Published in final edited form as:

Cytokine Growth Factor Rev. 2010 December ; 21(6): 425–434. doi:10.1016/j.cytogfr.2010.10.006.

Signal transduction pathways and transcriptional regulation in Th17 cell differentiation

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Abstract

Over the last decade, our understanding of helper/effector T cell differentiation has changed dramatically. The discovery of interleukin (IL-)17-producing T cells (Th17) and other subsets has changed our view of T cell-mediated immunity. Characterization of the signaling pathways involved in the Th17 commitment has provided exciting new insights into the differentiation of CD4⁺ T cells. Importantly, the emerging data on conversion among polarized T helper cells have raised the question how we should view such concepts as T cell lineage commitment, terminal differentiation and plasticity. In this review, we will discuss the current understanding of the signaling pathways, molecular interactions, and transcriptional and epigenetic events that contribute to Th17 differentiation and acquisition of effector functions.

Keywords

Th17; T cell differentiation; cytokines; signal transduction; transcription factor; epigenetics

1. Introduction

The selective production of cytokines by subsets of CD4⁺ T cells is a major mode of immunoregulation and an important mechanism by which CD4⁺ T cells orchestrate immune responses. Activated CD4⁺ T cells can be subdivided into lineages on the basis of the cytokines that they secrete, the specific transcription factors that they express and the immunological function that they mediate. CD4⁺ T helper (Th) cells were initially viewed as having two possible fates, Th1 and Th2 cells [1, 2]. Th1 cells, which express the transcription factor T-bet and secrete IFN- γ , protect the host against intracellular infections including viruses and *Toxoplasma* [3]. In contrast, Th2 cells, which express GATA-3 and secrete interleukin (IL-)4, IL-5 and IL-13, mediate host defense against helminths [4]. Recently, these two classical lineages have been joined by additional subsets that preferentially produce distinct cytokines. One includes cells that selectively produce IL-17 (Th17 cells) [5][6][7][8][9][10]. These cells are critical for host defense against extracellular pathogens [11] and have been implicated in a number of autoimmune diseases [12][13][14]

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[15][16][17]. Th17 cells can also produce IL-9, IL-10, IL-21, and IL-22; however, subsets of cells that selectively produce these cytokines have also been identified [18][19][20][21]. The other major lineage of CD4⁺ T cells includes those that express the transcription factor Foxp3. These immunosuppressive, regulatory T (Treg) cells comprise both natural (n)Tregs and induced (i)Tregs [22][23][24].

In this review, we examine the major factors that promote Th17 cell differentiation and discuss the signaling pathways and transcription factors that drive specification of this lineage. We then discuss how these factors influence epigenetic modifications of the *IL17* locus and the extent to which can influence stability versus plasticity of this subset. Finally, we will briefly review the relationship between the selective production of IL-17 and other cytokines and examine how this relates to lineage commitment versus flexibility of phenotype.

2. T cell receptor (TCR) signaling and IL-17 production

An obligate first step in the activation of CD4⁺ T cells is engagement of the T cell receptor. Not surprisingly, this is an important contributor to IL-17 regulation and for invariant NK T cells and $\gamma\delta$ T cells, TCR engagement is sufficient [25, 26]. For naïve CD4⁺ T cells, the “strength” of TCR signaling as determined by the concentration of cognate antigen is an important driver of Th1 versus Th2 differentiation [27]. Precisely how signal strength influences IL-17 regulation is also incompletely understood. Recently it has been found that high dose antigen-loaded dendritic cells induce Th17 generation [28]. The Tec family tyrosine kinase inducible T cell kinase (Itk) is a tyrosine kinase required for full TCR-induced phospholipase C- γ activation [29]. Itk^{-/-} mice show decreased IL-17 production [30]. Similarly, Raftlin, a protein located in lipid rafts, has been suggested to regulate the strength of TCR signaling [31]. Raftlin^{-/-} T cells produce less IL-17 and Raftlin^{-/-} mice show reduced severity of experimental autoimmune encephalomyelitis (EAE) [31]. Similarly, an E3 ubiquitin ligase denoted GRAIL (Gene related to anergy in lymphocytes) which regulates TCR-CD3 degradation, also influences IL-17 production [32]. These results are puzzling insofar as “strong” signals typically induce T-bet expression and Th1 differentiation. In human CD4⁺ T cells, it has been shown that “weak” TCR stimulation promoted Th17 responses [33]. It might be expected that upregulation of T-bet could attenuate IL-17 production. Recent work has revealed that mammalian target of rapamycin (mTOR) complex 2 (mTORC2) impaired differentiation into Th1 and Th2 cells without significant effect on Th17 differentiation [34]. Clearly more work is required in this area.

Signals from co-stimulatory molecules are also important for helper cell specification. But it is still controversial for Th17 differentiation; both ICOS and CD28 activation have been implicated in Th17 differentiation [6]. ICOS^{-/-} mice have exaggerated severity of Th17-mediated encephalomyelitis [35], while ICOS^{-/-} CD4⁺ T cells show no significant increase of IL-17 production *in vitro* [36]. More work is required in this area.

3. Role of cytokines in Th17 differentiation

Initial views of specification

IL-23 is a heterodimeric cytokine composed of p19 and p40, the latter subunit being shared with IL-12 [37]. IL-23 has long been recognized to be an inducer of IL-17 [38]. Moreover, gene targeting of p19 dramatically attenuated EAE, a disease that is dominated by Th cells that produce IL-17 [39]. However, more recently it has been appreciated that naïve T cells do not express the IL-23R [9, 15]. Rather, it needs to be induced. This has led to the notion that IL-23 cannot be the sole inducer of Th17 differentiation. Other cytokines such as IL-6 and IL-21 are implicated in the regulation/induction of IL-17 production [15, 38, 40] [41]. *In*

vitro, IL-6 is a potent inducer of Th17 cells but only when combined with other cytokines including transforming growth factor β 1 (TGF- β 1) [42][7, 8]. This led to the finding that the conjunction of TGF- β 1 with IL-6 is the initial driver of Th17 specification[7]. However, IL-6^{-/-} mice have reduced but not absent numbers of Th17 cells [7], suggesting that *in vivo* other cytokines can compensate for its actions. One candidate is IL-21 that is produced by Th17 and follicular helper T cells (Tfh). IL-21 induces IL-17, itself and [15, 40, 41, 43] IL-21 also induces the expression of IL-23R. However, in disease models that depend on Th17 specification IL-6 seems to be more relevant than IL-21. While IL-6^{-/-} mice are protected from EAE [44] the role of IL-21 in this model is less clear. *In vivo*, one study reported that the development of EAE was significantly reduced in IL-21^{-/-} mice [40]; however, two other studies showed that the development of EAE was not impaired by the absence of IL-21 signals [45, 46]. Thus, it appears that both IL-6 and IL-21 can promote Th17 differentiation, but neither appears to be absolutely necessary *in vivo*.

TGF- β as requisite inductive factor or a modulator?

The data are very clear that the combination of TGF- β and IL-6 is a very effective means of inducing naive T cells to become IL-17 producers. Consistent with this idea is that deleting TGF- β has also been reported to inhibit Th17 differentiation *in vivo* [47][7, 8]. Accordingly, transgenic overexpression of TGF- β in T cells was associated with increased susceptibility to the development of EAE in mice injected with MOG peptide [7]. Recently, it has been reported that the downstream signals of TGF- β , Smad2 is important for TGF- β mediated Th17 cell generation [48] [49][50]. Smad2^{-/-} mice showed less severe disease in a EAE model [50]. It has also been reported that CD4⁺ T cells lacking the molecular adaptor tumor necrosis factor receptor associated factor 6 (TRAF6) are more sensitive to TGF- β -induced Smad2/3 activation and exhibit a specific increase in Th17 differentiation [51].

However, imputing a role of TGF- β as an inductive factor in Th17 differentiation is complicated by the fact that mice lacking this cytokine or its receptor subunits, develop a fatal, systemic autoimmune disease [52–54]. Thus, the mechanism by which TGF- β induces Th17 development remains elusive and an essential role of TGF- β in Th17 differentiation has recently been challenged. Specifically, in mutant T cells lacking T-bet and STAT6, IL-6 alone was found to be sufficient to generate Th17 cells [55]. This suggests that TGF- β plays an indirect role in Th17 differentiation by suppressing the expression of T-bet and GATA3, and limiting differentiation to alternate fates.

The importance for TGF- β in the human Th17 differentiation has also complicated its potential role as an obligate inductive factor. Initial work showed that human Th17 differentiation could be induced in the absence of TGF- β ; indeed, TGF- β was noted to be an inhibitor of IL-17 production [56–58]. Another study showed that TGF- β in conjunction with IL-21 can induce Th17 differentiation from human naïve T cells, while TGF- β in conjunction with IL-6 failed to do so [59].

Flesh out in more detail more recently, we have found TGF- β -independent Th17 differentiation occurs in normal mouse T cells. Furthermore, we showed that mutant mice with impaired TGF β R signaling have residual Th17 cells within the gut [60]. As in human T cells the combination of IL-6 with IL-1 β and IL-23 is capable of inducing Th17 differentiation in mouse T cells [61]. This cell fate depends on IL-23R expression. IL-6 is sufficient to induce IL-23R expression and IL-23 further amplifies its receptor expression.

Amidst all this controversy, perhaps some lessons are emerging. First, is that there appears not to be a major, species-specific difference in the role of TGF- β in mice and humans [60]. Second, assuming that TGF- β is not an obligate specification factor for Th17 differentiation, one probable mode of action is the repression of T-bet expression [55]. Consistent with this

view, Th17 cells generated in the absence TGF- β express T-bet along with IL-17. Low concentrations of TGF- β promote Th17 differentiation and this would ensure that T-bet levels remain low and thereby attenuate the tendency of Th17 cells to become Th1 cells [62]. However, high concentrations of TGF- β inhibit Th17 differentiation [63]. Likely, this is because high concentrations of TGF- β induce expression of Foxp3. In addition, TGF- β also suppresses IL-23R expression [15][60].

So where does this leave us? One possibility is that Th17 cells are heterogeneous. IL-17-producing cells generated in the presence of TGF- β , which lack T-bet and express IL-10, may be more important for homeostasis in the gut. By contrast, IL-17 producing T cells that express T-bet in sites of inflammation may be the bad actors that need to be targeted for therapies to be effective in autoimmunity.

A new role for an old cytokine

The prototypic inflammatory cytokine, IL-1 acquired a new role with the recognition that it is a major promoter of Th17 development in both mouse and man, synergizing with IL-6 and IL-23 [61][60]. Accordingly, IL1R^{-/-} CD4⁺ T cells are severely impaired to differentiate towards Th17 cells *in vivo* and IL1R^{-/-} mice show reduced incidence of EAE [64, 65]. Furthermore, $\gamma\delta$ T cells activated by IL-1 β and IL-23 produce IL-17, IL-21 and IL-22 [66]. The IL-1R has homology to Toll-like receptors (TLR), and IL-1 engagement of its receptor results in the recruitment of the adapter MyD88 and IL-1 receptor-associated kinases (IRAK) [67]. A recent study has indicated that single Ig IL-1R-related molecule (SIGIRR), a negative regulator of IL-1R and TLR signaling, control Th17 cell differentiation and proliferation. The study also showed that IL-1-induced Th17 cell expansion is dependent on mTOR [68].

4. Regulation of IL-17 production by G-protein coupled receptors

S1P is an abundant lysophospholipid present in blood and lymph and signals via a G-protein coupled receptor (GPCR) denoted S1PR1. S1P regulates T cell trafficking and egress from lymphoid structures [69][70]. Addition of S1P to cultures of splenic CD4⁺ T cells has been reported to increase IL-17 and to reduce IFN- γ and IL-4 production [71]. Accordingly, transgenic overexpression of an S1P receptor resulted in enhanced IL-17 production [72]. Whether this is a critical means of regulating Th17 differentiation *in vivo* has not been established.

Prostaglandins are another class of lipids that signal via GPCR. Prostaglandin E2 (PGE2) is highly induced during inflammation. Interestingly, culture of human peripheral blood mononuclear cells with PGE2 and IL-23 resulted in high levels of production of IL-17 and C-C chemokine ligand 20 (CCL20), with less IFN- γ and IL-22 [73]. PGE2 acts through both prostaglandin EP2- and EP4- mediated signaling and cyclic AMP pathways to upregulate IL-23R and IL-1R expression resulting in synergistic induction of Th17 cells with IL-23 and IL-1 β [74]. *In vivo* administration of PGE2 induced IL-23-dependent IL-17 production and exacerbated collagen-induced arthritis (CIA) in mice [75].

5. Regulation of Th17 differentiation by transcription factors

5.1 Direct, critical role of STAT3 in Th17 specification

It has long been appreciated that a major means by which type I/II cytokines exert their effect is through signal transducers and activators of transcription (STATs) [76]. Notably, the major cytokines that induce IL-17, IL-23, IL-6, and IL-21, all activate STAT3. The critical role of STAT3 was established by the finding that deletion of STAT3 in T cells abrogated Th17 differentiation [77][78]. Conversely, retroviral overexpression of

constitutively active STAT3 was sufficient to induce IL-17 production [79]. Importantly, conditional deficiency of *Stat3* in CD4⁺ T cells impairs IL-17 production *in vivo* and limits IL-17-associated pathology [80][81][82].

The requirement for STAT3 in human IL-17 production was unraveled in patients with Hyper IgE syndrome (HIES). HIES is a primary immunodeficiency disorder due to dominant negative mutations of STAT3 [83][84]. We and others found that patients with HIES have severely impaired ability to produce Th17 cells [83] [84–87][88]. The impairment of STAT3 signaling and consequently attenuated Th17 generation may explain the patients' inability to clear bacterial and fungal infections.

The demonstration of the importance of STAT3 in mouse and man raised the issue of exactly how STAT3 contributes to Th17 cell development. STAT3 initially found to directly bind to the *Il-17* promoter by using chromatin immunoprecipitation (ChIP) assays [89]. However, new technology, namely ChIP and massive parallel sequencing (ChIP-seq) provides the opportunity to define the function of transcription factors on a genome-wide scale [90, 91]. The functional relevance of the binding can be ascertained by genome-wide transcriptional profiling and by assessing the transcription factor-dependent changes in epigenetic modifications. This has been accomplished for STAT3 in Th17 cells and it was found that STAT3 binds promoters and enhancers of many genes involved in Th17 development [82]. STAT3 binds throughout the *Il17* and *Il17f* locus. Interestingly, its binding is not restricted just within the promoter regions, STAT3 also binds the intergenic region (Figure 1).

STAT3 also binds and regulates the *Il21*, *Il21r* and *Il23r* genes [60]. Importantly, STAT3 directly controls expression of many of the other transcription factors that participate in Th17 differentiation including Ror γ t, IRF4, and Batf. Thus, this analysis revealed a greatly expanded role for STAT3 in Th17 differentiation. Interestingly, STAT3 has also been suggested to play a role in proliferation and survival of Th17 cells [82]. Furthermore, STAT3 is important both for the expression of IL-23R and the downstream signaling of this cytokine receptor complex [92].

5.2 Retinoid receptors and Th17 cells

Retinoid receptors comprise a family of nuclear receptors, only some members of which are regulated by metabolites of vitamin A. They are part of a larger family of steroid nuclear receptors and as such, have direct transcription factor properties. Retinoid receptors can be divided into three families: classical retinoic acid receptors (RAR α - γ), retinoid X receptors (RXR α - γ) and the retinoic acid orphan receptors (ROR α - γ). ROR γ t is a splice variant of the *Rorc* gene that results from initiation by a distinct promoter within the full length *Rorc* gene, resulting in products that differ in their amino terminus [93].

Whereas ROR γ is ubiquitously expressed, a transcript denoted, ROR γ t, is exclusively expressed in lymphoid cells. ROR γ is critical for T cell development and lymphoid organogenesis. This is evidenced by the fact that *Rorc*^{-/-} mice exhibit reduced numbers of double positive and CD4⁺ single-positive thymocytes. *Rorc*^{-/-} mice also lack lymph nodes, Peyer's patches and lymphoid tissue inducer cells [94][95][96]. *Rorc*^{-/-} mice also have reduced Th17 differentiation and have reduced disease severity in the EAE model [9]. Conversely, overexpression of ROR γ t promotes IL-17 expression; in this regard ROR γ t acts similarly to transcription factors such as T-bet and GATA3 in Th1 and Th2 differentiation respectively and therefore has been proposed to be a "master regulator" for Th17 differentiation. Multiple ROR γ t binding sites are present in the *Il17* promoter, and by the use of ChIP, ROR γ t has been found to bind the *Il17* gene [97] (Figure 1).

As indicated, STAT3 binds the *Rorc* gene and STAT3-deficient T cells have poor expression of ROR γ t [77–79][82]. However, overexpression of active STAT3 in *Rorc*^{−/−} cells resulted in poor IL-17 induction [15], arguing that STAT3 is necessary but not sufficient for IL-17 expression. Rather, STAT3 and ROR γ t appear to act cooperatively on the *Il17* locus to drive production of this cytokine.

Another related retinoic acid nuclear receptor, ROR α , is also preferentially expressed in Th17 cells [98]. In contrast to ROR γ t though, deletion of *Rora* had a minimal effect on IL-17 production. However, deficiency of both *Rora* and *Rorc* completely abolished IL-17 production and protected against EAE [98]. Co-expression of the two factors enhanced the number of IL-17-producing cells. Collectively, the findings suggest that there is some redundancy between the functions of ROR γ and ROR γ t.

5.3 Batf and Th17 differentiation

Batf is a member of the AP-1 transcription factor family that contains a basic region and leucine zipper, lacks a transcriptional activation domain and so has been considered as an inhibitor of AP-1 transcriptional activity [99]. Recently though, it has been shown that Batf is essential for Th17 cell differentiation [100]. *Batf*^{−/−} mice show a complete loss of Th17 differentiation and are resistant to the induction of EAE. However, Batf mRNA is not exclusively expressed in Th17 cells; it is expressed in Th1, Th2 and Th17 cells. Nonetheless, *in vitro* differentiation of Th1 and Th2 were apparently unaffected in the absence of Batf. Batf heterodimerizes with JunB in Th17 cells and binds the promoters of *Il17*, *Il17f*, *Il21* and *Il22* and the intergenic regions between *Il17* and *Il17f* (Figure 1).

5.4 IRF4 and Th17 differentiation

IRF4, a member of the interferon regulatory factor (IRF) family of transcription factors, was originally implicated as a key inducer of GATA3 expression in Th2 lineage differentiation [101]. While, *IRF4*-deficient T cells were shown to impair IL-17 production in response to TGF- β and IL-6 [102]. Recently, it has been shown that phosphorylation of IRF4 regulates IL-17 and IL-21 production [103]. Moreover, *IRF4*-deficient mice are resistant to EAE [102]. It has been suggested that IRF4 might co-operate with STAT3 to induce ROR γ t expression, but IRF4 also interacts with NFATc1 and c2 [104] [105]. In this manner, it may affect other Th cell lineages. It is of note that this is a rare example of a factor required for both Th17 and Th2 development, which is in contrast with the previously accepted links with Th17 and Th1 development via IL-23 [5] and Th17 and Treg development via TGF- β [7].

5.5 Runx transcription factors

Runx transcription factors represent another critical family of proteins that regulate CD4⁺ T helper cell differentiation. There are three mammalian Runt domain transcription factors, Runx1, Runx2, and Runx3. Runx1 is crucial for normal hematopoiesis including thymic T cell development [106]. Additionally, both Runx1 and Runx3 are reported to act as repressors of CD4 and *Zbtb7b* (the latter being encodes Th-POK, a transcription factor that regulates CD4⁺ differentiation), but at different stages of thymocyte development to affect the CD4/CD8 lineage choice [107][108]. Runx3 also functions as a CD4 silencer in CD8⁺ T cells and is involved in a feed-forward regulatory circuit in which T-bet induces Runx3 and then ‘partners’ with Runx3 to direct activation of *Ifng* and silencing of *Il4* [109][110]. In Treg cells, Runx1 binds the N-terminus of Foxp3. This interaction is required for Foxp3-mediated suppression of IL-2 production and suppression activity of Treg cells [111]. Recent work has indicated that Runx1 also plays a role in Th17 differentiation [112]. Both Runx1 and ROR γ t bind to the *Il17* promoters and by interaction with distinct functional

partners (ROR γ t versus Foxp3), Runx1 may contribute to both Treg and Th17 differentiation.

5.6 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) belongs to a bHLH-PAS family of transcription factors and is highly conserved in evolution [113]. AHR senses a wide range of small synthetic compounds and natural ligands, including metabolites of tryptophan and arachidonic acids, dietary flavonols and kaempferols. Importantly, environmental toxins such 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) bind AHR. Upon ligand binding in the cytosol, AHR undergoes conformational change, translocates to the nucleus and dissociates with its chaperone Hsp90 and p23 [114].

Th17 cells preferentially express AHR and tryptophan-derived photoproduct 6-formylindolo [3,2-b] carbazole (FICZ), promote expression of IL-17, IL-17F and IL-22 [115] [116]. It has been shown that not only Th17 cells but also $\gamma\delta$ T cells express AHR [117]. Mice lacking AHR have reduced severity in EAE; however, AHR is important for IL-22, but not IL-17 production [115]. Curiously, TCDD is profoundly immunosuppressive [118] and activation of AHR by TCDD expands CD25⁺ Foxp3⁺ Treg cells, which suppress EAE [119]. Recent work has indicated that activation of AHR also induces Foxp3⁺ Treg cells in human [120]. How different AHR agonists seem to have distinct effects is puzzling; however, AHR expression in Th17 cells provides an intriguing link as to how environmental toxins might contribute to the pathogenesis of autoimmune diseases.

5.7 Nuclear factor of activated T cells and NF- κ B family

One consequence TCR ligation is elevation of intracellular Ca²⁺, which leads to the dephosphorylation and activation of members of the nuclear factor of activated T cells (NFAT) transcription factor family. The proximal promoter of the human and mouse *IL17* genes contain multiple NFAT binding sites [30, 121]. There are four isoforms of NFAT with incomplete functional redundancy [122]. Recently, it has been shown that CD4⁺ T cells expressing hyperactivable NFAT1 produce more IL-17 [123]. Moreover, proteins that interfere with the DNA binding of NFAT can regulate IL-17 expression. It has been demonstrated that NR2F6, a nuclear zinc-finger orphan receptor, acts as a transcriptional repressor of Th17 cells, similar to zinc-finger proteins that repress GATA3 and Th2 cells [124, 125]. However, we have to know more about the specific roles of the individual NFAT isoforms and binding partners in IL-17 production.

IL-1, a major promoter of Th17 development, activates NF- κ B. A recent study has indicated that the NF- κ B family member I κ B ζ regulates Th17 development by cooperating with ROR nuclear receptors [126]. A further member of the NF- κ B family, c-Rel also plays a critical role in the differentiation of Th17 cells [127].

6. Negative Regulation of Th17 differentiation

6.1 Inhibition of Th17 differentiation by STAT proteins

In addition to positive roles in regulating Th17 differentiation, cytokines that bind type I/II cytokine receptors can negatively regulate Th17 differentiation. Consistent with work indicating that IFN- γ and IL-4 also antagonize Th2 and Th1 differentiation respectively, IFN- γ and IL-4 also inhibit IL-17 production [128][6]. IL-27 is an IL-12-related cytokine that consists of two subunits, p28 and EBI3 [129]. Like the IL-6R, the IL-27R complex contains gp130, but in addition it has a ligand-specific subunit termed WSX-1. Despite its similarities to IL-6, IL-27 has the opposite effect in regulating IL-17 expression; it is a critical negative regulator of Th17 differentiation [130][131]. Like IFN- γ , IL-27 activates

STAT1 and the inhibitory effect of IL-27 on Th17 differentiation is abrogated in STAT1-deficient mice [131]. Further investigation is needed to understand the exact mechanisms by which STAT1 activation inhibits Th17 generation.

Activated by IL-12, STAT4 is well known as a critical positive regulator of Th1 differentiation and IFN- γ production. As both IL-12 and IFN- γ suppress IL-17 production, in an analogy to STAT1, the expectation might be that STAT4 would negatively regulate IL-17 expression. However, two studies have reported that IL-17 production is decreased in STAT4-deficient T cells, arguing for a positive role of STAT4 for IL-17 production [79, 132]. The decreased IL-17 production in STAT4-deficient T cells might be related to the impaired IL-23 signaling, as IL-23 has been suggested to activate STAT4 [133]. It will be of interest to further examine the role of STAT4 in Th17 differentiation.

IL-2 is a well-known T cell growth factor *in vitro*, but at the same time the deficiency of IL-2 results in severe multi-organ autoimmune disease *in vivo* [134]. This is in part due to its role in promoting the differentiation of Tregs by STAT5, but recent work has shown that IL-2 also suppresses Th17 differentiation in a STAT5-dependent manner [78] [135]. Like IL2^{-/-} mice, STAT5-deficient mice suffer from inflammatory autoimmune disease that is associated with a loss of Treg cells and the simultaneous expansion of Th17 cells. STAT5a/b appear to be essential for Foxp3 expression and in constraining Th17 cells [78, 136]. However, the mechanisms underlying the blockade of Th17 differentiation by IL-2 via STAT5 are not entirely clear. One possibility is that this is mediated indirectly via induction of Foxp3, a factor known to bind and inhibit ROR γ t [137]. Alternatively, STAT5a/b might act as a direct repressor to inhibit *Il17a* expression [78].

6.2 Negative regulation of Th17 by nuclear receptors

In contrast to ROR γ t and ROR γ , other retinoic acid nuclear receptors have been suggested to inhibit Th17 differentiation. Several groups have shown that retinoic acid inhibits Th1, Th2 and Th17 differentiation *in vitro*. Retinoic acid downregulates expression of ROR γ t and enhances the expression of Foxp3 [138–140]. Conversely, a RAR antagonist inhibited Foxp3 expression. Foxp3 binds to the *Il17* promoter [137], thus Foxp3 and ROR γ t, directly interact and modify each other's function [137].

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of the nuclear receptor superfamily of transcription factors. Multiple natural and synthetic PPAR γ ligands have been described to exert anti-inflammatory effects on various levels [141]. Upon ligand binding, PPAR γ translocates into the nucleus and forms a heterodimer with the retinoid X receptor and then binds to PPAR γ response elements located in the promoter region of target genes. Recently the relevance of PPAR γ in Th17 differentiation has been demonstrated with the use of the synthetic ligand, pioglitazone (PIO) [142]. Th17 differentiation and expression of Th17-associated genes (*Il23r*, *Il21*, *Il17* and *Il17f*) were dramatically inhibited by treatment of CD4⁺ T cells with PIO, but this had no effect on Th1 differentiation. PIO ameliorated EAE clinical scores and mice had fewer IL-17 producing cells in the CNS. Consistent with this, the mice lacking PPAR γ in CD4⁺ T cells showed earlier onset and aggravated disease in the initial T cell-dependent phase of EAE. However, there was no difference in the later effector phase of disease.

6.3 Socs3

Suppressor of cytokine signaling (Socs3) is a cytokine-inducible negative regulator of STAT3; SOCS proteins are among the most prominent Stat-regulated genes [143] providing a negative feedback to STAT signaling. We first showed that T cell-specific deletion of Socs3 is associated with enhanced STAT3 phosphorylation and increased IL-17 production

[89]. Deletion of *Socs3* in the hematopoietic and endothelial cell compartment is associated with widespread autoimmune disease and increased susceptibility to both CIA and EAE [144].

6.4 MicroRNA and negative regulation of IL-17

MicroRNAs (miRNAs) are a large family of posttranscriptional regulators of gene expression that are approximately 21 nucleotides in length. Dicer is an RNaseIII-like enzyme that is required for generating miRNAs [145]. Specific deletion of Dicer in T cells resulted in impaired T cell development and aberrant IFN γ production [146]. Ets-1 is a transcription factor that associates with T-bet and binds the *Ifng* promoter. Of note, Ets-1 deficiency results in enhanced Th17 differentiation, along with increased levels of mRNA for IL-22 and IL-23R [147]. Ets-1's actions are likely to be indirect, as no binding of Ets-1 to the *Il17* promoter was observed. Rather, Ets-1 appears to negatively regulate Th17 differentiation through effects on IL-2. Specifically, Ets-1 deficient T cells were found to secrete less IL-2 and have impaired responsiveness to this cytokine. Ets-1 has been found to be a target of microRNA mir-326 and mir-326 has been reported to be upregulated in patients with multiple sclerosis and in murine Th17 cells generated *in vitro* [148]. Overexpression of mir-326 enhances Th17 generation and disease severity in EAE whereas blocking mir-326 reduces Th17 generation and ameliorates disease severity of the EAE. This has been explained by regulating expression of Ets-1.

Recently, miRNA-, mRNA, and ChIP-seq were performed to characterize the microRNome during lymphopoiesis within the context of the transcriptome and epigenome [149]. The authors showed that H3K27me3 inhibited expression of induced miRNAs during lymphopoiesis [149]. These studies reveal some of the epigenetic, transcriptional, and posttranscriptional strategies that help orchestrate cellular abundance of miRNAs during lymphopoiesis.

7. Relationship of Th17 cells to other subsets: influence of epigenetic modification

Cellular differentiation is associated with heritable changes in chromatin in daughter cells that preserve gene expression. Epigenetic modifications such as DNA methylation, chromatin remodeling, histone modifications and incorporation of histone variants can all contribute to regulate gene expression [150]. Cytokine genes in Th cell subsets are similarly regulated [151, 152].

The *Il17* gene is linked to the *Il17f* gene on chromosome 1 (human chromosome 6) in a tail-to-tail configuration. Expression of these two cytokines is linked, although there are some circumstances in which there is selective expression of one or the other [153][154]. The promoter regions of both the *Il17* and *Il17f* genes undergo histone H3 acetylation and K4 trimethylation upon Th17 differentiation, implying increased accessibility of the locus [77]. Moreover, there are eight conserved noncoding sequences in this locus, four of which reside in the intergenic region, which also undergo preferential histone acetylation [77]. In addition to the promoters, STAT3 binds in this intergenic region and the accessible histone marks are STAT3-dependent [82]. Some of STAT3 binding sites are shared with p300 binding [60].

Although the standard model of Th1 and Th2 differentiation implies that these subsets behave like terminally differentiated cells, recent findings indicate more flexibility than envisioned and recent findings provide mechanisms for flexibility in expression of key transcription factors [155–157]. While the histone methylation at the proximal promoters of cytokines genes showed reciprocal permissive (H3K4me3) and repressive (H3K27me3) marks in cell subsets that express the signature cytokines, the histone methylation patterns of

the key transcriptional factors for lineage specification exhibit bivalent modifications of those genes not expressed [156]. For example, the promoters of *Tbx21* and *Gata3* are modified by both H3K4me3 and H3K27me3 in Th17 cells suggesting that these genes are poised for expression. This is consistent with the reported transition of Th2 to produce IFN- γ following viral infection [155]. In this context, it is perhaps not surprising that Th17 cells generated *in vitro* are intrinsically unstable [158][159][160]. In contrast, isolated memory Th17 cells appear to be more stable [161].

8. Conclusions

The past several years have certified an outburst of information pertaining to the new lineage of CD4⁺ T cells. In addition to Th1 and Th2 cells, we know that CD4⁺ T cell fate includes Th17, Treg (natural and inducible) and T follicular helper cells. The major cytokines required for Th17 differentiation have been identified, key transcription factors involved in their generation are continuingly to be identified, their function in animal models of autoimmune diseases and in models of infectious diseases have been established. On the other hand, there are still many more questions remained to be answered. What is the real relationship between all these subsets of cytokine-producing cells? To what extent are these lineages interconvertible or are they terminally differentiated? How do these processes relate to immunologic memory? Based on what we know about Th1 and Th2 cells, it would be expected that interactions between transcription factors and modifications and epigenetic modifications, and possibly intra- and interchromosomal interactions, underlie selective regulation of cytokine genes. Understanding the molecular basis of these processes will be key to understanding the fates of differentiating T cells and the possibility of using specific lineages of cells as an attractive therapeutic target in the treatment of autoimmune and inflammatory diseases.

Acknowledgments

Work of the authors was supported by NIH/NIAMS Intramural Research Program (IRP).

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Fig 1. STAT3, p300, BATF and ROR γ t binding sites in the *Il17/Il17f* locus

In addition to the previously identified STAT3 binding sites in the promoters of *Il17* and *Il17f* in Th17 conditions [77], STAT3 have been found to bind strongly with multiple conserved non-coding sequences located in the intergenic region between *Il17* and *Il17f* [82]. Binding sites of p300 has been found in this locus by our study [60]. Binding sites of BATF and ROR γ t has been reported by others [100] [97]. The significance of those individual binding sites is of interest for further investigations.

Fig 2. Signaling pathways and transcription factors that regulate Th17 differentiation

TCR stimulation activates the transcription factor NFAT, which regulates IL-17 and IL-17F differentially. IL-6, IL-21 and IL-23 induce STAT3 activation, which in turn binds the *Il17*, *Il17f* and *Il21* genes. STAT3 binds to *Rorc* and *Irf4* genes and IRF4 co-operates with STAT3 to induce ROR γ t expression. TGF- β 1 signaling involves the activation of SMAD proteins, although the mechanism by which it promotes both iTreg and Th17 differentiation remains largely unknown. One possibility is that it does not provide inductive signals, but only attenuates expression of T-bet, GATA3 and other factors. IL-1 signals to potentiate IL-6-induced IRF4 expression thus promotes Th17 differentiation. Both ROR γ t and ROR α bind the *Il17* gene. In contrast, IL-2, IL-4, IL-27 and IFN- γ inhibit Th17 differentiation through STAT5, STAT6 and STAT1 activation respectively, although the underlying mechanism(s) are not completely understood. Reciprocally, STAT5 upregulates Foxp3 expression. Runx1 associates with both ROR γ t and Foxp3 and possibly regulates differentiation towards either the iTreg or Th17 lineage. Cytokines also upregulate Socs3, which attenuates STAT3 activation.

Table 1

Mediators and transcription factors that positively or negatively regulate Th17 differentiation

Factors positively regulating Th17 differentiation			
		Chapter	Ref.
Itk	Mouse	2	[30]
Raftlin	Mouse	2	[31]
IL-1	Mouse and Human	3	[60][61] [64, 65]
IL-6	Mouse and Human	3	[5][7][8][9][10][61]
IL-21	Mouse and Human	3	[15][40][41][59]
IL-23	Mouse and Human	3	[15][38][56]
S1P	Mouse	4	[71] [72]
PGE2	Mouse and Human	4	[73] [74] [75]
STAT3	Mouse and Human	5.1	[77] [78] [79] [80] [81] [82] [83] [84–87] [88]
ROR γ	Mouse	5.2	[9]
Batf	Mouse	5.3	[100]
IRF4	Mouse	5.4	[102] [103]
Runx1	Mouse	5.5	[112]
AHR	Mouse and Human	5.6	[115] [116]
I κ B ζ	Mouse	5.7	[126]
c-Rel	Mouse	5.7	[127]
Factors negatively regulating Th17 differentiation			
IL-2	Mouse	6.1	[78] [135]
IL-4	Mouse	6.1	[6]
IL-27	Mouse	6.1	[130][131]
IFN γ	Mouse	6.1	[6]
STAT5	Mouse	6.1	[78, 136]
Retinoic acid	Mouse	6.2	[138–140]
Socs3	Mouse	6.3	[89]
Mir-326	Mouse and Human	6.4	[148]