

Demonstration of circulating allergen-specific CD4⁺CD25^{high}Foxp3⁺ T-regulatory cells in both nonatopic and atopic individuals

Laura Maggi, BSc,* Veronica Santarlaschi, MD,* Francesco Liotta, MD, Francesca Frosali, BSc, Roberta Angeli, BSc, Lorenzo Cosmi, MD, Enrico Maggi, MD, Sergio Romagnani, MD, and Francesco Annunziato, PhD Florence, Italy

Background: CD4⁺CD25⁺Foxp3⁺ T-regulatory (Treg) cells play a fundamental role in the control of autoimmunity. Whether human CD4⁺CD25⁺Foxp3⁺ Treg cells that recognize foreign antigens also exist is less clear.

Objective: To investigate the existence in humans of circulating Treg cells able to recognize exogenous antigens, including allergens.

Methods: CD4⁺CD25^{high}Foxp3⁺ and CD4⁺CD25⁺Foxp3⁺ cells were purified from human peripheral blood and cultured for 15 days with autologous dendritic cells (DCs), unloaded, or loaded with Der p 1 allergen or the bacterial antigen streptokinase (SK). **Results:** CD4⁺CD25^{high}Foxp3⁺ circulating T cells obtained from healthy nonatopic subjects and cultured with Der p 1-loaded DCs, but not those cultured with either unloaded or SK-loaded DCs, suppressed the proliferative response to Der p 1 of autologous Der p 1-specific T cells generated from the CD4⁺CD25⁺Foxp3⁺ subset. The antigen specificity of either Der p 1-CD4⁺CD25^{high}Foxp3⁺ or SK-CD4⁺CD25^{high}Foxp3⁺ T cells was confirmed even at clonal level. Finally, under the same experimental conditions, functionally active Der p 1-specific Treg cells were obtained from the pool of circulating CD4⁺CD25^{high}Foxp3⁺ T cells of Der p 1-sensitive, atopic individuals.

Conclusion: These data provide undoubted demonstration of the existence of human CD4⁺CD25^{high}Foxp3⁺ circulating Treg cells specific for exogenous antigens, including the Der p 1 allergen, and indicate that CD4⁺CD25^{high}Foxp3⁺ Treg cells specific for Der p 1 are present and functionally active in both nonatopic and Der p 1-sensitive, atopic individuals.

Clinical implications: Caution should be advised in interpreting allergic disorders as simply resulting from defective Treg cell activity. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Allergy, immune suppression, T-regulatory cells

T-regulatory (Treg) lymphocytes are essential for the maintenance of host homeostasis because of their capacity to control the immune responses.¹ Different types of CD4⁺ Treg cells have been identified and classified as adaptive and natural Treg cells.² Classic adaptive Treg cells are those induced under particular experimental conditions, such as oral administration of autoantigen (T_H3),³ or immunization in presence of IL-10 (Tr1).⁴ By contrast, natural Treg cells are thought to arise during the normal process of maturation in the thymus as a distinct lineage and then colonize to secondary lymphoid organs.^{1,2,4,5} Natural Treg cells constitutively possess high levels of surface CD25 (CD25^{high}) and the expression of a unique transcription factor, Foxp3, which is required for their generation and the maintenance of suppressive activity.^{1,2,4,5} Natural Treg cells play a fundamental role in the control of autoimmunity, a function associated with their capacity to recognize self-antigens and to hamper effector responses against them. Whether natural Treg cells that recognize foreign antigens also exist, as well as the extent of their antigen repertoire, remains unclear. Recent reports have shown that CD4⁺CD25^{high}Foxp3⁺ cells can play an important role in the outcome of microbial infections.^{6,7} However, whether all CD4⁺CD25^{high}Foxp3⁺ Treg cells, including those specific for exogenous antigens, derive from a unique, distinct lineage or whether they result from the same populations responsible for effector responses is still unclear. By using MHC class II tetramers loaded with influenza hemagglutinin epitopes, CD4⁺CD25⁺ cells have been induced to generate antigen-specific CD4⁺CD25⁺ Treg cells *in vitro*.⁸ Nevertheless, the existence *ex vivo* of CD4⁺CD25^{high}Foxp3⁺ T cells specific for exogenous antigens has not been proven yet.

In this study, we demonstrate that the pool of human circulating CD4⁺CD25^{high}Foxp3⁺ T cells from nonatopic healthy subjects also contains cells specific for either Der p 1 or streptokinase (SK). More importantly, these cells were able to control, at least *in vitro*, the proliferation of autologous effector CD4⁺ T lymphocytes specific for the same antigen, whereas under identical conditions they did not exhibit the ability to suppress the proliferation

From the Excellence Center for Research, Transfer, and High Education DENOthe, University of Florence.

*These authors contributed equally to this work.

Supported by the Italian Ministry of Education (40%), the Italian Ministry of Health (AIDS Project), the Italian Association for Cancer Research, EU Projects SENS-IT-IV (FP6-LSBH-CT-2006-018861) and INNOCHEM (FP6-LSHB-CT-2005-518167), and Azienda Spaziale Italiana MoMa (WP reference, 1B1241-X5).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication December 15, 2006; revised May 3, 2007; accepted for publication May 3, 2007.

Reprint requests: Sergio Romagnani, MD, Dipartimento di Medicina Interna, Viale Morgagni 85, Firenze 50134, Italy. E-mail: s.romagnani@dmf.unifi.it. 0091-6749/\$32.00

© 2007 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2007.05.002

Abbreviations used

Ag:	Antigen
DC:	Dendritic cell
SK:	Streptokinase
Teff:	T-cell line generated from CD4 ⁺ CD25 ⁻ T cells
Teff-Der p 1:	T-cell line generated from CD4 ⁺ CD25 ⁻ T cells in the presence of Der p 1-loaded dendritic cells
Teff-SK:	T-cell line generated from CD4 ⁺ CD25 ⁻ T cells in the presence of streptokinase-loaded dendritic cells
Treg:	T-regulatory cell
Treg-Der p 1:	T-regulatory cell generated in the presence of Der p 1-loaded dendritic cells
Treg-SK:	T-regulatory cell generated in the presence of streptokinase-loaded dendritic cells

of effector T cells specific for an unrelated antigen. The antigen specificity of either CD4⁺CD25^{high}Foxp3⁺ Der p 1-specific or streptokinase-specific Treg cells was confirmed even at the clonal level. Of note, Der p 1-specific circulating T cells showing identical phenotypic and functional features were also found in Der p 1-sensitive atopic donors.

These findings provide undoubted demonstration on the existence of human circulating CD4⁺CD25^{high}Foxp3⁺ Treg cells specific for exogenous antigens, including a common allergen in both nonatopic and atopic individuals.

METHODS

Donors

Human PBMCs used in this study were obtained in accordance with the ethical standards of the responsible regional committee on human experimentation from 6 healthy nonatopic and 6 Der p-sensitive, atopic donors. All 6 Der p-sensitive patients had perennial rhinitis and showed a positive skin prick test to Der p and Der p-specific serum IgE antibodies (more than class 3).

Reagents and antibodies and ELISA for cytokines

See this article's Methods in the [Online Repository](http://www.jacionline.org) at www.jacionline.org.

Flow-cytometry analysis

Flow-cytometry analysis of cell suspensions was performed as detailed elsewhere.⁹ Foxp3 determination was performed accordingly to the manufacturer's instructions (eBiosciences, San Diego, Calif). Cells were analyzed on a BDLSRII cytofluorimeter using the Diva software (Becton Dickinson, Mountain View, Calif).

Isolation of CD14⁺ and of CD4⁺CD25^{high} cells

CD14⁺ and CD3⁺CD4⁺ T cells were selected from PBMCs by immunomagnetic cell sorting (Miltenyi Biotec, Bisley, Germany) as described elsewhere.¹⁰ The CD4⁺CD25⁻ and CD4⁺CD25^{high} populations were then purified from the CD3⁺CD4⁺ T-cell fraction by FACSARIA sorter (Becton Dickinson), taking care to avoid the recovery of CD25^{dim} cells.

Generation of dendritic cells

See this article's Methods in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org.

Antigen-specific T-cell lines and T-cell clones

See this article's Methods in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org.

Suppressive assays

See this article's Methods in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org.

Statistical analysis

Statistical analysis was performed using the Student *t* test.

RESULTS

Human CD4⁺CD25^{high}Foxp3⁺ T cells expanded in the presence of autologous dendritic cells and high IL-2 concentrations maintain in culture their suppressive phenotype

The experimental protocol used to assess the existence of human circulating CD25^{high}Foxp3⁺ T cells showing antigen-specificity is summarized in [Fig 1](#). First, CD14⁺ cells were sorted from PBMCs and induced to differentiate into dendritic cells (DCs) by culturing in presence of IL-4 and GM-CSF. On day 7, all CD14⁺ cells lost CD14 and acquired the expression of the DC marker CD1a ([Fig 1](#)). Accordingly, in the same cells, CD80 and HLA-DR expression was increased, whereas CD86 expression was decreased (data not shown). CD3⁺CD4⁺ T cells were also obtained from the CD14⁺ fraction of PBMCs from the same donors, and CD25^{high} or CD25⁻ cells were selectively recovered. After separation, virtually all purified CD4⁺CD25^{high} T cells consistently expressed Foxp3, whereas all CD4⁺CD25⁻ T cells were Foxp3⁻ ([Fig 1](#)).

We then assessed whether CD25^{high}Foxp3⁺ T cells could be expanded in culture and whether, despite their culturing, they retained their suppressive phenotypic and functional features. Therefore, both CD4⁺CD25⁻ and CD4⁺CD25^{high} cell fractions were cultured in presence of autologous DCs at 1:1 ratio, and on day 7 they were assessed for rate of proliferation, expression of Foxp3, and *in vitro* suppressive activity. Both CD4⁺CD25⁻ and CD4⁺CD25^{high} T cells showed no detectable proliferation when cultured in medium alone ([Fig 2, A](#)); however, when the same cell populations were cultured in presence of autologous DCs, with or without IL-2, a variable degree of proliferation was observed. There was very low proliferation by both cell populations in the absence of IL-2, whereas CD4⁺CD25⁻ T cells cultured with autologous DCs plus IL-2 showed a high degree of proliferation. A remarkable, even if lower, proliferative rate by CD25^{high} T cells cultured in the presence of DCs plus IL-2 was observed ([Fig 2, A](#)). Under the experimental conditions described, CD4⁺CD25^{high} T cells maintained the

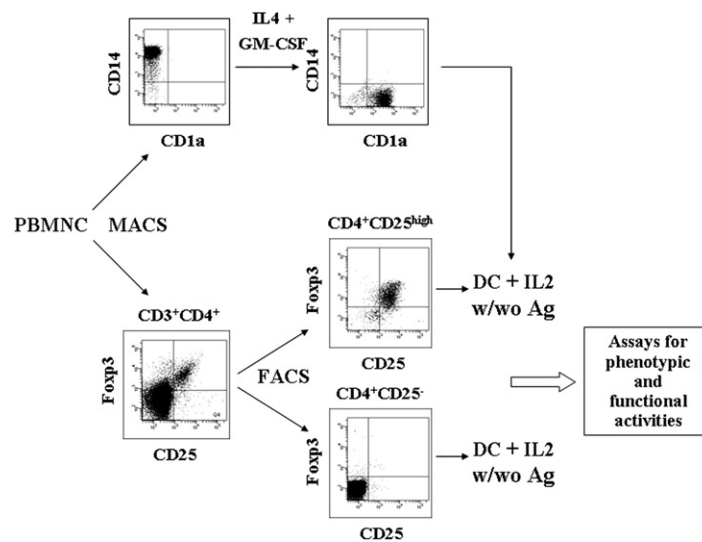


FIG 1. Experimental protocol used to assess antigen-specific Treg cells. Flow-cytometric evaluation of CD14 and CD1a in CD14⁺ monocytes on day 0 and after 7 days of culture in the presence of IL-4 and GM-CSF, as well as of CD25 and Foxp3 in CD3⁺CD4⁺, CD3⁺CD4⁺CD25⁻, or CD3⁺CD4⁺CD25^{high} purified cell fractions. *PBMNC*, Peripheral blood mononuclear cells; *MACS*, magnetic cell sorting; *FACS*, fluorescence-activated cell sorting; *w/wo*, with/without.

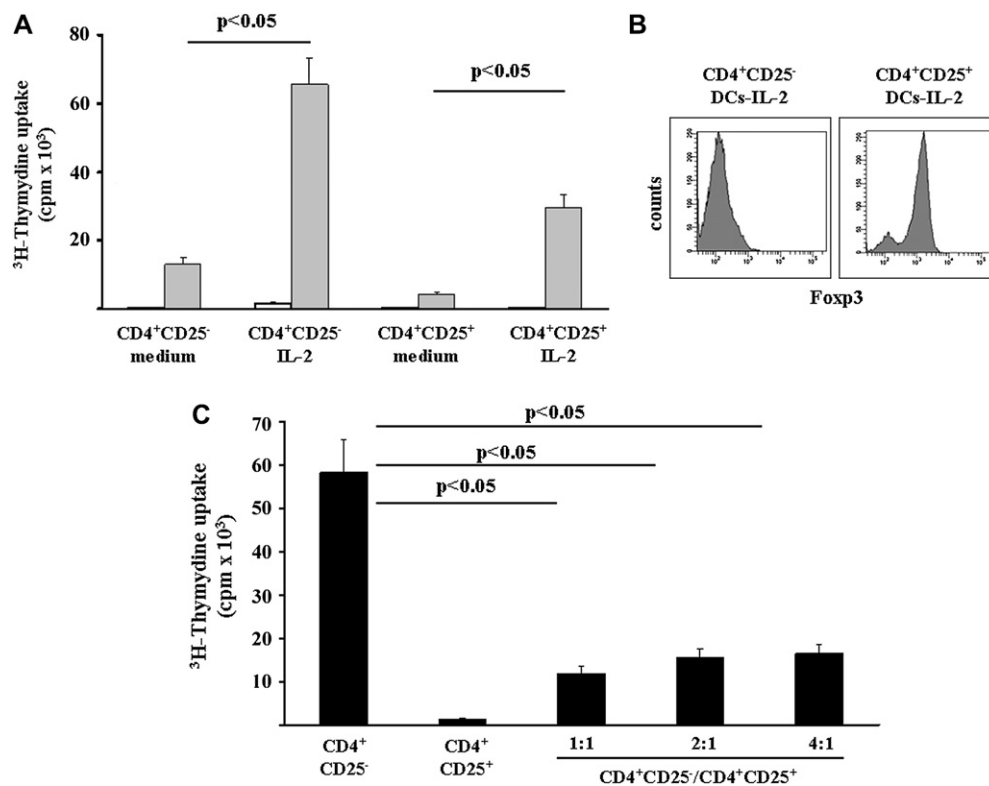


FIG 2. Cultured CD4⁺CD25^{high} Treg cells retain both phenotype and function. **A**, Proliferation (means $\pm$ SDs) of CD4⁺CD25^{high} and CD4⁺CD25⁻ T lymphocytes cultured with (gray columns) or without (white columns) autologous DCs ($n = 4$). **B**, Foxp3 expression by cultured CD4⁺CD25^{high} and CD4⁺CD25⁻ lymphocytes. **C**, Proliferation (means $\pm$ SDs) of CD4⁺CD25⁻ T cells to allogeneic stimulation with or without CD4⁺CD25^{high}Foxp3⁺ T lymphocytes ($n = 4$).

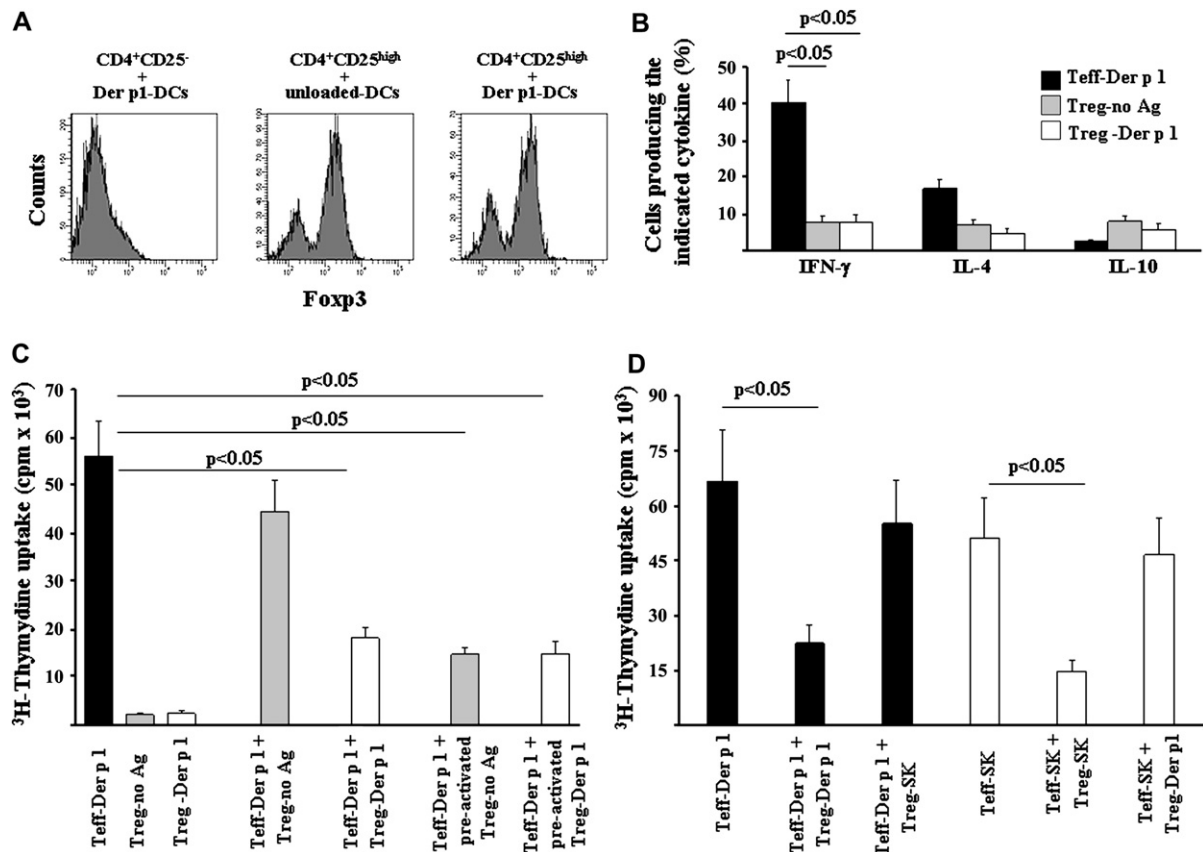


FIG 3. Expansion of antigen-specific CD4⁺CD25^{high} Foxp3⁺ T lymphocytes by Der p 1-loaded or SK-loaded autologous DCs. **A**, Flow-cytometric evaluation of Foxp3 expression. **B**, Cytokine-producing cells (mean percentages $\pm$ SDs) after polyclonal stimulation ($n = 3$). **C** and **D**, Effect of Treg-no Ag, Treg-Der p 1, or Treg-SK on the antigen-induced proliferation of Teff-SK or Teff-Der p 1 ($n = 5$, **C**; or $n = 3$, **D**, respectively).

expression of Foxp3, whereas virtually all CD4⁺CD25⁻ cells remained Foxp3⁻ (Fig 2, *B*). More importantly, CD4⁺CD25^{high} T cells retained their suppressive activity, inasmuch as they showed the ability to inhibit in a dose-dependent fashion the proliferative response of autologous CD4⁺CD25⁻ T cells in response to allogeneic stimulation (Fig 2, *C*). These findings allowed us to conclude that CD4⁺CD25^{high} T cells cultured in presence of autologous DCs and IL-2 can expand and retain their suppressive phenotype. This is in agreement with recently reported data showing that myeloid DCs are more efficient than other antigen-presenting cells for maintaining Foxp3⁺ Treg cells in culture.¹¹

In vitro coculturing of CD4⁺CD25^{high}Foxp3⁺ cells with antigen-loaded autologous DCs expands Treg cells showing antigen specificity

To demonstrate the existence of circulating CD25^{high}Foxp3⁺ T cells specific for exogenous antigens, CD4⁺CD25^{high}Foxp3⁺ T cells from healthy volunteers were purified as described in Fig 1 and cocultured in the presence of IL-2 with autologous DCs, which had been loaded with Der p 1 or streptokinase. Der p 1 and streptokinase were chosen because have been found to induce

T-cell proliferation in virtually all healthy adult subjects.¹² CD4⁺CD25⁻ cells derived from PBMCs of the same donors were also cultured under identical experimental conditions. After 15 days, both T-cell fractions were tested for Foxp3 expression, profile of cytokine production, antigen specificity, and suppressive function. Flow-cytometric analysis showed intracellular Foxp3 expression only in cultures of CD4⁺CD25^{high} T cells, irrespective of whether they had been established in presence of unloaded or antigen-loaded autologous DCs (Fig 3, *A*). Moreover, the great majority of cultured CD4⁺CD25^{high}Foxp3⁺ T cells did not show the ability to produce IL-4, IFN- γ , or IL-10 in response to phorbol 12-myristate 13-acetate plus ionomycin. By contrast, a significantly higher proportion of CD4⁺CD25⁻ T cells cultured in presence of Der p 1-loaded DCs produced IFN- γ and/or IL-4, with the proportions of IL-4-producing cells usually lower (Fig 3, *B*). As expected on the basis of the results of previous studies,¹⁰ more than 50% of T cells cultured in presence of streptokinase-loaded DCs produced IFN- γ , with IL-4-producing cells being virtually undetectable (data not shown). Both the maintenance of Foxp3 expression and the poor ability to produce cytokines strongly suggest that cultures of CD4⁺CD25^{high}Foxp3⁺ T cells performed in the presence of unloaded or antigen-loaded

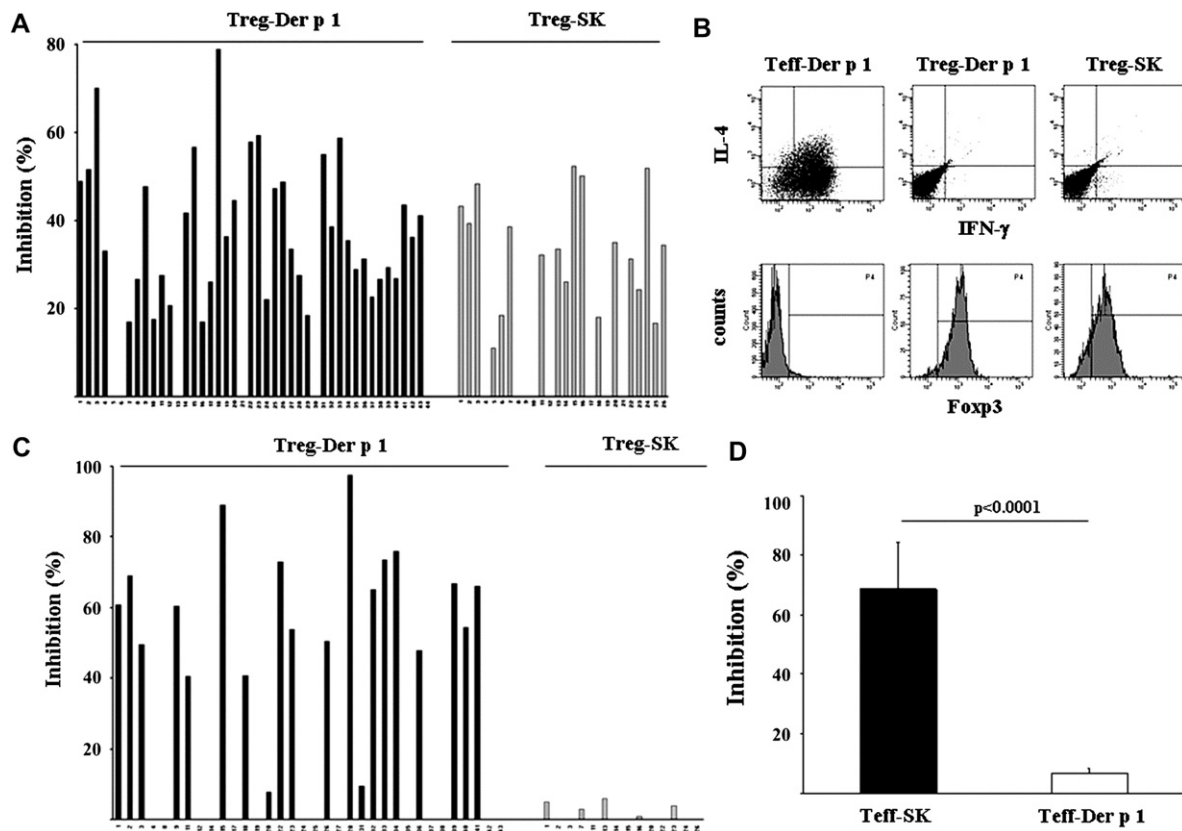


FIG 4. Treg-Der p 1, but not Treg-SK, clones suppress the Teff-Der p 1 proliferation. **A**, Effect of Treg clones on the proliferation of allogeneic-stimulated autologous T lymphocytes. **B**, Cytokines and Foxp3 expression by clones. **C**, Inhibition by Treg clones of antigen-induced proliferation of Teff-Der p 1 clones. **D**, Inhibition (mean percentages $\pm$ SDs) by Treg-SK clones of Teff clones proliferation ($n = 14$).

DCs mainly consist of Treg cells, whereas $CD4^+CD25^-$ T cells after culturing under the same experimental conditions predominantly express an effector phenotype. Therefore, this latter population is called T-effector (Teff) cells (Teff-Der p 1 and Teff-SK), whereas $CD4^+CD25^{high}Foxp3^+$ T cells cultured with unloaded DCs are defined as Treg-no antigen (Ag), and those cultured with DCs loaded with Der p 1 or SK are defined as Treg-Der p 1 and Treg-SK, respectively.

We then asked whether Treg cells had the ability to suppress the proliferation of autologous Teff cells and their ability to produce cytokines. To this end, Treg-no Ag or Treg-Der p 1 were tested for their ability to suppress the proliferation of autologous Teff-Der p 1 cells. As shown in Fig 3, C, Teff-Der p 1 vigorously proliferated to the recall Der p 1 stimulation, whereas neither Treg-Der p 1 nor Treg-no Ag, cells did. More importantly, the addition to Teff-Der p 1 of Treg-Der p 1 significantly suppressed their proliferation, whereas the addition of Treg-no Ag did not (Fig 3, C). Likewise, production of IFN- γ and IL-2, as assessed by ELISA into the supernatants of the same cell cultures, was strongly suppressed (data not shown). As expected, however, polyclonal preactivation with anti-CD3 plus anti-CD28 antibodies rendered Treg-no Ag cells able to suppress the antigen-induced

proliferation of Teff-Der p 1 cells (Fig 3, C). Of note, Treg-SK did not suppress the Der p 1-induced proliferation of Teff-Der p 1 cells, whereas they strongly suppressed the SK-induced proliferation of Teff-SK cells (Fig 3, D). Accordingly, Treg-Der p 1 cells did not suppress the SK-induced proliferation of Teff-SK cells, whereas even in this new series of experiments they strongly suppressed the Der p 1-induced proliferation of Teff-Der p 1 cells (Fig 3, D). Neither the suppressive activity induced by polyclonal stimulation on Treg-no Ag nor the suppression induced by antigen stimulation on Treg-Der p 1 was mediated by IL-10 production, inasmuch as in both culture systems, the addition of a neutralizing anti-IL-10 mAb did not have any detectable effect (preactivated Treg-no Ag + isotype control mAb $60\% \pm 5\%$ inhibition vs $56\% \pm 4\%$ inhibition in the presence of anti-IL-10 mAb; Treg-Der p 1 + isotype control mAb $56\% \pm 6\%$ inhibition vs $62\% \pm 5\%$ inhibition in the presence of anti-IL-10 mAb).

Antigen-specific suppression by Treg cells can also be observed at the clonal level

Both Teff-Der p 1 or Teff-SK and Treg-Der p 1 or Treg-SK cell suspensions do not exclusively consist of Der p 1-specific or SK-specific T cells. Therefore, the

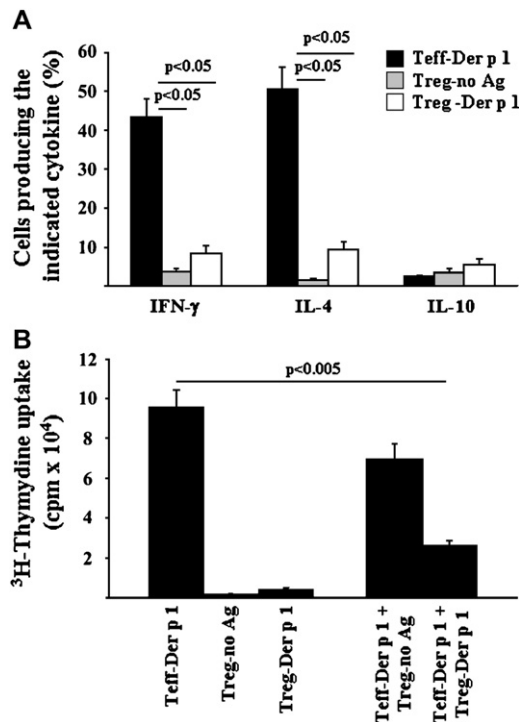


FIG 5. Circulating Der p 1-specific CD4⁺CD25^{high} Treg cells are also present and functionally active in donors with allergy. **A**, Percentages (means $\pm$ SDs) of cytokine-producing cells after polyclonal stimulation in the indicated population (n = 6). **B**, Effects of Treg-no Ag or Treg-Der p 1 on the antigen-induced proliferation (means $\pm$ SDs) of Teff-Der p 1 in Der p 1-sensitive subjects (n = 6).

effects we observed could be in some way influenced by the interference of antigen-nonspecific T cells. To provide more direct evidence of the antigen specificity of Treg-Der p 1 T cells, Teff-Der p 1, Treg-Der p 1, and Treg-SK culture suspensions derived from the same donor were cloned under limiting dilution. A total of 85 clones were obtained from Teff-Der p 1 (clonal efficiency, 45%), 44 from Treg-Der p 1 (clonal efficiency, 12%) and 26 from Treg-SK (clonal efficiency, = 7%) T-cell cultures. Twenty of the 85 Teff-Der p 1 clones (24%) showed marked proliferation (stimulation index > 20) in response to Der p 1-loaded autologous DCs, thus indicating they were homogenous populations of Der p 1-specific T cells (data not shown). The suppressive phenotype of the 70 Treg clones (44 Treg-Der p 1 and 26 Treg-SK) was then assessed by evaluating (1) their ability to inhibit the proliferative response of autologous CD4⁺CD25⁻ T cells to allogeneic stimulation, (2) the expression of Foxp3, and (3) the ability to produce cytokines after polyclonal stimulation. As shown in Fig 4, A, 34 of 44 Treg-Der p 1 (77%) and 14 of 26 Treg-SK (54%) clones exhibited a detectable, even if variable (between 20% and 80%), inhibitory activity on the proliferation of autologous CD4⁺CD25⁻ cells. Moreover, the Treg clones (34 Treg-Der p 1 + 14 Treg-SK), irrespective of their culture origin, were characterized by both high Foxp3 expression and the inability to produce IL-4 and IFN-γ, whereas all Teff-Der

p 1 clones were negative for Foxp3 and contained variable proportions of T-blasts able to produce IFN-γ and/or IL-4 (Fig 4, B). Therefore, the 34 clones obtained from Treg-Der p 1 and the 14 obtained from Treg-SK T-cell suspensions showing suppressive activity were tested for their capacity to inhibit in the presence of Der p 1-loaded autologous DCs the proliferation of a pool of autologous Teff-Der p 1 clones, previously assessed for their Der p 1 specificity. As shown in Fig 4, C, 13 of 34 (38%) Treg-Der p 1 clones exerted a suppressive activity higher than 50%, and another 5 clones (15%) between 20% and 50%, on the proliferation of Teff-Der p 1 clonal cell suspensions, whereas none of the Treg-SK clones exhibited any inhibitory capacity (consistently lower than 10%) on the proliferation of the same target cells. Of note, Treg-SK clones were able to inhibit the proliferation of autologous Teff-SK-specific but not Teff-Der p 1-specific clones when stimulated with SK-loaded or Der p 1-loaded autologous DCs, respectively (Fig 4, D).

Circulating Der p 1-specific CD4⁺CD25^{high}Foxp3⁺ Treg cells are also present and functionally active in atopic donors

It has been recently proposed that the atopic status may be a result of the lack in patients with allergy of Treg cells able to suppress the immune response to the specific allergens, whereas nonatopic subjects do not develop allergy because of the presence of Treg cells able to prevent allergen-specific T_H2 responses.¹³ However, the phenotype and mechanism of action of Treg cells involved in the prevention of allergy is still controversial. To verify whether allergen-specific CD4⁺CD25^{high}Foxp3⁺ Treg cells may be defective in atopic individuals, the experimental protocol described was used to assess both the presence and the functional activity of Der p 1-specific Treg cells in 6 Der p 1-sensitive atopic donors.

As expected, the cytokine production profile of Teff cells obtained from the CD4⁺CD25⁻ Der p 1-specific T cells exhibited a more T_H2 polarized phenotype than in nonatopic subjects, as shown by the higher numbers of IL-4-producing versus IFN-γ-producing cells (Fig 5, A). By contrast, and in agreement with the results obtained in healthy donors, either Treg cell cultures derived from CD4⁺CD25^{high}Foxp3⁺ T cells cocultured in the presence of Der p 1-loaded autologous DCs or CD4⁺CD25^{high}Foxp3⁺ cells cocultured in the presence of unloaded autologous DCs, contained significantly lower numbers of cells able to produce IL-4, IFN-γ, or IL-10 (Fig 5, A). Accordingly, when tested for their suppressive activity, only Treg-Der p 1, but not Treg-no Ag, cells showed the ability to inhibit the Der p 1-induced proliferation of Teff-Der p 1 cells (Fig 5, B), with the percentage of inhibition comparable to that observed in healthy donors (65% $\pm$ 31% in atopic donors, n = 6; vs 65% $\pm$ 18% in healthy donors, n = 6). Both Der p 1-specific and SK-specific T-cell clones were also obtained from 2 of these donors and assessed by using the same techniques described for nonatopic donors. When assessed for their

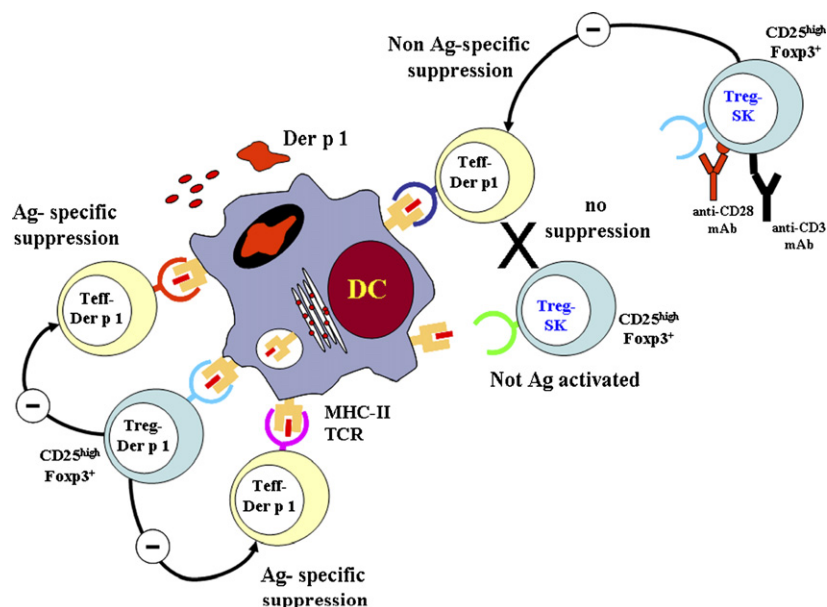


FIG 6. Scheme illustrating the *in vitro* suppression by Treg-Der p 1 cells. DCs present Der p 1 peptides to both Teff-Der p 1 and Treg-Der p 1 cells. Der p 1-induced Teff-Der p 1 cell proliferation is blocked by Der p 1-activated Treg-Der p 1 cells (antigen-specific suppression). Treg-SK cells do not inhibit the Teff-Der p 1 cell proliferation unless they are activated by anti-CD3/anti-CD28 mAbs (nonantigen-specific suppression). TCR, T-cell receptor.

suppressive activity, Der p 1–Treg clones derived from the 2 atopic donors exhibited a suppressive activity indistinguishable from that of either autologous Treg-SK clones or Treg-Der p 1 clones derived from nonatopic donors (data not shown). These results indicate that circulating Der p 1–specific Treg cells also exist in atopic patients and, more importantly, that these cells exhibit comparable activity in inhibiting the allergen-induced T-cell proliferation as in nonatopic subjects.

DISCUSSION

Treg cells certainly represent a highly heterogeneous family of cells. Natural $CD4^+CD25^{\text{high}}Foxp3^+$ Treg cells represent a distinct lineage that originates in the thymus and is probably devoted to avoid or hamper immune responses against self-antigens.^{1,2,5} Adaptive Treg cells specific for exogenous antigens have also been described. They include T_H3 and type 1 Treg,^{3,4} which are $Foxp3^-$ and do not constitutively express high CD25 levels; however, adaptive $CD4^+CD25^{\text{high}}Foxp3^+$ T cells have also been observed.^{6,7,14} The relationship between the latter cells and the natural $CD4^+CD25^{\text{high}}Foxp3^+$ T cells is still unclear. Likewise, direct evidence of whether human $CD4^+CD25^{\text{high}}Foxp3^+$ T cells showing specificity for exogenous antigens do really exist has not been provided yet.

The results of this study clearly demonstrate that within the pool of human circulating $CD4^+CD25^{\text{high}}Foxp3^+$ T cells, there are also cells able to recognize highly ubiquitous recall antigens such as Der p 1 and streptokinase. These cells can be expanded *in vitro* by culturing them

with antigen-loaded autologous DCs and high IL-2 concentrations. In addition, the results also clearly show that these cells retain their regulatory phenotype even after several rounds of *in vitro* proliferation and, once they are activated by the specific antigen, exhibit the ability to suppress the antigen-induced proliferation of autologous Teff cells. The existence of antigen-specific Treg cells was confirmed even at the clonal level. A scheme illustrating the events possibly occurring in the *in vitro* experimental system used is depicted in Fig 6. To our knowledge, this represents the first demonstration that antigen-specific Treg clones can be generated from the pool of circulating $CD4^+CD25^{\text{high}}Foxp3^+$ human T cells. The inhibitory mechanism of antigen-specific Treg cells was not mediated by the production of IL-10, because the suppressive activity could not be affected by the addition in culture of an anti-IL-10 neutralizing antibody. However, it cannot be excluded that either specific or nonspecific inhibitory mechanisms are operating, perhaps depending of different activation conditions, such as allergen concentration, type of allergen, and atopy status.¹³

Another finding emerging from this study is the demonstration that $CD4^+CD25^{\text{high}}Foxp3^+$ T cells specific for Der p 1 allergen exist in the circulation of either nonatopic or Der p 1–sensitive, atopic subjects. Recently, it has been suggested that a defect of allergen-specific Treg cells may be responsible for allergen-specific T_H2 responses and allergic inflammation in atopic subjects.¹³⁻¹⁵ Although we used a different experimental protocol to isolate allergen-specific Treg cells and the phenotype of these cells was different from that described by those authors,¹⁵ the results of this study provide evidence that at least allergen-specific

CD4⁺CD25^{high}Foxp3⁺ Treg cells are neither absent nor functionally inactive in atopic individuals. Obviously, given the high heterogeneity of Treg cell families, the possibility that other types of Treg cells (for example, those able to produce IL-10) are defective in atopic subjects cannot be excluded. Likewise, it should be taken into account that allergen-specific Treg cells described in such an *in vitro* model cannot have any pathophysiological significance *in vivo*. However, caution should be advised before considering allergic disorders as the simple consequence of defective T-cell regulatory mechanisms in the response to common environmental allergens.

A still unsolved question is whether Treg cells specific for exogenous antigens, including common environmental allergens, originate from the thymus as a distinct lineage as those specific for self-antigens do or whether they derive from Teff cells themselves, which are able to exhibit a plastic phenotype. Another point that needs to be clarified is the relationship between the Treg cells here described and others already reported.^{6,7,14} In any case, the possibility to expand *in vitro* antigen-specific CD4⁺CD25^{high}Foxp3⁺ Treg cells may represent a useful tool to investigate these points better, as well as the role of these cells in the pathogenesis and evolution of chronic immune disorders.

We thank Dr Gabriele Graziani for his help in recruiting Der p 1-sensitive patients.

REFERENCES

1. Sakaguchi S. Naturally arising CD4⁺ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004;22:531-62.
2. Bluestone J-A, Abbas A-K. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 2003;3:253-7.
3. Weiner H-L. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 2001;182:207-14.
4. O'Garra A, Vieira P-L, Vieira P, Goldfeld A-E. IL-10-producing and naturally occurring CD4⁺ Tregs: limiting collateral damage. *J Clin Invest* 2004;114:1372-8.
5. Fontenot J-D, Rudensky A. A well adapted regulatory contrivance: the forkhead family transcription factor Foxp3 and regulatory T cell development. *Nat Immunol* 2005;6:331-7.
6. Belkaid Y, Rouse B-T. Natural regulatory T cells in infectious disease. *Nat Immunol* 2005;6:353-60.
7. Suffia I-J, Reckling S-K, Piccirillo C-A, Goldszmid R-S, Belkaid Y. Infected site-restricted Foxp3⁺ natural regulatory T cells are specific for microbial antigens. *J Exp Med* 2006;203:777-88.
8. Walker M-R, Carson B-D, Nepom G-T, Ziegler S-F, Buckner J-H. De novo generation of antigen-specific CD4⁺CD25⁺ regulatory T cells from human CD4⁺CD25⁻ cells. *Proc Natl Acad Sci U S A* 2005;102:4103-8.
9. Annunziato F, Cosmi L, Liotta F, Lazzeri E, Manetti R, Vanini V, et al. Phenotype, localization, and mechanism of suppression of CD4⁺CD25⁺ human thymocytes. *J Exp Med* 2002;196:379-87.
10. Annunziato F, Cosmi L, Galli G, Beltrame C, Romagnani P, Manetti R, et al. Assessment of chemokine receptor expression by human Th1 and Th2 cells in vitro and in vivo. *J Leukoc Biol* 1999;65:691-9.
11. Banerjee D, Dhodapkar M-V, Matayeva E, Steinman R-M, Dhodapkar K. Expansion of FOXP3^{high} regulatory T cells by human dendritic cells (DCs) in vitro and after DC injection of cytokine matured DCs in myeloma patients. *Blood* 2006;108:2655-61.
12. Romagnani P, Maggi L, Mazzinghi B, Cosmi L, Lasagni L, Liotta F, et al. CXCR3-mediated opposite effects of CXCL10 and CXCL4 on TH1 or TH2 cytokine production. *J Allergy Clin Immunol* 2005;116:1372-9.
13. Bellinghause I, König B, Bottcher I, Knop J, Saloga J. Regulatory activity of human CD4⁺CD25⁺ T cells depends on allergen concentration, type of allergen and atopy status. *Immunology* 2005;116:103-11.
14. Ling EM, Smith T, Dao Nguyen X, Prodegeon C, Dallman M, Arbery J, et al. Relation of CD4⁺CD25⁺ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;363:608-15.
15. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567-75.

METHODS

Reagents, antibodies, and ELISA for cytokines

The cell culture medium used was RPMI 1640 (Seromed, Berlin, Germany), supplemented with 2 mmol/L L-glutamine, 1% nonessential amino acids, 1% pyruvate, and 2×10^{-5} mol/L 2-mercaptoethanol (Gibco Laboratories, Grand Island, NY). Fluorochromes-conjugated and isotype-matched mAbs were purchased from Becton Dickinson (Mountain View, Calif). The amounts of IL-2 (R&D Systems, Minneapolis, Minn) and IFN- γ (Endogen, Woburn, Mass) in the T-cell culture supernatants were evaluated by homemade sandwich ELISAs using commercial pairs of mAbs.

Generation of DCs

Dendritic cells were generated by culturing purified CD14⁺ cells in medium supplemented with 5% FCS (Hyclone, Logan, Utah), 50 ng/mL GM-CSF, and 20 ng/mL IL-4 (R&D Systems). On day 7, cells were analyzed by flow cytometry for CD1a, CD14, CD86, CD80, and HLA-DR expression.

Antigen-specific T-cell lines and T-cell clones

CD4⁺CD25⁻ T cells were stimulated with autologous DCs (1:1 ratio) in the presence of Der p 1 (10 μ g/mL) or streptokinase (1000 U/mL). On day 6, reactive cells were expanded with recombinant IL-2 (20 U/mL; Eurocetus, Milan, Italy) as previously described.^{E1} CD4⁺CD25^{high} cells were stimulated with autologous DCs (1:1 ratio) loaded or not with Der p 1 (10 μ g/mL) or streptokinase (1000 U/mL) in the presence of recombinant IL-2 (100 U/mL). T-cell blasts from Der p 1-specific T-cell lines obtained from CD4⁺CD25⁻ cells, from Der p 1-specific CD4⁺CD25^{high} cells, or from SK-specific CD4⁺CD25^{high} cells of the same donor were cloned under limiting dilution (0.3 cell/well), as previously reported.^{E1} Because the antigen-specific T-cell lines of all 3 donors showed comparable functional activities, the choice of one of them for limiting dilution studies was based on the highest availability of autologous DCs required for all subsequent experiments. Clonal efficiency was calculated as detailed elsewhere.^{E1}

Suppressive assays

Autologous CD4⁺CD25⁻ T cells 5×10^4 were cultured with 10^5 irradiated (9000 rad) T-cell-depleted allogeneic PBMCs in the presence of different numbers of CD4⁺CD25^{high} lymphocytes. To assess the capacity of antigen-specific CD4⁺CD25^{high} T cells to suppress the antigen-induced proliferation of autologous antigen-specific T-cell lines generated from CD4⁺CD25⁻ T cells, 2×10^4 of these latter cells were cultured with 4×10^3 irradiated (9000 rad) autologous antigen-loaded or antigen-unloaded DCs in the presence of 2×10^4 cells of T-cell lines generated from CD4⁺CD25^{high} T cells. The pools of Teff-Der p1 or Teff-SK clones were composed of 4 different clones, each of whom accounted for 25% of the total cell population. The selection of clones was made on the basis of their high proliferation rate in response to the Der p 1-loaded or SK-loaded autologous DCs—that is, with mitogenic index (ratio between counts per minute obtained in presence of antigen-loaded autologous DCs and counts per minute obtained with unloaded autologous DC alone) > 20.

In some experiments, T cells generated from CD4⁺CD25⁻ or CD4⁺CD25^{high} cells were activated at different ratios by anti-CD3 (5 μ g/mL) plus anti-CD28 (5 μ g/mL; BD Biosciences, Mountain View, Calif) mAbs. On day 4, after 8 hours of pulsing with 0.5 μ Ci ³H-TdR/well (Amersham, Little Chalfont, United Kingdom), cultures were harvested and radionuclide uptake measured by scintillation counting.

ELISA for cytokines

The amounts of IL-2 (R&D Systems) and IFN- γ (Endogen) in the T-cell culture supernatants were evaluated by homemade sandwich ELISAs using commercial pairs of mAbs.

REFERENCE

- E1. Annunziato F, Cosmi L, Galli G, Beltrame C, Romagnani P, Manetti R, et al. Assessment of chemokine receptor expression by human Th1 and Th2 cells in vitro and in vivo. *J Leukoc Biol* 1999;65:691-9.