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REVIEW

More stories on Th17 cells

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For more than two decades, immunologists have been using the so-called Th1/Th2 paradigm to explain most of the phenomena related to adaptive immunity. The Th1/Th2 paradigm implied the existence of two different, mutually regulated, CD4$^+$ T helper subsets: Th1 cells, driving cell-mediated immune responses involved in tissue damage and fighting infection against intracellular parasites; and Th2 cells that mediate IgE production and are particularly involved in eosinophilic inflammation, allergy and clearance of helminthic infections. A third member of the T helper set, IL-17-producing CD4$^+$ T cells, now called Th17 cells, was recently described as a distinct lineage that does not share developmental pathways with either Th1 or Th2 cells. The Th17 subset has been linked to autoimmune disorders, being able to produce IL-17, IL-17F and IL-21 among other inflammatory cytokines. Interestingly, it has been reported that there is not only a cross-regulation among Th1, Th2 and Th17 effector cells but there is also a dichotomy in the generation of Th17 and T regulatory cells. Therefore, Treg and Th17 effector cells arise in a mutually exclusive fashion, depending on whether they are activated in the presence of TGF-β or TGF-β plus inflammatory cytokines such as IL-6. This review will address the discovery of the Th17 cells, and recent progress on their development and regulation.

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Basis for the Th1/Th2 paradigm

An efficient adaptive immune response against pathogen antigen determinants is fundamental for their elimination by the host. At the same time, it is also crucial for host homeostasis that the immune system is able to tolerate self-components, as well as many foreign antigens, such as those from commensal bacteria and food. Uncovering the mechanisms that enable the adaptive immune system to accomplish these tasks has always (and still is) been a big challenge. That was exactly the challenge motivating Christopher Parish's research when he drew the basis for the later establishment of the Th1/Th2 paradigm.

Parish was employing antigen modification by acetoacetylation to induce tolerance. He found that acetoacetylated derivatives of flagellin (from *Salmonella adelaide*) were able to dramatically reduce a primary antigen response to unmodified flagellin in rats [1]. Unexpectedly, the same antigen modification led to an increased delayed-type hypersensitivity (DTH) response [2]. Thus, there was an inverse relationship between antigen response and DTH [3]. Although the term 'immune deviation' had been coined a little earlier, that was the first strong evidence showing that humoral- and cell-mediated immune responses could be cross-regulated [3]. One important question still remained: Are the T cells mediating DTH different from those helping B cells to produce antibodies? Although Parish and Liew performed experiments suggesting the existence of different T-cell populations orchestrating humoral- and cell-mediated responses [3, 4], a formal proof was still missing. It is important to keep in mind that at that time there were no monoclonal antibodies to surface markers and cytokines. Actually, the discovery of cytokines was about 10 years away and even distinguishing CD4 and CD8 cells was not as easy as it is today.

The Th1/Th2 paradigm

In the mid-1980s, the development of new techniques, such as the ability to clone T cells, and the MTT assay,

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a colorimetric assay for cell growth, allowed this question to be revisited (Figure 1). By combining these two new tools, Tim Mossman's lab was able to distinguish two different types of T cells producing different growth factors. While Th1 cells would mainly produce IL-2 and IFN-γ, Th2 cells would produce a weaker T-cell growth factor distinct from IL-2 [5, 6]. At the same time, Bob Coffman's lab had established a very sensitive and specific solid-phase assay for IgE, aiming at understanding how IgE production is regulated [5]. The two lines of research came along very nicely when they decided to test supernatants from the two different T cell types in their assay for IgE production. Surprisingly, supernatants from a Th2 clone added to LPS-stimulated B cells led to robust IgE responses, whereas supernatants containing IL-2 and IFN-γ from Th1 clones induced no IgE production [5, 7]. Importantly, when both supernatants were added together no IgE was detected, demonstrating the ability of a Th1 factor to block the Th2-induced IgE response. By using neutralizing antibodies (the only monoclonal antibody to a cytokine they had available at that time), they demonstrated that the Th1 factor responsible for inhibiting Th2-induced IgE production was IFN-γ [5, 7]. It was also found that the weaker T-cell growth factor released by Th2 clones that could induce IgE responses was actually IL-4, called B-cell stimulatory factor-1 (BSF-1) at that time [5, 8]. One year later, the last piece to build up the concept came when it was demonstrated that Th1 clones, but not Th2 clones, could mediate DTH responses [9].

The Th1/Th2 paradigm implies the existence of two different $CD4^+$ T helper subsets. One of them, Th1, drives cell-mediated immune responses involved in tissue damage and fighting infection against intracellular parasites and also provides help for B cells to produce certain isotypes of G immunoglobulin (Ig), specifically IgG2a [5, 10]. The other one, Th2, mediates IgE production and is largely involved in eosinophilic inflammation, allergy and clearance of helminthic infections [5, 10]. The concept also involved the notion that the two subsets are cross-regulated. Thus, cytokines released from cells of one subset had the ability to stimulate its own subset in an autocrine fashion and, at the same time, inhibit the other subset.

The studies on the Th1/Th2 paradigm rapidly evolved to understand better what determines the differentiation of each subset and also the transcription factors involved in their regulation. Accumulating evidence shows that IL-12 is crucial for Th1-cell differentiation through Stat4 (signal transducer and activator of transcription 4) and the activation of a unique transcription factor named T-bet (T-box expressed in T cells), which upregulates IFN-γ and downregulates IL-4 and IL-5 expression [11-13]. In contrast, IL-4 induces Th2-cell differentiation through Stat6 and activation of GATA3, which upregulates IL-4 and IL-5, but downregulates IFN-γ expression [11, 13].

The Th1/Th2 paradigm proposed by Mosmann and Coffman had a profound impact on the way immunologists perceived adaptive immune responses and the reciprocal relationships that might exist among T-cell subsets (Figure 1). It has also helped our better understanding of factors that regulate atopic diseases as well as host resistance and susceptibility to intracellular pathogens such as *Leishmania major* [14]. Following the "fall" of suppressor T cells [15], it was also proposed to explain peripheral tolerance to self-components [16].

Contradictions of the Th1/Th2 paradigm

The Th1/Th2 paradigm was not sufficient to explain

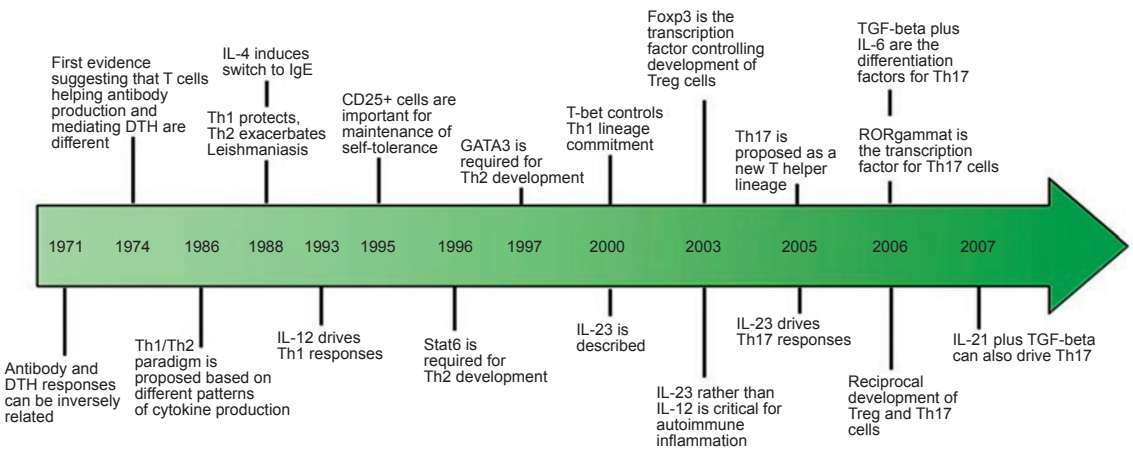

Figure 1 Timeline: advances on T helper research. Figure depicts some of the most relevant findings in the field of T helper research (based on the article written by FY Liew [133]).

a lot of experimental evidence coming particularly from studies on autoimmune diseases. Following the concept that Th1 cells play a major role in tissue damage, one would predict that administration of IFN-γ (the main effector cytokine produced by Th1 cells) would worsen autoimmune diseases and, conversely, that blocking IFN-γ by either using neutralizing antibodies or deleting IFN-γ gene would ameliorate autoimmune diseases. Those predictions could not be confirmed and experimental data suggested just the opposite. In an animal model for multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE), administration of IFN-γ reduced disease severity in susceptible strains of mice and rats [17-19]. Accordingly, treatment with neutralizing antibodies to IFN-γ rendered EAE-resistant strains susceptible to a very severe form of the disease [17, 20]. In a similar way, disruption of the gene encoding either IFN-γ or IFN-γ receptor converted otherwise EAE-resistant strains to a susceptible phenotype, suggesting a protective role for IFN-γ in EAE [21, 22]. In the BALB/c strain, IFN-γ disruption was associated with an enhanced T-cell response to MBP [22]. Moreover, animals lacking other molecules involved in Th1 differentiation, such as Stat1 and the IL-12 receptor β2, were also shown to be not only susceptible but to develop more severe disease [23, 24]. Altogether, these data challenged the concept that Th1 cells play an essential role in pathogenesis of autoimmune diseases.

Contradictory data supporting the Th1 relevance in EAE came from studies utilizing T-bet-deficient mice and transfer of myelin antigen-specific Th1 cells to naïve recipient animals. Deletion of the gene encoding the transcription factor T-bet was shown to confer resistance to EAE in mice immunized with MOG peptide [23]. Accordingly, it has been reported that upon transfer to naïve animals, activated Th1 cells are able to induce EAE in mice and rats [25, 26]. In addition, studies using IL-12 p40 gene-targeted animals and neutralizing antibodies to IL-12 p40 suggested that IL-12, the main inductor of Th1 responses, was necessary for EAE development [27, 28]. In summary, although experimental evidence suggested a role for Th1 cells in autoimmune diseases, it also demonstrated that Th1 cells alone could not fully explain autoimmune disease pathogenesis, thus implying that an important piece of the puzzle was missing.

Discovering IL-23 and Th17 cells

The discovery of IL-23 [29], a new member of the IL-12 cytokine family started to shed some light on the scene and clarify why autoimmune diseases could not be completely explained by the Th1/Th2 paradigm (Figure 1). IL-12 is a heterodimeric molecule formed by subunits p35 and p40. IL-23 is also a heterodimeric cytokine composed by the same subunit p40 but now paired with the unique p19 [29, 30]. IL-23, like IL-12, is mainly produced by cells of the innate immune system, such as dendritic cells (DCs) and tissue-resident macrophages. However, while some microbial products preferentially induce IL-12 expression, DC activation with PGE2, ATP or anti-CD40 antibodies elicits production of IL-23 [30-32]. Cua *et al.* [33] dissected the participation of IL-12 and IL-23 in EAE induction by using animals with gene disruption for each of the subunits forming IL-12 and IL-23: p19, p35 and p40. They were able to show that animals deficient in IL-23 ($p19^{-/-}$) and in both IL-12 and IL-23 ($p40^{-/-}$) were protected from EAE. In contrast, mice deficient only in IL-12 ($p35^{-/-}$) were highly susceptible to EAE induction [33]. Moreover, IL-23 gene transfer vectors delivered into the CNS reconstituted EAE in both $p19^{-/-}$ and $p40^{-/-}$ mice. Finally, IL-12 gene transfer into the CNS did not facilitate disease in $p40^{-/-}$ animals [33]. Thus, IL-23 rather than IL-12 is a crucial cytokine for the development of CNS autoimmune inflammation.

Besides being part of the same cytokine family and sharing the subunit p40, IL-23 and IL-12 also signal through similar receptors. IL-12 signals through a receptor complex composed of IL-12Rβ1 and IL-12Rβ2 [30]. IL-23 in turn signals through a heterodimeric receptor made of the sharing IL-12Rβ1 subunit plus a unique IL-23R subunit [30]. Since the two cytokines and their receptors are closely related, it was first predicted that IL-12 and IL-23 would exert similar functions. So, at first, it was proposed that IL-12 and IL-23 could play complementary roles, with IL-23 being essential to mediate or sustain late-stage chronic inflammation [33]. However, soon data appeared demonstrating that was not the case and that actually, IL-12 and IL-23 were responsible for driving different T-cell subsets (see below).

Daniel Cua's group extended their findings using a different animal model for autoimmune disease, the collagen-induced arthritis. Again using gene-targeted mice lacking only IL-12 ($p35^{-/-}$) or IL-23 ($p19^{-/-}$), they showed that the specific absence of IL-23 is protective, whereas the loss of IL-12 exacerbates collagen-induced arthritis [34]. IL-23 gene-targeted mice did not develop clinical signs of disease and were completely resistant to the development of joint and bone pathology [34]. Resistance correlated with the absence of IL-17-producing $CD4^+$ T cells despite normal induction of collagen-specific, interferon-γ-producing Th1 cells. In contrast, IL-12-deficient $p35^{-/-}$ mice developed more IL-17-producing $CD4^+$ T cells [34].

In fact, Aggarwal *et al.* [35] had demonstrated earlier

that activation of $CD4^+$ T cells in the presence of IL-23 leads to elevated IL-17 production, a phenotype distinct from those described previously by Mosmann and Coffman (Figure 1). In contrast, the main Th1 inductor cytokine IL-12 induced only marginal IL-17 production [35]. That was further confirmed and extended by Cua and colleagues [36]. They have shown that in contrast to IL-12, IL-23 does not promote the development of interferon-γ-producing Th1 cells, but is one of the essential factors required for the expansion of a distinct pathogenic $CD4^+$ T-cell population, which is characterized by the production of IL-17, IL-17F, IL-6 and tumor necrosis factor [36]. Gene expression analysis of IL-23-driven autoreactive T cells identified a unique expression pattern of proinflammatory cytokines and other novel factors, distinguishing them from IL-12-driven T cells. Using passive transfer studies, it was demonstrated that these IL-23-dependent $CD4^+$ T cells are highly pathogenic and essential for the establishment of organ-specific inflammation associated with central nervous system autoimmunity [36]. These findings were extended and IL-17-producing $CD4^+$ T cells were shown to play a fundamental role in different models of autoimmune diseases. Indeed, IL-17-deficient mice have reduced collagen-induced arthritis [37] and, when immunized with myelin antigens in CFA, develop EAE with delayed onset and diminished severity [38]. Consistently, treatment with an IL-17R antagonist attenuated adjuvant-induced arthritis in rats [39] and administration of blocking antibodies to IL-17 prevented chemokine expression in the brain and subsequent EAE development in mice [40].

In addition to their role in the development of autoimmune diseases, IL-17-producing $CD4^+$ T cells were also shown to constitute an important arm of adaptive immune responses conferring protection against extracellular pathogens [41]. It was reported that *in vitro* T-cell activation in the presence of conditioned media from *Klebsiella pneumoniae*-pulsed dendritic cells led to IL-17 production in an IL-23-dependent manner [42]. In addition, similar to IL-12 $p35^{-/-}$ animals, IL-23 p19-deficient mice are more susceptible to lung infection with *Klebsiella pneumoniae* [42]. Increased mortality in $p19^{-/-}$ animals was associated with dramatically reduced IL-17 production in the lungs and administration of exogenous IL-17 was able to restore bacterial control [42]. Consistently, IL-17R-deficient animals were reported to be exquisitely sensitive to intranasal *Klebsiella pneumoniae* with 100% mortality 48 h after infection [43]. The role played by IL-17-producing T cells in controlling certain extracellular pathogens may be of particular relevance in infections associated with immunodeficient conditions such as AIDS. In fact, it was recently demonstrated that in simian immunodeficiency virus (SIV)-infected rhesus macaques, T cell-driven IL-17 responses against *Salmonella typhimurium* were markedly blunted, which led to increased bacterial dissemination [44]. In the same context, IL-17-producing cells are also known to play an important role in the establishment of effective immune responses to *Mycobacterium tuberculosis* mainly through the recruitment of protective IFN-γ-producing $CD4^+$ T cells [45].

Then, besides Th1 and Th2, the third member of the effector T-cell trilogy, Th17, arises [46] (Figure 1). Two independent groups proposed that IL-17-producing $CD4^+$ T cells, so-called Th17, are a distinct lineage that does not share developmental pathways with either Th1 or Th2 cells [47, 48]. Hence, it was demonstrated that Th17 differentiation does not require any of the transcription factors involved in Th1 (such as T-bet, Stat4 and Stat1) or Th2 (such as Stat6 and c-Maf) development [47, 48]. Moreover, IL-17 expression was increased substantially when anti-IFN-γ and anti-IL-4 were added during T-cell differentiation, suggesting that IFN-γ and IL-4 negatively regulate the generation of IL-17-producing cells [47, 48]. Thus, it was proposed that in the absence of IFN-γ and IL-4, IL-23 induces naïve precursor cells to differentiate into Th17 cells [47]. However, it had been already shown that unlike memory cells, naïve T cells do not express the receptor for IL-23 [35]. Thus, it was unlikely that IL-23 would be the dominant factor required for Th17 differentiation. Indeed, independent studies demonstrated that a combination of the pro-inflammatory cytokine IL-6 and TGF-β could induce *in vitro* differentiation of truly naïve T cells into IL-17-producing cells [49, 50, 134].

The importance of this combination of cytokines for the development of Th17 cells *in vivo* was also documented. Upon *ex vivo* stimulation with antigen, $CD4^+$ T cells from mice bearing a transgenic TCR recognizing MOG and expressing TGF-β under the IL-2 promoter release high concentrations of TGF-β and can protect naïve recipients from EAE [51]. However, upon *in vivo* immunization with MOG in CFA, which leads to elevated IL-6 production by the innate immune system, those animals developed more severe EAE associated with increased IL-17 production by T cells [49]. Another important piece of data pointing to the importance of TGF-β signaling on induction of Th17 cells came from experiments utilizing CD4-DNTGFBRII mice. These animals, which express a dominant-negative mutant of TGF-β receptor II in CD4 cells, are deficient in Th17 cells and are more resistant to EAE [52]. The crucial participation of TGF-β in promoting differentiation of Th17 cells was surprising since TGF-β has long been recognized as an important molecule regulating adaptive immune responses [53]

and, particularly, as being directly responsible for *de novo* generation of peripheral Foxp3$^+$ regulatory T cells (iTreg) [54-57]. Altogether, the important concept of reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells [49] had been established. It seems that there is not only a functional antagonism between Th17 and T regulatory (Treg) cells but that there is a dichotomy in their generation as well. Therefore, Treg cells and Th17 effectors arise in a mutually exclusive manner, depending on whether they are activated in the presence of TGF-β or TGF-β plus IL-6 [49].

At the steady-state level or in the absence of any inflammatory insult, TGF-β produced in the immune system has the capacity to suppress the generation of effector T cells and induce Foxp3$^+$ regulatory T cells, thereby contributing to the maintenance of homeostasis. This pathway has particular relevance at mucosal surfaces such as the intestine, where both intense microbial load and production of TGF-β are constant under physiological conditions. In this regard, intestinal tissue has been shown to be highly effective at inducing iTregs (inducible Tregs). Lafaille's group, for instance, has shown by using mice lacking nTregs (natural Tregs) that iTregs are sufficient for oral tolerance induction [58, 59]. Belkaid and Powrie's groups confirmed and extended these findings by demonstrating that iTregs were preferentially induced in mesenteric LN (MLN) and lamina propria by a subpopulation of DCs, rather than in the spleen or peripheral lymph nodes, reinforcing that the intestine is a privileged site for Treg induction [60, 61]. Importantly, the intestinal cells also produce a "co-factor" for Treg development, the vitamin-A metabolite retinoic acid (RA) (discussed below) [60-63].

At the same time, the intestine also harbors high amounts of IL-17-producing T cells at steady state, which correlates with the fact that inflammatory cytokines are produced physiologically in the intestine [64-66]. Therefore, upon infection or inflammation, IL-6 produced by the activated innate immune system is able to suppress the generation of TGF-β-induced Treg cells and induce a pro-inflammatory T-cell response predominated by Th17 cells [49]. In a recent study, Ivanov *et al*. [66] reported that commensal bacteria are required for IL-17 production in the small intestine, since germ-free mice contained virtually no Th17 cells in the lamina propria. Moreover, upon introduction of bacteria from SPF mice (conventionalization), these formerly germ-free animals induced IL-17 production in the lamina propria. Surprisingly, neither Trif nor Myd88 were required for this "spontaneous" IL-17 production in the lamina propria, indicating that toll-like receptor signaling was not involved in this phenomenon [66]. An explanation for these findings could be found in the recent report by Atarashi *et al.* [67], who have shown that adenosine 5′-triphosphate (ATP) derived from commensal bacteria can activate a subset of lamina propria cells (CD70^{high}-CD11c^{low} cells) that are able to produce IL-6, IL-23 and TGF-β, triggering the differentiation of Th17 cells. A balance between these pro-inflammatory and anti-inflammatory functions of TGF-β is crucial to maintain immune tolerance to self or to the non-pathogenic non-self (microbiota and food antigens) and, at the same time, to keep an immune-tonus that generates efficient adaptive immune response against antigen determinants derived from pathogens.

Following the suggestion that IL-6 plays a pivotal role in dictating whether precursor cells in the presence of TGF-β will become either Treg or Th17 effector cells, one would predict that animals deficient in IL-6 do not mount efficient Th17 responses. IL-6-deficient mice had already been described as resistant to EAE induction, although the reasons for this were not clear [68, 69]. Consistent with the concept of reciprocal development of Treg and Th17 cells, upon immunization with MOG, IL-6-deficient mice fail to generate a Th17 response and present increased numbers of T regulatory cells in the peripheral repertoire [70]. These findings raised the question of whether the increased numbers of Tregs in IL-6-deficient animals are an important factor in protecting them from EAE. In fact, depletion of Tregs with an anti-CD25 antibody prior to MOG immunization rendered IL-6-deficient mice susceptible to EAE [70]. Surprisingly, however, there was a re-appearance of Th17 cells that could be isolated from the target organ in these animals [70], suggesting that there is an alternate pathway responsible for the generation of Th17 effector cells in the absence of IL-6. Two independent groups have shown that this alternate factor involved in Th17 generation is IL-21 [70, 71]. Thus, in the absence of IL-6, IL-21 together with TGF-β was shown to inhibit development of iTregs and to promote the differentiation of Th17 cells [70]. Moreover, IL-21-deficient animals are more resistant to EAE and even in the presence of IL-6 and TGF-β their naïve CD4 T cells poorly differentiate into Th17 [71]. Consistent with this, IL-21 receptor-deficient mice also generate decreased Th17 responses [70].

Although IL-23 is not involved in the initial steps driving the differentiation of naïve T cells into IL-17-producing cells, it plays a fundamental role in stabilizing the phenotypic features of the Th17 lineage. Without IL-23, T cells reactivated in the presence of only IL-6 plus TGF-β can produce high amounts of IL-17, but can not fully develop into pathogenic cells and acquire bystander

regulatory properties mediated by IL-10 production [72]. Thus, IL-23 is essential for Th17 cells to fully differentiate and exhibit effector function. Indeed, antigen-specific $CD4^+$ T cells activated in the presence of TGF-β and IL-6 not only are unable to induce disease upon transfer but they can protect mice from EAE when co-transferred with fully differentiated pathogenic Th17 cells driven by IL-23 [72]. These findings suggest that proliferation (*in vitro* and *in vivo*) and IL-17 production by T cells do not always correlate with their ability to induce inflammation and tissue damage. The difference in the ability of T cells activated in the presence of either IL-6 plus TGF-β or IL-23 to induce disease rather correlated with expression of chemokines such as IP-10, CCL2, CCL5, CCL22 and CXCL2 [72]. Moreover, these findings suggest that the same combination of cytokines driving initial commitment of the Th17 lineage may initiate a regulatory loop in which activated Th17 cells, by producing IL-10, constrain its own effector function. A similar self-regulatory circuit was also described for effector Th1 cells during infection with intracellular parasites such as *Leishmania major* and *Toxoplasma gondii* [73, 74]. During the course of the infection, under strong inflammatory conditions, IFN-γ-secreting T-bet^+ $Foxp3^-$ T helper type 1 (Th1) cells were found to be the major producers of IL-10 and paradoxically, displayed potent effector function against the parasite while also inducing profound suppression of IL-12 production by antigen-presenting cells [74].

Transcriptional control of the Th17 program

As mentioned above, the fact that Th17 cells can develop independently of transcription factors, such as Stat1, Stat4, Stat6, T-bet and c-Maf, indicated that they represent a distinct lineage of effector cells [47, 48]. Studies using two independent approaches led to the discovery of the RA-related orphan receptor (ROR) gammat as the key transcription factor for generation of Th17 cells. One involved comparison of gene expression profiles of activated T cells stimulated with IL-23 (Th17) and Th1 cells. While Th1 cells greatly expressed T-bet, in Th17 cells *rorcgamma*, the gene encoding RORγt, appeared as the best candidate among sequences for DNA-binding proteins [30, 75, 76]. The other approach involved mice expressing green fluorescent protein (GFP) along with expression of RORγt. Analysis of the GFP^+ cells in this mouse strain revealed that those were the cells expressing IL-17 [75, 76]. These findings established a clear association between RORγt and IL-17 expression. Further experiments using RORγt-deficient animals showed that expression of RORγt is both necessary and sufficient to drive the differentiation of Th17 cells [64]. $CD4^+$ T cells from RORγt-deficient animals are unresponsive to IL-23 upon stimulation *in vitro* and poorly differentiate into IL-17-producing cells [64]. Furthermore, forced expression of RORγt in naïve $CD4^+$ T cells was sufficient to induce expression of IL-17, IL-17F and IL-22. Finally, RORγt-deficient animals are resistant to EAE induction [64]. Recently, it was demonstrated that RORα synergizes with RORγt to promote differentiation of Th17 cells [77].

Although RORγt is crucial, other transcription factors, such as Stat3, are also required for full generation of the Th17 lineage [75, 78, 79]. The most recent model proposes that TGF-β and IL-6 initially drive the expression of IL-21 in a Stat3-dependent manner [75]. It was demonstrated that IL-21 expression induced by IL-6 depends on Stat3, but not on RORγt [75, 80]. IL-21 then starts a positive loop in which it induces its own expression and also the expression of RORγt and of IL-23 receptor. Accordingly, IL-23R expression is greatly reduced in IL-21R-deficient animals [75, 80]. IL-21-induced self-expression is only dependent on Stat3, while induction of the IL-23 receptor requires both Stat3 and RORγt [75, 80]. IL-23 induces further expression of its own receptor and of RORγt. Thus, IL-21, by inducing its own expression and RORγt expression, and IL-23, by driving expression of its own receptor and further inducing RORγt expression, are thought to represent two important loops expanding and stabilizing cells of the Th17 lineage [75, 80]. Interestingly, it was found that upon activation of naïve T cells IL-6 and IL-21 alone are able to drive IL-23R and some RORγt expression, but without TGF-β they are unable to induce high IL-17 and IL-17F production [71]. A number of studies have also proposed that Th17 induction in human cells was independent of TGF-β [81-83]. However, as it has been shown by Littman, Soumelis and Hafler's groups, these conclusions were jeopardized by two main drawbacks: contaminant TGF-β in the human serum and incomplete isolation of truly pure naïve $CD4^+$ T cells [84-86]. The signals downstream to TGF-β receptor cooperating with IL-6 and IL-21 to induce high levels of IL-17 in T cells remain to be elucidated.

The transcription factor aryl hydrocarbon receptor (AHR) was also recently shown to be a regulator of Th17 and Treg-cell differentiation. AHR is a ligand-dependent transcription factor with a promiscuous ligand-binding site, wherein structurally diverse synthetic and naturally occurring ligands have been identified [87]. Among these are halogenated aromatic hydrocarbons, non-halogenated polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and also natural ligands such as the tryptophan photoproduct, 6-formylindolo[3,2-b]carbazole (FICZ) [87]. AHR seems

to play opposite roles in Th17 and Treg-cell differentiation in a ligand-dependent fashion. Thus, while TCDD favored the development of Tregs and can protect mice from EAE [88], FICZ was shown to increase Th17 responses and to induce stronger EAE in mice [88, 89]. AHR has been reported to interact with different transcription partners depending on the ligand or on the activation pathway [90-92]. For instance, AHR is known to associate and regulate the activity of transcription factors such as the RA receptor [93] and the estrogen receptor [94], two receptors that influence Treg and Th17 differentiation [62, 95].

Interferon-regulatory factor 4 (IRF4) was also shown to play an important role in the differentiation of Th17 cells. IRF4-deficient animals are resistant to EAE and transfer of wild-type T helper cells into IRF4-deficient recipients rendered them susceptible to the disease [96]. In addition, T cells from IRF4-deficient mice failed to differentiate into IL-17-producing cells *in vitro*. Upon activation in the presence of TGF-β and IL-6, IRF4-deficient T cells did not downregulate Foxp3 expression and low levels of RORγt were detected [96]. Forced expression of RORγt partially rescued their ability to be converted into Th17. The authors concluded that the defective Th17 differentiation of Irf4$^{-/-}$ T helper cells could be partially attributed to a lack of IL-6-mediated downregulation of Foxp3 [96]. Indeed, it was recently found that Foxp3 inhibits Th17 differentiation, at least in part, by direct interaction with RORγt [97-99], which also helps to explain the reciprocal development of T regulatory and Th17 cells. Actually, RORγt and Foxp3 may coexist in the same cell [65, 98]. It was reported that, *in vivo*, an important fraction of RORγt T cells is comprised by cells with regulatory properties which also express Foxp3 and produce CCL20 and IL-10 [65]. In influenza A-virus-infected lungs, the amount of RORγt-expressing cells increased by more than 10-fold; however, the proportion between IL-17- and IL-10-producing (Foxp3$^+$) RORγt cells remained constant [65]. The authors proposed the existence of a robust mechanism maintaining the equilibrium between Th17 and Tregs within RORγt cells during infection [65]. Keeping the balance of IL-17 versus IL-10 production would promote inflammation, while limiting collateral damage, a necessary compromise between effective immunity and tissue integrity. Factors such as IL-6 and IL-23 twist the balance favoring Th17 responses, as the ratio of IL-17-producing to Foxp3$^+$ RORγt T cells decreased in IL-6- or IL-12Rβ1-deficient mice [65]. Conversely, Foxp3 and CCL20 skew the balance to the other side, favoring the Treg arm, as inferred by the increased ratio of Th17 to Tregs in scurfy and CCR6-deficient mice [65].

Negative regulators of Th17 development

The first cytokines described to negatively regulate Th17 generation were IFN-γ (Th1) and IL-4 (Th2). In fact, blocking antibodies to IFN-γ and IL-4 facilitate *in vitro* differentiation of IL-17-producing T cells [47, 48]. Accordingly, IFN-γ-deficient mice developed more severe antigen-induced arthritis associated with unrestricted IL-17 response [100]. Moreover, forced expression of T-bet, an important Th1 transcription factor, in naïve CD4 T cells blocked IL-17 production under Th17 polarizing conditions [75]. Similarly, T cells from mice overexpressing c-Maf, a Th2 transcription factor important for IL-4 expression, showed much less IFN-γ and IL-17 production upon activation [48]. Altogether, the data suggest some cross-regulation among Th1, Th2 and Th17 subsets.

IL-2 also emphasizes the reciprocal development of T regulatory and Th17 cells. IL-2 has been reported as essential for TGF-β conversion of naïve CD4$^+$ T cells into Foxp3$^+$ T regulatory cells [101, 102]. Moreover, IL-2 together with TGF-β seems to be important to stabilize phenotypic features of inducible Tregs by downregulating IL-6R expression and rendering them resistant to further Th17 conversion by IL-6 [103]. *In vivo*, IL-2 signaling also seems to be critically required for maintaining the homeostasis and competitive fitness of naturally occurring Treg cells [104]. In contrast, IL-2 signaling via Stat5 was shown to constrain Th17 generation upon activation of naïve T cells in the presence of IL-6 and TGF-β [105]. Stat5-dependent IL-2-mediated inhibition of Th17 differentiation seems to require Ets-1. Thus, Ets-1-deficient T cells presented increased resistance to the inhibitory effect of IL-2 on Th17 differentiation and this was associated with a defect downstream of Stat5 phosphorylation [106]. IL-2 deficiency was also shown to be associated with increased Th17 generation *in vivo* [105]. Interestingly, addition of IL-2 to T cell cultures in the presence of TGF-β and IL-6 was able to not only block conversion of naïve T cells into Th17 cells, but also drive generation of Treg cells [105]. Foxp3$^+$ T regulatory cells are thus thought to be great consumers of IL-2 and restricting IL-2 availability may be one means by which they can constrain Th1 and Th2 cells [107]. Following the same rationale, by restricting IL-2 availability, T regulatory cells could instead fuel the generation of Th17 cells. However, experimental evidence for this is still lacking.

In a similar way, RA produced by intestinal DCs has been shown to favor Foxp3$^+$ T regulatory cell generation and to constrain Th17 conversion [62] (reviewed in [108]). RA signaling through RAR receptors in the

T cells is able to block the inhibitory effects of inflammatory cytokines, such as IL-6, on the TGF-β-mediated Foxp3 induction, while efficiently suppressing primary and secondary development of IL-17-producing CD4 and CD8 T cells [62, 109]. Additionally, it was shown that RA directly inhibits TGF-β and IL-6-induced expression of RORγt in T cells [62, 110]. Additional mechanisms for this dual function of RA in T-cell differentiation were recently proposed by Xiao and coworkers. They suggested that RA can enhance TGF-β signaling by increasing the expression and phosphorylation of Smad3, and inhibit the expression of IL-6Rα and IL-23R [111], therefore simultaneously increasing the ability of TGF-β to induce Foxp3 and suppressing Th17 differentiation.

RA nuclear receptors RARs have been shown to form heterodimers with STATs, and particularly STAT5 and RARs can physically interact *in vivo* to promote RAR-mediated transcription [112], indicating that RA-mediated effects on Treg and Th17 differentiation could reflect this communication between STAT5 and RAR. Mucida *et al*. found that IL-2 signaling could play a role in the reciprocal regulation of Th17 and Treg differentiation mediated by RA, since both the enhancement of Foxp3 expression and suppression of IL-17 production were drastically reduced in the presence of high amounts of anti-IL-2 blocking antibodies or when IL-2-deficient naïve CD4 T cells were used. However, the direct effects of RA and IL-2 appear distinct: while the combination of IL-2 and TGF-β induced mostly $CD103^{-}Foxp3^{+}$ Treg cells, RA and TGF-β induced preferentially $CD103^{+}Foxp3^{+}$ cells [62]. Using similar *in vitro* approaches, Elias *et al.* [110] suggested that neither STAT3 nor STAT5 is required for the RA-mediated regulation. The authors found that in STAT3 or STAT5 conditional knockout CD4 T cells, RA still mediates enhancement of Foxp3 expression and suppression of IL-17 production, respectively. It is possible that the high dose of RA used in this study (1 μM) [110] could bypass the requirement for IL-2 [62], since depending on the dose of ligands, the balance between co-repressors (CoRs) and co-activators (CoAs) can alter the function of nuclear receptor ligands and therefore change their signaling cascade and the effects on the target genes [113, 114].

The dose of RA was also suggested to play a role in the suppression of Th17 differentiation. In contrast to previous studies, Uematsu *et al.* [115] have recently suggested that a low dose of RA promotes differentiation of antigen-specific Th17 and Th1 cells. This conclusion was based on experiments showing that the addition of RAR antagonist, LE540, inhibited an already modest IL-17 production [115]. The authors suggested that the discrepancy between this result and previously published data [62, 109, 110, 116] was due to the dose of RA, since addition of an extremely high dose of RA (10 μM) inhibited IL-17 production by $CD4^{+}$ T cells. However, previous studies that described the suppressive effects of RA on Th17 development performed dose-response experiments in which RA suppressed IL-17 responses in all doses examined, starting from 1 nM (the same used by Uematsu) to 100 nM [62] and 10 μM [110]. A typical dose-response curve of Th17 suppression by RA was also observed by Kattah and coworkers using a range from 1 nM to 1 μM in human cells [116]. More importantly, spontaneous production of RA by mucosal DCs in T/DC co-cultures also inhibited IL17 production and enhanced Foxp3 induction, while addition of RAR antagonist in these cultures reversed this effect [60-62], which indicates that "physiological production" of RA by gut-derived DCs would be enough to suppress, but not induce, IL-17 production, and enhance TGF-β-mediated Foxp3 induction. It is possible, however, that under certain conditions such as under TLR stimulation, RA might have dual effects on DCs and T cells. For example, simultaneous exposure of TLR ligands such as pIC and LPS led to synergistic effects on IL-6 production [117].

IL-27, another member of the IL-12 cytokine family, has been demonstrated to be a negative regulator of Th17 responses. IL-27 is a heterodimeric cytokine made of Epstein-Barr virus-induced gene 3 (EBI3) and p28 chains [30]. IL-27 signals through a receptor composed of the IL-27 receptor chain (also called WSX-1 or TCCR) and the gp130 chain, which is shared with the IL-6 receptor [30]. IL-27R-deficient animals are more susceptible to EAE and this is associated with higher IL-17 production by lymph node T cells upon *ex vivo* stimulation and more intense CNS infiltration by Th17 cells [118]. IL-27 via Stat1 was also found to prevent *in vitro* differentiation of Th17 cells [118, 119]. Consistently, $CD4^{+}$ T cells from EBI3-deficient mice produced higher levels of IL-17, IL-22, and RORγt upon stimulation under Th17 polarizing conditions [120]. Although IL-27 has been shown to favor T-bet expression and Th1 differentiation via Stat1 [121, 122], its effect on preventing Th17 generation was independent of T-bet and IFN-γ [118]. These findings suggest it is unlikely that IL-27 suppresses Th17 development by simply diverting naïve T cells into Th1, but rather it may do so by directly interfering with RORγt expression. Alternatively, *in vivo* IL-27 may also constrain Th17 responses by inducing IL-10-producing T cells [123-125]. It was already shown that modified IL-27-producing DCs drive the generation of IL-10-producing T cells [123].

Th17 responses were also reported to be constrained by Trif-dependent type I IFN production and its down-

stream signaling pathway. Mice with defects in Trif or type I IFN receptor (IFNAR) developed more severe EAE [126]. Notably, these mice exhibited marked CNS inflammation, as manifested by increased IL-17 production [126]. In addition, IFNAR-dependent signaling events were essential for negatively regulating Th17 development. Finally, IFN-β-mediated IL-27 production by innate immune cells was critical for the immunoregulatory role of IFN-β in the CNS autoimmune disease [126].

IL-25 (IL-17E), a member of the IL-17 cytokine family, was also demonstrated to be an important regulator of Th17 responses. IL-25-deficient mice are highly susceptible to EAE, which is associated with an increase of IL-23 in the periphery and increased numbers of inflammatory IL-17- and TNF-producing T cells that invade the central nervous system [127]. Consistently, treatment with recombinant IL-25 or IL-25 delivered by a viral vector system was effective in suppressing EAE in wild-type animals. IL-25 treatment induced elevated production of IL-13, which was required for suppression of Th17 responses by direct inhibition of IL-23, IL-1β and IL-6 expression in activated DCs [127]. In accordance with the observed IL-25-mediated IL-13 production, IL-25 is thought to be an important inducer of Th2 responses [128, 129].

Concluding remarks

Fine-tuning and tight control of ongoing adaptive immune responses are crucial for host immune homeostasis. A functional immune system is supposed to provide efficient protection against invading pathogens and transformed autologous cells and, at the same time, to tolerate self-components and non-hazardous non-self antigens. Although the Th1/Th2 paradigm represented a strong experimental and theoretical basis allowing significant advances in the immunology field, it was proven insufficient to fully explain certain immunological phenomena. Aberrant immune responses directed towards harmless non-self agents or normal endogenous cells can lead to severe autoimmune disorders, and just central deletion of auto-reactive T cells could not satisfactorily explain self-tolerance [130, 131]. Indeed, self-reactive Foxp3$^+$ regulatory T cells were also shown to be indispensable for preventing excessive and self-destructive immune responses [132]. The description of the Th17 subset also had an immediate impact in the way we depict autoimmune diseases and inflammatory T helper cells. The fact that the Treg and Th17 cells have reciprocal developmental pathways and, at the same time, opposite roles in the generation and control of inflammation provides a new framework that is certainly contributing to our comprehension of the adaptive immune system functioning. However, we should not expect that adding two new T-cell subsets will give us a complete picture and rather a lot more layers of complexity will be necessary to uncover the ways the adaptive immune system swings between tolerance and effector responses.

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