

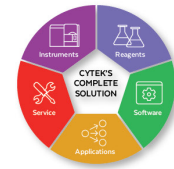


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Immune Regulation by Self-Reactive T Cells is Antigen Specific¹

Corinne Tanchot, Florence Vasseur, Christiane Pontoux, Corinne Garcia, and Adelaida Sarukhan²

Immune regulation plays an important role in the establishment and maintenance of self-tolerance. Nevertheless, it has been difficult to conclude whether regulation is Ag specific because studies have focused on polyclonal populations of regulatory T cells. We have used in this study a murine transgenic model that generates self-reactive, regulatory T cells of known Ag specificity to determine their capacity to suppress naive T cells specific for other Ags. We show that these regulatory cells can regulate the responses of naive T cells with the same TCR specificity, but do not inhibit T cell proliferation or differentiation of naive T cells specific for other Ags. These results demonstrate that immune regulation may be more Ag specific than previously proposed. *The Journal of Immunology*, 2004, 172: 4285–4291.

A variety of naturally occurring T cells with regulatory properties has been described and characterized in the recent literature. Among cells that have been reported to regulate autoimmunity in different disease models are CD4⁺CD45Rb^{low} cells, NK T cells, CD62L low cells, and $\gamma\delta$ T cells (reviewed in Ref. 1). The most studied of these cells are CD4⁺ T cells expressing high levels of the IL-2R (CD25⁺), although this population surely comprises a heterogeneous population in terms of their functional capacities (1). It has been shown that, although the majority of CD4⁺CD25⁺ T cells are generated in the thymus, T cells with regulatory capacities may be generated or experimentally induced in the periphery (2–4). In vitro, CD4⁺ T cells stimulated by their cognate Ag in the presence of IL-10 can also generate T cells with regulatory properties (5). Although some progress has been made concerning the ontogeny, phenotype, and gene expression profile of these different regulatory T cell populations, much uncertainty remains regarding their mode of action. This is in great part due to the fact that the Ag specificity of these cells is not known. Thus, an open question is whether these different cells only regulate immune responses toward the ligand they were selected on or whether they may regulate responses toward other epitopes in a bystander manner. In certain experimental models using T regulatory 1 cell clones (5) or Th3 cells induced by oral feeding of Ag (6), there has been evidence of bystander suppression in which cells specific for one Ag could suppress responses toward other unrelated Ags. In the case of CD25⁺ T cells, early in vivo experiments suggested a role for Ag specificity in immune regulation (7, 8). For example, peripheral T cells from athyroid rats were selectively unable to prevent thyroid-

specific autoimmune disease while retaining their capacity to prevent autoimmune diabetes (9). On the contrary, there has been some indication for non-Ag-specific regulation in vitro, using transgenic TCR mice (10, 11). However, these experiments were not conclusive because the regulatory and naive populations were not monospecific due to possible endogenous TCR rearrangements and dual TCR expression. Therefore, the models described at present in the literature do not allow direct and conclusive assessment of the Ag specificity of regulatory T cells.

In the present study, we have exploited a double-transgenic model that is unique in that it generates a peripheral population of self-reactive hemagglutinin (HA)³-specific T cells that have been shown to regulate, both in vivo and in vitro, immune responses by naive T cells expressing the same TCR (4, 12). Such regulatory T cells derived from TCR-HA \times IG-HA double-transgenic mice share several common features with other regulatory T cells described in the literature: they are anergic in terms of proliferative capacity, they secrete IL-10 (13), and they express high levels of CTLA4 and PD1 molecules (14) as well as glucocorticoid-induced TNF mRNA (A. Sarukhan, unpublished results).

Using these regulatory cells of known Ag specificity, we have developed a model that allows us for the first time to determine whether regulatory T cells with known Ag specificity are capable of mediating bystander suppression or whether their regulatory effect is Ag specific.

Materials and Methods

Mice

TCR-HA transgenic mice expressing a TCR $\alpha\beta$ specific for peptide 111–119 from influenza HA presented by I-E^d have been previously described (15) and are on the BALB/c background. For some experiments, TCR-HA mice expressing the Thy-1.1 allele were used, and in other experiments, Thy-1.2 TCR-HA mice deficient for the recombination-activating gene (RAG) were used. Similar results were obtained with RAG-deficient and wild-type TCR-HA transgenic mice. The 5CC7 mice express a TCR $\alpha\beta$ specific for peptide 81–104, pigeon cytochrome *c* (PCC), presented by I-E^k, and are on a RAG-deficient, B10.A background (16). TCR-LACK mice, kindly provided by N. Glaichenhaus, express a TCR $\alpha\beta$ specific for

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³ Abbreviations used in this paper: HA, hemagglutinin; BrdU, 5-bromo-2'-deoxyuridine; LACK, *Leishmania major* Ag; PCC, pigeon cytochrome *c*; RAG, recombination-activating gene.

peptide 156–173 of the immunodominant *Leishmania major* Ag, LACK, presented by I-A^d (17), and are on a BALB/c background. IG-HA mice, expressing the HA transgene under control of the Ig κ promoter and enhancer elements (18), were crossed with TCR-HA mice to generate TCR-HA \times IG-HA double-transgenic mice. Offspring were typed by PCR. (B10.A \times B10.D2)F₁ mice were used both as dendritic cell donors and recipients for the *in vivo* adoptive transfer experiments. B10.D2 mice were used instead of BALB/c to keep the H-2^d, but eliminate the Mtv-6 endogenous provirus that stimulates and deletes V β 3-positive cells. All mice were bred in our animal facilities in accordance with institutional guidelines.

Antibodies

The 6.5 Ab that recognizes the transgenic TCR-HA was produced in our laboratory and was used coupled to biotin, PE, or FITC. All other Abs were purchased from BD PharMingen (San Diego, CA).

Cell isolation

Naive CD4⁺ T cells were obtained from the lymph nodes of TCR-HA RAG^{-/-} or TCR-PCC RAG^{-/-} transgenic mice. For the naive TCR-HA cells expressing Thy-1.1, total lymph node cells from TCR-HA Thy-1.1 RAG-competent mice were first depleted of CD8⁺, CD19⁺, and CD11b⁺ cells using a mixture of mAbs and anti-rat Dynabeads (DynaL Biotech, Great Neck, NY) and then positively selected for expression of the transgenic TCR by magnetic sorting. For this, they were incubated with the biotinylated 6.5 mAb, washed, incubated with streptavidin-MACS microbeads (Miltenyi Biotec, Auburn, CA), and positively selected, according to manufacturer's instructions, with a purity always above 95%. For the naive LACK-specific T cells, total lymph node cells from TCR-LACK RAG-competent mice were depleted of CD8⁺, CD19⁺, and CD11b⁺ cells using a mixture of mAbs and anti-rat Dynabeads (DynaL Biotech).

Regulatory HA-specific T cells were obtained from the pooled spleens of 3- to 6-mo-old TCR-HA \times IG-HA mice. After magnetic sorting with the 6.5 mAb, cells were stained with CD4 CyChrome, 6.5 PE and sorted on a FACS Vantage (BD PharMingen) to eliminate the contaminating CD4⁺8⁻ cells expressing the transgenic TCR and to select cells expressing high levels of the transgenic TCR. In some cases, cells were stained with CD4 CyChrome, 6.5 FITC, and CD25 PE, and CD4⁺6.5⁺ cells were sorted in CD25⁻ and CD25⁺ populations. The percentage of CD25⁺ cells was ~5–8%. The purity after sorting was above 98%.

Dendritic cells were obtained from the spleen of BALB/c or (B10A \times B10.D2)F₁ mice after collagenase D digestion and magnetic sorting with CD11c microbeads (Miltenyi Biotec), according to manufacturer's instructions.

In vitro regulation assays

All assays were performed in complete IMDM, supplemented with 10% FCS. A total of 2×10^4 naive T cells was incubated with total irradiated splenocytes (5×10^5) or splenic-derived dendritic cells (5×10^4). In all experiments, APC were incubated with equimolar concentrations of both HA and PCC peptides, unless otherwise indicated. In some wells, FACS-sorted CD4⁺6.5⁺ regulatory cells were added at a 1:1, 1:2, or 1:5 ratio, as indicated. After 48 h of culture, supernatants were taken for cytokine measurement and thymidine was added for another 16-h pulse. In some cases, 5-bromo-2'-deoxyuridine (BrdU) and thymidine were added after 40-h culture and left for an additional 4-h period. BrdU incorporation was determined by flow cytometry, using BrdU FITC mAbs.

In vivo regulation assays

Splenic-derived DC were loaded *in vitro* for 1–2 h with no peptide or with 25 μ M concentration of HA and PCC or HA and LACK peptides. After washing, $2\text{--}3 \times 10^5$ dendritic cells were coinjected with 5×10^5 naive T cells that had been previously labeled with CFSE, as described (19). Injections were performed intrasplenically, in the absence or presence of 1×10^6 sorted CD4⁺6.5⁺ regulatory cells, as described (4). Recipient mice were sacrificed 4 days after transfer, and cells were analyzed by flow cytometry. HA-specific naive cells were detected by Thy-1.1, 6.5, and CD4 Abs. PCC-specific cells were detected by a combination of V β 3 and V α 11 Abs. When testing LACK-specific naive cells, recipients were Thy-1.1 BALB/c and transferred T cells were detected using the Thy-1.2 Ab.

Intracellular cytokine stainings

Naive T cells were stimulated *in vivo* for 4 days according to the protocol described above. Total splenocytes were recovered, depleted of CD19⁺

and CD8⁺ cells, and stimulated for 4 h with PMA (50 ng/ml), ionomycin (500 nM), and brefeldin A. Cells were then stained for surface markers (CD4, Thy-1.1, and 6.5, or CD4, V α 11, and V β 3) and then fixed, permeabilized, and incubated with IL-2 PE or IFN- γ PE Abs.

Results

*HA-specific regulatory T cells do not suppress *in vitro* proliferation of naive cells specific for another epitope*

We have used double-transgenic mice expressing a HA-specific TCR $\alpha\beta$ and the complete coding sequence of the HA protein under control of the Ig κ L chain promoter and enhancer. These mice express HA on B cells, but also on thymic epithelium and on dendritic cells. We have previously reported that CD4⁺ T cells expressing high levels of the transgenic (6.5) receptor accumulate in the periphery of TCR-HA \times IG-HA mice and are capable of suppressing proliferative responses of naive CD4⁺ T cells of the same Ag specificity, both *in vitro* and *in vivo* (4, 12). To determine whether such regulation is Ag specific or whether such HA-specific regulatory T cells are capable of regulating proliferative responses toward other Ags (i.e., bystander suppression), we developed the following system. As a source of HA-specific regulatory T cells, we used CD4⁺6.5⁺ T cells sorted from the spleen of TCR-HA \times IG-HA double-transgenic mice. As a source of naive CD4⁺ T cells, we used T cells from a RAG^{-/-} TCR-HA mouse (naive HA) or T cells from a RAG^{-/-} TCR-PCC mouse (naive PCC), specific for PCC_{81–104} and restricted to I-E^k molecules. As APCs we used either a mixture of H-2^d and H-2^k splenocytes or splenocytes from (k \times d) F₁ mice. In some cases, spleen-derived dendritic cells were used and gave similar results. The naive HA- or PCC-specific T cells were stimulated by APC loaded with an equimolar mixture of both peptides. We previously verified that the HA-specific T cells do not respond to PCC peptide and vice versa. Furthermore, we found that both types of cells respond well to the peptide mixtures without competitively inhibiting each other's response (data not shown). Naive HA or PCC T cells were stimulated in the absence or presence of HA-specific regulatory T cells. In some cases, Thy-1.1⁺ naive HA-specific T cells from RAG-competent mice were used to differentiate them by flow cytometry from the Thy-1.2⁺ HA reg cells, which express the same TCR. Such cells were sorted according to 6.5^{high} expression and gave similar results than T cells from RAG^{-/-} TCR-HA mice.

As previously reported (4) and as can be seen in Fig. 1A, HA reg cells inhibited very efficiently the thymidine incorporation of naive HA T cells at a naive:reg ratio of 1:1 or 1:5. Accordingly, the percentage of BrdU-positive naive HA-specific cells decreased significantly in the presence of HA reg cells. As expected, BrdU incorporation of HA reg cells was very low, because these cells proliferate poorly upon antigenic stimulation *in vitro*. In contrast, as can be seen in Fig. 1B, the proliferative response of naive PCC-specific T cells incubated in the presence of HA reg cells was not suppressed, despite the fact that the HA reg cells were being activated at the same time with their cognate peptide. These results were obtained even when favoring presentation of both HA and PCC peptides by the same APC by using (k \times d)F₁ APCs, as shown in Fig. 1B. All experiments shown hereafter were performed with (k \times d)F₁ APCs. Surprisingly, not only was no regulation of the PCC-specific naive T cells observed, but the total thymidine incorporation was significantly higher in the presence of HA reg cells. To determine whether this increase in thymidine incorporation was due to a greater proliferation of the naive PCC-specific T cells or to an increased proliferative response of the HA reg cells, we measured BrdU incorporation by flow cytometry, which permitted us to distinguish naive from HA reg cells by V β 3, V α 11 expression. As can be seen in Fig. 1B (right graph), the percentage

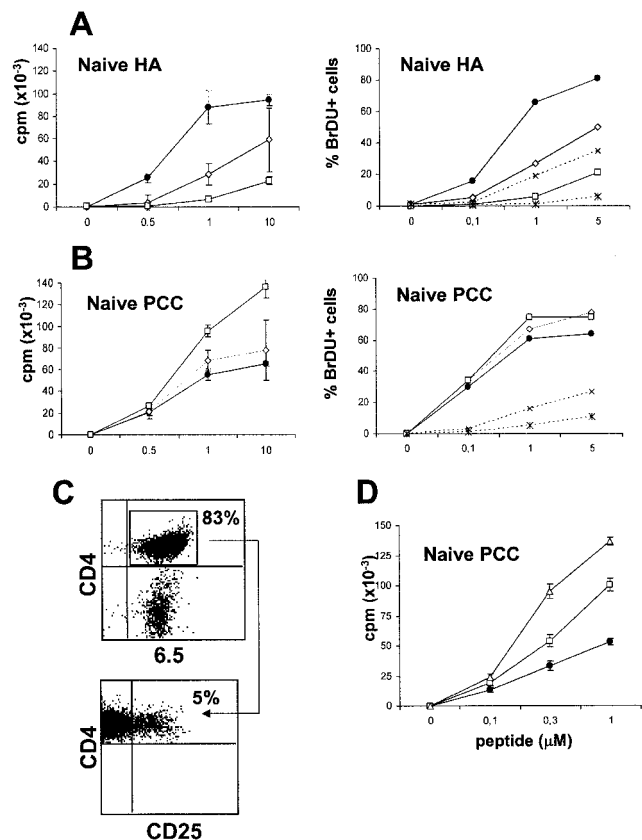


FIGURE 1. In vitro proliferation of naive T cells is regulated by CD4⁺ T cells specific for the same antigenic epitope. Naive CD4⁺6.5⁺ T cells from a RAG^{-/-} TCR-HA (A) or from a RAG^{-/-} TCR-PCC mouse (B) were stimulated in the absence or the presence of CD4⁺6.5⁺ T cells sorted from pooled spleens of TCR-HA × IG-HA mice (HA reg cells), at a naive: reg ratio of 1:1 or 1:5. APCs were (k × d)F₁ splenocytes (*left graphs*) or dendritic cells (*right graphs*) from a (B10D2 × B10A)F₁ mouse and were incubated with equimolar increasing concentrations of both HA and PCC peptides. In a parallel experiment, thymidine or BrdU was added for an additional 4 h after a 40-h culture. Thymidine (*left graphs*) and BrdU (*right graphs*) incorporation are shown for naive cells alone (●) or in the presence of HA reg T cells at a 1:1 or 1:5 ratio (◇ or □, respectively). Naive HA cells were distinguishable from the HA reg cells by Thy-1.1 expression, and naive PCC cells were distinguishable by Vβ3, Vα11 expression. BrdU incorporation was also determined for the HA reg cells and is shown in asterisks (1:1 ratio) or crosses (1:5 ratio). C, A low percentage of HA reg cells is CD25 positive. Splenocytes from TCR-HA × IG-HA mice were enriched for 6.5 by MACS and stained with CD4, 6.5, and CD25 Abs for FACS sorting. CD4⁺6.5^{high} cells were sorted according to their CD25 expression. Purity after sorting was above 98%. D, CD25⁺ HA reg cells do not function in a bystander manner. Naive PCC-specific CD4⁺ T cells were stimulated in absence (●) or presence of CD25⁺ (△) or CD25⁻ (□) HA reg cells at a ratio of 1:2, sorted according to C. APCs were (k × d)F₁ splenocytes. Results are the mean (+SD) cpm of triplicate cultures. Data in this figure represent one of two to three independent experiments with similar results.

of BrdU-positive naive PCC cells increased slightly in the presence of HA reg cells at the different ratios. However, the percentage of HA reg cells having incorporated BrdU was similar to that observed in Fig. 1A (*right graph*), arguing against the fact that the increase in total cpm was due to a significant reversal in the anergic phenotype of the HA reg cells. We did find, however, increased absolute numbers of HA reg cells recovered after incubation with PCC-specific T cells as compared with HA-specific T cells (data not shown). This, together with the slight increase in

BrdU incorporation by the PCC-specific T cells, would help explain the significant increase in total cpm that was reproducibly observed in all our in vitro experiments.

It has been reported that naive T cells can escape T reg-mediated suppression upon high antigenic doses and potent costimulation (20). To address the possibility that regulation would be observed upon high stimulation of HA reg cells and weaker stimulation of PCC-specific cells, we performed an experiment in which the HA peptide was maintained at high concentrations (5 μM), while the concentration of PCC peptide was decreased. No regulation was observed at any PCC peptide concentration ranging from 0.5 to 5 μM (data not shown).

HA-specific CD25⁺ regulatory T cells do not mediate bystander suppression

There exists compelling evidence that the CD25⁺ natural regulatory T cells are selected in the thymus (21, 22), and some in vitro experiments suggest that this population exerts regulation in a non-Ag-specific manner (11, 20). It has also been proposed that regulatory T cells generated in the thymus differ in their mode of action and specificity from those induced in the periphery (23). Furthermore, among T regulatory cells induced in periphery by nasal tolerization, CD25⁺ cells from tolerized mice could suppress CD4⁺ T cells in a nonspecific manner, while CD25⁻ acted in an Ag-specific fashion (24). In the TCR-HA × IG-HA double-transgenic model, CD25⁺ cells expressing high levels of the 6.5 TCR and with regulatory function were shown to be generated mainly in the thymus, where HA is expressed by thymic epithelial cells. On the contrary, a majority of CD25⁻6.5⁺ T cells were generated upon peripheral encounter with HA expressed on hemopoietic cells, but both subsets were capable of regulating HA-specific responses (4). We thus explored the possibility that the CD25⁻ and CD25⁺ cells in the TCR-HA × IG-HA mice could represent two different lineages, the former being Ag specific and the latter regulating in a non-Ag-specific manner. By flow cytometry, we sorted CD25⁺ and CD25⁻ CD4⁺6.5⁺ cells from the TCR-HA × IG-HA mice and tested them separately for their capacity to regulate PCC-specific responses. The CD25⁺ population represents only ~5–10% of the total CD4⁺6.5⁺ population (Fig. 1C). As shown in Fig. 1D, neither the CD25⁺ nor CD25⁻ TCR⁺IG⁺ cells were capable of regulating PCC-specific responses, and the presence of CD25⁺ cells resulted in even higher proliferative responses as compared with naive cells alone or in the presence of the CD25⁻ subpopulation. Both populations were capable of regulating responses by naive HA-specific T cells (data not shown), as previously reported (4). These results strongly suggest that, in our system, thymus-derived CD25⁺ cells expressing the self-reactive TCR and exerting regulatory functions are not capable of mediating bystander suppression in vitro, at least in terms of proliferative response.

HA-specific regulatory T cells do not suppress in vivo proliferation of naive cells specific for another epitope

Results obtained in vitro are to be taken with caution because they may be a poor reflection of what actually occurs in vivo (25). To study regulatory function in vivo, we performed adoptive transfers into immunocompetent hosts. Purified HA- or PCC-specific naive cells were labeled with CFSE and were coinjected with 2–3 × 10⁵ dendritic cells loaded with the corresponding peptides in the absence or presence of HA reg cells at a 1:2 ratio. Transferred dendritic cells were obtained from (k × d)F₁ mice to favor presentation of both peptides by the same APC. (B10.D2 × B10.A)F₁ mice instead of (BALB/c × B10.A)F₁ mice were used as recipients and dendritic cell donors to maintain the H-2^d, but avoid activation of

the $V\beta 3^+$, PCC-specific T cells by the Mtv-6 endogenous provirus present in BALB/c mice. Naive HA-specific T cells and PCC-specific T cells were distinguishable by expression of Thy-1.1 and of $V\beta 3$, $V\alpha 11$, respectively. Finally, to ensure that the appropriate ratio of naive and regulatory cells was maintained upon transfer, we performed intrasplenic injections, as described previously (4). Spleen cells from recipient mice were analyzed 4 days after transfer. Controls using DC not loaded with peptide were performed, and no CFSE loss was observed (data not shown). HA-specific naive T cells divided extensively in the presence of peptide-loaded DC. In contrast, when HA reg cells were coinjected, naive HA cell division was considerably abrogated (Fig. 2A). On the contrary, the PCC-specific naive T cells divided extensively in the absence and in the presence of the HA reg cells. Furthermore, we found no significant difference caused by the presence of HA reg cells in absolute numbers of naive PCC T cells recovered from the spleen 4 days after transfer (Fig. 2B).

The results obtained *in vitro* and *in vivo* with the PCC-specific T cells indicate that the HA reg cells are only capable of regulating proliferative responses to the same antigenic epitope, but not responses toward other Ags. However, to exclude that the absence of regulation was linked to this particular TCR specificity, we performed similar experiments this time using naive T cells transgenic for a TCR $\alpha\beta$ specific for LACK and restricted to I-A^d molecules (17). For the *in vitro* experiments, we used BALB/c splenocytes as APCs, incubated with equimolar concentrations of both HA and

LACK peptides. As shown in Fig. 3A, no regulation of the LACK-specific T cell proliferative response was observed (*right graph*), while, as expected, the different ratios of HA reg cells efficiently regulated proliferation of naive HA-specific T cells (*left graph*). Once again, as observed with the PCC-specific cells, there was an increase of total thymidine incorporation when the LACK-specific T cells were incubated in the presence of the HA reg cells. We sometimes observed that, at high peptide doses, the HA-specific regulation was lost, as can be seen in Fig. 3A (*left graph*). This loss of regulation upon high antigenic doses or strong stimulation confirms other data obtained *in vitro* (20).

For the adoptive transfer experiments, we intrasplenically injected HA-specific or LACK-specific Thy-1.2 naive cells into BALB/c Thy-1.1 recipients, together with BALB/c spleen-derived DC loaded with both peptides and in the absence or presence of HA reg cells at a 1:2 naive:reg ratio. Analysis of recipient mice 4 days after transfer showed regulation of HA-specific, but not LACK-specific T cell division in presence of HA reg cells (Fig. 3B). Altogether, these experiments confirm that the regulatory T cells generated in the TCR-HA \times IG-HA mice can only regulate proliferative responses of naive T cells specific for the same epitope.

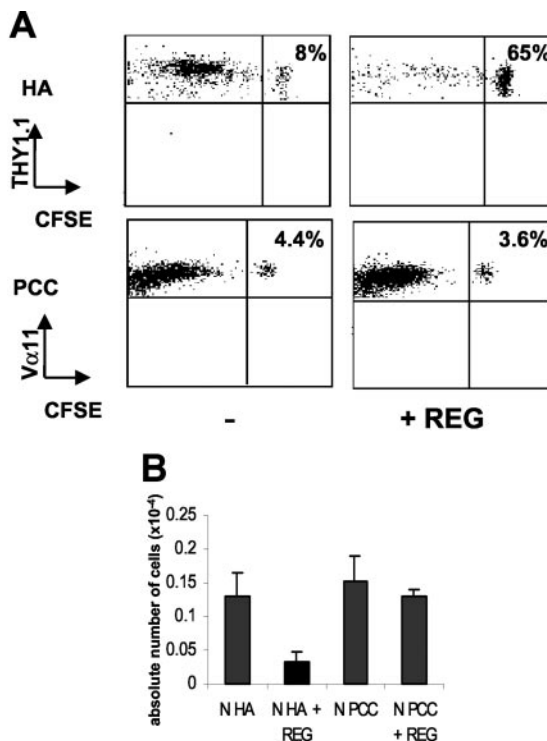


FIGURE 2. *In vivo* proliferation and expansion of PCC-specific CD4⁺ T cells are not suppressed by the presence of HA reg T cells. A total of 5×10^5 naive HA- or PCC-specific CFSE-labeled cells and $2-3 \times 10^5$ dendritic cells pulsed with both HA and PCC peptides was injected intrasplenically into (B10A \times B10.D2)F₁ recipients. Some of the recipients also received 1×10^6 sorted HA reg cells. Total splenocytes from recipient mice were analyzed 4 days after transfer. *A*, CFSE profiles for the gated naive cells represent one of three individual mice per group. *B*, Absolute numbers of naive cells recovered in the spleen 4 days after transfer in absence or presence of the HA reg T cells were calculated. Three independent experiments gave similar results.

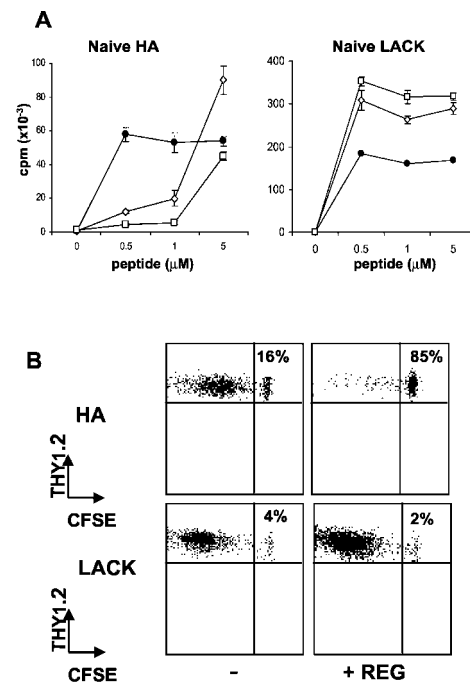
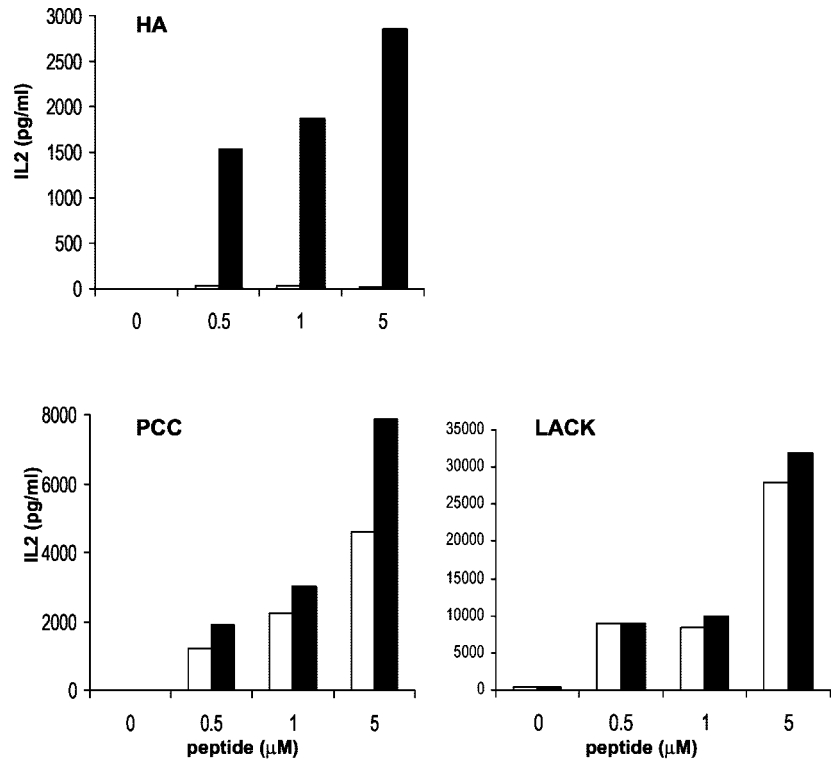


FIGURE 3. *A*, LACK-specific T cell proliferation *in vitro* is not suppressed in the presence of HA reg cells. Lymph node cells from a TCR-LACK transgenic mouse were depleted of CD8⁺, CD19⁺, and Mac-1 cells (99% purity). *In vitro* stimulation was done, as described above, using BALB/c splenocytes as APCs, incubated with equimolar increasing doses of both HA and LACK peptides. Results are the mean (\pm SD) cpm of triplicate cultures. *B*, *In vivo* expansion of naive LACK-specific CD4⁺ cells is not modified by the presence of HA reg cells. A total of 5×10^5 naive HA-specific or LACK-specific T cells (Thy-1.2) labeled with CFSE and $2-3 \times 10^5$ peptide-pulsed dendritic cells was injected intrasplenically in the absence or presence of 1×10^6 HA reg-sorted T cells (Thy-1.1) into Thy-1.1 BALB/c recipients. Spleen and lymph nodes of the recipient mice were collected and analyzed 4 days after transfer. Stainings with Thy-1.2, CD4, 6.5, and $V\beta 4$ Abs were performed. A representative CFSE profile is shown, corresponding to one mouse per group. Two independent experiments with two to three mice per group were performed, with similar results.

FIGURE 4. IL-2 production by naive PCC or LACK-specific CD4⁺ T cells is not significantly decreased by the presence of HA reg cells. Naive HA-specific, PCC-specific, or LACK-specific T cells were stimulated by increasing doses of HA and PCC peptides in absence (■) or presence (□) of HA reg cells. IL-2 was measured by ELISA from the pooled supernatant of a triplicate 48-h in vitro culture. Data shown are representative of two independent experiments.



Inhibition of cytokine production in vitro and in vivo is Ag specific

Although the HA-specific regulatory T cells exerted no effect on the proliferative capacities of naive T cells of different Ag specificity, there remained the possibility of an effect on their differentiation. Thus, we studied the effect of regulatory T cells on the capacity of naive T cells to secrete IL-2.

In vitro, the amount of IL-2 present in the culture supernatants was drastically decreased when the naive HA-specific T cells were incubated in the presence of HA reg cells. In contrast, no significant decrease of IL-2 was observed in the supernatant of naive PCC-specific or LACK-specific T cells incubated with HA reg cells, at any peptide dose (Fig. 4).

To determine whether these results were confirmed upon in vivo activation of the naive T cells, we performed adoptive transfers, as described above, recovered total splenocytes 4 days after transfer, and incubated them with PMA/ionomycin in vitro for 4 h, in the presence of brefeldin A. As can be seen in Fig. 5, the percentage of IL-2- or IFN- γ -producing Thy-1.1 HA-specific cells decreased in the presence of HA reg cells, while the percentage of V β 3, V α 11 PCC-specific T cells producing IL-2 or IFN- γ did not decrease in the presence of HA reg cells.

Overall, these results indicate that HA-specific regulatory T cells do not significantly regulate, in vitro or in vivo, differentiation of naive T cells into effector Th cells that are specific for another antigenic epitope.

Discussion

Naturally occurring T cells with regulatory function have been described in the recent literature and have aroused high expectations for their possible use in the control of autoimmune pathologies and allergies. It is now a widely accepted fact that immune regulation exists and that it plays an important role in keeping potentially autoreactive cells at bay. Nevertheless, it is still not certain whether there are special lineages committed to regulation

or whether it is a function that many T cells can perform, depending on yet undefined parameters such as their activation state and competition for survival signals (26). In any case, to efficiently manipulate T cells with regulatory capacities, it is important to determine their mode of action and their capacity to mediate bystander suppression. In the case of physiologically generated regulatory T cells, this has been a difficult task because the Ag specificity of these cells is not known.

In this study, we used HA-specific regulatory T cells that are generated in vivo and that were previously characterized in detail to address the question of the specificity of immune regulation.

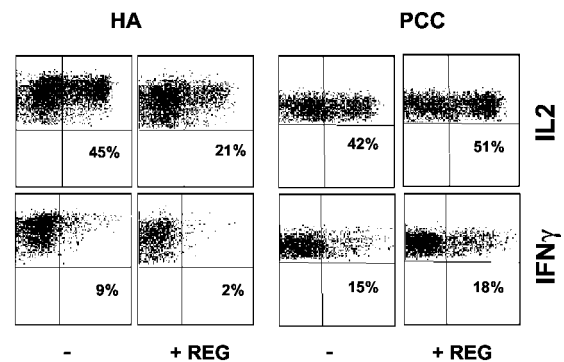


FIGURE 5. In vivo differentiation of naive PCC-specific T cells is not inhibited by HA reg cells. Intracellular production of IL-2 and IFN- γ by HA-specific or PCC-specific CD4⁺ T cells upon in vivo stimulation with peptide-pulsed dendritic cells in the absence or presence of HA reg cells was measured. Adoptive transfers were performed, as described above. Recipient mice were sacrificed 4 days after transfer. Total splenocytes corresponding to three mice per group were pooled, enriched for CD4⁺ T cells, and incubated for 4 h in presence of PMA/ionomycin and brefeldin A. Cells were stained with CD4 and Thy-1.1 or V β 3 Abs, and intracellular staining for cytokines was performed, as described in *Materials and Methods*. Two independent experiments were performed with similar results.

Our results obtained both in vitro and in vivo indicate that regulatory T cells specific for an HA epitope can efficiently regulate HA-specific responses in terms of proliferation and cytokine production, but cannot significantly regulate responses of naive T cells specific either for PCC or for LACK epitopes.

In accordance, some early experiments performed in vivo suggest that Ag specificity plays a role in immune regulation. Different groups have provided strong evidence that the CD25⁺CD4⁺ T cells are positively selected in the thymus by self ligands (22, 27–29). As such, it would be reasonable to consider that they will regulate immune responses toward such ligands in the periphery. In fact, it has been shown that T regulatory cells that prevent autoimmunity are generated in vivo only when the relevant self Ag is present, suggesting that the peripheral self Ag itself stimulates the generation of appropriate regulatory cells from emigrant thymic precursors. For example, CD4⁺ T cells from mice lacking the relevant organs are less effective in preventing disease development in those organs (7, 8), and peripheral T cells from athyroid rats were selectively unable to prevent thyroid-specific autoimmune disease, while retaining their capacity to prevent autoimmune diabetes (9).

Nevertheless, our results are in apparent contradiction with other reports addressing bystander suppression by natural CD25⁺CD4⁺ regulatory T cells in vitro. In these studies, CD25⁺ and CD25⁻ from TCR transgenic mice were shown to be capable of suppressing proliferative responses toward other antigenic epitopes in vitro (10, 11, 20, 30). One explanation for such a discrepancy could be that those experiments were performed using regulatory CD25⁺CD4⁺ and naive CD25⁻CD4⁺ T cells from TCR transgenic mice that were not on a RAG-deficient background. Furthermore, the cells were not selected for high levels of TCR transgenic expression. Thus, the authors could not rule out that among the CD25⁺CD4⁺ cells supposedly specific for one of the peptides, there were not T cells specific for the other peptide, and vice versa. This is especially true because it has been shown that the generation of CD25⁺CD4⁺ regulatory T cells in TCR transgenic mice depends on the rearrangement of endogenous TCRs (31). Thus, the great majority of CD25⁺CD4⁺ T cells may have more than one Ag specificity, although in one of the reports regulation was clearly dependent on the concentration of the peptide recognized by the regulatory T cells (10).

Another explanation for such apparent discrepancy could be that the regulatory cells used in the present study, including the CD25⁺ fraction, are different from the natural CD4⁺CD25⁺ T cells. In fact, it has been shown that it is possible to induce CD4⁺CD25⁺ regulatory T cells in mice that are deficient for the natural CD4⁺CD25⁺ T cells. Furthermore, they seem to differ from the latter in terms of modes of action and cytokine dependency (32).

What we call regulatory T cells very probably represent a large variety of heterogenous cells that have different phenotypic markers, that are generated in different ways, and that may exert their function by different mechanisms. It is very probable that the different cell subsets of regulatory cells may vary in terms of specificity and mechanisms. For example, it has been shown that in vivo stimulated autoreactive CD4⁺ T cells can protect from autoimmune diabetes via bystander suppression and in an apparently cytokine-dependent manner (33).

In our model, we show that regulation of T cell proliferation and differentiation is Ag specific. This does not close the door to protocols aiming at controlling autoimmune diseases via bystander suppression, but it is clear that further studies on the phenotype and mode of action of the different T cells with regulatory capacities are necessary to successfully manipulate such cells for therapeutic purposes. The results obtained in this

study underline the complexity of regulation, while demonstrating that immune regulation may be more Ag specific than previously proposed.

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