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*J Immunol* 2006; 176:6202-6210; doi: 10.4049/jimmunol.176.10.6202

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Modulation of Dendritic Cell Function by Naive and Regulatory CD4+ T Cells

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The consequences of interactions between dendritic cells (DCs) and either naive CD4+ T cells or regulatory CD4+CD25+ T cells on the expression of proinflammatory IL-6 and anti-inflammatory IL-10 in DC were examined over a period of 12 h, spanning the time frame during which stable T cell–DC interactions shape the development of tolerance and immunity in vivo. We demonstrate that the basal production of IL-6 and IL-10, which is initiated following DC stimulation with LPS, is modified in distinctly different ways by interaction with the two T cell populations. Naive CD4 T cells skew DC cytokine production toward IL-6 and suppress IL-10, whereas CD4+CD25+ T cells have the opposite effect. CD8 T cells or memory CD4 T cells do not influence basal cytokine production by stimulated DC. The effect of CD4+CD25+ T cells is dominant in coculture with naive CD4 T cells as long as inflammatory LPS is absent; the addition of LPS abrogates the suppression of IL-6. However, the modulating influence of CD4+CD25+ T cell populations following contact with pathogens are likely to influence the strength and quality of incipient immune responses in the local microenvironment. The Journal of Immunology, 2006, 176: 6202–6210.

The immune response is faced with two mutually exclusive requirements. It needs to deal efficiently with pathogenic threats by responding with great speed and potency, whereas simultaneously trying to avoid immune-pathology and inappropriate responses to benign stimuli. Dendritic cells (DCs) are central to the initiation and orchestration of immunity and tolerance (1, 2). Generated in the bone marrow, DC migrate to peripheral organs where they are distributed to optimize capture of Ags as an integral part of the innate immune system (3). Microorganisms are recognized through pattern-recognition receptors that directly bind conserved pathogen-associated molecular patterns, which upon ligation initiate DC maturation (4). Thereafter, DC migrate to the draining lymphoid tissues where they make contact with Ag-specific lymphocytes (3).

The function of the mediators of adaptive immunity, T and B cells, is under direct control of DC (5). They can polarize the adaptive immune response by biasing the development of CD4+ T cell subsets, providing help for cellular and humoral immunity (6). The encounter with microorganisms and subsequent maturation of DC involves up-regulation of surface MHC class II and costimulatory molecules, as well as the secretion of both pro- and anti-inflammatory cytokines. In addition to these stimuli, cell-cell contact, for example, the interaction between DC40 on DC and CD154 on CD4+ T cells, plays a critical role in the induction of inflammatory cytokines (7–9).

A growing body of evidence has implicated a particular subpopulation of CD4+ T cells in immune regulation (10, 11). Naturally occurring CD4+CD25+ regulatory T cells (Tregs), were originally identified by their ability to prevent autoimmunity (12). They have since been shown to attenuate antitumor immunity (13), prevent expansion of T cells in vivo (14), limit immune pathology in the face of chronic immune stimulation (15), and inhibit T cell activation in vitro (16). In vitro studies, that first defined the suppressor function of Treg, have shown their capacity to inhibit proliferation and IL-2 mRNA transcription of naive responder T cells (16). The inhibition of proliferation could be blocked in the presence of an inflammatory stimulus and attributed to the secretion of IL-6 by DC (17).

The precise mechanisms by which Tregs exert their suppressive capacity remain unknown and may vary depending on the nature of the immune response being regulated. Attention has focused on the cytokines IL-10 and TGFβ1, which are thought to regulate the differentiation and effector function of proinflammatory Th1 cells (18, 19). There is clear evidence (20–22) that each of these T cell-derived cytokines contributes to peripheral tolerance and in vitro suppression, even though suppression in vitro can occur independently of cytokines, suggesting that the immunosuppressive spectrum of Tregs is extensive. Furthermore, suppressive mechanisms include direct effects on the functionality of APC by affecting expression of costimulatory molecules and maturation markers, as shown for anergic CD4+ T cell clones (23, 24), and CD4+CD25+ Tregs cells in vitro (25–27) as well as in vivo (28).

In this study, we investigated the consequences of interactions between DC and either naive CD4+ or CD4+CD25+ Tregs on cytokine gene expression in DC spanning a time frame during which stable interactions between T cells and DC precede the development of both tolerance and immunity in vivo. The analysis was focused on two cytokines, IL-6 and IL-10, as examples for proinflammatory and anti-inflammatory cytokines, respectively. IL-6 is a critical cytokine directing transition from innate to adaptive immune responses through management of the acute phase response and enhancement of the expansion of Ag-specific CD4 T cells.
cells (29). IL-10, in contrast, is a known immunosuppressive cytokine essential in limiting immune responses to numerous pathogens and preventing immune pathology (30, 31).

We demonstrate that the basal production of IL-6 and IL-10, which is initiated following DC stimulation, is modified in dis-tinctively different ways by interaction with the two T cell populations. Our data suggest that the mutual interactions between DC and CD4^\textsuperscript+ but not CD8^\textsuperscript+ T cell populations are likely to influence the strength and quality of ensuing immune responses in the local microenvironment.

**Materials and Methods**

**Mice**  
C57BL/10, C57BL/6, C57BL/6 CD45.1, C57BL/6 IL-10^\textsuperscript−/−, and C57BL/6 MyD88^\textsuperscript−/− mice were bred under specific pathogen-free conditions, and experimental animals were kept in conventional, but pathogen-free, animal facilities at the National Institute for Medical Research (London, U.K.) in accordance with local guidelines.

**In vitro stimulation**  
The culture medium used was IMDM (Sigma-Aldrich) supplemented with 5% heat-inactivated FCS, 2 × 10^{-3} M l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^{-3} M 2-ME (all Sigma-Aldrich).

Cells were cultured in a 1 ml volume containing 5 × 10^5 bone marrow-derived DC (BMDC) with or without 7.5 × 10^3 naive CD4^\textsuperscript+ T cells with or without 7.5 × 10^4 CD4^\textsuperscript+CD25^\textsuperscript− T cells, 0.5 μg/ml anti-CD3 (45-2C11), 100 ng/ml LPS (from Salmonella minnesota R595, ultra pure TLR4 grade; Alexis Biochemicals), 200 nM CpG (1668; Invitrogen Life Technologies), 10 ng/ml TGFβ (Sigma-Aldrich), or 10 μg/ml αTGFβ (ID11.16; a gift from Dr. A. Cook, Department of Pathology, University of Cambridge, Cambridge, U.K.). Cells were harvested at indicated time points and lysed using TRIzol Reagent (Invitrogen life Technologies).

**Cell purification**  
Single-cell suspensions from spleens and lymph nodes were stained with anti-CD25-PE followed by anti-PE magnetic microbeads, and enriched by positive selection on an AutoMACS (Miltenyi Biotec) according to the manufacturer’s instructions. The positive fraction was then sorted on a MoFlo cytometer (DakoCytomation) to obtain pure populations of CD4^\textsuperscript+CD25^\textsuperscript− T cells (>99% purity), and the negative fraction was sorted into CD25^\textsuperscript+CD4^\textsuperscript+CD25^\textsuperscript− cells (>99.5% purity), whereas CD25^\textsuperscript−CD4^\textsuperscript+CD25^\textsuperscript− sorted cells were used as the memory/activated T cell fraction.

For the enrichment of splenic DC, spleens were treated for 30 min at 37°C with 0.4 mg/ml Liberase Cl (Boehringer Mannheim), followed by RBC lysis using 0.83% ammonium chloride. DC were stained with anti-CD11c magnetic microbeads (Miltenyi Biotec) and enriched by positive selection with specific cytokine Abs or isotype control. Cells were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences) and were further analyzed using FlowJo software (Tree Star). Dead cells were excluded using forward and side scatter.

**Results**

**Stimulation of DC results in pro- and anti-inflammatory cytokines**  
Before investigating the effect of different T cell populations on cytokine secretion by DC in vitro, we first determined the kinetics of basal cytokine gene expression by DC following stimulation. DC were generated from bone marrow cultures stimulated with GM-CSF for 6–8 days without addition of cytokines or LPS, promoting the generation of CD11c^\textsuperscript+CD11b^\textsuperscript+B220^−CD4^+CD8^− myeloid DC (33), whose CD11c and MHC class II profile indicated >95% DC purity (Fig. 1A). BMDC were cultured in vitro with either LPS or CpG to mimic inflammation through TLR ligation. Anti-CD3, which served to activate T cells in cocultures of T cells and DC, was also evaluated to control for any effect on cytokine expression by DC in the absence of T cells.

IL-6 and IL-10 were chosen as representatives of pro-and anti-inflammatory cytokines, respectively, and the kinetics of mRNA production for IL-6 and IL-10 were determined over a period of up to 14 h in 1- to 2-h intervals, spanning the time frame during which stable T cell–DC interactions precede the development of both tolerance and immunity in vivo (34). The results were normalized to show fold increase of cytokine mRNA expression over mRNA levels determined at time point 0. Anti-CD3 alone did not induce mRNA for IL-6 (Fig. 1B) or IL-10 (Fig. 1C). Stimulation via TLR4 with LPS induced high mRNA levels for both pro- and anti-inflammatory cytokines IL-6 (Fig. 1B) and IL-10 (Fig. 1C). Similar results were obtained following stimulation via TLR9 with CpG (data not shown).

To verify that BMDC represent DC resident in lymphoid organs, splenic cells were enriched for DC using MACS selection via CD11c (Fig. 1D), with a purity of around 60%. Ex vivo splenic DC were cultured with either anti-CD3 or LPS and, as shown in Fig. 1, E and F, their pro- and anti-inflammatory cytokine profile was similar to that of BMDC, although the reduced purity of the population resulted in lower signal amplification after stimulation. Given the difficulties in isolating large numbers of pure DC from lymphoid organs, the following experiments were all conducted using BMDC, which can be generated in sufficient quantities to allow kinetic analysis of cytokine mRNA expression.

**Cytokine detection using Luminex**  
Multiple cytokines were assayed on a Luminex SD with a Bioplex system (Bio-Rad) according to the manufacturer’s instructions. Using mouse IL-10 and IL-6–Beads and Beadlyte Mouse Multicytokine Beadmaster kit (all Upstate Biotechnology), 50 μl supernatants from cultured cells were assayed according to the manufacturer’s instructions; concentrations of cytokines were calculated against Multicytokine Standard 2 (Upstate Biotechnology).

**Abs and flow cytometry**  
Anti-CD4 aliphophycocyanin (RM4-5), anti-CD25 PE (PC61), anti-CD44 PE (IM7), anti-CD62L aliphophycocyanin (MEL14), anti-CD54.2 biotin (104), anti-IFN-γ FITC (XM1G1.2), and anti-CD11c PE (N418) were purchased from eBioscience; and streptavidin-aliphophycocyanin was purchased from Molecular Probes. Anti-CD3 (145-2C11) and anti-MHC class II (M5/114) were purified from hybridoma supernatant in our laboratory using standard procedures. For determination of intracellular cytokine production, cells were restimulated with 500 ng/ml phorbol dibutyrate, 500 ng/ml ionomycin, and 10 μg/ml brefeldin A for 4 h at 37°C. Cells were then stained for surface markers, fixed in 100 μl of 3% paraformaldehyde in PBS, and permeabilized with 0.1% Nonidet P-40 PBS for 3 min, followed by labeling with specific cytokine Abs or isotype control. Cells were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences) and were further analyzed using FlowJo software (Tree Star). Dead cells were excluded using forward and side scatter.
Naive CD4$^+$ T cells maintain a proinflammatory DC cytokine profile

Cell–cell interactions play an important role in immune regulation, providing bidirectional stimulatory signals that are important in the activation of specific T cells and in the regulation of bystander cells. This cross-talk between DC and T cell not only influences the surface expression of costimulatory molecules, but also regulates the production of cytokines (35).

We, therefore, investigated the kinetics of cytokine expression in DC in the presence of naive (CD62L$^{high}$CD44$^{low}$CD25$^-$) CD4$^+$ T cells that were activated by anti-CD3. Control cultures containing only BMDC were set up in parallel to allow comparison of the effects of T cells vs inflammatory stimuli on cytokine production by DC. It should be emphasized that, during the time frame we are focusing on, we are detecting cytokine expression in DC only, although the cocultured T cells are not removed before RT-PCR analysis. Stimulation of naive CD4$^+$ T cells with immobilized anti-CD3 and anti-CD28 in the absence of DC did not result in detectable levels of IL-6 or IL-10 mRNA during the 14-h culture period (data not shown).

In the absence of an inflammatory signal, coculture of BMDC with FACS-sorted naive CD4$^+$ T cells was found to marginally increase levels of IL-6 mRNA (Fig. 2A), while no IL-10 mRNA could be found (Fig. 2B). T cell activation in the context of...
invading microorganisms was mimicked with the addition of LPS, which provided the inflammatory signal in these cultures. In the presence of LPS, interaction of DC with naive CD4 \(^{+}\) T cells activated via anti-CD3 produced similar mRNA levels of the proinflammatory cytokine IL-6 (Fig. 2C), but lower mRNA levels of the anti-inflammatory cytokine IL-10 (Fig. 2D) when compared with BMDC cultured with LPS without T cells. In contrast with naive CD4 \(^{+}\) T cells, naive CD8 \(^{+}\) T cells did not influence IL-6- or IL-10 mRNA levels when cocultured with BMDC in the absence or presence of LPS (Fig. 3).

**CD4\(^{+}\)CD25\(^{+}\) T cells induce an anti-inflammatory DC cytokine profile**

Next, we asked whether CD4\(^{+}\)CD25\(^{+}\) T cells differentially influence the expression of DC-derived pro- and anti-inflammatory cytokines. In the absence of an inflammatory signal but with anti-CD3, cocultures of BMDC and CD4\(^{+}\)CD25\(^{+}\) T cells failed to induce any IL-6 mRNA (Fig. 4A), but a sharp increase in IL-10 mRNA could be shown, peaking at 2 h (Fig. 4B). Stimulation of CD4\(^{+}\)CD25\(^{+}\) T cells with immobilized anti-CD3 and anti-CD28 did not result in detectable levels of IL-6 or IL-10 mRNA during the first 14 h (data not shown). Cocultures of BMDC and CD4\(^{+}\)CD25\(^{+}\) T cells in the presence of the inflammatory signal LPS, resulted in decreased mRNA levels of IL-6 (Fig. 4E) but increased mRNA levels of IL-10 (Fig. 4F) when compared with BMDC cultured without T cells. Coculture of DC with CD4 T cells of a memory/activated phenotype (CD25\(^{+}\)CD44\(^{hi}\)) in the absence of an inflammatory signal increased levels of IL-6 mRNA (Fig. 4C), but lacked the sharp increase of IL-10 seen with cocultures containing CD4\(^{+}\)CD25\(^{+}\) T cells (cf. Fig. 4, D vs B). The increase in IL-6 mRNA is largely T cell derived, because stimulation of memory/activated T cells with anti-CD3/anti-CD28-coated beads in the absence of DC resulted in detectable IL-6 mRNA (data not shown). Activated/memory CD4 T cells, like naive CD4 T cells, but in contrast with CD4\(^{+}\)CD25\(^{+}\) T cells, did not strongly influence IL-6 mRNA induction in the presence of LPS and reduced the levels of IL-10 mRNA (Fig. 4, G and H).

**FIGURE 3.** Naive CD8\(^{+}\) T cells do not influence pro- or anti-inflammatory DC cytokines. A and B, C57BL/6 BMDC cultured with anti-CD3 alone (▲) or naive CD8\(^{+}\) T cells (●). C and D, The same experimental set up as A and B with the addition of LPS. mRNA for IL-6 (A and C) and IL-10 (B and D) was assessed at 1- to 2-h intervals. Values are plotted as fold increase over mRNA levels in DC at time point 0. The experiment shown was repeated twice with similar results.

**FIGURE 4.** CD25\(^{+}\) CD4\(^{+}\) T cells, but not memory/activated CD4\(^{+}\) T cells, induce an anti-inflammatory DC cytokine profile and suppress proinflammatory IL-6. Top panels, C57BL/6 BMDC cultured with anti-CD3 alone (▲), in the presence of naive CD4\(^{+}\) T cells (●), or in the presence of CD25\(^{+}\) CD4\(^{+}\) T cells (■) (A and B) or memory/activated CD44\(^{hi}\) CD4\(^{+}\) T cells (○) (C and D). Bottom panels, The same experimental set up with the addition of LPS. mRNA for IL-6 (A, C, E, and G) and IL-10 (B, D, F, and H) was assessed at 1- to 2-h intervals. Values are plotted as fold increase over mRNA levels in DC at time point 0. The experiments shown in A, B, E, and F were repeated three times; C, D, G, and H twice with similar results.
FIGURE 5. DC are the cellular source of IL-10 in cocultures with CD4+CD25+ T cells. A and B, mRNA for IL-10 in BMDC from C57BL/6 (■) or C57BL/6 IL-10−/− (□) stimulated with anti-CD3 in the presence of naïve CD4+ T cells (A) or CD25+CD4+ T cells (B). Values are plotted as fold increase over mRNA levels in DC at time point 0. The experiment shown was repeated three times with similar results. wt, Wild type.

DCs are the cellular source of IL-10 in cocultures with CD4+CD25+ T cells

The demonstration of increased IL-10 expression in cocultures of DC and CD4+CD25+ T cells raises the question of the cellular source of this cytokine. We have recently shown (36) that IL-10 protein in CD4+CD25+ T cells is only detectable after 6 days of culture and several rounds of cell division, which would be at odds with the induction of IL-10 mRNA peaking 2 h after stimulation. Furthermore, no IL-10 mRNA could be detected within 14 h of culture of CD4+CD25+ T cells stimulated with immobilized anti-CD3 and anti-CD28 in the absence of DC (data not shown). We, therefore, hypothesized that the IL-10 mRNA detected upon coculture with CD4+CD25+ T cells was derived from the DC input. This was confirmed using BMDC derived from C57BL/6 IL-10−/− mice. Cocultures of wild-type or IL-10−/− BMDC with naïve CD4+ T cells in the absence of an inflammatory signal failed to result in elevated levels of IL-10 mRNA (Fig. 5A). In contrast, CD4+CD25+ T cells triggered the expression of IL-10 in DC without the involvement of an inflammatory stimulus as shown by the induction of IL-10 mRNA in cocultures with wild-type DC, but not with IL-10−/− DC (Fig. 5B).

CD4+CD25+ induction of DC IL-10 is independent of MyD88 or TCR stimulation and TGFβ1

We showed that the induction of DC derived IL-10 via an inflammatory signal was strictly MyD88 dependent and questioned whether CD4+CD25+ T cells used a similar pathway to induce IL-10 in DC. To test this, C57BL/6 or C57BL/6 MyD88−/− BMDC were cocultured with CD4+CD25+ T cells and anti-CD3 without any addition of LPS, and IL-10 mRNA levels were determined during the first 10 h. Fig. 6A shows that CD4+CD25+ T cells are capable of inducing IL-10 mRNA with similar efficiency in wild-type and MyD88−/− BMDC.

In a series of in vitro studies, it has been shown that CD4+CD25+ T cells require TCR triggering to suppress T cell proliferation of naïve T cells (16, 37). We, therefore, determined whether CD4+CD25+ T cells require TCR stimulation to induce IL-10 mRNA expression in DC. CD4+CD25+ T cells were cocultured with BMDC in the presence or absence of anti-CD3. IL-10 mRNA was induced in DC with identical kinetics, but lower levels when CD4+CD25+ T cells were not activated by anti-CD3, suggesting that the induction of IL-10 mRNA expression in DC does not require the activation of CD4+CD25+ T cells (Fig. 6B).

Because induction of DC IL-10 was shown to be very rapid, peaking at 2 h after initiation of culture, but was independent of TCR triggering, we focused our attention on the role of immunoregulatory cytokines such as IL-10 and TGFβ1. IL-10 derived from CD4+CD25+ T cells was ruled out, because no IL-10 mRNA could be detected when coculturing CD4+CD25+ T cells and IL-10−/− BMDC (Fig. 5B). TGFβ1 was previously shown (38) to enhance the ability of macrophages to produce IL-10 and has been reported (39–41) to play an important role in the function of CD4+CD25+ T cells. Coculture of BMDC with anti-CD3 in the presence or absence of TGFβ, however, did not result in the induction of IL-10 mRNA (Fig. 6C) and addition of TGFβ along with an inflammatory signal (LPS) did not elevate IL-10 mRNA expression (Fig. 6D).

FIGURE 6. Induction of IL-10 in DC by CD25+CD4+ is independent of MyD88, TCR stimulation, or TGFβ. A, C57BL/6 (■) or C57BL/6 MyD88−/− (□) BMDC were cultured with anti-CD3 in the presence of CD25+CD4+ T cells. B, C57BL/6 BMDC were cultured in the presence of CD25+CD4+ T cells with (■) or without anti-CD3 (○). C, C57BL/6 BMDC were cultured in the absence (■) or presence (□) of TGFβ, in addition to anti-CD3 or to anti-CD3 and LPS (○). D, BMDC and CD25+CD4+ T cells were cultured with anti-CD3 in the absence (■) or presence (□) of anti-TGFβ. mRNA levels for IL-10 were assessed at 1- to 2-h intervals. Values are plotted as fold increase over mRNA levels in DC at time point 0. The experiments shown in A and B were repeated three times; C and D were repeated twice with similar results. wt, Wild type.
levels to those seen in the presence of CD4⁺CD25⁺ T cells (data not shown). Furthermore, the addition of anti-TGFβ1 to cocultures of BMDC with CD4⁺CD25⁺ T cells did not abrogate IL-10 mRNA expression (Fig. 6D).

Tregs have a dominant effect on the DC cytokine profile during cocultures with naive CD4⁺ T cells

The effect of naive or regulatory CD4⁺ T cells on DC cytokine profiles was so far only determined during coculture with one cell type. A recent report (26) suggested that CD40 ligation by CD4⁺ T cells releases DC from the control of CD4⁺CD25⁺ T cells with respect to expression of cell surface maturation markers. To address the combined influence of both naive CD4⁺ and CD4⁺CD25⁺ T cells on DC-derived cytokine expression, we set up cocultures of both T cell subsets with BMDC. Figure 7 shows that CD4⁺CD25⁺ T cells exert a dominant effect on the DC cytokine profile. In the absence of an inflammatory signal, there is little IL-6 gene transcription whether in the presence of naive CD4 T cells or coculture with CD4⁺CD25⁺ T cells (Fig. 7A). In contrast, IL-10 mRNA was markedly increased, when CD4⁺ T cells were cultured with BMDC in the presence of CD4⁺CD25⁺ T cells (Fig. 7B), with kinetics and levels similar to those seen in cultures of CD4⁺CD25⁺ T cells and BMDC alone. In cultures containing LPS in addition to the two T cell populations, IL-6 mRNA was increased whether or not CD4⁺CD25⁺ T cells were present (Fig. 7C). This was in contrast with the reduced levels of IL-6 mRNA seen in cultures of CD4⁺CD25⁺ T cells with BMDC and LPS on their own (cf. Fig. 7C vs Fig. 4E) and is in line with previous observations (17) that inflammatory stimuli override the suppressive effect of CD4⁺CD25⁺ T cells on this proinflammatory cytokine. However, a dominant effect of CD4⁺CD25⁺ T cells was observed on IL-10 mRNA that was increased irrespective of the presence of naive CD4⁺ T cells (Fig. 7D) and in comparable levels to those seen with CD4⁺CD25⁺ T cells alone in the presence of BMDC and LPS. Thus, our data indicate that CD4⁺CD25⁺ T cells exert a controlling influence on the transcription of some DC-derived cytokines but not others, even in the presence of strong inflammatory stimuli.

Cytokine production by DC mirrors mRNA profiles

The consequences of interactions between DC and naive or Treg populations had so far been determined on the level of gene expression in our study. Although this method is highly sensitive, it cannot account for potential posttranslational modification of cytokine expression. We, therefore, also determined protein levels for IL-6 and IL-10 in supernatants of DC that had been cultured in LPS in addition to the two T cell populations. IL-6 mRNA was increased whether or not CD4⁺CD25⁺ T cells were present (Fig. 7C). This was in contrast with the reduced levels of IL-6 mRNA seen in cultures of CD4⁺CD25⁺ T cells with BMDC and LPS on their own (cf. Fig. 7C vs Fig. 4E) and is in line with previous observations (17) that inflammatory stimuli override the suppressive effect of CD4⁺CD25⁺ T cells on this proinflammatory cytokine. However, a dominant effect of CD4⁺CD25⁺ T cells was observed on IL-10 mRNA that was increased irrespective of the presence of naive CD4⁺ T cells (Fig. 7D) and in comparable levels to those seen with CD4⁺CD25⁺ T cells alone in the presence of BMDC and LPS. Thus, our data indicate that CD4⁺CD25⁺ T cells exert a controlling influence on the transcription of some DC-derived cytokines but not others, even in the presence of strong inflammatory stimuli.

FIGURE 7. DC cytokine profiles during cocultures of naive and regulatory CD4⁺ T cells. A and B, C57BL/6 BMDC cultured with anti-CD3 in the presence of naive CD4⁺ T cells on their own (▲), Tregs on their own (■), or naive and regulatory CD4⁺ together (▲). C and D, The same experimental set up with the addition of LPS. mRNA for IL-6 (A and C) and IL-10 (B and D) was assessed at 1- to 2-h intervals. Values are plotted as fold increase over mRNA levels in DC at time point 0. The experiment shown was repeated three times with similar results.

FIGURE 8. DC cytokine production during cocultures with naive and Treg. A and B, C57BL/6 BMDC cultured with anti-CD3 (▲) or anti-CD3 and LPS (■) in the presence of indicated populations of FACS-sorted T cells. After 12 h, concentrations of IL-6 (A) and IL-10 (B) in the supernatants were determined by Luminex. Results are expressed as mean ± SD per ml per 10⁶ cultured DC of duplicate samples of two individual experiments.
the presence or absence of T cell subsets. Supernatants were taken at the 12-h end point for each culture condition and assessed by a Luminox assay. As shown in Fig. 8, protein production for both IL-6 and IL-10 accurately mirrors the mRNA data previously determined. In the absence of LPS, only the addition of naive CD4 T cells resulted in very low, but detectable levels of IL-6 protein, in agreement with the low levels of mRNA shown in Figs. 2A, 3A, 4C, and 7A. Cocultures with CD4+CD25+ T cells in the absence of LPS did not result in IL-6 production. Under the same culture conditions without an inflammatory stimulus, IL-10 protein was detected when DC were cocultured with CD4+CD25+ T cells irrespective of the presence or absence of naive responder CD4 T cells (Fig. 8B). Under inflammatory conditions in the presence of LPS, DC produced high levels of IL-6 protein corresponding to high mRNA levels (see Fig. 2C), which were not modified in the presence of naive CD4 or CD8 T cells, but drastically reduced in the presence of CD4+CD25+ T cells (Fig. 8A), again confirming mRNA data shown in Fig. 4E. However, in confirmation of the mRNA data shown in Fig. 7C, CD4+CD25+ T cells were unable to suppress IL-6 protein production in the presence of naive responder CD4 T cells. In contrast, IL-10 production under inflammatory conditions was increased in DC cocultures with CD4+CD25+ T cells irrespective of the presence or absence of naive CD4 T cells (Fig. 8B) as seen previously on the level of gene expression (Figs. 4F and 7D).

Discussion
Modulation of immune responses is essential for optimal protection against invading microorganisms, prevention of self-reactivity, and damage control preventing immune pathology due to excessive effector responses. Given how crucial regulation of T cell responses is, it stands to reason that there are several mechanisms operative which involve, for instance, differential action of cytokines. DC play a critical role in the balance between tolerance and immunity, bridging the innate and adaptive branches of the immune system via presentation of Ag and provision of costimulatory molecules together with stimulatory or inhibitory cytokines (3). We have investigated in particular the early time span of DC–T cell interaction, which precedes T cell activation and represents the branch point between innate and adaptive immune responses. In this study, we showed that the innate response by DC is influenced in characteristically different ways by the presence of either naive CD4 T cells or regulatory, CD25+CD4+ T cells, whereas memory/activated CD4 T cells or CD8 T cells did not play a role in modifying the innate response of DC. In contrast to many previous studies, we analyzed the effects of T cells on cytokine production by DC also in the presence of strong inflammatory signals, because this would more accurately reflect the in vivo scenario in which they would have to function. The two cytokines we focused on, IL-6 and IL-10, are typical representatives of pro- and anti-inflammatory cytokines.

IL-6 is a critical cytokine directing transition from innate to adaptive immune responses. Successful resolution of an inflammatory episode is characterized by the initial influx of neutrophils, followed by their clearance and subsequent replacement by mononuclear cells, a process that relies on IL-6. Although IL-6 does not directly effect the recruitment of neutrophils, it prevents their accumulation at sites of inflammation via promotion of apoptosis while directing mononuclear cell recruitment, activation, and survival (42–44), as well as promoting T cell migration, adhesion, and activation (45, 46). Chronic inflammation, defined by retention of activated mononuclear cells within the affected tissues, may result in a breakdown of IL-6 control. In accordance with this, mice carrying a deletion of the IL-6 gene remain resistant to the induction of a number of experimental autoimmune diseases (47–49), and inhibition of IL-6 or its receptor can successfully treat chronic diseases like rheumatoid arthritis and Crohn’s disease (47, 50, 51).

In this study, we show that in vitro initiation of an immune response in the absence of an inflammatory stimulus does not induce IL-6 transcription in DC. Under these conditions, there is unlikely to be cell recruitment in vivo, and subsequently no need to manage any immune response (52). Under inflammatory conditions, however, IL-6 is rapidly transcribed and maintained for several hours in DC. IL-6 is also produced in some T cells, notably memory/activated phenotype T cells, which accounted for the rise in IL-6 mRNA levels in cocultures with memory/activated CD4 T cells, but no T cell-derived IL-6 could be found in naive T cells during a 14-h culture in the absence of DC (data not shown). The in vitro addition of naive CD4+ or CD8+ T cells had no effect on IL-6 transcription or protein levels, suggesting the absence of a direct feedback loop of naive T cell control of IL-6. Under the same inflammatory conditions, however, the addition of CD4+CD25+ T cells to DC in vitro resulted in a strong block of IL-6 transcription (Fig. 4E) and protein production (Fig. 8A), in agreement with the role of these cells in down-regulation of inflammatory responses (11, 53–56). The importance of Treg–DC interactions is further illustrated by observations that spontaneous diabetes is exacerbated in CD80-, CD86-, and CD28-deficient NOD mice because these deficiencies also correlate with a profound decrease in CD4+CD25+ Treg numbers (57, 58). Under physiological conditions in vivo, however, it seems unlikely that an influx of mononuclear cells solely consists of CD4+CD25+ T cells. As described previously (17, 52) by Passare and Medzhitov, in the presence of both responder CD4+ and regulatory CD4+CD25+ T cells, TLR activation of DCs resulted in transcription and secretion of IL-6, thus allowing effective management of the transition from innate to an adaptive immune response by IL-6, resulting in resolution of acute inflammation. Nevertheless, modulation of bystander DC at a later stage of inflammation by CD4+CD25+ T cells would be in concordance with their role in preventing chronic disease.

Previous studies addressed the influence of CD25+CD4+ T cells on DC maturation following coculture with anergic T cell clones (23, 24), or Tregs (25, 27), and reported a failure to upregulate MHC class II, CD80, CD86, and CD40 on DC under non-inflammatory conditions. Although we similarly found reduced up-regulation of maturation markers on DC that had been preexposed to CD25+CD4+ T cells under non-inflammatory conditions, maturation markers were highly up-regulated under inflammatory conditions (LPS or CpG) as previously reported (Ref. 26 and data not shown). However, although CD25+CD4+ T cells were unable to prevent DC maturation or induction of IL-6 transcription in the presence of strong inflammatory stimuli, they were still able to modulate mature DC responses by enhancing transcription of the anti-inflammatory cytokine IL-10, whose immunosuppressive action in immune responses to numerous pathogens and immune pathologies is well documented (30, 31). Mice carrying a deletion of the IL-10 gene are highly susceptible to inflammatory bowel disease (59) and mount an excessive, host damaging response when infected with malaria parasites (60) or other organisms (31). Although IL-10 was originally described as a Th2 cytokine, it is now clear that it is produced by a variety of cells, including Treg, Th1, B cells, macrophages, and DC (31, 61). However, during the in vitro initiation of immune responses studied, no T cell-derived IL-10 could be detected (data not shown), in accordance with our previous observation (36) that T cell-derived IL-10 is found only after several days of culture. In contrast to IL-6, we
show that IL-10 can be transcribed and secreted by DC in the absence of an inflammatory stimulus. This process required the presence, but not the activation, of CD4+ CD25+ T cells (Fig. 6B) and was not inhibited in the presence of responder CD4+ T cells (Figs. 7B and 8B). The mechanisms underlying the effect of CD4+ CD25+ T cells on cytokine production by DC are currently unknown. Given the short time frame required to influence IL-10 immune responses induced by fully competent DC in vivo and the cannot be causally linked to an immature state because we found 68). However, the capacity of DCs to transcribe and secrete IL-10 transcription of IL-10 by DC. Our observations are consistent with their suppressive function because they support and enhance the of autocrine IL-10 in them. During an inflammatory immune re-

The authors have no financial conflict of interest.

Acknowledgments

We thank Jean Langhorne and Anne O’Garra for critical comments on this manuscript and for MyD88−/− and IL-10−/− mice.

Disclosures

The authors have no financial conflict of interest.

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