Outgrowth of CD4<sup>low/neg</sup>CD25<sup>+</sup> T Cells with Suppressor Function in CD4<sup>+</sup>CD25<sup>+</sup> T Cell Cultures upon Polyclonal Stimulation Ex Vivo

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Outgrowth of CD4<sub>low/neg</sub>CD25<sup>+</sup> T Cells with Suppressor Function in CD4<sup>+</sup>CD25<sup>+</sup> T Cell Cultures upon Polyclonal Stimulation Ex Vivo<sup>1</sup>

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CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) play an essential role in controlling autoimmunity and allograft rejection. Several ex vivo activation and expansion protocols have been developed to amplify cell numbers and suppressor function of murine and human Tregs. We demonstrate in this study that ex vivo activation and expansion of murine Tregs resulted in an enrichment of a CD4<sub>low/neg</sub>CD25<sup>+</sup> T cell population that was more than 20-fold more potent than expanded conventional Tregs in suppressing an in vitro CD4<sup>+</sup>CD25<sup>−</sup> T cell response to allo-Ag. The generation of CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells was independent of the presence of Tregs in the culture, and suppressor function was acquired only after activation and expansion. CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells expressed either an αβ or γδ TCR, had an activated phenotype, and did not express the transcription factor FoxP3. Despite expressing the cell surface Ags lymphocyte activation gene-3 (CD223) and CD103, neither was essential for suppressor cell function. Suppression by CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells was prevented by a semipermeable membrane and was independent of IL-10 and TGF-β. In summary, we describe in this study CD4<sub>low/neg</sub>CD25<sup>+</sup> FoxP3<sup>+</sup> T cells with highly potent suppressor cell function derived from cultures of an enriched population of CD4<sup>+</sup>CD25<sup>+</sup> T cells that may contribute to the suppressor activity of ex vivo expanded bone fide Tregs. The Journal of Immunology, 2008, 181: 8767–8775.

<sup>1</sup>Abbreviations used in this paper: Treg, CD4<sub>+</sub>CD25<sup>+</sup> regulatory T cells; DN, double negative; GVHD, graft-vs-host disease; LAG-3, lymphocyte activation gene-3; neg, negative; wt, wild type.

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<sup>5</sup>Abbreviations used in this paper: Treg, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell; DN, double negative; GVHD, graft-vs-host disease; LAG-3, lymphocyte activation gene-3; neg, negative; wt, wild type.

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mice were generated, as described (13). All mice were housed in a specific pathogen-free facility in microisolator cages, according to the National Institutes of Health guidelines.

**Cell purification**

Axillary, mesenteric, sacral, and inguinal lymph nodes were collected from 6- to 12-wk-old female mice into PBS containing 2% FCS (HyClone). CD4⁺ T cells were isolated, as described previously (14). The purity of the preparation was determined by FACS to be at least 95% CD4⁺ T cells. To enrich for CD4⁺CD25⁺ T cells, purified CD4⁺ T cells were incubated with anti-CD25 PE mAb (7D4), followed by streptavidin-PE (both from BD Pharmingen) or anti-CD25 PE (PC61). After incubation with MACS anti-PE MicroBeads, cells were positively selected on MS or LS MACS separation columns (both Miltenyi Biotec). Column separation was repeated until CD25 purity was at least 98%. CD4⁺CD25⁺ cells were used as responder cells in suppression assays. Similarly, separation of expanded CD4⁺CD25⁺ from CD4low/neg/CD25⁺ cells from expanded cultures was performed using an anti-CD4 PE mAb (RM4-5). Purity of both populations was over 99%. For separation of fresh CD4⁺CD25⁺ and CD4low/neg/CD25⁺ cells, CD4⁺CD25⁺ T cells were purified using MACS, as described above, stained with anti-CD4 CyChrome mAb (RM4-5), and sorted on a FACS DIVA or FACS Aria. Purity of sorted CD4⁺CD25⁺ and CD4low/neg/CD25⁺ cell populations was over 99%. For some experiments, expanded cells were stained with anti-CD8 PE mAb and anti-CD103 PE mAb or anti-LAG-3 PE mAb or anti-CD103 PE mAb, followed by sorting on a FACS Aria.

**CD4⁺CD25⁺ in vitro activation and expansion**

Enriched CD4⁺CD25⁺ T cells were suspended at a final concentration of 0.3–0.7 × 10⁶ cells/ml in DMEM complete medium and cultured in 24- or 48-well plates (Costar) (15). CD4⁺CD25⁺ cells were activated for 3 days with 0.5 μg/ml plate-bound anti-CD3e mAb (145-2C11). Cultures were maintained at 0.5–2 × 10⁶ cells/ml and supplemented with 100 U/ml human IL-2 (AmpGen) throughout the culture period every 2–3 days.

**In vitro suppression assay**

Freshly isolated CD4⁺CD25⁻ T cells were mixed with T cell-depleted irradiated (30 Gy) bm12 splenocytes (as previously described) at a 1:1 ratio at a final concentration of 1.5 × 10⁶ total cells/ml in DMEM complete medium (14). Suppressor cells were added at various concentrations, as indicated. Cultures were incubated at 37 °C and 10% CO₂ in 200 μl well in 96-well plates (Costar) in at least triplicates. After 6 days of culture, cells were pulsed with tritiated thymidine (1 μCi/well; Amersham Life Sciences) for 16–18 h, the cells were harvested, and tritiated thymidine uptake was assessed using a gas-operated 96-well plate reader (Packard Instrument). A total of 100 μg/ml anti-TGF-β mAb (clone 1D11.16.8, mouse IgG1; American Type Culture Collection) was added to suppression assays using IL-10−/− T cells. For suppression assays including Transwell membranes, CD4⁺CD25⁺ cells were mixed 1:1 with irradiated T cell-depleted bm12 splenocytes at a concentration of 3 × 10⁶ cells/ml and cultured in the bottom well (600 μl/ml) of 24-well Transwell plates (Costar). Suppressor cells were adjusted to a concentration of 10 × 10⁶ cells/ml and cultured in the Transwell insert (100 μl/ml) of 24-well plates. Tritiated thymidine uptake was assessed on day 6, and after 16–18 h cells from the bottom wells were transferred into 96-well plates and harvested, and tritiated thymidine uptake was assessed.

**Quantitative RT-PCR**

RNA was isolated using TRIzol reagent (Life Technologies), according to manufacturer’s protocol, followed by cDNA synthesis using SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen). Real-time PCR was performed using TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems). GAPDH-specific primers and probe were purchased from Applied Biosystems. Afterward, CD4-specific primers and probe were used for quantitative RT PCR, as follows: CD4 forward (5'-GCA GCA TGG CAA AGG TGT ATT-3'), CD4 reverse (5'-TGG CCC TTT TTT GGA ATC AA-3'), and CD4 probe (5'-56-FAM/AGG TTC GCC TTC GCA GTT TGA TCG T/3BHQ_1/-3') (IDT). An ABI Prism 7600 real-time PCR machine was used for acquisition (Applied Biosystems).

**Flow cytometry and Abs**

Mouse-specific Abs were purchased from BD Pharmingen or ebioscience, and staining was performed, according to manufacturer’s protocol. Anti-granzyme B mAb was purchased from Caltag Laboratories. Acquisition was performed using a FACScalibur (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

**Surface and intracellular cytokine staining**

Cells were harvested and plated on anti-CD3 mAb-coated 96-well plates at a concentration of 5–10 × 10⁶ cells/ml for 4–5 h in the presence of IL-2 and monensin at 37°C and 10% CO₂. Surface staining was performed for 20 min. Cells were fixed with fixation/permeabilization buffer (BD Pharmingen) for 20
sorted CD4 cells. CD4 surface expression of freshly isolated and separately expanded T cells before and after FACS sorting.

Results

In vitro activation and expansion of bead-isolated Tregs result in an enrichment of a potent CD4low/negCD25+ suppressor population that is independent from CD4+CD25+ T cells

We used an established protocol for isolation of Tregs consisting of a negative selection method for CD4+ T cell enrichment and positive selection using magnetic beads for isolation of CD25+ cells. CD4+ T cell purity routinely was over 95%, and CD25+ purity was over 98%, with a combined purity of CD4+CD25+ cells of 90–92% (data not shown). The higher proportion of CD25+ vs CD4+ T cells suggests that CD4low/negCD25+ T cells may be positively selected during the isolation procedure. After 3 days of anti-CD3 mAb exposure and an additional 5–8 days in IL-2 alone, cells were subjected to FACS analysis. As shown in Fig. 1A, there was an enrichment of a CD4low/neg CD25+ T cell population up to 60% of the final culture. When separated from CD4+CD25+ T cells and tested in an in vitro suppression assay, CD4low/negCD25+ T cells were more potent than CD4+CD25+ T cells in suppressing freshly isolated allogenic stimulated CD4+CD25− effector T cells in a dose-dependent manner (Fig. 1B). In contrast to the suppressor cell function of these two cell types, the same activation and expansion protocol used in cultures containing CD4+CD25+ T cells resulted in mildly augmented proliferation in an allogeneic MLR culture (data not shown).

To determine whether CD4low/negCD25+ T cells are derived from CD4+CD25+ T cells, we highly purified freshly isolated Tregs into CD4+CD25+ T cells and CD4low/negCD25+ T cells by FACS sorting (purity of both populations > 99%), and then expanded both populations ex vivo in separate cultures (Fig. 2, A and B). As compared with cultures containing both cell populations, CD4low/negCD25+ T cells expanded 17-fold after 11 days of culture in the absence of CD4+CD25+ T cells (data not shown). Importantly, CD4+CD25+ T cells did not give rise to CD4low/negCD25+ T cells during culture (Fig. 2B). In studies in which sorted CD4low/negCD25+ T cells and congenic CD4+CD25+ T cells were mixed together to achieve the same ratio as was present in cells before sorting, we observed no conversion of CD4low/negCD25+ T cells to CD4+CD25+ T cells or vice versa under these conditions (Fig. 2D). In addition, CD4low/negCD25+ T cells derived in the absence of CD4+CD25+ T cells were highly and comparably suppressive as CD4low/negCD25+ T cells expanded in the presence of CD4+CD25+ T cells (Fig. 2C). These results suggested that CD4low/negCD25+ T cells do not derive from or depend on CD4+CD25+ T cells during the culture.

We next tested the ability of freshly isolated CD4low/negCD25+ T cells to suppress an alloreponse. Freshly isolated CD4low/negCD25+ T cells enhanced allo-MLR proliferation (Fig. 2E), which required the presence of both responder and stimulator cells (data not shown). Based on these results, we conclude that CD4low/negCD25+ T cell suppressor function is induced during ex vivo activation and culture.

CD4+CD25+ CD45.2+ and CD4low/negCD25+ CD45.1+ T cells were isolated as in A and mixed at a ratio similar to unsorted freshly isolated Tregs. Day 11 cultures were stained with CD4 and CD45.1 mAbs. E, Suppressor function of freshly isolated CD4+CD25+ and CD4low/negCD25+ T cells. A total of 2.5 × 10^6 freshly isolated CD4+CD25+ and CD4low/negCD25+ T cells was cocultured with CD4+CD25+ T cells and irradiated allogeneic splenic stimulators. Proliferation was determined by adding [3H]thymidine at day 6, and cells were harvested 16–18 h later. Average plus SEM of triplicate samples from one of three similar experiments is shown (C and D).
As compared with cultures containing CD4+CD25+ T cells, we observed that CD4low/neg/CD25+ T cells expanded in the absence of CD4+CD25+ T cells had lower CD4 surface expression levels than when cultured in the presence of CD4+CD25+ T cells (Fig. 3A). To exclude the possibility that FACS sorting changed the properties of the sorted cells, CD4+CD25+ and CD4low/neg/CD25+ T cells were sorted and then recombined to the original ratio present before sorting. We did not observe a significant difference in the phenotype (based on CD4 and CD25) and function between nonsorted and reconstituted expanded cultures (Fig. 3A and data not shown). To further determine whether CD4low/neg/CD25+ T cells indeed express low levels of CD4, we looked at CD4 mRNA expression. As shown in Fig. 3, B and C, CD4low/neg/CD25+ T cells did not express CD4 message as determined by quantitative RT-PCR. To ensure that the amount of cDNA from CD4low/neg/CD25+ T cells used for the CD4 quantitative RT-PCR was not below the level of detection, we performed a titration experiment using different amounts of cDNA isolated from CD4low/neg/CD25+ T cells. As shown in Fig. 3C, a positive signal was still detectable at 1 ng, whereas 0.1 ng of cDNA was below the threshold of detection. A total of 500 ng of cDNA from CD4low/neg/CD25+ T cells did not result in a positive signal by 45 cycles, which suggests an at least 500- to 5000-fold difference in CD4 expression, which is significantly different from what would be expected based on the FACS results. Thus, we conclude that CD4low/neg/CD25+ T cells are positive for surface CD4, but do not express CD4 mRNA at detectable levels.

CD4low/neg/CD25+ Tregs with suppressive potential are activated, FoxP3+ T cells

Because T cells up-regulate CD25 surface expression upon stimulation through the TCR, the activation status of CD4low/neg/CD25+ T cells and CD4+CD25+ T cells was determined. Tregs were isolated as in Fig. 1, and CD4low/neg/CD25+ T cells and CD4+CD25+ T cells were analyzed separately based on differential CD4 expression for activation Ag expression by FACS. As determined by size and activation Ag expression (CD62L, CD69, CD44, CD45RB), a higher percentage of freshly CD4low/neg/CD25+ T cells was activated as compared with CD4+CD25+ T cells (Fig. 4A). FoxP3 expression was undetectable in CD4low/neg/CD25+ T cells. Similarly, when Tregs were activated and expanded ex vivo and CD4low/neg/CD25+ T cells and CD4+CD25+ T cells were analyzed separately based on their differential CD4 expression by FACS, CD4low/neg/CD25+ T cells maintained an activated phenotype, whereas CD4+CD25+ T cells largely maintained a naive phenotype. Most activation markers on fresh and expanded CD4low/neg/CD25+ T cells were similar, with the exception of CD45RB, which was up-regulated after culture and appeared to reflect the increase in suppressor function of freshly isolated and expanded CD4low/neg/CD25+ T cells. FoxP3 expression was not induced in CD4low/neg/CD25+ T cells during the culture period (Fig. 4B). When gated on CD4+CD25+ T cells after expansion, only ~30% of this cell population was FoxP3+ (Fig. 4B). In contrast, ~80–85% of CD4+CD25+ T cells in the starting Treg culture were FoxP3+ (Fig. 4A). Intracellular cytokine expression can be used to further determine the activation status as well as the type of Tcell generated upon activation. Freshly isolated and expanded Treg cultures were restimulated with plate-bound anti-CD3 mAb in vitro, and intracellular cytokine staining was determined in CD4low/neg/CD25+ and CD4+CD25+ T cells (freshly isolated; Fig. 4C) or CD4low/neg/CD25+, CD4+CD25+, FoxP3+, and CD4+CD25+ FoxP3− T cells (ex vivo expanded; Fig. 4D) and separately analyzed based on the differential expression of CD4 and FoxP3. The vast majority restimulated freshly isolated CD4low/neg/CD25+ and CD4+CD25+ T cells did not produce Th1 effector cytokines even though CD4low/neg/CD25+ T cells had an activated surface phenotype (Fig. 4C). As shown in Fig. 4D, upon restimulation, CD4low/neg/CD25+ T cells expressed Th1 cytokines, including
FIGURE 4. Characterization of CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells. A, FACS analysis of freshly isolated Tregs gated on CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells. B, FACS analysis of expanded Treg cultures gated on CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells. C, Intracellular cytokine staining of freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> were isolated, as described in Fig. 1. Cells were activated with plate-bound anti-CD3 mAb for 4 h in the presence of 100 U/ml IL-2 and monensin. Cytokine expression gated on CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells is shown. D, Intracellular cytokine staining of expanded CD4<sub>low/neg</sub>CD25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells. Expanded Treg cultures were restimulated as in D, and cytokine expression gated on CD4<sub>low/neg</sub>CD25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells is shown. E, FACS analysis of Treg cultures gated on CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells (dot plots), CD4<sup>+</sup>CD25<sup>+</sup>, and CD4<sub>low/neg</sub>CD25<sup>+</sup> T cells (CD3 histogram) or CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sub>low/neg</sub>CD25<sup>+</sup>γδ<sup>+</sup>, and CD4<sub>low/neg</sub>CD25<sup>+</sup>γδ<sup>+</sup> T cells (β TCR histogram). Representative plots of at least three experiments are shown.
IL-2, IFN-γ, and TNF-α. Interestingly, both CD4⁺CD25⁺FoxP3⁻ and CD4⁺CD25⁺FoxP3⁺ T cells expressed IL-2, IFN-γ, and TNF-α (Fig. 4D). The CD4⁺CD25⁺FoxP3⁺ T cell population could potentially contain induced FoxP3⁺ T cells that express effector cytokines and are not suppressive, as described in human studies (16). None of the ex vivo activated and expanded populations expressed IL-4 and IL-10 (data not shown).

Several types of naturally occurring and induced T cells with regulatory potential have been described (17). To further characterize the CD4⁺low/negCD25⁺ T cells, Abs against a panel of cell surface Ags were used in FACS analysis. As shown in Fig. 4E, all subpopulations were positive for CD3ε and expressed either an αβ or γδ TCR. Some γδ T cells coexpressed CD8 or NK1.1; however, γδ⁺NK1.1⁺ T cells were DX5⁻ (Fig. 4E and data not shown).

Importantly, expression of activation markers and cytokines and suppressor function was similar in individual subpopulations of CD4⁺low/negCD25⁺ T cells (data not shown).

**Suppression by CD4⁺lowCD25⁺ Tregs occurs independently of IL-10 and TGF-β and requires close proximity to effector T cells and APCs**

Some Tregs require IL-10 and TGF-β for their suppressor function, whereas others are thought to suppress independently of these cytokines. To address the requirement for IL-10 and TGF-β in suppressor cell function in CD4⁺low/negCD25⁺ T cells, Tregs from B6 or IL-10⁻/⁻ mice were isolated and expanded ex vivo. CD4⁺low/negCD25⁺ T cells from B6 or B6.IL-10⁻/⁻ cultures were separated from CD4⁺CD25⁺ T cells at the end of the culture using MACS and were used in a suppression assay. To block TGF-β...

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**FIGURE 5.** Suppressor mechanisms. A, CD4⁺low/negCD25⁺ T cells or CD4⁺CD25⁺ T cells from wt or IL-10⁻/⁻ mice were separated after expansion and were cocultured with 10⁶ per well CD4⁺CD25⁻ T cells and 10⁶ per well irradiated allogeneic splenic stimulators at the indicated ratios in the absence or presence of 100 μg/ml anti-TGF-β mAb. Proliferation was determined by adding [³H]thymidine at day 6, and cells were harvested 16–18 h later. Average plus SEM of triplicate samples from one of three similar experiments is shown. B, CD4⁺low/negCD25⁺ T cells or CD4⁺CD25⁺ T cells were separated after expansion and were cocultured with CD4⁺CD25⁻ T cells and irradiated allogeneic splenic stimulators in direct contact or separated by a semipermeable membrane. Proliferation was determined by adding [³H]thymidine at day 6, and cells were harvested 16–18 h later. Average plus SEM of triplicate samples from one of three similar experiments is shown.

**FIGURE 6.** Expression and role of Treg molecules in CD4⁺low/negCD25⁺ T cells. A, FACS analysis of expanded Treg cultures gated on CD4⁺CD25⁺ and CD4⁺low/negCD25⁺ T cells. B, CD4⁺CD25⁺ T cells, CD4⁺low/negCD25⁺LAG-3⁺, and CD4⁺low/negCD25⁺LAG-3⁻ T cells were sorted by FACS after expansion and were cocultured with 10⁶ per well CD4⁺CD25⁻ T cells and 10⁶ per well irradiated allogeneic splenic stimulators at the indicated ratios. Proliferation was determined by adding [³H]thymidine at day 6, and cells were harvested 16–18 h later. Average plus SEM of triplicate samples from one experiment is shown. C, CD4⁺low/negCD25⁺ T cells or CD4⁺CD25⁺ T cells from wt or LAG-3⁻/⁻ mice were separated after expansion and were cocultured with 10⁶ per well CD4⁺CD25⁻ T cells and 10⁶ per well irradiated allogeneic splenic stimulators at the indicated ratios. Proliferation was determined by adding [³H]thymidine at day 6, and cells were harvested 16–18 h later. Average plus SEM of triplicate samples from one of two identical experiments is shown. D, CD4⁺CD25⁺ T cells, CD4⁺low/negCD25⁺CD103⁺, and CD4⁺low/negCD25⁺CD103⁻ T cells were sorted by FACS after expansion and were cocultured with 10⁶ per well CD4⁺CD25⁻ T cells and 10⁶ per well irradiated allogeneic splenic stimulators at the indicated ratios. Proliferation was determined by adding [³H]thymidine at day 6, and cells were harvested 16–18 h later. Average plus SEM of triplicate samples from two similar experiments is shown.
signaling, an anti-TGF-β-neutralizing mAb was added to the IL-10-deficient CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells during the suppression assay. Fig. 5A shows that IL-10 and TGF-β were not required for suppressor function of CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells in vitro.

Naturally occurring Tregs are thought to suppress in a contact-dependent manner or require close proximity to effector T cells and/or APCs for suppressor function. To test whether CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells suppress effector T cells in a similar fashion, CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells were separated from CD4<sup>+</sup> CD25<sup>-</sup> T cells based on CD4 expression at the end of the culture using MACS and were used in a suppression assay separated from responder T cells and APCs by a semipermeable membrane. These results showed that contact or close proximity between CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells and responder T cells and APCs was required for their suppressive effect (Fig. 5B).

**CD4<sup>low/neg</sup>CD25<sup>+</sup> Tregs express cell surface Ags implicated in Treg phenotype and function**

Several surface and effector molecules have been shown to be involved in suppressor function of Tregs, including LAG-3 and CD103 (18, 19). To determine whether CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells express some of these suppressor molecules, we expanded Tregs ex vivo and analyzed CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells and CD4<sup>-</sup>CD25<sup>+</sup> T cells separately based on their differential CD4 expression. The majority of CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells expressed LAG-3 on their surface, and a subpopulation expressed CD103 (Fig. 6A). In contrast, neither LAG-3 nor CD103 was detected on expanded CD4<sup>-</sup>CD25<sup>+</sup> T cells. Expression of ICOS and intracellular CTLA-4 was comparable on CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells and CD4<sup>-</sup>CD25<sup>+</sup> T cells. Expression of the TNFR family members OX40 and glucocorticoid-induced TNFR-related protein was higher on CD4<sup>-</sup>CD25<sup>+</sup> T cells compared with CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells. CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells expressed slightly higher levels of intracellular granzyme B, consistent with their cytokine profile. Expression of molecules shown in Fig. 6A did not correlate with any subpopulation of T cells shown in Fig. 4E, and expression levels were similar.

To test whether LAG-3 is required for the suppressor function in CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells, we sorted expanded cultures into CD4<sup>-</sup>CD25<sup>-</sup>, CD4<sup>low/neg</sup>CD25<sup>+</sup> LAG-3<sup>-</sup>, and CD4<sup>low/neg</sup>CD25<sup>+</sup> LAG-3<sup>+</sup> populations using FACS sorting. As shown in Fig. 6B, CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells that do not express LAG-3 were equally potent as their LAG-3<sup>-</sup> counterparts in an in vitro suppression assay. To further test whether LAG-3 is involved in CD4<sup>low/neg</sup>CD25<sup>+</sup> T cell development, activation, or expansion, we isolated Tregs from B6 and LAG-3<sup>-/-</sup> mice and activated and expanded these cells ex vivo. At the end of culture, CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells were separated from CD4<sup>-</sup>CD25<sup>+</sup> T cells based on CD4 expression by MACS and used in an in vitro suppression assay. No significant difference was observed in the expansion rate between B6 and LAG-3<sup>-/-</sup> cultures (data not shown).

As shown in Fig. 6C, we did not observe a significant difference in CD4<sup>low/neg</sup>CD25<sup>+</sup> T cell suppression isolated from wild-type (wt) or LAG-3<sup>-/-</sup> mice. To test whether suppressor function correlated with CD103 expression, we sorted expanded cultures into CD4<sup>-</sup>CD25<sup>-</sup>, CD4<sup>low/neg</sup>CD25<sup>+</sup> CD103<sup>-</sup>, and CD4<sup>low/neg</sup>CD25<sup>+</sup> CD103<sup>+</sup> populations using FACS sorting. Interestingly, only CD4<sup>low/neg</sup>CD25<sup>+</sup> CD103<sup>-</sup> T cells were able to suppress an alloresponse very potently (Fig. 6D).

**Discussion**

We show that CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells pre-existing at low frequency could outgrow conventional T cells and acquire potent suppressor function, independent of the presence of CD4<sup>-</sup>CD25<sup>+</sup> T cells, during ex vivo culture. Even though CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells appeared to express low levels of CD4 by FACS, quantitative PCR indicated that these cells did not express mRNA for CD4, suggesting passive acquisition of low levels of CD4 Ag from CD4<sup>-</sup> cells during culture. CD4<sup>low/neg</sup>CD25<sup>+</sup> T cell suppressor function is induced during ex vivo activation and culture, although it remains possible that there is an outgrowth of a highly potent suppressor cell population underrepresented in the initial CD4<sup>low/neg</sup>CD25<sup>+</sup> T cell population that accounts for the suppressor function seen after ex vivo culture. CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells showed an activated phenotype and consisted of several distinct populations, including γδ, CD8, and NKT cells, each of which had similar cell surface Ag expression and cytokine production as well as suppressor cell function. Suppression by CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells required close proximity to effector T cells and APCs because Transwells precluded inhibition of effector T cell proliferation and suppression by CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells was not dependent upon the effects of IL-10 or TGF-β regulatory cytokines. Even though a subset of CD4<sup>low/neg</sup>CD25<sup>+</sup> T cells expressed CD103 and the vast majority expressed LAG-3, neither was required for their suppressor function.

Because freshly isolated Tregs are present at low frequency, ex vivo expansion of Tregs often will be required for therapeutic use in patients and for more detailed characterization of Treg suppression mechanisms. Although several methods for Treg isolation and expansion have been reported, common problems that can hinder these uses include Treg purity and maintenance of potent suppressor function at the end of ex vivo culture. Because T cells other than naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> Tregs can acquire regulatory properties in vitro or in vivo (17), the finding that suppressor cell function is observed in cultures initiated with a high proportion of naturally occurring Tregs and designed to polyclonally expand T cells does not ensure that suppression is only due to CD4<sup>+</sup>CD25<sup>+</sup> T cells, as we have demonstrated. For example, the reduction in graft-vs-host disease (GVHD) lethality capacity of polyclonally expanded alloreactive CD4<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> T cells (20) may be due to the presence of other T cell populations such as those reported in this study. Others have shown that activated CD4<sup>+</sup> T cells maintained in medium supplemented with IL-2 in vitro can be rendered unresponsive to Ag restimulation and can suppress naive T cell (21). Similarly, in our studies, other activated T cell population can acquire suppressor function during in vitro activation and expansion in the presence of exogenous IL-2.

Polyclonal Treg expansion conditions often are used for preclinical studies and will be incorporated into many therapeutic trials, especially in instances in which there are numerous or unknown target peptides that can be loaded onto APCs used to drive Ag-specific Treg expansion, or there are undesirable effects of using host APCs, as might be an instance in which the patient has a hematopoietic malignancy. Therefore, the need to develop expansion of polyclonal Tregs, as used in this study, has been a major focus of the field. For clinical applications, our data raise the question as to whether it always is advantageous to initiate cultures with a highly purified population if bystander cells could contribute to suppression either directly or via support Treg expansion. Furthermore, our data highlight the need for detailed characterization of ex vivo activated and expanded Treg cultures in rodent and human systems. The use of ex vivo expanded cells for studies designed to characterize Tregs may be compromised by the reliance exclusively upon suppressor cell function as the major readout of preservation of Treg function at the end of culture because other non-Treg populations clearly can dominate the suppressor cell assay response. Limited flow cytometry analysis of ex vivo expanded Treg-containing populations may be insufficient, especially considering that murine Tregs can reduce or lose FoxP3 protein expression during culture.
or FoxP3− cells can outgrow ex vivo cultures, whereas activated human CD4+ CD25− T cells can acquire FoxP3 expression (16, 22, 23). The use of highly purified, cell-sorter isolated FoxP3-GFP transgenic CD4+ CD25high cells could be used to avoid such complications of expansion, although this method cannot be applied to human Tregs. Drugs such as rapamycin that favor the development of suppressor cell function warrant investigation in cultures such as these to determine the extent to which the increased potency of suppression is due to effects on preformed Tregs.

Although several groups have identified NK1.1+ γδ T cells, this is the first report to show that this population can be propagated from ex vivo CD4+ CD25+ T cell enriched cultures (24–27). Drobyski et al. (28) showed that ex vivo activated γδ T cells could delay GVHD when administered 2 wk before effector T cell infusion, suggesting a regulatory function of expanded γδ T cells in vivo. Double-negative (DN) αβ TCR Tregs have been identified in rodents and humans (29). A recently published report showed that DN Tregs could be derived from Tregs expanded ex vivo and in vivo (30). These Tregs could be derived DN-Tregs that lost FoxP3 expression, and their suppressor function was dependent on perforin. In mice, such cells, present at very low numbers in peripheral lymphoid tissues, can be expanded in vitro and, upon adoptive transfer into irradiated allogeneic recipients, will prevent graft rejection and GVHD. In our study, we generated activated T cells with suppressor function that were independent of Tregs. Together, these studies highlight the complexity of Treg phenotypes as well as mechanisms of suppressor functions of suppressor T cells expanded ex vivo.

The suppressor mechanisms of naturally occurring Tregs are still largely unknown. In vitro suppression is thought to be contact dependent or rather dependent on close proximity to effector T cells and APCs. Most induced Tregs secrete IL-10 and TGF-β or secrete Th2-type cytokines (17). The CD4low/−/CD25+ T cells described in this study produce a large amount of Th1-type cytokines involved in cytotoxic immune responses. Similar to our findings with CD4low/−/CD25+ T cells, neither IL-10 and TGF-β (31) is required for in vitro suppression by naturally occurring Tregs, although this is not uniformly the case for Tregs in vivo (32–34). CD4low/−/CD25+ T cells could limit alloresponses by targeting alloreactive T cells or allo-APCs directly by releasing these cytokines or through granzyme B and perforin (33, 34). Although blocking LAG-3 has been shown to abrogate suppressor function in natural Tregs and Ag-specific transgenic Tregs (18), and even though a large fraction of CD4low/−/CD25+ T cells was LAG-3+.

LAG-3 expression was not required for their suppressor function. Our data also showed that CD4low/−/CD25+ CD103+ T cells did not suppress as well as their CD103− counterpart. CD103 is a recently identified marker for a subset of Tregs that is important for immune regulation in vivo. Because CD103 does not seem to have suppressor function itself, but rather is important in Treg homing or retention (19, 35), it is likely that the expression of CD103 demarcates a subpopulation of CD4low/−/CD25+ T cells and itself does not influence suppressor cell function.

In conclusion, we report the identification and characterization of CD4low/−/CD25+ T cell population that consisted of subpopulations of T cells expressing either an αβ or γδ TCR. Such cells had an activated phenotype and did not express the transcription factor FoxP3, and, despite the expression of LAG-3 and CD103, suppressor cell function was not dependent upon LAG-3 or CD103 expression. Because CD4low/−/CD25+ T cells were more than 20-fold more potent than expanded conventional Tregs in suppressing an in vitro CD4−CD25− T cell response to allo-Ag, ex vivo expansion cultures should be carefully monitored by flow cytometry for the existence of non-Treg populations, and further indicate that suppression potency can be strikingly augmented by non-Treg populations present at low frequency at the time of culture initiation.

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Disclosures

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References


