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TGFβ in the Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of IL-17-Producing T Cells

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Summary

We describe de novo generation of IL-17-producing T cells from naive CD4 T cells, induced in cocultures of naive CD4 T cells and naturally occurring CD4$^+$ CD25$^+$ T cells (Treg) in the presence of TLR3, TLR4, or TLR9 stimuli. Treg can be substituted by TGFβ1, which, together with the proinflammatory cytokine IL-6, supports the differentiation of IL-17-producing T cells, a process that is amplified by IL-1β and TNFα. We could not detect a role for IL-23 in the differentiation of IL-17-producing T cells but confirmed its importance for their survival and expansion. Transcription factors GATA-3 and T-bet, as well as its target Hlx, are absent in IL-17-producing T cells, and they do not express the negative regulator for TGFβ signaling, Smad7. Our data indicate that, in the presence of IL-6, TGFβ1 subverts Th1 and Th2 differentiation for the generation of IL-17-producing T cells.

Introduction

CD4$^+$ T cells can be divided into different subsets with distinct differentiation profiles and functional characteristics. The best-characterized subsets are Th1, Th2, and Treg, whose development is specified by the transcription factors Tbet, GATA-3, and Foxp3, respectively (Fontenot et al., 2005; Szabo et al., 2000; Zheng and Flavell, 1997). A unique subset characterized by production of IL-17 and crucially involved in the pathogenesis of certain autoimmune diseases such as rheumatoid arthritis and EAE as well as allergen-specific responses was recently identified (Cua et al., 2003; Kolls and Linden, 2004; Langrish et al., 2005; Nakae et al., 2002). IL-17 promotes granulopoiesis and neutrophil accumulation, and, while it plays an active role in inflammatory diseases, also has been shown to protect mucosal barrier functions due to stimulation of tight junction formation and mucin secretion (Chen et al., 2003b; Kinugasa et al., 2000). Thus, neutralization of IL-17 enhances dextran sulfate sodium (DSS)-induced inflammatory bowel disease (Ogawa et al., 2004).

It has so far been unclear how de novo differentiation of IL-17-producing T cells is stimulated. Although DC-derived IL-23 was shown to increase IL-17 secretion from activated CD4$^+$ T cells (Aggarwal et al., 2003; Langrish et al., 2005), IL-23 is not sufficient to induce differentiation of IL-17-producing cells from naive CD4$^+$ T cells in vitro, suggesting the involvement of additional or other factors in the de novo development of this T cell subset. The rationale underlying the existence of multiple subsets of CD4$^+$ T cells is their crucial role in orchestration and control of immune responses. Cytokine-mediated crossregulation of each other's responses is an important principle of immune regulation (O'Garra et al., 2004), involving IL-4-mediated downregulation of IL-12Rβ2 (Szabo et al., 1997) or IFN-γ mediated inhibition of Th2 development (Fitch et al., 1993). In addition, interference strategies involving suppressive cytokines such as TGFβ1 (Letterio and Roberts, 1998) or IL-10 (Moore et al., 2001) play an important role in the control of immune responses. These immunoregulatory functions are strongly influenced by interactions with dendritic cells that act as go betweens for the innate and adaptive immune system through integration of pathogen-derived signals and release of immune-modulating mediators (Reis e Sousa, 2004). The naturally occurring CD4$^+$ CD25$^+$ T cell subset (Treg) has been suggested to be dedicated to the control of immune pathology and autoimmunity, substantiated by the finding that absence of the Foxp3 transcription factor that controls the generation of this subset results in a fatal autoimmune disorder (Fontenot and Rudensky, 2005; Sakaguchi, 2005). Treg suppress the expansion of potentially pathogenic T cells in vivo (Almeida et al., 2002; Annacker et al., 2000; Maloy and Powrie, 2001) and inhibit the proliferation and IL-2 transcription of naive CD4$^+$ T cells in vitro (Thornton and Shevach, 1998). Interestingly, however, it appears that Treg do not suppress T cell proliferation in vitro in the presence of an inflammatory stimulus such as LPS. The blockade of suppression under these conditions was attributed to the secretion of IL-6 and other unidentified cytokine(s) by LPS-stimulated dendritic cells acting on the responder CD4$^+$ T cells (Pasare and Medzhitov, 2003). In this paper, we show that, despite restoration of proliferative function in CD4 T cells cultured with Treg in the presence of LPS, their IL-2 and especially their IFN-γ responses are still profoundly suppressed. However, the presence of Tregs in a proinflammatory cytokine milieu resulted in differentiation of naive CD4$^+$ T cells to IL-17 production. We provide evidence that the component involved in differentiation of naive CD4 T cells to IL-17 producers is TGFβ1, which, together with DC-derived IL-6, is essential for de novo differentiation of IL-17-producing T cells from naive CD4 T cells in vitro, a process that is amplified by IL-1β and TNFα. IL-17-producing T cells are distinct from Th1 T cells, as shown by absence of IFN-γ transcription as well as absence of the transcription factor T-bet and its target Hlx. Our data indicate that a critical balance of pro- and

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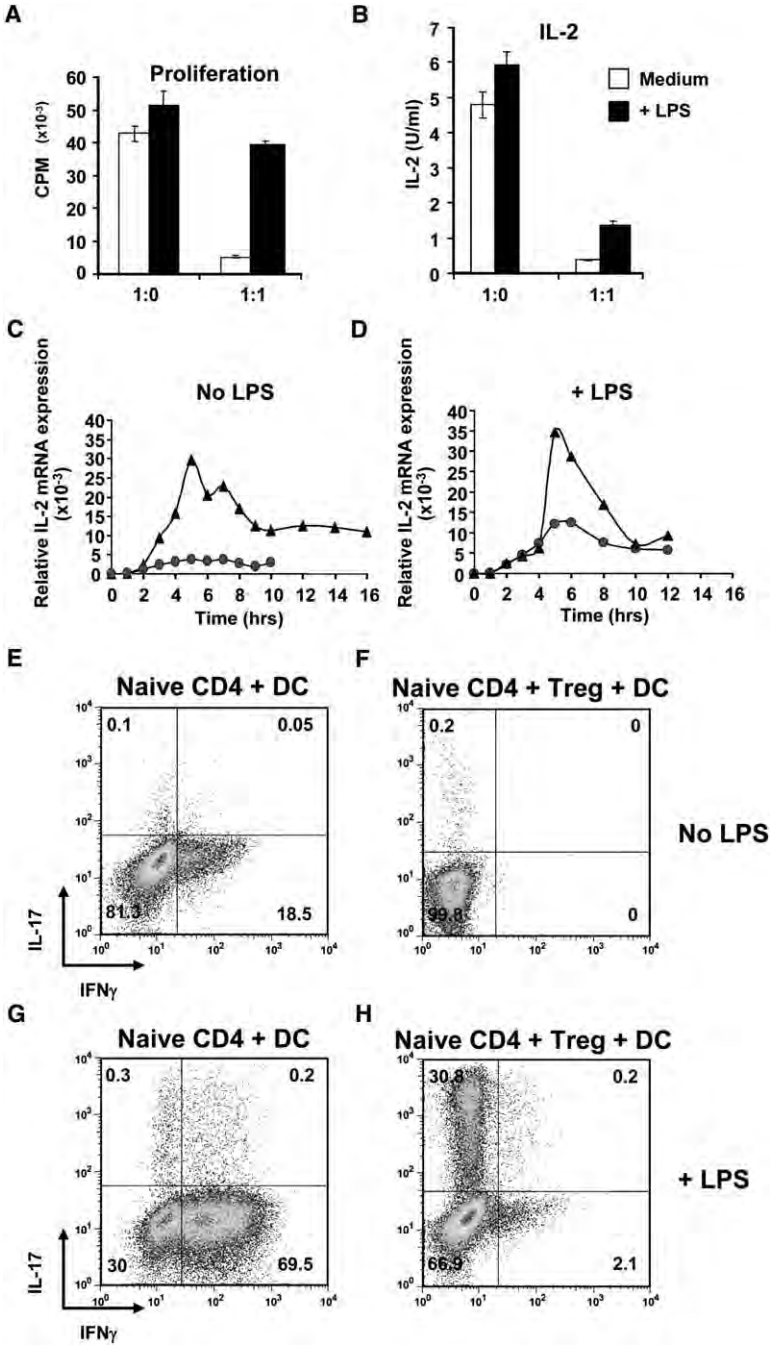

Figure 1. LPS Stimulation Overcomes Treg-Mediated Inhibition of Proliferation but Not IL-2 and IFN-γ and Results in IL-17 Production

FACS-sorted naive CD4$^+$ T cells and BMDC were stimulated with anti-CD3 alone or in a 1:1 mixture with FACS-sorted Treg in the absence (white bars) or presence (black bars) of LPS. (A) Proliferation was assessed after 48 hr by ^{3}H-thymidine incorporation; (B) IL-2 protein in culture supernatants assessed by CTLL-2-assay. (C and D) mRNA for IL-2 assessed at 1–2 hr intervals, of BMDC and naive CD4$^+$ T cells alone (triangles) or mixed with Tregs (circles), in the absence (C) or presence (D) of LPS. Values are plotted as fold increase over mRNA levels in DC at time point 0. (E), (F), (G), and (H) show intracellular staining for IFNγ and IL-17 of CD4$^+$ T cells (gated on naive T cell input), cultured for 4 days as described above. Shown are naive cells alone (E), mixed with Treg (F), naive alone in presence of LPS (G), and mixed with Treg (H). Error bars represent SD values.

antiinflammatory cytokines is conducive to differentiation of IL-17-producing T cells, thereby potentially tipping the balance toward more severe inflammatory reactions that might aggravate immune pathology.

Results

Coculture of Naive CD4 T Cells with Treg in the Presence of LPS Induces IL-17 Production

Coculture of naive CD4 T cells and Treg at a 1:1 ratio in the presence of DC and LPS abrogates the suppressive function of Treg on T cell proliferation, as previously shown (Pasare and Medzhitov [2003] and Figure 1A). However, the IL-2 response remained suppressed, as indicated by the reduced amounts of IL-2 protein in the supernatant whether or not LPS was present in the cultures (Figure 1B). Since it is difficult to quantitate IL-2 protein levels in these cocultures due to the substantial consumption of IL-2 by Treg (Barthlott et al., 2005; de la Rosa et al., 2004), we assessed levels of mRNA for IL-2. While, in cocultures of naive CD4 T cells and Treg in the presence of DC and LPS, mRNA levels for IL-2 were partially restored compared with cocultures without LPS, the major peak of IL-2 message around 6 hr after activation remained absent (Figures 1C and 1D). Furthermore, the suppression of IFN-γ-producing cells, which is evident in the presence of

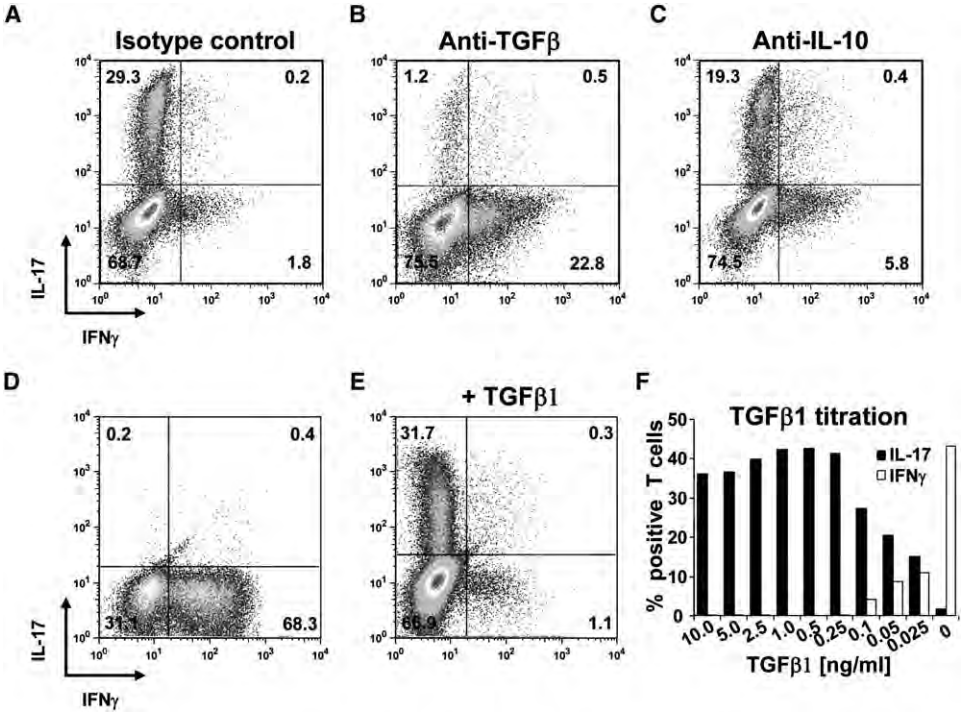

Figure 2. Treg Provide TGFβ for Differentiation of IL-17-Producing CD4$^+$ T Cells

FACS-sorted naive CD4$^+$ T cells were mixed 1:1 with FACS-orted Treg and cultured for 4 ays with BMDC, anti-CD3, nd LPS and (A) isotype control antibody, (B) anti-TGFβ, or (C) anti-IL-10. Naie CD4$^+$ T cells cultured under identical conditions as above, but in the absence of Treg, are shown in the absence (D) or presence (E) of TGFβ. Dot plots show intracellular staining for IFNγ and IL-17 of CD4$^+$ T cells, gated on the naie CD4$^+$ T cell input. (F) Naive CD4$^+$ T cells cultured as above, in presence of indicated concentrations of TGFβ1. Percentages of IL-17-negative (closed bars) or IFNγ-positive (open bars) T cells are shown.

Treg (Figure 1E versus Figure 1F) was still enforced in the presence of LPS (Figure 1, GvsH). However, instead of IFN-γ, a substantial proportion of CD4 T cells cocultured with Treg in the presence of LPS secreted IL-17 (Figure 1H versus Figure 1G), and this effect titrated with the number of Treg present in the culture (see Figure S1 in the Supplemental Data available with this article online).

The Component Involved in Differentiation of IL-17 Producers Is TGFβ1

Tregs are known to be a potent source of TGFβ1, an immunosuppressive cytokine produced by multiple cell types (Maloy and Powrie, 2001). IL-10 is another suppressive cytokine implicated in the action of Treg, but its delayed induction in T cells in vitro (Barthlott et al., 2005) makes it a less likely candidate in the differentiation of IL-17 producers. In order to test the involvement of TGFβ1 or IL-10 in this process, neutralizing antibodies specific for either TGFβ or IL-10 were added to cocultures of naive CD4$^+$ T cells, Treg, and DC in the presence of LPS. As shown in Figure 2B, the addition of anti-TGFβ suppressed the generation of IL-17-producing T cells, whereas anti-IL-10 only caused a slight reduction in the proportion of IL-17-producing T cells (Figure 2C). Furthermore, addition of as little as 25 pg/ml of TGFβ1 to cultures of naive T cells with DC and LPS without any Treg was sufficient to deviate differentiation from Th1 T cells (Figure 2D) to IL-17-producing T cells (Figures 2E and 2F).

DCs Stimulated by Different TLR Ligands in a MyD88-Dependent Fashion Are Crucial for CD4 Differentiation to IL-17 Production

Given that generation of IL-17-producing CD4 T cells depended on the presence of LPS, the involvement of TLR4 triggering of DC seemed essential for this pathway. In order to test whether other TLR ligands could substitute for LPS, we added either CpG or polyI:C, triggering TLR9 or TLR3, respectively, to cultures containing naive CD4 T cells, Treg, and DC. As shown in Figures 3A and 3B, ligands for TLR3 and -9 were also able to induce differentiation of IL-17-producing T cells. The absence of IL-17-producing T cells in cultures containing DC from MyD88$^{-/-}$ mice (Figure 3D) compared with wild-type DC in the presence of LPS (Figure 3C) suggests the involvement of inflammatory cytokines rather than mere upregulation of costimulatory molecules on DC, a process that is MyD88 independent (Kaisho et al., 2001). In order to determine whether cell-cell contact between DC and T cells was essential for differentiation to IL-17 or whether soluble mediators secreted by DC were sufficient, DC-free cultures were set up, and activation of naive CD4 T cells was achieved by addition of beads coated with anti-CD3 and anti-CD28. Figures 3E and 3F show that addition of LPS to naive CD4 T cells on their own or in the presence of Treg does not suffice to induce either IFN-γ or IL-17 differentiation, ruling out a direct effect of LPS on T cells and illustrating the importance of DC for T cell differentiation processes. The addition of conditioned medium (CM) from LPS-stimulated

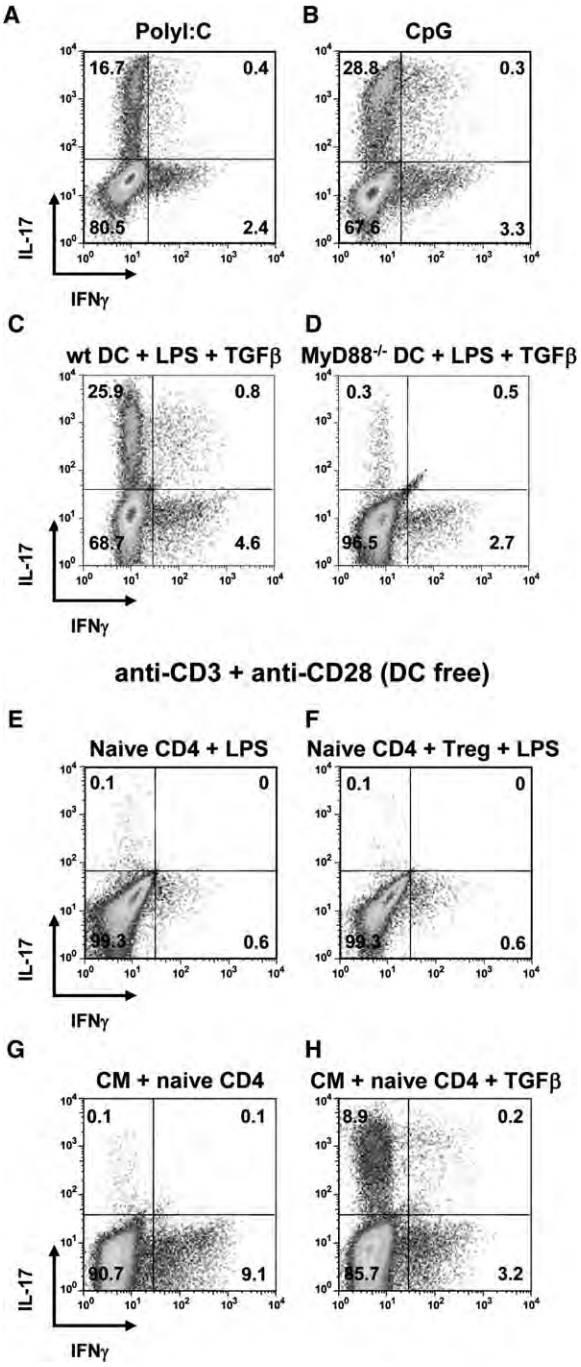

Figure 3. Differentiation of IL-17-Producing CD4$^+$ T Cells Requires a DC Factor or Factors that Are Soluble, MyD88 Dependent, and Induced by TLR Ligation

Panels show intracellular staining for IFNγ and IL-17 of CD4$^+$ T cells, gated on the naive CD4$^+$ T cell input after 4 days of culture. FACS-sorted naive CD4$^+$ T cells were mixed 1:1 with FACS-sorted Treg and cultured with BMDC, anti-CD3 and either Polyl:C (A) or CpG (B). Purified naive CD4$^+$ T cells were cultured with either C57BL/6 BMDC (C) or C57BL6 MyD88$^{-/-}$ BMDC (D), and anti-CD3, LPS, and TGFβ. Sorted naive CD4$^+$ T cells were cultured with anti-CD3 and anti-CD28 coated beads and LPS in the absence (E) or presence of equal numbers of sorted Treg (F). CM was obtained from BMDC cultured for 12–14 hr in the presence of LPS. Sorted naive CD4$^+$ T cells were cultured in this medium with anti-CD3- and anti-CD28-coated beads in the absence (G) or presence of TGFβ (H).

DC on its own caused differentiation of IFN-γ producers (Figure 3G). However, in the presence of TGFβ1, CM from LPS stimulated DC-induced IL-17-producing CD4$^+$ T cells and suppression of IFN-γ-producing Th1 cells (Figure 3H). Thus, soluble mediator(s) from DC together with TGFβ1 are important for differentiation of IL-17-producing T cells.

Identification of DC-Derived Cytokines Involved in Differentiation of IL-17 Producers

Previous reports suggested that IL-23 is a crucial cytokine in the development of IL-17-producing CD4$^+$ T cells, although as yet no studies have shown that IL-23 will direct the differentiation of naive CD4 T cells into IL-17-producing T cells (Langrish et al., 2005; Murphy et al., 2003; Vanden Eijnden et al., 2005). We show here that the addition of anti-p40 antibody, which blocks both IL-12 and IL-23, to cultures of naive CD4$^+$ T cells with TGFβ1 and CM from LPS-stimulated DC does not suppress IL-17 production (Figure 4C). IL-6 is a likely candidate for the generation of IL-17-producing T cells, because we first detected their development in the experimental system described by Pasare et al. in which IL-6 was described to overcome the proliferative defect of naive CD4 T cells when cocultured with Treg (Pasare and Medzhitov, 2003). Furthermore, IL-6 and IL-23p19 are closely related members of the subfamily of helical cytokines, exhibiting a wide range of often overlapping biological functions (Oppmann et al., 2000). Indeed, addition of a neutralizing antibody specific for IL-6 to cultures of naive CD4$^+$ T cells and DC-conditioned medium abolished the generation of IL-17 producers (Figure 4D), thus supporting the assumption of its involvement. In a DC free culture system with anti-CD3- and anti-CD28-coated beads, addition of IL-6 together with TGFβ1 to naive CD4 T cells stimulated by antibody-coated beads induced a dose-dependent increase in IL-17 differentiation (Figure 4E, panels). The differentiation of IL-17-producing T cells was further augmented by the presence of two additional DC-derived proinflammatory cytokines, TNFα and IL-1β (Figure 4F).

Addition of IL-23 induced a low degree of Th1 development but no IL-17, whether alone or in combination with TGFβ1 (Figures 4G and 4H), thus making it unlikely that this cytokine is responsible for de novo generation of IL-17-producing T cells. Various blocking antibodies and additional cytokine combinations were tested (shown in Figure S2), further emphasizing the obligatory role IL-6 and TGFβ1 are playing in the differentiation of IL-17-producing T cells and illustrating the accessory function of IL-1β and TNF-α.

Distinction of Th1 and IL-17-Producing T Cells and an Active Role for TGFβ1

In order to study the differentiation of IL-17-producing CD4$^+$ T cells and their relation to Th1 cells, we made use of IFN-γ gene knockin bicistronic reporter mice (Yeti) expressing a yellow fluorescent protein (eYFP) (Stetson et al., 2003). Modification of the IFN-γ locus allowing access to transcription factors correlates with the presence of mRNA and is reported by YFP staining. While Yeti mice faithfully report the presence of cytokine mRNA, translation of the downstream fluorescent reporter is less stringently regulated, and cells that have

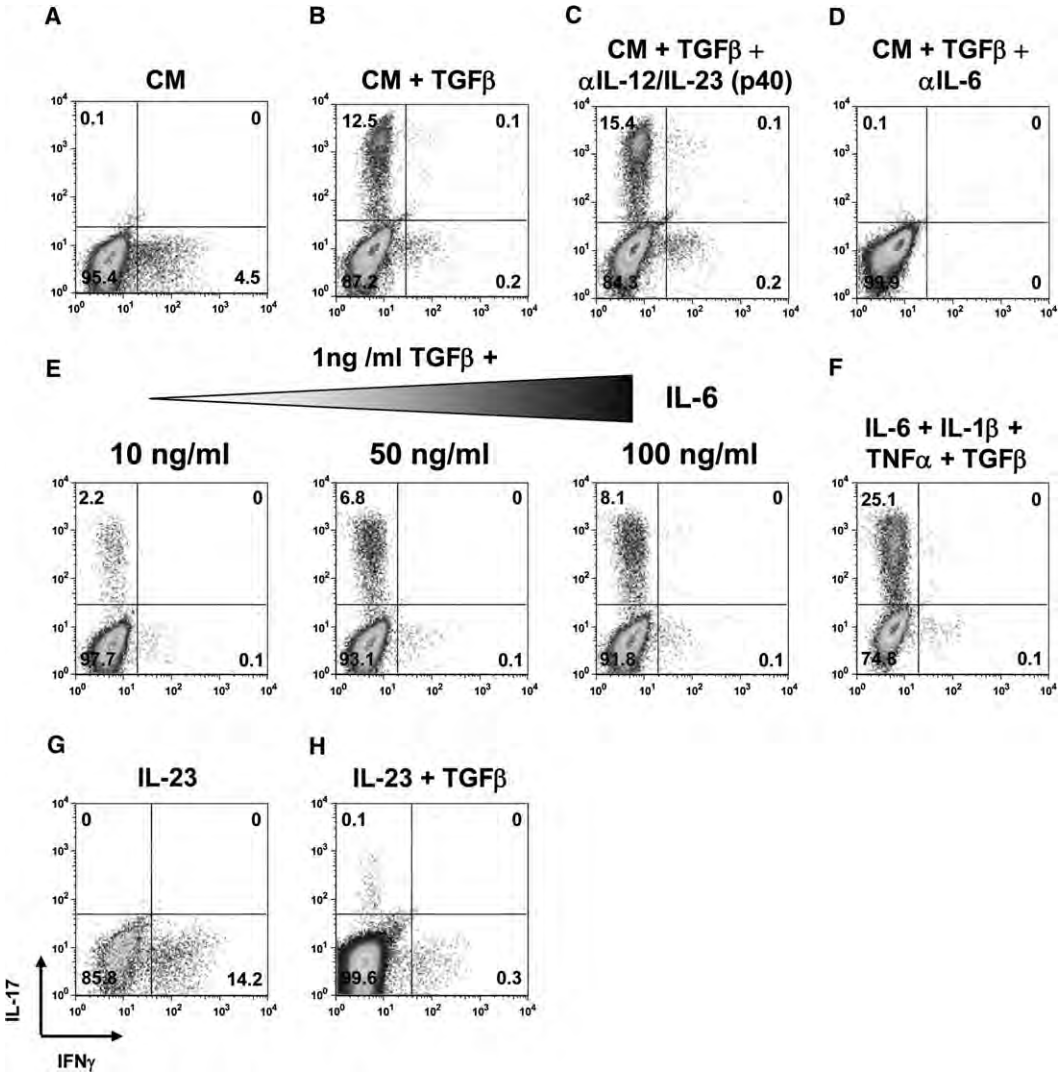

Figure 4. Differentiation of IL-17-Producing CD4$^+$ T Cells Requires Combinations of DC-Derived Proinflammatory Cytokines

Dot plots show intracellular staining for IFNγ and IL-17 of sorted naive CD4$^+$ T cells after 4 days of culture with anti-CD3- and anti-CD28-coated beads. (Top row) CD4$^+$ T cells cultured in CM from DC stimulated with LPS for 12–14 hr (A), CM supplemented with TGFβ (B), or supplemented with TGFβ and anti-IL-12/IL-23 (C), or TGFβ and anti-IL-6 (D). (Second row) CD4$^+$ T cells cultured with anti-CD3- and anti-CD28-coated beads and TGFβ, and indicated concentrations of IL-6 (E), or IL-6, IL-1β, and TNFα (F). (Third row) CD4$^+$ T cells cultured with anti-CD3- and anti-CD28-coated beads with IL-23 (G), or IL-23 in combination with TGFβ (H).

once upregulated IFN-γ remain YFP positive. Therefore, it is possible to ascertain whether an IL-17-producing T cell has ever opened the IFN-γ locus during differentiation and thus would allow us to see whether Th1 and IL-17-producing T cells are related, as has previously been suggested (Bettelli et al., 2004). In vitro stimulation of YFP-negative sorted naive CD4$^+$ T cells from Yeti mice with DC in the presence of LPS induced YFP-positive (IFN-γ producing) T cells (Figure 5A), whereas the addition of TGFβ induced IL-17 production but no YFP-positive T cells (Figure 5B), indicating that differentiation to IL-17 progresses without opening of the IFN-γ locus.

In fact, the addition of neutralizing antibodies to IL-12/23, IFNγ, and IL-4 without addition of TGFβ1 was sufficient to induce the differentiation of IL-17-producing T cells in the presence of LPS-stimulated DC (Figure 5C). This result is in line with a role for TGFβ1 in preventing Th1 and Th2 differentiation in the presence of IL-6, thus allowing the diversion to differentiation of IL-17-producing T cells. However, in a DC-free system, addition of the Th1/Th2 blocking antibody cocktail could not substitute for TGFβ1 and thus did not result in IL-17-producing T cells (Figure 5D). The apparent paradox is resolved by the finding that addition of anti-TGFβ1 to cultures containing DC prevented the appearance of IL-17-producing T cells despite the presence of neutralizing antibodies to IL-12/23, IFNγ, and IL-4 (Figure 5F). Thus, LPS-stimulated DC produce TGFβ1 themselves, albeit in amounts that cannot support IL-17 differentiation without further blockade of cytokines that promote differentiation of the other T cell subsets. We observed that addition of IL-12, IL-18, or IL-4 to primary cultures containing IL-17-promoting cytokines resulted in dose = -dependent reduction of IL-17-producing

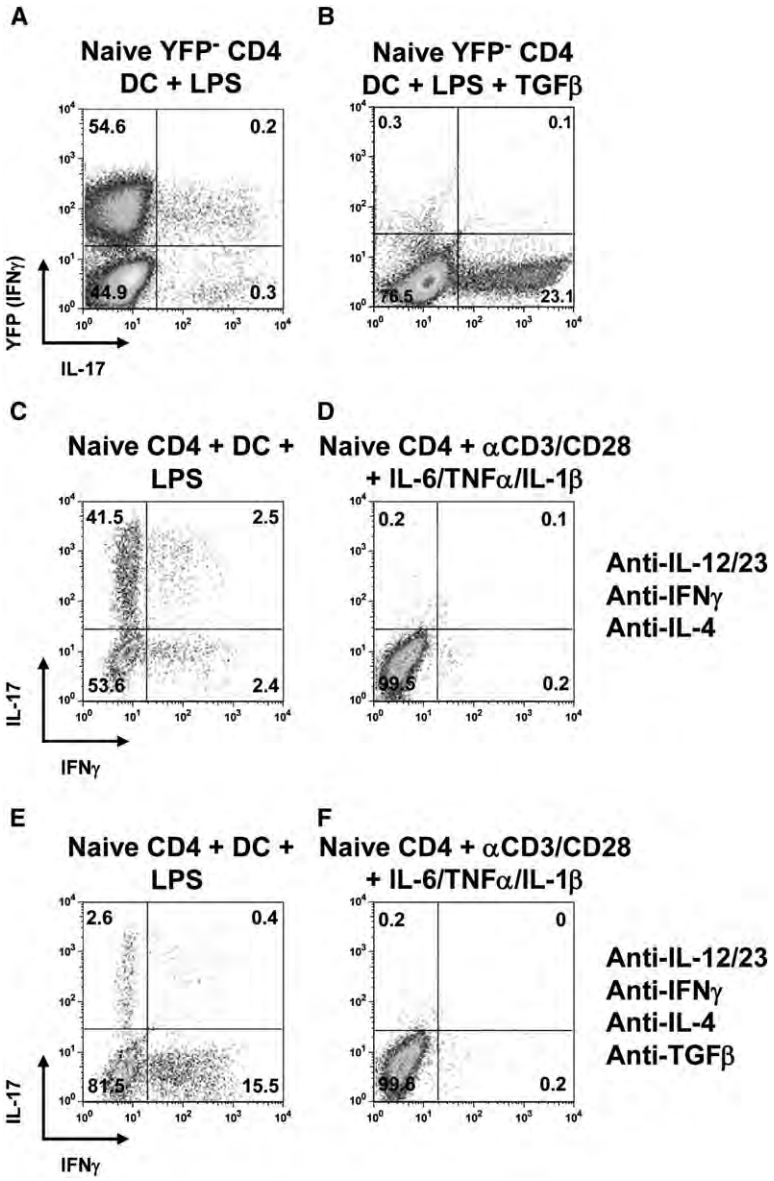

Figure 5. Blockade of Th1 and Th2 Allows Differentiation of IL-17-Producing T Cells

Dot plots show intracellular staining for IFN-γ or YFP expression and IL-17 of sorted naive CD4$^+$ T cells after 4 days of culture. Yeti YFP$^-$ naive CD4$^+$ T cells cultured with DC stimulated with LPS (A), and TGFβ (B). Naive CD4$^+$ T cells cultured with DC stimulated with LPS (C) or naive T cells stimulated in the absence of DC with anti-CD3, anti-CD28, IL-6/TNFα, and IL-1β (D), cultured in the presence of anti-IL-4, anti-IL12/23 (p40), and anti-IFNγ. (E) The response of naive T cells cultured in the presence of DC and LPS with the antibodies shown in (C) and (D) and, in addition, anti-TGFβ. (F) Response of naive T cells cultured in the absence of DC (conditions as outlined in [D]) and including the antbodies outlined for (E).

T cells (data not shown), indicating sensitive crossregulation of CD4 T cell subsets.

IL-17-Producing T Cells Do Not Express Th1 or Th2 Transcription Factors or Smad7

Restimulation of primary cultures containing IL-17-producing T cells with IL-2 resulted in outgrowth of IFN-γ producers (Figure 6B). In contrast, the addition of IL-23 promoted further expansion of IL-17-producing T cells (Figure 6C). This confirms the reported role of IL-23 in supporting growth and survival of activated IL-17 producers (Aggarwal et al., 2003; Langrish et al., 2005). Finally, analysis of the expression of transcription factors showed clearly that IL-17-producing T cells express neither GATA-3 nor T-bet or its target Hlx (Figures 6D–6F), which further sets them apart as a distinct subset. TFGβ signaling is subject to negative regulation via transcriptional induction of Smad7, which competes for receptor-regulated phosphorylation of Smad2 and Smad3 by the activated TGFβ receptor complex (Nakao et al., 1997) and enhances receptor degradation (Shi and Massague, 2003). As shown in Figure 6G, the inhibitor of TGFβ signaling, Smad 7 is induced in Th1 T cells but absent in Th2 and IL-17-producing T cells.

Discussion

This paper describes the in vitro differentiation of IL-17-producing CD4$^+$ T cells from naive CD4$^+$ T cell precursors and provides evidence for the involvement of a uniquely balanced combination of pro- and antiinflammatory cytokines. Dendritic cells stimulated via different Toll-like receptors provide a range of proinflammatory cytokines that promote the development of Th1 T cells, notably IL-12 and IL-18. However, a similar stimulus in the presence of TGFβ1, a pleiotropic cytokine that is made by multiple cells types (Gorelik and Flavell, 2002), deviates differentiation to IL-17-producing T

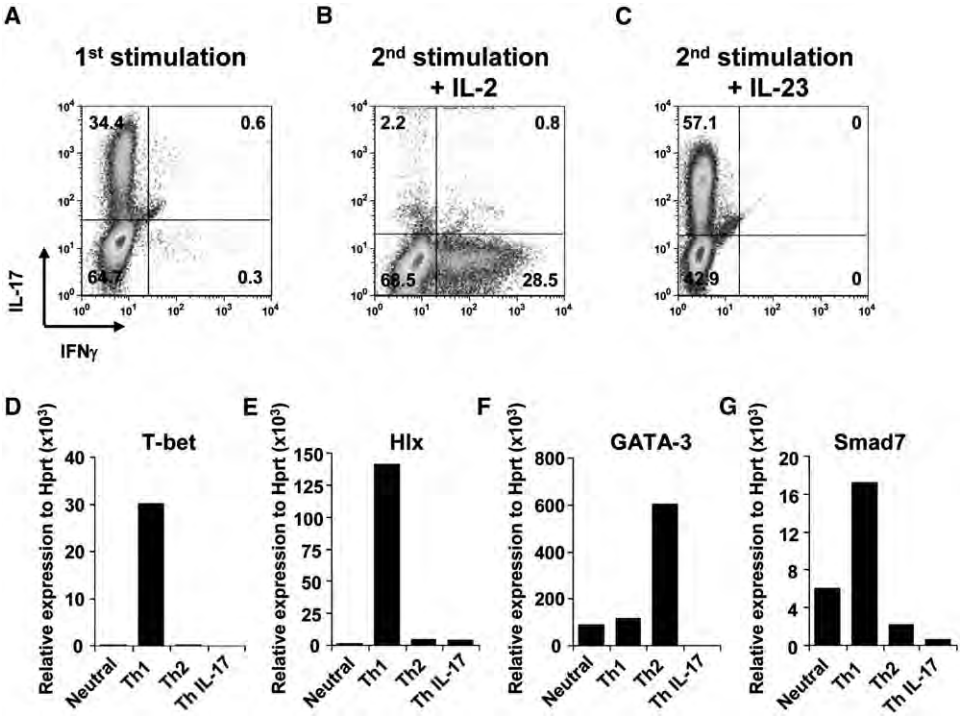

Figure 6. IL-17-Producing CD4+ T Cells Are Distinct from Th1 and Th2 Subsets and Are Maintained by IL-23

Dot plots show intracellular staining for IFN-γ and IL-17 of sorted naive CD4+ T cells after 4 days of culture. Naive CD4+ T cells were activated with plate bound anti-CD3 and anti-CD28 for 3 days in the presence of TGFβ, IL-6, IL-1β, and TNFα and rested for 3 days (A), then restimulated with plate bound anti-CD3 and anti-CD28 for 3 days in medium supplemented with IL-2 (B) or IL-23 (C) and rested for 4 days before analysis. mRNA levels of the transcription factors T-bet (D), Hlx (E), and GATA-3 (F), and Smad inhibitor Smad7 (G) were determined in CD4+ T cells stimulated with plate bound anti-CD3 and anti-CD28 for 3 days under neutral, Th1 (IL-12), Th2 (IL-4), or Th IL-17 (cytokine mix) polarizing conditions and rested for 3 days before RNA isolation.

cells. TGFβ1 has a critical function as an antagonist of Th1 development affecting IFN-γ as well as T-bet (Gorelik et al., 2002; Lin et al., 2005), but also interferes with Th2 differentiation (Chen et al., 2003a; Gorelik et al., 2000; Heath et al., 2000). Indeed, a combination of anti-IL-12/23, anti-IFN-γ, and anti-IL-4 was recently shown to support the development of IL-17-producing T cells (Harrington et al., 2005; Park et al., 2005). Our study confirmed that this combination of antibodies in the presence of LPS-stimulated DC allows generation of IL-17-producing T cells, and it should be noted that we detected IL-17 differentiation in the absence of IL-23. This is in agreement with a role of TGFβ1 in blocking Th1 and Th2 differentiation, thus allowing the diversion to IL-17 T cell differentiation.

However, our data indicate that there may be an additional, more active role for TGFβ1 in this process, since, even in the presence of antibodies that strongly inhibit Th1 and Th2 generation, TGFβ was essential to allow IL-17 differentiation. Antibodies to TGFβ abrogated the positive effect of antibodies to IL-12/23, IFNγ, and IL-4 on differentiation of IL-17-producing T cells, implicating DC as the cellular source of TGFβ1 in these cultures. However, it appears that the amount of TGFβ1 they produce in primary cultures is obviously not sufficient to promote IL-17 differentiation without further blockade of Th1/Th2-promoting cytokines. A critical balance between IFNγ- and TGFβ-mediated signals crucially influences the extent of Smad3 activation (Ulloa et al., 1999), so that any interference with the IL-12/IFNγ axis will shift the balance in favor of TGFβ.

Since disruption of TGFβ1 signaling in T cells results in inflammation and autoimmunity (Gorelik and Flavell, 2000; Kulkarni et al., 1993; Shull et al., 1992), it may at first appear counterintuitive to invoke TGFβ1 in the initiation of a highly proinflammatory T cell subset. However, it has long been recognized that TGFβ1 not only has antiinflammatory potential but can, under certain circumstances, also potentiate inflammation (Wahl, 1994). This dichotomy of action depends on local versus systemic expression (Allen et al., 1990; Wahl et al., 1993), as well as concentration and timing between TCR-triggered activation and signaling through the TGFβ receptor (McKarns and Kaminski, 2000). Notably, local administration of TGFβ1 precipitated inflammatory responses and recruitment of neutrophils in experimental arthritis models (Allen et al., 1990; Fava et al., 1991). These experimental models predate the discovery of IL-17-producing T cells, but since they evoke typical characteristics of this proinflammatory T cell subset, it would in fact be very interesting to reevaluate these models in light of our findings concerning the role of TGFβ1 in the differentiation of IL-17-producing T cells.

In agreement with a recent publication (Harrington et al., 2005), we did not find the Th1 lineage transcription factor T-bet and its target, the homeoprotein Hlx (Mullen et al., 2002), expressed in IL-17-producing T cells. However, T-bet-deficient mice are protected from EAE

(Bettelli et al., 2004; Lovett-Racke et al., 2004), although this autoimmune disease appears to be mostly driven by IL-17-producing T cells (Cua et al., 2003). While this may indicate involvement of Th1 T cells in EAE, it is also worth considering that Tbet expression in dendritic cells is important for their function in innate immunity (Lugo-Villarino et al., 2005) and thus may play a role in the differentiation of IL-17-producing T cells. The absence of Smad 7, an inhibitor of TGFβ signaling (Hayashi et al., 1997; Nakao et al., 1997), also sets IL-17-producing T cells apart from Th1 T cells.

IL-6, the other obligatory component in the differentiation of IL-17-producing T cells, has an important role in many aspects of immune responses (Taga and Kishimoto, 1997). Notably, its absence results in decreased induction of EAE as well as collagen-induced arthritis (Alonzi et al., 1998; Eugster et al., 1998; Ohshima et al., 1998; Okuda et al., 1998; Rochman et al., 2005), both diseases in which IL-17 is strongly involved. Despite the fact that TGFβ1 and IL-6 alone were capable to drive the initial differentiation of IL-17-producing T cells, their numbers were amplified by IL-1β and TNF-α. Synergistic roles of IL-6, TNF-α, and IL-1β have been described previously, and deficiency in any one component reduces onset of severity of pathology (Hata et al., 2004). Since IL-17 itself has been implicated in induction of TNF-α, IL-1β, and IL-6 (Fossiez et al., 1998; Langrish et al., 2005), it is likely that there is positive feedback control also involving cytokine production from T cells once IL-17-producing cells have been generated. However, in the case of TNF-α, it seems clear that it is DC-derived, not T cell-derived, TNF-α that is instrumental in driving IL-17 differentiation, since IL-17 production in recipients of adoptively transferred T cells from TNF-α-deficient mice was found to be normal (Nakae et al., 2002).

IL-23, which has been implicated in the generation of IL-17 responses from preactivated $CD4^+$ T cells, does not appear to play a role in de novo generation of IL-17-producing T cells. The protection against induction of EAE and CIA in p19 knockout mice, together with the absence of IL-17-producing T cells in such mice, was thought to reflect a direct involvement of IL-23 in the generation of IL-17-producing T cells (Langrish et al., 2005; Murphy et al., 2003). While $p19^{-/-}$ mice were reported to have elevated levels of TNFα, IL-6, and IL-1β early after immunization with MOG (Cua et al., 2003), the levels of these cytokines were reduced in $p19^{-/-}$ mice 42 days after immunization with collagen, when control mice showed chronic disease (Murphy et al., 2003). Our data suggest that $p19^{-/-}$ mice are capable of generating IL-17-producing T cells, but the absence of IL-23 may compromise their survival and thereby limit the positive feedback loop that upregulates IL-6, IL-1β, and TNF-α. The importance of IL-23 in driving the survival and expansion of activated IL-17 producers as previously suggested (Aggarwal et al., 2003; Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005) was confirmed in our study.

Although it is clear that there are many cellular sources for TGFβ1 (Fahlen et al., 2005; Letterio, 2005), an intriguing feature in the generation of IL-17-producing T cells was the apparent involvement of Treg in the differentiation of IL-17-producing T cells in our study. What could be the contribution of Treg to this phenomenon? Treg have been shown to secrete TGFβ1 (Belghith et al., 2003), and some of them express TGFβ1 on their surface (Nakamura et al., 2001), but the role of TGFβ1 in their suppressive function is somewhat controversial (Piccirillo et al., 2002). Given that DCs appear to be able to secrete some TGFβ themselves, an alternative explanation for the function of Treg may be their strong suppression of both Th1 and Th2 responses via the inhibition of IFNγ and IL-4. In addition, Treg may increase the production of TGFβ1 by DC. Furthermore, we have recently shown that Treg induce MyD88-independent IL-10 production in DC (M.V. and B.S., unpublished data), whose suppression of IL-12 might aid the development of IL-17-producing T cells. While anti-IL-10 was not sufficient to suppress IL-17 differentiation, it nevertheless caused a reduction in the proportion of T cells that were able to produce IL-17 (Figure 2C).

The perceived role of Treg is in downregulation of inflammatory responses, whereas IL-17 is thought to be highly proinflammatory. Under physiological conditions in vivo, the number of Treg present in the vicinity of an ongoing infection may not be sufficient to skew T cell differentiation. Furthermore, there may be a strict temporal regulation of the effect, since delaying the addition of Treg to naive T cells for 6 hr abrogated the generation of IL-17-producing T cells (data not shown). Nevertheless, the potential connection of Treg that play a well-documented protective role in inflammatory bowel disease (Powrie, 1995) and IL-17, which is beneficial at mucosal sites, represents another intriguing aspect of immune regulation.

Taken together, our data suggest an important role for TGFβ, which together with IL-6 can mediate the de novo differentiation of IL-17-producing T cells from naive precursors. While part of the effect of TGFβ1 consists of suppressing Th1 and Th2 differentiation, thus allowing diversion to IL-17 differentiation, the fact that blocking of TGFβ in the context of Th1/Th2 antibody blockade abolishes IL-17 production suggests that it also has a more direct role in the differentiation of IL-17-producing T cells. Our data furthermore provide a logical explanation for the apparent paradox that TGFβ can play both anti- and proinflammatory roles in the immune system.

Experimental Procedures

Mice

C57BL/6, C57BL/6 Ly5.1, C57BL/6 Yeti (Stetson et al., 2003), C57BL/6 $MyD88^{-/-}$, and C57BL/6 $Rag1^{-/-}$ mice were bred under SPF conditions, and experimental animals were kept in conventional but pathogen-free animal facilities at the National Institute for Medical Research (London, United Kingdom) in accordance with local guidelines.

Cell Purification

Single-cell suspensions from spleens and lymph nodes were stained with anti-CD25-phycoerthrin (PE) followed by anti-PE magnetic microbeads (Miltenyi Biotec, Surrey, United Kingdom) and enriched by positive selection on an AutoMACS (MACS, Miltenyi Biotec, GmbH), according to the manufacturer's instructions. The positive fraction was then sorted on a Moflo cytometer (Cytomation, Fort Collins, Colorado) to obtain pure populations of $CD4^+CD25^+$ T cells (>99% purity); the negative fraction was sorted into $CD25^-$ $CD44^{lo}$ $CD62L^{hi}$ $CD4^+$ T cells (>99.5% purity).

Generation of Bone-Marrow-Derived DC

Bone-marrow-derived DCs (BMDC) were generated as described previously (Stockinger and Hausmann, 1994). Briefly, femurs and

tibia were flushed with culture medium, and 3×10^6 bone marrow cells were cultured in petri dishes (NUNC) in 10 ml culture medium containing 10% supernatant of Ag8653 myeloma cells transfected with murine granulocyte macrophage colony stimulating factor (GM-CSF) cDNA. On day 4 of culture, nonadherent granulocytes were removed and GM-CSF medium replaced. Loosely adherent cells were transferred to a fresh dish on day 6 of culture and used from day 6 to day 8 as the source of dentritic cells (>95% purity).

In Vitro Stimulation and Exogenous Cytokines

The culture medium used was Iscove's modified Dulbecco medium (IMDM) (Sigma) supplemented with 5% heat-inactivated FACS, 2×10^{-3} M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-3} M mercaptoethanol (all Sigma).

Cells were cultured in 2 ml volume, containing 1×10^5 BMDC, 2.5×10^5 sorted naive $CD4^+$ T cells with or without 2.5×10^5 $CD4^+CD25^+$ T cells, stimulated with soluble 0.5 μg/ml anti-CD3 (45-2C11), or immobilized anti-CD3 (1 μg/ml) and 10 μg/ml anti-CD28 (37.51) or 5 μl anti-CD3 and anti-CD28-coated beads (Dynal Biotech, Oslo, Norway). TLR stimuli used were 100 ng/ml LPS (from Salmonella Minnesota R595, ultrapure TLR4 grade) (Alexis Biochemicals, Lausen, Switzerland), 200 nM CpG (1668) (Invitrogen), 100 μg/ml PolyI:C (Amersham Biosciences, New Jersey). Exogenous cytokines used were TGFβ (1 ng/ml) (Sigma), IL-1β (10 ng/ml), IL-4 (10 ng/ml), IL-6 (20 ng/ml), TNFα (10 ng/ml), (All ImmunoTools, Germany), IL-12 (5 ng/ml) (Preprotech EC Ltd, United Kingdom), and IL-23 (10 ng/ml) (kind gift from Dr. A. O'Garra).

Quantitative mRNA Analysis of Cytokines and Transcription Factors

RNA was extracted using 1-bromo-3-chloro-propane (Sigma) and reverse transcribed with oligo $d(T)_{16}$ (Applied Biosystems, Branchburg, New Jersey) according to the manufacturer's protocol. The cDNA served as template for the amplification of genes of interest and the housekeeping gene (Hprt) by real-time PCR, using TaqMan Gene Expression Assays (Hprt, Mm00446968_m1; IL-2, Mm00434256_m1; Tbet, Mm00450960_m1; GATA3, Mm00484683_m; Hlx, Mm00468656_m1; Smad7, Mm00484741_m1) (all Applied Biosystems, Foster City, California), universal PCR Master Mix (Applied Biosystems, Warrington, United Kingdom) and the ABI-PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, California). Target gene expression was calculated using the comparative method for relative quantitation upon normalization to Hprt gene expression.

Antibodies and Flow Cytometry

Anti-CD4 APC (RM4-5), anti-CD25 PE (PC61), anti-CD44 PE (IM7), anti-CD62L APC (MEL14), anti-CD45.2 Biotin (104), anti-IFNγ FITC (XMG1.2), and anti-IL-6 (MP5-20F3) were purchased from eBioscience (San Diego, California); Streptavidin APC was purchased from Molecular Probes (Eugene, Oregon); and anti-IL-17 PE from BD Biosciences (United Kingdom). Anti-TGFβ (1D11.16) and isotype control (Ox-1) were a kind gift from Dr. A. Cooke. Anti-CD3 (145-2C11), anti-CD28 (37.51) anti-IFNγ (R46A2), anti-IL-4 (11B11) anti-IL-12/IL-23 p40 (C17.8), and anti-IL-10 (JES5 16E3) were purified from hybridoma supernatant in our laboratory using standard procedures. All antibodies were used at 10 μg/ml for blocking. For determination of intracellular cytokine production, cells were restimulated with 500 ng/ml PdBu, 500 ng/ml ionomycin, and 1 μg/ml BrefeldinA (all Sigma) for 4 hr at 37ºC. Cells were then stained for surface markers, fixed in 1% paraformaldehyde in PBS, and permeabilized with 0.1% NP40 PBS, followed by labeling with specific cytokine Abs or isotype controls. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, California) using Cellquest software (Becton Dickinson) and further analyzed using FlowJo software (Tree Star, Inc).

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.immunity.com/cgi/content/full/24/2/179/DC1/.

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