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From interleukin-23 to T-helper 17 cells: human T-helper cell differentiation revisited

Katia Boniface[1], **Bianca Blom**[1,*], **Yong-Jun Liu**[1,†], and **René de Waal Malefyt**[1]
[1]Department of Immunology, Schering-Plough Biopharma (Formerly DNAX Research), Palo Alto, CA, USA

Summary

Protracted inflammation leading to dysregulation of effector T-cell responses represents a common feature of a wide range of autoimmune diseases. The interleukin-12 (IL-12)/T-helper 1 (Th1) pathway was thought to be responsible for the pathogenesis of multiple chronic inflammatory diseases, including psoriasis, inflammatory bowel disease, arthritis, or multiple sclerosis, mainly through their production of interferon-γ and its effects on macrophage activation and chemokine production. However, this initial concept of T-cell-mediated chronic inflammation required an adjustment with the discovery of an IL-12-related cytokine, designated IL-23. IL-23 was rapidly recognized for its involvement in the establishment of chronic inflammation and in the development of a Th cell subset producing IL-17, designated Th17, which is distinct from the previously reported Th1 and Th2 populations. This review aims to describe the characterization of IL-23 and its receptor, its biological activities, as well as its involvement in the development of human Th17 cells and autoimmunity.

Keywords

IL-23; Th17 cells; inflammation; autoimmunity; human; cytokines

Interleukin-23, a novel heterodimeric cytokine involved in inflammation

Over 20 years ago, Mosmann and Coffman (1) described in mice the presence of two distinct populations of T-helper (Th) cells, designated Th1 and Th2, which were characterized by a specific cytokine signature. While interleukin-12 (IL-12) induces the development of Th1 cells that produce IL-2, interferon-γ (IFN-γ), and lymphotoxin-α, and elicit cell-mediated immunity against intracellular pathogens, Th2 cells differentiate in response to IL-4, produce IL-4, IL-5, and IL-13, and are involved in humoral immunity against parasites and allergy (2). Although it was relatively easy with the availability of T-cell receptor (TCR) transgenic mouse strains to polarize naive mouse T cells in vitro into pure Th1 and Th2 effector cells and to isolate effector T-cell clones from lymph nodes and organs of mice subjected to strongly polarizing disease models, studies on Th1 and Th2 cells in human were more challenging. A large percentage of T cells isolated from the blood of healthy individuals produce a mixed cytokine phenotype upon activation. Eventually human Th1 and Th2 cells were defined as differentiation protocols and culture conditions were

Correspondence to: René de Waal Malefyt, Department of Immunology, Schering-Plough Biopharma, 901 California Avenue, Palo Alto, CA 94304, USA, Tel: + 1 650 496 1164, Fax: + 1 650 496 1200, rene.de.waal.malefyt@spcorp.com.
*Current address: Department of Cell biology and Histology, Amsterdan Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
†Current address: Center or Cancer Immunology Research M. D. Anderson Cancer Center, Houston, TX, USA.

optimized for human T cells with multiple rounds of priming (3–5). In addition, it turned out to be important that for the isolation of T-cells clones with polarized phenotypes, T cells were isolated from the actual site of inflammation from patients suffering from infectious or allergic diseases. The discovery of additional cytokines and mediators, such as thymic stromal lymphopoietin, IL-25, IL-33, IL-18, IFN-α, and Notch ligands that have the ability to modulate Th1/Th2 differentiation, further illustrates the complexity of the T-cell development process within and between different species (6, 7).

Protracted inflammation leading to dysregulation of effector T-cell responses represents a hallmark of a wide range of autoimmune diseases. Th1 cells have been associated with the development and maintenance of chronic inflammatory diseases, such as psoriasis, inflammatory bowel disease (IBD), multiple sclerosis (MS), and rheumatoid arthritis (RA), through their production of IFN-γ and its effects on macrophage activation and chemokine production. Enhanced expression levels of IFN-γ, IL-12, and other critical components of the Th1 pathway have indeed been demonstrated in these human inflammatory diseases and their appropriate corresponding mouse models (2, 8). However, this initial concept of T-cell-mediated chronic inflammation required an adjustment with the unexpected discovery that mice deficient in IFN-γ or IFN-γ receptor were not resistant to experimental autoimmune encephalomyelitis (EAE) but were actually more susceptible to central nervous system autoimmunity (9–11). Observations in mice with targeted disruptions in the genomic regions encoding the IL-12 subunits further questioned the association between IL-12 and inflammatory disorders. IL-12 was the first identified cytokine with a heterodimeric protein structure and is composed of a soluble cytokine receptor-like 40 kDa subunit p40 that is covalently linked to a cytokine-like 35 kDa subunit p35 (12). Surprisingly, mice with a targeted disruption of the gene encoding p35 were more susceptible to disease in models of chronic inflammation, whereas mice lacking the p40 subunit of IL-12 were resistant (8, 13). Conversely, IL-12p40-deficient mice were more susceptible than IL-12p35-deficient mice with respect to *Cryptoccocus neoformans* and *Listeria monocytogenes* bacterial infections (14, 15). Several years later, the identification of another cytokine-like binding partner for IL-12p40 would provide the first plausible explanation for these unexpected findings (16).

Based on a computational screen of cDNA and expressed sequence tag databases with structure-based algorithms modeled on the IL-6 helical cytokine family, we identified a novel cytokine called p19. Characterization of p19 protein proved difficult at first, as the protein was inefficiently secreted from transfected cells and did not show biological activity in various *in vitro* bioassays. However, when we realized that p19 could be part of another heterodimeric cytokine complex and evaluated potential binding partners in this family, we demonstrated that p19 could form a p19–p40 heterodimer. Furthermore, we showed that the p19–p40 heterodimer was expressed and secreted by primary dendritic cells (DCs) upon activation, and that this heterodimer had biological activity on T cells, which, all together, justified its designation as IL-23 (16).

The discovery of IL-23 has had a tremendous impact on our understanding of the cytokines and T-cell pathways that govern chronic inflammation. Many previous studies that explored the influence of IL-12 on chronic inflammation were based on the use of antibodies neutralizing the IL-12p40 chain or of mice deficient in the IL-12p40 gene and needed to be revisited, because this approach neutralized the biological activities of IL-12 and IL-23. That IL-23, rather than IL-12, is crucial during the pathogenesis of autoimmune diseases became clear when p19 and p35 subunits were targeted. IL-23p19-deficient but not IL-12p35-deficient mice were resistant to EAE and collagen-induced arthritis (CIA) (17, 18). Cua and colleagues (19) further showed that IL-23p19-deficient mice were still able to mount a Th1 response but failed to produce the proinflammatory cytokine IL-17 (reviewed in 20).

Characterization of IL-23-induced signal transduction

IL-23 exerts its biological activities through the interaction with a heterodimeric receptor complex composed of IL-12Rβ1 and IL-23R (21, 22) (Fig. 1). IL-23R is mainly expressed by T cells, natural killer cells, and to a lower extent by monocytes and DC populations (21). Like IL-12, IL-23 can directly bind the IL-12Rβ1 chain through its interaction with the IL-12p40 subunit. Whereas IL-12 uses IL-12Rβ2, IL-23 requires IL-23R as heterodimeric partner to allow signal transduction to occur. IL-23 and IL-12 activate the same Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling molecules. Non-receptor protein tyrosine kinase-2 is associated with IL-12Rβ1, and JAK2 is constitutively associated with the IL-23R chain. Binding of IL-12 and IL-23 to their receptor leads to phosphorylation of STAT1, STAT3, STAT4, and STAT5. However, STAT4 phosphorylation induced by IL-23 is much weaker than that induced in response to IL-12, and the formation of DNA-binding complexes are different, with mostly STAT4 homodimers formed in response to IL-12 and STAT3 homo- and heterodimers formed in response to IL-23. The responsiveness of cells to either IL-12 or IL-23 is thus determined by the respective expression of IL-12Rβ2 and IL-23R (21). Based on this pattern of receptor expression and signal transduction pathways, it can be expected that IL-12 and IL-23 would have overlapping but also unique biological activities.

Characterization of IL-23 bioactivity

In the initial characterization of IL-23 bioactivity, we compared the effects of IL-12 and IL-23 on sorted naive and memory mouse T cells from IL-10-deficient mice in the presence of anti-IL-2 monoclonal antibodies (16). Whereas CD4 CD45RB^{high} naive T cells proliferated in response to anti-CD3 stimulation and IL-12 but not IL-23, CD4 CD45RB^{low} memory T cells proliferated in response to IL-23 but not to IL-12. These results are in agreement with the observation that CD45RB^{high} cells expressed high levels of IL-12Rβ2 and lacked IL-23R, whereas CD45RB^{low} cells expressed IL-23R but low levels of IL-12Rβ2, and thus indicate that IL-23 preferentially acts as a growth factor for memory T cells (21). IL-10-deficient mice spontaneously develop enterocolitis resembling Crohn's disease (CD), which can be blocked by treatment with anti-IL-12p40 monoclonal antibodies (23). In a follow-up study, we showed that IL-12p35 × IL-10-deficient mice but not IL-23p19 × IL-10-deficient mice spontaneously developed IBD, supporting a role of IL-23 in promoting intestinal inflammation (24). Furthermore, administration of IL-23 in a T-cell transfer model of colitis accelerated disease development irrespective of whether naive or memory cells from diseased IL-10-deficient mice were transferred into recombination-activating gene knockout recipients. Presumably, the naive T cells were converted to memory T cells in this inflammatory environment, making them responsive to IL-23. Furthermore, as shown in the EAE model (19), IL-23 promoted the production of IL-17 and IL-6 from the memory-activated T cells (24). These IL-17-producing T cells were present in the gut of IL-10-deficient mice but not in IL-10 × IL-23p19-deficient mice. Similar results on the role of IL-23 and production of IL-17 have also been described in other models of IBD and are discussed in (25) by Powrie *et al.* These results thus clearly identified a role for IL-23 in the proliferation of murine memory T cells, production of IL-17, and development of inflammation.

In our initial characterization of the effects of IL-23 on human T cells, we compared the activities of IL-23 and IL-12 on the proliferation and IFN-γ production by 7-day-old PHA blasts, a classical bioassay for IL-12 activity (12). Both IL-12 and IL-23 enhanced proliferation and IFN-γ production of anti-CD3- and anti-CD28-activated PHA blasts, which was blocked by anti-IL-12Rβ1 and anti-IL-12p40 monoclonal antibodies (16). However, the IL-23-induced activity could not be neutralized with anti-IL-12p35

monoclonal antibodies. We also compared the activities of IL-12 and IL-23 on fluorescence-assisted cell sorter (FACS)-sorted $CD4^+CD45RA^+$ naive and $CD4^+CD45RO^+$ memory T cells isolated from peripheral blood. In these experiments, T cells were activated by plate-bound anti-CD3 and soluble anti-CD28 in the presence of IL-2 and assessed for IFN-γ production at days 3 and 6. $CD45RO^+$ memory T cells responded to IL-23 and IL-12 at both time points with enhanced IFN-γ production, although the levels were lower in response to IL-23. $CD45RA^+$ naive T cells produced IFN-γ in response to IL-23 at day 6 but not at day 3, whereas IL-12 enhanced IFN-γ production at both time points (16). IL-23R is expressed on human memory T cells but is barely detected on naive cells (26, 27), suggesting that TCR activation of naive human T cells leads to an upregulation of low levels of IL-23R expression, rendering the cells sensitive to IL-23. In addition, IL-23 increases its own receptor expression on activated naive T cells (27). This is analogous to the TCR-mediated induction of IL-12Rβ2 expression in Th1 commitment (28) and further upregulation by IL-27 (29). In the next series of experiments, we extended these observations using purified cord blood $CD4^+CD45RA^+$ T cells in a culture system that was set up to study human Th1 and Th2 differentiation (4). T cells were cultured with anti-CD3 monoclonal antibody crosslinked onto irradiated $FcγRII^+$ (CD32), $CD58^+$, and $CD80^+$ transfected L-cell fibroblasts in the presence of IL-2 and IL-23, IL-12, or IL-4, and expansion and differentiation was determined following restimulation with plate-bound anti-CD3, soluble anti-CD28, and IL-2 after 6 and 12 days. The addition of IL-2 to these cultures only affected cell survival and proliferation of T cells and did not affect the differentiation of the $CD4^+$ T cells. Clonal expansion of naive $CD4^+$ T cells stimulated in the presence of IL-23 was slightly higher compared with control cultures containing IL-2 only after 6 and 12 days of culture. The increase in cell number induced by IL-12 was higher compared with that induced by IL-23 or IL-2 but lower compared with that induced by IL-4. Culture of naive $CD4^+$ T cells in IL-2 alone induced little IFN-γ after 6 or 12 days of stimulation, and addition of IL-4 to the cultures abolished the induction of IFN-γ production (Fig. 2). Strikingly, IL-23 induced the production of IFN-γ from naive $CD4^+$ T cells after 6 days, and this was even more pronounced after 12 days of stimulation. Addition of IL-12 to the $CD4^+$ T cells induced production of the highest levels of IFN-γ, both after 6 and 12 days. Interestingly, IL-23 also induced the production of tumor necrosis factor-α (TNF-α) from $CD4^+$ T cells, which again was slightly lower compared with the IL-12-cultured cells. No significant effect of IL-23 on the production of the Th2 cytokines IL-4, IL-5, and IL-10 could be observed. Flow cytometric analysis was performed following intracellular cytokine staining to determine the effect of IL-23 on cytokine production at the single cell level. Clearly, after 6 days of priming, IL-23 induced a population of $CD4^+$ T cells to produce IFN-γ (Fig. 3). Consistent with the results obtained by enzyme-linked immunosorbent assay, IFN-γ induction by IL-23 was more pronounced after 12 days of culture. IL-12 was a more potent inducer of IFN-γ-producing cells at both culture time points. IL-23 induced higher numbers of both IL-2 and TNF-α-producing T cells compared with the control-stimulated T cells, although prolonged stimulation with IL-23 was required to observe this effect. Similar observations were made in the presence of IL-12, with a more pronounced effect than IL-23. To determine the specificity of the IL-23-induced cytokine induction, experiments were carried out with neutralizing antibodies directed against IL-12p40, IL-12Rβ1, or IL-12p35 present during the priming conditions. Addition of anti-IL-12p40 monoclonal antibody and anti-IL-12Rβ1 polyclonal antibodies significantly blocked the IL-23- and IL-12-induced IFN-γ production (Fig. 4). The anti-IL-12p35 monoclonal antibody was not capable of blocking the IFN-γ production induced by IL-23 but did block IL-12-induced IFN-γ secretion. Taken together, the results of these early experiments on the biological activities of IL-23 in human indicated that it could act on both memory and naive T cells. The effects of IL-23 on naive T cells from peripheral blood and cord blood required prolonged exposure, consistent with the need to further upregulate the IL-23R on these cells. The read-out in these earlier experiments was the production of IFN-γ, mainly as we were

comparing IL-23's activity with that of IL-12. It is not uncommon for cytokines to have redundant activities such as IFN-γ production, especially for those that share receptor components and thus intracellular signaling pathways. There are many examples available, e.g. IL-4/IL-13 for immunoglobulin E (IgE) production, IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 as T-cell growth factors, IL-3/granulocyte-macrophage colony-stimulating factor for myeloid cell growth. However, the most important activity of IL-23 that distinguishes it from IL-12 is its ability to induce the production of IL-17, and differentiation of Th17 cells.

Discovery of a third Th cell subset: Th17 cells

IL-17 (30) was originally identified by Rouvier et al. (31) in rodent T-cell hybridoma clones as cytotoxic T-lymphocyte-associated antigen 8 and the human version was cloned from a $CD4^+$ T-cell library (32). IL-17 is the founding member of a newly identified cytokine family comprising IL-17 (IL-17A), IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. IL-17 is mostly known for its pro-inflammatory activities, both *in vitro* and *in vivo*, and its expression is increased in inflammatory tissues (33).

Initial findings showed that *Borrelia burgdorferi* induced IL-17 production by human T cells independently of Th1 or Th2 cytokine production (34). However, the concept that T cells producing IL-17 should be classified as a distinct Th cell subset emerged from mouse models of autoimmunity, such as EAE, IBD, or CIA, following the discovery of IL-23 (16, 17, 19, 35). Th17 cells are now recognized as a third T-effector cell subset (36–39), and the IL-23/IL-17 pathway is linked to mucosal host defense against extracellular pathogens (40–42) and to the induction and progression of a number of inflammatory diseases, including psoriasis, IBD, arthritis, and MS (17–19, 24, 26, 43–46). Following their identification in the mouse, we set out to define the development, cytokine profile, and function of human IL-17-producing T cells (26). The characterization of specific surface markers and cytokines defining human Th17 cells is critical to identify and 'track' those cells in the tissue during inflammation. Furthermore, the understanding of the cytokines involved in Th17 cell development and/or regulation is central in the perspective of new target discovery to cure inflammatory disorders.

Cytokine profile of human Th17 cells

Culture of naive T cells in the presence of IL-23 and or IL-1β induced the differentiation of human IL-17-producing T cells. In addition, based on the expression of the IL-23R, we identified human Th17 cells as a subpopulation of $CD45RO^+$ T cells in the blood from normal healthy donors. Both the *in vitro*-derived Th17 cells and the in vivo-occurring Th17 cells were found to express a signature cytokine profile consisting of IL-17, IL-17F, IL-22, IL-26, and CCL20. In addition, a significant proportion of these cells coexpressed IL-17 and IFN-γ (26). This cytokine profile of human Th17 cells producing inflammatory cytokines, such as IL-17, IL-17F, and IL-22, was observed by other groups (26, 27, 40, 47–49) and was previously also shown in the mouse system (19, 35, 50, 51). Two groups independently isolated IL-17-producing $CD4^+$ memory T cells from peripheral blood or intestine of healthy individuals as well as patients with active CD (40, 46), and these cells were found in both central ($CCR7^+$) and effector ($CCR7^-$) memory $CD4^+$ pools (40, 52). Both IL-17 and IL-17F have major pro-inflammatory effects on epithelial cells and are important for the recruitment of neutrophils (26, 33, 51, 53). IL-22 is a member of the IL-10 family, largely described for its pro-inflammatory activities on keratinocytes and its upregulated expression during inflammatory disorders (51, 54–59). Moreover, IL-22 was recently shown to be a crucial cytokine during IL-23-induced dermal inflammation and acanthosis (59). We showed that human Th17 cells also express IL-26 (26), an IL-10 family member recently reported to induce inflammatory genes expression in intestinal epithelial cells and to be upregulated in

colonic retinoid-related orphan receptor γt (RORγt)-expressing Th17 cells in CD patients (60). The work of Liu and Rohowsky-Kochan (52) suggests that the majority of IL-17 producers also express TNF-α and IL-6.

Importantly, as we discussed above from our early IL-23 experiments, human Th17 cells can also express IFN-γ (26, 27, 40, 46, 61). IFN-γ has been largely described for its inflammatory activities, and its expression is elevated in a number of inflammatory diseases, such as psoriasis and IBD (62–64). Moreover, IL-17 IFN-γ^- (Th17) and IL-17^+ IFN-γ^+ (Th17/Th1) cells were identified in the intestine of patients with active CD (46). A preliminary study addressing this question showed that Th17 and Th17/Th1 populations expressed a similar chemokine receptor expression profile, at least for CCR4, CCR6, CXCR3, and CXCR6 (65). A more extensive characterization of the surface markers and cytokine profile expressed by these populations may improve the understanding of the specific function of these two subsets of IL-17 producers.

Phenotype of human Th17 cells

Chemokines are differentially expressed in inflamed tissues by epithelial and immune cells and induce the recruitment of specialized effector cells through the expression of specific chemokine receptors. Whereas Th1 cells specifically express CXCR3, CCR5, and CXCR6, Th2 cells express CCR4, CCR8, the prostaglandin D2 receptor CRTh2, and to a lesser extent CCR3 (66). It is now well documented that IL-17-producing cells express CCR6 (40, 46, 47, 52, 65, 67). CCR6 expressed by Th17 cells is functional, as its ligand CCL20/macrophage inflammatory protein-3a induced calcium influx in Th17 but not Th1 clones (46). Acosta-Rodriguez *et al.* (40) further showed that expression of CCR6 and CCR4 defines a population of Th17 cells expressing IL-17 but not IFN-γ, whereas cells expressing CCR6 and CXCR3 produce IL-17 and IFN-γ (Th1/ Th17) or IFN-γ only (Th1). CCR6 and CCL20 are highly expressed in inflammatory tissues and are involved in the recruitment of pathogenic T cells in MS, RA, CD, and psoriasis (46, 68–72).

Whether CCR2 could be used as a ‘marker’ of Th17 cells remains unclear. Sato et al. (73) described IL-17 producers as memory $CD4^+$ T cells expressing CCR2. These cells secreted IL-17 and IFN-γ. Further analyses using CCR5 indicated that $CCR2^+$ $CCR5^-$ T cells expressed high levels of IL-23R and IL-17 but not of IFN-γ. In contrast, $CCR2^+CCR5^+$ T cells produced IFN-γ but not IL-17 (73). Singh *et al.* (67) also observed an increased frequency of IL-17 producers in the $CCR2^+$ $CCR5^-$ T cells compared with $CCR2^+$ $CCR5^+$ T cells; however, only a minority of $CCR2^+$ $CCR5^-$ T cells could be induced to make IL-17. In addition, few Th17 cells from peripheral blood were found within the $CCR2^+CCR5^-$ subset. Whether the CCR6 Th17 population overlaps with $CCR2^+CCR5^-$ T cells was not examined.

It was recently shown that *in vitro*-derived Th17 cells have a higher expression of CCR9 and CXCR6 as compared with Th1 and Th2 cells (67). Thus, CCR9 and CXCR6 may also be expressed preferentially on Th17 cells. In line with this observation, $CCR9^+$ lymphocytes from the *lamina propria* have been described to produce IL-17 (74), and CCR9 together with its ligand CCL25 have been reported to play an important role in small bowel immunity and inflammation (75, 76).

Besides expression of a specific set of chemokine receptors, we and others identified IL-23R as a specific marker for the Th17 population (26, 46, 47). In contrast, the specific IL-12 receptor subunit IL-12Rβ2 was expressed by all Th cell subsets (46). We showed that circulating IL-$23R^+$ memory $CD4^+$ T cells isolated from healthy donors produced higher levels of IL-17 than their IL-$23R^-$ counterparts, whereas IFN-γ levels were similar. IL-23R–expressing cells also express the ‘signature’ cytokine profile of Th17 cells, including

IL-17F, IL-22, IL-26, and CCL20 (26). Thus, as for Th1 and Th2 subsets, Th17 cells are characterized by expression of specific chemokine receptors inducing their recruitment to inflammatory sites expressing their specific ligands. In addition, the expression of lineage specific surface markers, such as IL-23R, allows us to identify and track those cells in inflamed tissues as well as in the periphery.

Human Th17 cell differentiation

Although initial studies showed a crucial role of IL-23 in production of IL-17 and Th17-mediated autoimmunity, it became evident that IL-23 did not drive differentiation of naive T cells into Th17 cells in the mouse system (77–79). These observations were not unexpected, as naive T cells do not express IL-23R (21). Instead, transforming growth factor-β (TGF-β) was identified as a critical factor for mouse Th17 cell differentiation. Indeed, whereas TGF-β alone induces the development of regulatory T cells (Tregs) expressing Forkhead box protein 3 (FoxP3) (79, 80), the presence of IL-6 or IL-21 will prevent the generation of $FoxP3^+$ cells and lead to the development of Th17 cells and upregulation of the IL-23R (77–79, 81–83). Consequently, IL-23 is not required for early Th17 development; it is however strictly necessary for the maintenance and pathogenicity of Th17 cells (50).

Several studies assessed the different cytokines possibly involved in the differentiation of human Th17 cells (Fig. 5); however, it remains difficult to obtain a consensus between groups, reminiscent of the early controversy with Th1 and Th2 cells in humans. The section below highlights the main findings observed by different groups.

IL-1β and IL-23

We and others (26, 27, 49, 61, 84) have shown that the combination of TGF-β and IL-6 does not drive human Th17 development. Instead, the culture of peripheral blood naive T cells in the presence of IL-23 or IL-1β was sufficient to drive Th17 development (26, 27, 61). Moreover, Acosta-Rodriguez *et al.* (61) further showed that IL-6 could enhance IL-1β-driven IL-17 production. Furthermore, we (26) and O'Shea's group (27) also described IL-23 as a potent promoter of Th17 development. In vitro-derived Th17 cells also express RORγt, IL-22, IL-17F, IL-26, CCL20, CCR6, and IL-23R. In addition, these cells are characterized by the presence of both $IL\text{-}17^+$ and $IL\text{-}17^+$ /IFN-γ^+ producers.

TGF-β or no TGF-β?

All the studies published so far agree that in contrast to mice, the combination of TGF-β and IL-6 alone was not sufficient to drive human Th17 cell differentiation. Whether TGF-β is required for human Th17 cell differentiation is still a matter of controversy. Indeed, three independent groups (48, 49, 84) recently reported an absolute necessity of TGF-β to drive Th17 cell development in human. Volpe *et al.* (48) detected IL-17 production when naive T cells were cultured in the presence of IL-23, IL-1β, IL-6, and TNF-α, but observed that IL-17 levels were strongly increased when TGF-β was added to this inflammatory cytokine cocktail. This was true for naive T cells selected by magnetic cell sorting or FACS-sorted from peripheral blood and cord blood cells. Manel *et al.* (49) showed that the combination of TGF-β, IL-1β, and IL-23 was optimal to induce IL-17 producers from naive cord blood $CD4^+$ T cells and that endogenous levels of IL-6 or IL-21 were not required for Th17 cell development. Th17 cells expressed increased levels of IL-17, IL-17F, IL-26, and IL-23R. In contrast, IL-22 production was downregulated in the presence of TGF-β (49). In their study, Volpe *et al.* (48) showed that IL-17 and IL-6 were associated with Th17 cells, whereas IL-22, TNF-α, IL-21, and IFN-γ were expressed by both Th17 and Th1 cells. However, Th17 cells polarized in the presence of TGF-β were IL-17 single producers, and no $IL\text{-}17^+$ IFN-γ^+ cells were detected. As both $IL\text{-}17^+$ IFN-γ^- and $IL\text{-}17^+$ IFN-γ^+ have been identified

in inflamed tissues, for example the brain of mice after EAE induction (50, 85) or the intestine of patients with active CD (46), one could ask whether in vitro Th17 cells polarized in the presence of TGF-β reflect the Th17 populations present in vivo at sites of inflammation. The work of Yang *et al.* (84) suggests that in a serum-free medium, the combination of TGF-β and IL-21 drives the development of Th17 cells expressing increased levels IL-17 but not IFN-γ or IL-22 from peripheral blood and cord blood naive T cells. In contrast, Manel *et al.* (49) failed to see any induction of IL-17 production in the presence of TGF-β and IL-21 from naive cord blood cells cultured in medium either containing serum or not. They further showed that the neutralization of IL-21 during Th17 cell development did not regulate the proportion of IL-17 producers. We also failed to detect any upregulation of IL-17 production when peripheral blood naive T cells were stimulated in the presence of TGF-β and IL-21 (unpublished data). The recent observation that IL-21 plays an important role in the generation of T-follicular helper cells and is a potent inducer of Ig production would challenge its physiological importance for Th17 biology (86, 87). Interestingly, we and others (26, 27, 61, 77) showed that TGF-β inhibited IL-17 production induced by IL-23 or by the combination of IL-1β and IL-6.

How can the discrepancies observed in human Th17 development between groups be explained? One possibility could be the presence of endogenous TGF-β in cultures containing serum. Indeed, the use of serum-free medium leads to an increased proportion of IL-17 producers compared with medium containing serum (49), suggesting that TGF-β present in the serum can downregulate to some extent IL-17 production. Volpe *et al.* (48) showed that the use of medium with or without serum required the same cytokines for Th17 development, although IL-17 levels were lower in a medium containing serum. Another explanation could be the time of culture. The effects of IL-1β and/or IL-23 without addition of exogenous TGF-β were analyzed in a 2-week culture assay. In contrast, cultures performed in the presence of TGF-β were usually harvested after a 5-day culture period. Despite the differences observed among the different studies, IL-23 and IL-1β emerge as critical factors in the induction of IL-17-producing cells.

Transcription factors associated with human Th17 cell development

As T-bet/STAT4 and GATA-3/STAT6 are respectively related to Th1 and Th2 cells, Ivanov et al. identified RORγt as a transcription factor specifically expressed in mouse and human Th17 cells (26, 88, reviewed in 20). Transduction of human cord blood $CD4^+$ T cells with RORγt is sufficient to induce IL-17 production, and conversely knockdown of RORγt results in much lower IL-17 production (49). As previously shown in mice (89), the involvement of other ROR family members is conceivable, as RORα also induces IL-17 expression when overexpressed in primary human T cells (49). Interestingly, forced expression of RORγt is not sufficient to induce IL-22 production (49), suggesting the requirement of additional transcription factors. In this regard, Veldhoen *et al.* (77) identified the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor, as a critical factor in the induction of IL-22 production in mice. They further showed that human Th17 cells express AHR. In addition to RORγt, human Th17 cells express also T-bet, which is consistent with the observed production of IFN-γ (46).

An increasing body of evidence shows the requirement of STAT3 for Th17 cell development. Indeed, in mice STAT3 is absolutely required for the induction of IL-17, IL-17F, and RORγt (90–92). Moreover, patients suffering from autosomal dominant hyper-IgE syndrome, associated with negative mutations in STAT3, had impaired Th17 cell differentiation (93–96). These subjects secreted much lower IL-17 and IL-22 than control individuals, showing the requirement of STAT3 to induce the production of these cytokines *in vivo* in human. Thus, although there is still debate on the cytokines involved in Th17

differentiation in mice and human, there is a consensus that STAT3 activation is essential. In this light, it is relevant that IL-6, IL-21, and IL-23 are all able to induce STAT3 phosphorylation.

Negative regulators of human Th17 cell development

When looking at the strong potential of Th17 cells to cause damage to the host, it was expected that this subset would be subjected to many regulatory processes. Initial studies assessing lineage commitment of naive $CD4^+$ T cells into Th1 and Th2 cells revealed that these subsets cross-regulate each other through their specific signature cytokines IFN-γ and IL-4, respectively (97–99). The same principle applies to Th17 cell regulation both in the mouse and in the human systems. We showed that IL-4 and IL-12 prevented Th17 differentiation induced by IL-23 from human naive $CD4^+$ T cells (26).

Regulation of Th17 responses can also happen in a cytokine-independent context. For example, retinoic acid, an active metabolite of vitamin A, is a potent inhibitor of Th17 commitment in mice, while enhancing $FoxP3^+CD4^+$ Tregs (100–103). In contrast, sphingosine 1-phosphate can enhance development of Th17 cells in mice (104). Thus, non-cytokine immunomodulatory agents produced during inflammation also play an important role in regulating Th17 responses, and further studies will undoubtedly help to better understand how such agents positively or negatively modulate Th17 function.

Function of human Th17 cells

The link of Th17 cells to pathologic inflammation is much more established than our understanding of their role in normal immune defense mechanisms. Several lines of evidence support their involvement in mucosal immunity in mice, particularly against extracellular bacterial infections. For example, IL-17, IL-22, and IL-23 are all necessary to elicit full immune response to *Klebsiella pneumoniae* (105–107); mice deficient in IL-17RA have enhanced susceptibility to *Toxoplasma gondii and Candida albicans* infection but not to *Mycobacterium tuberculosis* or *L. monocytogenes* (106, 108, 109), and IL-22 is critical for host defense against *Citrobacter rodentium* infection (110). However, while little is known regarding the role of IL-23, IL-17, and IL-22 in resistance to infection in humans, what has emerged in the last few years is indirect evidence that patients suffering from diseases associated with various infections, including hyper-IgE syndrome, chronic mucocutaneous candidiasis, or Mendelian susceptibility to mycobacterial diseases, have a defect in Th17 cells (93–95, 111). In addition, both IL-17 and IL-22 induce anti-microbial peptides production from various epithelial cell types in human (26, 51, 57, 107, 112), suggesting their participation in host defense. Napolitani and colleagues (40) showed that human memory T cells specific for *C. albicans* were mainly $CCR6^+CCR4^+$ Th17 subset, whereas T cells specific for *M. tuberculosis* were present in the $CCR6^+CXCR3^+$ Th1 population, suggesting that Th17 and Th1 cells exhibit different immune functions in response to pathogens. *C. albicans* in the hyphal form primed Th17 responses *in vitro* and induced production of IL-23 but not IL-12 by human DCs (40).

Annunziato *et al.* (46) showed that Th17 cells exhibit poor proliferative capacity, low cytotoxicity, and reduced susceptibility to suppressive activity of $CD4^+FoxP3^+$ Tregs compared with Th1 and Th2 cells. They are also able to help B cells to induce the production of IgG, IgM, and IgA, but not IgE (46). Accordingly, a recent study showed that human Th17 cells, but not Th1 or Th2 cells, expressed B-cell chemoattractant CXCL13 (113). Thus, the Th17/B-cell interaction could lead to the production of Igs by B cells, which would help in resolving the infection.

IL-23/Th17 and/or IL-12/Th1-mediated autoimmune diseases?

While nature's plan certainly envisioned a contribution of Th17 cells to host defense mechanisms, this inflammatory Th cell subset is better known for its role in promoting destructive tissue inflammation. Th17 cells are key mediators of chronic inflammation in various animal models (17–19, 24, 45). However, even after the establishment of Th17 cells as pathologic mediators, evidence remained for a contribution of the Th1 pathway during inflammatory processes, reflecting the complexity of inflammatory diseases. For example, IFN-γ producers are found in the brain of mice during EAE, inhibition of T-bet expression by RNA interference ameliorates EAE, and IFN-γ has been described for its pathogenic role in different mouse models of inflammation (50, 114–116).

The situation in human inflammatory diseases comes with an even higher degree of complexity: Pène *et al.* (47) isolated $CD4^+$ T cells from lesions of patients suffering from chronic diseases, including psoriasis, CD, RA, and severe asthma, and identified Th1, Th2, as well as Th17 cells in inflamed tissues. Th17 clones, selected for their elevated IL-17 and IL-22 production, expressed higher levels of RORγt, IL-17F, IL-26, CCL20, TNF-α, CCR6, IL-1R1, and IL-23R than Th1 and Th2 clones. Interestingly, Th17 clones contained low levels of IFN-γ. To further understand the mechanisms of human inflammatory diseases, it becomes then critical to determine the importance of the distinct Th cell subsets in each particular disease.

A new line of evidence favors a role of IL-23/Th17 rather than IL-12/Th1 pathway during the pathogenesis of autoimmune disorders, for example in MS. Increased levels IL-17 were found in the cerebrospinal fluid and blood of MS patients (117, 118), and IL-23 but not IL-12 production was increased in monocyte-derived DCs isolated from MS patients (118). Prat and colleagues (44) directly investigated the involvement of human Th17 cells in central nervous inflammation. IL-23-driven Th17 cells generated from peripheral blood $CD4^+$ T cells from healthy donors migrated more efficiently across the blood–brain barrier than did IL-12-driven Th1 cells. These Th17 cells were IL-17, IL-22, or IL-17/ IL-22 producers. $IL\text{-}17^+IL\text{-}22^+$ cells preferentially expressed granzyme B and had enhanced cytolytic activity toward neuronal cells isolated from fetal brain compared with nonactivated lymphocytes (44).

If psoriasis was traditionally associated with IL-12/Th1-exacerbated responses, this conclusion was mainly based on the upregulation of IL-12p40 expression in lesional skin. However, we and others (26, 119–121) reported that IL-23 but not IL-12 expression is increased in psoriatic lesions, as shown by upregulated expression of IL-23p19, IL-12p40, but not IL-12p35. The expression of IL-1β, RORγt, and Th17 cytokines IL-17, IL-22, IL-17F, IL-26, and of IFN-γ was also upregulated in psoriatic skin (26, 56, 58, 122, 123). Overexpression of Th17 cytokines also led to the production of anti-microbial peptides and chemokines. All together, this inflammatory milieu sets the stage for pathology (Fig. 6). The involvement of IL-23 during cutaneous inflammation is also corroborated *in vivo*, as intradermal injection of IL-23 in mice induced a psoriasis-like phenotype (59, 119, 124). The most compelling evidence indicating a major role of the IL-23/Th17 pathway during psoriasis came from the improvement in psoriasis area-and-severity index in patients when anti-IL-12p40-neutralizing antibodies were administered (125–129). We now know that neutralization of IL-12 by blocking IL-12p40 also neutralizes the function of IL-23, raising the question for the actual mechanism of action. Cooper and colleagues (125) further showed that the clinical improvement was associated with reduced expression of IL-12p40 and IL-23p19 but not of IL-12p35. Furthermore, genetic studies revealed an association between IL23R and IL12B genes and susceptibility to psoriasis (130–132), making it likely that the IL-23/Th17 pathway plays a dominant role in this disease.

Mounting evidence supports a major role of IL-23 and Th17 cells in the pathogenesis of IBD. IL-12, IFN-γ, IL-23, and IL-17 expression is increased in the colonic *lamina propria* of CD patients (62, 63, 133–136). Clinical trials using anti-IL-12p40-neutralizing antibodies are showing promising results in the treatment of CD (137, 138). Investigating how IL-12p40 neutralization modulates the inflammatory response in CD patients, Mannon and colleagues (133) identified reduced secretion of IL-23, IL-12, IL-17, and IL-6 by mononuclear cells in the colonic *lamina propria* of patients that received anti-IL-12p40-neutralizing antibodies.

Strong evidence for the importance of IL-23 in CD pathogenesis emerged from genetic studies. Performing genome-wide analysis of single nucleotide polymorphisms in healthy subjects and CD patients, Cho and colleagues (139) established the association between *IL23R* gene polymorphisms and susceptibility to CD. Several studies performed in different patient cohorts confirmed those results (139–145). In addition, it was recently reported that *IL23R* gene polymorphisms correlate with IL-22 serum levels in CD patients (146). The established link between IL-23 and CD raises the question for a biological involvement of Th17 cells. Two groups recently cloned Th17 cells from the inflamed *lamina propria* of CD patients and compared them to Th1 or Th2 clones isolated from the same tissue (46, 47). These elegant studies were instrumental in characterizing the phenotype of human Th17 cells. The study of T-cell clones, however, provided only limited insight into the involvement of Th17 cells in the inflammatory process itself. The increasing knowledge about the phenotypical features of Th17 cells should soon enable studies of this important immune pathway in health and disease.

Conclusion

The discovery of IL-23 has led to the identification of a new Th cell subset that complements the Th1/Th2 paradigm. It is now clear that this Th17 pathway, with all its complexity, is essential for protection against infectious agents but also for the pathogenesis of inflammatory disorders. However, many questions still need to be answered. The precise role played by TGF-β during mouse and human Th17 cell development remains to be analyzed at a molecular level to get full insight in the importance of this factor. In addition, we have not discussed in this review the interaction and relationship between human Th17 and Treg cells. It is known that the regulation of FoxP3 expression is different between mice and humans (147) and that the interplay between FoxP3 and RORγt is important for the decision of mouse Th17 versus Treg cell lineage commitment (148). In this respect it is interesting that human Treg cells seem to be able to differentiate into IL-17-producing cells when stimulated with IL-2 and IL-15 in the presence of monocytes (149).

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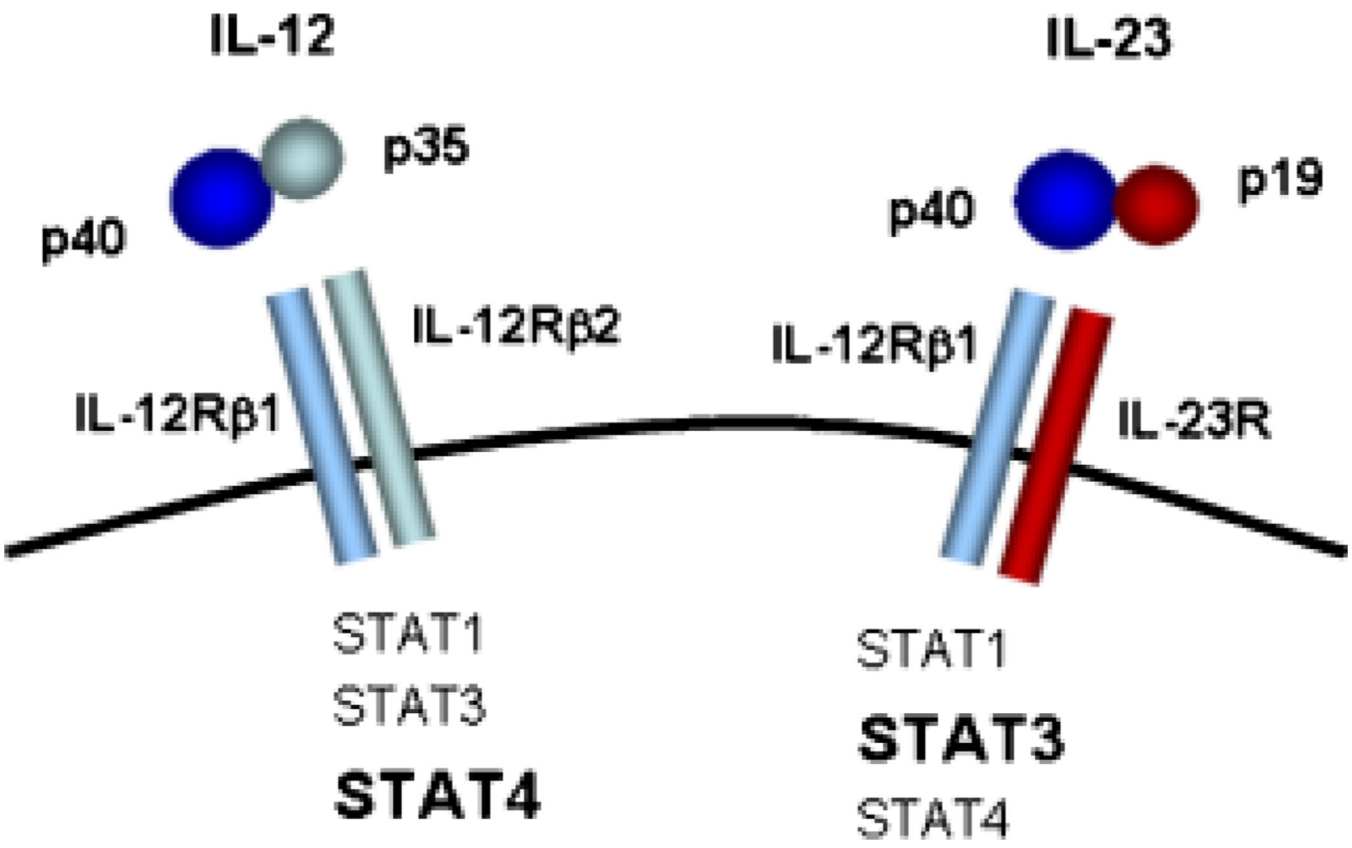

Fig. 1. Overview of IL-12 and IL-23 ligand and receptor complexes

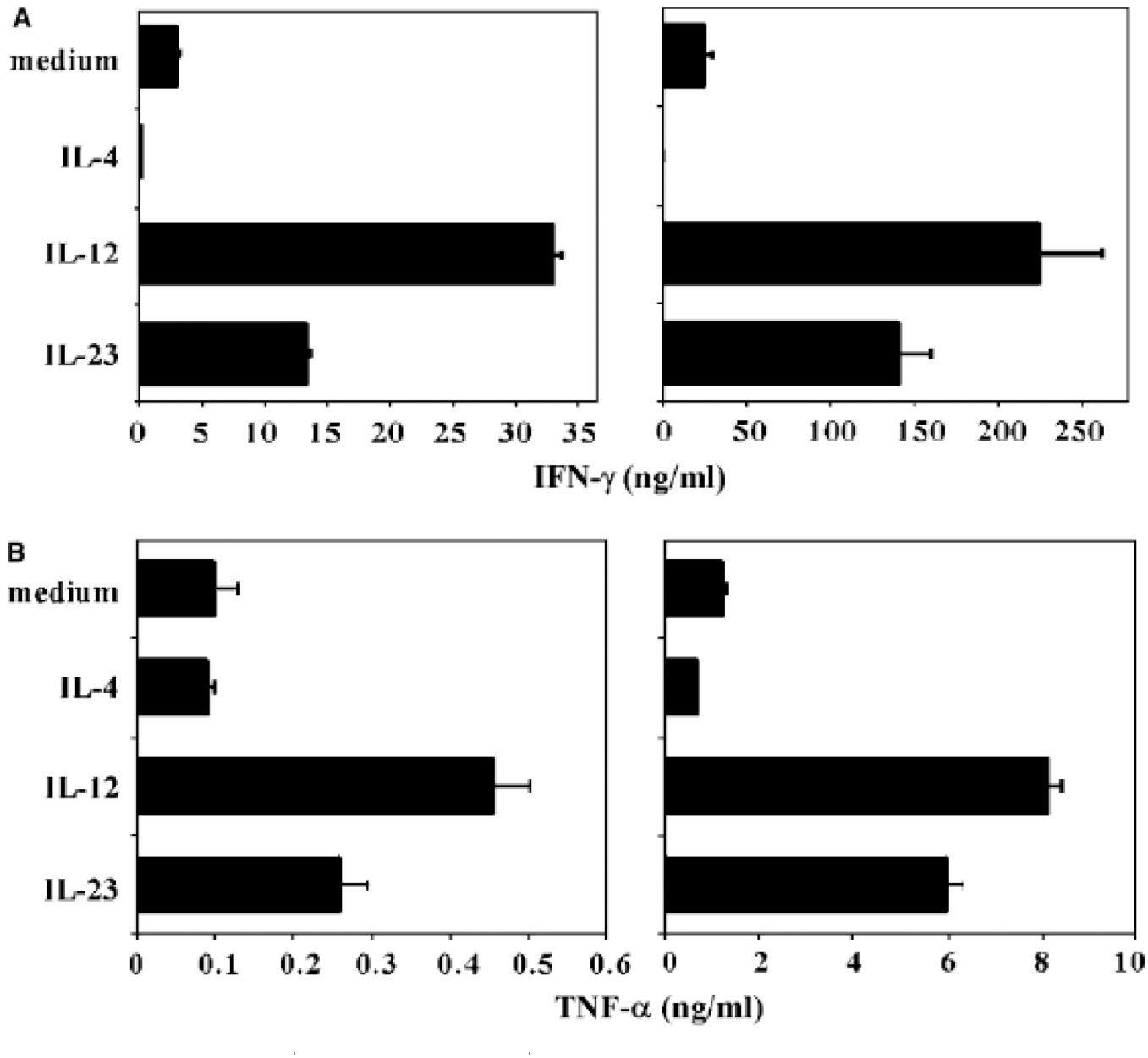

Fig. 2. IL-23 induces polarization of naive CD4$^+$ T cells

Naive CD4$^+$ T cells were isolated from cord blood, primed for 6 days, and recultured for an additional 6 days in the presence of the indicated cytokines, anti-CD3, IL-2, and irradiated CD32/CD58/CD80-transfected L cells. Cells were harvested at day 6 (left) and day 12 (right) and stimulated for 24 h.

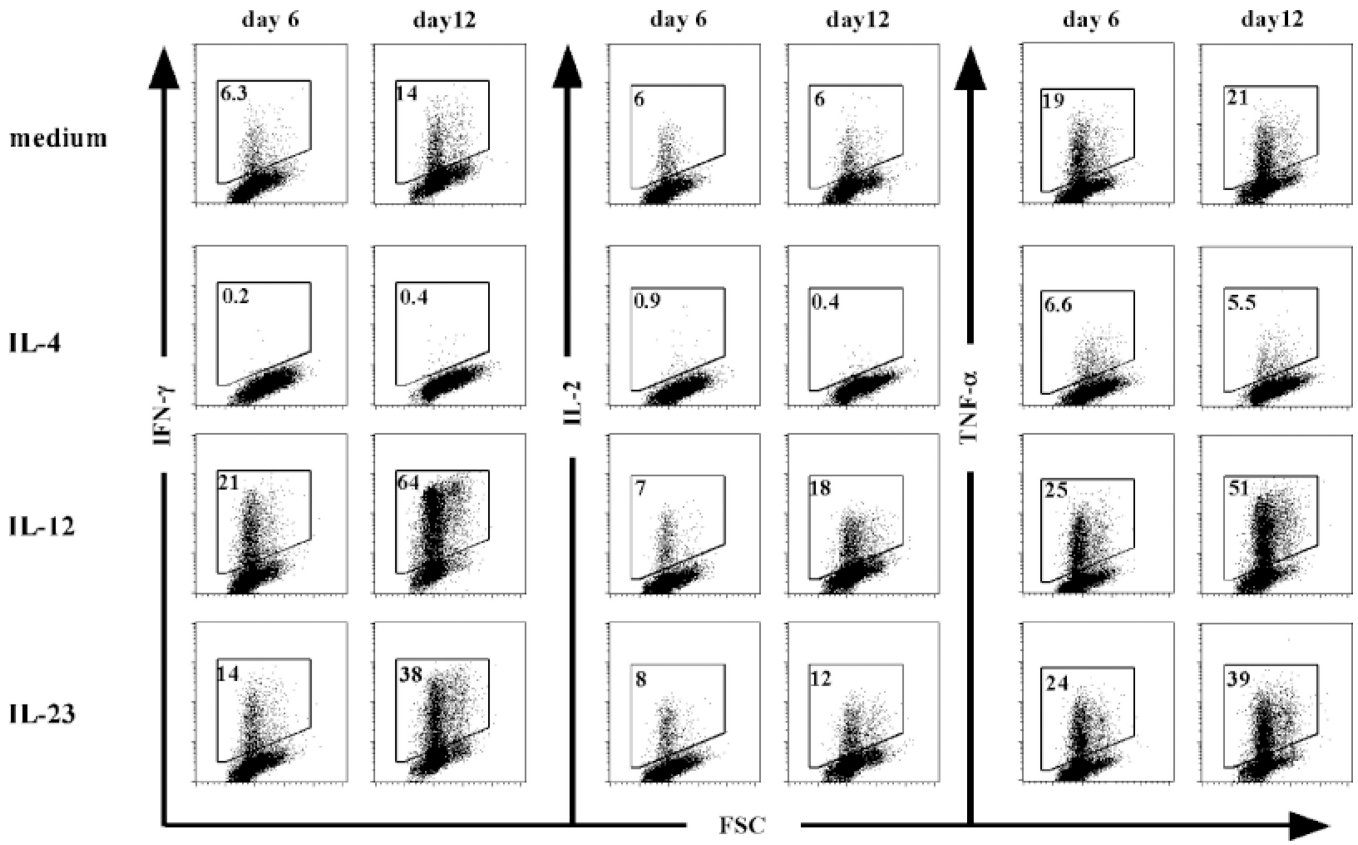

Fig. 3. IL-23 induces polarization of naive CD4$^+$ T cells

Naive CD4$^+$ T cells were isolated from cord blood, primed for 6 days, and recultured for an additional 6 days in the presence of the indicated cytokines, anti-CD3, IL-2 and irradiated CD32/CD58/CD80-transfected L cells. Cells were harvested at days 6 and 12 and stimulated for 6 h.

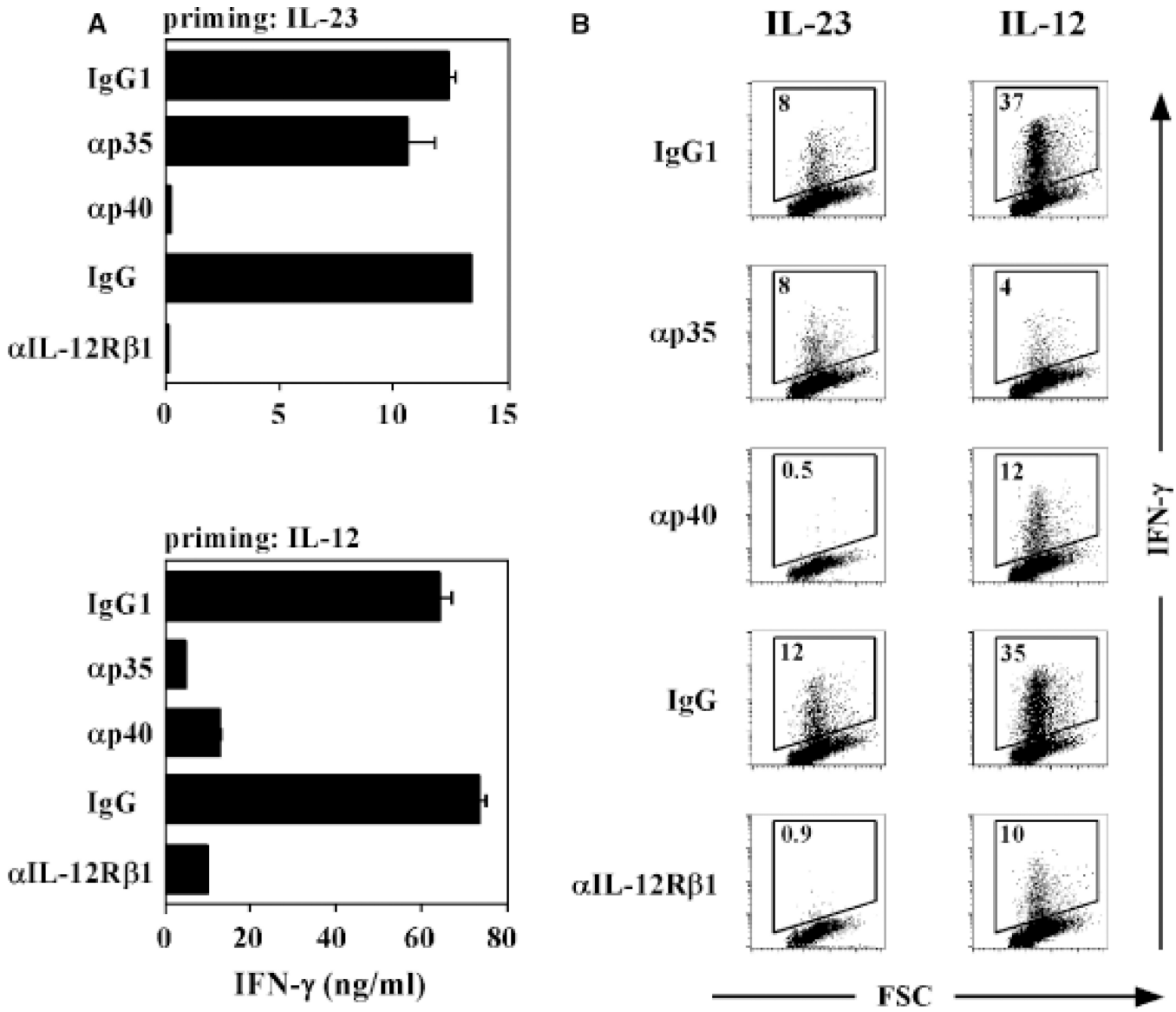

Fig. 4. IL-23-induced polarization is inhibited by neutralizing antibodies against p40 and IL-12Rβ1 but not p35

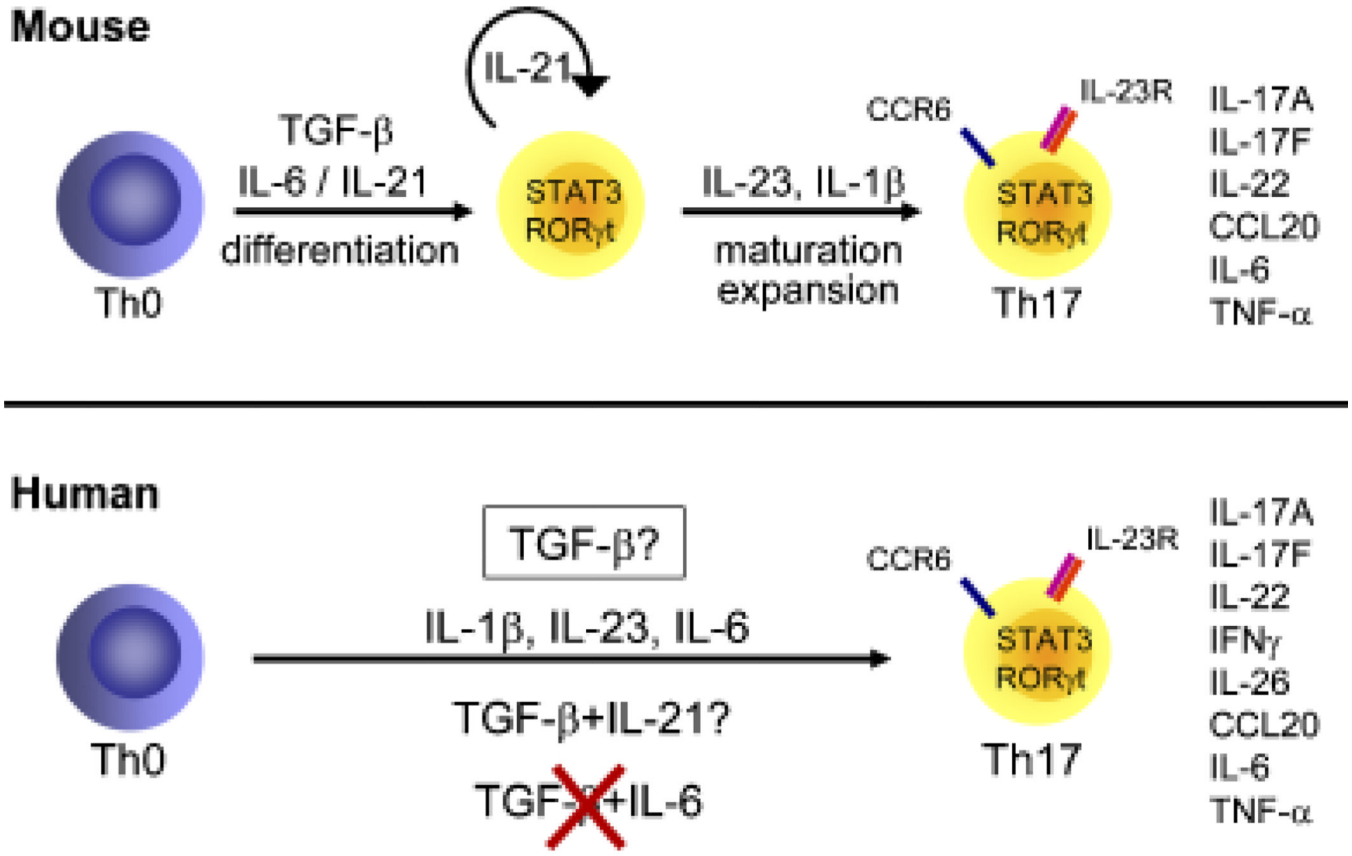

Fig. 5. Th17 cell differentiation in mouse and human

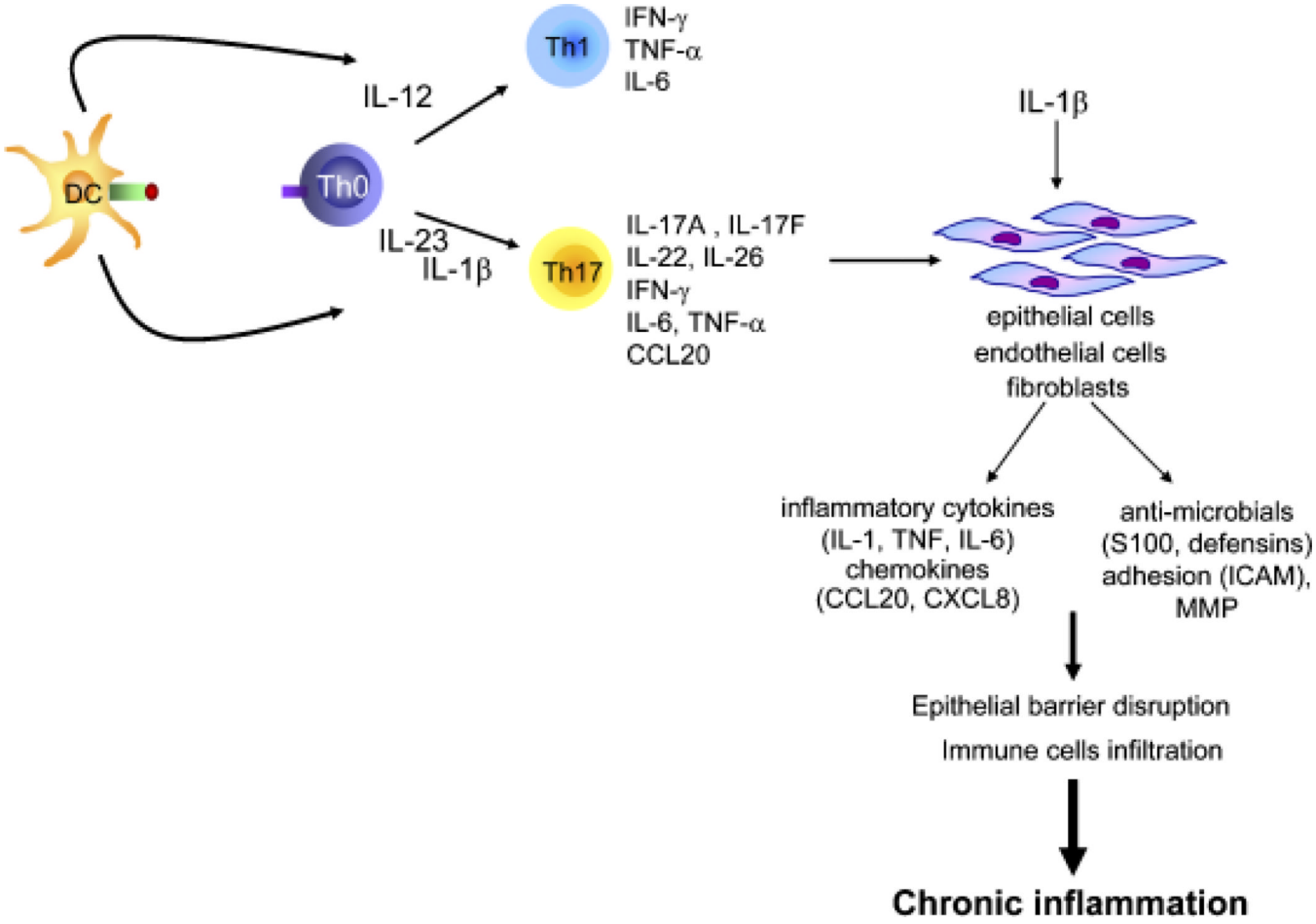

Fig. 6. IL-23/Th17 pathway and inflammation

In response to inflammatory stimuli, dendritic cells produce IL-23 and IL-1β, which induce development of Th17 cells that produce IL-17, IL-22, IL-17F, and other proinflammatory cytokines. This inflammatory milieu, through its action on epithelial cells, will contribute to epithelial barrier disruption and recruitment of immune cells.