

Transcriptional regulatory networks in Th17 cell differentiation

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Upon encountering antigen in the context of antigen presenting cells, naïve CD4⁺ T cells undergo differentiation into effector T helper (Th) cells, which can secrete high levels of cytokines and other immunomodulators to mediate host defense and tissue inflammation. During the past three years, the immunology field has witnessed an explosion of research advances in the biology of Th17 cells, the most recently described subset of T helper cells, which play crucial roles in host immunity and inflammation. Here we review emerging data on transcriptional regulatory networks that govern the differentiation program of Th17 cells, and focus on how the orphan nuclear receptor ROR γ t coordinates this process in concert with diverse cytokine-induced transcription factors.

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Introduction

The T helper cell (Th) paradigm, introduced by Mosmann and Coffman more than two decades ago, has been used to explain how different adaptive immune responses are elicited in the host organism for the purpose of eradicating infections with diverse microbial pathogens [1]. Th1 and Th2 cells, described in the original studies, have now been joined by Th17 cells that make the signature cytokines IL-17, IL-17F, and IL-22 [2^{••},3[•],4[•]]. These cells are involved in clearance of extracellular pathogens, particularly at mucosal surfaces [5[•],6,7], where IL-17 induces recruitment and differentiation of neutrophils and IL-22 is required for production of anti-microbial defensins.

Excessive or persistent effector T cell responses can drive the onset of inflammatory diseases. It is now clear that Th17 cells, along with Th1 cells, are often responsible for autoimmune disease, and may also contribute to tumor

progression due to the role of their cytokines in inflammation and tissue repair [8]. Another subset of CD4⁺ T cells, the regulatory T cells (Tregs), suppress effector T cell responses and prevent their potentially pathogenic effects [9]. There are two major categories of Foxp3⁺ Tregs, the naturally occurring CD4⁺ CD25⁺ Tregs (nTregs) that arise in the thymus and the TGF- β -induced Tregs (iTregs) produced in the periphery. Both types of Tregs are likely to be important in maintaining peripheral tolerance and preventing autoimmunity, but their individual contributions have not yet been established *in vivo* [10]. The T helper cell differentiation program is largely controlled by cytokines produced in response to microbial products by innate immune cells. These include IL-12/IFN γ for Th1 differentiation, IL-4 for Th2 differentiation, and IL-23 that has been linked to pathogenesis ascribed to Th17 cells both in mice and humans [11]. However, differentiation of IL-17 producing murine T helper cells *in vitro* does not require IL-23, but is dependent instead on a unique cytokine combination, IL-6 plus TGF- β [5[•],12^{••},13^{••}]. Differentiation of human Th17 cells was initially thought to be independent of TGF- β , but has recently been shown to also require this cytokine [14–16].

The differentiation of each effector T cell subset requires the induction and/or function of a series of transcriptional regulators that interact with each other in complex networks and thus orchestrate the functional program of the cells. For each T helper cell differentiation program, single transcription factors have been identified as pivotal regulators, named by some ‘master regulators’ because their overexpression can induce transcription of the relevant cytokine genes, presumably by directly binding to their *cis*-regulatory elements. These transcription factors include T-bet for Th1 cells and GATA3 for Th2 cells [17]. Foxp3 was identified as a specific regulator for Tregs and controls the expression of multiple genes that mediate Treg cell functions [18,19]. Retinoid-related orphan receptor (ROR) γ t, but not T-bet or GATA3, was identified as a Th17 specific transcription factor, further demonstrating that Th17 cells have an unique T effector program [20^{••}]. However, ROR γ t does not function in isolation, but coordinates the activity of a series of other essential transcription factors in guiding the differentiation of Th17 cells. The roles of ROR γ t in the context of other transcriptional regulators will be discussed in this review.

Unique cytokine environment for Th17 cell differentiation

Th17 cells were discovered after IL-23-deficient mice (lacking expression of the p19 polypeptide unique to this cytokine) were found to be resistant to multiple models of

autoimmune disease and also to have markedly reduced expression of IL-17 by their activated T helper cells. It has been proposed that IL-23 is required for Th17 cell function *in vivo* through control of their expansion and/or maintenance, but this has not been demonstrated in experimental models [21,22]. Moreover, recent reports have indicated that the frequency of IL-17⁺ T helper cells in the intestine at steady state or after infection with *Citrobacter rodentium* was not affected by the absence of IL-23 [5^{*},23]. However, there is compelling evidence that production of IL-22 by T helper cells is dependent on IL-23 and survival of animals after *C. rodentium* infection requires both IL-23 and IL-22 [5^{*},6]. Therefore, it will be important to evaluate the roles of both IL-17 and IL-22 in autoimmune diseases dependent on IL-23. It is also possible that IL-23 influences other effector functions that are crucial to inflammatory diseases mediated by T helper cells [24]. Thus, the precise mechanisms underlying the contribution of IL-23 to Th17 cell differentiation and function *in vivo* remain to be elucidated.

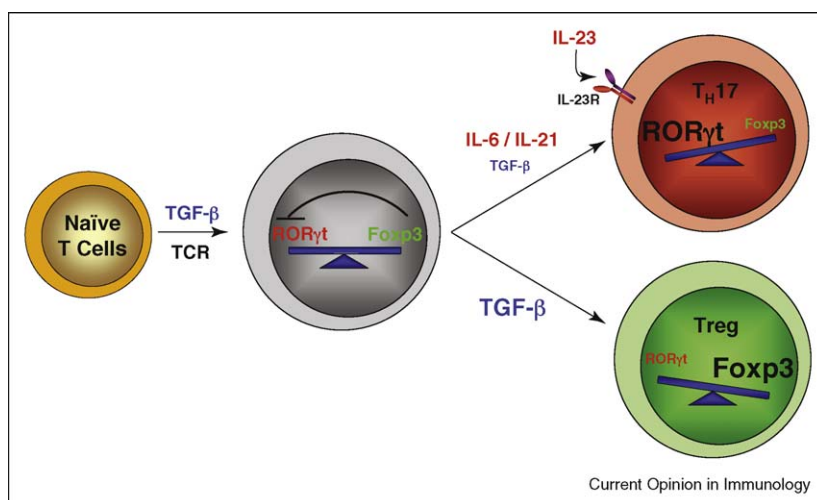
Our current limited understanding of the roles of IL-23 and other cytokines in the differentiation of Th17 cells is largely derived from *in vitro* studies. In conjunction with T cell antigen receptor (TCR) activation, the proinflammatory cytokine IL-6 induces the expression of IL-21 in naïve CD4⁺ T cells. IL-21, in turn, induces its own expression in an autocrine manner, acting through the γ_c -family member IL-21 receptor that is induced by TCR stimulation. IL-21 (but not IL-6) also induces expression of the IL-23R. Together with TGF- β , IL-21, independently of IL-6, can induce IL-17 in mice and in humans

[14,25^{*}–27^{*}]. In line with these findings, IL-21R-deficient cells expressed about threefold less IL-17 upon stimulation of IL-6 plus TGF- β , and IL-6-induced IL-23R expression was markedly reduced in IL-21R-deficient cells. These results suggested that IL-21 contributes to Th17 cell differentiation and serves as an intermediary mediator for IL-6-initiated signaling in the induction of IL-23R [25^{*}]. Intriguingly, the absence of IL-21 signaling appears to have little effect on IL-17⁺ Th17 cell differentiation *in vivo*, suggesting that some factors (e.g. IL-6) may compensate for the loss of IL-21 signaling [23,25^{*},28^{*},29^{*}]. Since IL-21 is essential for IL-23R expression *in vitro* and IL-23 signaling is required for *in vivo* expression of IL-22, it will be important to determine whether IL-21R signaling is required for T cells to produce IL-22 *in vivo* [30].

Lack of IL-23R expression on naïve murine CD4⁺ T cells explains why IL-23 alone has no effect on Th17 induction *in vitro*. Once IL-23R is upregulated by cytokines such as IL-21, then IL-23 plus TGF- β are capable of inducing IL-17 expression [25^{*}]. IL-23 further upregulates IL-23R expression, therefore imposing another amplifying loop and contributing to induction of Th17 cells [31^{**}]. All together, these data suggest that IL-23 may function at a late stage of Th17 cell differentiation after initial induction by other proinflammatory cytokines (e.g. IL-6 and IL-21).

It was recently shown that mice deficient for *gp130* (a shared receptor subunit for IL-6) mounted efficient Th17 responses and developed experimental autoimmune encephalomyelitis (EAE) after depletion of Treg cells,

Figure 1



TGF- β orchestrates *in vitro* Th17 and Treg cell differentiation in a concentration-dependent manner. In the presence of TGF- β , TCR-activated CD4⁺ T cells express both ROR γ t and Foxp3, but ROR γ t function is antagonized by Foxp3. Such cells can differentiate into either Th17 or Treg cells dependent on the cytokine environment. In the presence of proinflammatory cytokines and low concentrations of TGF- β , ROR γ t expression is further upregulated, whereas Foxp3 expression and function are inhibited, thus tipping the balance in favor of the Th17 cell fate. ROR γ t-induced IL-23R expression on T cells confers responsiveness to IL-23, which further promotes Th17 cell differentiation. By contrast, in the absence of proinflammatory cytokines, high concentrations of TGF- β favor Foxp3 expression and result in Treg cell differentiation.

suggesting that IL-6 signaling was dispensable for the induction of pathogenic Th17 cells *in vivo*, at least in the absence of Treg cells [32]. These results highlight the complexity of the cytokine-induced Th17 differentiation program, suggesting that *in vivo* Th17 cell differentiation can be driven by multiple proinflammatory cytokines (IL-6, IL-21, IL-23, and/or other yet unidentified factors).

TGF- β has been extensively characterized as being required to maintain immunological tolerance, acting in both differentiation and maintenance of Foxp3⁺ Tregs that restrain effector T cell responses [33]. Why TGF- β is required for both proinflammatory Th17 cell and anti-inflammatory Treg differentiation remains paradoxical. Most recently, we showed that TGF- β orchestrates *in vitro* Th17 and Treg cell differentiation programs in a concentration-dependent manner (Figure 1) [31^{••}]. At lower concentrations, together with IL-6 or IL-21, TGF- β synergistically induces IL-23R and thus promotes Th17 cell differentiation in the presence of IL-23. However, at higher concentrations, TGF- β inhibits IL-23R, IL-22, and IL-17 expression and favors induction of Foxp3 and, thus, Treg lineage differentiation. In spite of the absolute requirement of TGF- β , little is known about its precise signaling pathways in Th17 and Treg cell differentiation. Since Smad4 (a co-smad in the Smad dependent TGF- β pathway) appears not to be required for Th17 cell differentiation [34], the involvement of Smad-dependent or Smad-independent pathways in Th17 and Treg cell differentiation needs to be carefully examined. It will be important to determine if modulation of active TGF- β concentration or sensitivity to TGF- β *in vivo* results in differential loss of Treg versus Th17 cells. On this basis, polymorphisms in TGF- β signaling pathway genes may also contribute to human autoimmune diseases.

Th17 transcriptional regulatory networks

Th17 cell lineage specification requires ROR γ t, which was earlier identified as a critical transcription factor for early T cell and lymphoid organ development [35–37]. During a gene profiling analysis of Th17 cells, ROR γ t was identified as one of the most highly upregulated transcription factors. Accordingly, in the small intestinal lamina propria, ROR γ t⁺ T cells but not ROR γ t⁻ T cells express IL-17. *In vitro*, IL-6 plus TGF- β treatment-induced IL-17 expression requires induction of ROR γ t, and forced expression of ROR γ t is sufficient to induce IL-17 expression in the absence of any exogenous cytokines. In line with its crucial roles in Th17 cell differentiation, ROR γ t-deficient mice develop less severe autoimmune diseases and specifically lack Th17 cells in the inflammatory tissues [20^{••},38]. Interestingly, another ROR family member, ROR α , is also upregulated during *in vitro* Th17 cell differentiation [39^{••}]. Although forced expression of ROR α is sufficient to induce IL-17, lack of ROR α has only a minor effect on Th17 cell differen-

tiation (L Zhou and DR Littman, unpublished data). However, complete loss of lamina propria Th17 cells was observed in animals harboring compound mutations of ROR γ t and ROR α , suggesting that these two closely related transcription factors, which presumably share the same DNA binding sequence, may have similar functions in Th17 cell differentiation (L Zhou and DR Littman, unpublished data) [39^{••}]. Accordingly, chromatin immunoprecipitation assay (ChIP) has suggested that *il17a* is a direct target gene of ROR γ t [39^{••},40^{••}].

IL-6, IL-21, and IL-23 signaling all utilize the Jak-Stat pathway and activate Stat3 [41]. In Stat3-deficient CD4⁺ T cells, induction of IL-21 and IL-23R was barely detected. Accordingly, IL-17 expression induced either by IL-6 plus TGF- β or IL-21 plus TGF- β was also greatly reduced, suggesting that Stat3 plays an essential role in Th17 cell differentiation [25[•],27[•],42]. Stat3 binds to the *il17a* promoter directly as shown by ChIP [43]. Stat3 is also required for induction of ROR γ t by cytokines. Forced expression of ROR γ t can partially rescue IL-17 expression in Stat3-deficient cells, suggesting that ROR γ t may function downstream of Stat3 to induce IL-17 expression (L Zhou and DR Littman, unpublished data). Stat3 and ROR γ t can also act together to induce maximal IL-17 expression, suggesting that they may also form a complex and/or cooperatively bind to the *cis*-elements of the *il17a* locus [25[•]].

In the course of IL-6 dependent Th17 cell polarization, there is a strong correlation between downregulation of Foxp3 and upregulation of IL-17 [13^{••}]. This finding can be explained by the ability of Foxp3 to suppress Th17 cell differentiation through antagonism of ROR γ t activity [31^{••}]. TGF- β induces both ROR γ t and Foxp3, but is unable to induce IL-17 unless combined with proinflammatory cytokines (IL-6, IL-21, or IL-23) (Figure 1) [31^{••}]. Foxp3 has the potential for physical interaction with ROR γ t and ROR α and can thus inhibit their transcriptional activities [31^{••},44]. Accordingly, murine Foxp3 lacking amino acids encoded by exon 2 (Foxp3 Δ Exon2) cannot inhibit ROR γ t function owing to a loss of interaction with ROR γ t. The interaction between ROR γ t and Foxp3 has been confirmed by several other groups [34,40^{••},45,46], but it remains unclear if it is direct or in the context of a larger complex. Intriguingly, the inhibitory effect of Foxp3 on IL-17 induction was largely circumvented in the presence of IL-6 or IL-21, even though the levels of Foxp3 and ROR γ t proteins were not affected [31^{••}]. This result suggests that the inhibition of ROR γ t by Foxp3 was relieved by proinflammatory cytokines through a post-translational mechanism. Although it is clear that the interaction between ROR γ t and Foxp3 is crucial for *in vitro* Th17 cell differentiation, *in vivo* relevance of this interaction is yet to be defined. Notably, neither the natural function of Foxp3 Δ Ex2, an isoform found only in humans, nor the precise mechanism

underlying Foxp3-mediated inhibition of ROR γ t is known. Most recently, ROR γ t was shown to interact with Runx1, a transcription factor upregulated during TCR stimulation and required both for differentiation of Th17 cells and for Foxp3 function [40^{**},47] (M Chong and DR Littman, unpublished data). Binding of ROR γ t and Runx1 together to the *il17a* locus leads to increased expression of IL-17, whereas Foxp3 inhibits both Runx1 and ROR γ t activity [40^{**}]. It is possible that Runx1 can modify ROR γ t/Foxp3 complexes in the presence of proinflammatory cytokines, therefore relieving the inhibition of ROR γ t activity by Foxp3. Alternatively, Runx1 may differentially associate with ROR γ t or Foxp3, and thus participate in mediating their respective transcriptional activating or repressing activities, according to the cytokine-initiated signals.

IRF-4, a transcription factor previously shown to be important for Th2 cell differentiation, was also discovered to be essential for Th17 cell differentiation. IRF4-deficient mice were protected from EAE and T cells from these animals failed to differentiate into Th17 cells. IRF-4, which regulates expression of IL-21 and IL-23R, is in turn inhibited by IRF-4-binding protein (IBP) [48^{**},49,50^{*}]. ROR γ t and ROR α induction were impaired in IRF4-deficient T cells, but their forced expression could partially restore induction of IL-17, suggesting that IRF-4 may function upstream of the nuclear receptors [48^{**},49]. Since rescue was only partial, it is likely that a complex transcriptional network, rather than a linear process, governs the Th17 cell differentiation program. Furthermore, IRF4-deficient T cells had increased Foxp3 expression, highlighting the importance of the ROR γ t-Foxp3 balance in Th17/Treg cell differentiation. Th17 cells have also been shown to express c-Maf, a transcription factor involved in regulation of Th2 cell differentiation. Genetic loss of c-Maf resulted in a defect in IL-21 production, IL-23R expression, and consequently, in fewer Th17 cells [51].

The discovery of the involvement of aryl hydrocarbon receptor (AhR) in the regulation of transcription of Th17 cytokines has added another layer of complexity to this field [52^{**},53^{**}]. AhR, a mediator of the effects of environmental toxins (e.g. dioxin, a polycyclic aromatic hydrocarbon xenobiotic compound), is a ligand dependent transcription factor that is structurally distinct from the nuclear receptor superfamily. Upon binding to a ligand (such as dioxin or FICZ, a UV photoproduct of tryptophan), cytosolic AhR translocates into the nucleus, heterodimerizes with its partner aryl hydrocarbon receptor nuclear translocator (ARNT), and turns on transcription of its target genes [54]. The findings of the involvement of AhR in Th17 cell differentiation suggest a potential link between environmental pollution and inflammation. However, the precise contribution of AhR to Th17 cell differentiation is unclear. Analysis of AhR-deficient cells has shown that it is required for IL-22 and, to a lesser extent,

IL-17 expression in Th17 polarizing conditions in the presence of either dioxin or FICZ [52^{**}] (L Zhou and DR Littman, unpublished data). However, a requirement for AhR in IL-17 expression is still controversial [55]. Thus, one study suggested that different AhR ligands (dioxin versus FICZ) exert opposite effects on Th17 and Treg cell differentiation [53^{**}], whereas another showed no difference between individual AhR ligands [55]. Thus, dioxin was found to suppress progression of EAE [53^{**}], while FICZ exacerbated EAE [52^{**},53^{**}]. The reasons behind these discrepancies remain unclear and may be due to different culture conditions and animal housing environments [56]. We have found that AhR cooperates with ROR γ t to induce maximal amounts of IL-17 and IL-22 and also inhibits TGF- β -induced Foxp3 expression, thus highlighting the antagonism between Th17 and Treg cell differentiation (L Zhou and DR Littman, unpublished data). Together, these results suggest that ROR γ t may function as a node in the Th17 cell transcriptional network and may interact either positively or negatively with other factors to influence lineage specification.

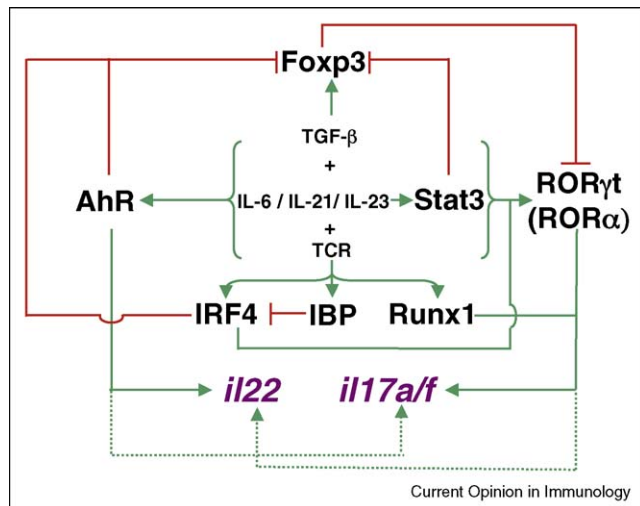
***In vivo* relevance and implications**

The local cytokine environment greatly influences immune system homeostasis through regulation of various transcription factors. Local TGF- β concentration, together with proinflammatory cytokines, may determine the balance between Foxp3 and ROR γ t (and probably other Th17 transcription factors as well), leading to different T cell fates. This is especially relevant in the gut environment where, upon encountering commensal bacteria, dendritic cells secrete proinflammatory cytokines. High concentrations of TGF- β , together with retinoic acid (RA), may be required for induction of Foxp3⁺Tregs to suppress potentially detrimental inflammatory Th17 cell responses [57–62]. The inhibition of IL-6 receptor (IL-6R α) expression by RA, suggested by a recent study, provides at least partly an explanation for how RA antagonizes Th17 cell differentiation [63]. Foxp3 inhibits ROR γ t-directed IL-17 expression in mouse T cells, but it remains to be determined whether this also occurs in humans. This is relevant because FOXP3 Δ Exon2 is a natural spliced isoform that was only identified in humans. Thus, the balance between full-length FOXP3 versus FOXP3- Δ Exon2 may influence the frequency of Th17 and Treg cells and susceptibility to autoimmunity in humans. Modulation of the interaction between ROR γ t (together with other Th17 transcription factors) and Foxp3 may thus maintain immune homeostasis and permit control of inflammatory responses in different disease settings.

Conclusions

Over the past three years, there have been remarkable advances in our understanding of Th17 cell differentiation and function. Transcriptional regulatory networks that specify Th17 differentiation have begun to be elucidated, but our understanding is probably still superficial

Figure 2



Transcriptional regulatory network that governs the Th17 cell differentiation program. The inflammatory cytokine (IL-6)-initiated signaling cascade, together with TGF- β signaling, induces ROR γ t expression in a Stat3-dependent manner. In the absence of proinflammatory cytokines, TGF- β -induced Foxp3 inhibits ROR γ t (and ROR α) activity, and thus promotes Treg cell differentiation. AhR is also induced during Th17 cell polarization and it downregulates Foxp3 expression. Ligand activated AhR cooperates with ROR γ t to induce maximal amounts of IL-17 and IL-22 and also to inhibit TGF- β -induced Foxp3 expression, ensuring full progression of Th17 cell differentiation. TCR-induced IRF-4 upregulates ROR γ t expression and inhibits Foxp3 expression, and thus promotes Th17 cell differentiation and antagonizes Treg cell differentiation. TCR-activated IBP inhibits IRF-4 function by sequestering IRF-4. TCR-induced Runx1 influences Th17 cell differentiation by inducing ROR γ t expression and by binding to and acting together with ROR γ t to direct *il17* transcription.

(Figure 2). Little is known about precise molecular mechanisms whereby these interactions determine the Th17 differentiation program. Furthermore, aside from several effector cytokine genes, the targets of these transcriptional regulators in the Th17 program are unclear. In addition, the mechanism by which ROR γ t is regulated by a yet unknown ligand remains to be characterized. ROR γ t function and its interaction with Foxp3 may be manipulated pharmacologically using small molecule compounds. Our knowledge of the interplay among different T helper cell lineages at the molecular level will shed light on human disease pathogenesis and may eventually provide novel means for treating inflammatory and autoimmune diseases.

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