). The reason why the bioactivity of CD86 on T cells is different from that on APC is unclear; however, DCs are capable of selecting the receptor of B7 by recruiting either CD28 or CTLA-4 into immunological synapses (35). In contrast, it is unlikely that Treg cells have the ability to develop immunological synapse with effector T cells to select receptors. Moreover, it was demonstrated that CD86 expressed on T cells had a hypoglycosylated form and showed no detectable binding activity to CD28 with preserved binding to CTLA-4 (36). Thus, it is possible that CD86 on T cells preferentially give negative signaling through CTLA-4 (37).

$CD62L^{high}CD4^+CD25^+$ T cells regulated $CD8^+$ T cell functions via mechanisms other than the CD86-CTLA-4 interaction, because the inhibitory mAb against CD86 or CTLA-4 did not abolish the suppression. Because neutralizing Ab against TGF-β partially inhibited the induction of suppression, it is likely that membrane-bound TGF-β participated in $CD8^+$ T cell suppression (data not shown).

These results indicate that Treg cells for tumor Ags are primed in draining LNs during tumor progression and that the balance between $CD62L^{low}$ effector T cell priming and $CD62L^{high}CD4^+CD25^+$ Treg cell priming in secondary lymphoid organs determines the outcome of antitumor immune reactions. Our data also indicate that promoting antitumor effector $CD62L^{low}$ T cell priming while eliminating $CD62L^{high}CD4^+CD25^+$ Treg cells or inhibiting regulatory mechanisms such as CD86-CTLA-4 interaction is critical to establishing effective antitumor immunotherapy. Furthermore, it might be possible to orchestrate adaptive immune reactions by manipulating the balance of effector and regulatory T cell priming against acquired Ags, such as infectious pathogens, alloantigens, and allergens.

Disclosures

The authors have no financial conflict of interest.

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