

Characterization of Dendritic Cells that Induce Tolerance and T Regulatory 1 Cell Differentiation In Vivo

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Summary

Active suppression is mediated by a subpopulation of CD4⁺ T cells that prevents autoimmunity. However, the mechanisms involved in their differentiation in vivo are currently under intensive research. Here we show that in vitro culture of bone marrow cells in the presence of IL-10 induces the differentiation of a distinct subset of dendritic cells with a specific expression of CD45RB. These CD11c^{low}CD45RB^{high} DCs are present in the spleen and lymph nodes of normal mice and are significantly enriched in the spleen of IL-10 Tg mice. These natural or in vitro-derived DCs display plasmacytoid morphology and an immature-like phenotype, and secrete high levels of IL-10 after activation. OVA peptide-pulsed CD11c^{low}CD45RB^{high} DCs specifically induce tolerance through the differentiation of Tr1 cells in vitro and in vivo. Our findings identify a natural DC subset that induces the differentiation of Tr1 cells and suggest their therapeutic use.

Introduction

The induction of tolerance is critical for the maintenance of immune homeostasis. In the thymus, central tolerance is a well-established mechanism that involves deletion of self-reactive T cells. In the periphery, the mechanisms involved include the induction of cell death, the development of a state of unresponsiveness (anergy) of T cells, and active suppression by T regulatory (Tr) cells (Groux et al., 1997). However, the mechanisms by which Tr cells arise in vivo and exert their immunoregulatory effects remain to be defined and are the subject of intensive investigations.

We have previously 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, termed Tr1 (T regulatory 1) cells (Groux et al., 1997). These T cells have a poor proliferative response, secrete high levels of IL-10, and regulate Th1 and Th2 responses in vitro and in vivo (Cottrez et al., 2000; Groux et al., 1997; Cong et al., 2002). We also showed that the effect of IL-10 on the differentiation of Tr1 cells was not

a direct effect on T cells but involved a modification of APC function (Wakkach et al., 2001).

Dendritic cells (DCs) are professional APCs, specialized in the capture, processing, and transport of the antigen to lymphoid organs where they sensitize antigen-specific naive T cells (Banchereau and Steinman, 1998). DCs also have an important role in the control of the adaptive immune response (Moser and Murphy, 2000). Recently, several studies have also reported a role for DCs in the induction of peripheral tolerance (Steinman et al., 2000). In vitro experiments have demonstrated that DCs with an immature phenotype induce the differentiation of anergic/suppressive T cells (Jonuleit et al., 2000). On the basis of these data, the tolerogenic potential of DCs has been proposed to be correlated with the absence of activation of DCs with proinflammatory signals. On the other hand, in vivo experiments have suggested that induction of T cell tolerance is mediated by a subtype of specialized DCs (Akbari et al., 2001). Therefore, though experimental evidence supports the existence of tolerogenic DCs, their nature is still largely unknown.

In this study, we demonstrate the ability of IL-10 to induce the differentiation of a distinct subset of DCs which express specifically the CD45RB marker and secrete IL-10. This CD11c^{low}CD45RB^{high} DC subset was also isolated from the spleen of IL-10 transgenic (Groux et al., 1999) or BALB/c and C57Bl/6 mice. They display an immature-like phenotype even after in vitro activation with LPS or CpG oligodeoxynucleotides (ODN). In addition, CD11c^{low}CD45RB^{high} DCs induce the differentiation of Tr1 cells, and adoptive transfer of peptide-pulsed CD11c^{low}CD45RB^{high} DCs induced OVA-specific unresponsiveness in recipient mice.

Results

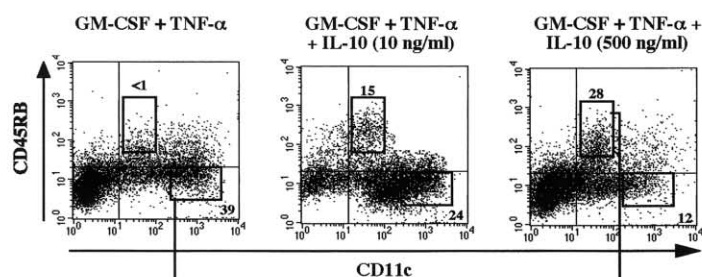
IL-10 Induces the Differentiation of a Distinct DC Subset

We have shown that IL-10 induces the differentiation of Tr1 cells through an indirect effect (Wakkach et al., 2001). We therefore analyzed the effect of IL-10 on the differentiation of DCs from bone marrow cells cultured in the presence of GM-CSF and TNF- α (preliminary experiments have shown that the presence of TNF- α did not influence the phenotype and function of DCs; however, it helped to expand the yield and viability of DC populations (data not shown). The addition of IL-10 at day 0 of the culture induces the differentiation of a population of DCs with a low expression of CD11c and a high expression of CD45RB (Figure 1A). After FACS cell sorting based on the specific expression of CD11c and CD45RB, in vitro-differentiated CD11c^{low}CD45RB^{high} DCs displayed plasmacytoid morphology with a smooth plasma membrane and an eccentric nucleus (Figure 1B, 1) in contrast to CD11c^{high}CD45RB^{low} DCs (Figure 1B, 3). After further maturation with LPS, both populations acquired morphology of fully mature DCs (Figures 1B, 2 and 4). A population of DCs with a plasmacytoid mor-

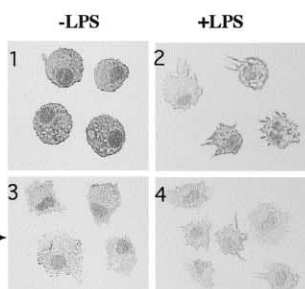
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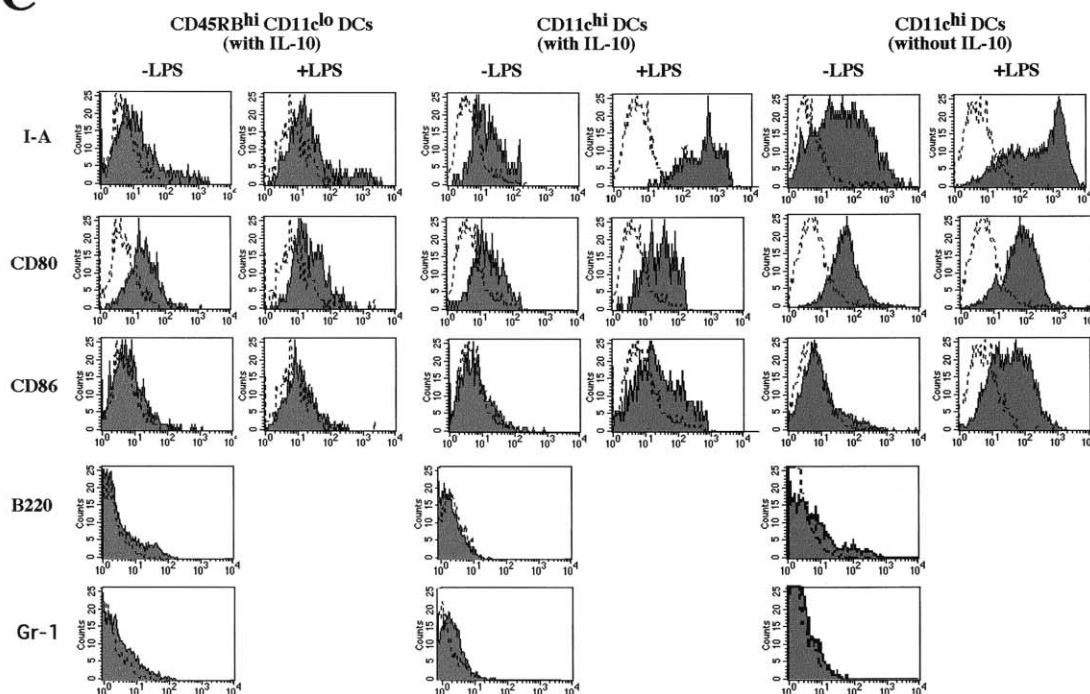
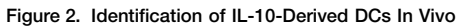


Figure 1. IL-10 Induces the Differentiation of CD11c^{low}CD45RB^{high} DCs

(A) Bone marrow cells were cultured with GM-CSF and TNF- α in the presence or absence of IL-10 (10 or 500 ng/ml) as indicated for 6 days and then activated for 24 hr with LPS. BM-DCs were analyzed for CD11c and CD45RB expression. Data represent the results of one representative experiment out of seven.

(B) DCs were sorted according to the expression of CD11c and CD45RB as indicated in Figure 1A. Sorted cells were cytocentrifuged and stained with May Grünwald Giemsa. (1) CD11c^{low}CD45RB^{high} DCs after 6 days of differentiation. (2) CD11c^{low}CD45RB^{high} DCs after LPS activation. (3) CD11c^{high}CD45RB^{low} DCs after 6 days of differentiation. (4) CD11c^{high}CD45RB^{low} DCs after LPS activation.

(C) Sorted DCs were stained with the indicated FITC-antibodies. Sorted DCs were also stimulated with LPS (1 μ g/ml) for 24 hr, stained with the indicated mAbs, and reanalyzed. Open histogram dotted lines represent cells stained with isotype-matched control mAbs. Data represent the results of one representative experiment out of three.



(A) Splenic DCs from IL-10 transgenic, IL-10^{-/-}, and BALB/c or C57Bl/6 mice were isolated and enriched by cell depletion with a cocktail of antibodies containing anti-B220, anti-Gr1, and anti-CD3 mAbs, and surface expression of CD11c was analyzed by flow cytometry.

(B) DC preparations enriched by cell depletion with a cocktail of antibodies containing anti-B220, anti-Gr1, and anti-CD3 mAbs were isolated from the spleen of IL-10 transgenic, IL-10^{-/-}, and BALB/c or C57Bl/6 mice and analyzed for expression of CD11c-cy5 and CD45RB-PE. The percentages of cells in the different quadrants are indicated. Results are typical of over ten experiments.

(C) Sorted CD11c^{low}CD45RB^{high} (1) and CD11c^{high}CD45RB⁺ (3) DCs isolated from the spleen of BALB/c mice were cytocentrifuged and stained with May-Grünwald-Giemsa. Sorted CD11c^{low}CD45RB^{high} (2) and CD11c^{high}CD45RB⁺ (4) DCs were also stimulated with LPS (1 μg/ml) in the presence of GM-CSF for 24 hr.

(D) Enriched DC preparations from IL-10 Tg, BALB/c, or C57Bl/6 mice were FACS sorted based on CD11c^{low}CD45RB^{high} or CD11c^{high}CD45RB⁺ expression and reanalyzed as shown. Sorted cells were then stained with the FITC-labeled third marker and analyzed by cytofluorometry (open histograms). Sorted DCs were also stimulated with LPS (1 μg/ml) in the presence of GM-CSF for 24 hr, stained with the indicated mAbs, and reanalyzed (closed histograms). Dotted histograms represent isotype-matched control mAbs.

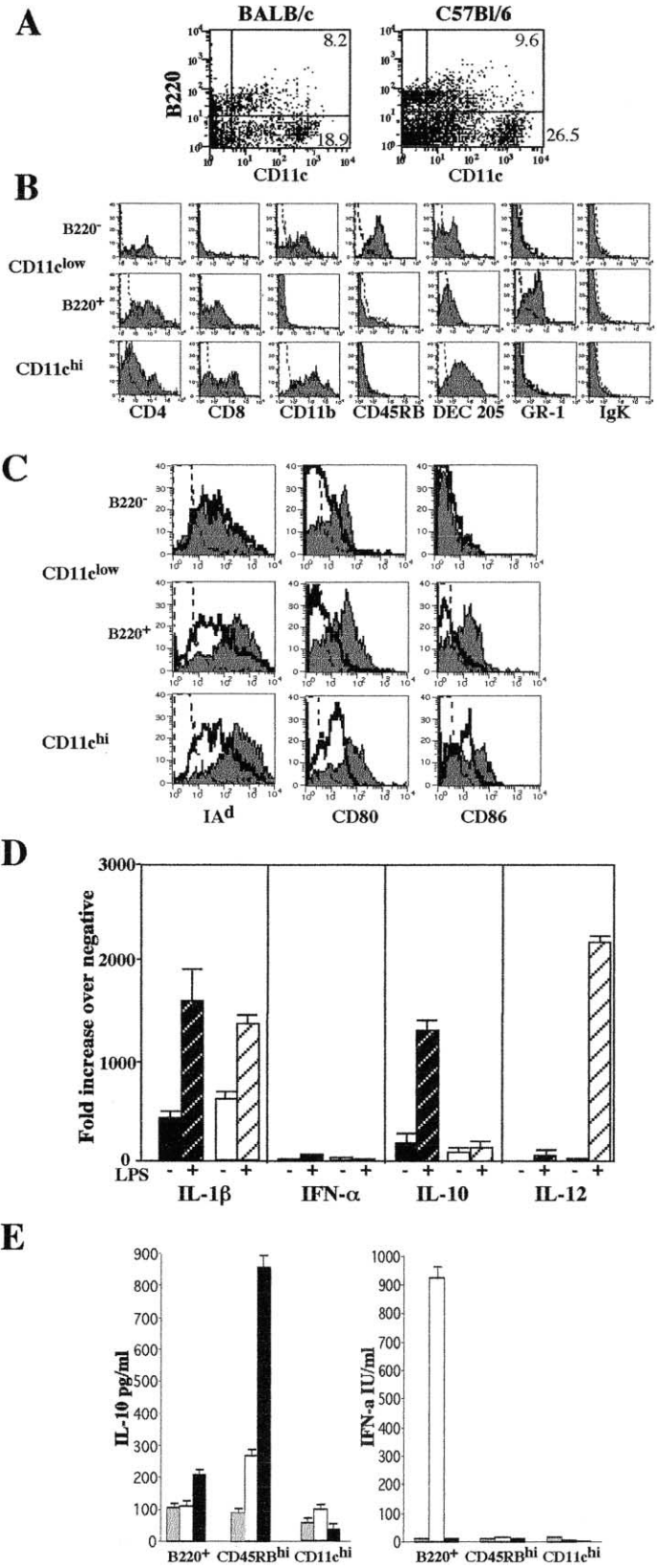


Figure 3. Phenotypic Characterization and Cytokine Secretion of the CD11c^{low}CD45RB^{high}/B220⁻ DC Subset

(A) To compare CD11c^{low}CD45RB^{high} populations with previously described plasmacytoid cells expressing low levels of CD11c but expressing the B220 and Gr1 markers, analyses were performed on DC preparations enriched by cell depletion with a cocktail of antibodies containing anti-CD19 and anti-CD3 mAbs. DCs isolated from the spleen of BALB/c or C57Bl/6 mice were analyzed for expression of CD11c-cy5 and B220-PE.

(B) Enriched DC preparations from BALB/c mice were sorted based on CD11c^{low}B220⁺, CD11c^{low}B220⁻, or CD11c^{high} expression as indicated, and staining for the third marker was analyzed on the different populations. Dotted histograms represent isotype-matched control mAbs.

(C) Similar sorted cells were stained with the FITC-labeled third marker and analyzed by cytofluorometry (open histograms). Sorted DCs were also stimulated with CpG ODN (1 μ M) in the presence of GM-CSF for 24 hr, stained with the indicated mAbs, and reanalyzed (closed histograms). Dotted histograms represent isotype-matched control mAbs.

(D and E) Cytokine profiles of the different DC subpopulations. (D) In a first set of experiments, CD11c^{high}CD45RB⁻ (white bars) and CD11c^{low}CD45RB^{high} (black bars) DC subsets were sorted out based on CD11c and CD45RB expression from splenic DC populations enriched with a cocktail containing anti-CD3, anti-B220, and anti-Gr1 mAbs. Sorted CD11c^{high}CD45RB⁻ (white crosshatched bars) and CD11c^{low}CD45RB^{high} (black crosshatched bars) cells were also stimulated with LPS (1 μ g/ml) in the presence of GM-CSF for 24 hr. Values are expressed as fold increased expression above a negative sample and represent mean $\pm$ SD of three separate sample experiments. (E) DC populations were also prepared with a cocktail containing anti-CD3 and anti-CD19 mAbs and sorted according to CD11c and B220 expression. Cells were not stimulated (gray bars) or stimulated with CpG ODN (1 μ M, white bars) or LPS (1 μ g/ml, black bars), and supernatants were analyzed at 24 hr for secretion of IFN- α or 48 hr for IL-10 measurements.

phology that expresses low levels of CD11c has already been described (Asselin-Paturel et al., 2001; O'Keeffe et al., 2002). However, CD11c^{low}CD45RB^{high} DCs do not

express B220 and Gr-1 (Figure 1C), which have been described as specific markers of plasmacytoid DCs, suggesting that they represent distinct populations of

DCs. Analysis of costimulatory and MHC class II molecule expression revealed low expression levels of CD80, CD86, and I-A molecules on CD11c^{low}CD45RB^{high} DCs (Figure 1C) as compared to CD11c^{high}CD45RB^{low} DCs. Maturation of DCs with LPS did not modify the phenotype of CD11c^{low}CD45RB^{high} DCs (Figure 1C) whereas it enhanced expression of CD80, CD86, and I-A molecules on CD11c^{high}CD45RB^{low} DCs. These results show that IL-10 induces the differentiation of a distinct subset of DCs characterized by the specific high expression of CD45RB that displays an immature-like phenotype that cannot be modified by the stimulation with LPS.

Isolation of the Natural Counterpart of IL-10-Derived DCs

We therefore addressed the influence of IL-10 *in vivo* on the differentiation of DCs. Enriched splenic DCs isolated from IL-10 Tg, IL-10^{-/-}, syngeneic BALB/c, or C57Bl/6 mice were analyzed for the expression of CD11c (Figure 2A). In IL-10 Tg mice, we observed a higher number of DCs expressing low levels of CD11c as compared to BALB/c mice (Figure 2A). Similar to the CD11c^{low} DCs differentiated *in vitro* in the presence of IL-10, the splenic CD11c^{low} DCs highly expressed the CD45RB marker (Figure 2B). Moreover, both *in vitro* IL-10-derived DCs and splenic CD11c^{low}CD45RB^{high} DCs showed plasmacytoid morphology (Figure 2C, 1) in contrast to CD11c^{high}CD45RB^{low} DCs (Figure 2C, 3). After maturation with LPS, both populations of splenic DCs differentiated into DCs with long dendrites (Figure 2C, 2 and 4). In contrast to the results obtained in IL-10 Tg mice, the analysis of CD11c^{low}CD45RB^{high} DCs in IL-10^{-/-} did not reveal a specific breakdown of this DC subset (Figures 2A and 2B) suggesting that IL-10 is not absolutely required for the differentiation of CD11c^{low}CD45RB^{high} DCs but rather specifically enhances it.

We next examined the expression of MHC molecules I-A and costimulatory molecules CD80 and CD86 on FACS-sorted CD11c^{low}CD45RB^{high} and CD11c^{high}CD45RB^{low} splenic DC subsets from IL-10 Tg, control BALB/c, or C57Bl/6 mice. As shown in Figure 2D, we observed that *ex vivo* purified CD11c^{low}CD45RB^{high} DCs expressed low levels of MHC class II, CD80, and CD86 molecules, as compared to CD11c^{high}CD45RB^{low} DCs. Moreover, after *in vitro* maturation with LPS, CD11c^{low}CD45RB^{high} DCs still retained poor expression levels of MHC class II and CD86 molecules, suggesting that their immature-like DC phenotype is stable except for an enhanced expression of the CD80 molecule, in contrast to the CD11c^{high}CD45RB^{low} DCs (Figure 2D). Altogether, these results showed that CD11c^{low}CD45RB^{high} DCs isolated from the spleen represent the *in vivo* counterpart of the IL-10-derived DCs.

Phenotypic Characterization and Comparison of CD11c^{low}CD45RB^{high} DC Subset with IFN- α Secreting B220⁺ Plasmacytoid DCs

As mentioned above, another population of DCs with plasmacytoid morphology has been previously described. These plasmacytoid DCs secrete high levels of IFN- α after viruses or CpG ODN activation and specifically express B220 and Gr-1 (Asselin-Paturel et al., 2001). In our experiments, these cells were depleted from our purified DCs as we used, according to the most

current protocols for mouse DC isolation, a cocktail of antibodies containing anti-B220 and anti-Gr-1 in the enrichment step. We therefore performed other experiments with another cocktail of depleting antibodies (anti-CD3 and anti-CD19). The expression analysis of CD11c and B220 on this new DC preparation revealed the existence of three distinct subsets, CD11c^{low}B220⁺, CD11c^{low}B220⁻, and CD11c^{high} DCs in both BALB/c and C57Bl/6 mice (Figure 3A). To further characterize these different DC subsets, we analyzed the expression of a set of previously described DC markers on the sorted CD11c^{low}B220⁺, CD11c^{low}B220⁻, and CD11c^{high} DC populations from BALB/c (Figure 3B) or C57Bl/6 (data not shown) mice. Again, the IL-10-derived DC subset, which represents the CD11c^{low}B220⁻ DCs expressed specifically high levels of CD45RB, whereas the CD11c^{low}B220⁺ subset, which has been described as plasmacytoid DCs, expressed low levels of CD45RB. CD11c^{low}CD45RB^{high} B220⁻ DCs expressed intermediate levels of CD11b, CD4, and DEC 205 and no CD8 α , B220, Gr-1, and Igk light chain, a phenotype which clearly showed that they were different from plasmacytoid CD11c^{low}B220⁺, or classical CD8⁺CD11c^{high}CD11b⁺ DCs, and that they did not represent another B cell or T cell subpopulation.

We have shown above (Figures 1C and 2D) that LPS stimulation did not modify the immature-like phenotype of CD11c^{low}CD45RB^{high} DCs; we therefore tested the effect of CpG ODN stimulation on the phenotypes of CD11c^{low}B220⁺, CD11c^{low}B220⁻, and CD11c^{high} DCs isolated from BALB/c mice. Figure 3C shows that CpG ODN stimulation induced the maturation of B220⁺ plasmacytoid DCs as previously reported (Asselin-Paturel et al., 2001; O'Keeffe et al., 2002), whereas it did not modify the immature-like phenotype of CD11c^{low}CD45RB^{high}B220⁻ DCs, showing that these DCs have a stable phenotype with low expression levels of MHC class II and CD86 after activation with LPS or CpG ODN.

The specific function of each DC subset is also dictated by distinct cytokine profiles. In a first set of experiments, we analyzed by quantitative RT-PCR the relative expression of several important cytokines on freshly sorted or LPS-activated CD11c^{low}CD45RB^{high} and CD11c^{high}CD45RB^{low} DCs obtained after depletion with anti-B220 and anti-Gr-1 antibodies. After activation with LPS, CD11c^{low}CD45RB^{high} DCs secreted IL-10 whereas CD11c^{high}CD45RB^{low} DCs secreted IL-12. Both populations secreted IL-1 β , which was further enhanced by LPS activation, but did not secrete IFN- α , even after activation for 24 hr with LPS (Figure 3D). We then measured by ELISA the secretion of IL-10 and IFN- α on freshly isolated, LPS-stimulated or CpG ODN-stimulated CD11c^{low}B220⁺, CD11c^{low}B220⁻, and CD11c^{high} DC populations from BALB/c mice (Figure 3E). Again, after LPS activation, high IL-10 secretion was detected in the CD11c^{low}CD45RB^{high}B220⁻ population as compared to the B220⁺ or CD11c^{high}CD45RB^{low} populations. CpG ODN stimulation induced high levels of IFN- α in the B220⁺ population as previously reported (O'Keeffe et al., 2002), whereas it had no significant effect on the CD11c^{high}CD45RB^{low} population and induced a slight but significant amount of IL-10 in the CD11c^{low}CD45RB^{high} population. These results showed that CD11c^{low}CD45RB^{high}B220⁻ DCs represent a distinct subset of DCs that predom-

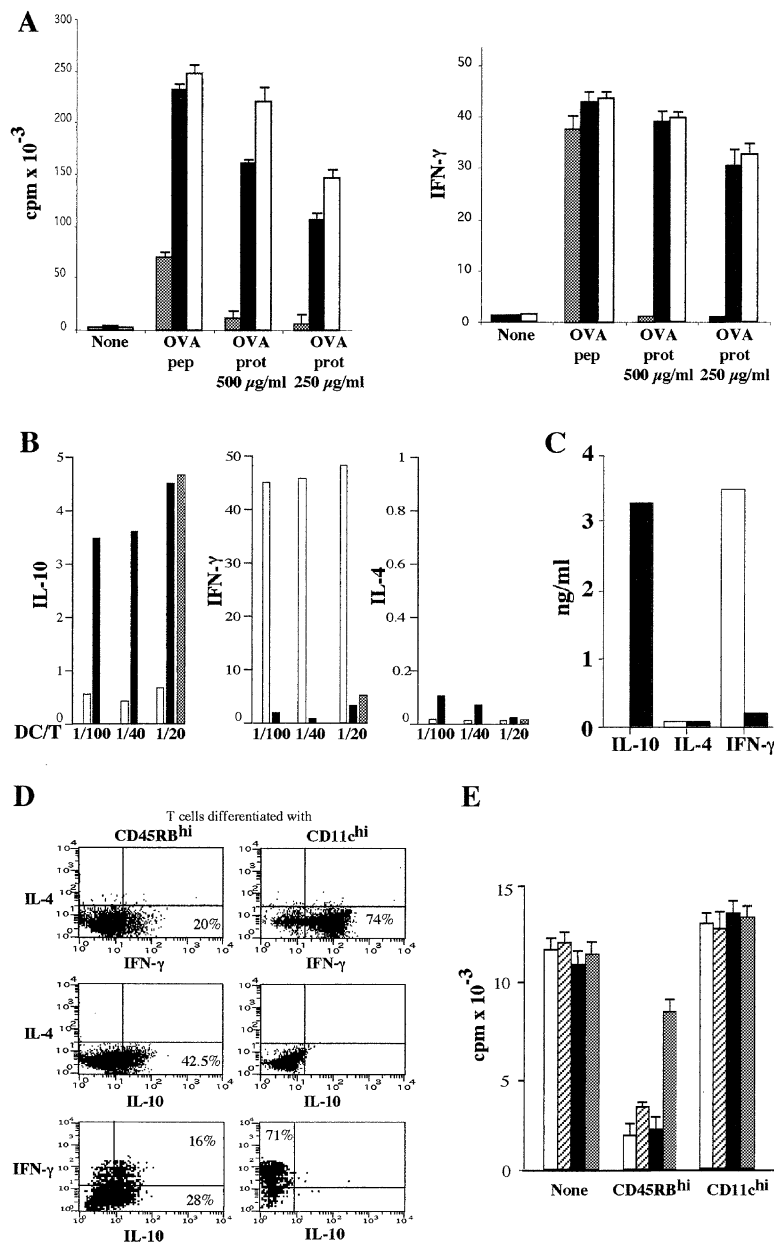


Figure 4. CD11c^{low}CD45RB^{high} DCs Induce the Differentiation of Regulatory Tr1 Cells In Vitro

(A) CD11c^{low}CD45RB^{high} DCs process and present antigen in vitro. A Th1 cell population from DO11-10 mice was stimulated without APCs (gray bars) or with sorted CD11c^{low}CD45RB^{high} (black bars) or CD11c^{high}CD45RB⁻ (white bars) DCs in the presence of OVA peptide (0.6 μM) or OVA protein (500 or 250 μg/ml as indicated). Proliferative response or IFN-γ secretion was analyzed after stimulation at 72 or 48 hr, respectively. Results represent the mean of triplicate determinations and are expressed in ng/ml for IFN-γ. Data are representative of two separate experiments with similar results.

(B) Analysis of cytokines secreted by CD4⁺ T cell populations activated with ex vivo sorted DCs. Naive OVA TCR transgenic T cells were differentiated for 3 weeks with sorted CD11c^{high}CD45RB⁻ (white bars) or CD11c^{low}CD45RB^{high} (black bars) DCs in the presence of OVA peptide. DCs were isolated from BALB/c mice and mixed at different DC/T ratios as indicated. Differentiation of T cells was also performed in the presence of anti-IL-10 (10 μg/ml) at a 1/20 ratio (gray bars). After the 3 weeks culture, T cells were collected and stimulated with irradiated BALB/c splenocytes and OVA peptide (0.3 μM). Forty-eight hours later, cytokines were analyzed by ELISA. Results represent the mean of triplicate determinations and are expressed in ng/ml. Data is representative of five separate experiments with similar results.

(C) Cytokines secreted by CD4⁺ T cell populations differentiated with in vitro-derived DCs. DCs were obtained from cultures of bone marrow cells with GM-CSF and TNF-α and IL-10 (500 ng/ml). DCs were sorted into CD11c^{high}CD45RB⁻ and CD11c^{low}CD45RB^{high} DC subsets. Sorted DCs were used to differentiate DO11-10 naive CD4⁺ T cells, at 1:20 DC/T ratio in the presence of OVA peptide (0.6 μM). After 1 week, T cells were collected and stimulated with irradiated splenic APCs and OVA peptide (0.3 μM). Forty-eight hours later, cytokines were analyzed by ELISA. Results represent the mean of triplicate determinations and are expressed in ng/ml. Data are representative of two separate experiments with similar results.

(D) Intracellular cytokine assay for naive DO11-10 T cells stimulated with OVA peptide and sorted CD11c^{low}CD45RB^{high} or CD11c^{high}CD45RB⁻ DCs. Both DC subsets were isolated from BALB/c mice, FACS sorted, and cocultured with naive DO11-10 T cells in the presence of OVA peptide. Seven days later, cells were stimulated and stained for intracellular cytokine assay using FITC- or PE-conjugated specific mAbs as indicated. One representative experiment out of three is shown.

(E) Regulatory function of T cells differentiated with CD11c^{low}CD45RB^{high} DCs. In the bottom well of a transwell system, purified CD4⁺ T cells isolated from BALB/c mice were stimulated with irradiated BALB/c splenocytes and anti-CD3 mAbs (white bars). In the upper compartment, CD4⁺ T cells differentiated by a single stimulation with either CD11c^{low}CD45RB^{high} DCs or CD11c^{high}CD45RB⁻ DCs were stimulated with OVA peptide and irradiated splenocytes. Coculture experiments were also performed in the presence of blocking mouse anti-IL-10 mAb (10 μg/ml) (white crosshatched bars), mouse anti-TGF-β mAb (40 μg/ml) (black bars), or both (gray bars). Three days later, the basket was removed and the proliferative response of the bystanders CD4⁺ T cells was measured after a pulse with 0.5 μCi of ³H-thymidine during the final 12 hr of the 72 hr culture. Results represent mean ± SD of triplicates from one representative experiment out of three.

inantly secrete IL-10 after activation with LPS or CpG ODN.

CD11c^{low}CD45RB^{high} DCs Differentiate Tr1 Cells In Vitro

First, to analyze the ability of CD11c^{low}CD45RB^{high} DCs to process and present antigens, we tested their ability

to stimulate a Th1 cell population. Analysis of the proliferative response and IFN-γ secretion showed that both sorted CD11c^{low}CD45RB^{high} and CD11c^{high}CD45RB⁻ DCs processed and present the ovalbumin protein to stimulate an OVA-specific T cell population, although the CD11c^{high}CD45RB⁻ DCs induced a more vigorous proliferation than the CD11c^{low}CD45RB^{high} DCs (Figure 4A). To

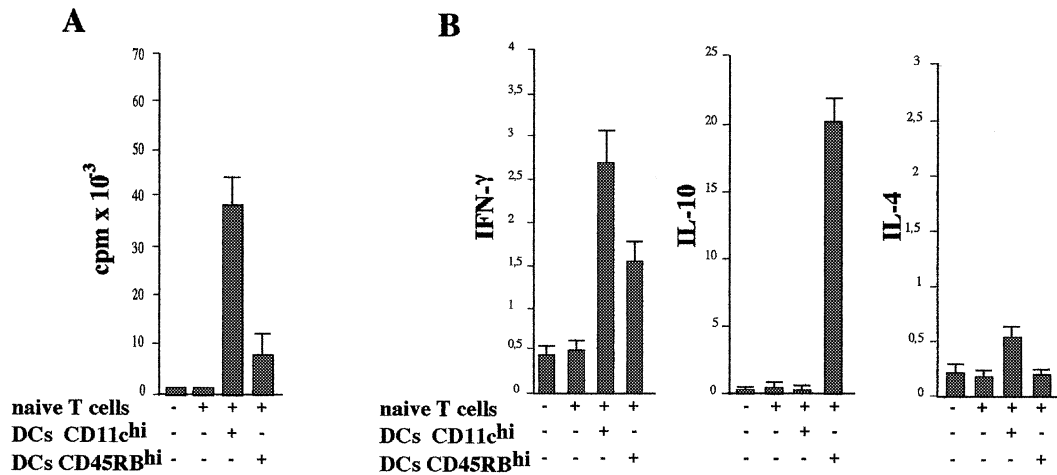


Figure 5. CD11c^{low}CD45RB^{high} DCs Induce the Differentiation of Tr1 Cells In Vivo

Sorted CD11c^{low}CD45RB^{high} or CD11c^{high}CD45RB^{low} DCs isolated from BALB/c mice were loaded with OVA peptide in vitro and injected intravenously into mice previously transferred with OVA peptide-specific T cells. One week later, proliferative response (A) and cytokine production (B) of splenic CD4⁺ T cells stimulated with OVA peptide (0.3 μM) and irradiated splenic APCs were tested. For cytokine measurements, results represent means of triplicate determinations and are expressed in ng/ml. Data are representative of three separate experiments with similar results.

analyze the influence of the different DC subsets on the priming and differentiation of naive OVA specific T cells, multiple rounds of stimulations of T cells were established with sorted CD11c^{low}CD45RB^{high} or CD11c^{high}CD45RB^{low} DCs isolated from the spleen of BALB/c mice (Figure 4B) or differentiated in vitro in the presence of IL-10 (Figure 4C). Our results showed that both ex vivo purified or in vitro differentiated CD11c^{low}CD45RB^{high} DCs, regardless of the DC/T ratio used, induced the differentiation of Tr1 cells which secreted high levels of IL-10, low levels of IFN-γ, and no or negligible amounts of IL-4 (Groux et al., 1997) (Figures 4B and 4C). In contrast to CD11c^{low}CD45RB^{high} DCs, CD11c^{high}CD45RB^{low} DCs primed Th1 cells which secreted high levels of IFN-γ (Figures 4B and 4C). As a control, we also tested the function of in vitro-derived DCs cultured in the absence of IL-10; as expected the CD11c^{high}CD45RB^{low} population induced the differentiation of Th1 cells (data not shown), whereas not enough CD11c^{low}CD45RB^{high} DCs were obtained to test their function. Altogether, these results are correlated with the cytokine profile of the DC subpopulations, i.e., CD11c^{low}CD45RB^{high} DCs, which differentiate Tr1 cells and secrete IL-10, whereas IL-12 secreted by CD11c^{high}CD45RB^{low} DCs induces the differentiation of Th1 cells.

Moreover, the specific differentiation of Tr1 cells with the CD11c^{low}CD45RB^{high} DCs was observed after a single stimulation as shown by the intracytoplasmic analysis of cytokine secretion in the different T cell populations (Figure 4D). The phenotype of these Tr1 cells was highly stable as even when restimulated with CD11c^{high}CD45RB^{low} DCs, a situation that is likely to occur in vivo, they conserved their cytokine profile (data not shown). To analyze the functional properties of these Tr1 cells, transwell experiments were performed. T cells obtained after a single stimulation with CD11c^{low}CD45RB^{high} DCs from BALB/c mice suppressed the proliferation of bystander CD4⁺ T cells stimulated with irradiated spleno-

cytes and anti-CD3 mAb (Figure 4E). Furthermore, the addition of blocking mouse anti-IL-10 and anti-TGF-β mAbs reverted the suppressive effect of these T cells, confirming the differentiation of Tr1 cells both phenotypically and functionally (Figure 4E). As a control, Th1 cell populations (induced by CD11c^{high}CD45RB^{low} DCs) had no inhibitory effect (Figure 4E). Altogether, these results showed that CD11c^{low}CD45RB^{high} DCs induced the differentiation of Tr1 cells in vitro.

CD11c^{low}CD45RB^{high} DCs Induce Antigen-Specific Tolerance In Vivo

To analyze the in vivo function of CD11c^{low}CD45RB^{high} DCs, we used a mouse model where naive splenic CD4⁺ OVA-specific T cells from DO11-10 mice were transferred into normal BALB/c mice. These transferred mice were then treated with purified subsets of DCs pulsed with OVA peptide in vitro. One week later, we measured the proliferative response and the secretion of cytokines in response to OVA peptide stimulation of purified splenic CD4⁺ T cells from the transferred mice. Similar to the results obtained in vitro, Figure 5 shows that the injection of CD11c^{low}CD45RB^{high} DCs induced the differentiation of OVA peptide-specific Tr1 cells in vivo. These cells were characterized by a poor proliferative response (Figure 5A) and a high secretion of IL-10 (Figure 5B). As a control, the injection of OVA peptide-pulsed CD11c^{high}CD45RB^{low} DCs induced the differentiation of T cells with a high proliferative response and a high secretion of IFN-γ.

We therefore analyzed the ability of CD11c^{low}CD45RB^{high} DCs to induce tolerance in vivo. Sorted CD11c^{low}CD45RB^{high} or CD11c^{high}CD45RB^{low} DCs, either isolated from the spleen of BALB/c mice or obtained after in vitro differentiation in the presence of IL-10, were pulsed with OVA peptide and injected into naive OVA-specific T cell-transferred BALB/c mice. One week later, tolerance induction was challenged by two 15 day interval immuni-

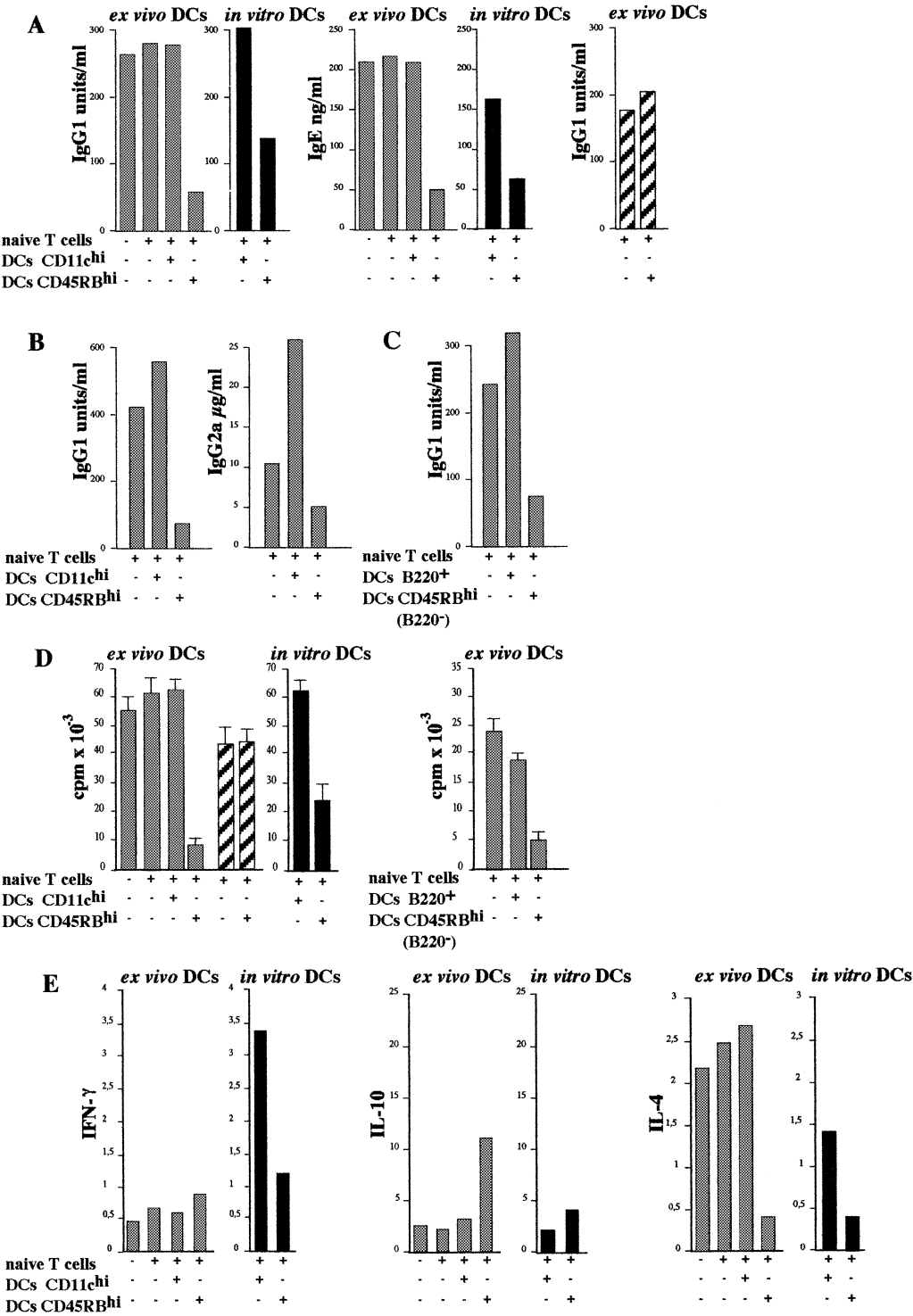


Figure 6. CD11c^{low}CD45RB^{high} DCs Induce Tolerance In Vivo

Sorted CD11c^{low}CD45RB^{high} or CD11c^{high}CD45RB⁻ DCs either isolated from BALB/c mice or differentiated in vitro as indicated were loaded with OVA peptide in vitro and injected intravenously into mice previously transferred with OVA peptide-specific T cells. One week later, mice were immunized with OVA/alum, TT/alum, or OVA/CFA and boosted 2 weeks later. Mice were analyzed 4 weeks after the first immunization. (A) OVA-specific (plain bars) or TT-specific (striped bars) IgG1 and IgE were measured by ELISA on pooled sera from each group of five mice immunized with OVA or TT in alum and expressed as means of triplicate assays. One representative experiment out of three is shown. (B) OVA-specific IgG1 and Ig2a were measured by ELISA on pooled sera from each group of five mice immunized with OVA in CFA and expressed as means of triplicate assays. (C) Sorted CD11c^{low}B220⁻ or CD11c^{low}B220⁺ DCs isolated from normal BALB/c mice were loaded with OVA peptide in vitro and injected intravenously into mice previously transferred with OVA peptide-specific T cells. Mice were immunized with OVA/alum 1 week later and were

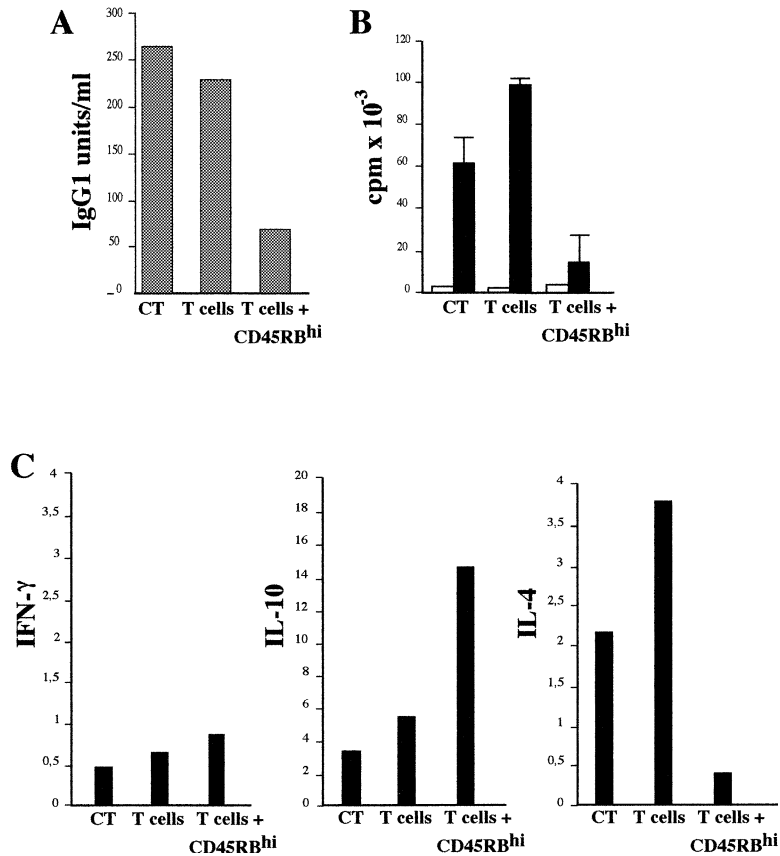


Figure 7. CD4⁺ T Cells Isolated from Mice Tolerized with CD11c^{low}CD45RB^{high} DCs Are Able to Transfer the Tolerance to the Antigen BALB/c mice transferred with naive OVA-specific T cells were treated with CD11c^{low}CD45RB^{high} DCs pulsed with OVA peptide. As a control, some mice were transferred with naive T cells alone. One week after transfer, splenic purified CD4⁺ T cells from injected mice were adoptively transferred into normal BALB/c mice. As indicated, untreated mice or mice transferred with purified CD4⁺ T cells were then immunized with OVA/alum. Immunization was boosted 2 weeks later, and mice were analyzed 4 weeks after the first immunization.

(A) OVA-specific IgG1 were measured by ELISA on pooled sera from each group of five mice and expressed as means of triplicate assays. One representative experiment out of three is shown.

(B) Purified splenic CD4⁺ T cells from transferred and immunized mice were stimulated in vitro with OVA protein (250 μg/ml) (black bars) or medium alone (white bars). The proliferative response was measured by ³H-thymidine incorporation during the last 12 hr of a 72 hr culture. Data are representative of three separate experiments.

(C) Forty-eight hours after restimulation, as described in (B), culture supernatants were collected and cytokines were measured by ELISA. Results represent means of triplicate determinations and are expressed in ng/ml. Data are representative of three separate experiments with similar results.

zations, with the whole ovalbumin protein in alum or CFA. Two weeks after the last immunization, analysis of serum OVA-specific IgE and IgG1 for alum immunization or IgG1 and IgG2a for CFA immunization revealed a marked reduced response in mice treated with in vitro-differentiated or ex vivo purified CD11c^{low}CD45RB^{high} DCs, as compared to control immunized mice (Figures 6A and 6B) in both types of immunization. Injection of OVA-pulsed CD11c^{high}CD45RB⁺ DCs boosted the Th1-type response (especially the IgG2a response) after the immunization with CFA (Figure 6B), but their priming effect (as observed in Figure 5) was minimized after the Th2-mediated responses induced by the alum immunization (Figure 6A). The induction of tolerance was antigen specific as the immunization with tetanus toxoid (TT) in alum induced the same amount of TT-specific antibodies in the sera of mice treated or not with CD11c^{low}CD45RB^{high} DCs (Figure 6A). In similar experiments, we also compared the in vivo effects of CD11c^{low}B220⁺ and B220⁺ DCs obtained after depletion with anti-CD3 and anti-CD19. As shown in Figure 6C,

after immunization with OVA in alum, a marked reduced response was observed in mice treated with ex vivo purified CD11c^{low}B220⁺ (CD45RB^{high}) DCs, as compared to control or CD11c^{low}B220⁺ DC treated mice, supporting a specific role of the CD11c^{low}CD45RB^{high} DCs in inducing antigen-specific tolerance as compared to other DC subpopulations.

The induction of tolerance was confirmed by the analysis of the proliferative response to ovalbumin of CD4⁺ T cells isolated from the spleen of immunized mice. Indeed, CD4⁺ T cells isolated from mice treated with CD11c^{low}CD45RB^{high} DCs proliferated poorly after stimulation with ovalbumin as compared to the proliferative response of CD4⁺ T cells isolated from the spleen of immunized mice previously untreated or treated with CD11c^{high}CD45RB⁺ or B220⁺ DCs (Figure 6D). Finally, the analysis of cytokine secretion by splenic CD4⁺ T cells confirmed the induction of tolerance. Immunization of mice with ovalbumin in alum resulted in a Th2-like response with a high IL-4 and a low IFN-γ secretion (Figure 6E). In contrast, in mice treated with CD11c^{low}

boosted 2 weeks later. Mice were analyzed 4 weeks after the first immunization. OVA-specific IgG1 were measured by ELISA on pooled sera from each group of five mice and expressed as means of triplicate assays.

(D) Purified splenic CD4⁺ T cells, from mice immunized with OVA/alum (plain bars) or TT/alum (striped bars), were stimulated in vitro with OVA protein (250 μg/ml). The proliferative response was measured by ³H-thymidine incorporation during the last 12 hr of a 72 hr culture. Data are representative of three separate experiments.

(E) Forty-eight hours after restimulation, as described in (D), culture supernatants were collected and cytokines were measured by ELISA. Results represent means of triplicate determinations and are expressed in ng/ml. Data are representative of three separate experiments with similar results.

CD45RB^{high} DCs, T cells secreted low amounts of IFN- γ and IL-4 and some IL-10.

Tolerance Induced by CD11c^{low}CD45RB^{high} DCs Is Transferable with Splenic CD4⁺ T Cells

Our results suggest that CD11c^{low}CD45RB^{high} DCs induce tolerance through the differentiation of antigen-specific Tr1 cells *in vivo*. Therefore, we next evaluated whether splenic CD4⁺ T cells isolated from mice exposed to CD11c^{low}CD45RB^{high} DCs had the capacity to transfer tolerance. CD11c^{low}CD45RB^{high} DCs isolated from the spleen of BALB/c mice, pulsed with OVA peptide *in vitro*, were injected into naive OVA-specific T cell-transferred mice. Seven days later, purified splenic CD4⁺ T cells were adoptively transferred into untreated BALB/c mice. To assess for tolerance induction, transferred mice were subsequently immunized twice with OVA/alum. Figure 7 shows that mice transferred with CD4⁺ T cells isolated from mice treated with tolerogenic CD11c^{low}CD45RB^{high} DCs were unable to mount a correct immune response to antigenic challenge, as measured by OVA-specific antibodies in the serum and poor recall response *in vitro*. Indeed, CD4⁺ T cells isolated from these mice displayed a low proliferative response to stimulation with the antigen (Figure 7B) and a modest secretion of IL-4 and IFN- γ , as compared to control mice (Figure 7C). However, CD4⁺ T cells isolated from tolerant mice secreted higher levels of IL-10 (Figure 7C). These results showed that CD11c^{low}CD45RB^{high} DCs induced tolerance through the differentiation of antigen-specific regulatory T cells that transferred tolerance to normal mice.

Discussion

In this study, we describe a population of tolerogenic DCs characterized by a low expression level of CD11c and the selective high expression of CD45RB. These DCs are highly potent to induce tolerance as a single injection of 3×10^5 OVA peptide-pulsed CD11c^{low}CD45RB^{high} DCs is sufficient to dramatically decrease the ability of the treated mice to mount a T and B cell-mediated immune response directed against the whole ovalbumin protein in the presence of different adjuvants. Moreover, our results show that the induction of tolerance induced by CD11c^{low}CD45RB^{high} DCs is mediated by the differentiation of regulatory Tr1 cells. *In vitro*, a single stimulation of naive T cells with CD11c^{low}CD45RB^{high} tolerogenic DCs induces the differentiation of T cells characterized by the secretion of high levels of IL-10, some IFN- γ , no IL-2, and no IL-4. Moreover, *in vitro* functional assays using a transwell system have confirmed the differentiation of Tr1 cells. *In vivo*, the treatment of mice with OVA peptide-pulsed CD11c^{low}CD45RB^{high} tolerogenic DCs also induced the differentiation of antigen-specific Tr1 cells characterized by their cytokine profile (Figure 5). Indeed, the ability to transfer tolerance from mice treated with the CD11c^{low}CD45RB^{high} tolerogenic DCs to normal mice with the purified CD4⁺ T cells indicates that the differentiation of Tr1 cells *in vivo* accounts for the observed induction of tolerance (Figures 6 and 7).

The CD11c^{low}CD45RB^{high} tolerogenic DCs represent a subset of DCs, located in the spleen and lymph nodes

(data not shown) and characterized by low expression levels of CD11c, CD11b, and DEC-205 and no expression of Gr-1, CD8, and B220. This phenotype and the percentage of cells in the different tissues analyzed appear similar to the myeloid CD4⁺CD8⁺CD205⁺CD11b⁺ population of DCs previously described (Shortman and Liu, 2002). Moreover, although they have the typical morphology of plasmacytoid cells, CD11c^{low}CD45RB^{high} tolerogenic DCs are different from the B220⁺ plasmacytoid cells which secrete IFN- α (Asselin-Paturel et al., 2001; O'Keeffe et al., 2002). The CD11c^{low}CD45RB^{high} tolerogenic DCs are distinct from B220⁺ DCs by the absence of IFN- α secretion after activation with CpG ODN and a specific high secretion of IL-10. Moreover, while activation with LPS and CpG ODN enhances expression of MHC class II and costimulatory molecules on human and mouse IFN- α secreting plasmacytoid DCs (Liu et al., 2001), no CD86 and MHC class II molecules were induced on CD11c^{low}CD45RB^{high} tolerogenic DCs after activation with LPS or CpG ODN. This discrepancy is also consistent with their different functions *in vitro* as IFN- α -secreting plasmacytoid DCs have been shown in several reports to induce the differentiation of effector T cells, either Th2 (Rissoan et al., 1999) or Th1 (Cella et al., 2000), based on the different microenvironments and types of activation, whereas CD11c^{low}CD45RB^{high} tolerogenic DCs induce the differentiation of Tr1 cells.

The observation that tolerogenic DCs secrete IL-10 may be linked to the stability of their immature-like phenotype. IL-10 has been shown to inhibit MHC class II and costimulatory molecules on APCs (Ding et al., 1993; Koppelman et al., 1997), and, *in vitro*, the addition of IL-10 onto bone marrow precursors allows enrichment in DCs expressing CD11c^{low}CD45RB^{high} markers in populations differentiated in the presence of GM-CSF. In the absence of IL-10, the population of DCs differentiated with GM-CSF has a very low percentage of DCs expressing the CD45RB marker (<1%), whereas these cells represent approximately 30% of the cells in the presence of IL-10. The fact that IL-10 acts by a direct effect on the differentiation of tolerogenic DCs and not by the inhibition of the differentiation of other populations is supported by the observation that equivalent numbers of cells were recovered, even when TNF- α was added to enhance the yield and survival of DCs. Moreover, the positive effect of IL-10 on the differentiation of tolerogenic DCs was confirmed in IL-10 Tg mice where higher proportions of these CD11c^{low}CD45RB^{high} tolerogenic DCs were observed in the spleen. However, if IL-10 appears to enhance the differentiation of CD11c^{low}CD45RB^{high} tolerogenic DCs, the analysis of IL-10^{-/-} mice DCs suggests that IL-10 is not absolutely required for the differentiation of these tolerogenic DCs and that other pathways could be used for their differentiation in the absence of IL-10.

Until now, immature DCs were thought to induce T cell anergy or regulatory T cells (Jonuleit et al., 2000; Lutz et al., 2000) whereas the same cells, transformed after stimulation into immunogenic mature DCs, represented the unique inducers of effector T cells (Banchereau and Steinman, 1998). However, several reports have described populations of mature (or not fully immature) DCs that induce tolerance *in vivo* (Akbari et al., 2001; McGuirk et al., 2002; Menges et al., 2002). These data

challenge the bimodal concept and raise the question of the true nature of tolerogenic DCs and the mechanisms of tolerance induced by these different populations of DCs, deletion, anergy, or active suppression. Immature DCs are resident resting sentinel cells present in peripheral tissues that endocytose apoptotic materials. However, endocytosis alone does not induce maturation and migration of these cells (Rubartelli et al., 1997). Therefore, DCs remain immature and tissue resident, and express low amounts of MHC class II molecules and no costimulatory molecules, a condition that leads to T cell anergy. However, spontaneous migration of DCs is also observed under healthy, steady-state conditions. These steady-state migrating DCs, loaded with self- and commonly encountered antigens, are observed in different tissues like the skin (Hemmi et al., 2001), the vagina, and cervix (Parr et al., 1991), the gut (Huang et al., 2000), or the lung (Akbari et al., 2001) and have been shown to induce tolerance in vivo (Akbari et al., 2001). These migrating DCs are different from immature DCs. First, they downregulate the expression of chemokine receptors expressed by immature DCs (CCR5, CCR6) and gain expression of CCRs that drive their migration to lymphoid tissues (CXCR4 and CCR7) (Allavena et al., 2000). Indeed, we observed that CD11c^{low}CD45RB^{high} tolerogenic DCs express CCR7 and CXCR4 (data not shown). Moreover, transport, processing, and presentation for tolerance induction require maturation signals to partially enhance upregulation of MHC and costimulatory molecules (Allavena et al., 2000). These maturation signals are obviously different from microbial or inflammatory stimuli that induce the differentiation of other types of DCs expressing high levels of MHC and costimulatory molecules and secreting IL-12. Our results suggest that IL-10 could be one of these maturation signals that drives the differentiation of tolerogenic DCs with an intermediate phenotype called semimature (Lutz and Schuler, 2002) or quiescent (Shortman and Liu, 2002). Altogether, our results suggest that CD11c^{low}CD45RB^{high} DCs represent a population of DCs that have matured to display a stable tolerogenic phenotype with a unique molecular design by the downregulation of T cell activation partners. Under steady-state conditions, these cells, loaded with self- and commonly encountered antigens, are driven to lymphoid organs by endogenous factors to induce the differentiation of Tr1 cells and tolerance.

Finally, the ability to isolate tolerogenic DCs opens new therapeutic perspectives for the use of tolerogenic DCs in autoimmune/inflammatory diseases and allogeneic transplantation. Indeed, in vitro pulsing of tolerogenic DCs with self- or alloantigens followed by in vivo injection could lead to the specific differentiation of regulatory T cell populations able to downregulate self/alloreactivity mediated by both Th1 or Th2 T cells.

Experimental Procedures

Mice

Homozygous DO11-10 mice were a generous gift from Dr. S.D. Hurst (DNAX, Palo Alto, CA). BALB/c IL-10 Tg mice were described (Groux et al., 1999). BALB/cAnN, C57Bl/6, and C57Bl/6 IL-10^{-/-} mice were purchased from Charles River Laboratory (IFFA CREDO, L'Arbresle, France). All mice were raised in common mouse pathogen-free con-

ditions and were 4- to 8-week-old females at the beginning of each experiment.

Medium, Antibodies, and Reagents

The medium used for T cell cultures was Yssel medium (Yssel et al., 1984). DCs were cultured in RPMI 1640 supplemented with 10% FCS (Roche, Meylan, France), 2 mM L-Glutamine, 1% sodium pyruvate, 2×10^{-5} M β 2 mercaptoethanol all from Invitrogen (Cergy-Pontoise, France).

Recombinant mouse GM-CSF, TNF- α , and IFN- γ were purchased from R&D Systems (Abington, United Kingdom). Recombinant mouse IL-10 and IL-4 were a generous gift from Dr. R.L. Coffman, DNAX. Recombinant mouse IFN- α was obtained from PBL Biomedical Laboratories (New Brunswick, NJ).

Anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD19 (1D3), anti-CD28 (37.51), anti-I-Ad (AMS-32.1), anti-I-Ab (AF6-120.1), anti-CD62L (Mel-14), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-B220 (RA3-6B2), CD45RB (16A), anti-Gr1 (RB6-8C5), anti-IgK (187.1) (all from Pharmingen Becton Dickinson, Le Pont de Claix, France), and DEC-205 (NLDC-145) (Serotec, United Kingdom) were used for purification and characterization of DCs.

For cytokine assays, we used anti-IL-4-PE or -FITC (11B11), anti-IFN- γ -PE (XGM1.2), anti-IL-10-FITC (JES5-16E3), anti-IL-4 (11B11), anti-IL-10 (2A5), anti-IFN- γ (XGM1.2), biotin anti-IL-4 (24G2), anti-IL-10 (SXC1), anti-IFN- γ (R4-6A2) (Pharmingen Becton Dickinson), anti-IFN- α (RMMA-1), and rabbit anti-mouse-IFN- α (PBL Biomedical Laboratories).

Anti-IgE (R35-72), anti-IgG1 (A85-1), and anti-IgG2a (R19-15) used for analysis of OVA-specific Ig were from Pharmingen Becton Dickinson.

Lysis buffer, saponin, metrizamide, LPS, OVA ₃₂₃₋₃₃₉ peptide, ovalbumin, alum, and Complete Freund's Adjuvant (CFA) were from Sigma (Saint Quentin Fallavier, France). Tetanus toxin was a gift from H. Yssel (INSERM U454, France). CpG oligodeoxynucleotide-1668, 5'-TCCATGACGTTCTCTGATGCT-3', was obtained from MWG-Biotech AG Ebersberg (Germany). Brefeldin A was purchased from Molecular Probes (Leiden, The Netherlands).

DCs Derived from Bone Marrow

DCs derived from bone marrow (BM-DCs) were generated from bone marrow cells, as described (Inaba et al., 1992). Cells were cultured in complete RPMI medium supplemented with 10 ng/ml GM-CSF and 2.5 ng/ml TNF- α with or without IL-10 (10 to 500 ng/ml). BM-DCs were harvested on day 6.

Purification of Splenic DCs

Spleens were cut into small fragments and digested with collagenase D (1 mg/ml) in HBSS for 20 mn at 37°C. The cells were then layered over RPMI 1640/14.5% metrizamide and centrifuged for 10 mn at 600 g. T cells, B cells, and granulocytes were depleted by treating the recovered low-density cells for 30 mn at 4°C with a monoclonal antibody mixture containing anti-CD3, anti-B220, and anti-Gr-1 or alternatively anti-CD3, anti-CD19, and anti-rat Ig-coated magnetic beads.

Differentiation of TCR Transgenic T Cells by DCs

Naive CD4⁺ T cells from DO11.10 OVA TCR transgenic mice were prepared as described (Groux et al., 1997). Primary stimulation cultures were established by activation of purified naive CD4⁺ T cells (2.5×10^5) with sorted DC populations pulsed with the OVA ₃₂₃₋₃₃₉ peptide (0.6 μ M). After one week, cells were restimulated with fresh sorted DC populations plus 0.3 μ M of the OVA peptide. The same procedure was performed for the third differentiation round. After differentiation, T cells were restimulated with 0.3 μ M OVA ₃₂₃₋₃₃₉ and irradiated total splenic APCs. Proliferation of T cells was assayed by incorporation of ³H-thymidine during the last 12 hr of the 72 hr incubation. The production of IL-4, IL-10, and IFN- γ was measured by ELISA in supernatants collected at 48 hr. Transwell experiments were performed as described (Groux et al., 1997).

Cytology

Sorted DCs generated in vitro or isolated from spleens of BALB/c mice were incubated for 24 hr with LPS (1 μ g/ml) in the presence of GM-CSF (10 ng/ml). DCs were cytocentrifuged (Cytospin2, Shandon) and stained with May-Grünwald-Giemsa.

Real-Time Quantitative RT-PCR Assay

Total RNA from sorted DC subsets (1×10^6 ; purity >99%) was prepared using TRIzol (Life Technologies) as previously described (Cottrez et al., 1994), and any potential contaminating chromosomal DNA was digested with DNase I according to the manufacturer's instructions (Gene Hunter, Nashville, TX). The real-time quantitative PCR was performed with the SYBR Green PCR Core Reagents Kit in an ABI PRISM 5700 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Primers (MWG Biotech) were designed to span exon-intron junctions to prevent amplification of genomic DNA and to result in amplicons between 100 and 150 bp. All primers were used in conditions that prevented the formation of dimers, and accuracy of amplified products was tested by electrophoresis and restriction enzyme digest maps. Expression of target genes was measured after normalization of RNA concentrations with four different housekeeping genes, and values are expressed as fold increased expression above a theoretical negative sample.

Immunization and Adoptive Transfer of OVA-Specific CD4⁺ T Cells and DC Subsets

2.5×10^6 naive OVA-specific T cells were transferred intravenously into BALB/c mice. 3×10^5 sorted DCs were incubated with OVA peptide at 4°C for 2 hr, washed, and injected intravenously into the T cell-transferred mice. These mice were immunized at day 7 with 50 μ g/mouse of ovalbumin or tetanus toxoid (TT) in alum (4 mg/mouse) or CFA injected intraperitoneally or subcutaneously, respectively. Two weeks later, immunization was boosted with 25 μ g/mouse of ovalbumin or TT in alum or 10 μ g/mouse of ovalbumin in CFA.

Analysis of the tolerance induced by DCs was monitored by proliferation assays and cytokine secretion analysis of stimulated purified splenic CD4⁺ T cells with irradiated splenocytes (2500 rad) and OVA protein (250 μ g/ml) or OVA₃₂₃₋₃₃₉ peptide (0.3 μ M). OVA-specific antibodies were measured by ELISA on sera collected every week after the first immunization as previously described (Cottrez et al. 2000).

Transfer of Tolerance

Splenic CD4⁺ T cells from control or tolerized mice were purified by negative depletion with magnetic beads. 10^6 cells were then adoptively transferred intravenously into normal BALB/c mice. Transferred mice were subsequently immunized with OVA/alum as described above.

Cytokine Assays

Sandwich ELISA were used to measure IL-4, IL-10, and IFN- γ as described (Cottrez et al. 2000). IFN- α production was determined by ELISA as described (Asselin-Paturel et al., 2001).

Analysis of OVA-Specific Serum IgE, IgG1, and IgG2a

OVA-specific Ig levels were measured using a two-step sandwich ELISA as described (Cottrez et al., 2000).

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