

CD4⁺ CD25⁺ Regulatory T Cells Control T Helper Cell Type 1 Responses to Foreign Antigens Induced by Mature Dendritic Cells In Vivo

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Abstract

Recent evidence suggests that in addition to their well known stimulatory properties, dendritic cells (DCs) may play a major role in peripheral tolerance. It is still unclear whether a distinct subtype or activation status of DC exists that promotes the differentiation of suppressor rather than effector T cells from naive precursors. In this work, we tested whether the naturally occurring CD4⁺ CD25⁺ regulatory T cells (Treg) may control immune responses induced by DCs in vivo. We characterized the immune response induced by adoptive transfer of antigen-pulsed mature DCs into mice depleted or not of CD25⁺ cells. We found that the development of major histocompatibility complex class I and II-restricted interferon γ -producing cells was consistently enhanced in the absence of Treg. By contrast, T helper cell (Th)2 priming was down-regulated in the same conditions. This regulation was independent of interleukin 10 production by DCs. Of note, splenic DCs incubated in vitro with Toll-like receptor ligands (lipopolysaccharide or CpG) activated immune responses that remained sensitive to Treg function. Our data further show that mature DCs induced higher cytotoxic activity in CD25-depleted recipients as compared with untreated hosts. We conclude that Treg naturally exert a negative feedback mechanism on Th1-type responses induced by mature DCs in vivo.

Key words: primary response • T helper cell type 1/type 2 balance • regulation • inflammation • Toll-like receptors

Introduction

Regulatory T cells (Treg)* are diverse and include at least three populations that differ by their phenotype, cytokine secretion profile, and suppressive mechanism. Tr1 cells have been shown to produce high levels of IL-10 and TGF- β , and require IL-10 for their differentiation and function. Th3 cells produce TGF- β , IL-10, and IL-4, and suppress via a TGF- β -dependent mechanism. CD4⁺ CD25⁺ cells constitutively express the IL-2R α (CD25), inhibit IL-2 production, and promote cell cycle arrest in CD4⁺ and CD8⁺ T cells by a mechanism that requires cell to cell contact, is largely independent on IL-10, and partially dependent on TGF- β . Once activated through their

T cell receptor, these cells suppress in an antigen-nonspecific manner (for review see references 1–3).

T cells constitutively expressing CD25 are stably anergic and suppressive, and control various autoimmune diseases such as gastritis, thyroiditis, and autoimmune diabetes in NOD mice (4). They arise spontaneously during ontogeny and are present in the periphery of normal naive mice. Therefore, CD4⁺ CD25⁺ T cells are “naturally occurring” regulatory cells, whereas Tr1 are induced mainly in the presence of IL-10 and TGF- β . Levings et al. (5) have clearly demonstrated at the clonal level that Tr1 and CD4⁺ CD25⁺ T cells represent two distinct subsets of regulatory cells with different cytokine production profiles.

It is still unclear whether a distinct subtype or activation status of DC exists that promotes the differentiation of regulatory rather than effector T cells from naive precursors. In vitro and in vivo observations suggest that immature DCs may induce the differentiation of Treg in mice and

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*Abbreviations used in this paper: CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; TLR, Toll-like receptor; Treg, regulatory T cells.

humans. However, these Treg seem to involve IL-10–induced Tr1 cells rather than the naturally occurring CD4⁺ CD25⁺ population.

It is intriguing that differentiation of effector cells and homeostasis of CD25–expressing Treg are similarly dependent on TCR triggering, costimulatory signaling, CD40 engagement, and/or IL-2 production. Stimulation via TCR is required for Treg to exert suppression. Of note, very low doses of antigen are required, suggesting that Treg display higher antigen sensitivity than effector T cells (6). IL-2 seems to have a crucial role in the development and maintenance of the CD4⁺ CD25⁺ population *in vitro* and *in vivo* (7) and in the induction of their protective function in a mouse model of spontaneous experimental autoimmune encephalomyelitis (8). Kumanogoh et al. (9) have shown that the CD4⁺ CD25⁺ subpopulation is markedly reduced in CD40-deficient mice and that CD40–CD40L defective interactions can lead to autoimmunity. The essential role of B7/CD28 costimulation in the homeostasis of Treg has been illustrated in NOD mice. Indeed, spontaneous diabetes appears exacerbated in both B7-1/B7-2– and CD28-deficient NOD mice and correlates with a profound decrease in the number of CD4⁺ CD25⁺ T cells (4). Consistent with this finding, agonistic anti-CD28 mAbs have been shown to drive expansion of Treg *in vitro* and *in vivo* (10).

These observations prompted us to investigate whether Treg control immune responses induced by mature DCs that express high levels of antigen–MHC complexes and costimulatory molecules, secrete IL-2 themselves, and induce IL-2 production by T lymphocytes.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were purchased from Harlan Nederland. OT-II mice, transgenic for $\alpha\beta$ -TCR reactive with the I-A^b-restricted 323–339 peptide of OVA, and OT-I mice, transgenic for $\alpha\beta$ -TCR reactive with the H-2K^b-restricted 257–264 peptide of OVA, were provided by P. Dubois (GlaxoSmithKline, Rixensart, Belgium) with the permission of W.R. Heath (The Walter and Eliza Hall Institute, Victoria, Australia). All mice were housed in our pathogen-free facility and used at 6–9 wk of age. The experiments were performed in compliance with the relevant laws and institutional guidelines.

Reagents and Antibodies

The OT-II OVA peptide (chicken OVA peptide 323–339 ISQAVHAAHAEINEAGR) and the OT-I OVA peptide (chicken OVA peptide 257–264 SIINFEKL) were purchased from Neosystem. KLH was purchased from Calbiochem. LPS from *Escherichia coli* (serotype 055:B5) was purchased from Difco Laboratories and 1826 CpG ODN (5'-TCC ATG ACG TTC CTG ACG TT-3') was purchased from Eurogentec Bel S.A. The mAbs used were PC61 (rat IgG1 anti-mouse CD25; produced in house; reference 11), 7D4 (rat IgM anti-CD25; BD Biosciences), N418 (hamster anti-CD11c; produced in house), and GL-1 (rat IgG2a anti-CD86; produced in house). Propidium iodide was

purchased from Sigma-Aldrich. 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes.

Purification of Splenic DCs

Mice were injected i.p. with 10 μ g recombinant human Fms-like tyrosine kinase 3 ligand (human Chinese hamster ovary cell-derived; produced at Amgen) for 9 consecutive days. Spleen cells were digested with collagenase type 3 (Worthington Biochemicals), further dissociated in Ca²⁺-free medium, and separated into low and high density fraction on a Nycodenz gradient (Nycomed).

Mature (Cultured) DCs. Low density cells were cultured for 2 h in RPMI 1640 containing 2% Ultrosor HY (Life Technologies), nonadherent cells were eliminated by vigorous pipetting, and adherent cells were further cultured overnight at 37°C. After overnight culture, nonadherent cells contained at least 90% of DCs, as assessed by morphology and specific staining (unpublished data). For the experiment depicted in Fig. 2 C (comparison of immature and mature DCs), immature DCs, purified as described below, were induced to mature during overnight culture in presence of 20 ng/ml mouse GM-CSF.

Immature (Freshly Isolated) DCs. The low density fraction was separated according to CD11c expression by incubation with anti-CD11c-coupled microbeads followed by one passage over a MACS column (Miltenyi Biotec).

Antigen Pulsing

DCs were incubated with 30 μ g/ml KLH during overnight culture of low density spleen cells; with OVA peptide at the end of purification steps for 1 h and 30 min at 4°C (5 μ g/ml OT-I peptide and 50 μ g/ml OT-II peptide).

FACS[®] Analysis

Purified DCs were double stained for CD11c expression using PE-conjugated N418 and for CD86 expression using FITC-conjugated GL1. Peripheral blood mononuclear cells, splenocytes, and LNs were double stained for CD4 expression using PE-conjugated L3T4 (BD Biosciences) and for CD25 expression using FITC-conjugated 7D4 (BD Biosciences). The cells were gated based on characteristic forward and light scatter, and analyzed on a FACSort[™] (Becton Dickinson).

Immunization Protocol

Antigen-pulsed DCs were administered at a dose of 3×10^5 cells into the hind and/or fore footpads according to a protocol described by Inaba et al. (12). When indicated, some groups of animals received one i.p. injection of 1 mg anti-CD25 mAb (PC61) 3–35 d before immunization. Draining popliteal and axillary LNs were harvested 5 d after immunization.

When indicated, recipient mice were transferred 3 d before immunization with CFSE-labeled OT-II (equivalent of 10^6 transgenic T cells) or OT-I (equivalent of 5×10^5 transgenic T cells) cells. CFSE labeling: splenic and LN cells from transgenic mice (10^7 cells/ml) were incubated with CFSE (0.5 μ M in RPMI) for 10 min at 37°C. Cells were washed in RPMI containing 10% FCS and then in PBS, and injected i.p.

Antigen-specific T Cell Response after *In Vivo* Priming

LN cells were cultured in Click's medium supplemented with 0.5% heat-inactivated mouse serum and additives, with or without antigen (KLH or OVA peptide). The proliferation was measured as thymidine incorporation during the last 16 h of a 3-d culture. Culture supernatants were assayed for IFN- γ , IL-4, and

IL-5 after 72 h of incubation. No IL-4 nor IL-5 was detected in recipients which were transferred with transgenic T cells.

In Vivo CTL Assay (13)

Splenocytes and LN cells from C57BL/6 mice were pulsed or not with 5 μ g/ml OT-I peptide and labeled with CFSE. Peptide-pulsed cells were labeled with CFSE at a final concentration of 5 μ M and unpulsed cells were labeled at a 10-fold lower concentration by incubation for 10 min at 37°C. The two cell populations were mixed at 1:1 ratio and 4–5 $\times 10^7$ cells were injected i.v. into recipient mice. The percentage of antigen-specific lysis *in vivo* is calculated as follows: (1 – number of CFSE^{hi} cells/number of CFSE^{lo} cells) $\times 100$.

Results

***In Vivo* Depletion of CD4⁺ CD25⁺ Population.** Naturally occurring Treg have been shown to constitutively express IL-2R α (CD25) chain and represent 5–10% of peripheral blood CD4⁺ cells in mice. As previous studies have shown that anti-CD25 mAb was capable of depleting CD25⁺ cells *in vivo* (14, 15), 1 mg PC61 was injected i.p. into C57BL/6 mice. Depletion of the IL-2R α ⁺ cells was assessed by flow cytometry using a mAb specific for a distinct epitope (7D4), which confirmed that very few of them remained (from 10% to 1–2%; Fig. 1). Kinetic studies revealed that injection of anti-CD25 mAbs led to a selective loss of CD4⁺ CD25⁺ T cells in blood for at least 30 d and that a replenishment of the population was observed 50 d after treatment (Fig. 1 A). Further analysis of CD4⁺ CD25⁺

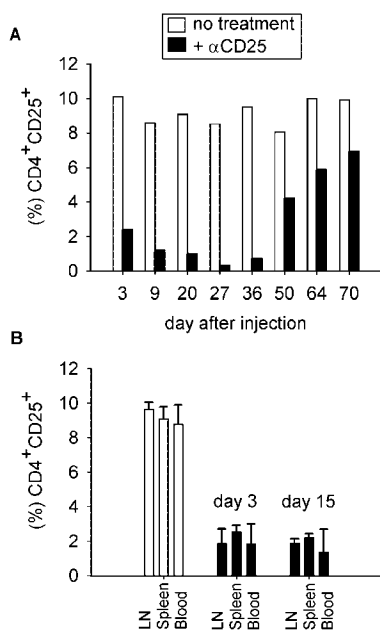


Figure 1. Injection of anti-CD25 mAb results in selective loss of CD25⁺ cells. Mice were injected with 1 mg anti-CD25 mAb (PC61) and analyzed by flow cytometry at the indicated days after treatment. Data are expressed as percentage of CD4⁺ CD25⁺ cells amongst CD4⁺ cells in blood (A) or in LN, spleen, or blood (B). Two (A) and three (B) experiments were performed with similar results. Data are shown for individual mouse (A) or as mean \pm SD of three mice tested individually (B).

population in lymphoid organs showed that these cells were markedly depleted from spleen and LNs on days 3 and 15 after injection of 1 (Fig. 1, B) or 0.25 mg (unpublished data) anti-CD25 mAb. FACS[®] analysis of splenic populations indicated that CD4, CD8, B, NK, granulocytes, DC, and macrophage populations were unaltered in CD25-treated animals (unpublished data). Furthermore, CD4 T cells from CD25-depleted mice displayed higher reactivity when stimulated *in vitro* with allogeneic mature DCs as compared with CD4 T cells from untreated animals (unpublished data). The response is similar to the one of sorted CD4⁺ CD25⁻ cells and is down-regulated by the addition of purified CD25⁺ cells, suggesting that Treg were selectively depleted in PC61-treated animals (unpublished data).

***In Vivo* Depletion of CD25⁺ Cells Enhances the Development of IFN- γ -producing CD4⁺ T Cells by Mature DCs *In Vivo*.** To test whether Treg control the induction of T cell development by mature DCs *in vivo*, we transferred splenic DCs that have undergone spontaneous maturation in culture. In a first set of experiments, splenic DCs, pulsed with protein antigen (KLH) during the purification steps, were injected into the footpads of syngeneic animals that were depleted or not of CD25⁺ cells 7 d earlier. The immune response was analyzed 5 d later in the draining LNs. The data in Fig. 2 A indicate that administration of KLH-pulsed mature DCs in treated and untreated recipients resulted in similar T cell priming as assessed by KLH-dependent proliferation in culture (left). Analysis of the cytokines released by LN cells upon antigenic challenge *in vitro* revealed that mature DCs sensitized cells producing IFN- γ , IL-4, and IL-5 in untreated recipients, as shown previously (16). Of note, injection of DCs in CD25-depleted mice induced the activation of cells secreting higher levels of IFN- γ and lower levels of IL-4 and IL-5 (Fig. 2 A and data not depicted for IL-5). Similarly, a polarized Th1 response was observed in recipients that were treated 35 d earlier with anti-CD25 mAbs (Fig. 2 B), ruling out any possible interference of residual anti-CD25 mAb on T cell activation or DC function. It is noteworthy that T cells from CD25-treated mice displayed an increased IFN- γ production in response to low antigen doses (see the shift in the dose response curve in Fig. 2, A and C) while retaining similar proliferative capacities. The increase in antigen sensitivity ranged between 10- and 100-fold in over 12 independent experiments.

Next, we tested the immune response induced by immature versus mature DCs. To enhance the frequency of peptide-specific CD4⁺ T lymphocytes, unirradiated mice were transferred with OT-II transgenic T cells, the majority of which express a TCR specific for the OVA peptide 323–339 in the context of I-A^b. Transferred recipients were treated or not with anti-CD25 mAbs and injected with immature or mature DCs pulsed with OVA peptide. Immature DCs were freshly isolated from spleen and expressed lower levels of CD86 as compared with mature DCs (Fig. 2 C, insert). Injection of immature splenic DCs failed to induce Th1 priming *in vivo*. Therefore, we tested whether depletion of Treg would restore Th1 priming by immature DCs. The data in Fig. 2 C clearly demonstrate that injection

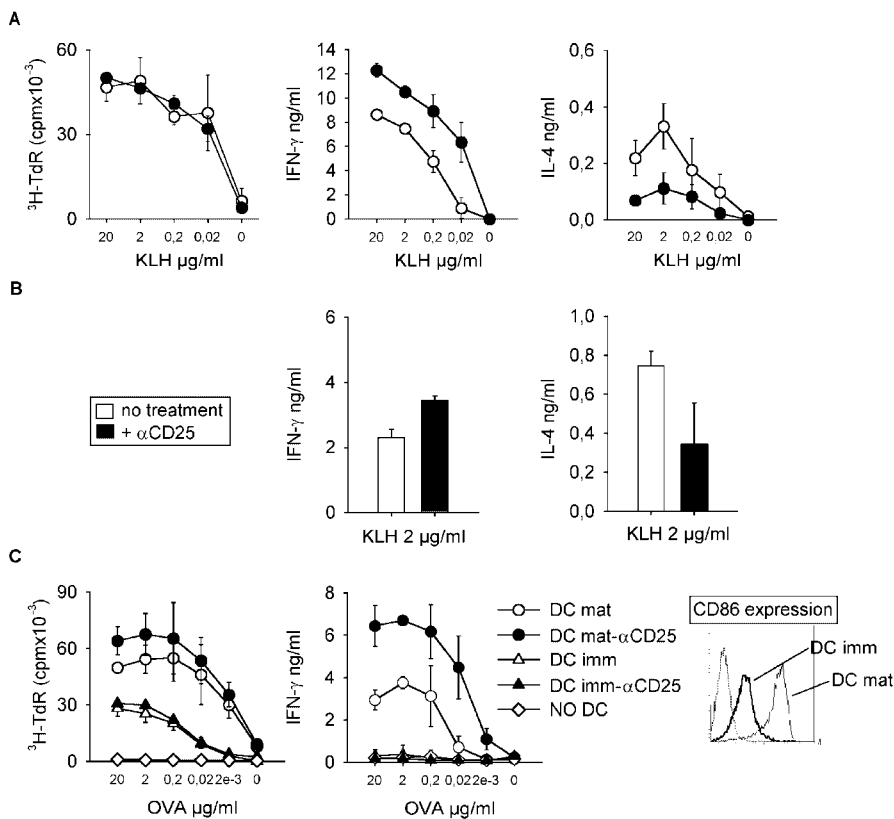


Figure 2. Depletion of Treg results in enhanced development of IFN- γ -producing CD4 $^+$ T cells by mature DCs in vivo. KLH-pulsed mature DCs were injected into the footpads of syngeneic mice that were either untreated (open symbols/bars) or injected with 1 mg PC61 (solid symbols/bars) 7 (A) or 35 d (B) earlier. Draining LNs were harvested 5 d later and cultured with graded doses (A) or 2 $\mu\text{g/ml}$ (B) KLH. Proliferation and lymphokine production were measured as indicated in Materials and Methods. (C) C57BL/6 mice were transferred with T cells from TCR transgenic OT-II mice and immunized s.c. with OVA peptide-pulsed immature or mature DCs (3×10^5 DC/footpad). Proliferation and IFN- γ production were measured in the supernatant of LN cells harvested 5 d after immunization and cultured with graded doses of OVA peptide. The insert in C shows the CD86 expression by immature versus mature DCs. Five (A) and three (B and C) experiments were performed with similar results. Data are shown as mean \pm SD of three individual mice in each group.

tion of freshly isolated DCs in untreated or depleted mice did not sensitize Th1 cells. Collectively, these observations show that Treg selectively down-regulate Th1 responses induced by mature DCs.

DC-derived IL-10 Is Not Required for Treg Function. IL-10 has been shown to be a critical factor for the differentiation of IL-10-producing Treg of Tr1 type. Therefore, we tested whether IL-10 production by donor DCs was required for the regulatory function of CD4 $^+$ CD25 $^+$ T cells. There is evidence that among splenic DCs, the CD8 α^- , but not CD8 α^+ , population produces IL-10 (17, 18). We immunized mice by injection of KLH-pulsed CD8 α^- DCs, a protocol that has been shown to direct the development of Th2-type cells (16). Of note, the polarized Th2-type response (high IL-4) induced by WT CD8 α^- DCs was skewed toward a polarized Th1-type response (high IFN- γ) in the absence of Treg (Fig. 3 A, DC wt). CD8 α^- DCs from IL-10 KO mice induced a mixed Th1/Th2 response in untreated recipients and a polarized Th1 response in PC61-treated mice (Fig. 3 A, DC IL-10 KO). These observations indicate that induction and/or function of Treg in the LNs is not directed by DCs that secrete IL-10.

The Increased Th1 Responses in Treg-depleted Recipients Do Not Require IL-12 nor IFN- γ Production by DCs. Next, we tested whether IL-12 and IFN- γ production by DCs were required for enhanced Th1 priming in deleted recipients. We immunized mice with KLH-pulsed CD8 α^- DCs (to enhance the amplitude of the Th2 to Th1 shift) isolated from mice deficient for IL-12 or IFN- γ , a protocol that in-

duces Th2 priming (Fig. 3 B). Treatment with PC61 mAb resulted in the development of a polarized Th1 response in mice injected with IL-12- or IFN- γ -deficient DCs, showing that production of these cytokines by DCs themselves is not required for Th1 priming in these conditions (Fig. 3 B).

Role of Toll-like Receptor (TLR) Engagement. Interestingly, recent studies have suggested that ligation of TLRs on DCs may overcome CD4 $^+$ CD25 $^+$ T cell-mediated suppression. Pasare and Medzhitov (19) have reported that LPS-treated DCs produce a cytokine (IL-6) that acts on responder T cells and renders them refractory to Treg-mediated suppression in vitro and in vivo. Similarly, George et al. (20) have shown that Treg suppression of T helper proliferation is broken with potent stimulation, i.e., high antigen dose and activated DCs. These observations prompted us to test whether ligation of TLR on DCs may induce an immune response that would not be under the control of Treg. The data in Fig. 4 show that injection of untreated or LPS-treated (unseparated splenic) DCs, pulsed with KLH, induced the development of a mixed immune response and that CpG-treated DCs induced a polarized Th1 response. Depletion of Treg further enhanced the differentiation of IFN- γ -producing cells and down-regulated the production of IL-4 by LN cells from mice primed with untreated or LPS-treated DCs.

Depletion of Treg Enhances the Development of IFN- γ -producing CD8 T Cells and CTLs by Mature DCs In Vivo. Next, we tested whether CD8 T cell priming by mature DCs is similarly under the control of Treg. To study the

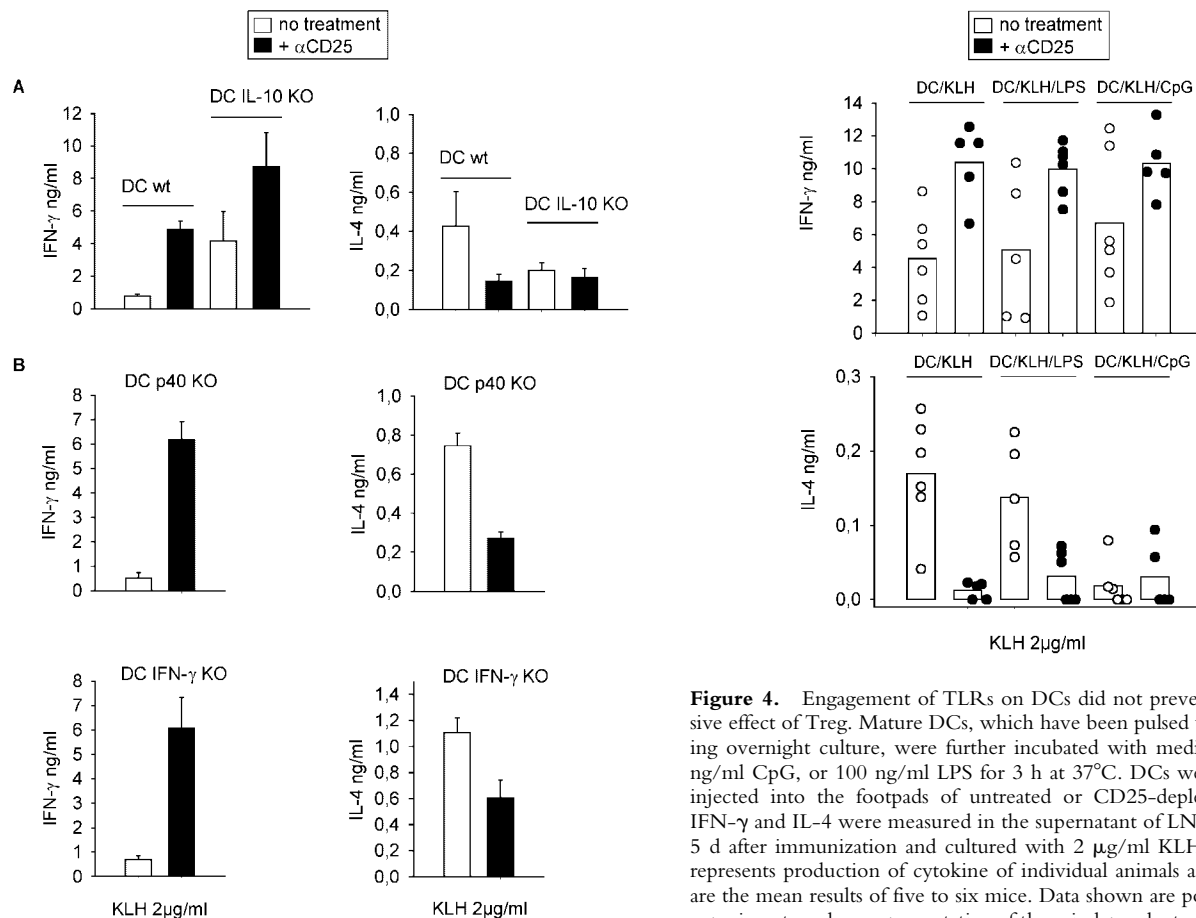


Figure 3. (A) DC-derived IL-10 is not required for Treg function. CD8 α^- DCs from WT or IL-10-deficient mice were pulsed with KLH and injected into the footpads of untreated or depleted WT recipients. 5 d later, LN cells were harvested and cultured with 2 μ g/ml KLH. The IFN- γ and IL-4 content was measured in the supernatant at 72 h. (B) The enhanced Th1 priming is independent of DC-derived IL-12 and IFN- γ . Purified CD8 α^- DC from IL-12 or IFN- γ KO mice were pulsed overnight with KLH and injected into the footpads of WT recipients treated or not with anti-CD25 mAb. IFN- γ and IL-4 were measured in the supernatant of LN cells harvested 5 d after immunization and cultured with 2 μ g/ml KLH. Four (A and B) and three (C) experiments were performed with similar results. Data are shown as mean \pm SD of three individual mice in each group.

activation of MHC class I-restricted CD8 $^+$ T lymphocytes, unirradiated mice were transferred with OT-I transgenic T cells, the majority of which express a TCR specific for the OVA peptide 257–264 in the context of H-2K b . Transferred recipients were treated or not with anti-CD25 mAbs and injected with mature DCs pulsed with OVA peptide. The data in Fig. 5 show that antigen-specific IFN- γ production was strongly enhanced upon PC61 treatment (Fig. 5 A) and that LN cells from CD25-treated mice were hyperreactive as indicated by enhanced responsiveness to low antigen stimulation. To monitor the differentiation of OVA-specific CTLs in vivo, untransferred mice were immunized by injection of mature DCs, pulsed with OT-I peptide, into the hind footpads. 5 d later, CTLs were assayed in vivo by a method originally described by Aichele

Figure 4. Engagement of TLRs on DCs did not prevent the suppressive effect of Treg. Mature DCs, which have been pulsed with KLH during overnight culture, were further incubated with medium alone, 500 ng/ml CpG, or 100 ng/ml LPS for 3 h at 37°C. DCs were washed and injected into the footpads of untreated or CD25-depleted recipients. IFN- γ and IL-4 were measured in the supernatant of LN cells harvested 5 d after immunization and cultured with 2 μ g/ml KLH. Each symbol represents production of cytokine of individual animals and vertical bars are the mean results of five to six mice. Data shown are pooled from two experiments and are representative of three independent experiments.

et al. (13). In brief, splenocytes, pulsed or not with OT-I peptide, were labeled with distinct intensities of CFSE fluorescence, mixed 1:1, and transferred i.v. as indicator cells into naive or primed recipients. Fluorescence-activated cell sorter analysis of LN and spleen revealed two CFSE-labeled indicator cells of comparable size in naive mice (Fig. 5 B) or in mice injected with unpulsed DCs (not depicted). Percentage of peptide-specific lysis was calculated by comparing the relative loss of peptide-pulsed versus unpulsed indicator cells and revealed a lysis of 0–10% in the absence of immunization. By contrast, the OVA peptide-loaded CFSE $^{\text{hi}}$ cell population was strongly reduced in numbers when compared with the unloaded CFSE $^{\text{lo}}$ population in the draining LNs of mice primed with mature DCs, treated or not with PC61. Of note, the reduction of OVA peptide-pulsed indicator cells was more pronounced in non-draining LNs and spleens of CD25-treated recipients as compared with untreated mice (Fig. 5 B).

Discussion

The data presented herein underscore the crucial role of DCs in the regulation of immunity and show that the function of mature DCs is under the control of the naturally occurring CD4 $^+$ CD25 $^+$ cells in vivo. Our observations

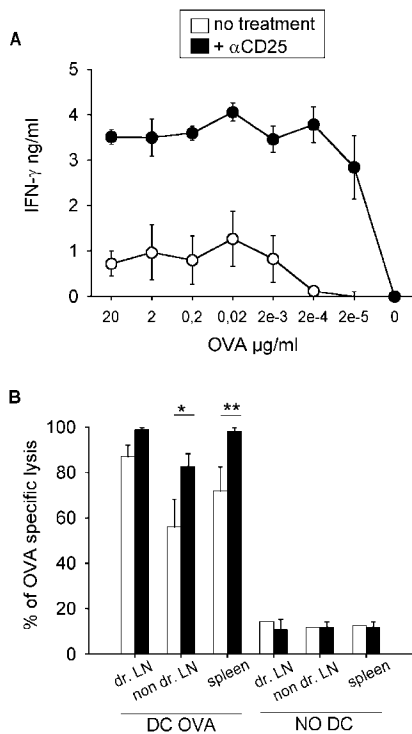


Figure 5. Enhanced development of IFN- γ -producing CD8 T cells and CTLs by mature DCs in CD25-depleted recipients. (A) Mature splenic DCs, pulsed with OT-I OVA peptide, were injected into the footpads of syngeneic animals, which were transferred 2 d earlier with cells from OT-I transgenic mice. A group of mice was treated 7 d earlier with PC61. LN cells were harvested 3 d after DC injection and cultured with graded doses of OVA OT-I peptide. IFN- γ production was measured after 72 h of culture. Data are shown as mean of cytokine production by three individual mice \pm SD. Four experiments were performed with similar results. (B) Mature DCs, pulsed with OT-I peptide, were injected into the hind footpads of untransferred recipients. The cytotoxic activity was assayed in vivo 5 d after immunization. Data represent the relative loss of antigen-pulsed indicator cells (CFSE^{hi}) as compared with unpulsed indicator cells (CFSE^{lo}). Data are shown as mean of the percent antigen-specific lysis by three to four individual mice \pm SD. The groups indicated by asterisks are significantly different from control. Student's *t* test, *, $P < 0.02$ or **, $P < 0.005$. Three experiments were performed with similar results.

suggest that Treg exert a negative feedback mechanism on immune responses specific for nonself antigens and influence the character of T cell differentiation by selectively dampening Th1-type responses and CTLs. As the effector mechanisms that protect the host from invading microorganisms can induce immune-mediated pathologies if not properly regulated, Treg may constitute a physiological homeostatic checkpoint.

Recent evidence suggests that DCs are involved in immunity and tolerance, depending on their maturation state. DCs in the steady state seem to transport antigen from the trachea and the intestine to the draining LNs, inducing a state of tolerance (21, 22). Targeting an antigen on nonactivated DCs resulted in abortive immune responses leading to a state of antigen-specific unresponsiveness in a murine model (23). Activation of DCs restored the immunostimu-

latory properties of DCs in the same conditions. In humans, injection of immature DCs pulsed with influenza peptide into volunteers rendered them unable to respond to a subsequent immunogenic stimulus (24). Based on these observations, it was suggested that immature DCs constitutively expressing self-antigens would directly or indirectly induce a state of peripheral tolerance. Indirect mechanisms of tolerance may include the differentiation/activation of Treg.

However, although the type of Treg has not been identified in all studies, there is evidence that immature DCs produce IL-10 and induce a state of tolerance by triggering the differentiation and function of Tr1 cells in mice and humans (25, 26). By contrast, our observations suggest that mature DCs induce a regulatory mechanism mediated by CD4⁺ CD25⁺ T cells. Depletion of Treg did not restore Th1 priming by immature DCs in our experimental setting, suggesting that the lack of differentiation is not due to CD4⁺ CD25⁺ cells. Although we cannot rule out the possibility that Treg are induced by immature DCs at a time when anti-CD25 mAbs are no longer present, we found that the number of CD4⁺ CD25⁺ T cells was not enhanced in the blood of mice that received three to five injections of immature DCs (unpublished data). Experiments are underway to test whether Tr1 cells are induced in recipients primed with one injection of immature DCs.

It is noteworthy that both DCs and Treg express several TLRs, suggesting that their function can be regulated by microbial products. An interesting study has suggested that DCs, activated by TLR ligand, would induce an immune response that is refractory to inhibition by Treg (19). We found, however, that splenic DCs that have matured spontaneously in culture or have been treated with LPS or CpG activate immune responses that remain sensitive to Treg function. Caramalho et al. (27) have further shown that Treg are activated by LPS. Collectively, these observations suggest that the feedback mechanism mediated by Treg is enhanced by microbial agents.

Our results show that Treg control immunity to foreign antigens (KLH protein and OVA class I and II peptides). These observations are consistent with reports demonstrating pathogen-specific Treg in viral, bacterial, fungal, and parasitic infection. In particular, during infection by *Leishmania major*, CD4⁺ CD25⁺ T cells accumulate in the dermis where they prevent elimination of the parasite by effector cells. Interestingly, the persistence of parasite appears essential for immunity to reinfection (28). Whether depletion of Treg may affect the onset of memory responses will be an important issue to address in future experiments.

The molecular mechanisms responsible for regulatory activity are still a matter of speculation. Thornton and Shevach (29) have demonstrated that Treg suppress the proliferation of conventional T cells by specifically inhibiting the production of IL-2. The role of cytokines (IL-10, TGF- β) and cell-associated molecules (CTLA-4, GITR) remains controversial in vivo (for review see reference 2). IL-10-dependent and -independent mechanisms have been found to promote parasite persistence in mice infected with *L.*

major (28). A role of IL-10 would be consistent with the selective inhibitory effect of Treg on Th1 responses. Our data further show that the enhanced Th1 responses in CD25-depleted mice are independent from IL-12 and IFN- γ production by transferred DCs. These findings were unexpected as we have previously shown that the activation of Th1 responses by DCs correlates with their production of IL-12p70 heterodimer and IFN- γ (17).

There is little information about the trafficking of Treg in vivo. CD4⁺ CD25⁺ T cells were found in recipient lymphoid tissues after transplantation and at the graft site (30). Our data show that Treg limit the development of Th1 cells in the LNs draining the site of injection. Additionally, these cells may migrate to the inflammatory site where that may inhibit the effector function of differentiated Th1 cells.

Our data show that both CD4 and CD8 lymphocytes are under the control of CD4⁺ CD25⁺ Treg. The IFN- γ production by MHC class I-restricted CD8 T cells was strongly enhanced upon Treg depletion. The CTL activity was assessed by an in vivo assay and was significantly increased in the spleen and nondraining LNs. Our observations are consistent with recent reports demonstrating that the numbers of CD4⁺ CD25⁺ T cells inversely correlated with the numbers of memory phenotype CD8⁺ T cells with age in mice (31) and that CD4⁺ CD25⁺ T cells regulate human CD8⁺ T cell function in vitro (32).

In conclusion, CD4⁺ CD25⁺ Treg not only inhibit immune responses against self-antigens (33, 34), but also suppress those against microbes (28) and innocuous antigens (this paper). It has been suggested that the stage of activation of DCs may tune the degree of suppressive activity of Treg, with resting DCs activating Treg and mature DCs suppressing Treg (35). However, our results indicate that Treg dampen the immune responses induced by fully competent DCs in vivo. We believe that DCs that are able to discriminate innocuous self from microbial nonself may regulate the immune responses to self and nonself indirectly through distinct populations of suppressor cells. Thus, immature DCs would induce the differentiation of IL-10-producing Tr1 cells, whereas mature DCs would promote the survival and/or function of naturally occurring CD4⁺ CD25⁺ Treg. The combined action of DCs in the thymus and immature DCs in the periphery (via Tr1) would strongly limit the number of precursor T cells specific for self-antigens. Therefore, CD4⁺ CD25⁺ Treg would have a strong suppressive effect on "low" self-specific responses, thereby avoiding autoimmunity. The same Treg would limit "strong" Th1 responses specific to microbial antigens to avoid excessive inflammatory responses and serious damage to the host. The IL-10-induced Tr1 cells and constitutive CD4⁺ CD25⁺ Treg would therefore differentially regulate effector immune responses whether they are specific to a self-constituent (presented by immature DCs) or to a microbe (presented by maturing DCs).

Our observations suggest that presentation of tumor antigen by mature DCs and silencing of CD4⁺ CD25⁺ Treg may synergistically augment tumor-specific immunity and

open novel opportunities to optimize DC-based immunotherapy in cancer patients.

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References

1. Shevach, E.M. 2002. CD4⁺ CD25⁺ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2:389–400.
2. Wood, K.J., and S. Sakaguchi. 2003. Regulatory lymphocytes: regulatory T cells in transplantation tolerance. *Nat. Rev. Immunol.* 3:199–210.
3. Maloy, K.J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol.* 2:816–822.
4. Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4⁺ CD25⁺ immunoregulatory T cells that control autoimmune diabetes. *Immunity.* 12:431–440.
5. Levings, M.K., R. Sangregorio, C. Sartirana, A.L. Moschin, M. Battaglia, P.C. Orban, and M.G. Roncarolo. 2002. Human CD25⁺ CD4⁺ T suppressor cell clones produce transforming growth factor β , but not interleukin 10, and are distinct from type 1 T regulatory cells. *J. Exp. Med.* 196:1335–1346.
6. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182:18–32.
7. Papiernik, M., M.L. de Moraes, C. Pontoux, F. Vasseur, and C. Penit. 1998. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. *Int. Immunol.* 10:371–378.
8. Furtado, G.C., M.A. Curotto de Lafaille, N. Kutchukhidze, and J.J. Lafaille. 2002. Interleukin 2 signaling is required for CD4⁺ regulatory T cell function. *J. Exp. Med.* 196:851–857.
9. Kumanogoh, A., X. Wang, I. Lee, C. Watanabe, M. Kamanaka, W. Shi, K. Yoshida, T. Sato, S. Habu, M. Itoh, et al. 2001. Increased T cell autoreactivity in the absence of CD40-CD40 ligand interactions: a role of CD40 in regulatory T cell development. *J. Immunol.* 166:353–360.
10. Lin, C.H., and T. Hunig. 2003. Efficient expansion of regulatory T cells in vitro and in vivo with a CD28 superagonist. *Eur. J. Immunol.* 33:626–638.
11. Lowenthal, J.W., P. Corthesy, C. Tougne, R. Lees, H.R.

- MacDonald, and M. Nabholz. 1985. High and low affinity IL 2 receptors: analysis by IL 2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. *J. Immunol.* 135:3988–3994.
12. Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J. Exp. Med.* 172:631–640.
 13. Aichele, P., K. Brduscha-Riem, S. Oehen, B. Odermatt, R.M. Zinkernagel, H. Hengartner, and H. Pircher. 1997. Peptide antigen treatment of naive and virus-immune mice: antigen-specific tolerance versus immunopathology. *Immunity.* 6:519–529.
 14. McHugh, R.S., and E.M. Shevach. 2002. Cutting edge: depletion of CD4⁺ CD25⁺ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J. Immunol.* 168:5979–5983.
 15. Taguchi, O., and T. Takahashi. 1996. Administration of anti-interleukin-2 receptor alpha antibody in vivo induces localized autoimmune disease. *Eur. J. Immunol.* 26:1608–1612.
 16. Maldonado-Lopez, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. CD8 α^+ and CD8 α^- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med.* 189:587–592.
 17. Maldonado-Lopez, R., C. Maliszewski, J. Urbain, and M. Moser. 2001. Cytokines regulate the capacity of cd8alpha(+) and cd8alpha(-) dendritic cells to prime th1/th2 cells in vivo. *J. Immunol.* 167:4345–4350.
 18. Legge, K.L., R.K. Gregg, R. Maldonado-Lopez, L. Li, J.C. Caprio, M. Moser, and H. Zaghoulani. 2002. On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. *J. Exp. Med.* 196:217–227.
 19. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4⁺ CD25⁺ T cell-mediated suppression by dendritic cells. *Science.* 299:1033–1036.
 20. George, T.C., J. Bilsborough, J.L. Viney, and A.M. Norment. 2003. High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression in vitro. *Eur. J. Immunol.* 33:502–511.
 21. Huang, F.P., N. Platt, M. Wykes, J.R. Major, T.J. Powell, C.D. Jenkins, and G.G. MacPherson. 2000. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J. Exp. Med.* 191:435–444.
 22. Vermaelen, K.Y., I. Carro-Muino, B.N. Lambrecht, and R.A. Pauwels. 2001. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J. Exp. Med.* 193:51–60.
 23. Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J.V. Ravetch, R.M. Steinman, and M.C. Nussenzweig. 2001. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* 194:769–779.
 24. Dhodapkar, M.V., R.M. Steinman, J. Krasovsky, C. Munz, and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med.* 193:233–238.
 25. Jonuleit, H., E. Schmitt, G. Schuler, J. Knop, and A.H. Enk. 2000. Induction of interleukin 10-producing, nonproliferating CD4⁺ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192:1213–1222.
 26. Menges, M., S. Rössner, C. Voigtlander, H. Schindler, N.A. Kukutsch, C. Bogdan, K. Erb, G. Schuler, and M.B. Lutz. 2002. Repetitive injections of dendritic cells matured with tumor necrosis factor α induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med.* 195:15–21.
 27. Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* 197:403–411.
 28. Belkaid, Y., C.A. Piccirillo, S. Mendez, E.M. Shevach, and D.L. Sacks. 2002. CD4⁺ CD25⁺ regulatory T cells control Leishmania major persistence and immunity. *Nature.* 420:502–507.
 29. Thornton, A.M., and E.M. Shevach. 1998. CD4⁺ CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188:287–296.
 30. Graca, L., S.P. Cobbold, and H. Waldmann. 2002. Identification of regulatory T cells in tolerated allografts. *J. Exp. Med.* 195:1641–1646.
 31. Murakami, M., A. Sakamoto, J. Bender, J. Kappler, and P. Marrack. 2002. CD25⁺ CD4⁺ T cells contribute to the control of memory CD8⁺ T cells. *Proc. Natl. Acad. Sci. USA.* 99:8832–8837.
 32. Camara, N.O., W.F. Ng, M. Hernandez-Fuentes, E. Eren, and R.I. Lechler. 2002. Human CD4(+)CD25(+) T cells regulate CD8(+) T-cell activation. *Transplant. Proc.* 34:2858–2860.
 33. Suri-Payer, E., A.Z. Amar, A.M. Thornton, and E.M. Shevach. 1998. CD4⁺ CD25⁺ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* 160:1212–1218.
 34. Mottet, C., H.H. Uhlig, and F. Powrie. 2003. Cutting edge: cure of colitis by CD4(+)CD25(+) regulatory T cells. *J. Immunol.* 170:3939–3943.
 35. Sakaguchi, S. 2003. Control of immune responses by naturally arising CD4⁺ regulatory T cells that express Toll-like receptors. *J. Exp. Med.* 197:397–401.