The IL-4 Receptor α-Chain-Binding Cytokines, IL-4 and IL-13, Induce Forkhead Box P3-Expressing $CD25^+CD4^+$ Regulatory T Cells from $CD25^-CD4^+$ Precursors[1]

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The mechanisms underlying the extrathymic generation of $CD25^+CD4$ regulatory T cells (Tregs) are largely unknown. In this study the IL-4R α-chain-binding cytokines, IL-4 and IL-13, were identified as inducers of $CD25^+$ Tregs from peripheral $CD25^-CD4$ naive T cells. IL-4-induced $CD25^+$ Tregs phenotypically and functionally resemble naturally occurring Tregs in that they are anergic to mitogenic stimulation, inhibit the proliferation of autologous responder T cells, express high levels of the Forkhead box P3 and the surface receptors glucocorticoid-induced TNFR family-related protein and CTLA-4, and inhibit effector T cells in a contact-dependent, but cytokine-independent, manner. The IL-4-induced generation of peripheral Tregs was independent of the presence of TGF-β or IL-10, but was dependent on Ag-specific stimulation and B7 costimulation. The significance of the IL-4Rα-binding cytokines in the generation of Ag-specific Tregs was emphasized in a mouse model of oral tolerance, in which neutralization of IL-4 and IL-13 in mice transgenic for the TCR specific for OVA completely inhibited the expansion of OVA-specific Tregs that can be induced in untreated mice by feeding the nominal Ag. Together, our results demonstrate that IL-4 and IL-13 play an important role in generating Forkhead box P3-expressing $CD25^+$ Tregs extrathymically in an Ag-dependent manner and therefore provide an intriguing link between the well-established immunoregulatory capacity of Th2 cells and the powerful $CD25^+$ Treg population. Moreover, our findings might provide the basis for the design of novel therapeutic approaches for targeted immunotherapy with Tregs to known Ags in autoimmune diseases or graft-vs-host reactions. ***The Journal of Immunology,*** **2005, 175: 6107–6116.**

Thymus-derived $CD25^+CD4$ regulatory T cells (Tregs;[3] so-called naturally occurring $CD25^+$ Tregs) constitute 5–10% of peripheral CD4 T cells in mice and humans (1–4). They are widely believed to play an important role in controlling self-reactive T cells and maintaining immunologic self-tolerance (5, 6). Injection of peripheral T cells depleted of $CD25^+CD4$ T cells into athymic animals results in a high incidence of organ-specific autoimmune diseases (1, 7). The critical ability of $CD25^+$ Tregs to control autoimmune diseases has sparked much interest concerning whether such cells develop or expand in the periphery and, if so, which mechanisms underlie this process. Several factors have been identified that make the development of $CD25^+$ Tregs in the periphery tenable. First, soluble factors, for example, a combination of TGF-β and IL-2, promote the expansion of $CD25^+CD4$ T cells with regulatory capacities from activated human naive CD4 T cells (8). Suboptimal costimulation during Ag presentation using immature or pharmacologically treated APC has also favored the induction of $CD25^+$ Tregs in vivo and in vitro (9–11).

Considerable evidence has been accumulated for the anti-inflammatory ability of Th2 cytokines, in particular of IL-4, in vivo as well as in vitro. For example, amelioration of autoimmune diabetes in NOD mice is associated with increased expression of the Th2-derived cytokines, IL-4 and IL-5 (12, 13). Pancreatic expression of IL-4, moreover, completely prevents diabetes in NOD mice (14). Injection of IL-4-transduced dendritic cells (DC) or T cells reduces the incidence and severity of collagen-induced arthritis (15) and experimental autoimmune encephalomyelitis (16). Furthermore, treatment of proteoglycan-induced arthritis with rIL-4 induces a switch from a Th1-type to a Th2-type response and prevents the onset of disease (17). In vitro, IL-4 has been shown to have a direct inhibitory effect on the development of human Th1 cells (18). Recent studies aiming to dissect the impact of new treatment approaches on human autoimmune diseases have revealed that clinical benefit may be associated with enhanced Th2 cell differentiation in vivo (19–21). Moreover, administration of IL-4 to patients with psoriasis resulted in improvement of skin disease (22).

Given the potency of Th2 cytokines as regulators of inflammatory immune processes and the crucial role of the $CD25^+CD4$ T cell population as $CD25^+$ Tregs in the periphery, we investigated whether Th2 cytokines might induce the development of $CD25^+$ Tregs, providing physiologic amplification of the regulation of Th1-mediated immune responses by Th2 cytokines.

Materials and Methods

Reagents and Abs

The following mAbs were used for purification, stimulation, and staining of human cells: anti-CD16 (3g8FcIII), anti-CD3 (OKT3), anti-CD8 (OKT8), anti-

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[3] Abbreviations used in this paper: Treg, regulatory T cell; DC, dendritic cell; Foxp3, Forkhead box P3; γc, common γ-chain; GITR, glucocorticoid-induced TNFR family-related protein; NHS, normal human serum; Tg, transgenic.

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CD45RO (UCHL-1), and anti-HLA-DR (L243; American Type Culture Collection); FITC-conjugated anti-CD3, PE-labeled anti-CD4, and FITC-labeled anti-CD4 (Sigma-Aldrich); FITC-labeled anti-CD14 (Cymbus Biotechnology); anti-CD19 and FITC-labeled anti-HLA-DR (Dako Diagnostika); PE-labeled anti-CD25, PE-labeled anti-CD83, FITC-labeled anti-CD80, PE-labeled anti-CD86, PE-labeled anti-CTLA-4, PE-labeled mouse IgG2a, and PE-labeled mouse IgG2b (BD Pharmingen); PE-labeled anti-glucocorticoid-induced TNFR family-related protein (anti-GITR; R&D Systems); polyclonal goat anti-mouse Ig (ICN Biomedicals); neutralizing mAb to IL-10, TGF-β, and IL-2 (R&D Systems); and neutralizing mAb to IL-4 (Endogen). Human CTLA-4-Ig was provided by Bristol-Myers Squibb. Human rIL-4 was obtained from Endogen, and IL-5, IL-9, and IL-13 were purchased from R&D Systems. Human rGM-CSF, rIL-4, rIL-6, rTNF, and rIL-1β, used for DC maturation, were obtained from CellGenix. PGE_2 was purchased from Pharmacia Biotech. Isotype-matched control Abs (mouse IgG2a for anti-IL-2 and anti-HLA-DR, mouse IgG2b for anti-IL-10 and anti-TGF-β) were purchased from BD Pharmingen.

For staining of murine cells, FITC-labeled anti-CD4, PE-labeled anti-CD25 (both from BD Pharmingen), and PE-Cy5-conjugated anti-clonotype-specific mAb KJ1-26 (Caltag Laboratories) were used. For the neutralization experiments, anti-IL-4 (11B11; American Type Culture Collection), anti-IL-13 (R&D Systems), and complete rat IgG (Sigma-Aldrich) were used. OVA was purchased from Sigma-Aldrich.

Mice

OVA TCR-transgenic (Tg) mice on the BALB/c background (clone DO11.10) were purchased from The Jackson Laboratory. The animals were housed under pathogen-free conditions in the animal facility of the Nikolaus Fiebiger Center. Mice were used at 6–12 wk of age throughout the studies.

Cell purification

PBMC were obtained from heparinized venous blood donated by healthy individuals by centrifugation over a Ficoll-Hypaque gradient (Sigma-Aldrich). For isolation of T cells, PBMC were incubated with SRBC as described previously (23). The rosette-negative cells were used as T cell-depleted PBMC. The rosette-positive cells were further purified by negative selection panning with mAbs to CD8, CD16, CD56, CD19, HLA-DR, and CD45RO as described previously (24). The recovered naive CD4 cells stained brightly for CD45RA and were negative for CD45RO. CD25$^+$ and CD25$^-$CD4 cell populations were isolated from the naive CD4 T cells using CD25 microbeads from Miltenyi Biotec according to the manufacturer's instructions. The homogeneity and purity of all isolated populations were routinely controlled by flow cytometry.

Generation of CD25$^+$ CD4 Tregs

All cell cultures were conducted in RPMI 1640 medium supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM; all from Invitrogen Life Technologies), and 10% normal human serum (NHS) at 37°C in a humidified atmosphere containing 5% CO_2. Purified naive CD4 T cells were incubated for 10 days at a concentration of 1×10^6/ml in the presence of 1×10^6/ml irradiated autologous T cell-depleted PBMC in a final volume of 2 ml in 24-well cell culture plates (Costar). IL-4 at a final concentration of 6.25 ng/ml was added to the cultures. Where indicated, IL-4 was replaced by IL-5 (10 ng/ml), IL-9 (10 ng/ml), or IL-13 (50 ng/ml); anti-IL-10, anti-TGF-β, anti-IL-2, anti-HLA-DR, and CTLA-4-Ig (all 10 μg/ml) were added to the IL-4-containing cultures. Control cultures were incubated with the appropriate isotype-matched Abs. After the 10-day culture, cells were harvested, counted, analyzed for surface expression of CD4 and CD25, and processed for CD25$^+$ and CD25$^-$ T cell isolation.

Generation of DC

DC were generated as described previously with minor modifications (25). In brief, T cell-depleted PBMC were plated in six-well cell culture plates (Costar) at a density of 10×10^6/well in 10% NHS/RPMI 1640. After 1–2 h of incubation, the nonadherent fraction was removed, and the adherent fraction was allowed to stand overnight in fresh medium. Starting the next day, cells were fed every second day (days 1, 3, and 5) with 800 U/ml GM-CSF and 250 U/ml IL-4. On day 6, maturation cytokine mixture containing IL-1β, TNF, IL-6, and PGE_2 was added at final concentrations of 2 ng/ml IL-1β, 10 ng/ml TNF, 1000 U/ml IL-6, and 1 μg/ml PGE_2. Two days later, cells were visually monitored with a microscope, harvested, and analyzed by flow cytometry.

Proliferation assay

CD25$^+$ and CD25$^-$ T cells (25×10^3/well) were cultured together or separately in triplicate for 3 days in 96-well, round-bottom plates (Corning) in the presence of soluble anti-CD3 mAb (1 μg/ml) and in the presence of 50×10^3 irradiated T cell-depleted PBMC. In the experiments assessing the Ag specificity of the generated CD25$^+$CD4 T cells, T cell-depleted PBMC were replaced by irradiated DC. Transwell experiments were conducted in 24-well plates in the presence of soluble anti-CD3 mAb (1 μg/ml); irradiated T cell-depleted PBMC were added to both the Transwell (Millipore) and the well. Neutralizing anti-IL-10, anti-IL-4, or anti-TGF-β Abs (all at 10 μg/ml) were added where indicated. Incorporation of [^{3}H]TdR (1 μCi/well) by proliferating lymphocytes during the last 16 h of the culture was measured using a liquid scintillation counter.

Cytokine determination

CD25$^+$ and CD25$^-$CD4 T cells were stimulated at a concentration of 1×10^6/ml with ionomycin (1 mM; Calbiochem) and PMA (20 ng/ml; Sigma-Aldrich) for 24 h. Levels of IL-10, IL-4, TNF, and IFN-γ were measured in supernatants using commercially available ELISA kits (R&D Systems).

Preparation of total RNA and amplification of cDNA

Total RNA was extracted using the RNeasy Minikit (Qiagen) with an additional DNA digestion step (RNase Free DNase Set; Qiagen). One microgram of mRNA was transcribed to cDNA for 1 h at 42°C in a total volume of 20 μl containing 1× avian myoblastosis virus reverse transcriptase buffer (Promega), 1 mM dNTPs (all from Amersham Biosciences), 100 ng/ml oligo(dT)$_{12-18}$ (Amersham Biosciences), and 0.25 U/μl avian myoblastosis virus reverse transcriptase (Promega). Real-time PCR was performed in duplicate using the Universal PCR Master Mix and Assays-on-Demand Gene Expression Products for Forkhead box P3 (Foxp3) and elongation factor-1α in the ABI PRISM 7000 Sequence Detection System (all from Applied Biosystems). Foxp3 mRNA expression was normalized to the expression of the housekeeping gene, elongation factor-1α.

Induction of CD25$^+$CD4 Tregs in response to oral Ag administration

OVA TCR-Tg mice were fed 20 mg/ml OVA in the drinking water for 5 days following the protocol described previously (26). On day −1, animals were treated i.p. with anti-IL-4, anti-IL-13, or complete rat IgG (0.5 mg/mouse). Mice were killed on day 6, and splenocytes were analyzed.

Statistical analysis

Results were analyzed by one-way ANOVA, followed by the Tukey or Bonferroni test.

Results

IL-4-induced development of CD25$^+$ Tregs from human naive CD4 T cells

To investigate the effect of IL-4 on the development of CD25$^+$ Tregs, we used a cell culture system in which human naive CD4 T cells were cultured in 10% NHS/RPMI 1640 in the presence of autologous T cell-depleted irradiated PBMC as APC and in the presence or the absence of IL-4. After 10 days of culture, the frequencies of CD25$^+$ cells were significantly increased independent of the presence of IL-4 (Fig. 1, *A* and *Bb*). However, when the absolute T cell numbers before and after the 10-day culture were compared, IL-4 was found to induce significant expansion of CD4 T cells (Fig. 1*Ba*). Accordingly, significantly larger numbers of CD25$^+$ T cells were obtained from IL-4-stimulated compared with control cultures (Fig. 1*Bc*). Analysis of the proliferative capacity of the recovered purified CD25$^+$ and CD25$^-$CD4 T cells revealed that CD25$^+$ T cells isolated from IL-4-stimulated cultures were anergic to anti-CD3 stimulation (Fig. 1*Bd*). In contrast, CD25$^+$ T cells recovered from control cultures (i.e., in the absence of IL-4) proliferated effectively and even more vigorously than CD25$^-$ T cells from the same cultures in response to anti-CD3 stimulation (Fig. 1*Bd*). Not only were CD25$^+$ T cells isolated from IL-4-stimulated cultures anergic, they also suppressed the proliferation of CD25$^-$ T cells (Fig. 1*Bd*). These findings demonstrate that the Th2

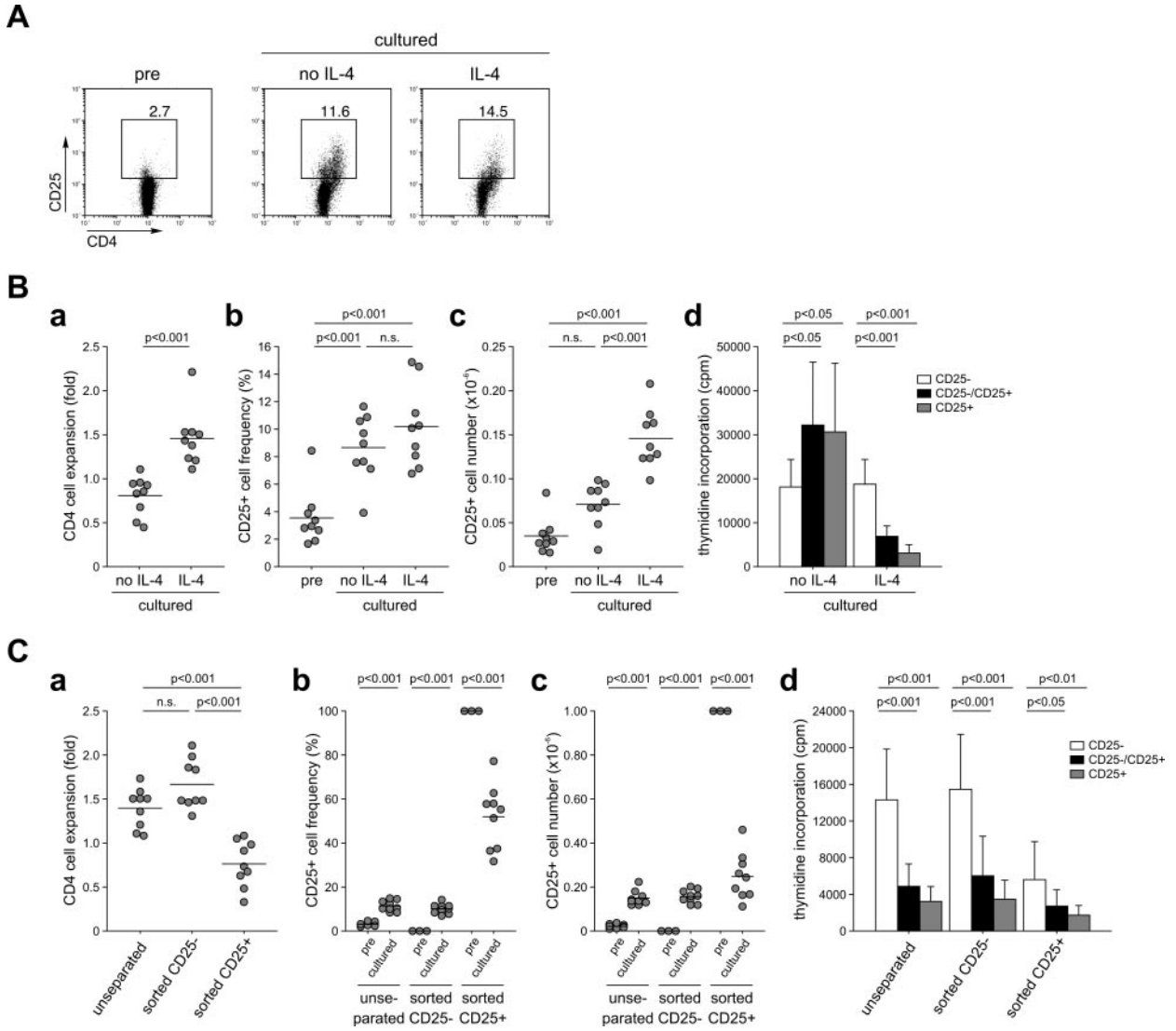

FIGURE 1. Development of anergic CD25$^+$ Tregs in response to IL-4. Freshly isolated human naive CD4 T cells, either unfractionated or sorted into CD25$^+$ and CD25$^-$ T cells where indicated, were cultured in the presence of autologous APC and in the presence or the absence of IL-4. After 10 days of culture, CD4 T cells were analyzed for the extent of expansion, as calculated by comparison of cell numbers before and after culture, for the surface expression of CD25, for the absolute number of CD25$^+$ cells generated from an initial 1×10^6 CD4 T cells, and for the proliferative capacity of isolated CD25$^+$ and CD25$^-$ T cells in response to anti-CD3. *A*, Representative staining patterns of freshly isolated naive CD4 T cells (pre) and naive CD4 T cells that had been cultured for 10 days in the absence or the presence of IL-4 are demonstrated. *B*, Expansion of CD4 T cells (*a*), the frequencies (*b*), and the absolute numbers (*c*) of recovered CD25$^+$CD4 T cells before (pre) and after the 10-day cell culture in the presence or the absence of IL-4, and the proliferation of recovered CD25$^+$ and CD25$^-$ cells in response to anti-CD3 (*d*) are shown. The results of nine independent experiments using cells from different donors (indicated by circles or as the mean ± SD) are summarized. *C*, Freshly isolated naive CD4 T cells were sorted into CD25$^-$ (sorted CD25−) and CD25$^+$ (sorted CD25+) T cells or were left unfractionated (unseparated), and the proliferative and functional responses of these cells to IL-4 were examined. Data show the fold expansion of CD4 T cells (*a*), the frequencies (*b*) and the absolute numbers (*c*) of recovered CD25$^+$CD4 T cells, and the proliferation of recovered CD25$^+$ and CD25$^-$ cells in response to anti-CD3 (*d*). The results of nine independent experiments using cells from different donors (indicated by circles or as the mean ± SD) are shown. n.s., nonsignificant.

cytokine, IL-4, was able to induce the development and/or expansion of anergic CD25$^+$CD4 T cells with regulatory capacity. The effect of IL-4 on the attainment of the anergic and regulatory phenotype of CD25$^+$ T cells was concentration dependent (data not shown).

To delineate whether IL-4 induces the development of CD25$^+$ Tregs from CD25$^-$ T cells or, rather, the expansion of the small pool of CD25$^+$ Tregs pre-existing in the pool of naive CD4 T cells, freshly isolated human naive CD4 T cells were sorted into CD25$^+$ and CD25$^-$ T cells, and the ability of both populations to expand or to generate CD25$^+$ T cells in response to IL-4 and autologous APC was assessed. Analysis of T cell expansion revealed that T cells from the highly pure, initially CD25$^-$ population (≥99.5% CD25$^-$ cells) and from the unseparated population proliferated to a similar extent, whereas the CD25$^+$ T cell population did not proliferate at all (Fig. 1*Ca*) and, in fact, their absolute number even decreased in the culture (Fig. 1*Cc*). In contrast, the initially CD25$^-$ T cell population generated a significant number of CD25$^+$ T cells after stimulation with IL-4 (Fig. 1, *Cb* and *Cc*), confirming the hypothesis that these cells were generated de novo from CD25$^-$ T cells. The novel CD25$^+$ T cells that developed from the initially CD25$^-$ T cell population showed an anergic phenotype and were able to inhibit the proliferation of CD25$^-$ T cells to an extent comparable to that of the CD25$^+$ T cells isolated from unseparated control cultures (Fig. 1*Cd*). Together, in the

presence of autologous APC, IL-4 favored the induction and growth of CD25$^+$CD4 Tregs from CD25$^-$ T cells.

IL-4-induced CD25$^+$CD4 Tregs resemble naturally occurring CD25$^+$ Tregs

The IL-4-induced CD25$^+$CD4 Tregs generated from both unseparated and CD25$^+$ cell-depleted naive CD4 T cells inhibited the proliferation of CD25$^-$ T cells in a concentration-dependent fashion and expressed high levels of mRNA for the Foxp3 transcription factor (Fig. 2*A*). The inhibitory effect of these cells could be abolished by addition of exogenous IL-2 (data not shown). CD25$^+$, but not CD25$^-$, T cells constitutively expressed detectable levels of CTLA-4 and GITR and additionally up-regulated the expression of both proteins in response to anti-CD3 stimulation (Fig. 2*B*). Finally, in response to mitogenic stimulation, CD25$^+$ T cells that were generated from naive CD4 T cells in the presence of IL-4 produced high levels of the immunomodulatory cytokines, IL-10 and IL-4, but low levels of the effector cytokines, IFN-γ and TNF (Fig. 2*C*). However, despite this, the inhibition of proliferation was independent of IL-4, IL-10, and TGF-β, but was dependent on cell-cell contact (Fig. 2*D*). Together, the phenotypic and functional characteristics of the IL-4-induced CD25$^+$CD4 Tregs were comparable to those of the naturally occurring CD25$^+$ Tregs isolated directly from peripheral blood (data not shown).

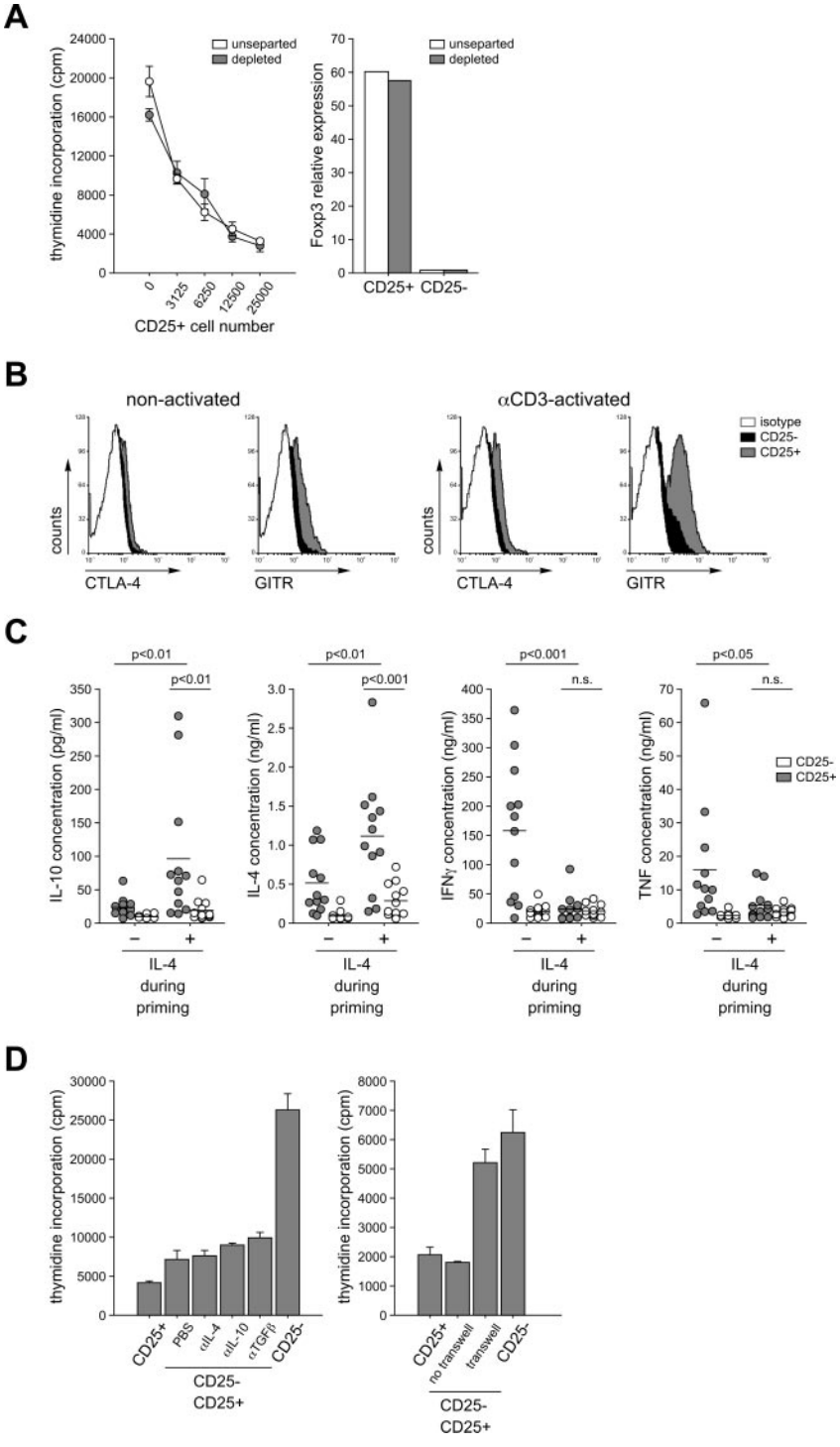

FIGURE 2. Characterization of IL-4-generated CD25$^+$ Tregs. Freshly isolated human naive CD4 T cells either unfractionated or depleted of CD25$^+$ T cells where indicated were cultured in the presence of autologous APC and in the presence or the absence of IL-4. After 10 days of culture, CD4 T cells were sorted into CD25$^-$ and CD25$^+$ T cells using a magnetic bead CD25$^+$ cell depletion system. *A*, CD25$^+$ and CD25$^-$ T cells isolated from IL-4-treated cultures of naive CD4 T cells either initially depleted of CD25$^+$ cells (depleted) or unfractionated (unseparated) were analyzed for their proliferative capacity in response to anti-CD3 and for the expression of Foxp3 mRNA. The *left panel* demonstrates proliferation of 25×10^3 CD25$^-$ T cells in the absence or the presence of increasing numbers of CD25$^+$ T cells in response to anti-CD3. The *right panel* shows Foxp3 mRNA expression by CD25$^+$ and CD25$^-$ T cells relative to expression by CD25$^-$ T cells. The results of one representative experiment of six performed for proliferative capacity and five performed for Foxp3 mRNA levels are shown. *B*, Expression of CTLA-4 and GITR by CD25$^+$ and CD25$^-$ T cells isolated from IL-4-treated cultures either directly after purification (nonactivated) or after 24-h stimulation with anti-CD3 (αCD3-activated). *C*, CD25$^+$ and CD25$^-$ T cells isolated from cells cultured in the presence or the absence of IL-4 were stimulated with PMA/ionomycin for 24 h. Levels of IL-10, IL-4, IFN-γ, and TNF were determined in supernatants by ELISA. The results of 12 independent experiments using cells from different donors (demonstrated by circles) are shown. n.s., nonsignificant. *D*, The proliferative capacity of CD25$^+$ and CD25$^-$ T cells isolated from cultures of IL-4-stimulated cells in response to anti-CD3 was assessed. The *left panel* shows inhibition of the proliferation of CD25$^-$ T cells by CD25$^+$ T cells in the presence of neutralizing Abs to IL-4, IL-10, and TGF-β. The *right panel* demonstrates proliferation of CD25$^-$ cells when CD25$^+$ cells were kept in a Transwell. A representative experiment of five (*left panel*) and four (*right panel*) performed is shown.

IL-4-induced differentiation of CD25$^+$ Tregs is not mediated by endogenous TGF-β or IL-10

To investigate whether the effect of IL-4 on the differentiation of CD25$^+$ Tregs was mediated by endogenously produced TGF-β or IL-10 that might have been secreted in response to IL-4, we cultured human naive CD4 T cells for 10 days in the presence of autologous APC and IL-4 and in the presence or absence of neutralizing Abs to TGF-β or IL-10. Neutralization of TGF-β or IL-10 affected neither the IL-4-induced growth of CD4 T cells nor the frequency or numbers of CD25$^+$CD4 T cells in the T cell populations after the cultures compared with the control cell cultures containing isotype-matched Abs (Fig. 3, *Aa*, *Ab*, and *Ac*). Purified CD25$^+$ T cells recovered from all cell cultures were anergic and were able to inhibit the proliferation of the respective CD25$^-$ T cell populations in response to anti-CD3 (Fig. 3*Ad*). Notable, however, was the finding that the extent of the inhibition by CD25$^+$ T cells isolated from anti-TGF-β cultures was modestly, but significantly, less than the inhibition by CD25$^+$ T cells isolated from isotype-treated as well as anti-IL-10-treated cultures (62.45 ± 18.60, 53.15 ± 23.05, and 66.89 ± 16.73% inhibition in control, anti-TGF-β-treated, and anti-IL-10-treated cultures, respectively). These results indicate that endogenously produced IL-10 was not involved, and TGF-β was only minimally, if at all, involved in the effect of IL-4 on the attainment of a regulatory phenotype by CD25$^+$ T cells.

Endogenous IL-2 is required for the outgrowth of CD25$^+$ Tregs

Next, we analyzed the role of endogenously produced IL-2 in the IL-4-induced differentiation of CD25$^+$ Tregs. Although IL-2 neutralization did not influence the expansion of the naive CD4 T cell population in response to IL-4 (Fig. 3*Ba*), it was critical for expansion of the CD25$^+$ T cell subpopulation, as demonstrated by the decreased CD25$^+$ cell frequencies and numbers in anti-IL-2-containing cultures compared with those using control Ab (Fig. 3, *Bb* and *Bc*). However, IL-2 had no effect on the attainment of the anergic or the regulatory phenotype of CD25$^+$ T cells (Fig. 3*Bd*). These results underline the importance of IL-2 as a growth factor for CD25$^+$ Tregs.

IL-13 shares with IL-4 the ability to induce CD25$^+$ Tregs

The next experiments delineated whether induction of CD25$^+$ Tregs is a unique feature of IL-4 or is a common characteristic of Th2 cytokines. IL-5 and IL-13, but not IL-9, were able to induce the expansion of CD4 T cells over the culture period to an extent similar to that induced by IL-4 (Fig. 4*A*). The frequencies of CD25$^+$ T cells after the 10-day culture were similar in all priming conditions (Fig. 4*B*). Although modestly lower numbers of CD25$^+$ T cells were recovered from the IL-5- and IL-9-treated cultures compared with the IL-4-stimulated cultures, IL-13 stimulation resulted in similar recoveries of those cells (Fig. 4*C*). A striking difference was observed, however, in the effect of the cytokines on

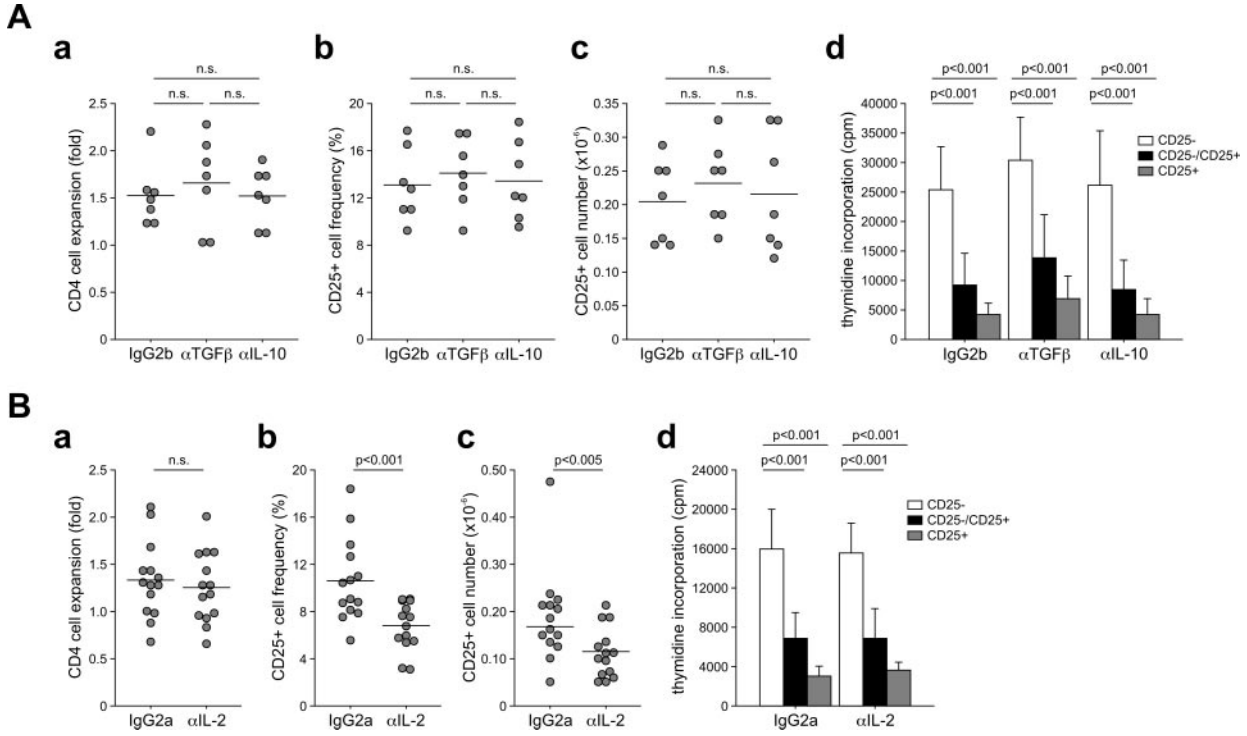

FIGURE 3. Endogenously produced cytokines in the IL-4-induced differentiation of CD25$^+$ Tregs. Freshly isolated human naive CD4 T cells were cultured in the presence of autologous APC, IL-4, and neutralizing Abs to TGF-β, IL-10, or IL-2 or the appropriate isotype-matched Abs. After 10 days of culture, CD4 T cells were analyzed for the extent of expansion, as calculated by comparison of cell numbers before and after the 10-day culture, for the frequency of CD25$^+$ cells, for the absolute number of CD25$^+$ cells generated from an initial 1×10^6 CD4 T cells, and for the proliferative capacity of CD25$^+$ and CD25$^-$ T cells in response to anti-CD3. *A*, The effects of endogenous TGF-β and IL-10 on the expansion of CD4 T cells (*a*), the frequencies (*b*) and absolute numbers (*c*) of recovered CD25$^+$CD4 T cells, and the proliferation of recovered CD25$^+$ and CD25$^-$ cells isolated from cultured cells in response to anti-CD3 (*d*) were investigated by addition of neutralizing anti-TGF-β (αTGFβ), anti-IL-10 (αIL-10) or isotype-matched (IgG2b) Abs. The results of seven independent experiments using cells from different donors (demonstrated by circles or as the mean ± SD) are shown. *B*, The effects of endogenous IL-2 on the expansion of CD4 T cells (*a*), the frequencies (*b*) and the absolute numbers (*c*) of recovered CD25$^+$CD4 T cells, and the proliferation of recovered CD25$^+$ and CD25$^-$ cells isolated from cultured cells in response to anti-CD3 (*d*) were investigated by addition of neutralizing anti-IL-2 (αIL-2) or isotype-matched (IgG2a) Abs. The results of 14 independent experiments using cells from different donors (demonstrated by circles or as the mean ± SD) are shown. n.s., Nonsignificant.

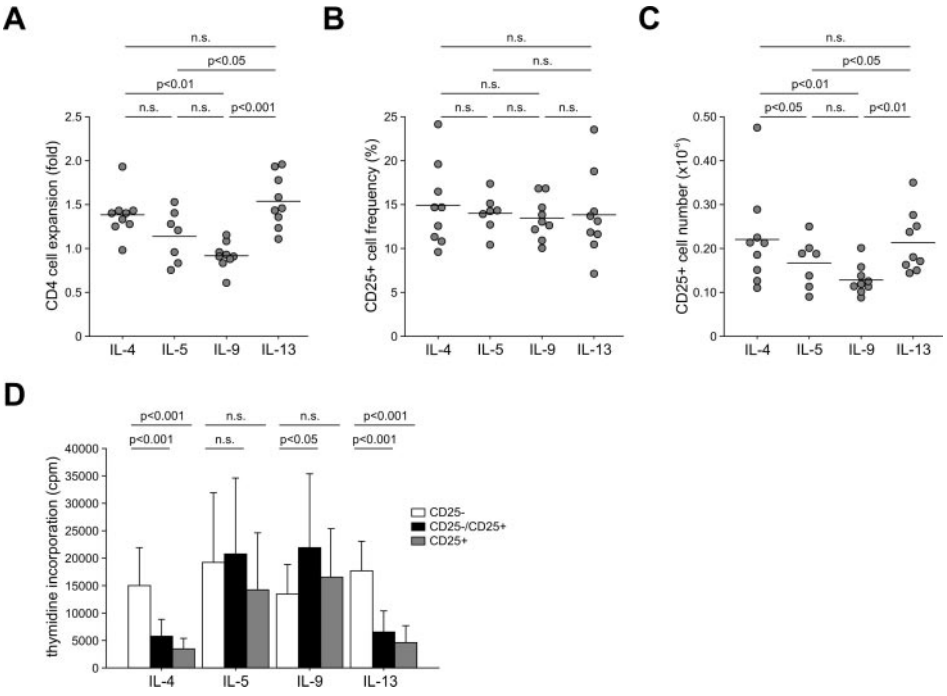

FIGURE 4. Effect of Th2 cytokines on the induction of CD25+ Tregs. Freshly isolated human naive CD4 T cells were cultured in the presence of autologous APC and in the presence of IL-4, IL-5, IL-9, or IL-13. After 10 days of culture, CD4 T cells were analyzed for the extent of expansion, as calculated by comparison of the cell numbers before and after the culture (*A*), for the frequency (*B*) and the absolute numbers (*C*) of recovered CD25+ cells, and for the proliferative capacity of CD25+ and CD25− T cells isolated from cultured cells in response to anti-CD3 (*D*). The results of nine independent experiments using cells from different donors (demonstrated by circles or as the mean ± SD) are shown. n.s., Nonsignificant.

the attainment of the anergic and regulatory phenotype of CD25+ T cells. Although CD25+ T cells isolated from IL-4- and IL-13-treated cultures showed an anergic phenotype and the ability to inhibit proliferation of CD25− T cells, CD25+ T cells sorted from cells that were initially cultured with IL-5 or IL-9 were neither anergic nor inhibitory (Fig. 4*D*). Notably, CD25+ T cells isolated

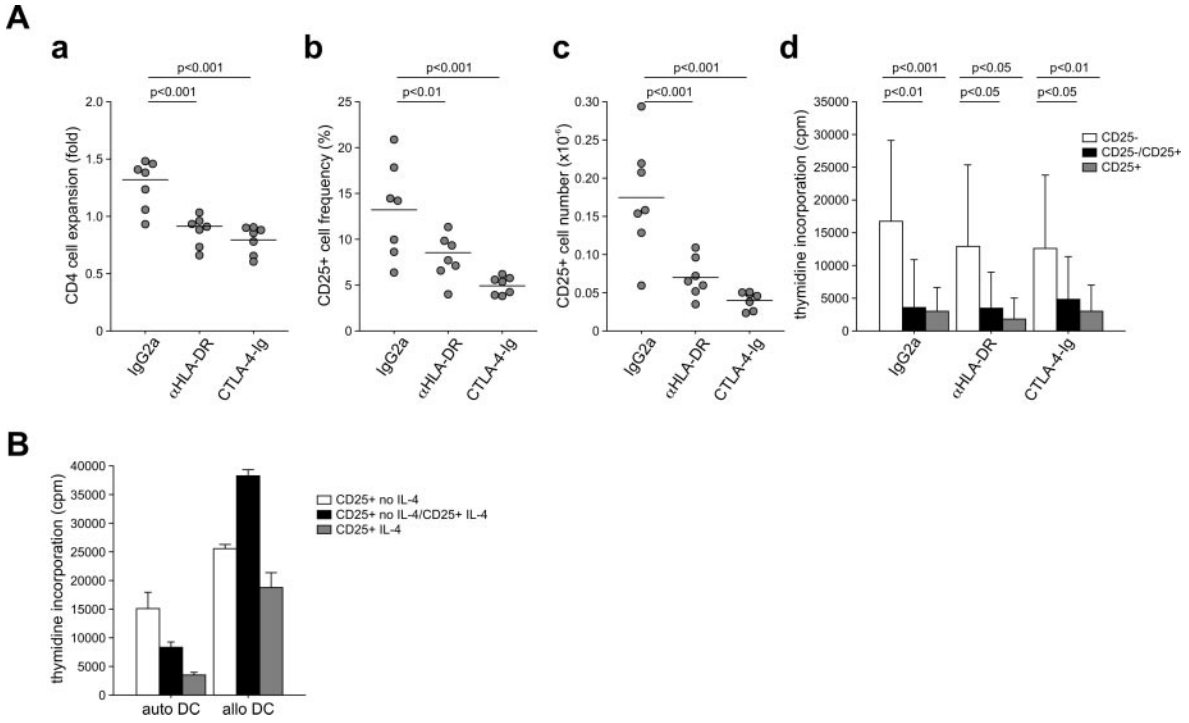

FIGURE 5. Analysis of the Ag specificity of IL-4-induced CD25+ Tregs. Freshly isolated human naive CD4 T cells were cultured for 10 days in the presence of autologous APC and in the presence or the absence of IL-4. *A*, The requirement for TCR and costimulatory signaling in the IL-4-mediated generation of CD25+ T cells was investigated by the addition of neutralizing anti-HLA-DR (αHLA-DR), CTLA-4-Ig (CTLA-4-Ig), or irrelevant mouse IgG2a (IgG2a). After 10 days of culture, CD4 T cells were analyzed for the extent of expansion, as calculated by comparison of cell numbers before and after culture (*a*), for the frequency of CD25+ cells (*b*), for the absolute numbers of CD25+ cells generated from an initial 1×10^6 CD4 T cells (*c*), and for the proliferative capacity of CD25+ and CD25− T cells isolated from cultured cells in response to anti-CD3 (*d*). The results of seven independent experiments using cells from different donors (demonstrated by circles or as the mean ± SD) are shown. n.s., nonsignificant. *B*, The Ag specificity of IL-4-induced CD25+ Tregs was tested by assessing the proliferative capacity of CD25+ T cells generated in the absence of IL-4 (CD25+ no IL-4) and the presence of IL-4 (CD25+ IL-4) in response to autologous (auto DC) or allogeneic (allo DC) irradiated DC, alone or in coculture. The results of one representative experiment performed in triplicate of 11 independent experiments using cells from different donors are shown.

from both IL-4- and IL-13-treated cultures inhibited proliferation of the respective CD25$^-$ T cell populations to a similar extent. Therefore, IL-4 and IL-13 shared the ability to induce CD25$^+$ anergic Tregs, suggesting that within the Th2 cytokines, those sharing the use of the IL-4Rα uniquely have the capacity to induce the development of CD25$^+$ Tregs.

CD25$^+$ T cells induced by IL-4 in the presence of autologous APC show an Ag-specific inhibition pattern

To address the question of whether IL-4 induces CD25$^+$CD4 Tregs with antigenic specificity, we first analyzed whether they were generated in an Ag-specific way by examining the impact of a blocking mAb to HLA-DR and of inhibiting CD28 costimulation. Prevention of TCR ligation or CD28 engagement during the culture inhibited the expansion of total CD4 cells (Fig. 5*Aa*) and, moreover, markedly reduced the frequencies of CD25$^+$CD4 T cells in the recovered populations (Fig. 5*Ab*) without influencing their regulatory function (Fig. 5*Ad*). Accordingly, a significantly diminished number of CD25$^+$ T cells was isolated from cells cultured in the presence of the anti-HLA-DR Abs or CTLA-4-Ig compared with that from cells cultured with irrelevant mouse IgG2a (Fig. 5*Ac*), indicating that the generation of CD25$^+$ Tregs in response to IL-4 was dependent on the presentation of Ag by autologous APC in the culture.

If IL-4-induced CD25$^+$ Tregs performed their function in an Ag-specific way, these Ags should be the same as those used for generation. Because CD25$^-$ cells from the priming cultures in which T cells were primed in response to autologous Ags were not reactive to autologous Ags, as indicated by their persistent CD25 negativity, we could not use these CD25$^-$ T cells as responder cells to repeated stimulation with autologous Ags, but had to use CD25$^+$ T cells that were purified from the T cell cultures that had been primed in the absence of IL-4, because these CD25$^+$ T cells were identified as effector T cells in the previous experiments (as shown, for example, in Fig. 1*Bd*). Therefore, we next generated CD25$^+$ T cells, as described above, in the presence of autologous APC and in the presence or the absence of IL-4 and investigated the capacity of the CD25$^+$ T cell populations to mount a proliferative response to DC from the same (autologous response) and from a different (allogeneic response) donor. CD25$^+$ T cells isolated from IL-4-stimulated cultures showed an anergic phenotype compared with CD25$^+$ T cells isolated from cells cultured in the absence of IL-4 in response to stimulation with autologous DC (Fig. 5*B*; $p < 0.001$). Moreover, CD25$^+$ T cells isolated from IL-4-stimulated cultures inhibited the proliferative response induced by autologous DC of CD25$^+$ T cells that had been generated in the absence of IL-4 (Fig. 5*B*; $p < 0.01$). In marked contrast, when stimulated with allogeneic DC, CD25$^+$ T cells generated in the presence of IL-4 showed neither an anergic phenotype nor an inhibitory capacity (Fig. 5*B*). These results demonstrate that CD25$^+$ Tregs generated in the presence of IL-4 and autologous APC exhibited their regulatory potential only when stimulated by autologous APC; this indicates that these cells are Ag specific.

Neutralization of IL-4 and IL-13 completely prevents the generation of CD25$^+$ CD4 Tregs in vivo in response to Ag

To ascertain whether IL-4 is involved in the generation of Ag-specific CD25$^+$ Tregs in vivo, we took advantage of an oral tolerance model, described previously (26), in which CD25$^+$CD4 T cells with regulatory capacity increase in number in the spleen and lymph nodes of OVA TCR-Tg mice in response to oral administration of OVA. In concordance with published data, OVA feeding resulted in a significant increase in total as well as clonotype-positive CD25$^+$CD4 T cell frequencies in the spleen (Fig. 6, *Aa*, *Ab*, and *Ba*) accompanied by a pronounced decrease in the frequency of CD4$^+$ T cells (Fig. 6*Ab*). These CD25$^+$CD4 T cells exhibited a regulatory function (data not shown), confirming the original report (26). When the effect of IL-4/IL-13 neutralization during OVA feeding was analyzed, we found that splenic clonotype-positive CD4 T cells from animals that had received either Ab alone contained lower frequencies of CD25$^+$ T cells compared with cells from control animals (Fig. 6*Ba*). Inhibition of the increase in frequency of KJ1-26$^+$CD25$^+$ T cells was even greater when both cytokines were neutralized (Fig. 6*Ba*).

Because the increased percentage of clonotype-positive CD25$^+$CD4 T cells in OVA-fed mice could be simply related to a decrease in CD4 T cells, in particular, those not expressing CD25, it was important to analyze the absolute numbers of KJ1-26$^+$CD25$^+$CD4 T cells obtained from spleens of these animals. Significantly higher numbers of KJ1-26$^+$CD25$^+$CD4 T cells were obtained from the OVA-fed animals treated with irrelevant Ig than from the unfed animals (Fig. 6*Bb*). Treatment with either neutralizing Ab markedly inhibited the increase in KJ1-26$^+$CD25$^+$CD4

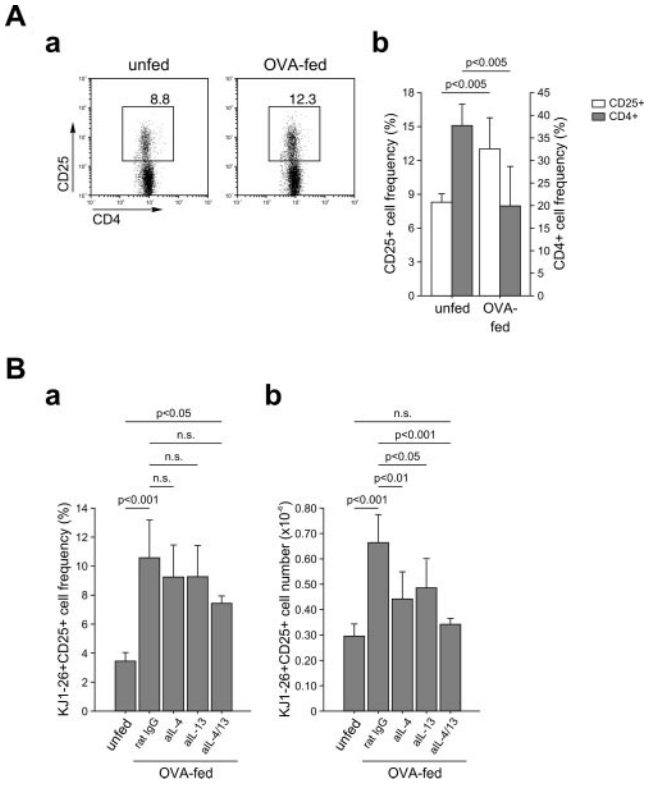

FIGURE 6. Effects of IL-4 and IL-13 on CD25$^+$ Tregs in response to oral Ag administration. OVA TCR-Tg mice were fed OVA in the drinking water for 5 days, and splenocytes were analyzed by flow cytometry on day 6. Where indicated, the animals were treated. *A*, Frequencies of total CD25$^+$ T cells in CD4 T cells and CD4 T cells in the lymphocyte gate were analyzed. *a*, A representative expression pattern of CD25 by CD4 T cells isolated from unfed or OVA-fed mice is demonstrated. *b*, Mean ± SD percentage of CD25$^+$ T cells within the CD4 population and of CD4 T cells within lymphocytes in unfed and OVA-fed mice. The data shown are the results of one of two independent experiments (five mice per group) with similar results. *B*, Animals were treated with Abs neutralizing IL-4 (αIL-4) or IL-13 (αIL-13) alone, with the combination of both (αIL-4/13), or, as a control, with complete rat IgG (rat IgG), as described in *Materials and Methods*, and the frequencies (*a*) and absolute numbers in the spleen (*b*) of clonotype-specific KJ1-26$^+$CD25$^+$CD4 T cells were investigated. The data shown are the results of one of two independent experiments (five mice per group) with similar results.

T cells. When both cytokines were neutralized, the increase in the number of KJ1-26$^+$CD25$^+$CD4 T cells was completely prevented (Fig. 6*Bb*). Together, these data demonstrate that IL-4 and IL-13 are necessary for the development of Ag-specific CD25$^+$ Tregs in response to oral tolerization, providing a strong indication for a critical role of both cytokines in the generation of Ag-specific CD25$^+$ Tregs in vivo.

Discussion

We have found that the Th2 cytokines, IL-4 and IL-13, were able to induce the development of CD25$^+$CD4 T cells with regulatory capacity in an Ag-specific manner from peripheral, naive, CD25$^-$CD4 precursors and, moreover, that both cytokines play a prominent role in the development of Ag-specific CD25$^+$CD4 Tregs in vivo.

The immunomodulatory effect of Th2 cytokines is well recognized. A large body of data is available assessing the mechanisms by which these cytokines display their immunosuppressive potential (27). In the current study we propose an alternative pathway by which Th2 cytokines, in particular, IL-4 and IL-13, might accomplish their immunomodulatory functions, identifying a novel role for IL-4 and IL-13 in generating CD25$^+$ Tregs in the periphery during antigenic stimulation. The biological effects of IL-4 on target cells are mediated by the IL-4R, which consists of two separate chains: the IL-4-binding chain, IL-4Rα, and the common γ-chain, γc. The IL-4R α-chain is a high affinity receptor for IL-4 that is also involved in IL-13 signaling. Once IL-13 binds to its high affinity receptor, IL-13Rα, IL-4Rα associates with the IL-13/IL-13Rα complex and mediates the biological effects of IL-13. Specific signaling of both receptor complexes (IL-4Rα/γc and IL-4Rα/IL-13Rα) is transmitted from the IL-4R α-chain mainly via STAT6 and/or insulin receptor substrate 1/2 (28). Because both IL-4 and IL-13, the only two cytokines using the IL-4R α-chain, were able to induce CD25$^+$CD4 T cells with regulatory capacity, it is highly likely that signaling through the IL-4R α-chain plays an essential role in the generation of Ag-induced CD25$^+$ Tregs in the periphery. The finding that oral administration of OVA did not induce CD25$^+$ Tregs in IL-4Rα-knockout mice Tg for the OVA-specific TCR, in contrast to their IL-4Rα-expressing littermates, supports this conclusion (data not shown). Moreover, of the analyzed cytokines whose receptors use the γc, only IL-4, but not IL-2 (data not shown) or IL-9, was able to induce the generation of CD25$^+$ Tregs, confirming the hypothesis that the IL-4R α-chain, not the γc, might be the signal-transducing molecule involved in CD25$^+$ Treg generation.

To our knowledge, this is the first identification of the involvement of IL-4 and IL-13 in the generation of CD25$^+$CD4 Tregs, phenotypically resembling naturally occurring CD25$^+$ Tregs. The naturally occurring, thymic-derived, CD25$^+$ Tregs are anergic, which is closely linked to the suppressive property of these cells (29). In terms of mechanisms of action, they require activation via the TCR to become suppressive, acting via inhibition of IL-2 production by the effector cells (30, 31). Moreover, ligation of CTLA-4 as a costimulatory molecule seems to be necessary for functional activation of CD25$^+$ Tregs (32, 33). The expression of another surface molecule, GITR, has been suggested to be characteristic of CD25$^+$ Tregs (34, 35). In response to stimulation, CD25$^+$ Tregs produce immunomodulatory cytokines, such as IL-10 and TGF-β, although the involvement of these cytokines in mediating the biologic effects of CD25$^+$ Tregs has not been definitively delineated (2, 3, 31, 36–38). Recently, expression of the transcription factor, Foxp3, has been reported to be a more accurate marker for CD25$^+$ Tregs (39). Based on their aggregate characteristics, CD25$^+$CD4 Tregs generated from peripheral naive CD4 T cells, as reported in this study, in response to IL-4Rα-binding cytokines are comparable to those generated in the thymus, suggesting a similar function in the immune response.

CD25$^+$ Tregs naturally occurring in the thymus represent a unique T cell subpopulation engaged in the maintenance of self-tolerance and in preventing the development of autoimmune diseases (1, 7, 29, 40, 41). This T cell population evidently has the potential for therapeutic use. However, the small number of such cells, their anergic phenotype, and, moreover, their unknown Ag specificity hamper their successful therapeutic exploitation. Extensive efforts, therefore, have been focused on delineating the mechanisms involved in expanding naturally occurring CD25$^+$ Tregs or generating inducible CD25$^+$ Tregs with defined Ag specificity. From in vivo findings, it is obvious that under certain conditions, naturally occurring CD25$^+$ Tregs are capable of Ag-specific expansion despite their anergy in vitro (42, 43). TGF-β and IL-2 have been identified as factors necessary for the expansion of CD25$^+$ Tregs with potent regulatory capacity (8, 44–46). Stimulation of CD25$^+$ Tregs via their TCR by using either high dose Ag-loaded DC or Abs to CD3 in the presence of CD28 costimulation and IL-2 facilitates the expansion of CD25 Tregs that retain their phenotype and suppressive activities (47–49). Alternatively, it has been reported that CD25$^+$ Tregs can be expanded using mitogenic anti-CD28 Abs (superagonistic anti-CD28) (50). From in vivo studies, there are also some indications that CD25$^+$ Tregs might arise from CD25$^-$CD4 precursors in a thymus-independent manner in response to a novel Ag (51, 52). However, with the exception of B7 costimulation (53), the mechanisms involved in this process have not been identified. In this study we identified IL-4 and IL-13 as novel molecules involved in generating CD25$^+$ Tregs from CD25$^-$ precursors. The finding that neither TGF-β, IL-10 (another cytokine with recognized immunomodulatory functions; data not shown), IL-5, nor IL-9 was able to induce the development of such cells emphasizes that this is a unique feature of IL-4 and IL-13. Although necessary for the expansion of newly generated CD25$^+$ Tregs, IL-4 and IL-13 failed to induce the expansion of naturally occurring CD25$^+$ Tregs. This is consistent with the observation that IL-4- as well as IL-4Rα-knockout mice have naturally occurring CD25$^+$ Tregs in the usual frequency and with a comparable suppressive activity in vitro as their IL-4Rα-expressing littermates (data not shown). This suggests that different mechanisms might be involved in vivo in the development of CD25$^+$ Tregs derived in the thymus compared with those generated in the periphery in response to antigenic stimulation. To further support the hypothesis that the IL-4 induced CD25$^+$ Tregs identified in this study derive from CD25$^-$CD4 T cells and not from a preferential outgrowth of the small number of CD25$^+$ T cells in the cultures in response to IL-2, we have conducted priming cultures in which the starting T cell population was primed with autologous APC in the presence of IL-4 and increasing concentrations of rIL-2 (data not shown). In these cultures, vigorous T cell proliferation occurred; however, the resulting cells expressed no regulatory capacity, indicating that in these cultures, IL-2 augmented the proliferation of effector cells that outgrew the Treg population at the end of the priming culture. However, whether IL-4/IL-13 preferentially affects the CD25$^-$ pre-existing precursors of CD25$^+$ Tregs or, alternatively, induces de novo generation of CD25$^+$ Tregs remains to be elucidated.

Although cytokines engaging the IL-4R α-chain were the decisive molecules in the generation of CD25$^+$ Tregs in vitro, alone they were not sufficient for the induction of a regulatory phenotype in CD4 T cells. Thus, 10-day incubation of human naive CD4 cells with IL-4 alone in the absence of APC did not result in the induction of regulatory cells (data not shown). Therefore, other signals

involved in the generation and outgrowth of $CD25^+$ Tregs must have been provided either directly by the APC or indirectly by the T cells themselves. For example, signaling through the TCR and endogenous IL-2 present in culture have been identified as critical factors for the outgrowth of $CD25^+$ Tregs. Moreover, in concordance with previous publications (54, 55), we found that blockade of CD28-B7 interactions during the culture period using a fusion CTLA-4-Ig protein completely prevented the development of $CD25^+$ Tregs in response to IL-4. Together, these data indicate that the thymus-independent generation of $CD25^+$ Tregs in response to IL-4 or IL-13 is the result of a complex integration of a variety of signals in which IL-4 or IL-13 play a decisive role. The apparent complexity of this mechanism makes it conceivable, on the one hand, that similar mechanisms might be involved in the development of $CD25^+$ Tregs from $CD25^-CD4$ precursors in vivo. Support for this consideration might be taken from the results of the model of oral tolerance, in which neutralization of both IL-4 and IL-13 completely prevented the increase in clonotype-positive $CD25^+$ Tregs in response to Ag administration. On the other hand, an interesting hypothesis concerning the biological role of the autologous MLR can be derived from our observations. The dependency of IL-4-induced generation of $CD25^+$ Tregs in vitro on the interaction between T cells and autologous APC in the absence of nominal Ag and the ability of these de novo-generated $CD25^+$ Tregs to perform their suppressive function only in response to autologous APC suggest that the physiologic role of the autologous MLR may be related to the continuous generation of peripheral autoantigen-specific $CD25^+$ Tregs, thereby contributing to the maintenance of peripheral tolerance.

Finally, a major challenge for the therapeutic application of in vitro-expanded $CD25^+$ Tregs is the Ag-independent manner of their suppressive function (8, 49). As shown in this study, however, the $CD25^+CD4$ Tregs generated in vitro in response to autologous APC in the presence of IL-4 or IL-13 express their function in an Ag-dependent way. Moreover, the experiments with OVA TCR-Tg mice indicate that IL-4/IL-13 play an essential role in the generation of Ag-specific $CD25^+$ Tregs that are known to carry out their suppressive functions in an Ag-dependent manner (26). Because many T cell Ags have been identified to contribute to disease development, such as the cartilage glycoprotein, gp39, in rheumatoid arthritis or myelin basic protein in multiple sclerosis, the system described in this study makes it conceivable to generate and expand $CD25^+$ Tregs with defined Ag specificity for the precise targeting of Ag-specific, pathogenic, immune responses.

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Disclosures

The authors have no financial conflict of interest.

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