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Signal transduction and Th17 cell differentiation

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Abstract

The paradigm of effector T helper cell differentiation into either Th1 or Th2 lineages has been notably shaken by the discovery of a third lineage of cells that selectively produce interleukin (IL)-17. Characterization of this new subset, referred to as Th17, has provided exciting new insights into immunoregulation, host defense and the pathogenesis of autoimmune diseases. Additionally, the discovery of this T cell subset has offered a fresh look at such concepts as lineage commitment and terminal differentiation. The transcriptional regulatory events and epigenetic modifications that control these processes are diverse and complex, and despite the rapid pace at which data continues to accumulate, many questions remain to be answered. Here we review our current understanding of the signaling pathways, molecular interactions and transcriptional events that lead to Th17 differentiation and effector function, as well as the epigenetic modifications that accompany them.

Keywords

Th17; IL-17; Signal transduction; Transcription factor; Lymphocyte differentiation; Cytokines; Chromatin

Introduction

Successful host defense relies on communication between cells of the innate and adaptive immune system. The seminal studies initiated by Coffman and Mossman and expanded by many others, provided the first clues of how helper T cells orchestrate proper innate and adaptive responses to microbial pathogens [1–3]. These studies also provided considerable insight into our understanding of T cell activation and the selective regulation of gene expression, providing robust models for understanding mechanisms of gene regulation and T cell fate determination [2,4,5] [6]. Despite its utility, a simple, static Th1/Th2 view of T cell differentiation implies rather inflexible responses, but recent discoveries have emphasized that T cells are anything but static in their responses.

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We now appreciate that IL-17-producing helper T cells or Th17 cells represent another subset of CD4⁺ T cells. These cells have critical roles in host defense against extracellular bacteria and also play pivotal roles in the pathogenesis of autoimmune disease. Th17 cells produce a range of cytokines including TNF, IL-17A, IL-17F, IL-21 and IL-22, but a simplistic view of these cells as merely another “lineage” of T cells unravels somewhat when one considers a number of points. First, it is apparent *in vitro* and *in vivo* that some T cells can produce both IFN- γ and IL-17; exactly where these cells fit in the spectrum of Th17 and Th1 cells is unclear. In humans, IL-17/IFN- γ double producers and IL-17 single producers can be distinguished by their chemokine receptor expression [7]. Second, although IL-21 and IL-22 are produced by Th17 cells, they can also be produced in the absence of IL-17 [8–10]. Third, we now recognize that Th17 cells, as well as other T cell subsets produce IL-10; as a result, not all Th17 cells are “pathogenic” [11]. Finally, there is evidence that another critical subset of CD4⁺ T cells, regulatory T (Treg) cells, is related to Th17 cells [12]. Treg cells are CD4⁺ CD25⁺ cells found in the thymus and in the periphery, which express the forkhead box protein transcription factor, Foxp3. We now know that some of the factors that contribute to the differentiation of Tregs also drive Th17 differentiation, and there is evidence indicating that Treg cells can become pathogenic Th17 cells. Additionally, there are data indicating that Th17 cells can express Foxp3 and that the respective Th17 and Treg lineage “master regulators” interact.

In this review, we will discuss the receptors and signaling pathways that promote the differentiation of Th17 cells, bearing in mind that the concept of a Th17 cell may be a moving target. Th17 cells are capable of producing a range of cytokines and may not always constitute a stable “lineage”. Consequently, understanding the regulation of IL-17 is not synonymous with “lineage commitment” or terminal differentiation of Th17 cells – if indeed, this concept is appropriate.

T cell receptor signaling

For any T cell, the first steps in the activation process occur as consequences of antigen binding to the T cell receptor (TCR). For naïve CD4⁺ T cells, TCR signal strength is a well-known factor influencing Th1 versus Th2 differentiation [13]. Strong TCR signaling upregulates the Th1-associated transcription factor T-bet, which is a major regulator of IFN- γ production, whereas weak signals favor IL-4 production. TCR-dependent signals also play an important role in Th17 differentiation and IL-17 production, as exemplified by the findings that invariant NK T cells, gamma-delta T cells, and memory CD4⁺ T cells all produce IL-17 in response to TCR occupancy [14–16]. However, the importance of “strong” versus “weak” TCR signals in Th17 differentiation has not been established, and T-bet has been reported to have contrasting roles in Th17 differentiation. Mathur *et al.* showed that T-bet^{-/-} memory CD4⁺ T cells had a greater propensity to produce IL-17 in response to IL-23 [17]. Accordingly, ectopic T-bet expression promoted IFN- γ secretion but inhibited IL-17 production. Similarly, in a model of autoimmune myocarditis, T-bet was found to suppress production of IL-17 [17,18]. Paradoxically though, administration of a small interfering RNA specific for T-bet in a model of experimental autoimmune encephalomyelitis was associated with reduced numbers of IL-17 producing cells. It was also reported that T-bet positively regulated expression of the IL-23 receptor (IL-23R), potentially enhancing the response to IL-23, a signal promoting Th17 differentiation. [19]. Lastly, and most recently reported, CD4⁺CD62L^{High} cells from T-bet^{-/-} and control mice at 3 and 7 days post-stimulation had similar fractions of Th17 cells [20].

Of note is that Th17 cells isolated from humans can produce both IL-17 and IFN- γ ; such cells also express T-bet [7,16,21]. Thus, while one might predict that T-bet's role in promoting IFN- γ production would naturally lead to antagonized IL-17 production, the data suggest that there may be subtleties that are not yet appreciated. Closely related to T-bet is the transcription factor

Eomesodermin (Eomes). In CD8⁺ T cells Eomes is the major regulator of IFN- γ production. CD8⁺ T cells doubly deficient in Eomes and T-bet, showed marked up-regulation of the Th17 associated factors ROR γ t, IL-17, IL-21, IL-22 and IL-23R in the context of a lymphocytic choriomeningitis virus (LCMV) infection. Additionally, mice with T cells lacking both T-bet and Eomes developed a progressive inflammatory and wasting syndrome characterized by multi-organ infiltration of neutrophils, a hallmark of Th17-mediated disease. CD4⁺ and CD8⁺ cells deficient in both factors inappropriately polarized into IL-17 secreting cells [22].

A major pathway activated by the TCR is the production of intracellular calcium and the activation of the transcription factor nuclear factor of activated T-cells (NFAT). Not surprisingly, the proximal promoter of the human *IL17A* gene contains two NFAT binding sites that appear to be important in the regulation of IL-17 [23]. Four isoforms of NFAT exist with incomplete redundancy [24]. Mice deficient in both NFATc2 and NFATc3 massively overproduce Th2 cytokines [25,26], but relatively little has been done to investigate the roles of NFAT isoforms in IL-17 production. It has been found that mice deficient in NFATc2 produced splenic and lymph node cells that exhibited increased secretion of IL-4 and IL-5 in response to infection by *N. brasiliensis* [27]. Accordingly, these mice did not develop oxazolone-mediated colitis and had impaired IL-6 and IL-17 production [28]. TCR signaling also induces the activation of the transcription factors NF- κ B and AP-1. AP-1 is known to form a complex with NFAT proteins, but the role of these transcription factors in IL-17 production is not known. Nonetheless, it would be surprising if they did not play important roles.

Co-stimulatory signaling, especially through molecules like ICOS, also contributes to the regulation of IL-17 expression as CD3-induced IL-17 production is enhanced when anti-CD28 is added [29,30]. However, the pathways and transcription factors responsible are poorly understood.

Stat family transcription factors in Th17 differentiation

In addition to presenting antigen and activating T cells through their antigen receptors, dendritic cells (DCs) also modulate the adaptive immune response through the production of cytokines. Indeed, this is critical for immunoregulation, and the importance of the cytokine milieu for CD4⁺ T cell differentiation was well established in classic studies on Th1 and Th2 cells. Specifically, IL-12, produced by DCs in response to Toll receptor ligands or other pattern recognition receptors, is a major factor that drives Th1 differentiation. Conversely, IL-4, produced by other innate immune cells such as basophils and mast cells, promotes Th2 differentiation.

It is now generally agreed that IL-6, which can also be produced by activated DCs, is a key factor in promoting Th17 differentiation of naïve CD4⁺ T cells. IL-6^{-/-} mice have reduced but not absent Th17 differentiation [31]. Another cytokine IL-21, which is selectively produced by Th17 cells, also contributes to Th17 differentiation and may have a role in sustaining Th17 cells [9,10,31,32], but more recent findings have cast doubt on the relative importance of IL-21 in vivo [33,34]. IL-23 was initially thought to be the critical inducer of Th17 differentiation; it is now recognized that this is not so. Supporting this concept is the finding that naïve CD4⁺ T cells do not express IL-23R. This raises the question, if IL-6 induces Th17 cells and IL-21 sustains them, what is the role of IL-23? Memory T cells express the IL-23 receptor, and IL-23 is now viewed as being more important for the in vivo pathogenicity of Th17s [30,35, 36].

Type I and II cytokines such as IL-12, IL-4, IL-6, IL-21 and IL-23 all activate Janus family kinases (Fig. 1). Tyk2 is important for signaling by IL-12 and IL-23, and mice lacking this kinase have been generated. Although responses to IL-23 have not been carefully analyzed, IL-12 responsiveness is dramatically affected by loss of Tyk2 [37,38]. Notably, a single human

with a severe primary immunodeficiency syndrome was found to have Tyk2 mutations and was noted to have profound impairments in IL-12 signaling [39,40].

The Stat (signal transducer and activator of transcription) family of DNA binding proteins is critical for transmitting signals from Type I/II cytokine receptors. The importance for Stat1, Stat4 and Stat6 for IFNs, IL-12 and IL-4 expression, respectively, is well-established. Functionally, they have critical roles in mediating anti-viral responses and immunoregulation including Th1/Th2 differentiation [41,42]. As expected, Stat targets include cytokine genes. Stat4, for instance, is known to bind the *Ifng* gene [43]. Other Stat4 target genes include *Map3k8* and *Furin*. *Map3k8* is induced by TCR and cytokine signals. It promotes IFN- γ production by enhancing expression of T-bet and Stat4 [44]. In contrast, furin is essential for maintenance of peripheral tolerance through its essential role in processing TGF β -1 in effector and Treg cells [45]. A number of other Stat target genes have been identified, but with the advent of ChIP-on-Chip (chromatin immunoprecipitation and microarray) and ChIP-Seq (chromatin immunoprecipitation and massive parallel sequencing), we will soon have a comprehensive understanding of direct Stat targets [46,47]. With the genetic tools available (Stat-deficient mice), this will unquestionably continue to be a robust system to define the roles of transcription factors in programming cellular responses.

IL-6, IL-21, and IL-23 all preferentially activate Stat3 (Fig. 1). The first evidence of the importance of Stat3 in regulating IL-17 production came from the demonstration that the *Il17A/F* locus has multiple putative Stat binding sites. Using chromatin immunoprecipitation (ChIP) assays, it was determined that Stat3 directly binds to this promoter [48]. Furthermore, *in vitro* Th17 differentiation is greatly impaired in Stat3-deficient T cells [17,49,50]. Conversely, retroviral over-expression of constitutively active Stat3, results in increased production of IL-17 [17]. Thus Stat3 appears to be a direct regulator of IL-17. Additionally though, Stat3 may have other roles in promoting Th17 differentiation. IL-6-mediated production of IL-21 is also Stat3 dependent, and Stat3 appears to be a direct regulator of IL-21 production as it binds to the *Il21* promoter [32]. Furthermore, IL-6, IL-21 and IL-23 can up-regulate expression of the IL-23R, and this too appears to be Stat3 dependent [9,10,16]. At present, Stat3 is a critical factor for Th17 differentiation in at least four ways: regulation of IL-17, IL-21, IL-23R and ROR γ t, a critical transcription factor for Th17 cells (discussed below); the genes encoding the first two factors appear to be direct Stat targets. We have performed microarray analysis on Th17 cells and have noted ~500 genes to be preferentially expressed [32]; of these, 50% are Stat3 dependent. Clearly, in the future it will be important to carefully define direct Stat3 targets in Th17 cells and dissect the interactions between Stat3 and other factors that are important for this subset of T cells.

In general, the factors that regulate human Th17 cells are similar to the factors that drive mouse Th17 differentiation, although there are some inconsistencies among the various cytokine recipes. This is not surprising given the inherent variability between human donors and the difficulty of obtaining naïve T cells. However, despite the inconsistencies, a common feature between groups is the importance of including a Stat3 dependent cytokine (IL-6, IL-21 or IL-23) in cultures [7,51,52]. While Stat3 was known to be essential for mouse Th17 differentiation, until recently it was not possible to assess the importance of Stat3 in human Th17 differentiation.

Hyper IgE syndrome (HIES) is a primary immunodeficiency disorder characterized by the susceptibility to fungal and staphylococcal infections, elevated IgE serum concentration, dermatitis and bony abnormalities [53]. HIES has recently been found to be due to heterozygous mutations of Stat3, which produce a dominant negative protein that can block binding of the wild type Stat3 to candidate DNA binding sites [54,55]. We and others have shown that patients with HIES Stat3 mutations are unable to generate Th17 cells [56]. How much this inability to

produce Th17 cells accounts for the patients' inability to clear bacterial and fungal infections remains to be determined.

Suppressor of cytokine signaling (Socs3) is a cytokine-inducible negative regulator of Stat3; indeed Socs proteins are among the most prominent Stat-regulated genes [57]. Interestingly, deletion of Socs3 is associated with enhanced Stat3 phosphorylation and increased IL-17 production [48,58]. Deletion of Socs3 is also associated with multiorgan inflammatory disease characterized as neutrophil infiltration and overexpression of factors regulated by IL-17 (G-CSF, chemokines, IL-6). These findings further support the critical functions of Stat3 in regulating IL-17.

In addition to positive roles in regulating Th17 differentiation, cytokines that bind Type I/II cytokine receptors also have important roles in negatively regulating Th17 differentiation. In the early studies in which Th17 cells were proposed as a new Th lineage, data were provided indicating that IFN- γ and IL-4 inhibit IL-17 production. This counter-regulation was consistent with previous work indicating that IFN- γ and IL-4 also antagonize Th2 and Th1 differentiation respectively. IL-27 is an IL-12-related cytokine that consists of two subunits, p28 and EB13. Like IL-6, its receptor is composed of gp130, but in addition 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 [59,60]. IL-2 is a well-known T cell growth factor *in vitro*, but the deficiency of IL-2 results in severe multi-organ autoimmune disease. This is due in part to its role in promoting the differentiation of Tregs, but it is also now clear that another essential role of IL-2 is to negatively regulate Th17 differentiation [49,61].

As indicated, IFN- γ activates Stat1. In contrast to IL-6, IL-27 also activates Stat1 and in Stat1-deficient mice the inhibitory effect of IL-27 is abrogated [59,60]. Of note is that Stat1-deficient helper T cells have a greater propensity to produce IL-17 [36]. Additionally, after viral infection, mice lacking Stat1 develop airway disease associated with increased IL-17 expression [62]. IFN- γ and IL-27 also up-regulate T-bet [Lighvani, 2001 #169[63,64], and T-bet expression is significantly reduced in Th17 cells [36,65–67]. As indicated above, T-bet may be a negative regulator of Th17 cells, but this issue has not been fully resolved [17,19, 36,68].

Strongly 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, similar 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 [69,70]. The decreased IL-17 production in Stat4-deficient T cells might be related to the impaired IL-23 signaling, as IL-23 also activates Stat4. It will be of interest to further examine the role of Stat4 in Th17 differentiation.

Stat5a/b are two related transcription factors that are critical for IL-2 signaling. Like *Il2*^{-/-} mice, Stat5-deficient mice have autoimmune disease that is associated with a loss of Treg cells and expansion of Th17 cells. Thus, Stat5a/b appear to be essential for Foxp3 expression and in constraining Th17 cells [49,71]. However, the mechanisms underlying the blockade of Th17 differentiation by IL-2 via Stat5 are not entirely clear. It is possible that this is mediated indirectly via Foxp3; however, Stat5 also binds to the *Il17A* gene [49]. So it is possible that Stat5a/b might directly repress *Il17A* transcription. While it appears that some Stats (Stat3) directly activate genes involved in Th17 differentiation (*Il17A*, *Il17F*, and *Il21r*), it has not been established that others (e.g. Stat1 and Stat5a/b) directly repress these genes; this issue is

also clearly worthy of additional investigation. Other possibilities are that Stat5a/b might negatively regulate ROR γ t, which in turn would downregulate IL-17 expression.

Ets-1 is a transcription factor that associates with T-bet and can directly bind to 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 [72]. However, Ets-1's actions do not appear to be direct, 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.

Complex role of TGF β signaling in Th17 cells

There are three isoforms of transforming growth factor β , TGF β 1–3, with TGF β 1 being the most immunologically relevant. The essential immunosuppressive functions of TGF β 1 are best illustrated in mice lacking the cytokine, its receptor subunits, or key signaling molecules, which have fatal, systemic autoimmune disease [73–75]. Yet despite these essential immunosuppressive functions, it is also clear that this cytokine serves a proinflammatory role, being critical for Th17 differentiation [67,76]. This begs the question as to exactly how TGF β signaling contributes to the regulation of IL-17 expression.

The receptor for TGF β is structurally very different from those of Type I/II cytokine receptors. Its receptor comprises two chains, TGF β type 1 receptor (TGF β RI) and TGF β type 2 receptor (TGF β RII), both of which are receptor serine-threonine kinases. Two of each receptor chains associate with the ligand to initiate signaling. This activates receptor-associated latent cytosolic transcription factors known as Smads (Smad2 or Smad3), which become phosphorylated and dimerize with Smad4 – also referred to as a Co-Smad – to form heterodimers. This complex binds Smad-binding elements of its target genes, but in addition, Smads can associate with many other DNA-binding cofactors including Jun and Stat3.

Mice expressing a mutated form of the TGF β receptor (CD4dnTGFRII) fail to generate Th17 cells in vitro and are protected from the development of EAE [77]. Despite the clear role in Th17 differentiation in vitro and in vivo, how TGF β regulates Th17 differentiation is unknown. At present, there are no data indicating that Smads directly bind to the *Il17A*, *Rorc*, *Il21*, or *Il23r* genes. While Smads are critical signal transducers of most TGF β signaling pathways, Smad-independent pathways do exist (e.g. MAPK, Rho, and PI3K), but their roles in Th17 regulation have also not been determined [78]. Regardless though, stimulation of cells with TGF β -1 enhances ROR γ t expression. While it has been reported that activated Stat3 or Ror γ t alone are poor inducers of IL-17 in the absence of TGF β -1, other work argues against this possibility [10,70]. At this point, it is not certain how TGF β and Stat signals are coordinated.

Whereas stimulation of cells with TGF β and IL-6 induces Ror γ t, the combination of IL-2 and TGF β upregulates Foxp3. Foxp3 directly binds a number of genes, serving as a putative activator or repressor [79], but can also associate with other transcription factors. In this regard, it is notable that Foxp3 also interacts with Ror γ t and that Ror γ t and Foxp3 are co-expressed in lamina propria T cells and in naive CD4⁺ T cells exposed to TGF β [80,81]. In this way, TGF β can regulate Th17/Treg polarization in a concentration-dependent manner. Specifically, at low concentrations, TGF β synergizes with IL-6 and IL-21 to promote IL-23R expression. Additionally, IL-6 represses Foxp3 in a Stat3-dependent manner [71,76]. Conversely, high concentrations of TGF β repress IL-23R expression and enhance Foxp3 expression. In addition, TGF β down-regulates expression of both the Th1 and Th2 associated “master-regulators” T-bet and Gata3 [65,82], and also inhibits IL-2 production, both of which could favor Th17 differentiation [49]. Also of note is that IL-17 and IL-22 appear to differ in their requirement for TGF β [83].

Despite its role in murine Th17 differentiation, TGF β has been reported to be dispensable for human Th17 differentiation and has been reported to inhibit IL-17 production [7,51]. This conundrum may be resolved with the recognition of the importance of the concentration of TGF β in regulating the Th17/Treg balance [52,84]. These results have additional implications. Since TGF β -1 can promote both Th17 and Treg differentiation, and TGF β produced by Treg cells can also promote Th17 differentiation [67,85], it appears that the balance between Treg and Th17 cells is more fluid than we initially considered. Again, this speaks to a very different model from the simplistic Th1/2 dichotomy, which implies more options and more flexibility for differentiating T cells. This more complex view of T cells also might provide a better conceptual framework for understanding autoimmune disease but is also a cautionary note for the therapeutic use of Tregs [86,87].

Production and secretion of TGF β is more complex than many other cytokines. It is synthesized as a pre-cursor protein, called pro-TGF β , which must be proteolytically cleaved and activated by the endoprotease furin in the Golgi apparatus prior to secretion [88]. The cleaved TGF β remains associated with Latency associated protein, which must also be removed to generate the active cytokine. Failure to properly process TGF β results in widespread autoimmune disease [45,89].

IL-1 and Th17 differentiation

Another inflammatory cytokine reported to positively regulate Th17 development is IL-1. This has been documented in both mouse and human cells where it synergizes with IL-6 and IL-23 [67,90]. Whereas IL-6 alone causes transient induction of Ror γ t, the combination of IL-1 and IL-6 results in sustained induction. Accordingly, IL-1RI $^{-/-}$ mice have impaired Th17 cell differentiation and reduced incidence of EAE associated with failure to induce autoantigen-specific Th17 cells [91].

The IL-1 receptor has homology to Toll-like receptors, and IL-1 engagement of its receptor results in activation of a complex that comprises the adapter MyD88 and IL-1 Receptor-associated kinases (IRAK). This complex recruits another adapter molecule, tumor necrosis factor receptor associated factor (TRAF)-6. This leads to the activation of other kinases including Jun kinase (Jnk) and inhibitor of kappa-B kinases (IKKs). Jnk phosphorylates the transcription factor Jun, which dimerizes with Fos to form the transcription factor AP-1. IKKs phosphorylate the inhibitor of kappa B, which is then degraded, allowing NF- κ B to translocate to the nucleus. The role of these various factors in Th17 differentiation has not been determined but is clearly an important area of ongoing research.

Retinoid receptors and Th17 cells

ROR γ t belongs to the superfamily of steroid nuclear receptors and is most closely related to a retinoic acid receptor (RAR) subfamily of transcription factors [92]. The family consists of nine members grouped into three sub-families, RAR's, RXR's and ROR's. The first two members bind metabolites of retinoic acid (RA), the active metabolite of vitamin A, whereas the ligand(s) for ROR members are unknown.

ROR γ is important in lymphoid organogenesis and in thymopoiesis as evidenced by the fact that *Rorc* $^{-/-}$ mice exhibit reduced numbers of double positive and CD4 $^{+}$ single-positive thymocytes. *Rorc* $^{-/-}$ mice also show a lack of lymph nodes and Peyer patches [93–95]. ROR γ t is a splice variant of ROR γ that results from initiation by a distinct promoter, resulting in products that differ in their amino termini [96]. In the normal state, ROR γ t is predominantly expressed in lamina propria T cells. However, *Rorc* $^{-/-}$ mice have reduced Th17 differentiation and are less susceptible to experimental autoimmune encephalomyelitis, indicating that this transcription factor is also important in disease pathogenesis [97]. 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 and therefore has been proposed to be a “master regulator” for Th17 differentiation.

Precisely how ROR γ t regulates IL-17 production is only beginning to be understood. Multiple functional ROR γ t binding sites are present in the *Il17A* promoter, and through the use of chromatin immunoprecipitation, ROR γ t has been found to bind the *Il17A* gene [81]. How *Rorc* and its splice variant are regulated is also not clear. One important means of regulating ROR γ t expression is through antigen receptor stimulation in the presence of Th17 inducing cytokines including IL-6. Stat3 mediates IL-6 regulation of ROR γ t, but it has not been established that Stat3 binds the *Rorc* gene. Overexpression of active Stat3 in *Rorc*-deficient cells resulted in poor IL-17 induction, indicating that Stat3 is necessary but not sufficient for IL-17 expression. However, overexpression of ROR γ t failed to induce IL-17 secretion, suggesting that these two transcription factors act in parallel to some extent [80]. Identification of ROR γ t targets is unquestionably an important challenge in understanding the biology of Th17 cells.

Another related retinoic acid nuclear receptor, ROR α , has also been found to be up-regulated in Th17 cells. ROR α deletion in mice had minimal effect on IL-17 production, however deficiency of both ROR α and ROR γ t completely abolished IL-17 production and protected against EAE [98]. Co-expression of the two factors together promoted enhanced numbers of IL-17 producing cells. Collectively, the findings suggest that, though not the major player, ROR α seems to synergize with ROR γ t to promote Th17 differentiation.

ROR γ t and ROR α are not the only members of the retinoic acid receptor family to influence the induction of Th17 cells, however. A series of recent studies show that RA produced by mucosal DC reciprocally regulates Th17 and Treg differentiation [99–103]. That is, addition of RA inhibits Th1, Th2 and Th17 differentiation. RA also down-regulates expression of ROR γ t, and enhances the expression of Foxp3. Moreover, T cells cultured in RA and TGF β are functionally immunosuppressive even in the absence of IL-2 *in vitro*. Conversely, an RAR antagonist inhibited Foxp3 expression, indicating that expression of this key transcription factor may be dependent upon endogenous production of RA and the positive effect of RARs. These new data suggest that the balance of Treg vs. Th17 differentiation might be influenced by the actions of RAR and ROR γ t and modulated by the production of RA and other unknown ligands. The data also suggest that the induction of Foxp3 in developing Th17 cells might curtail differentiation of this lineage. Supporting this is evidence that Foxp3 binds to the *Il17A* promoter [80]. IL-17 and IL-21 are down-regulated in Foxp3-expressing cells [104], but it is not known if Foxp3 also directly binds the *Rorc* or *Il21* promoters.

As mentioned, we know the ligands that bind and activate some retinoid receptors, but in the case of ROR γ t the ligand is unknown. This begs the obvious question as to whether a secreted product of immune cells can activate this transcription factor, and in so doing, regulate T cell differentiation. It is of note in this regard that dendritic cells from the gut-associated lymphoid organs tend to be immunosuppressive. Importantly, mucosal DCs also produce retinoic acid (RA) which, among other actions, enhances the expression of α 4 β 7 integrin and CCR9 on activated T cells, promoting gut tropism [99–101,105]. DC-derived RA negatively regulates Th17 differentiation and so one might hypothesize that other DCs could produce ROR γ t ligands and promote Th17 differentiation.

Arylhydrocarbon receptor

The aryl hydrocarbon receptor (AHR) belongs to a basic-helix-loop-helix-PER-ARN-SIM family of transcription factors. AHR in its ligand-free state is a cytosolic transcription factor, associated with the chaperone Hsp90. AHR has a number of ligands, some endogenous (also

referred to as 'natural') and other exogenous (also referred to as 'synthetic'). The natural ligands include metabolites of tryptophan and arachadonic acids, as well as dietary flavonols and kaempferols [106,107]. Synthetic ligands include both planar and aryl hydrocarbons, found in environmental toxins. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic member of a large group of structurally similar halogenated aromatic hydrocarbons polychlorinated dibenzodioxin congeners (PCDDs) as well as polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs). Dioxins are most infamously known as products of the defoliant, Agent Orange. Upon ligand binding, AHR dissociates with its chaperone and translocates to the nucleus. Here, AHR associates with the aryl receptor nuclear translocator (ARNT) and the resulting heterodimeric complex is responsible for regulating gene expression [108].

There is a large body of literature related to the effects of TCDD immune responses and many reports indicate that the compound is profoundly immunosuppressive [109]. Recently, it has been reported that activation of AHR by TCDD expands CD25⁺ Foxp3⁺ Treg cells, which suppress EAE [110]. Conversely though, it has been found that Th17 cells preferentially express AHR. In contrast to the effect of TCDD, the tryptophan-derived photoproduct 6-formylindolo[3,2-*b*] carbazole (FICZ), a high affinity endogenous ligand for the AHR, upregulated expression of IL-17A, IL-17F and IL-22 [111]. Accordingly, mice lacking AHR were resistant to EAE. T cells from these mice were able to develop into Th17 cells, but they were unable to express IL-22 [112]. How two different AHR agonists, TCDD and FICZ could have such distinct effects remains a puzzle, but it is possible that 'synthetic' and 'natural' ligands could have distinct functions [113]. How AHR is regulated in Th17 cells remains to be fully delineated; but regardless of the existing controversies, the discovery that Th17 cells preferential express the AHR provides new clues as to how environmental toxins might contribute to the pathogenesis of autoimmune disease. This is particularly intriguing, considering that factors such as cigarette smoking are associated with an increased risk of autoimmune diseases like RA [114,115]

An additional new wrinkle is the finding that AHR selectively associates with Stat1 and Stat5, but not with Stat3 or Stat6. AHR deficiency was associated with prolonged Stat1 phosphorylation, so it is conceivable that induction of AHR provides a counter-regulatory mechanism that would attenuate the effects of cytokines like IFN- γ and IL-27 that activate Stat1[116].

Runx transcription factors

Runx transcription factors represent another family of proteins that regulate CD4⁺ helper cell differentiation. There are three mammalian Runt domain transcription factors, Runx1, Runx2, and Runx3 [117]. These factors form heterodimers with an obligatory non-DNA-binding partner termed Cbfb protein. AML1/Runx1 is crucial for normal hematopoiesis including thymic T cell development [118]. Additionally, both Runx1 and Runx3 are reported to serve as repressors of CD4 and Zbtb7b (a transcription factor that regulates CD4⁺ differentiation), but at different stages of thymocyte development [118–120]. 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* [121,122].

AML1/Runx1 also binds the N-terminus of Foxp3. Disrupting this interaction impairs the Foxp3-dependent suppression of IL-2 production and attenuates suppressive activity without affecting the binding of Foxp3 to NFAT [123]. Recent work has indicated that Runx1 also interacts with Ror γ t to drive Th17 differentiation [124]. In this way, distinct functional partners associated with Runx1 may decide the cell fate in a stimulus-dependent manner. AML1/Runx1

has been reported to bind the *Ii2* promoter at a site upstream of NFAT, AP-1, and NF-κB binding sites.

IRF4 and Th17 differentiation

The interferon regulatory factor (IRF) family of transcription factors is yet another group of proteins that influence T helper differentiation. IRF4 was originally implicated as a key inducer of GATA3 expression and Th2 lineage differentiation [125–127]. More recently, deficiency of IRF4 was shown to impair IL-17 production despite stimulation with TGFβ-1 and IL-6 [128]. Moreover, *IRF4*^{-/-} mice are resistant to EAE, which is overcome by the transfer of wild type CD4⁺ T cells, indicative of an intrinsic T cell defect. However, *IRF4*^{-/-} mice show a degree of resistance to EAE in excess of IL-17, IL-23, RORγt or Stat3 null animals, implying that there are additional effects of IRF deficiency. It has been suggested that IRF4 might co-operate with Stat3 to induce RORγt expression, but IRF4 is also known to interact with NFATc1 and c2 [125,127] suggesting its effects on Th17 cells may be related to its effects on 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 [36] and Th17 and Treg development via TGFβ [76].

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

Sphingosine 1-phosphate (S1P) is an abundant lysophospholipid in blood and lymph that regulates T cell trafficking and egress from lymphoid structures [129]. The importance of this mechanism is underscored by the utility of the immunosuppressive drug FTY720 (fingolimod). Addition of S1P to culture of splenic CD4⁺ T cells has been reported to increase IL-17 and reduce IFN-γ and IL-4 production [130]. Accordingly, transgenic overexpression of an S1P receptor was also associated with enhanced IL-17 production [131]. S1P receptors are members of the seven-transmembrane, or G-protein coupled, receptor family. Whether S1P is a physiologically important regulator of Th17 differentiation has yet to be determined; nonetheless, the findings raise the intriguing possibility that ligands for other G-protein coupled receptors might also influence T cell fate determination. In this regard, it is possible that chemokines utilize this class of receptors and might have similar actions. Ligand binding to G-protein coupled receptors activates a number of biochemical pathways, but there is no information presently on how signaling influences IL-17 expression.

Prostaglandins are another class of lipids that regulate cell function by acting on GPCRs. Prostaglandin E2 (PGE2) has been reported to promote the expansion of IL-17-producing cells. 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 [132]. In vivo administration of PGE2 induced IL-23-dependent IL-17 production of human cells and exacerbated collagen-induced arthritis and IL-17 production in mice [133]. It has been argued that PGE2 promotes IL-23 production in DCs, so it is unclear at this time whether the effect of PGE2 is direct or indirect [134,135].

Epigenetic regulation of Th17 cells

While transcription factors play critical roles in regulating gene expression, work from many systems has emphasized that the acquisition of heritable traits in cell differentiation can be highly regulated by epigenetic modifications of genes. In the nucleus, DNA forms nucleosomes through its association with histone proteins; this complex of DNA and protein is referred to as chromatin. Epigenetic regulation refers to DNA methylation, chromatin remodeling, histone modifications and incorporation of histone variants that alter gene expression.

The heritable selective expression of cytokine genes in activated CD4⁺ T cells represents an excellent example of how transcription factors and epigenetic regulation influence gene expression. A key feature of Th1 and Th2 cells is the silencing of the gene encoding the signature cytokine of the opposing fate – in this respect, Th1 and Th2 cells appear to be terminally differentiated. As a result, Th1 and Th2 cells develop into mature effectors with rather stable phenotypes. Generally, once a CD4⁺ T cell has committed to one of these two lineages, limited conversion to the other lineage occurs. The *Ifng* locus has been studied for a number of years and we have a reasonably comprehensive understanding of the regulatory elements that are spread over a region of about 100 kilobases flanking the gene [136,137]. These include multiple DNase hypersensitivity sites and conserved noncoding sequences (CNS), which include sites that bind Stats, NFAT and T-bet. Epigenetic marks, such as histone modification and DNA methylation throughout the *Ifng* locus, are critical in maintaining the gene in a transcriptionally accessible state, and it appears that Stat4 is essential for these modifications [138]. Over-expression of T-bet also promotes epigenetic modifications [139]. Conversely, silencing of the *Ifng* locus in differentiating Th2 cells is dependent upon GATA3 and Stat6 [136,137,140]. Similarly, the Th2 locus, which encompasses 140 kb and includes genes encoding IL-4, IL-13, Rad50, and IL-5, also undergoes a number of lineage-specific epigenetic modifications. In addition, the *Ifng* and *Il4*, *Il5*, *Il13* loci undergo intra- and interchromosomal interactions [141].

Our understanding of the epigenetic control of the *Il17* locus is quite primitive. The *Il17A* 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. The promoter regions of both the *Il17A* and *Il17F* genes have been found to undergo histone H3 acetylation and K4 tri-methylation in response to TGFβ and IL-6, implying increased accessibility of the locus [142]. In addition, there are eight conserved non-coding sequences in this locus, four of which reside in the intergenic region, which also undergo preferential histone acetylation. The mechanisms through which TGFβ, in combination with IL-6, promotes accessibility to this locus are entirely unknown. Additionally, whether and how Rorγt and Stat3 contribute to the chromatin modifications in the *Il17A-Il17F* locus has not been determined. One also wonders whether interchromosomal interactions between the *Il17A-Il17F* locus and the *Ifng*, Th2, or *Foxp3* loci also exist and how interchromosomal and intrachromosomal interactions might change with the activation and differentiation status of T cells.

In contrast to the signature cytokine loci, relatively little is known about the epigenetic regulation of the “master regulators” that control Th differentiation. It will obviously be important to understand how T-bet, GATA3, Rorγt and Foxp3 themselves are regulated, such that lineage specification is preserved or if flexibility is permitted.

Conclusions

The past several years have witnessed an explosion of information pertaining to CD4⁺ T cell differentiation. In addition to disrupting the classic Th1/Th2 paradigm with the inclusion of Treg (natural and inducible) and Th17 cells, we have now learned that a number of cytokines once thought to be lineage specific (IFN-γ, IL-10, IL-22) can be produced by several different cell types. These findings have brought to the forefront questions of ‘plasticity’ and the inter-relatedness of various cytokine-producing subsets. Studies on Th17 differentiation have fueled this fire, showing that TGF-β regulates Th17/Treg polarization in a concentration dependent manner. Further interconnecting these two lineages are the findings that RORγt and Foxp3 can not only interact, but also are co-expressed, and that Runx1 has been shown to interact with both factors. Additionally, ligands for both the RAR and AHR can drive differentiation into either lineage.

However, most of our understanding of these phenomena is only superficial. We have a severely limited understanding of the mechanism of TGF- β action and whether or not Smads are direct regulators of IL-17, IL-21, IL-23R or Foxp3. Additionally, the regulation of the supposed 'master-regulator', ROR γ t, has not been elucidated. Other factors that we now understand to promote Th17 differentiation could be working through two or more mechanisms; e.g. IRF4 could be working through either NFAT-mediated TCR signals or Stat-mediated cytokine signals. Stats appear to be involved in AHR regulation, but the mechanism is unknown. Finally, regulation of the IL-17 locus is becoming increasingly complicated with both inhibitory (Stat5, Foxp3) and stimulatory (Stat3, ROR γ t) factors being recruited to it. Answering these questions and understanding the molecular basis of these processes will be key to gaining a better understanding of T cell differentiation and to ultimately applying it to questions of 'plasticity' and lineage commitment as they relate to host defense and the immune response.

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Fig 1. Jak/STAT targets of cytokines involved in T helper cell differentiation

The receptors of Type I and Type II cytokines associate with Janus kinases (Jaks). Cytokine binding activates the Jaks, which phosphorylate the receptors, allowing the recruitment of signal transducers and activators of transcription (STATs). Recruited STATs are phosphorylated and subsequently form dimers. STAT dimers translocate to the nucleus, where they regulate gene expression. Many cytokines are known to activate more than one STAT pair. The receptor components and downstream Jak/STAT targets of IL-2, IL-4, IL-6, IL-12, IL-21 and IL-23 are diagrammed above. Major and minor signaling pathways are represented with solid and dashed arrows respectively.

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

In helper T cells, TCR stimulation activates NFAT, which is thought to directly regulate IL-17 expression. Cytokines such as IL-6, IL-21 and IL-23 activate STAT3, which in turn binds the *IL17A*, *IL17F* and *IL21* genes. STAT3 is also thought to regulate ROR γ t expression and is inhibited by the cytokine inducible Socs3. Although uncertain, IRF4 is thought to co-operate 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 unknown. The mechanism by which IL-1 signaling via MyD88 promotes Th17 differentiation is also unknown; one possibility is via NF- κ B. ROR γ t and ROR α regulation of IL-17 production is poorly understood, although ROR γ t has been found to bind the *IL17A* gene. Binding of the AHR ligand FICZ causes dissociation of the receptor with cytoplasmic chaperones, enabling it to translocate to the nucleus, heterodimerize and promote IL-17 and IL-22 expression. Discouraging Th17 differentiation, IL-2, IL-4, IL-27 and IFN- γ inhibit STAT 3 activity through STATs 5,6 and 1 respectively. Notably, STAT5 also regulates Foxp3 expression. RA, acting through its cognate receptors, downregulates ROR γ t expression and up-regulates Foxp3 expression. Runx1 associates with both ROR γ t and Foxp3 and possibly regulates differentiation towards either the iTreg or Th17 lineage. Abbreviations: AHR. aryl hydrocarbon receptor; ARNT. Aryl receptor nuclear translocator; Co-SMAD. Co-mediator SMAD; FICZ. 6-formylindolo[3,2-b] carbazole; R-SMAD. receptor-regulated SMAD; RA (R). retinoic acid (receptor)