Foxp3\(^+\)CD25\(^+\) T Regulatory Cells Stimulate IFN-\(\gamma\)-Independent CD152-Mediated Activation of Tryptophan Catabolism That Provides Dendritic Cells with Immune Regulatory Activity in Mice Unresponsive to Staphylococcal Enterotoxin B

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*J Immunol* 2007; 179:910-917; 
doi: 10.4049/jimmunol.179.2.910

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Foxp3⁺CD25⁺ T Regulatory Cells Stimulate IFN-γ-Independent CD152-Mediated Activation of Tryptophan Catabolism That Provides Dendritic Cells with Immune Regulatory Activity in Mice Unresponsive to Staphylococcal Enterotoxin B¹

Pascal Feunou,* Sophie Vanwewinkel,* Florence Gaudray,* Michel Goldman,* Patrick Matthys, † and Michel Y. Braun²*‡

Mice made unresponsive by repeated injection of staphylococcal enterotoxin B (SEB) contained SEB-specific CD25⁺CD4⁺TCRBV8⁺ T cells that were able to transfer their state of unresponsiveness to primary-stimulated T cells. About one-half of these cells stably up-regulated the expression of CD152. We undertook the present study to determine whether CD152high cells seen in this system were T regulatory cells responsible for suppression or whether they represented SEB-activated CD4⁺ T effector cells. Our results show that, among SEB-specific TCRBV8⁺ T cells isolated from unresponsive mice, all CD152highCD25⁺CD4⁺ T cells expressed Foxp3, the NF required for differentiation and function of natural T regulatory cells. Moreover, suppression by CD25⁺CD4⁺TCRBV8⁺ T cells was fully inhibited by anti-CD152 Abs. Following stimulation by soluble CD152-Ig, dendritic cells (DC) isolated from unresponsive mice strongly increased the expression and the function of indoleamine-2,3-dioxygenase (IDO), the enzyme responsible for the catabolism of tryptophan. This capacity to activate IDO was independent of IFN-γ production by DC because CD152-Ig stimulation of DC isolated from SEB-treated IFN-γ-deficient animals activated IDO expression and function. Finally, adding 1-methyl-tryptophan, an inhibitor of tryptophan catabolism, increased substantially the capacity of DC from unresponsive animals to stimulate primary T cell response toward SEB. Thus, we conclude that IFN-γ-independent CD152-mediated activation of tryptophan catabolism by Foxp3⁺CD25⁺ T regulatory cells provides DC with immune regulatory activity in mice unresponsive to SEB. The Journal of Immunology, 2007, 179: 910–917.

B ased on their origin, two types of CD4⁺ T regulatory cells have been described, some of them are induced in response to antigenic challenge, whereas others are considered natural regulators (1). Inducible regulatory T cells can develop from conventional CD4⁺ T cells that are exposed to specific stimulatory conditions, such as altered activation signals or in the presence of modulating cytokines. Natural T regulatory cells, however, are selected by self-peptides in the thymus and survive in the periphery as regulatory cells. The unique transcription factor Foxp3 represents the most specific marker of natural regulatory cells identified so far and is required for their generation (2–4). Inducible or natural regulatory cells can also be distinguished depending on the way they modulate T cell responses. Whereas inducible CD4⁺ T cell-mediated immune regulation occurs preferentially through the release of soluble factors, such as the suppressive cytokines IL-10 and TGFβ, cell contact appears to be required for the suppressive function of natural T regulatory cells (5–9). In particular, accessory molecules such as CD152 and glucocorticoid-induced TNFR expressed at the surface of natural T regulatory cells have been directly implicated in vitro in their suppressive function (10–13).

Indoleamine 2,3-dioxygenase (IDO)³ is a tryptophan-catabolizing enzyme considered to play a role in T cell tolerance (14). Tryptophan depletion or the presence of tryptophan catabolites in the extracellular environment appear to arrest activated T lymphocytes in the G1 phase, thereby promoting tolerance (15, 16). Direct evidence has emerged that CD152-positive T regulatory cells could represent a key cell population responsible for activating IDO in B7-expressing cells, including dendritic cells (DC) (17–19). Recent studies, however, suggest that IDO expression by DC contribute little to the regulation of T cell responses. Indeed, Terness et al. (20) showed that human DC do not constitutively express IDO and, even after induction of expression by IFN-γ treatment, possess only limited immunoregulatory function. Thus, the precise role played by IDO in mediating the suppressive activity of T regulatory cells has yet to be defined.

We have recently shown that the spleen of BALB/c mice tolerant to staphylococcal enterotoxin B (SEB) contains SEB-specific CD4⁺TCRBV8⁺ T cells exerting an immunoregulatory function on SEB-specific primary T cell responses (21, 22). Two types of suppressor cell populations were identified in this system. Whereas CD4⁺CD25⁺ T regulatory cells were required for the induction of tolerance, CD4⁺CD25⁻ T cells exerted their regulatory activity at

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Received for publication September 20, 2005. Accepted for publication May 4, 2007.

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¹ This work was supported by the Télévie and the Fonds National pour la Recherche Scientifique of Belgium.

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³ Abbreviations used in this paper: IDO, indoleamine 2,3-dioxygenase; DC, dendritic cell; SEB, staphylococcal enterotoxin B; 1-MT, 1-methyl-tryptophan; SE, staphylococcal enterotoxin; Dn, division number.

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the maintenance stage of specific unresponsiveness. Interestingly, in tolerant animals, most of CD4⁺CD25⁺ TCRBV8⁺ T regulatory cells strongly and specifically up-regulated their expression of CD152. In this study, we considered the possibility that repeated TCR-mediated stimulation of CD4⁺CD25⁺ TCRBV8⁺ T regulatory cells could induce CD152-dependent regulatory activity in DC, making them tolerogenic for primary T cell responses.

Materials and Methods

Animals and tolerizing protocol

The protocols used in this study were reviewed and approved by the Committee of Ethics on Animal Experimentation of the Institute for Medical Immunology, and the experiments were conducted according to the Guidelines of the Administration de la Santé Animale and de la Qualité desProduitsAnimaux of the Ministère de l’Agriculture de Belgique.

Four- to 8-wk-old BALB/c female mice, bred and kept at the pathogen-free animal facilities of the Institute for Medical Immunology, were used in this study. For the experiments analyzing the role of IFN-γ in immune suppression, 8-wk-old IFN-γ−/− BALB/c mice (23) and wild-type controls, bred in the Rega Institute for Medical Research, were kept at the Institute for Medical Immunology quarantine facilities for the whole experimental procedure. Induction of unresponsiveness was performed by three i.p. injections of 10 µg of SEB (Toxin Technology) on days 0, 2, and 4 (21, 22).

Flow cytometry

Cells were immunostained with various combinations of fluorescence-conjugated Abs and analyzed by flow cytometry. Fluorochrome- and biotin-conjugated Abs were all obtained from BD Pharmingen (Erembodegem), except for the rat anti-mouse Foxp3 Ab that were purchased from eBioscience. Abs were ECD-conjugated rat anti-mouse CD4 (clone RM4-5), FITC-conjugated mouse anti-mouse TCRBV8 (clone P22H11), PE-conjugated hamster anti-mouse CD152 (clone UC16-4F10; cells 11), PE-conjugated rat anti-mouse CD255 (7D4), and allophycocyanin-conjugated rat anti-mouse Foxp3 (clone FJK-16s). Intracytoplasmic immunostaining for Foxp3 and CD152 was conducted according to the manufacturer’s protocol. Samples were analyzed on a Cyan ADP flow cytometer (DakoCytomation) using Summit 4.2 computer software.

For CFSE labeling, cells were incubated with CFSE (1 µM in PBS) for 10 min at 37°C. The reaction was then stopped by adding a large volume of FCS to the cell suspension. After centrifugation, the cells were plated in culture medium.

Purification of T cell and DC

Two days after the last SEB injection, spleens were removed and prepared into single-cell suspensions by gently crushing the organs without collagenase treatment. CD4⁺ T cells were isolated using magnetic separation (22). Briefly, cells were incubated with FITC-conjugated rat anti-mouse CD8 (clone 53-6.7; BD Pharmingen), FITC-conjugated rat anti-mouse B220 (clone RA3-6B2; BD Pharmingen), FITC-conjugated rat anti-CD11b (clone M1/70; BD Pharmingen), FITC-conjugated rat anti-mouse CD11c (clone HL3; BD Pharmingen), and FITC-conjugated mouse anti-mouse Ig (clone M5/114; BD Pharmingen) mAbs. Cells were then washed and incubated with anti-FITC microbeads (Miltenyi Biotec). Depletion of CD11b⁺, CD11c⁺, CD8⁺, Igα, and B220⁺ cells was conducted by magnetic separation with MACS columns according to the suggested protocol (Miltenyi Biotec). The CD4⁺ T cell-enriched population (>95%) always contained <0.3% CD11b⁺, CD11c⁺, CD8⁺, Igα, and B220⁺ cells as assessed by flow cytometry. For some experiments, CD4⁺ T cell-enriched spleen cells were separated according to their expression of CD25 (22). Cells were incubated with biotinylated rat anti-mouse CD25 mAbs (clone PC61; BD Pharmingen). They were washed, then incubated with streptavidin-conjugated microbeads, and magnetic separation was conducted on MACS columns (Miltenyi Biotec). The CD4⁺CD25⁺ T cells passed through the column. The retained cells were eluted as CD4⁺CD25⁻ T cells.

For purification of DC, spleens were prepared into single-cell suspensions. Cells were then immunomabeled with FITC-conjugated anti-mouse CD11c Abs. After washing, they were incubated with anti-FITC Ab-coupled microbeads (Miltenyi Biotec), and the bound cells were isolated using MACS columns (>95% CD11c⁺).

In vitro assays

Suppression was assayed on the capacity of normal spleen cells to proliferate in response to in vitro stimulation with SEB. Serial dilutions of spleen cells or purified subsets of T cells from mice made unresponsive to SEB were mixed with normal BALB/c spleen cells (1 × 10⁵/well) in U-shaped 96-well culture plates (final volume, 200 µl) and stimulated for 60 h with SEB (1 µg/ml). Proliferation for the last 16 h of culture was assessed using the [³H]TdR incorporation assay. In some experiments, the activity of IDO was inhibited by adding 1-methyl-tryptophan (1-MT; Sigma-Aldrich) to the cultures. The 1-MT stock solution was made in a 30 mM HCl solution.

Purified CD11c⁺ DC from normal or unresponsive mice were analyzed for their capacity to stimulate purified CD4⁺ T cells. Serial dilutions of isolated DC (1.5 × 10⁵–5 × 10⁵ cells/well) were mixed with 5 × 10⁴ purified CD4⁺ T cells. Cultures were stimulated with 1 µg/ml SEB for 60 h. Proliferation for the last 16 h of culture was assayed using the [³H]TdR incorporation assay. Culture supernatants were also analyzed for their content in IFN-γ and IL-10 by ELISA according to the manufacturer’s instructions (R&D Systems).

Western blotting

Purified CD11c⁺ DC were analyzed for their production of IDO. The cells (0.5 × 10⁶) were cultured overnight in the presence of recombinant mouse IgG1Fc fusion protein (30 µg/ml; R&D Systems) or control recombinant human IgG1Fc fusion protein (30 µg/ml; R&D Systems). In some experiments, the effect of IFN-γ on IDO expression was analyzed and rIFN-γ (100 ng/ml; R&D Systems) was added to DC cultures. Cell lysates were then prepared and subjected to immunoblot analyses according to standard procedures (24) using anti-human IDO mAbs (1/2000; Bioconnect) and anti-G3PDH mAbs (1/1000; Biodesign International). Transfected 293 cells expressing rat IDO (gift from Dr J. Anegon and M. Hill, Université de Nantes, Nantes, France) were used to generate positive control cell lysates.

HPLC

Tryptophan is catabolized by IDO to N-formylkynurenine which is rapidly converted to kynurenine (25). Measurement of kynurenine levels can provide evidence of functional IDO activity. Purified CD11c⁺ DC were stimulated for 17 h with soluble CD152-lg (30 µg/ml) and/or rIFN-γ (100 ng/ml; R&D Systems). Culture supernatants were harvested and kynurenine was quantified by HPLC. Briefly, 25 µl of the clarified sample was injected into a Waters C₁₈ monomeric column and eluted with KH₂PO₄ buffer (0.01 M KH₂PO₄ and 0.15 mM EDTA, pH 5.0) containing 10% methanol at a flow rate of 1.0 ml/min. The spectrophotometer was set at 254 nm. The retention time as well as standard concentration curves were previously determined with standard solutions.

Results

Suppression of primary T cell response by SEB-induced T regulatory cells depends on CD152 expression and the production of IL-10

Others and we (21, 22, 26) have previously shown that the repeated injection of staphylococcal enterotoxins (SE) in mice induced specific immune unresponsiveness among SE-reactive T cells. This state was characterized by the induction of CD4⁺ T regulatory cells that, once adoptively transferred in naive syngeneic recipients, mediated suppression of primary T cell response (21, 22). Suppression could also be observed in vitro, where spleen cells from unresponsive mice suppressed the primary proliferative response of SE-specific T cells (Ref. 22 and Fig. 1A). To investigate the molecular mechanism used by these T regulatory cells to modulate T cell activity, culture supernatants harvested from suppression assays were added to primary cultures of SEB-stimulated spleen cells. As shown in Fig. 1A, these were able to inhibit in a dose-dependent manner the proliferation of primary-stimulated spleen cells. This observation led us to postulate that soluble factors were involved in SEB-induced immune suppression. Among soluble immunoregulatory proteins, IL-10 and TGF-β have been often linked to T
cell-mediated suppression (5, 27). Adding anti-mouse IL-10-neutralizing Abs to our assay slightly inhibited the suppression mediated by unresponsive spleen cells (Fig. 1B). On the contrary, the presence of soluble TGF-βR-Ig alone or in combination with IL-10 neutralization had no or little effect on suppression (data not shown). An important cell surface protein in maintaining T cell tolerance is the CTLA-4 (CD152) (28). T regulatory cells express CD152 constitutively and CD152 has been reported to be important in maintaining regulatory cell function (10, 11). Moreover, several studies have reported that combining IL-10 neutralization and blockade of the interaction between CD152 and its ligands (CD80 and CD86) fully inhibited T regulatory cell-mediated suppression (29, 30). We therefore analyzed the effect of adding both anti-IL-10 mAbs and/or anti-CD152 mAbs in SEB-mediated suppression assays. As shown in Fig. 1B, blocking CD152/CD80–86 interactions partially restored the proliferative capacity of suppressed spleen cells. However, when combined with neutralizing anti-IL-10 Abs, the same treatment fully inhibited the suppression mediated by unresponsive spleen cells. Thus, immune suppression by T regulatory cells isolated from mice unresponsive to SEB is mediated through mechanisms that involve soluble IL-10 and membrane CD152. Interestingly, the levels of proliferation reached in the presence of anti-IL-10 and anti-CD152 mAbs were constantly higher than those produced in cultures containing responder cells alone (Fig. 1B), as if these two reagents were also able to inhibit spontaneous endogenous suppression mediated by cell population of normal spleen.

CD152highFoxp3+CD25+CD4+ T regulatory cells differentiate in the spleen of unresponsive mice

As mentioned before, we have previously shown that the spleen of BALB/c mice receiving repeated injections of SEB contained CD25+CD4+ T cells that were able to mediate suppression of primary T cell response (22). Interestingly, CD25highCD4+ T suppressor cells also contained a subpopulation of CD152high cells (22). This pattern of CD152 expression was restricted to SEB-specific CD4+ T cells because it was observed within cells displaying TCR bound by SEB, such as TCRVB8+ T cells, and not in TCRVB6+ T cells (22). CD25+CD4+ T cells and CD8+ T cells from SEB-treated mice did not contain CD152high cells. CD152highCD25+CD4+TCRBV8+ T cells were long-lived in SEB-treated mice because they were specifically detected for >10 days after the last injection of SEB (22). Because both CD25 and CD152 molecules are also expressed by recently activated T cells (31, 32), it was difficult to assess whether CD152highCD25+CD4+TCRBV8+ T cells present within the spleen of SEB-treated mice represented T regulatory cells responsible for suppression or were T effector cells activated by SEB. To address this question, we analyzed by flow cytometry the expression of Foxp3, the NF specifically expressed by T regulatory cells, in CD152highCD25+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3. Moreover, the majority of Foxp3+CD4+TCRBV8+ T cells present in the spleen of tolerant mice. As depicted in Fig. 2A, CD152high cells were the only cell subset among CD4+TCRBV8+ T cells to express Foxp3.
CD25⁺CD4⁺ T cells and CD25⁻CD4⁺ T cells could mediate immune suppression (22). Suppressive activity of CD25⁺CD4⁺ T cells was less inhibited by treatment with anti-CD152-blocking Abs (Fig. 2B). Adding neutralizing anti-IL-10 Abs, however, inhibited only the suppression mediated by CD25⁺CD4⁺ T cells (Fig. 2B). Thus, Foxp3⁺CD152⁺CD25⁺CD4⁺ T regulatory cells and Foxp3⁺CD152⁻CD25⁺CD4⁺ T regulatory cells can be segregated on the basis of their suppression pathways, the former

![FIGURE 2.](image)

DC isolated from unresponsive mice have reduced stimulatory capacities. DC isolated from naive or tolerant mice were used to stimulate SEB-specific responses of CD4⁺ T cells purified from naive or tolerant mice. Proliferation was assessed by [³H]TdR incorporation, whereas IFN-γ or IL-10 production within culture supernatants was quantified by specific ELISA. Data shown are means ± SD of culture triplicates. Results presented are representative of two independent experiments.

![FIGURE 3.](image)
CD11c donor to stimulate CD4 cell lines. However, the ability of DC isolated from unresponsive panels showed the production of kynurenine by DC isolated from IFN-γ primary response of CD4 cells (Fig. 5). Thus, DC isolated from SEB-treated animals appeared to have acquired regulatory function. Whereas both types of cell stimulated equally the production of regulatory factors such as IL-10.

**DC isolated from the spleen of unresponsive mice have acquired immunoregulatory capacities**

DC have been shown to acquire immunoregulatory function through CD152-dependent activation of tryptophan catabolism (17–19). Because suppression mediated by CD25+CD4+ T cells in our model depended on the interaction of CD152 with its ligands (CD80/CD86), we investigated whether DC from unresponsive mice could modulate primary T cell responses. This hypothesis was also supported by our preliminary observation that the spleen of unresponsive BALB/c mice contained relatively more CD8+CD11c+ DC (17 ± 1.2% among CD11c+ cells), known to exhibit immunoregulatory properties (19), than that of untreated animals (11.5 ± 0.9% among CD11c+ cells). Fig. 3 compares the primary response of CD4+ T cells stimulated by SEB in the presence of normal DC or DC isolated from unresponsive mice. Both types of DC stimulated CD4+ T cell proliferation and IFN-γ production. However, the ability of DC isolated from unresponsive donor to stimulate CD4+ T cells was half that of normal DC, whereas both types of cell stimulated equally the production of IL-10 (Fig. 3). Thus, DC isolated from SEB-treated animals appeared to have acquired regulatory function.

As mentioned before, one mechanism by which DC regulate T cell responses is through the expression of IDO that degrades the essential amino acid tryptophan. We therefore investigated whether the regulatory function of DC present in the spleen of unresponsive mice could be correlated with increased IDO activity. IDO activity can be triggered in DC by IFN-γ and/or by engagement of the membrane proteins CD80/CD86 by their ligand CD152 (17–19, 33, 34). Isolated DC from normal or unresponsive mice were cultured alone or stimulated with soluble rIFN-γ and/or CD152-Ig for 17 h and IDO expression within the cells was investigated by Western blotting. As shown in Fig. 4, weak IDO expression was detected in unstimulated DC. Incubating with rIFN-γ, marginally increased the expression of IDO in both cell types. Stimulating DC with soluble CD152-Ig, however, induced strong expression of IDO in both normal DC and DC from unresponsive mice (Fig. 4). On the contrary, IDO expression was not induced in isolated CD11c+ DC incubated with control recombinant human IgG1 Fc protein (data not shown). Because it has been reported that both CD80/CD86 expression and IFN-γ production by DC are required for inducing tryptophan catabolism by DC (17), we analyzed the effect of stimulating by both CD152-Ig and rIFN-γ on IDO expression in isolated DC. As depicted in Fig. 4, applying both stimuli on normal DC or on unresponsive animal-derived DC did not modify its expression.

One intriguing observation was that, in opposition to what Falzarino and colleagues (17) had previously reported, IFN-γ could not be detected by ELISA in cultures of DC following stimulation with soluble CD152-Ig (data not shown). Thus, in our hands, stimulation of SEB-treated DC with CD152-Ig appeared to induce IDO expression in DC does not require IFN-γ secretion. Analysis of IDO (upper photograph) and NAPDH (lower photograph) expression by Western blotting in splenic CD11c+ DC isolated from normal (A) or IFN-γ-deficient (B) BALB/c mice cultured overnight alone (lane 1), with IFN-γ (lane 2), soluble CD152-Ig (lane 3), or both IFN-γ and CD152-Ig (lane 4), and spleen CD11c+ DC isolated from SEB-treated BALB/c mice (lanes 5–8) cultured overnight alone (lane 5), with IFN-γ (lane 6), soluble CD152-Ig (lane 7), or with both IFN-γ and CD152-Ig (lane 8). Results presented are representative of three independent experiments. IDO/GAPDH expression ratios were calculated using a Gel Doc EQ camera and Quantity One computer software (Bio-Rad) for the measurement of signal intensity (C).

**FIGURE 4.** CD152-mediated induction of IDO expression in DC does not require IFN-γ secretion. Analysis of IDO (upper photograph) and NAPDH (lower photograph) expression by Western blotting in splenic CD11c+ DC isolated from normal (A) or IFN-γ-deficient (B) BALB/c mice cultured overnight alone (lane 1), with IFN-γ (lane 2), soluble CD152-Ig (lane 3), or both IFN-γ and CD152-Ig (lane 4), and spleen CD11c+ DC isolated from SEB-treated BALB/c mice (lanes 5–8) cultured overnight alone (lane 5), with IFN-γ (lane 6), soluble CD152-Ig (lane 7), or with both IFN-γ and CD152-Ig (lane 8). Results presented are representative of three independent experiments. IDO/GAPDH expression ratios were calculated using a Gel Doc EQ camera and Quantity One computer software (Bio-Rad) for the measurement of signal intensity (C).

**FIGURE 5.** Induction of immune unresponsiveness conditions CD11c+ DC for their capacity to mediate tryptophan catabolism upon CD152-mediated activation. A. Shown is the production of kynurenine by splenic CD11c+ DC isolated from normal or SEB-treated BALB/c mice cultured overnight alone, with IFN-γ, soluble CD152-Ig, or both IFN-γ and CD152-Ig as indicated. The results of two independent experiments are represented. B. The left panels show the production of kynurenine by DC isolated from IFN-γ-deficient animals and submitted to the same stimulation as in A.
expression in the absence of IFN-γ. Because one could argue that, although undetectable in our experiments, IFN-γ was produced at very low, but sufficient, levels by CD152-Ig-stimulated DC, we analyzed IDO expression in DC populations isolated from IFN-γ-deficient mice. As shown in Fig. 4B, normal IFN-γ−/− DC and DC derived from SEB-treated IFN-γ−/− animals could not be differentiated based on their expression of IDO. Both types of cells, like those isolated from IFN-γ+/+ competent mice, expressed constitutively low levels of IDO. Stimulation with soluble CD152-Ig, in contrast, strongly up-regulated the expression of the enzyme. Thus, IDO expression in DC does not require IFN-γ production and, in our hands, CD152-Ig stimulation does not appear to induce the production of IFN-γ by isolated DC.

Normal DC and DC derived from SEB-treated animals were, however, very different in their ability to modulate tryptophan catabolism. As shown in Fig. 5A, whereas rIFN-γ induced the production of kynurenine within the cultures of DC isolated from normal mice, it did not stimulate IDO function in DC isolated from SEB-treated mice. On the contrary, stimulating DC from unresponsive mice with soluble CD152-Ig induced a strong IDO activity that was not observed after CD152-mediated stimulation of DC generated from normal mice. Finally, induction of IDO activity by CD152-Ig was also investigated in DC isolated from IFN-γ-deficient mice. As seen in Fig. 5B, CD152-mediated stimulation of SEB-treated IFN-γ−/− DC also induced a strong IDO activity.

The last experiment was performed to demonstrate the link between IDO activity of DC isolated from SEB-treated mice and the suppression mediated by CD25+CD152+ T regulatory cells. CFSE-labeling technology and flow cytometry were used to assess the extent of suppression on the proliferation of primary T cell responses. Cell cultures were set up containing CFSE-labeled naïve CD4+CD25− T responder cells and CD11c+ cells isolated from SEB-unresponsive mice. We have shown that, compared with those isolated from untreated mice, CD11c+ DC from unresponsive mice have a reduced capacity to stimulate SEB-specific primary T cell responses (Fig. 3). As shown in Table I, the addition of SEB to the cultures stimulated a modest, but reproducible, proliferation of the CFSE-labeled responder T cells. The relative number of T cells that underwent proliferation following SEB stimulation dropped, however, by at least a 3-fold following the addition of CD25+CD152+ T regulatory cells isolated from SEB-unresponsive mice. Remarkably, inhibiting the activity of IDO by 1-MT restored the level of proliferation of CD4+CD25− T responder cells, even slightly above that observed in the absence of regulatory cells. Taken together, these results demonstrate that DC isolated from unresponsive animals acquire immune regulatory activity following interaction with CD25+CD152+ T regulatory cells through activation of tryptophan catabolism.

### Discussion

We observed that repeated exposure to SEB increased the expression of CD152 on SEB-specific Foxp3+CD25+CD4+ T cells and provided the cells with the capacity to regulate and control the strong primary T cell response stimulated by SEB. This regulatory function was dependent on the activation of tryptophan catabolism and could be inhibited by neutralization of CD152-B7 interactions. In addition, we also observed that CD11c+ DC from unresponsive animals acquired regulatory activity through CD152-mediated stimulation of tryptophan catabolism. DC isolated from normal or IFN-γ-deficient BALB/c mice were equally effective in up-regulating the molecule as well as in inducing tryptophan catabolism following CD152-Ig exposure. These results, in agreement with previous observations made by others (19), indicated that IFN-γ signaling was not necessary for IDO expression and activity. We conclude that repeated TCR-mediated stimulation of Foxp3+CD25+CD4+ T regulatory cells within a short period of time activates their immunoregulatory function which mostly consists in inducing IDO-dependent tryptophan catabolism in APC through CD152/B7 signaling.

These results confirm those of previous studies that linked directly the activity of CD25+CD4+ T regulatory cells with the induction of tryptophan catabolism in APC (17, 18). Our study shows also that DC isolated from mice unresponsive to SEB can acquire immunoregulatory properties, as previously suggested by Muraille et al. (35). Indeed, although both normal and SEB-treated DC expressed similar levels of IDO protein upon stimulation with soluble CD152-Ig, only DC isolated from unresponsive mice could exhibit IDO activity. Because DC are known to be competent APC for the presentation of bacterial superantigens to T cells, our study suggested that, in unresponsive animals, DC acquired their regulatory function through repeated superantigen-mediated interactions with Foxp3+CD25+CD4+ T regulatory cells. Repeated in vitro stimulation with soluble CD152-Ig has been shown to induce suppressive function in rat DC (36). Therefore, we can reasonably suggest that DC suppressive function is acquired through repeated engagement of surface B7 molecules by CD152 overexpressed at

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**Table I. Blocking IDO activity inhibits the suppression mediated by CD25+CD4+ T cells and CD11c+ cells isolated from SEB-unresponsive mice**

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>CD4+ T cells (%)</th>
<th>CD4+ T cells (%)</th>
<th>CD4+ T cells (%)</th>
<th>CD4+ T cells (%)</th>
<th>CD4+ T cells (%)</th>
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</thead>
<tbody>
<tr>
<td>Division No.</td>
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<td>Initial (%)</td>
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<td>Initial (%)</td>
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<td>4.1</td>
<td>2.0</td>
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<tr>
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<td>5.4</td>
<td>1.3</td>
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<tr>
<td>% of CD4+ T cells</td>
<td>0.7</td>
<td>5.1</td>
<td>2.2</td>
<td>6.0</td>
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a Calculation of the percentage of CD4+ T cells that underwent SEB-stimulated division. The data presented are representative of two independent experiments.

b CFSE-labeled CD4+CD25− cells (500 × 10³) cultured in the presence of SEB (1 µg/ml).

c CFSE-labeled CD4+CD25− cells (500 × 10³) cocultured with CD11c+ cells (200 × 10³/well) isolated from SEB-unresponsive animals in the presence of SEB (1 µg/ml).

d CFSE-labeled CD4+CD25− cells (500 × 10³) cocultured with CD11c+ cells (200 × 10³/well) and CD25+CD4+ T cells (250 × 10³/well) isolated from SEB-unresponsive animals in the presence of SEB (1 µg/ml) and vehicle.

e CFSE-labeled CD4+CD25− cells (500 × 10³) cocultured with CD11c+ cells (200 × 10³/well) and CD25+CD4+ T cells (250 × 10³/well) isolated from SEB-unresponsive animals in the presence of SEB (1 µg/ml) and l-MT.

f Percentage of cells in a given cycle (Dn) within CD4+ T cells. Mean of triplicates.
the cell surface of activated T regulatory cells. Moreover, our data in Fig. 5, showing strong induction of IDO expression in both naïve and SEB-treated DC but IDO activity only in SEB-treated DC upon CD152-Ig stimulation, support the conclusion that SEB treatment makes DC more sensitive to CD152-B7 interactions.

One major observation made in our study was that induction of IDO expression did not necessarily activate tryptophan catabolism. Previous studies have shown that adding IFN-γ or soluble CD152-Ig induces IDO expression in DC (37–39). Whereas in our study IFN-γ did not increase IDO expression but stimulated weak IDO function within DC isolated from untreated BALB/c mice, adding soluble CD152-Ig to cultures of DC-stimulated strong IDO expression, but did not stimulate its activity as assessed by the absence of kynurenine production within the cultures. In our hands, only DC isolated from SEB-treated animals exhibited strong IDO activity following treatment with soluble CD152-Ig. This activity did not result from higher IDO expression since both types of DC, resting and SEB-treated DC, could not be distinguished based on their level of IDO expression. Thus, IDO expression does not necessarily indicate IDO activity. A similar conclusion was reached by others in IFN-γ-treated macrophages where IDO activity was not observed despite IDO expression (40).

Although induction of IDO activity appeared to be dependent on IFN-γ activity in resting DC, this was not observed in SEB-induced tolerogenic DC. Indeed, adding IFN-γ to cultures of SEB-treated DC did not induce IDO activity. Moreover, CD152-Ig treatment, though inducing IDO activity, did not stimulate the production of IFN-γ by DC. Even more interesting was the inhibitory effect of exogenous IFN-γ on CD152-Ig-stimulated IDO activity (but not production of IDO) in DC from SEB-treated mice. This observation strongly suggested the possibility that, following increased CD152-B7 signaling, IFN-γ acts in a negative feedback loop to control IDO activity in DC. Taken together, our results clearly show that there are two pathways for induction of IDO expression: one of them is mediated by IFN-γ and is active in resting DC; the other one requires CD152-B7 interaction and is effective in immunomodulated DC. These two pathways also appeared to be mutually exclusive since applying both IFN-γ and CD152-Ig stimuli inhibited IDO activity. To our knowledge, this is the first report of such duality in the induction of tryptophan catabolism in immunoregulatory DC.

Results presented in Fig. 2A show that ~20% of Foxp3+CD4+ TCRBV8+ T cells in mice unresponsive to SEB were CD25+. Thus, the anergic state and suppressive activity of CD25+CD4+ TCRBV8+ T cells following SEB injection could then be attributed to the activity of these Foxp3+CD25+ TCRBV8+ T cells, which could suppress Foxp3−CD25− TCRBV8−CD4+ T cells. This conclusion is supported by our observation that, if not all, Foxp3+ TCRBV8+CD4+ T cells in SEB-unresponsive mice did express CD152 and the addition of blocking anti-CD152 Abs partly blocked the suppressive activity of CD25+CD4+ TCRBV8+ T cells on SEB-specific primary responses. However, whereas the suppressive activity of CD25−CD4+ TCRBV8− T cells could be entirely blocked by anti-CD152 Abs, suppression mediated by CD25+CD4+ TCRBV8+ T cells also depended on neutralization of IL-10. This observation supports the hypothesis that, depending on their pathway of suppression, there are two types of suppressor cells in mice unresponsive to SEB. The first expresses Foxp3, includes both CD25+ and CD25− T cells, and has its suppressive function dependent on CD152 expression. The second is Foxp3+CD25− and relies on IL-10 secretion for its regulatory activity.

Our results also suggest that Foxp3+CD25−CD4+ T regulatory cells need to be continuously activated through their TCR to mediate their function. Indeed, SEB-specific Foxp3+CD25+CD4+ TCRBV8+ T regulatory cells isolated from SEB-pretreated mice specifically up-regulated cell surface CD152 expression and controlled more efficiently SEB-specific primary T cell responses (this study and Ref. 22) than cells isolated from untreated mice. Previous studies by Salomon and colleagues (41) have shown that CD25+ T regulatory cells isolated from resting BALB/c mice could be divided into two populations according to their state of activation. Some T cells remained quiescent, whereas other T cells were dividing extensively and expressed multiple activation markers (41). Transfer experiments suggested that these activated T cells were autoreactive and were continuously activated by tissue self-Ags. Although the study suggested that these cells represented T regulatory cells, it failed to show, however, whether they could modulate the activity of T effector cells.

Because their revival by immunologists in the mid-1990s and despite extensive investigation, T regulatory cells have yet to find practical application in the clinic. This is particularly true in the field of organ transplantation where high recipient anti-donor T cell precursor frequencies make allogeneic T cell responses difficult to control. Simple adoptive transfer of alloantigen-specific T regulatory cells has revealed itself to be poorly efficient at transferring immune unresponsiveness. In the same way, IDO-expressing DC have been shown to possess only limited T cell regulatory function in humans (20), and injection of immunoregulatory DC has shown limited therapeutic potential in mouse models (42–44). Our results support the idea that combining the transfer of T regulatory cells along with that of immunomodulated DC could well substantially improve the potential of T regulatory cell therapy.

Acknowledgments

We are very thankful to E. Depret and B. Diallo for their help with HPLC and V. Vercruyse for mouse DC isolation.

Disclosures

The authors have no financial conflict of interest.

References
