

T Regulatory Cells 1 Inhibit a Th2-Specific Response In Vivo¹

Françoise Cottrez,* Steven D. Hurst,[†] Robert L. Coffman,[†] and Hervé Groux^{2*}

We recently described a new population of CD4⁺ regulatory T cells (Tr1) that inhibits proliferative responses of bystander T cells and prevents colitis induction in vivo through the secretion of IL-10. IL-10, which had been primarily described as a Th2-specific cytokine inhibiting Th1 responses, has displayed in several models a more general immune suppression on both types of effector T cell responses. Using an immediate hypersensitivity model in which BALB/c mice immunized with OVA (alum) normally generate Th2-dominated responses, we examined the ability of OVA-specific Tr1 T cell clones to inhibit OVA-specific cytokines and Ab responses. In contrast to Th2 or Th1 T cell clones, transfer of Tr1 T cell clones coincident with OVA immunization inhibited Ag-specific serum IgE responses, whereas IgG1 and IgG2a synthesis were not affected. This specific inhibition was mediated in part through IL-10 secretion as anti-IL-10 receptor Abs treatment reverted the inhibitory effect of Tr1 T cell clones. Although specifically targeted to IgE responses, Tr1 clones' inhibitory effects were more profound as they affected Ag-specific Th2 cell priming both in term of proliferative responses and cytokine secretion. These results suggest that regulatory T cells may play a fundamental role in maintaining the balance of the immune system to prevent allergic disorders. *The Journal of Immunology*, 2000, 165: 4848–4853.

Immediate hypersensitivity is a major chronic health problem in developed nations. Its incidence and that of closely related conditions, such as asthma, has risen dramatically in recent decades, so that ~20–25% of the population is affected. It represents one of the most common examples of in vivo activation of inappropriate pattern of Th2-type cytokines synthesis (1).

Th2 responses are mediated by CD4⁺ T cells that secrete cytokines, such as IL-4, IL-5, and IL-13, which are known to play a central role in allergic responses (2–4). In contrast to healthy subjects, allergic patients develop specific IgE directed against sensitizing allergens that play a key role in the physiopathology of allergic diseases (5). Induction of IgE switching requires two primary signals. The first one, given by IL-4 or IL-13, induces the expression of the sterile ϵ transcript (6–8). The second one, provided by the triggering of CD40 ligand, induces the expression of the mature ϵ transcript encoding for IgE (9, 10). Several cytokines modulate IL-4-dependent IgE production. IFN- α , IFN- γ , TGF- β , and IL-12 have inhibitory effects, whereas IL-2, IL-5, IL-6, and TNF- α enhance IL-4-induced IgE synthesis.

IL-10 is a cytokine produced by numerous cell types, including activated T cells, mast cells, and macrophages. By blocking Ag-presenting capacities of monocytes/macrophages, IL-10 plays a major role in suppressing immune and inflammatory responses (11). IL-10 also acts on human B cells activated by anti-CD40 mAb by enhancing the switching to IgA, IgG1, and IgG3 isotypes (12, 13), the short-term proliferation (14), and the differentiation of

B-cells into Ig-secreting plasma cells (15). However, IL-10 specifically decreases IgE production by IL-4-stimulated PBMC in vitro (16).

We have recently shown that both human and mouse CD4⁺ T cells repeatedly stimulated in the presence of IL-10 differentiate into a new subset of CD4⁺ T cells different from the classical Th1 and Th2 T cell clones (17). These cells, termed T regulatory 1 (Tr1)³ have a poor proliferative response and secrete no IL-2 or IL-4, but produce high levels of IL-10 and inhibit the proliferative response of bystander cells both in vitro and in vivo (17). Using OVA-immunized BALB/c mice in the presence of alum that generate a Th2-type response characterized by substantial IL-4 and IL-5 production, we examined the impact of Tr1 cells in the regulation of IgE and CD4⁺ T cells responses to OVA. These experiments could also give evidence of potential helper function of Tr1 cells on B cell activation and differentiation.

Materials and Methods

Animals

BALB/cAnN mice were obtained from CERJ (Le Genest Saint Isle, France), and homozygous DO11-10 mice were a generous gift from Dr. A. O'Garra (DNAX Research Institute, Palo Alto, CA). All mice were raised free of common mouse pathogen conditions in our animal facility. They were all female mice, 6- to 8-wk-old at the beginning of each experiment.

Cytokine ELISA

Sandwich ELISAs were used to measure IL-4, IL-5, IL-10, and IFN- γ as previously described (18). In brief, ELISA plates were coated with the appropriate anti-cytokine Abs (11B11, TRFK4, 2A5, and XGM1.2 for IL-4, IL-5, IL-10, and IFN- γ , respectively) and incubated at 4°C overnight. After incubation, plates were blocked for 30 min at room temperature by adding 150 μ l of 20% FCS/PBS containing 0.04% Tween 20 to each well. Supernatants from in vitro-stimulated purified splenocytes were diluted in 5% FCS Yssel's medium and added at a volume of 50 μ l/well. Plates were incubated overnight at 4°C then washed and the second-step Ab (24G2, TRFK5, SXC1, and R4-6A2 for IL-4, IL-5, IL-10, and IFN- γ , respectively) was added at 50 μ l/well. Plates were incubated for 1 h at room temperature then washed and the enzyme conjugate was added to each well. Plates remained at room temperature for 1 h, after which they were washed and 100 μ l/well of substrate containing 1 mg/ml 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (Sigma, St. Louis, MO), 0.0003% H₂O₂ in Na₂HPO₄,

*Institut National de la Santé et de la Recherche Médicale Unité 343 Hôpital de l'Archet, Nice, France; and [†]DNAX Research Institute, Palo Alto, CA 94304

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² Address correspondence and reprint requests to Dr. Hervé Groux, Institut National de la Santé et de la Recherche Médicale Unité 343 Hôpital de l'Archet, Route de Saint Antoine de Ginestiere, 06000 Nice, France. E-mail address: groux@unice.fr

³ Abbreviation used in this paper: Tr1, T regulatory 1.

and 0.05 M citric acid was added. After the substrate was developed, applying 50 μ l of 0.2 M citric acid solution to each well stopped the reaction. The plates were read on an ELISA reader (Labsystems iEMS reader, Helsinki, Finland). Abs for ELISAs were purified from serum-free hybridoma supernatants as previously described (18).

Analysis of OVA-specific serum IgE

OVA-specific serum IgE was determined using a two-step sandwich ELISA without depleting for IgG as described (19). The coating Ab was a monoclonal anti-IgE Ab called EM95. The second step was a digoxigenin-coupled OVA that was prepared according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). In brief, plates were coated with 2 μ g/ml of EM95 and incubated overnight at 4°C. The serum samples were added and, subsequently, the digoxigenin-coupled OVA was added to the wells. Antidigoxigenin-Fab coupled to peroxidase (Boehringer Mannheim) were added. As described above, after 1 h of incubation, 0.1 ml of substrate was added to each well.

Analysis of OVA-specific IgG1 and IgG2a

ELISA plates were coated overnight at 4°C with 10 μ g/ml OVA in PBS. The detecting Ab for IgG1 that was used at 0.5 μ g/ml was a biotinylated rabbit anti IgG1. The detecting Ab for the IgG2a was a rabbit anti-IgG2a coupled with the nitroiodophenyl hapten. After incubation and washing, peroxidase-conjugated streptavidin was added to the wells of the IgG1 ELISA. The nitroiodophenyl-labeled anti-IgG2a was revealed with a HRP conjugate of a rat monoclonal anti-nitroiodophenyl Ab. Finally, plates were developed as described above. Standards for OVA-specific IgG1 were pooled from sera from hyperimmunized BALB/c mice. The concentration of OVA-specific IgG1 was estimated by comparison to an IgG1 standard run in parallel on anti-IgG1-coated plates. This method was also used for the quantification of OVA-specific IgE and IgG2a in the ELISA.

Cell lines, culture, and reagents

All assays were conducted in Yssel's medium (20) supplemented with 10% FCS. CD4⁺ T cells were purified from the lymph nodes of mice by negative depletion using anti-B220, anti-Mac-1, anti-CD8, and KJ1-26 mAbs (PharMingen, San Diego, CA) and sheep anti-rat-coated Dynabeads (Dyna, Oslo, Norway). In brief, cells were incubated with the different Abs (10 μ g/ml) for 30 min at 4°C; after washing, 500 μ l of beads for 5×10^7 cells were added for 30 min at 4°C. Cells were negatively purified upon application of a magnetic field. The T cell clones were obtained from DO11-10 mice after in vitro differentiation as previously described (21). Naive (MEL-14^{bright}) CD4⁺, KJ1-26⁺ cells were stimulated repeatedly for 3 wk with OVA peptide 323–339 in the presence of IL-4 and anti-IL-12, IL-12 and anti-IL-4, or IL-10 for Th2, Th1, or Tr1 cells, respectively. The populations obtained were cloned at 1 cell/well by cytofluorometry (FACSvantage SE; Becton Dickinson, Mountain View, CA) and stimulated with irradiated splenocytes (4500 rad) and OVA peptide. Clones were then expanded and analyzed for cytokine secretion after activation with APCs and OVA peptide. Selected clones were then cultured with stimulation with irradiated splenocytes and OVA peptide every 2 wk and further expanded with 10 ng/ml IL-2 (R&D Systems, Minneapolis, MN). T cell clones were used at least 10 days after the last stimulation. Several T cell clones were used. A-10-9 and A-10-11 were previously described (17), Nice-1 and Nice-2 were cloned after in vitro differentiation of KJ1-26⁺ cells in the presence of IL-10 and selected based on proliferation and cytokine secretion. D4-6, D4-15, and D4-19 were Th2 T cell clones isolated after differentiation of KJ1-26⁺ cells in the presence of IL-4 and anti-IL-12. A-7 and A-21 were Th1 T cell clones isolated after differentiation of KJ1-26⁺ cells in the presence of IL-12 and anti-IL-4 as described (17).

Anti-IL-10 receptor-blocking mAbs (1B1.2) were kindly provided by K. Moore (DNAX Research Institute, Palo Alto, CA). Abs were injected i.p. at day 0 (1 mg/mouse), and injection was repeated every week at 0.5 mg/mouse.

To measure cytokines released by CD4⁺ T cells contained in mesenteric lymph nodes, purified cells were stimulated in 1-ml cultures. The culture medium consisted of Yssel's medium with 10% heat-inactivated FCS (Roche, Meylan, France), 0.05 mM 2-ME (Sigma), and penicillin/streptomycin (Life Technologies, Gaithersburg, MD). Purified CD4⁺ T cells were stimulated at 2×10^6 cells/ml in culture medium containing 0.25 mg/ml OVA and irradiated splenocytes (5×10^6 cells/ml). The supernatant was harvested at 48 h.

Immunization and adoptive transfer of OVA-specific CD4⁺ T cell clones

Mice were immunized with 10 μ g/mouse of OVA with alum (both from Sigma) injected i.p. at day 0 and day 21. OVA-specific T cell clones (10^6 cells/mouse) were injected i.p. 2 h before OVA injection at day 0 and day 21.

Flow cytometry

For analysis, splenocytes were stained with FITC- or PE-conjugated mAbs (PharMingen). Flow cytometry analysis was performed on a FACScan flow cytometer (Becton Dickinson) and analyzed with the CellQuest software.

Results

Specific inhibition of IgE secretion in mice treated with Tr1 clones

To evaluate the capacity of Ag-specific Tr1 clones to either promote B cell help or inhibit a Th2 response, OVA-specific T cell clones were transferred into BALB/c mice 2 h before OVA (alum) immunization. In contrast to the situation where Th1 and Th2 T cell clones were transferred, no helper activity on Ig secretion was detected after injection of Tr1 T cell clones. Instead, the OVA-specific IgE response was inhibited by 90% by the transferred Tr1 clones, whereas OVA-specific IgG1 and IgG2a responses were not inhibited (Fig. 1). This specific effect of Tr1 clones on IgE production was completely reversed after injection of blocking anti-IL-10R Abs, confirming the importance of this cytokine in the regulatory effect of Tr1 clones (17). In contrast to Tr1 clones, no modification in IgE, IgG1, and IgG2a responses was observed after the transfer of an OVA-specific Th1 clone, and a slight enhancement of the specific IgE response was observed after transfer of an OVA-specific Th2-specific clone (Fig. 1).

Tr1 clones inhibit activation of OVA-specific T cells in vivo

We analyzed the effect of Tr1 clones on the activation of Ag-specific T cells in vivo by testing the recall in vitro proliferative response to OVA of CD4⁺ T cells previously depleted of the injected clones by using the anti-clonotype Ab KJ1-26. Injection of Tr1 cells resulted in a decreased recall proliferative response to OVA of CD4⁺ T cells isolated from mesenteric lymph nodes, whereas injection of a Th1- or a Th2-specific clone resulted in an enhancement in the in vivo priming for Ag-specific cells (Fig. 2). Again, addition of IL-10R blocking Abs reverted the effect of Tr1 cells on the priming of OVA-specific T cells in vivo (Fig. 2).

The inhibition of the activation of CD4⁺ T cells was confirmed by analyzing the percentage and the accumulation of CD25⁺, KJ1-26⁺ T cells in draining lymph nodes 2 days after the second immunization. Eight percent of activated CD4⁺ T cells were detected by cytometry analysis in the draining lymph nodes (Fig. 3). Addition of a Tr1 clone inhibited the number of activated (CD4⁺, CD25⁺, KJ1-26⁺) T cells present in mesenteric lymph nodes, whereas addition of Th1 or Th2 OVA-specific clones slightly enhanced the number of activated T cells.

Cytokine profiles of CD4⁺ T cells after immunization in the presence of different types of T cell clones

To determine the cytokine profile of OVA-specific host CD4⁺ T cells induced by immunization of mice receiving different types of Ag-specific T cell clones, lymph nodes were taken 7 days after the second immunization with alum and OVA. Draining lymph nodes' T cells were depleted of KJ1-26⁺ T cells, restimulated in vitro, and their supernatants were assayed for cytokines. Mice immunized with OVA in alum mounted a strong Th2 response as indicated by the significant levels of IL-4, IL-5, and IL-10, and undetectable levels of IFN- γ (Table I). The presence of Tr1 clones at the time

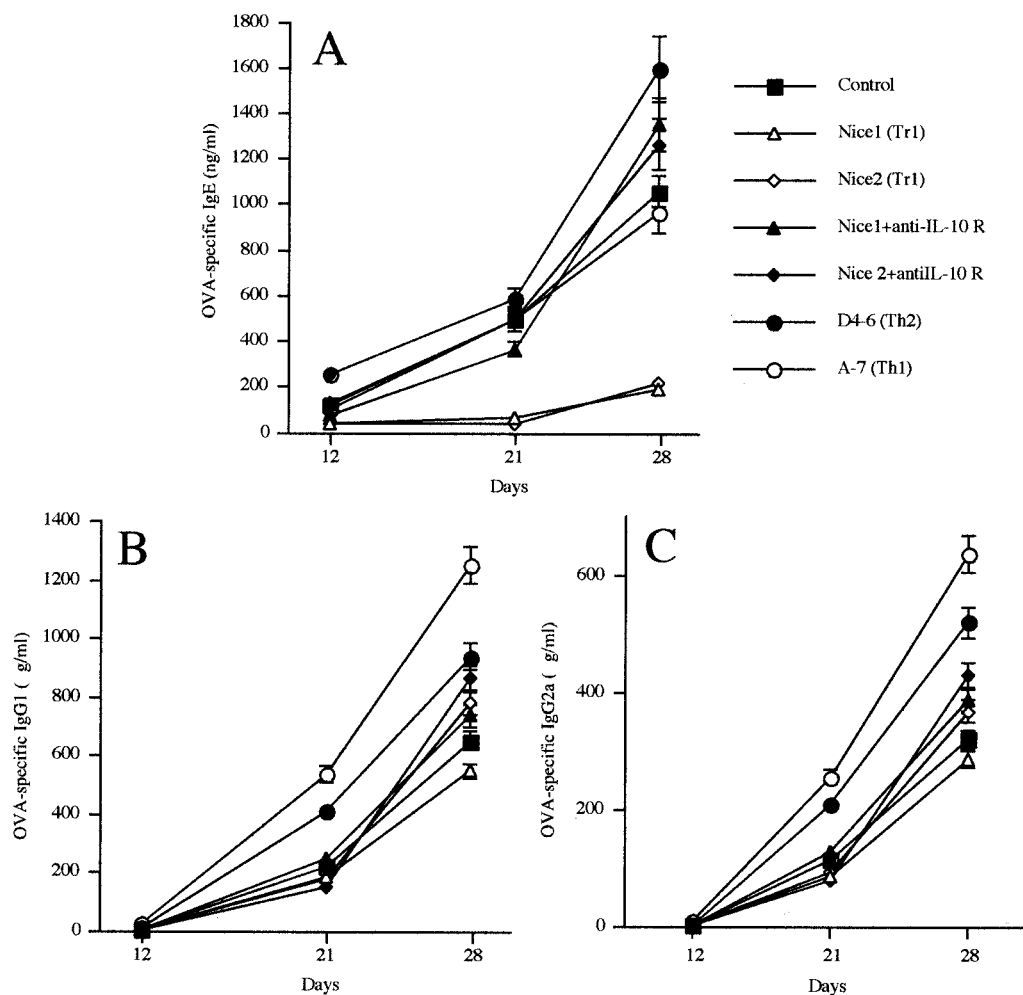


FIGURE 1. Kinetics of IgE, IgG1, and IgG2a synthesis of BALB/c mice transferred with different T cell clones before OVA/alum immunization. Effect of anti-IL-10 receptor treatment. Twice at 1-wk intervals, BALB/c mice were transferred with different T cell clones (as indicated) followed 2 h later with immunization (as described above) and then bled at days 12, 21, and 28 after the beginning of the treatment. One group of mice transferred with Tr1 clones was also treated with anti-IL-10R Abs. OVA-specific Abs were estimated by ELISA on pooled serum samples for five mice per group and expressed as mean of triplicate $\pm$ SD. Statistical analysis was performed with a Student *t* test. One representative experiment of four is shown. Experiments performed with different Tr1 clones (A-10-9, A-10-11), Tr1 cell lines, and several different Th1 and Th2 clones and cell lines gave similar results.

of immunization dramatically inhibited the differentiation of IL-4-secreting cells and promoted the differentiation of a Tr1 cell type population secreting no IL-4, high IL-10, and some IL-5 (Table I). The presence of a Th2 clone at the time of immunization enhanced the priming of naive CD4 T cells toward a Th2-type response with increased levels of secreted IL-4 and IL-5. Finally, the presence of a Th1 clone reduced the amounts of Th2-type cytokines (IL-4, IL-5, and IL-10) secreted by primed CD4⁺ T cells, but enhanced the differentiation of IFN- γ -secreting Ag-specific T cells.

Discussion

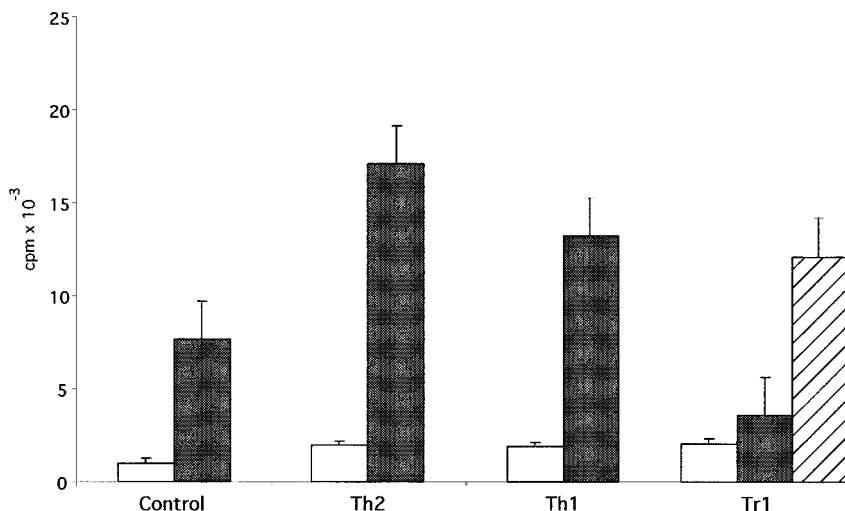
We have recently characterized a new subset of CD4⁺ T cells different from the classical Th1 and Th2 T cell clones. These cells, termed Tr1 cells secrete no IL-2 or IL-4, but do produce high levels of IL-10 (17). Both human and mouse Tr1 clones were shown to suppress immune responses *in vitro*. Ag-induced proliferation of naive CD4⁺ T cells was dramatically reduced following coculture with activated Tr1 clones, which were separated from the responding T cells by a *trans*-well insert. Suppression was reversed by addition of anti-TGF- β and IL-10 mAbs, implicating these cytokines in the mechanism of immune suppression. Suppression was a characteristic specific for Tr1 clones, as OVA-spe-

cific Th1 or Th2 clones had no suppressive effects, but rather enhanced OVA-induced proliferation of naive CD4⁺ T cells.

More importantly, Tr1 cells were shown to be immune suppressive *in vivo* in a typical Th1-mediated inflammation. Indeed, a colitis induced in *scid* mice by transfer of CD45RB^{high}CD4⁺ T cells was prevented by cotransfer of murine OVA-specific Tr1 clones. Immune suppression was dependent on Ag-induced activation of Tr1 cells *in vivo* as these cells only inhibited colitis in recipients that received OVA in their drinking water. Similar to *in vitro* experiments, we recently observed that *in vivo* suppression mediated by Tr1 clones was completely abrogated when mice were treated with anti-IL-10 receptor Abs (F. Cottrez and H. Groux, manuscript in preparation), confirming in this different model, the importance of IL-10 in the function of Tr1 clones.

In this study, the role of Ag-specific Tr1 cells in modulating Th2 responses *in vivo* was compared with Th1 and Th2 clones expressing the same specificity. Preliminary experiments using Th1 and Th2 clones have shown that injection of the T cell clones 2 h before immunization allows the analysis of both stimulatory and inhibitory effects of the T cells on Ig secretion. Intraperitoneal injection of OVA in alum induced OVA-specific IgE in naive

FIGURE 2. OVA-specific proliferative response of CD4⁺ T cells isolated from draining lymph nodes of BALB/c mice immunized with OVA/alum and transferred with different types of OVA-specific T cell clones. One week after the second immunization in the presence of a Th1 (A-7), Th2 (D4-6), or Tr1 (Nice-1) (as indicated), CD4⁺ T cells isolated from mesenteric lymph nodes were purified by negative purification using specific mAbs and magnetic beads and further depleted of KJ1-26⁺ T cells. Cells were stimulated with 100 μ g/ml OVA (■) or culture medium alone (□) in the presence of irradiated splenocytes. One group of mice transferred with the Tr1 clone was treated with anti-IL-10 receptor Abs (1 mg/mouse at 1-wk intervals starting at day 0) (▨). Results shown are mean $\pm$ SD cpm of triplicate cultures from the pooled cells of five mice.



BALB/c mice, but not in mice previously transferred with OVA-specific Tr1 clones. The same clones did not suppress OVA-specific IgG1 and IgG2a responses. No inhibition of IgE synthesis was observed in mice transferred with a Th1 clone, whereas a slight enhancement in IgE levels was observed in mice treated with an OVA-specific Th2 clone, as expected. The inhibition of IgE synthesis induced by Tr1 clones was mediated by their capacity to secrete high levels of IL-10 as treatment of mice with anti-IL-10 receptor Abs completely reverted the amounts of IgE detected in the serum of immunized mice.

IL-10 was originally described as a mouse Th2 cell factor, inhibiting cytokine synthesis by Th1 cells (22). However, increasing evidence suggest that IL-10 also acts as an inhibitor of Th2 cell responses both in vitro and in vivo (23–25). In particular, IL-10 was found to down-regulate IL-5 production by human resting T

cells and in human Th0 and Th2 clones (25, 26). The inhibitory action of IL-10 on IL-5 synthesis was confirmed in a murine model of allergic eosinophilic peritonitis and airway eosinophilia in which IL-10 administration suppressed both IL-5 production and eosinophil recruitment (24, 25). Finally, in mice, IL-10 administration before allergen treatment induces Ag-specific tolerance (27). We confirm in this report the direct effect of IL-10 in specifically inhibiting IgE synthesis in vivo. It has been previously reported that IL-10 decreases ϵ transcript expression and IgE production by IL-4- or IL-13-stimulated PBMC (16, 28). However, the inhibitory effect of Tr1 clones seems to be more profound than a specific inhibition at the B cell level.

Indeed, direct examination of the OVA-specific T cell recall response in vitro revealed that Tr1-treated mice did not develop significant CD4⁺ T cell responses, suggesting that specific loss of

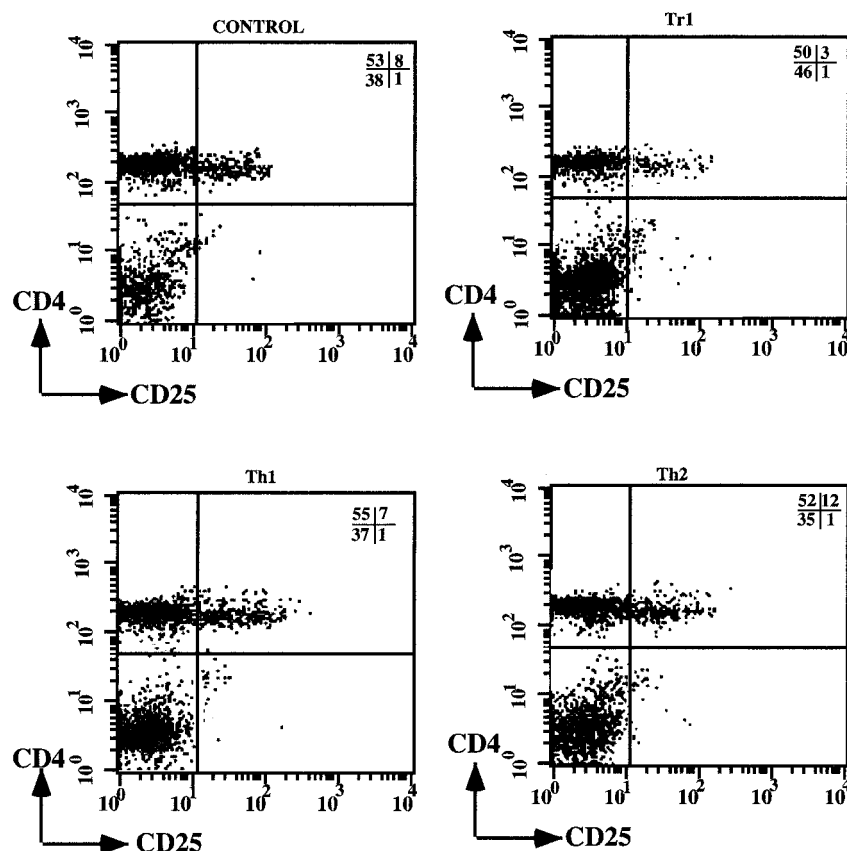


FIGURE 3. Analysis of the percentage of activated T cells in the draining lymph nodes. Two days after the second immunization, mesenteric lymph nodes were isolated and the cells analyzed by immunofluorescence using CD4-PE and CD25-FITC mAbs. KJ1-26⁺ T cells were gated out using a biotinylated Ab revealed by streptavidin-tricolor. Percentage in each quadrant is indicated.

Table I. Cytokine production after in vitro restimulation with OVA of CD4⁺ T cells from BALB/c mice transferred with different OVA-specific T cell clones

Injected Cells ^a	IL-4 (pg/ml)	IL-5 (ng/ml)	IL-10 (U/ml)	IFN- γ (ng/ml)
None	320 $\pm$ 87	8.8 $\pm$ 2.3	17.8 $\pm$ 3.5	ND
Tr1 (Nice 1)	ND	0.5 $\pm$ 0.01	34.1 $\pm$ 5.4	ND
Th2 (D4-6)	774 $\pm$ 102	11.5 $\pm$ 3.8	21.3 $\pm$ 3.2	ND
Th1 (A7)	25 $\pm$ 1.8	2.8 $\pm$ 0.8	12.3 $\pm$ 2.8	5.1 $\pm$ 1.2

^a Tr1, Th1, or Th2 OVA-specific T cell clones were injected into BALB/c mice 2 h prior to i.p. immunization with OVA in alum. Cell transfer and immunization were repeated twice at 1-wk intervals. Two days after the second immunization, draining lymph nodes were collected. Purified CD4⁺ T cells isolated from mesenteric lymph nodes were depleted of KJ1-26⁺ cells using magnetic beads and stimulated in vitro with 100 μ g/ml OVA in the presence of irradiated splenocytes. After 2 days, supernatants were collected and cytokine levels were analyzed by ELISA. Results represent mean $\pm$ SD of triplicate culture of one representative experiment of five.

IgE responses in Tr1-treated mice reflects a more fundamental inhibition in the activation of OVA-specific T cells by the regulatory T cells. Similar specific inhibition of IgE responses in vivo and induction of anergy in CD4⁺ T cells has been reported in different human (29) and mouse (19, 30) models of tolerance induction. In humans, specific immunotherapy is an efficient treatment for allergic diseases and is used most effectively in allergic reactions to insect venom and allergic rhinitis (31). It has recently been shown that administration of high allergen doses, as applied in immunotherapy, enhances endogenous production of IL-10 in specific T cells similar to Tr1 clones (29). Similarly, we recently demonstrated that exposure to inhaled OVA induced a state of unresponsiveness of CD4⁺ T cells that results in a prolonged loss of IgE and eosinophil responses to subsequent challenges (19). Whether this T cell unresponsiveness reflects the action of a regulatory population has not yet been determined by us; however, previous experiments with this model in both rats and mice suggest that an active suppression is involved (32, 33).

In a similar model, it has been suggested that the TCR- γ/δ ⁺ T cells are the principal mediators of IgE suppression (33). Adoptive transfer of TCR- γ/δ ⁺ T cells from aerosol OVA-primed mice suppressed OVA-specific IgE secretion in mice immunized with OVA (alum). However, our recent results showing that mice deficient in TCR- γ/δ ⁺ T cells have the same degree of IgE-specific unresponsiveness after aerosol priming and immunization with OVA argue against a unique role of these cells in establishing IgE unresponsiveness (19). The same group (34) and others (35) have also shown that CD8⁺ T cells, through the secretion of IFN- γ , were also important in suppressing IgE response. However, injection of Th1 T cell clones secreting IFN- γ did not result in inhibition of IgE secretion (Fig. 1). Moreover, we have recently shown in the model described above using aerosol-primed mice depleted of CD8⁺ T cells with specific Abs or mice deficient for the β_2 -microglobulin molecule that CD8⁺ T cells do not have a major role in aerosol-induced IgE unresponsiveness to soluble protein Ag. Our experiments do not rule out the possibility that CD8⁺ T cells could transfer an IgE-specific suppression, but simply show that CD8⁺ T cells are not required to suppress IgE synthesis as previously described (36).

Similar analysis has been done to study the unresponsiveness that occurs after oral Ag ingestion. Investigators have shown that the feeding of mice transgenic for OVA-specific TCR with high doses of OVA can inhibit airway eosinophilic inflammation induced by intratracheally administered OVA. This inhibitory effect was adoptively transferred by splenic CD4⁺ T cells, demonstrating that it is an active mechanism (37). Overall, there are clear similarities between the results obtained when tolerance is induced by multiple Ag challenges or when mice are treated with Tr1 clones, suggesting that tolerance induction in these models operates through the differentiation of Tr1-type response.

In summary, the data presented above demonstrate that Tr1 clones actively modulate a Th2-type response in vitro through the secretion of IL-10, thus strengthening the role of IL-10 as a general immunomodulator of immune responses. Improved knowledge of the differentiation mechanisms and effector function of Tr1 cells should provide a crucial insight into their role in the allergic response in vivo and help us to better understand the dysregulation of the immune response resulting in allergic disorders.

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