Annals of Medicine. 2008; 40: 322–335

informa
healthcare

REVIEW ARTICLE

An insight into molecular mechanisms of human T helper cell differentiation

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Abstract
Selective activation of T helper (Th) cell subsets plays an important role in immune response to pathogens as well as in the pathogenesis of human allergy and inflammatory diseases. Th1 cells along with the recently discovered Th17 cells play a role in the pathogenesis of autoimmune diseases. Th2 cytokines lead to series of inflammatory processes characteristic for asthma and other atopic diseases. To understand the pathogenesis of immune-mediated diseases it is crucial to dissect pathways and regulatory networks leading to the development of distinct Th subsets. Such knowledge may lead to better strategies for developing diagnostics and therapies for these diseases. The differentiation of Th1, Th2, and Th17 effector cells is driven by signals originating from T cell and costimulatory receptors as well as cytokines in the surroundings of activated naive T helper cells. There are several proteins involved in the regulation of this differentiation process. Most of the data on T helper cell differentiation have been acquired using mouse. In this review, we have summarized what is known about human T helper differentiation. In addition, selected differences between human and mouse will be discussed.

Key words: *Caspase, epigenetic regulation, GATA3, IFN-γ, IL-12, IL-4, NFAT, T cell differentiation, T-bet*

Introduction

The differentiation of naive $CD4^+$ T helper (Th) cells into Th1, Th2, or Th17 effector cells is a finely balanced process that is controlled by T cell receptor (TCR) activation, costimulatory molecules on the surface of the antigen-presenting cell, and polarizing cytokines in the vicinity of the T cell. There are several reviews describing the key molecules and mechanisms driving T helper cell differentiation (1–3). Differentiated T helper cells are characterized by a specific set of cytokines that they secrete upon the restimulation. Th1 cells produce mainly interferon-γ (IFN-γ), but also tumor necrosis factor-α (TNF-α), and lymphotoxin, whereas Th2 cells produce interleukin-4 (IL-4), IL-13, IL-9, and IL-5 (1,2).

T helper cells have a profound role in a variety of immune responses. Through their cytokine production, effector Th1 cells activate macrophages, natural killer cells, and cytotoxic $CD8^+$ T cells, and stimulate the production of immunoglobulin (Ig-) G antibodies that are involved in opsonization and phagocytosis. Th1 cells are important for the eradication of intracellular pathogens, including bacteria, parasites, yeast, and viruses. Th1 cells, along with the newly discovered Th17 subset, are also associated with autoimmune diseases, such as type I diabetes and multiple sclerosis (MS) (1,4). Effector Th2 cells activate mast cells and eosinophils, and their cytokines induce B lymphocytes to switch to IgE-producing cells. Th2 cells are important in the defense against certain helminths and other extracellular parasites, but a predominant Th2 response is also linked to atopic diseases and allergies (2).

Recently, a lineage of $CD4^+$ T cells producing IL-17 was described and accordingly designated as

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ISSN 0785-3890 print/ISSN 1365-2060 online
DOI: 10.1080/07853890802068582

Key messages

- T helper (Th) cells are important in immune response to pathogens as well as in the pathogenesis of human immune-mediated diseases.
- Th cells can be divided into functionally distinct subsets, such as Th1, Th2, Th17, and Th regulatory cells, all of which originate from a common naive precursor cell.
- Th cell differentiation is a finely balanced process regulated by T cell receptor activation, cytokines, costimulatory molecules and the strength and quality of each stimulus.

Abbreviations

c-FLIP	cellular Fas-associated death domain-like interleukin-1β-processing enzyme (FLICE) inhibitory protein
CNS	conserved nucleotide sequence
CRTH2	chemoattractant receptor-homologous molecule expressed on TH2 cells
DC	dendritic cell
ERK	extracellular signal regulated kinase
ERM	Ets-related molecule, PEA3-like
GATA3	GATA-binding protein 3
HLX	H2.0-like homeobox 1
ICOS	inducible costimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MAPK	mitogen-activated protein kinase
MS	multiple sclerosis
NDFIP2	Nedd4 family interacting protein 2
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-kappa B
NKT cells	natural killer T cells
PBMCs	peripheral blood mononuclear cells
RORγt	RAR-related orphan receptor gamma t
shRNA	small hairpin RNA
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TBX21	T-box 21
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin
Txk	TXK tyrosine kinase

Th17 cells (5). These proinflammatory T cells have been mostly studied in the mouse. Th17 cells exhibit a cytokine profile distinct from Th1 and Th2 cells, producing cytokines, such as IL-17 and IL-22, and express a characteristic transcription factor retinoic acid receptor (RAR)-related orphan receptor gamma t (RORγt) (6). In addition to its proinflammatory role in autoimmune diseases, IL-17 has been shown to be important for host defense against pathogens such as *Klebsiella pneumoniae* and *Bacteroides fragilis* (3). The detection of IL-17-producing T cells in humans with inflammatory diseases, such as MS, contact dermatitis, rheumatoid arthritis, and Lyme arthritis (4), suggests that in humans these cells have a proinflammatory role similar to that described in mouse models. Th17 cells were also isolated from gut mucosa of patients with Crohn's disease, and interestingly a novel population of Th cells producing both IL-17 and IFN-γ was detected in these patients (7).

In this review we focus on findings on human effector Th cell differentiation. Data on molecular mechanisms of human Th cell differentiation are limited compared to that in mouse. Moreover, several differences between human and mouse systems, e.g. in the expression of CRTH2 (chemoattractant receptor-homologous molecule expressed on TH2 cells) (8,9), and the induction of Th17 development (3,10), underscore the importance of elucidating the molecular mechanisms of human T helper cell differentiation for a better understanding of T cell-mediated human diseases.

Cytokines driving T helper cell development

IL-12 is considered to be the main cytokine driving Th1 differentiation (Figure 1). IL-12 is produced mainly by macrophages and dendritic cells (DCs), but also by monocytes, neutrophils, and B cells in response to different pathogens (11). Naive Th cells are unresponsive to IL-12 due to the lack of IL-12 receptor β2 (IL-12Rβ2) expression, which forms IL-12 receptor together with IL-12Rβ1 (1). In human $CD4^+$ T cells, TCR signaling and STAT4 (signal transducer and activator of transcription 4) are important for the induction of IL-12Rβ2 expression (12). In mouse it has been shown that in naive Th cells also IFN-γ signals through STAT1 and T-box expressed in T cells (T-bet), resulting in IL-12Rβ2 expression (13).

In addition to IL-12, other cytokines, such as IL-27, IL-18, IFN-γ, and IFN-α, also play a role in Th1 differentiation. IL-27 is a novel cytokine that is structurally related to IL-12 and induces Th1 differentiation through phosphorylation of STAT1 both in mouse and human Th cells (14). IL-18 has a

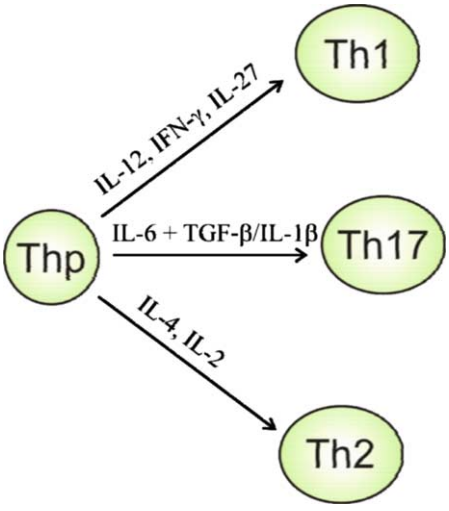

Figure 1. Cytokines driving T helper cell differentiation. IL-12 and IL-4 are the main cytokines polarizing the Th cells into Th1 and Th2 direction, respectively. IL-27 and IFN-γ can also polarize cells into Th1 direction, and IL-2 is needed for proper Th2 development in mouse. IL-6 and TGF-β induce the development of Th17 cells in mice, and IL-1β together with IL-6 or IL-23 in human. IL = interleukin; IFN = interferon; TGF = transforming growth factor; Th = T helper.

dual role in controlling Th cell cytokine production: it enhances the effect of IL-12 in inducing IFN-γ production by both human and murine Th1 cells (15,16), whereas in mouse studies in the absence of IL-12 it is able to promote Th2 cytokine production (17). IFN-γ produced in response to bacteria and viruses promotes Th1 cell differentiation through STAT1 signaling (1). IFN-α, on the other hand, is produced mainly by dendritic cells in response to viral infections. IFN-α was originally shown to induce IFN-γ production and promote Th1 differentiation in human T cells, although this has not been detected in mouse (18). In subsequent experiments, however, it was found that while IFN-α alone could induce T-bet expression in human T cells, the levels of IFN-γ produced were low (12,19). Nonetheless, IFN-α plays a role in Th1 differentiation at least through making the cells prone to Th1-inducing factors, since it has been shown to induce STAT4 phosphorylation (18) and IL-12Rβ2 expression in human Th cells (20).

Murine Th cells can be directed towards the Th17 lineage by IL-6 and transforming growth factor (TGF)-β (21), whereas this combination of cytokines is incapable of generating IL-17-producing cells in humans (10,22,23). In human Th cells, IL-1β has recently been found to be crucial for differentiation of IL-17-producing Th cells, and its effect was enhanced by IL-6 (10) or IL-23 (23). In addition, prolonged IL-23 stimulation of human Th cells increases IL-17 production more effectively in the absence than in the presence of IL-6 or TGF-β (22). In mouse cells, IL-23 was first reported to regulate the differentiation of Th17 cells, but it is currently believed to be involved in the expansion of committed Th17 cells rather than promoting their differentiation (21). In addition, TGF-β was found to inhibit the differentiation of human Th17 cells (10,22). These results imply that the differentiation of Th17 cells is regulated differently in human and mouse.

Signaling through IL-4 receptor (IL-4R) is needed for adequate differentiation into Th2 direction and proper Th2 responses. IL-4 can be produced by many cell types, such as mast cells, basophils, eosinophils, natural killer T (NKT) cells and differentiated Tc2 and Th2 cells (24). Yet the source of IL-4 in the lymph node during the initial activation of naive T helper cells is presently unclear. $CD4^+$ T helper cells produce IL-4 in a STAT6-independent manner in the lymph nodes during the initial activation, and they are the main producers of IL-4 in the lymph node at that stage (25,26). IL-4 produced by activated naive T helper cells might be necessary for driving Th2 development, since Th2 responses can be mounted in mice in which only $CD4^+$ cells are capable of producing IL-4 (27). Nonetheless, whether the amount of IL-4 produced by activated CD4+ T cells in the lymph node is sufficient to generate potent Th2 responses remains a matter of debate (25). Intriguingly, in this connection, Th2 development of human and mouse T cells has been shown to occur in the absence of any exogenous IL-4 at least in *in vitro* cultures (28,29).

In addition to IL-4, there are also a number of other cytokines involved in Th2 differentiation and Th2 effector functions. First of all, the significant role of thymic stromal lymphopoietin (TSLP) in Th2-mediated disease states, such as asthma and allergies, has been well reported. Human and mouse TSLP regulate T helper cell differentiation mainly by triggering dendritic cells to induce Th2 development, which is mediated at least partly by OX40L-OX40, OX40L = OX40 ligand interaction between DCs and $CD4^+$ T cells (30). In addition, IL-2 and STAT5a, which is activated in response to IL-2 stimulation, are needed for proper Th2 differentiation and Th2-mediated responses in mouse (31,32). This is partly due to a direct regulation of the IL-4 locus by STAT5a, which binds to and opens up the chromatin in the IL-4 locus during Th2 development (32). IL-18 has been shown to promote mouse Th2 differentiation in an IL-4/STAT6-dependent

manner (17). IL-6 has been suggested to play a role in Th2 differentiation by enhancing IL-4 production in a NFAT1 (nuclear factor of activated T cells)-dependent manner during the initial TCR activation (33). However, IL-6 is not needed for proper Th2 differentiation, since Th2 development is not impaired in IL-6-deficient mice (34). Similarly in humans, IL-6 increases the production of IL-4 and IL-5 in activated naive T helper cells, but it does not increase Th2 development or Th2 cytokine production at the effector phase (35). IL-25, a member of the IL-17 family of cytokines, induces production of Th2 cytokines in human and mouse memory Th cells (36,37). However, IL-25 is not crucial for Th2 differentiation since Th2 differentiation is not impaired in IL-25-deficient mice (36).

Cytokine-regulated factors involved in T helper cell differentiation

There are several proteins involved in regulating the fate of differentiating T helper cells. In the following chapters, we will concentrate on those factors that have been reported to play a role also in the human system and discuss their role in T helper cell differentiation.

Factors regulating Th1 differentiation

STAT4. IL-12 signaling through STAT4 is important for Th1 cell differentiation (Figure 2). Mice deficient in STAT4 exhibit a diminished Th1 response, IFN-γ production, and increased susceptibility to infection (11). However, STAT4-deficient Th cells, when cultured in Th1-promoting conditions, produce some IFN-γ, indicating the existence of an additional, STAT4-independent, pathway for Th1 differentiation (38). STAT4 mediates its function by regulating several genes important for Th cell differentiation such as IL-12Rβ2, IL-12-responsive ERM (Avian erythroblastosis virus E26 (v-ets) oncogene homolog (Ets)-related molecule, polyomavirus enhancer activator 3 (PEA3)-like), and IL-18R, and promotes the expression of Th1 hallmark cytokine IFN-γ (39–41). In mice, a possible mechanism for IFN-γ regulation by STAT4 is through binding of STAT4 to its target sites in the *IFNG* gene locus (42).

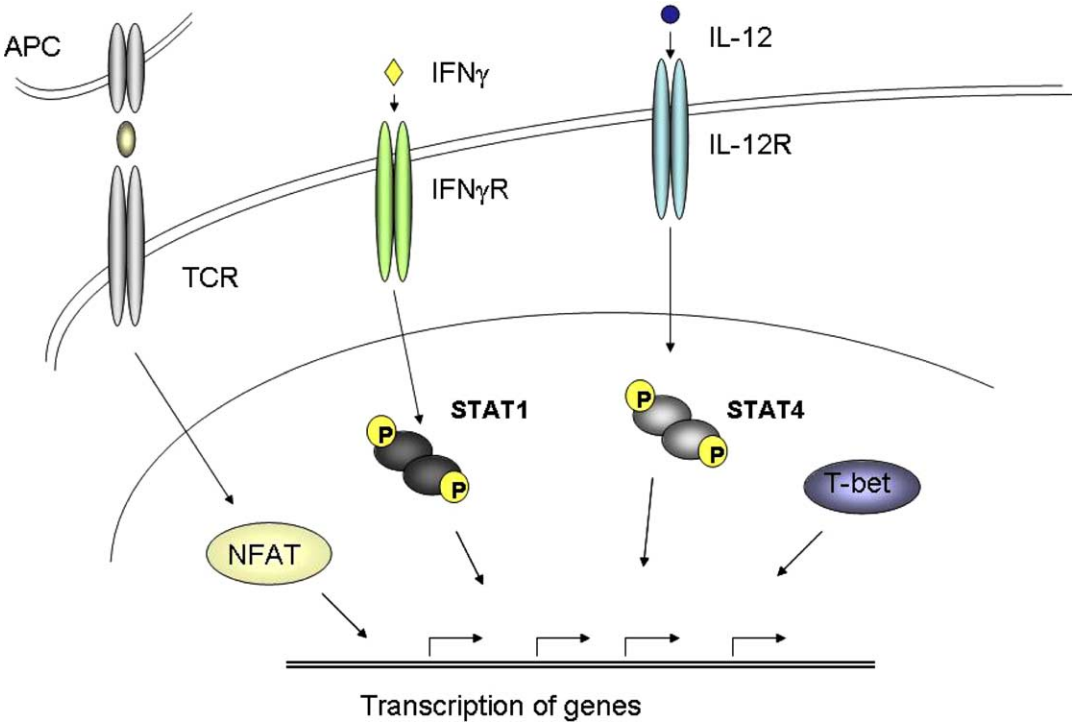

Figure 2. Signals leading to Th1 cell differentiation. IL-12 binding to IL-12 receptor (IL-12R) leads to activation of STAT4. Similarly, binding of IFN-γ to its receptor (IFN-γR) activates STAT1 which in turn upregulates expression of transcription factor T-bet. T cell receptor (TCR) stimulation activates many intracellular signaling pathways and leads to activation of NFAT proteins. NFAT proteins and Th1 specific transcription factors T-bet, STAT1 and STAT4 upregulate the transcription of several Th1 specific genes in the nucleus of Th cell. IL = interleukin; IFN = interferon; NFAT = nuclear factor of activated T cells; STAT = signal transducer and activator of transcription; T-bet = T-box expressed in T cells; Th = T helper; APC = antigen presenting cell.

In human Th cells, knocking down STAT4 by small interfering RNA (siRNA) oligonucleotides strongly reduces expression of IL-12Rβ2. The role of STAT4 in the regulation of IL-12Rβ2 gene expression was further characterized by identification of an IL-12Rβ2 promoter enhancer element that recruits STAT4 during the early stages of Th1 polarization. TCR activation was required for STAT4 binding to this element, and STAT4 binding was further increased by IL-12 and IFN-α signaling (20). Induction of IL-12Rβ2 expression enables the cells to respond to IL-12, the main cytokine driving Th1 differentiation.

In addition to IL-12, IFN-α signaling also leads to phosphorylation of STAT4 on tyrosine residues and to its activation in human Th cells (18). While IFN-α was shown to be the most potent inducer of IL-12Rβ2 expression during the early Th1 differentiation, IL-12 was a more efficient inducer of IFN-γ production and IL-12Rβ2 expression during the later stages of differentiation (20). In addition to this, IL-12 is more efficient than IFN-α in driving Th1 differentiation in humans, and this is probably due to the fact that IL-12-induced STAT4 activation is more long-lasting than that induced by IFN-α (12).

T-bet. T-bet, also called TBX21, was originally cloned from mouse T helper cells as a Th1-specific transcription factor that represses the production of Th2 cytokines and mediates production of the Th1 hallmark cytokine, IFN-γ (43,44). T-bet expression is induced by signals through T cell receptor and IFN-γ/STAT1 signaling both in human and mouse (13,19). Studies using T-bet knockout mice have shown that, together with IL-12/STAT4 signaling, T-bet is essential for Th1 cell differentiation (44). Besides IFN-γ, in human $CD4^+$ T cells both T-bet mRNA and protein levels are also upregulated by IL-12 in a manner that is independent of IFN-γ (19). In the latter study, IL-12 induced some STAT1 tyrosine phosphorylation in human Th cells, which is likely to activate T-bet expression.

T-bet is a member of the T-box family of transcription factors and binds to DNA through its T-box DNA binding domain. It regulates Th1 cell differentiation by activating IFN-γ production and upregulating IL-12Rβ2 expression, thereby enabling IL-12/STAT4 signaling (13,43). T-bet is bound to the T-box half-site in the IFN-γ promoter and activates the promoter in the Jurkat T cell line (45). In addition, T-bet also modulates the *IFNG* gene locus by binding to conserved enhancer sequences (46,47) thus inducing IFN-γ expression. Another Th1-specific T-bet target gene, Hlx1, is a transcription factor that also acts as a cofactor of T-bet to induce optimal IFN-γ production (48). While this has been demonstrated in mouse Th cells, in humans Hlx1 has also been found to be expressed at higher levels in Th1 than in Th2 cells (49).

In addition to its role in inducing Th1 differentiation, T-bet also plays a role in suppressing Th2 lineage commitment. Both in human and mouse Th2 cells, retroviral transduction of T-bet is able to repolarize these cells towards the Th1 direction (43,50). Mouse studies have revealed one possible mechanism of T-bet in Th2 lineage repression: tyrosine-phosphorylated T-bet is able to interact with GATA3, a transcription factor important for Th2 differentiation, and thus prevent it from binding to its target sequences (51).

Because T-bet is an important regulator of Th cell differentiation, it is likely to be associated with immune-mediated conditions. Indeed, mice deficient of T-bet are resistant to many autoimmune diseases such as experimental autoimmune encephalomyelitis (52). T-bet knockout mice have been shown to exhibit a phenotype resembling human asthma and, consistently, asthmatic patients show reduced expression of T-bet in their airways (53). In humans, either weak or moderate associations of T-bet single nucleotide polymorphism (SNP) variants have been linked to aspirin-induced asthma (54), asthma susceptibility, or airway hyperresponsiveness in asthmatic children (55). In other studies, no association between T-bet SNPs and asthma has been found (56,57). In addition, none of these studies found association between high serum IgE-levels and T-bet variants, and no strong association with T-bet SNPs and asthma other than that related to aspirin intolerance has been found so far.

Recently described IFN-γ-inducing factors. In addition to molecules described above, some novel factors influencing IFN-γ production in human T cells have recently been discovered. Thus, NDFIP2 (Nedd4 family interacting protein 2), a protein proposed to have a role in protein trafficking and activation of NF-κB signaling, was shown to regulate Th1 differentiation by promoting IFN-γ production (58). This was revealed from experiments using NDFIP2-specific small hairpin RNA (shRNA) in polarizing human Th1 cells. Recently furin, a proprotein convertase, was shown to be induced by IL-12 during early Th1 differentiation, and this process was dependent on STAT4 (40,59). Pesu et al. also showed that specific inhibition of furin, either by α_1-antitrypsin variant or by furin-specific siRNA, diminishes IL-12-stimulated IFN-γ production by human Th cells, suggesting a positive role for furin in the regulation of IFN-γ expression.

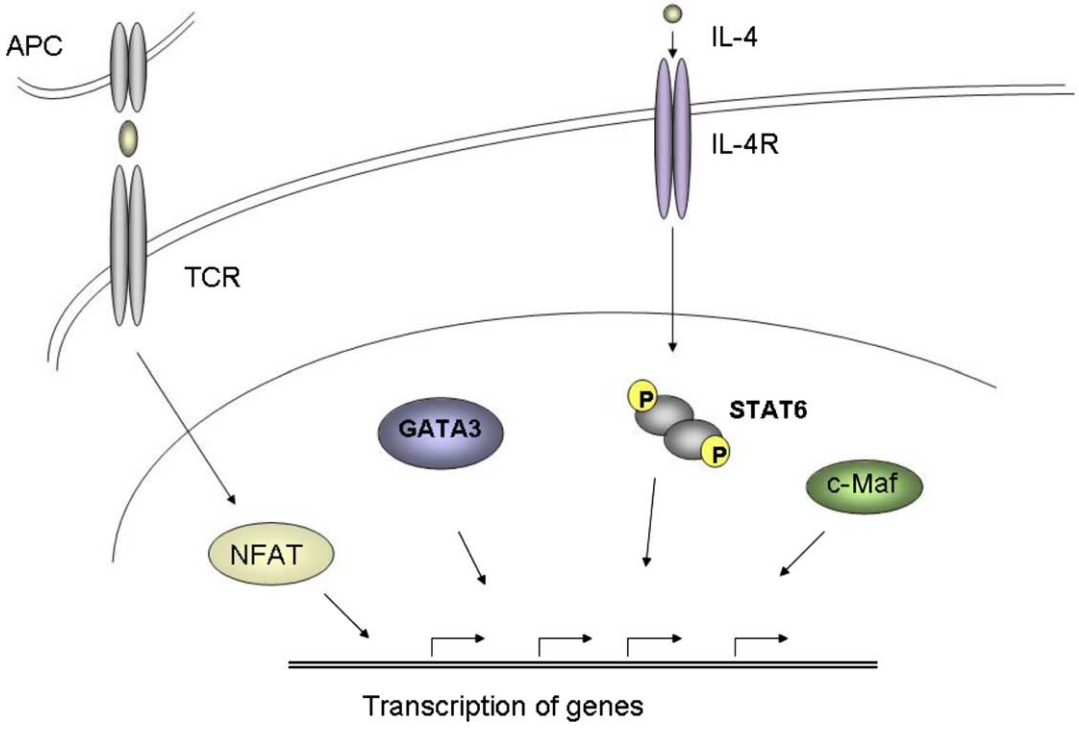

Figure 3. Signals driving Th2 differentiation. IL-4 binding to its receptor (IL-4R) activates STAT6. T cell receptor (TCR) activation activates many intracellular signaling pathways and leads to stimulation of NFAT proteins. NFAT proteins and Th2 specific transcription factors STAT6, GATA3, and c-Maf upregulate transcription of several Th2-specific genes in the nucleus of Th cell. GATA3 = GATA-binding protein 3; IL = interleukin; NFAT = nuclear factor of activated T cells; STAT = signal transducer and activator of transcription; Th = T helper; APC = antigen presenting cell.

Factors regulating Th2 differentiation

STAT6. As IL-4 binds to its receptor, STAT6 becomes tyrosine-phosphorylated, dimerizes, and is localized into the nucleus, where it regulates the transcription of its target genes (Figure 3) (60). The importance of STAT6 for Th2 differentiation has been shown in studies with STAT6-deficient mouse strains (61) and also by knocking down STAT6 in human primary T helper cells (62). In addition, certain STAT6 SNP variants have been linked to asthma and high IgE in some studies but not in others (63). STAT6 mediates some of its functions by upregulating GATA3 expression in developing Th2 cells in both human and mouse cells (58,64,65), but it also binds directly to the Th2 cytokine locus at least in mouse (66). Although the mechanism of STAT6 phosphorylation and its role in Th cell development has been well characterized, less attention has been paid to the regulation of STAT6 levels during T helper cell differentiation. According to our results, STAT6 is proteolytically downregulated in response to oxidative stress in activated primary human Th cells. We have also identified Preli as a protein that is upregulated by TCR stimulation and induces oxidative stress leading to STAT6 degradation and decreased Th2 differentiation (J. Tahvanainen et al., unpublished observations).

GATA3. Following TCR activation, GATA3 mRNA and protein levels are induced by IL-4 in both humans and mice, and this induction is dependent on STAT6 (58,64,65). However, STAT6 does not appear to be obligatory either for GATA3 induction or Th2 development, since in mice low frequencies of Th2 type cells can be generated in the absence of either STAT6 or IL-4R (67). Both STAT6 and IL-4R-deficient murine cells show increased expression of GATA3, suggesting the presence of other upstream regulators for GATA3 (68). One possibility for this upstream regulator of GATA3 came from two recent studies that revealed GATA3 as a target of Notch signaling (69,70). T cell-specific deletions of Notch signaling partners in mouse revealed that Notch *trans* activates *Gata3* gene and drives Th2 differentiation (69). Impaired Notch signaling resulted also in defects of IL-4 production and GATA3 expression (70). These data indicate that GATA3 is a downstream target of Notch signaling and that Notch signaling together with GATA3 is needed for proper Th2 differentiation at least in mouse.

GATA3 binds to several sites in the Th2 cytokine locus and controls there DNA conformation and accessibility (66,71). In humans, the overexpression

of GATA3 can induce or enhance the production of IL-4, IL-5, and IL-13 in developing Th2 cells and in memory cells. GATA3 also downregulates the expression of Th1 type chemokine receptor CXCR3 and upregulates Th2 type chemokine receptor CCR4 (50). The importance of GATA3 in Th cell differentiation is also supported by studies of GATA3 SNP variants and defective GATA3 expression in humans. One GATA3 haplotype has been associated with asthma-related traits, especially to high serum IgE (72). In addition, individuals with defective GATA3 expression have decreased proportions of IL-4-secreting memory Th2 cells. Their serum IgE and IgG4 levels are decreased and IgG1 levels increased, implicating a defective Th2 response as well (73). Consistently, knockdown of GATA3 by siRNA decreases the production of Th2 cytokines in primary human memory cells and malignant Jurkat cells (73,74). In mouse Th2 cells, GATA3 is required for the optimal IL-4 production, but IL-4 production is not totally dependent on it: deletion of GATA3 in differentiated Th2 cells has only a slight effect on the proportion of IL-4-producing cells. However, the amount of IL-4 produced by these cells is significantly decreased, whereas the effect of GATA3 deletion on IL-13 and IL-5 secretion is more severe (75,76).

In addition to inducing Th2 development, GATA3 also inhibits Th1 differentiation independently of STAT6 or IL-4 (64,68). GATA3 is reported to mediate its negative effect on IFN-γ production by downregulating STAT4 both in mouse and in Jurkat cell lines (74,77). Both in human and mouse, the competence of GATA3 to induce Th2 phenotype or to shut down IFN-γ expression weakens progressively as T helper cells differentiate into Th1 type effector cells (50,78).

Despite GATA3 expression being markedly stronger in mouse Th2-differentiated cells than in Th1 cells at the 7 day time point, it is more similarly expressed between polarized human Th1 and Th2 cells (19,73,79). The lower degree of GATA3 upregulation in human Th2 cells compared to mouse cells correlates with the lower production of IL-4 in human than in mouse Th2 cells (79). In humans, GATA3 is able to upregulate the expression of chemokine receptor CRTH2 (50), which is differentially expressed between human but not mouse Th1 and Th2 cells (8,9). In humans, stimulation of CRTH2 with prostaglandin D_2 leads to an enhanced Th2 response *in vitro* (80). This is in line with the results showing that the expression of human CRTH2 correlates strongly with the production of IL-4 and IL-13 as well as the decreased expression of Th1 markers T-bet and IFN-γ (79). Although the mRNA and protein levels of GATA3 are also increased in CRTH2-positive cells, they are still lower in human $CRTH2^+$ Th2 cells than in mouse Th2 cells (79). The lower GATA3 expression and IL-4 production in human Th2 cells implies that Th2 differentiation *in vitro* is less efficient in human than in mouse. Of course one has to take into consideration that inbred mouse strains, which are used to study Th2 differentiation, are already Th2-prone, whereas the genetic background is more diverse in humans.

In addition to the IL-4-induced increase of GATA3 transcription, there are also posttranslational mechanisms involved in the regulation of GATA3 expression or activity. Thus, it has been reported that the Ras-extracellular signal regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) signaling cascade in human and mouse stabilizes the GATA3 protein by decreasing ubiquitin-mediated GATA3 degradation (81). In addition, both human and mouse friend of GATA proteins as well as tyrosine-phosphorylated mouse T-bet have been shown to suppress both GATA3 activity and GATA3-mediated Th2 cytokine production by binding to GATA3 (51,82).

c-Maf and ICOS. v-maf musculoaponeurotic fibrosarcoma oncogene homolog (c-Maf) was originally found to be differentially expressed between mouse Th1 and Th2 cell clones and to induce IL-4 production synergistically with NFAT1 (83). In humans, c-Maf is upregulated at the level of mRNA or protein in bronchial biopsies and in induced sputum of asthmatic patients (84,85). c-Maf mRNA expression is also increased in bronchoalveolar cells of asthmatics after allergen exposure, and the expression correlates strongly with the amount of IL-4-producing $CD4^+$ T cells in the alveoli (86). Furthermore, c-Maf mRNA expression is increased by IL-4 in differentiating human Th2 cells (87). In mice, c-Maf is needed for proper IL-4 production, but it does not directly regulate the expression of two other Th2 cytokines, IL-13 or IL-5 (83,88). c-Maf has been shown to bind to several sequences within the Th2 cytokine locus upon restimulation of mouse Th2 effector cells (89). Mouse c-Maf is also capable of inducing IL-4 transcription in a Th1 clone AE7 and in M12 B lymphoma that do not spontaneously produce IL-4, and this induction is synergistically increased by NFAT1 (83). IL-4 production is similarly induced by c-Maf and NFAT1 in a human Jurkat T cell line not producing IL-4, and IL-4 production is further increased by the NIP45 protein (90).

As there is still a rather limited amount of data about the role of c-Maf in human T helper cell

differentiation, its relevance for human Th2 differentiation needs to be clarified further. However, the positive role of c-Maf in Th2 differentiation has been indirectly supported by studies on a protein called inducible costimulator (ICOS). The production of IL-4 and IL-13 by T helper effector cells is selectively reduced in ICOS knockout mice (91). Consistently, activation of wildtype (wt) $CD4^+$ T cells with ICOS ligand B7h-deficient APCs leads to impaired expression of IL-4 and IL-13 upon restimulation of Th cells (92). In humans, allergic sensitization and the production of Th2 cytokines IL-4, IL-13, and IL-5 are enhanced in individuals with alternative SNP variants in the promoter region of the ICOS gene leading to increased ICOS expression (93). The effect of ICOS on IL-4 production by effector cells might be mediated by c-Maf. TCR-induced expression of c-Maf, but not that of JunB, T-bet, or GATA3, is reduced in ICOS-deficient T helper cells, and c-Maf overexpression restores the normal IL-4 levels in differentiated ICOS knockout cells (94). If activated ICOS-deficient T helper cells are cultured in the presence of IL-4, both the induction of c-Maf and the IL-4 production by effector cells are restored (91,94). These data indicate that ICOS and c-Maf have a role in IL-4 production in mouse Th cells.

TCR-regulated factors involved in T helper cell differentiation

The strength of TCR induction influences T cell differentiation, usually strong TCR activation promoting Th1 and weak Th2 differentiation (95). There are a number of proteins involved in TCR-signaling pathways that regulate the T helper cell differentiation. In this review, we will discuss the role of NFAT proteins, Tec kinases, and the caspase pathway in T helper cell differentiation.

NFAT proteins

NFAT proteins become activated through signaling mediated by TCR and costimulatory molecules. There are three NFAT proteins that regulate Th cell differentiation: NFAT1, NFAT2, and NFAT4 (96). Mouse knockout studies indicate that NFAT1 induces Th1 differentiation and represses Th2 cell differentiation since deletion of NFAT1 leads to mild bias towards Th2 and to reduced Th1 cell differentiation (97,98). Deletion of NFAT1 also decreases the production of IFN-γ, and, consistently, NFAT1 has been shown to bind to mouse *IFN-γ* promoter *in vivo* (98,99). Mice that lack both NFAT1 and NFAT4 have exacerbated Th2 responses and produce elevated levels of Th2 cytokines (100). In contrast, NFAT2 knockout mouse lymphocytes produce decreased levels of Th2 cytokines implicating that NFAT2 is a positive regulator of Th2 cell differentiation (101). However, the role of NFAT proteins in Th cell differentiation seems to be complex, since NFAT1 can induce the production of both IL-4 and IFN-γ, depending on cytokine stimuli and other factors (98,102). NFAT1 can bind both to IL-4 and IFN-γ promoters in naive Th cells, but only to IL-4 promoter in activated mouse Th2 cells and to IFN-γ promoter in activated Th1 cells (66). In addition, NFAT proteins have been reported to cooperate with many different transcription factors depending on the cell type and stimuli. In human T cell line Jurkat, NFAT1 together with Th1-specific transcription factor T-bet were found to bind to IFN-γ enhancer element (47). On the other hand, NFAT1 has also been reported to bind to IL-4 enhancer element together with GATA3 in differentiated mouse Th2 cells and in murine Th2 cell clone D10 (99). Thus the NFAT proteins seem to have redundant roles in Th cell differentiation, and their effect on gene transcription depends on the cytokines, other transcription factors, and state of the cell (Th1/Th2/naive). The significance of NFAT proteins in controlling the epigenetic changes is demonstrated mainly in murine T cells—the role of NFAT proteins in human Th cell differentiation is an interesting topic for further investigation.

Tec kinases

Txk is a non-receptor tyrosine kinase that belongs to the Tec family of tyrosine kinases. It is expressed in human Th1 and Th0 cells but not in Th2 cells and promotes IFN-γ production as shown by antisense oligonucleotides and overexpression experiments (103,104). Its expression is upregulated by IL-12 (103). In human T cells, Txk has been shown to form a protein complex that binds to IFN-γ promoter and activates gene transcription (105). Txk binding sequences similar to that on the IFN-γ promoter can also be found in other Th1-specific genes (104). This suggests that this kinase might also regulate other Th1-specific genes. In addition, Txk has also been shown to be involved in autoimmune Behcet's disease (BD). Patients show increased Txk expression in $CD4^+$ cells, resulting in skewed Th1 response, and this seems to be important in the pathogenesis of BD (106). Thus Txk is likely to have a role in human Th1 differentiation *in vivo*.

The expression of Itk, another kinase involved in T helper cell functions, is selectively increased in

developing mouse Th2 cells *in vitro* and in peripheral blood T cells from patients with atopic dermatitis (107,108). Itk is needed for proper TCR activation, and its deficiency leads to diminished Th2 responses (109). This is mainly due to the fact that Itk-deficient Th2 cells are incapable of mounting the proper cytokine response upon restimulation (110,111). Human TIM-1 (T cell, Ig domain, and mucin domain-1), an orthologue for mouse Tim-1 and Tim-2, is reported to act in the same pathway upstream of Itk, implicating than it might have a similar role in effector T cell functions (112). More generally, several members of the TIM gene family has been associated with the regulation of Th1 and Th2 responses, for example in mouse Tim-3 is linked to Th1 and Tim-2 to Th2 cell responses (113).

Caspase pathway

Caspase activity is generally associated with apoptotic cell death, but it appears that certain caspases are also needed for proper T cell activation and development (114). During the initial activation of primary human T cells, caspase-3, -6, -7, and -8 become activated in a manner that is linked to selective substrate cleavage and is not related to apoptosis of these cells (115). Similar observations have also been made in mouse effector cells, and, in this case, Th2 cells show both higher caspase activity and better survival than Th1 cells (116). In developing Th cells, caspase activity promotes Th1 differentiation, since caspase inhibition or stimulation leads to increased IL-4 or IFN-γ production, respectively (117,118). More specifically, caspase-8 activity prevents Th2 responses, and active caspase-8 is required for effective T cell-mediated immunity against the intracellular parasite *Trypanosoma cruzi* (119). On the other hand, transgenic mice expressing the long form of cellular Fas-associated death domain-like interleukin-1β-processing enzyme (FLICE) inhibitory protein (c-FLIP) show elevated caspase-8 activity and enhanced Th2 responses: Th2 differentiation is augmented, allergic inflammation is enhanced, and mice are protected from Th1 type experimental autoimmune encephalomyelitis (120). Caspase-8 and c-FLIP long are also needed for proper T cell activation and TCR-induced proliferation (121,122). Activity of another caspase, caspase-3, is decreased by IL-4 in activated primary naive human T helper cells (123). Consistently, IL-4 is able to regulate other proteins in the caspase pathway. Thus, it decreases Fas receptor expression and increases the levels of c-FLIP short (c-FLIPs), Bcl-2, and Bcl-xL (123). The upregulation of c-FLIP short by IL-4 is of particular interest since c-FLIPs is known to bind caspase-8 and inhibit its activation (124), potentially promoting Th2 differentiation. These findings provide an exciting basis for further studies to define the role of c-FLIP or effector caspases in human T helper cell differentiation.

Epigenetic regulation of T cell differentiation

In addition to expression of lineage-specific and other transcription factors, the chromatin structure of the cytokine loci of differentiating Th1 and Th2 cells is altered. The changes in the chromatin structure include formation of DNaseI hypersensitive sites and associated epigenetic changes such as DNA demethylation and histone modifications. The accessibility of promoters and other regulatory elements for transcription factors is controlled by these epigenetic changes. During Th1 and Th2 differentiation lineage-specific epigenetic changes occur across the IFN-γ gene and Th2 cytokine loci encoding for IL-4, IL-5, and IL-13 genes as well as in other sites in chromatin (125–127). In naive activated T cells, the chromatin structure of both IFN-γ and IL-4 loci is open, and thus these cells are able to produce both of these cytokines (128). However, during the differentiation of Th1 or Th2 cells specific regions of the chromatin become accessible while others are repressed by modifications (125). More specifically, if Th cells are activated under Th2 conditions, modifications seen in naive activated IL-4 locus are stabilized, whereas under Th1 conditions, the permissive modifications are replaced with the repressive modifications (129). In human cells, there is a histone hyperacetylation at the IL-4 promoter region in memory Th2 cells required for the induction of IL-4 gene (130). Moreover, the promoter region modifications of the IFN-γ gene locus in human Th2 cells are related to silent chromatin structures whereas the promoter is modified to be active in Th1 cells (131,132). The epigenetic regulation of genes is an important area of research, and together with transcription factors it has a crucial role in regulation of cytokine and other gene expression during Th cell differentiation. As many other aspects of Th cell differentiation, also the epigenetic regulation of the IFN-γ and Th2 locus genes are more studied in mouse than in human cells. Therefore, unraveling the chromatin changes involved in the regulation of these genomic regions along with the transcription factors involved in those changes during human Th cell differentiation is an important area of future research.

Genome-wide characterization of human T helper cell differentiation

Recent advances in genome projects and development of genome-wide analysis methods have revealed genes and proteins differentially regulated during various stages of Th cell polarization and potentially important for human Th cell differentiation (40,41,65,87,133–136) or at the effector phase (58,65,87,123,137–142). Integration of such genome-wide analysis with siRNA approach (58,62) and efficient data mining and pathway analysis tools along with data-driven modeling exercises (143,144) will make it feasible to build a holistic view of Th cell differentiation and signaling and the gene regulatory networks involved. We are currently actively exploiting such an approach to generate and test novel hypotheses to improve our understanding of human Th cell differentiation and immune-mediated diseases.

Understanding the molecular mechanisms of lymphocyte differentiation to functional effector cells is crucial for understanding the pathogenesis of immune-mediated diseases or factors important in host defense to microbes. In spite of the wide research activity carried out in the mouse system, relatively little is known about the molecular basis of Th differentiation in human. Exploitation of cutting-edge technologies is likely to reveal novel players and molecular mechanisms involved as well as lead to identification of key pathways and points of potential intervention to modify immune responses.

Acknowledgements

Kirsi J. Rautajoki and Minna K. Kyläniemi have contributed equally to this work.
We acknowledge the Academy of Finland, The Sigrid Jusélius Foundation, DBT The Government of India, CIMO/Sitra, The National Technology Agency of Finland, The Juvenile Diabetes Research Foundation, Turku Graduate School of Biomedical Sciences, The National Graduate School in Informational and Structural Biology, Turku University Hospital Research Fund for their financial support. We would like to thank Robert Moulder and Anu Neuvonen for the language revision of the manuscript.

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