

Human CD4⁺CD25⁺ T cells derived from the majority of atopic donors are able to suppress T_H1 and T_H2 cytokine production

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Background: Recently, it has been established that CD4⁺CD25⁺ T cells with regulatory capacity are present in human peripheral blood, inhibiting allogeneic proliferation and cytokine production of preactivated CD4⁺CD25⁻ responder T cells.

Objective: The aim of this study was to analyze in an allergen-specific setting whether such regulatory CD4⁺CD25⁺ T cells also exist and function normally in atopic individuals, especially concerning the inhibition of T_H2 cytokines.

Methods: For this purpose, CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells from donors allergic to grass or birch pollen (mainly with rhinitis) or from healthy nonatopic donors were stimulated in the presence of autologous, mature, monocyte-derived, allergen-pulsed dendritic cells, and the preactivated CD4⁺CD25⁺ T cells were added to CD4⁺CD25⁻ T cells during restimulation. **Results:** CD4⁺CD25⁺ T cells from the nonatopic donors and from the majority of the patients investigated proliferated poorly, produced fewer cytokines, and inhibited the proliferation and T_H1 (IFN- γ) and T_H2 (IL-4 and IL-5) cytokine production of CD4⁺CD25⁻ T cells but not IL-10 production. The suppression of CD4⁺CD25⁻ T cells by CD4⁺CD25⁺ T cells was at least partially antigen unspecific and not reversible with anti-IL-10, anti-transforming growth factor β , or anti-cytotoxic T lymphocyte-associated antigen 4 mAb but was reversible with IL-2. In some atopic patients preactivated CD4⁺CD25⁺ T cells reproducibly showed strong proliferative responses, produced higher amounts of IL-4 and IL-10 than CD4⁺CD25⁻ T cells, and suppressed only the IFN- γ production of CD4⁺CD25⁻ T cells.

Conclusion: These data indicate that regulatory CD4⁺CD25⁺ T cells are present and functional in most atopic patients with allergic rhinitis and are able to inhibit T_H1, as well as T_H2, cytokine production. (J Allergy Clin Immunol 2003;111:862-8.)

Key words: Human regulatory T cells, CD25, T_H1/T_H2, allergy, dendritic cells

Immunologic tolerance is achieved through a variety of mechanisms, and animal models have shown that a distinct subset of T cells is particularly involved in this process.¹⁻⁴ These so-called suppressor or regulatory T cells can prevent autoimmunity or allograft rejection by

Abbreviations used

CTLA: Cytotoxic T lymphocyte-associated antigen
DC: Dendritic cell
ECP: Eosinophil cationic protein
IMDM: Iscove modified Dulbecco medium
PE: Phycoerythrin
TGF: Transforming growth factor

means of their ability to inhibit the activation and expansion of peripheral T cells. In mice regulatory CD4⁺ T cells are characterized by the expression of CD25⁺CD45RB^{low} and production of high amounts of transforming growth factor (TGF) β and IL-10.³ Several groups have shown in an experimental system of inflammatory bowel disease in lymphocyte-deficient mice that these cells protected the recipients from wasting disease induced by naive CD45RB^{high}CD4⁺ T cells depending on TGF- β , IL-10, and cytotoxic T lymphocyte-associated antigen (CTLA) 4.⁵⁻⁷ However, prevention of disease and control of peripheral CD4⁺ T-cell expansion are largely independent processes because CD25⁻CD45RB^{low}CD4⁺ T cells are also able to prevent inflammatory bowel disease but not to inhibit the accumulation of cotransferred CD45RB^{high}CD4⁺ T cells.⁸ Additionally, many in vivo and in vitro studies have shown that CD4⁺CD25⁺ T cells do not produce cytokines and suppress T-cell responses in an antigen-nonspecific manner, requiring direct cell-cell contact.^{3,9,10}

Recently, regulatory CD4⁺CD25^{high} T cells have also been described in human peripheral blood.¹¹⁻¹⁶ They express CD45RO, MHC class II (HLA-DR), and intracellular CTLA-4 and represent 1% to 6% of the total peripheral CD4⁺ T-cell population.^{13,16} In contrast to the murine system, in which the whole population of CD4⁺CD25⁺ T cells expressing both low and high CD25 levels has regulatory properties, in human subjects only the CD25^{high} cells do not proliferate and are able to inhibit proliferation and cytokine production by activated CD4⁺CD25⁻ responder cells, whereas the CD25^{low} cells, which represent up to 16% of the total CD4⁺ T-cell population, are nonregulatory.^{8,14} The inhibitory effect of human regulatory CD4⁺CD25⁺ T cells is antigen nonspecific but activation dependent, requires cell-cell contact, and can be abrogated by addition of IL-2, whereas IL-10, TGF- β , and CTLA-4 appear not to be involved in vitro.^{12,15,16}

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Furthermore, CD4⁺CD25⁺ T cells retain their suppressive properties after expansion in the presence of exogenous IL-2, indicating that the CD4⁺CD25⁺ cell population is a stable cell lineage.^{11,15} In the present study we investigate the role of this regulatory T-cell population in allergic T_H2 immune responses. We demonstrate that CD4⁺CD25⁺ regulatory T cells also exist in the majority of atopic individuals and that they can suppress both T_H1 and T_H2 cytokines of the responding T cells. However, there are some patients whose preactivated CD4⁺CD25⁺ T cells proliferate well and produce high amounts of IL-4 and IL-10, resulting in the suppression of T_H1 cytokine production by CD4⁺CD25⁻ T cells only.

METHODS

Blood samples

Heparinized blood was obtained from nonatopic healthy donors or from atopic donors with allergic rhinoconjunctivitis (all) and partially from asthma to grass pollen or birch pollen. Specific sensitization was documented on the basis of positive skin prick test responses to the respective allergen and detection of allergen-specific IgE in the sera (RAST class ≥ 2). Total IgE and eosinophil cationic protein (ECP) levels were also measured in the sera, and a complete blood count, including a differential white blood cell count, was performed.

Cell culture reagents

Iscove modified Dulbecco medium (IMDM) with L-glutamine and 25 mmol/L HEPES (Life Technologies GmbH, Karlsruhe, Germany) supplemented with 3024 mg/L sodium bicarbonate, 100 μ g/mL streptomycin, 100 U/mL penicillin, and 1% heat-inactivated autologous plasma was used for the culture of dendritic cells (DCs) and with 5% autologous plasma for the coculture of T cells and DCs.

Human recombinant IL-4, IL-1 β , and TNF- α were purchased from Strathmann Biotech GmbH (Hannover, Germany), and IL-2 (Proleukin) was purchased from Chiron GmbH (Ratingen, Germany). GM-CSF (Leucomax) was obtained from Sandoz AG (Nürnberg, Germany), and prostaglandin E₂ (Minprostin) was obtained from Pharmacia & Upjohn GmbH (Erlangen, Germany). The grass pollen and birch pollen allergen extracts were provided by ALK-Scherax (Hamburg, Germany). Phorbol 12-myristate 13-acetate, ionomycin, and monensin were obtained from Sigma Aldrich (Deisenhofen, Germany).

Antibodies

The following mAbs were used. Mouse IgG was anti-CD80 (MAB104; Immunotech, Hamburg, Germany), anti-CD83 (HB15a, Immunotech), and anti-CD86 (BU63; Camon, Wiesbaden, Germany). Rat IgG was anti-HLA-DR (YE2/36HLK, Camon) and mouse and rat subclass-specific isotypes (Immunotech). Conjugated secondary antibodies were fluorescein (DTAF)-conjugated goat anti-rat IgG (Dianova, Hamburg, Germany) and phycoerythrin (PE)-conjugated donkey anti-mouse IgG (Dianova). FITC- or PE-conjugated antibodies for staining of MACS-sorted T cells were anti-CD4 (RPA-T4), anti-CD25 (M-A251), anti-CD45RA (HI100), anti-CD45RO (UCHL1), anti-CTLA-4 (BNI3), anti-IFN- γ (4S.B3), anti-IL-4 (MP4-25D2; all from BD PharMingen, San Diego, Calif), and FITC- and PE-conjugated mouse or rat IgG isotype control (Immunotech). For blocking experiments, 1 μ g/mL anti-IL-10 (JES3-19F1, BD PharMingen), 2 μ g/mL anti-TGF- β 1 (MAB240; R&D Systems, Wiesbaden, Germany), and 10 μ g/mL anti-CTLA-4 (ANC152.2/8H5, Ancell Corp, Bayport, Minn) were added to the coculture of T cells and DCs.

Generation of monocyte-derived human DCs

PBMCs were isolated from heparinized blood by means of Ficoll-Paque 1.077 (Biochrom, Berlin, Germany) density centrifugation. To enrich CD14⁺ monocytes, 1×10^7 PBMCs per well were incubated for 45 minutes in a 6-well plate (Costar, Bodenheim, Germany) in IMDM supplemented with 3% autologous plasma at 37°C. After washing the nonadherent cells with prewarmed PBS, the remaining monocytes (purity >90%) were incubated in 3 mL of IMDM per well supplemented with 1% heat-inactivated autologous plasma, 1000 U/mL IL-4, and 800 U/mL GM-CSF. Cells were fed with fresh medium every other day. On day 7, the resulting immature DCs were pulsed with 10 μ g/mL grass pollen or birch pollen allergen extracts and further stimulated with 1000 U/mL TNF- α , 2000 U/mL IL-1 β , and 1 μ g/mL prostaglandin E₂ to induce their full maturation. Mature DCs were harvested 48 hours after stimulation, washed twice, and used for T-cell stimulation assays.

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMCs by using antibody-coated paramagnetic MultiSort MicroBeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), according to the protocol of the manufacturer. After detaching, CD4⁺ T cells were stained with CD25 MicroBeads (Miltenyi Biotec), and CD4⁺CD25⁺ T cells were positively selected. Separation was controlled by means of flow cytometry (purity of >98% CD4⁺ T cells and >90% CD25⁺CD4⁺ T cells).

Coculture of T cells and autologous allergen-pulsed DCs

For proliferation assays, 1×10^5 CD4⁺CD25⁻ T cells were cocultured in 96-well plates (Costar) in triplicate with 1×10^4 autologous allergen-pulsed DCs with or without the addition of titrated numbers of CD4⁺CD25⁺ T cells in 200 μ L of IMDM supplemented with 5% heat-inactivated autologous plasma. In some experiments 1 μ g/mL anti-IL-10, 2 μ g/mL anti-TGF- β 1, 10 μ g/mL anti-CTLA-4, or 100 U/mL IL-2 were added from the beginning of the coculture. After 5 days, the cells were pulsed with 37 kBq per well of tritiated thymidine ([methyl-³H]thymidine; ICN, Irvine, Calif) for 6 hours, and tritiated thymidine incorporation was evaluated in a beta counter (1205 Betaplate; LKB Wallac, Turku, Finland).

For cytokine production assays, 1×10^6 CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells were cultured in 24-well plates in the presence of 1×10^5 autologous allergen-pulsed DCs in 1 mL of IMDM supplemented with 5% heat-inactivated autologous plasma. At day 5 of culture, 10 U/mL IL-2 was added. On day 7, T cells were washed, and 5×10^5 CD4⁺CD25⁻ cells were restimulated in 48-well plates with 5×10^4 newly generated autologous allergen-pulsed DCs with or without the addition of titrated numbers of CD4⁺CD25⁺ T cells, and supernatants were collected 24 hours later. For intracellular detection of IL-4 and IFN- γ , T cells were further stimulated with 10 ng/mL phorbol 12-myristate 13-acetate, 0.5 μ mol/L ionomycin, and 2.5 μ mol/L monensin for 6 hours.

Flow cytometric analysis

Surface phenotyping was performed by staining 5×10^5 T cells or 5×10^4 DCs with specific mAbs for 20 minutes at 4°C. After being washed with PBS containing 0.1% BSA, the cells were incubated with DTAF- or PE-conjugated secondary antibodies for 20 minutes at 4°C, washed, and analyzed in a FACSCalibur (Becton Dickinson, Mountain View, Calif) equipped with CELLQuest software. For determination of intracellular CTLA-4, IFN- γ , and IL-4 production, 5×10^5 T cells were fixed with 4% paraformaldehyde for 10 minutes on ice. Then cells were permeabilized with 0.5% saponin diluted in PBS for 10 minutes before staining.

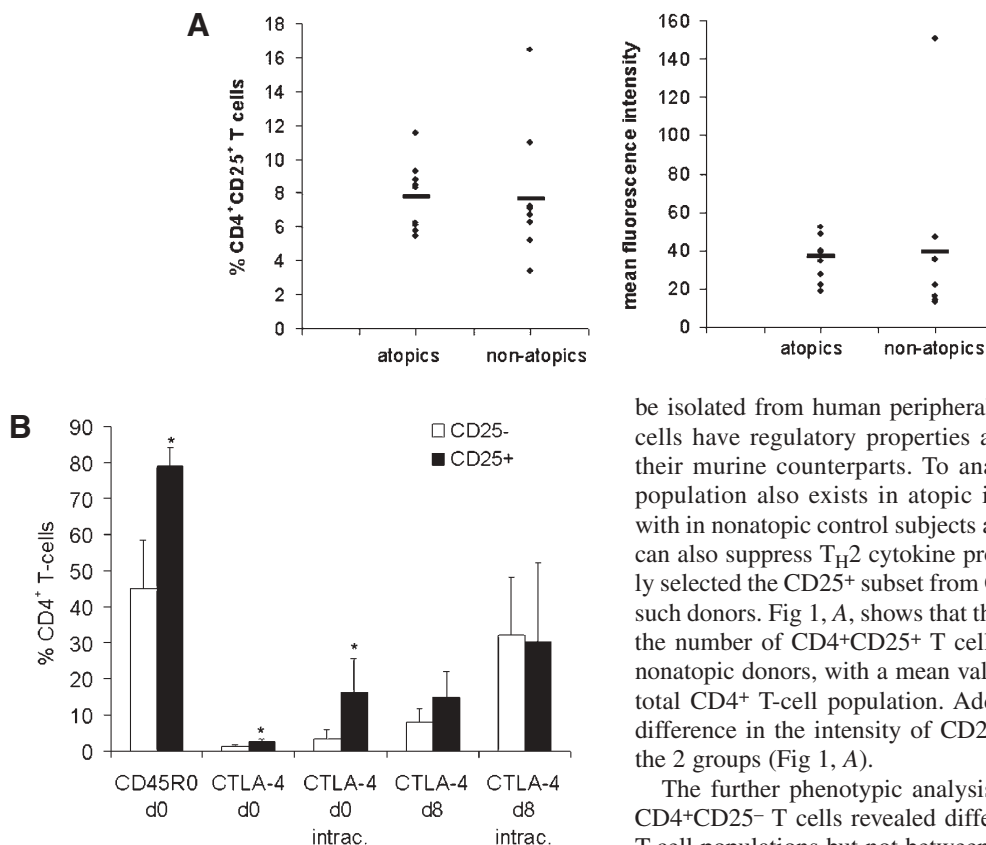


FIG 1. Phenotypic characterization of CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells from atopic and nonatopic donors. **A**, Purified CD4⁺ T cells were stained with anti-CD4 FITC and anti-CD25 PE. Shown are the individual data points (filled circles), as well as the means (black line) of the percentage and mean fluorescence intensity of CD4⁺CD25⁺ T cells from 9 atopic and 9 nonatopic donors. **B**, CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells from atopic donors were stained with the indicated FITC- or PE-conjugated mAbs directly after isolation (d0) or after stimulation with allergen-pulsed DCs for 1 week, as described in the Methods section (d8). The data represent the mean $\pm$ SD from 6 independent experiments. *Statistically significant differences between CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells ($P \leq .05$).

Quantification of cytokine production by means of ELISA

Human IL-4, IL-5, IL-10, and IFN- γ levels were measured by means of ELISA according to the instructions of the distributors of the used pairs of antibodies (BD PharMingen), as described previously.¹⁷ The detection limit was 8 pg/mL for IL-4 and 32 pg/mL for all other cytokines.

Statistics

The Student *t* test was used to test the statistical significance of the results. *P* values of .05 or less was considered significant.

RESULTS

Phenotypic characterization of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from atopic and nonatopic individuals

Recently, it was shown that CD4⁺CD25⁺ T cells can

be isolated from human peripheral blood and that these cells have regulatory properties analogous to those of their murine counterparts. To analyze whether such a population also exists in atopic individuals compared with in nonatopic control subjects and whether such cells can also suppress T_H2 cytokine production, we positively selected the CD25⁺ subset from CD4⁺ T helper cells of such donors. Fig 1, A, shows that there is no difference in the number of CD4⁺CD25⁺ T cells between atopic and nonatopic donors, with a mean value of about 8% of the total CD4⁺ T-cell population. Additionally, there is no difference in the intensity of CD25 expression between the 2 groups (Fig 1, A).

The further phenotypic analysis of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells revealed differences between the 2 T-cell populations but not between atopic and nonatopic donors. Freshly isolated CD4⁺CD25⁺ T cells from atopic or nonatopic donors expressed higher levels of CD45R0 and of intracellular CTLA-4 than CD4⁺CD25⁻ T cells. Surface CTLA-4 was only weakly expressed in both T-cell populations but still significantly higher in CD4⁺CD25⁺ compared with CD4⁺CD25⁻ T cells (Fig 1, B). Furthermore, the expression of surface CTLA-4 was increased in CD4⁺CD25⁺ T cells measured 1 week after stimulation with allergen-pulsed autologous mature monocyte-derived DCs (Fig 1, B). At this time point, there was no difference in expression of intracellular CTLA-4 between the 2 T-cell populations.

Low proliferation and cytokine production of freshly isolated CD4⁺CD25⁺ T cells derived from the majority of atopic donors

Analysis of proliferation in this allergen-specific setting revealed that it was significantly reduced in the CD25⁺ subset compared with in CD4⁺CD25⁻ T cells from all nonatopic and from most of the examined atopic donors (Fig 2). The CD4⁺CD25⁺ T cells also produced significantly less IL-4, IL-5, IL-10, and IFN- γ (IL-4/IL-10^{low}) than the CD25⁻ subset (Fig 3). Additionally, the number of IL-4-producing and IFN- γ -producing cells was also reduced in the CD25⁺ subset (19.93% $\pm$ 9.11% IL-4-producing and 9.83% $\pm$ 4.42% IFN- γ -producing CD4⁺CD25⁻ T cells vs 9.28% $\pm$ 4.72% IL-4-producing and 4.10% $\pm$ 2.74% IFN- γ -producing CD4⁺CD25⁺ T cells [*n* = 5]). However, in some atopic donors prolifera-

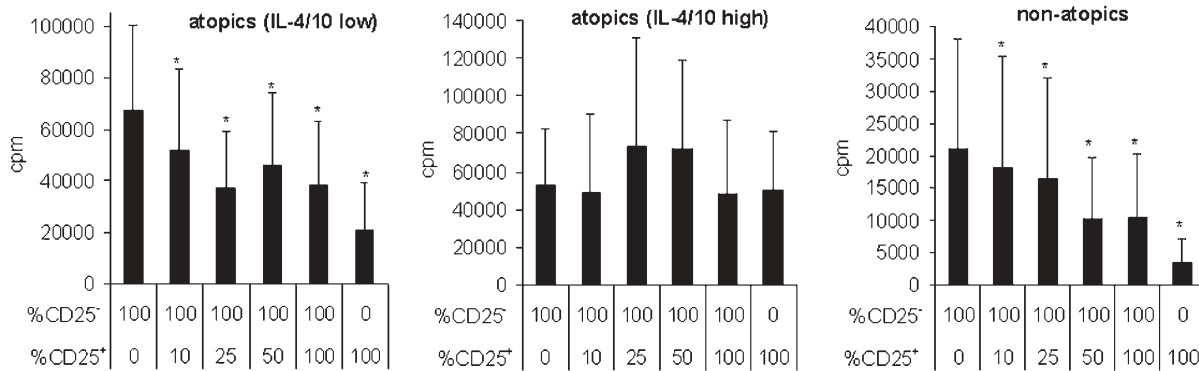


FIG 2. CD4⁺CD25⁺ T cells from all nonatopic and from the majority of atopic donors proliferate poorly and inhibit the proliferation of CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells (1×10^5 ; 100%) were cultured alone or in the presence of titrated numbers of CD4⁺CD25⁺ T cells as indicated and stimulated with 1×10^4 allergen-pulsed autologous DCs. After 5 days, the cells were pulsed with tritiated thymidine for 6 hours before harvesting. Shown are the means $\pm$ SD from 8 (for IL-4/IL-10^{low} atopic donors), 6 (for IL-4/IL-10^{high} atopic donors), and 8 (nonatopic donors) independent experiments. *Statistically significant differences between CD4⁺CD25⁻ T cells alone and CD4⁺CD25⁻ T cells in coculture with CD4⁺CD25⁺ T cells ($P \leq .05$).

tion of CD4⁺CD25⁺ T cells was less reduced, and the secretion of IL-4 and IL-10 of these cells was enhanced (IL-4/IL-10^{high}) compared with the CD25⁻ subset, whereas IFN- γ production was low (Figs 2 and 3). In these cases we detected fewer IFN- γ -producing but a substantial number of IL-4-producing CD25⁺ T cells ($31.26\% \pm 18.22\%$ IL-4-producing and $6.32\% \pm 2.48\%$ IFN- γ -producing CD4⁺CD25⁻ T cells vs $18.05\% \pm 10.74\%$ IL-4-producing and $4.79\% \pm 1.12\%$ IFN- γ -producing CD4⁺CD25⁺ T cells [$n = 3$]). This difference from the majority of the atopic donors was reproducible in independent experiments at different time points in these donors. These particular donors were not characterized by special severity of their atopic disease nor did their other laboratory findings show unusual values (total IgE level, ECP level, and complete-differential blood count). However, CD4⁺CD25⁺ T cells with increased IL-4 (and IL-10) production were not found among nonatopic donors.

Dose-dependent suppression of proliferation and cytokine production of CD4⁺CD25⁻ T cells by low-proliferating, less-cytokine-producing CD4⁺CD25⁺ regulatory T cells

To investigate whether the low-proliferating, less-cytokine-producing CD4⁺CD25⁺ T cells from the nonatopic and from most of the atopic donors have regulatory properties, we cocultured CD4⁺CD25⁻ T cells together with different amounts of CD4⁺CD25⁺ T cells. As shown in Fig 2, CD4⁺CD25⁺ T cells strongly suppressed proliferation of the CD4⁺CD25⁻ population, even at a ratio of 1:10. Additionally, T_H1 (IFN- γ), as well as T_H2 (IL-4 and IL-5), cytokine production of CD4⁺CD25⁻ T cells was dose dependently and significantly reduced after restimulation with allergen-pulsed autologous DCs in the presence of preactivated CD4⁺CD25⁺ T cells,

whereas the production of IL-10 was not affected (Fig 3). When CD4⁺CD25⁻ T cells were cocultured with high IL-4/IL-10-producing CD4⁺CD25⁺ T cells, IL-4 and especially IL-10 production were enhanced in such cocultures, whereas the production of IFN- γ was significantly inhibited already at a ratio of 1:4. Proliferation and IL-5 production remained unchanged (Figs 2 and 3). Addition of the same numbers of CD4⁺CD25⁻ T cells to CD4⁺CD25⁻ T cells for control purposes (crowding) did not yield any inhibiting effect and led to an increase of proliferation and cytokine production (data not shown).

Inhibition of proliferation and cytokine production of CD4⁺CD25⁻ T cells by CD4⁺CD25⁺ regulatory T cells is not mediated by IL-10, TGF- β , or CTLA-4 and is mainly antigen and allergen unspecific

To address the question of whether the inhibitory molecules IL-10, TGF- β , or CTLA-4 are responsible for the suppression of proliferation and cytokine production of CD4⁺CD25⁻ T cells by low-proliferating, less-cytokine-producing CD4⁺CD25⁺ regulatory T cells, we blocked these molecules with neutralizing antibodies that were added from the beginning of the cocultures or during restimulation at concentrations established to block the respective molecules in previous experiments. However, the inhibition of none of these factors alone or in combination significantly restored the proliferative responses (Fig 4) and cytokine production (data not shown) of CD4⁺CD25⁻ T cells cocultured in the presence of CD4⁺CD25⁺ regulatory T cells. Only the addition of high concentrations of exogenous IL-2 (100 U/mL) reversed the inhibitory effects (Fig 4). Furthermore, the suppressed proliferation and cytokine production of CD4⁺CD25⁻ T cells in coculture with CD4⁺CD25⁺ T

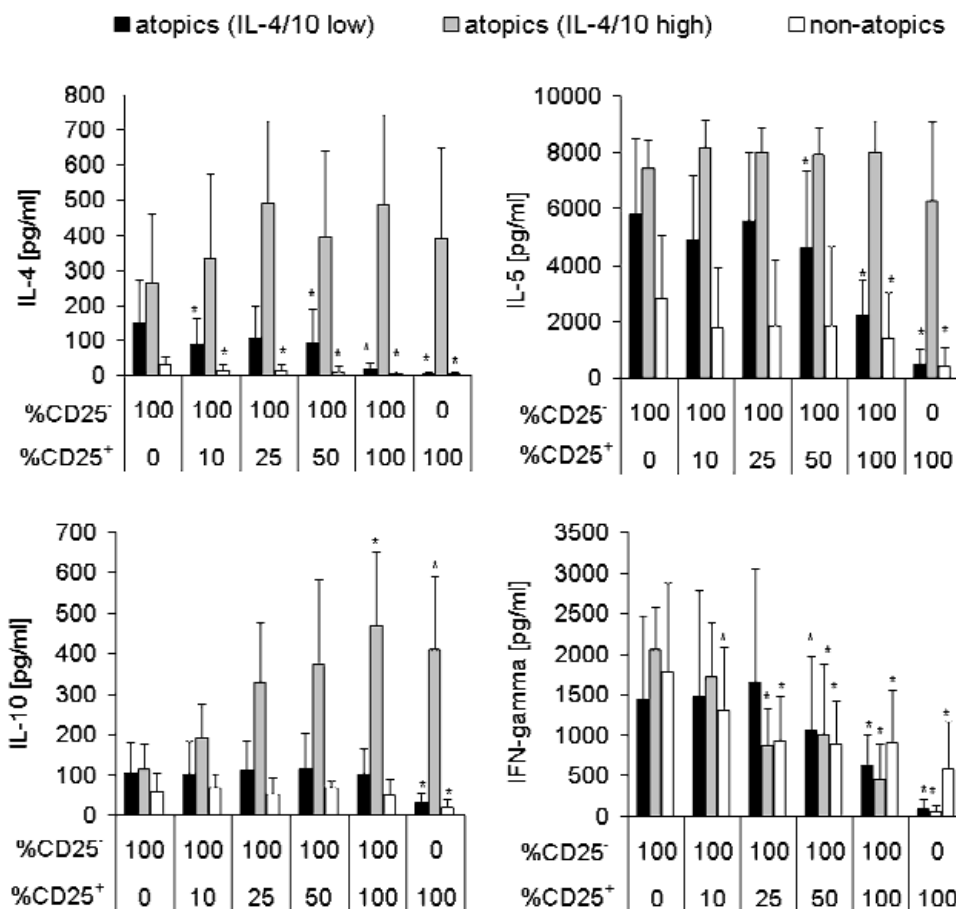


FIG 3. CD4⁺CD25⁺ T cells from all nonatopic and from the majority of atopic donors produce less cytokines and suppress the cytokine production of CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells (1×10^6) were stimulated with 1×10^5 allergen-pulsed autologous DCs for 1 week. Ten units per milliliter of IL-2 was added during the last 48 hours of culture. On day 7, 5×10^5 CD4⁺CD25⁻ T cells (100%) were restimulated with 5×10^4 newly generated allergen-pulsed autologous DCs in the presence or absence of titrated numbers of CD4⁺CD25⁺ T cells as indicated. Supernatants were collected 24 hours later and analyzed for IL-4, IL-5, IL-10, and IFN- γ content by means of ELISA. The data represent the means $\pm$ SD from 11 (for IL-4/IL-10^{low} atopic donors), 6 (for IL-4/IL-10^{high} atopic donors), and 7 (for nonatopic donors) independent experiments. *Statistically significant differences between CD4⁺CD25⁻ T cells alone and CD4⁺CD25⁺ T cells in coculture with CD4⁺CD25⁺ T cells ($P \leq .05$).

cells was not fully dependent on preactivation of the CD4⁺CD25⁺ T cells with the same antigen-allergen because we observed a similar but slightly weaker effect using CD4⁺CD25⁺ T cells preactivated with autologous DCs pulsed with a different allergen (data not shown).

DISCUSSION

Regulatory T cells play an important role in the inhibition of autoreactive T cells and the maintenance of self-tolerance. A population of CD4⁺ T cells expressing the IL-2 receptor α chain (CD25) has been demonstrated in animal models to be of particular importance in this respect.^{18,19} More recently, CD4⁺CD25⁺ T cells with regulatory properties were also found in human peripheral blood.¹²⁻¹⁶ In the present study we investigated the role of these regulatory T cells in allergic immune responses. We demonstrate that in comparison with nonatopic donors, atopic or allergic

patients also harbor regulatory CD4⁺CD25⁺ T cells, which show no or only little proliferative activity and cytokine production and suppress the proliferative, as well as cytokine, responses of peripheral CD4⁺CD25⁻ T cells. In a subset of atopic-allergic patients, CD4⁺CD25⁺ T cells showed less reduced proliferation and higher amounts of IL-4 and IL-10 production compared with CD4⁺CD25⁻ T cells. Consequently, this population did not suppress proliferation and T_H2 cytokine production of CD4⁺CD25⁻ T cells. The production of IFN- γ , however, was inhibited by these cells already at very low ratios, probably because of the additional inhibitory effect of IL-4 on the production of IFN- γ .²⁰ These donors had no particular profile of allergic disease and no particularly striking other laboratory findings (total IgE levels, ECP levels, and numbers of lymphocytes, monocytes, neutrophils, eosinophils, and basophils). High IL-4-producing (and IL-10-producing) CD4⁺CD25⁺ T cells were not found among nonatopic donors.

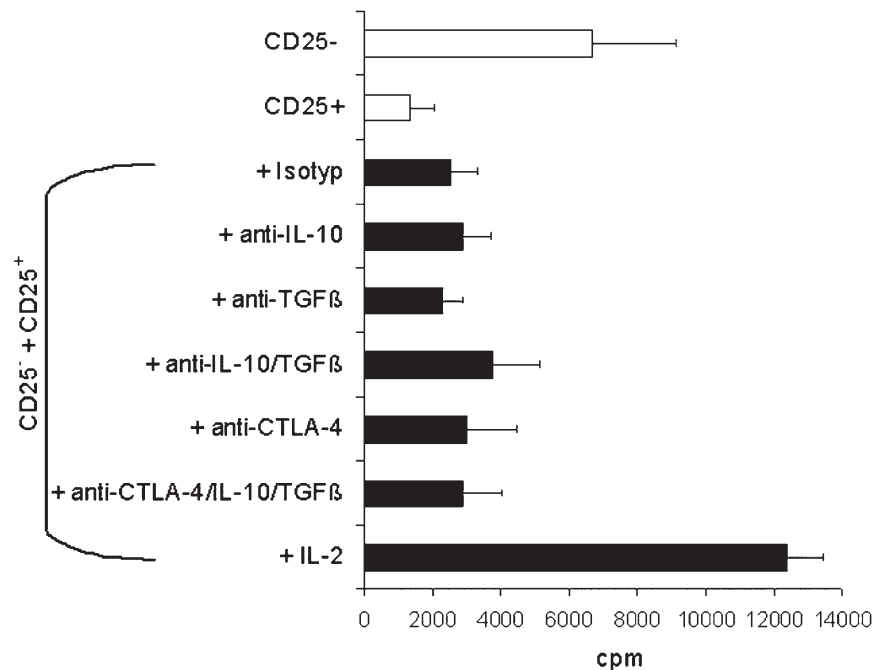


FIG 4. The suppressive property of CD4+CD25+ T cells is not dependent on IL-10, TGF-β, or CTLA-4. CD4+CD25- T cells (1×10^5) were stimulated with 1×10^4 allergen-pulsed autologous DCs in the presence or absence of 5×10^4 CD4+CD25+ T cells and with or without the addition of anti-IL-10, anti-TGF-β1, anti-CTLA-4, or 100 U/mL IL-2. After 5 days, the cells were pulsed with tritiated thymidine for 6 hours before harvesting. Shown are the means $\pm$ SD from 5 independent experiments.

Concerning the regulatory properties of low-proliferating, less-cytokine-producing CD4+CD25+ T cells, our results are in line with those of other studies showing that this T-cell population strongly suppresses the activation and proliferation of CD4+ and CD8+ T cells to alloantigens or on polyclonal stimulation with anti-CD3 and irradiated syngeneic PBMCs.^{11,13,14,16} However, we describe, for the first time, the regulatory properties of CD4+CD25+ T cells in an antigen (or allergen)-specific system and demonstrate that these cells are not only able to inhibit human T_H1 but also human T_H2 cell responses. In the mouse the inhibition of a T_H2-specific response was also observed by Cottrez et al²¹ *in vivo* by using a type of regulatory T cells designated T_R1 cells, which secrete IL-10 and TGF-β and suppress immune responses through these cytokines.^{21,22} In contrast to T_R1 cells, the *in vitro* suppression of peripheral T_H1 and T_H2 cell responses by CD4+CD25+ regulatory T cells is not mediated by IL-10, TGF-β, or CTLA-4 because neutralization of these factors did not abrogate the suppressive effects in our allergen-specific settings, as well as in response to alloantigens.^{11,13,16} Thus the negative regulator of T-cell activation, CTLA-4, seems not to be essential for the mediation of the inhibitory properties of human CD4+CD25+ regulatory T cells, although freshly isolated CD4+CD25+ T cells produce more intracellular CTLA-4 than CD4+CD25- T cells and express more CTLA-4 on their surfaces after activation. Similar results were obtained with murine regulatory T cells by Thornton and Shevach,⁹ whereas another group demonstrated that anti-CTLA-4 treatment completely restored the proliferative response.²

The observation that CD4+CD25+ T cells from a subset of atopic individuals produced high levels of IL-4 and IL-10 and do not suppress T_H2 cytokine production by CD4+CD25- T cells is of special interest. This might indicate that such patients might not possess CD4+CD25+ regulatory T cells that are also able to inhibit T_H2 cells. Alternatively, the CD4+CD25+ population of such patients might consist of regulatory CD4+CD25+ T cells that are still able to inhibit T_H2 cytokine production but are contaminated by a particularly high frequency of activated (and therefore CD25+) normal CD4+ T cells with no regulatory capacity, which proliferate and produce cytokines and therefore mask the effect of the CD4+CD25+ regulatory T cells. As long as there is no marker that discriminates activated (CD25+) normal CD4+ T cells from CD4+CD25+ regulatory T cells, this question cannot be answered. High IL-4 and IL-10 production among CD4+CD25+ T cells was not observed by us in nonatopic healthy donors. In a recent study by Stephens et al¹² CD4+CD25+ peripheral blood lymphocytes from donors not specified concerning their atopy status were also described to produce higher levels of IL-4 and similar amounts of IL-10 than CD4+CD25- cells. In contrast to our allergen-specific data, cytokine production was measured in response to mitogenic stimulation, and the status of the donors concerning atopic-allergic disease-sensitization was not analyzed. In the murine system Suto et al²³ have demonstrated that CD4+CD25+ T cells can upregulate allergic immune responses because antigen-induced eosinophil recruitment and IL-4 and IL-5

production in the airways was decreased in mice that received CD4⁺CD25⁺ T cell-depleted splenocytes compared with mice that received unfractionated splenocytes.

Taken together, we demonstrate, for the first time, that the majority of atopic-allergic individuals with mainly rhinitis also possess CD4⁺CD25⁺ regulatory T cells in their blood, with no major differences concerning frequency and functionality compared with nonatopic-nonallergic donors. CD4⁺CD25⁺ T cells with high IL-4 (and IL-10) production, however, were only found among atopic-allergic donors and only in a subpopulation of such donors. No other laboratory or clinical markers could be correlated with the status of high IL-4 (and IL-10) production of CD4⁺CD25⁺ T cells thus far. Further studies with higher numbers of patients and evaluation of particular subsets of atopic diseases, such as eczema, or high severity or poor responsiveness to certain therapies are necessary to fully explore the importance of CD4⁺CD25⁺ regulatory cells for atopic-allergic diseases. The fact that this cell population has the capacity to suppress also allergen-specific responses, and the production of T_H2 cytokines has been already established in this investigation and might open new possibilities of immune diagnostic monitoring and therapy of atopic-allergic diseases. On the other hand, the presence of atopic-allergic disease (in this investigation mainly rhinitis) in spite of the normal frequency and function of CD4⁺CD25⁺ regulatory T cells might indicate that these cells are not sufficiently able to suppress clinical manifestation of atopic-allergic diseases. However, the subset of atopic-allergic patients identified in this study who harbor CD4⁺CD25⁺ T cells that proliferate well, produce high amounts of IL-4 (and IL-10), and do not inhibit T_H2 cytokine production might point to a defect or disturbance in the population of CD4⁺CD25⁺ regulatory T cells, at least in a subpopulation of atopic-allergic patients. Furthermore, normal frequencies and functions of CD4⁺CD25⁺ T cells in the majority of atopic-allergic donors in vitro do not rule out a dysfunction in vivo, which might also be due to an aberrant function of other cell types (eg, involving expression of glucocorticoid-induced TNF receptor ligand or other factors of the local inflammatory micromilieu).^{24,25}

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