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Hidehiro Yamane
William E. Paul

Early signaling events that underlie fate decisions of naive CD4$^+$ T cells toward distinct T-helper cell subsets

Authors' address
Hidehiro Yamane[1], *William E. Paul*[1]
[1]Cytokine Biology Unit, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.

Correspondence to:
Hidehiro Yamane
Building 10/Room 11N314
10 Center Drive
Bethesda, MD 20892-1892, USA
Tel.: +1 301 594 9818
Fax: +1 301 480 7352
e-mail: hyamane@niaid.nih.gov

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Summary: CD4$^+$ T-helper (Th) cells are a major cell population that play an important role in governing acquired immune responses to a variety of foreign antigens as well as inducing some types of autoimmune diseases. There are at least four distinct Th cell subsets (Th1, Th2, Th17, and inducible T-regulatory cells), each of which has specialized functions to control immune responses. Each of these cell types emerge from naive CD4$^+$ T cells after encounter with foreign antigens presented by dendritic cells (DCs). Each Th cell subset expresses a unique set of transcription factors and produces hallmark cytokines. Both T-cell receptor (TCR)-mediated stimulation and the cytokine environment created by activated CD4$^+$ T cells themselves, by 'partner' DCs, and/or other cell types during the course of differentiation, play an important role in the fate decisions toward distinct Th subsets. Here, we review how TCR-mediated signals in collaboration with the cytokine environment influence the fate decisions of naive CD4$^+$ T cells toward distinct Th subsets at the early stages of activation. We also discuss the roles of TCR-proximal signaling intermediates and of the Notch pathway in regulating the differentiation to distinct Th phenotypes.

Keywords: *T-helper cells, TCR signals, cytokines, transcription factors, Notch pathway*

Introduction

When naive CD4$^+$ T cells recognize a foreign antigen-derived peptide presented in the context of major histocompatibility complex (MHC) class II on dendritic cells (DCs), they undergo massive proliferation and differentiation into distinct T-helper (Th) cell subsets. There is still considerable uncertainty as to the number of these subsets, the precursor-product relationships among them, and their ability to convert from one to another. At least four different Th cell subsets [Th1, Th2, Th17, and induced T-regulatory (iTreg) cells] have been studied in great detail. Each cell subset expresses a unique set of transcription factors as well as hallmark cytokines. Indeed, the cytokine environment created by activated CD4$^+$ T cells themselves, by 'partner' DCs, and/or other cell types during the course of differentiation

is one of the key determinants for the fate decision toward distinct Th subsets (1).

The analysis of how various signaling pathways impinge on Th differentiation is best understood in the context of a two-phase process, consisting of a T-cell receptor (TCR)-driven induction phase, in which key transcription factors are induced or activated, and a cytokine-driven polarization phase, in which the expression of the key factors is amplified and their differentiation completed. Indeed, each subset utilizes a master regulatory transcription factor and a particular signal transducer and activator of transcription (STAT). The relationships are as follows: Th2, GATA-binding protein 3 (GATA-3)/STAT5; Th1, T-box transcription factor expressed in T cells (T-bet)/STAT4; Th17, retinoid orphan receptor γt (RORγt)/STAT3; iTreg, forkhead box protein 3 (Foxp3)/STAT5. Recent studies suggest that Tfh cells may also fit the paradigm with the factors being B-cell lymphoma 6 (Bcl-6)/STAT3. Interestingly, in many instances, the STAT involved also plays a role in the induction of the master transcriptional regulator (*Fig. 1*). In this review, we discuss how the signals mediated by the TCR in collaboration with the cytokine environment influence the fate decisions of naive $CD4^+$ T cells toward distinct Th subsets at the early stages of activation as well as the roles of TCR-proximal signaling intermediates and of the Notch pathway in regulating Th subset differentiation.

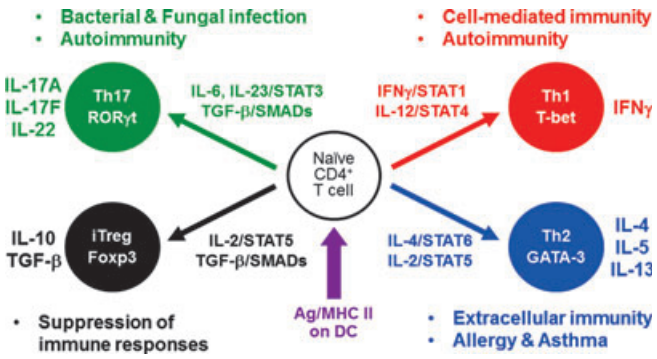

***Fig. 1.* T-helper cell subsets.** Upon T-cell receptor (TCR) recognition of a foreign antigen-derived peptide presented in the context of major histocompatibility complex class II on dendritic cells, naive $CD4^+$ T cells undergo massive proliferation and differentiation into distinct T-helper (Th) cell subsets. There are at least four different Th cell subsets (Th1, Th2, Th17, and iTreg) that have distinct functions to regulate immune responses. Each cell subset expresses a unique set of transcription factors as well as hallmark cytokines. The cytokine environment created by activated $CD4^+$ T cells themselves, by dendritic cells, and/or other cell types during the course of differentiation plays a critical role in the Th fate decisions. It still remains controversial whether Tfh cells (not depicted in the figure) originate directly from naive $CD4^+$ T cells as a distinct subset similar to Th1, Th2, Th17, and iTreg cells or whether Tfh cells emerge from $CD4^+$ T cells that have already determined their fates toward either Th1, Th2, or Th17 cell subset.

TCR signal strength

Early decision between Th1 and Th2 subsets

Strength of TCR signaling during *in vitro* differentiation regulates Th1/Th2 polarization. In general, weak signaling favors Th2 differentiation, and stronger signaling leads to Th1 differentiation (2). Priming AND TCR-transgenic $CD4^+$ T cells, specific for moth cytochrome C (MCC), with the altered peptide ligand K99R, which has a weaker affinity for the AND TCR than does the MCC peptide, preferentially induces Th2 differentiation (3). Jorritsma *et al.* (4) showed that K99R stimulates weak and transient extracellular signal-regulated kinase (ERK) activation compared to the MCC peptide. The reduced activation of ERK by stimulation with K99R is associated with early IL-4 production by naive AND $CD4^+$ T cells and with a distinct pattern of DNA-binding activity of AP-1 to the *Il4* promoter, dominated by a JunB homodimer (4). This is consistent with a previous report showing that JunB directly binds to the *Il4* promoter and synergizes with c-Maf to activate an *Il4* luciferase reporter gene (5).

We observed that when naive $CD4^+$ T cells of the 5C.C7 TCR-transgenic mouse (also specific for MCC) are stimulated with low concentrations of a peptide from the related protein pigeon cytochrome C (PCC), ERK activation is weak and transient. These 'low concentration-stimulated' T cells rapidly induce the expression of the Th2 master regulatory transcription factor GATA-3 and produce IL-2 that in turn activates STAT5. GATA-3 and STAT5 synergize to result in TCR-dependent, IL-4-independent early IL-4 transcription (the induction phase). These low concentration-stimulated T cells undergo the completion of their differentiation into Th2 cells by responding to the endogenously produced IL-4 and continued STAT5 activation (the polarization phase) (6) (*Fig. 2*). By contrast, stimulating naive 5C.C7 $CD4^+$ T cells with high concentrations of cognate peptide results in failure to undergo Th2 differentiation. TCR-dependent IL-4-independent early GATA-3 expression is suppressed and IL-2R-mediated STAT5 activation is transiently blocked, resulting in failure to produce early IL-4. Under these stimulation conditions, strong and prolonged ERK activation is observed. Blockade of the ERK pathway with a MEK inhibitor allows T cells stimulated with high concentrations of PCC peptide to express early GATA-3 and to respond to endogenously produced IL-2, leading to early IL-4 production, completion of the induction phase and subsequent Th2 polarization process. Thus, strong ERK activation prevents early GATA-3 production and 'desensitizes' the IL-2 receptor, thus blocking the Th2 induction phase (*Fig. 3*).

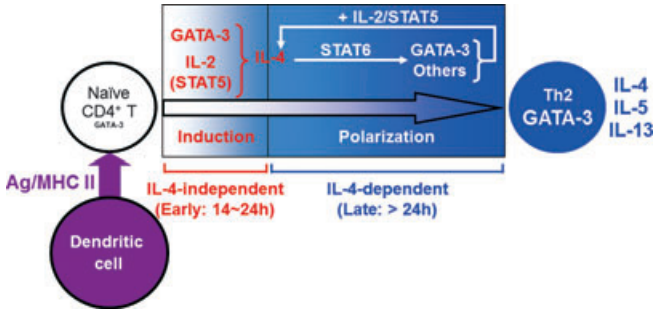

Fig. 2. Two-step regulation of Th2 differentiation initiated by weak T-cell receptor (TCR) signals. When naive $CD4^+$ T cells receive weak TCR signals, these 'weakly stimulated' T cells rapidly induce the expression of the Th2 master regulatory transcription factor GATA-3 in an IL-4/STAT6-independent manner and produce IL-2 that in turn activates STAT5. GATA-3 cooperates with STAT5 to induce TCR-dependent, IL-4/STAT6-independent early IL-4 production that occurs 14–24 h after activation (the induction phase). The endogenously produced IL-4 acts on the weakly stimulated T cells through IL-4R to further upregulate GATA-3 expression in a STAT6-dependent manner. The upregulated GATA-3, in collaboration with continued STAT5 activation, amplifies the production of IL-4. This positive feedback loop leads to the completion of differentiation into Th2 cells (the polarization phase).

Although these 'high concentration-stimulated' T cells express basal levels of GATA-3 and become able to strongly activate STAT5 in response to endogenously produced IL-2 after 40 h of stimulation, they still fail to produce IL-4, implying some mechanism(s), possibly epigenetic gene regulation, through which high concentration stimulation 'permanently' silences the Th2 differentiation program.

IL-2 also upregulates IL-4Rα expression on activated $CD4^+$ T cells in a STAT5-dependent manner, enhancing the capacity of IL-4 to induce signals in developing Th2 cells. Leonard and colleagues (7) showed that IL-2 activation of STAT5 results in the binding of STAT5 to the IFNγ-activated sequence 3 and 5 motifs in the *Il4ra* locus during the early stage of Th2 differentiation. IL-4 itself also upregulates IL-4Rα expression, so that the relative importance of IL-2 and IL-4 in enhancing the developing $CD4^+$ T cell's sensitivity to IL-4 will almost certainly depend upon the timing of the availability of these cytokines during the Th2 differentiation process.

The physiological relevance of TCR signal strength-mediated regulation of *in vivo* Th1/Th2 differentiation has been an open question. Two recent studies revealed that Omega-1, a T2 ribonuclease derived from *Schistosoma mansoni* egg antigen, acts on DCs to suppress IL-12 production and to diminish intensity of TCR-mediated signals that naive $CD4^+$ T cells receive, implying that manipulating the function of DCs may result in weak TCR signals even if antigen amount is not limiting, thus favoring *in vivo* Th2 differentiation (8, 9).

What is the biological significance of strong TCR signal-mediated suppression of Th2 differentiation? We propose that this could be a mechanism by which naive $CD4^+$ T cells are prevented from undergoing excessive Th2 differentiation

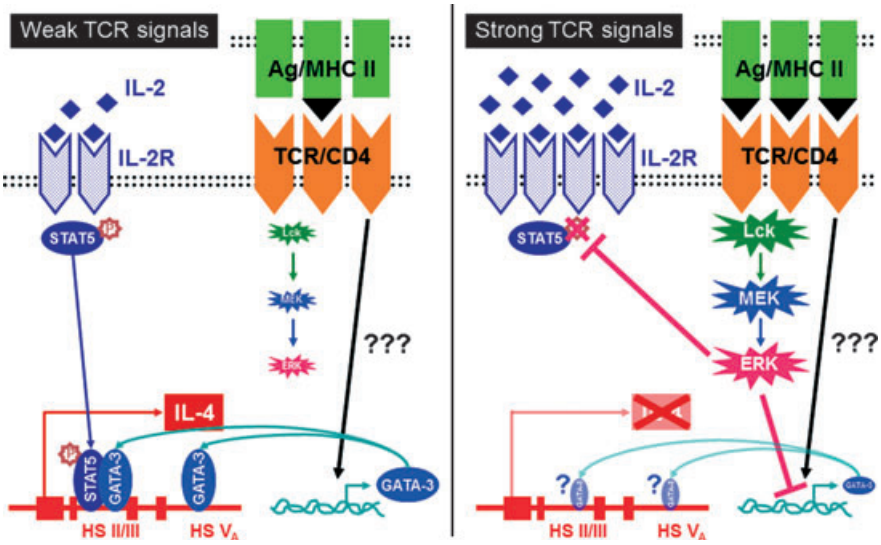

Fig. 3. Early Th2 fate decision regulated by strength of T-cell receptor (TCR) signals. When naive 5C.C7 $CD4^+$ T cells receive strong TCR signals, strong and prolonged ERK activation is induced. This intense ERK activation results in failure to produce early IL-4 by suppressing IL-4-independent TCR-driven early GATA-3 expression and by transiently blocking IL-2R-mediated STAT5 activation (right). By contrast, when naive 5C.C7 $CD4^+$ T cells receive weak TCR signals, the level of ERK activation is not so strong as to suppress early GATA-3 expression or to inhibit the IL-2/STAT5 pathway. GATA-3 and STAT5 subsequently translocate into the nucleus and bind to the DNase hypersensitivity sites II/III and V_A in the *Il4* gene locus, leading to the induction of early IL-4 production, completion of the induction phase and subsequent Th2 polarization process (left).

when they encounter large amounts of foreign antigen that often lead to massive T-cell proliferation and could induce a dangerously high level of allergic sensitization.

Early decision between Th17 and iTreg subset differentiation

Th17 cells play an important role in protection from bacterial and fungal infection and in the development of autoimmunity. It has been proposed that there are three steps that control the differentiation of naive $CD4^+$ T cells into Th17 cells: (i) TGF-β/IL-6-induced differentiation, (ii) IL-21-driven amplification, followed by (iii) IL-23-mediated stabilization (10, 11). We would map the differentiation step as equivalent to the induction phase described above and the amplification and stabilization steps as making up the polarization phase. During the differentiation step, TGF-β and IL-6 in combination with TCR stimulation cause naive $CD4^+$ T cells to express the IL-23R and to induce the major Th17 master regulatory transcription factor RORγt, as well as to produce IL-17A, IL-17F, and IL-21 (12–15). Induction of IL-21 production depends on IL-6-driven STAT3 activation and inducible T-cell costimulator (ICOS) stimulation (16, 17). During the amplification step, IL-21 cooperates with TGF-β to further upregulate IL-17 production and IL-23R expression in a STAT3-dependent manner. Deletion of either IL-21 or the IL-21R results in diminished Th17 differentiation both *in vitro* and *in vivo* (16, 18, 19). If Treg cells are depleted by anti-CD25 treatment prior to immunization, endogenous IL-21 is capable of inducing Th17 responses *in vivo* even in the absence of IL-6, although to a lesser degree than if IL-6 is available (18). Thus, IL-21 appears to play an important role in the positive feedback regulation of Th17 differentiation through its activation of STAT3. However, this conclusion has been challenged by a study reporting that mice deficient in either IL-21 or the IL-21R are still capable of mounting Th17 immune responses *in vivo*, implying a dispensable role for IL-21 in Th17 differentiation when pro-inflammatory cytokines such as IL-6, IL-1, and TNF-α are abundantly available (20).

The IL-2/STAT5 pathway has been demonstrated to block differentiation to the Th17 fate. When IL-2 is exogenously provided to Th17-polarizing cultures that contain IL-6 and TGF-β, the generation of IL-17-producing cells is impaired, while there is an increase in the frequency of cells that express Foxp3, the master regulatory transcription factor for Treg cells (21). Indeed, TGF-β is essential for the conversion of peripheral naive $CD4^+$ T cells into $Foxp3^+$ cells with regulatory capacity, often referred to as induced Treg (iTreg) cells (22); IL-2 is generally required for such TGF-β-mediated iTreg differentiation (23). Naive $CD4^+$ T cells from *Rag1*$^{-/-}$ OT-II TCR-transgenic mice on a *scurfy* background, which lack functional Foxp3, still fail to undergo Th17 differentiation under Th17-polarizing conditions if IL-2 is added to the culture (24). STAT5 has been shown to compete with STAT3 for binding to multiple sites within the *Il17a-Il17f* locus; STAT5 binding to these sites is associated with repressive epigenetic marks across the *Il17a* promoter region and enhancer elements (24), suggesting that STAT5-binding blocks STAT3 function, thus providing a mechanism through which IL-2 directly represses Th17 differentiation.

Similar to the regulation of the Th1/Th2 fate decision, TCR signal strength has been shown to be a key factor in determining the choice between iTreg and Th17 differentiation. Indeed, if naive $CD4^+$ T cells receive weak TCR signals, they fail to differentiate into Th17 cells, but instead express Foxp3, even though they are exposed to Th17-inducing cytokines (25–27). As discussed earlier, strong TCR signals have been demonstrated to transiently inhibit IL-2-driven STAT5 activation despite abundant production of IL-2 by activated $CD4^+$ T cells, thereby blocking Th2 development (6, 28). Thus, it is conceivable that the early fate decision toward the Th17 subset may require strong TCR signals to block the IL-2/STAT5 pathway so that the inhibitory effects of IL-2 can be abrogated. It will be of great importance to clarify the molecular basis underlying TCR signal strength-mediated control of IL-2/STAT5 pathway to better understand the delicate fate balance between Th17 and iTreg subsets that are reciprocally regulated.

TGF-βR-mediated phosphorylation of the receptor-regulated SMAD proteins (R-SMADs) SMAD2 and SMAD3 consists of two aspects: (i) TGF-β-induced phosphorylation of C-terminal serines, leading to association with SMAD4, inducing transcription of TGFβ target genes, and (ii) mitogen-activated protein kinase (MAPK)-mediated phosphorylation of a short region that links N-terminal and C-terminal MAD homology domains of R-SMAD suppressing the TGF-β signaling pathway (29–32). MAPK kinase kinases MEKK2 and MEKK3 are the upstream regulators of ERK, p38, and c-Jun N-terminal protein kinases. Su and colleagues (33) reported that in naive $CD4^+$ T cells from mice in which both MEKK2 and MEKK3 have been conditionally deleted, Foxp3 is induced more efficiently in response to limiting amounts of TGF-β than in such cells from wildtype mice. Loss of MEKK2 and MEKK3 in $CD4^+$ T cells results in the impaired phosphorylation of the R-SMADs at their short

linker regions in response to TCR/CD28 stimulation, resulting in increased serine phosphorylation at their C-terminus domain in response to TGF-β (33). Moreover, Su and colleagues (33) showed that ERK is responsible for the MEKK2/3-mediated phosphorylation of the short linker region of the R-SMADs, suggesting a negative regulation of the TGF-β signaling pathway by strong TCR-mediated stimulation. This implies that during the differentiation into iTreg cells, weak TCR stimulation more efficiently allows naive $CD4^+$ T cells to receive signals through both the IL-2R and TGF-βR for induction of Foxp3.

Early decision toward Tfh cells

T-follicular helper (Tfh) cells 'help' T-cell-dependent humoral immune responses by promoting germinal center formation, immunoglobulin class switching, affinity maturation of antibody-secreting B cells and long-lived antibody responses (34). Tfh cells express the transcriptional repressor Bcl-6 as their master regulator (35–37), the CXC-chemokine receptor 5 (CXCR5) (38, 39), large amounts of programmed cell death 1 (40), B and T-lymphocyte attenuator (41), SLAM-associated protein (SAP) (42), and ICOS (38, 39), but downregulate B lymphocyte-induced maturation protein 1 (Blimp-1) (36). It still remains controversial whether Tfh cells originate directly from naive $CD4^+$ T cells as a distinct subset similar to Th1, Th2, Th17, and iTreg cells or whether Tfh cells emerge from $CD4^+$ T cells that have already determined their fates toward either Th1/Th2, or Th17 cell subset. One key issue regarding the acquisition of Tfh identity is to clarify the timing and the mechanism by which responding $CD4^+$ T cells acquire expression of Bcl-6 and CXCR5. CXCR5 expression is essential for activated $CD4^+$ T cells to migrate to the follicles. In the follicles, the $CD4^+$ T cells interact with B cells that express the same cognate peptide that had been presented by DCs in the priming process that led to their becoming functional Tfh cells.

Mice with a T-cell-specific deletion of *Stat3* ($Stat3^{fl/fl}$ × *CD4Cre*-transgenic) exhibit a substantial diminution in the frequency of $CD4^+CXCR5^+$ cells *in vivo* in response to immunization with KLH in complete Freund's adjuvant, a phenotype resembling that seen in IL-6 or IL-21 deficiency (43). By contrast, mice deficient in either IL-6 or IL-21 have recently been reported to generate Tfh cells normally upon infection with lymphocytic choriomeningitis virus (LCMV), indicating a redundant role of these cytokines in Tfh cell development (44). Although the combined absence of IL-6 and IL-21 results in the failure to secrete antigen-specific IgG upon LCMV infection, such mice display only a modest reduction in Tfh cell generation (45). Since STAT3 dependence of LCMV-generated Tfh cells has not been determined, it is uncertain as to whether IL-6 and IL-21 may be replaced by another STAT3 activator in this response or whether a STAT3-independent pathway for Tfh generation exists.

Two days after LCMV infection of mice that have received $CD4^+$ T cells expressing a SMARTA TCR specific for an LCMV glycoprotein, the donor $CD4^+$ T cells fall into two subpopulations: $CD25^{hi}$ cells, expressing high levels of Blimp1, undergoing a program of T-effector (Teff) cell differentiation, and $CD25^{int}$ cells, expressing Bcl-6 and CXCR5, undergoing differentiation to Tfh cells (46). The failure of $CD25^{hi}$ cells to become Tfh cells suggests an inhibitory role for IL-2-generated signals in the development of Tfh cells *in vivo*. Subsequently, four groups (47–50) independently reported that the IL-2/STAT5 pathway blocks the generation of Tfh cells by inducing Blimp-1, resulting in suppression of Bcl-6 expression. Interestingly, this crucial role for the IL-2/STAT5 pathway in the fate decision toward either Teff or Tfh cells through regulation of the expression of the two mutually exclusive transcriptional regulators, Blimp-1 and Bcl-6, resembles that observed for the differentiation into either iTreg or Th17 subset, in which Foxp3 and RORγt reciprocally regulate one another. Given the evidence shown by McHeyzer-Williams and colleagues (51) that the generation and function of Tfh cells depends on the strength of TCR binding to a foreign peptide/MHC class II complex, TCR signal strength appears to be one of the key determinants for the early fate decision toward Tfh cells rather than Teff cells as well through the transient blockade of IL-2-driven STAT5 activation, thereby abrogating the inhibitory effects of IL-2 on Bcl-6 expression and preventing Blimp-1 from being induced.

TCR-proximal signaling intermediates

Src-family tyrosine kinases Lck and Fyn

Lck and Fyn are Src-family tyrosine kinases involved in optimal T-cell activation. Lck associates with the cytoplasmic tail of CD4 and becomes activated upon TCR ligation, leading to phosphorylation of tyrosine residues in the CD3ζ chain to which ζ-chain-associated protein kinase 70-kDa (ZAP-70) is recruited via its Src homology region 2 (SH2) domain. $Lck^{-/-}$ mice exhibit a prominent but incomplete developmental arrest at the transition from the double negative to the double positive stage of thymocyte development. The few peripheral T cells that do develop have markedly impaired responses to TCR stimulation (52). The impor-

tance of Lck in T-cell development makes difficult the assessment of the role of Lck in peripheral T-cell activation in such mice. To inactivate the function of Lck in peripheral T cells without affecting thymocyte development, a transgenic mouse line was generated that expressed a dominant-negative enzymatically inactive form of Lck under the control of the distal *Lck* promoter (DLGKR); DLGKR is expressed in peripheral T cells, but not in thymocytes. Nakayama and colleagues (53) reported that $CD4^+$ T cells from DLKGR transgenic mice exhibit impaired Th2 differentiation *in vitro*. Moreover, $CD4^+$ T cells from mice expressing a transgene encoding a dominant-negative H-Ras under the control of the proximal *Lck* promoter show a phenotype similar to that of DLGKR-expressing cells, leading the authors to conclude that TCR-mediated Lck/Ras/ERK activation is required for Th2 differentiation through enhancing STAT6 activation in response to IL-4 (54) and/or preventing ubiquitin/proteasome-mediated degradation of GATA-3 by inhibiting the activity of Mdm2, an E3 ubiquitin ligase that acts on GATA-3 (55). These results provide a conflicting view of the role of ERK function in Th2 differentiation to that described in the previous section of this review and need to be considered in terms of our two-phase model of Th2 polarization. Indeed, studied in detail, naive $CD4^+$ T cells from the DLKGR transgenic mice actually produce significantly more IL-4 than do those from the littermate control mice during the Th2 induction phase (53), indicating an inhibitory role of the Lck/Ras/ERK cascade in TCR-driven IL-4 production. However, IL-2 production by naive $CD4^+$ T cells from the DLKGR transgenic mice is significantly diminished (53). Reduced IL-2 production could account for the poorer Th2 differentiation observed in such cells, since STAT5 activation is essential for both the induction and polarization phases of Th2 differentiation; the degree of STAT5 activation may have fallen below the threshold level during the polarization phase in cells in which Lck or Ras is not functional. Indeed, this result is consistent with data we have reported showing that blockade of the ERK pathway leads to a substantial diminution in IL-2 production by 5C.C7 $CD4^+$ T cells stimulated with low concentrations of PCC peptide (6) and that in the presence of a MEK inhibitor, Th2 differentiation initiated by weak TCR signals requires addition of IL-2. Interestingly, the expression of Mdm2 is aberrantly induced by loss of the *Stat5* gene in $CD4^+$ T cells, implying that the IL-2/STAT5 pathway suppresses Mdm2 expression, protecting GATA-3 from degradation during Th2 differentiation (7), and that Mdm2-mediated degradation of GATA-3 could be secondary to the diminished IL-2 production in cells treated with a MEK inhibitor.

Fyn also plays a critical role in signal transduction through the TCR. $Fyn^{-/-}$ mice show virtually no abnormality in thymocyte development and in the size of the compartment of peripheral T cells (56, 57). Nariuchi and colleagues reported that naive $CD4^+$ T cells from $Fyn^{-/-}$ C57BL/6 mice have enhanced Th2 polarization upon TCR/CD28 stimulation under neutral conditions, where no exogenous polarizing cytokines are added, although the molecular basis for Th2 skewing by loss of Fyn was not identified in their study (58). DeFranco and colleagues (59) found that $CD4^+$ T cells from $Fyn^{-/-}$ DO11.10 TCR-transgenic BALB/c mice have greater skewing toward IL-4-producing cells than cells from wildtype DO11.10 mice upon stimulation with cognate peptide *in vitro*. These reports imply that Fyn plays an inhibitory role in Th2 differentiation.

By contrast, Schwartzberg and Veillette and their colleagues (60, 61) reported that $Fyn^{-/-}$ naive $CD4^+$ T cells are poorer at undergoing Th2 differentiation compared to wildtype counterparts. A possible explanation of their results is based on the recruitment of Fyn to the SH2 domain of SAP when it is bound to the SLAM family receptors that are homotypically engaged during a cognate interaction between a $CD4^+$ T cell and a DC. Fyn then phosphorylates the cytoplasmic tail of the SLAM family receptor, recruiting protein kinase Cθ (PKCθ) and leading to the translocation of the NF-κB1 (NF-κB p50) subunit to the nucleus. $CD4^+$ T cells from $Nfkb1^{-/-}$ or $Prkcq^{-/-}$ mice have been reported to be substantially defective in Th2 differentiation *in vitro* and *in vivo* due to impaired GATA-3 expression (62, 63).

Since NF-κB1 has no transactivation domain, it must form a complex with other protein(s) to activate NF-κB1-dependent gene expression. Boothby and colleagues (64) identified Bcl-3, which belongs to the IκB family proteins and possesses a transactivation domain, as the partner of NF-κB1 for binding to the κB-like DNA consensus sequence located at 310–301 bp upstream of the *Gata3* transcription initiation site, indicating that NF-κB1 and Bcl-3 may be important in regulating cell/cell interaction-driven GATA-3 expression (65) and thus indirectly TCR-driven GATA-3 expression. However, given the critical role for the NF-κB pathway in IL-2 production and CD25 expression (66), entities also essential for Th2 differentiation, a careful study is needed to determine the relative importance of NF-κB-mediated GATA-3 upregulation versus enhanced STAT5 phosphorylation during Th2 cell differentiation induced by this pathway. Similarly, the disagreements regarding the effect of

deleting Fyn on Th2 differentiation require resolution; we are actively studying this problem.

Lat

Linker for activation of T cells (LAT) is a transmembrane adapter protein that assembles many substrates of ZAP-70 including SH2 domain-containing leukocyte protein of 76-kDa (SLP-76) and phospholipase Cγ1 (PLC-γ1), leading to T-cell development, activation, survival, and homeostasis. Love's and Malissen's groups (67, 68) independently reported that mice with a point mutation converting tyrosine 136 of LAT to phenylalanine (Lat^{Y136F} mice), which abrogates its interaction with PLC-γ1, exhibit abnormal thymocyte development with impaired positive and negative selection but later develop lymphoproliferative disorders with massive Th2 immune responses in which T cells produce large amounts of Th2 cytokines and B cells secrete high levels of IgG1 and IgE and of autoantibodies. T cells from the Lat^{Y136F} mice have severe defect in TCR-induced activation of PLC-γ1, nuclear factor of activated T cells (NFAT), Ca^{2+} influx, IL-2 production, and cell death, whereas TCR-induced ERK activation is intact. These data have been interpreted to indicate that LAT plays a critical role in many signaling intermediates downstream of TCR and that LAT contributes to the suppression of Th2 differentiation.

Given that the phenotype of the Lat^{Y136F} mice resembles that of *Scurfy* mice, it was possible that the Lat^{Y136F} mice might not have Treg cells. Indeed, Zhang and colleagues (69) reported that the Lat^{Y136F} mice lack $Foxp3^+$ Treg cells in the thymus as well as in the peripheral lymphoid organs. Lat^{Y136F} bone marrow (BM) cells that were co-transferred with wildtype BM cells into $Lat^{-/-}$ mice fail to develop into $Foxp3^+$ Treg cells. Moreover, adoptive transfer of wildtype $CD4^+CD25^+$ T cells into neonatal Lat^{Y136F} mice protects the recipients from lymphoproliferative disorders. These results indicated that the LAT/PLC-γ1 interaction plays a critical role in the development of $Foxp3^+$ Treg cells (69). However, Malissen and colleagues (70) challenged this conclusion by showing that Lat^{Y136F} mice on a Foxp3-eGFP reporter background still have $Foxp3^+$ Treg cells in the thymus although threefold diminished in absolute cell number compared to wildtype Foxp3-eGFP reporter mice. Moreover, they showed that adoptive transfer of wildtype Foxp3-$eGFP^+$ Treg cells into neonatal Lat^{Y136F} mice failed to rescue the recipient mutant mice from lymphoproliferative disorders, implying that Lat^{Y136F} $CD4^+$ T cells are capable of escaping from the suppression by wildtype $Foxp3^+$ Treg cells (70).

Although we cannot provide a reasonable explanation for the discrepancy between these two studies, we would like to propose a different interpretation for the massive Th2 lymphoproliferative responses in Lat^{Y136F} mice. IL-2 production by T cells from Lat^{Y136F} mice is abrogated given the critical role of LAT/PLC-γ1 interaction in the activation of signaling intermediates essential for the induction of IL-2 production (67, 68). Although we discussed in the previous section that the IL-2/STAT5 pathway play a critical role in the differentiation of naïve $CD4^+$ T cells into Th2 subset, this IL-2 function can be substituted by other STAT5-activating cytokines such as IL-7 and thymic stromal lymphopoietin (TSLP). However, IL-2 is indispensable for $Foxp3^+$ Treg cells to exert their suppressor function to responder T cells (71); neither IL-7 nor TSLP can replace IL-2's role in activating the suppressor function of $Foxp3^+$ Treg cells. Indeed, mice lacking either the *Il2* or *Il2ra* gene, in which $Foxp3^+$ Treg cells are present but not functional, develop abnormalities resembling those observed in the Lat^{Y136F} mice (72–74). Thus, the abrogated IL-2 production by responder T cells carrying the LAT^{Y136F} mutation could lead to the failure of adoptively transferred wildtype $Foxp3^+$ Treg cells to control lymphoproliferative disorders of the neonatal Lat^{Y136F} recipient mice.

Itk

T cells express three Tec-family kinases Itk, Rlk, and Tec. The role of Itk (IL-2-inducible T-cell kinase) during T-cell activation and Th1/Th2 differentiation has been extensively studied. Following TCR ligation, Itk is recruited to LAT/SLP-76/PLC-γ1 multi-molecular complex where it activates PLC-γ1, inducing hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate and diacylglycerol leading to Ca^{2+} release from the endoplasmic reticulum and to PKCθ activation, respectively (75). $Itk^{-/-}$ mice have diminished *in vivo* Th2 responses to *Leishmania major*, and $CD4^+$ T cells from these mice show impaired Th2 differentiation *in vitro* due to a deficit in the Ca^{2+}/NFATc1 pathway (76). Indeed, T cells from mice lacking NFATc1 in the lymphoid system generated by blastocyst complementation exhibit impaired Th2 cytokine production. Sera from these mice display reduced IgG1 and IgE levels, indicating a critical requirement of NFATc1 for Th2 immune responses (77, 78).

Berg and colleagues reported that $CD4^+$ T cells from $Itk^{-/-}$ 5C.C7 TCR-transgenic $Rag2^{-/-}$ mice show aberrant expression of T-bet and polarize toward the Th1 fate in response to weak TCR signals that would normally induce a

Th2 response, further arguing for a critical role for Itk in Th2 differentiation (79). Recently, Schwartzberg and colleagues (80) found that when naive *Itk*$^{-/-}$ CD4$^+$ T cells are stimulated under Th17-polarizing conditions, a defect in the Ca^{2+}/NFATc1 pathway leads to substantial diminution in IL-17A production without affecting IL-17F production or RORγt expression.

A caveat to all of these studies is that a null mutation in the *Itk* gene leads to abnormal thymocyte development (81). As a consequence, the number of peripheral CD4$^+$ T cells is very low in *Itk*$^{-/-}$ mice, and the frequency of cells of activated/memory phenotype, as judged by the surface expression of CD44 and CD62L, is greatly increased. Moreover, even if naive CD4$^+$ T cells are carefully prepared by cell sorting, these cells express fewer TCRs than do their wildtype counterparts. The impaired expression of TCR resulting from loss of Itk is corrected by crossing *Itk*$^{-/-}$ mice onto 5C.C7 TCR-transgenic mice on a *Rag2*$^{-/-}$ background. We carefully purified naive CD4$^+$ T cells from *Itk*$^{-/-}$ 5C.C7 transgenic *Rag2*$^{-/-}$ mice and stimulated them with low and high concentrations of PCC peptide presented by CD11c$^+$CD49b$^-$ DCs under neutral conditions, where no exogenous polarizing cytokines are added. *Itk*$^{-/-}$ cells polarized toward the Th2 phenotype comparably to wildtype cells at low peptide concentration, but those cells underwent Th2 differentiation at high peptide concentration that induced robust Th1 differentiation in wildtype cells (manuscript in preparation by Pamela Schwartzberg and colleagues). Our results conflict with those reported by Berg and colleagues, but ours appear to make more sense given the critical role of Itk in activating PLC-γ1, which subsequently activates many signaling intermediates including the ERK pathway that is responsible for strong TCR signal-mediated suppression of Th2 differentiation. Generation of conditional *Itk*-deficient mice will provide an improved picture of the role of Itk in Th subset differentiation.

Notch pathway

The Notch pathway has been demonstrated to play a crucial role in a number of biological events including development of the central nervous system and the vascular system. The mammalian Notch family has four members: Notch1, 2, 3, and 4. They are expressed on the cell surface following various post-translational modifications, including fucosylation by GDP-fucose protein *O*-fucosyltransferase 1, glucosylation by lunatic, manic, and radical Fringes, and S1-cleavage by a Furin-like protease. In mammals, there are five Notch ligands: Jagged (Jag) 1 and 2, and Delta-like (Dll) 1, 3, and 4. When a Notch interacts with a Notch ligand, the γ-secretase complex proteolytically releases the Notch intracellular domain (NICD). NICD translocates into the nucleus, where it displaces a co-repressor complex from CSL (CBF1/RBP-Jκ in vertebrates, Suppressor of Hairless in *Drosophila*, Lag1 in *C. elegans*) and recruits a co-activator complex leading to Notch-dependent gene transcription (82).

In the immune system, Notch has been extensively studied in thymic T-cell differentiation and in the development of marginal zone B cells (83). There is accumulating evidence showing that Notch also plays an important role in regulating differentiation of naive CD4$^+$ T cells into distinct Th subsets (*Fig.* 4). Yasutomo and colleagues (84) first reported that the interaction of Notch3 on CD4$^+$ T cells with the Dll1 ligand plays a critical role in Th1 differentiation. Nussenzweig and colleagues (85) found that lipopolysaccharide (LPS) induces myeloid differentiation factor 88 (MyD88)-dependent Dll4 expression on the CD8α^- DC subset and that these Dll4-expressing DCs direct Th1 differentiation in an IL-12-independent but a Notch-dependent manner. Pearce and colleagues (86) reported that enforced expression of either Dll1 or Dll4 on IL-12 p40$^{-/-}$ BM-derived DCs promotes Th1 differentiation in a T-bet-dependent manner and suppresses Th2 development. Osborne and colleagues (87, 88) found that Notch1 ICD (N1ICD) can form a complex with NF-κB1 and c-Rel, allowing these NF-κB isoforms to be retained in the nucleus and to activate IFNγ expression as a result of the binding of the N1ICD/NF-κB complex to the *Ifng* promoter. They also showed that the N1ICD binds to the *Tbx21* promoter and claimed that T-bet gene expression is directly regulated by the Notch1 pathway (89).

Several reports have indicated that Notch also has a role in directing Th2 lineage commitment. CD4$^+$ T cells from mice with a conditional deletion of *Rbpj*, encoding CSL, by Cre-recombinase driven by the *CD4*-promoter, fail to undergo Th2 differentiation under non-polarizing conditions (90, 91). Flavell and colleagues (91) proposed a model in which Notch-mediated signals instruct naive CD4$^+$ T cells to undergo differentiation to distinct Th fates by recognizing different Notch ligands with the Dll- and Jag-ligands favoring Th1 and Th2 differentiation, respectively. They identified a CSL-binding site in the HS V site of the *Il4* locus and found that the binding of a retrovirally overexpressed N1ICD to this site upregulates *Il4* gene expression (91). Moreover, Flavell's and Pear's groups (92, 93) independently found that the N1ICD binds to an alternative *Gata3* promoter, approximately 10 kb upstream of a translational start site of the *Gata3* gene, and proposed that such

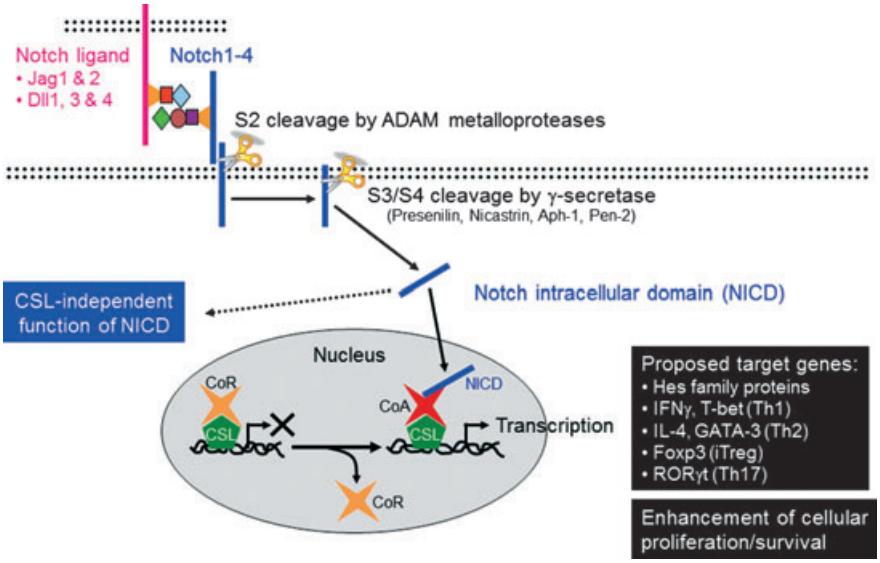

Fig. 4. Canonical and noncanonical Notch pathways. In mammals, there are four Notch receptors, Notch 1-4, and five Notch ligands, Jagged (Jag) 1 and 2, and Delta-like (Dll) 1, 3, and 4. When a Notch interacts with a Notch ligand, the γ-secretase complex proteolytically releases the Notch intracellular domain (NICD). NICD translocates into the nucleus, where it displaces a co-repressor complex from CSL and recruits a co-activator complex leading to Notch-dependent gene transcription. CSL-independent functions of NICD have been also reported. The Notch pathway has been proposed to govern differentiation of naive $CD4^+$ T cells into each of the Th fates by directly controlling the expression of unique master regulatory transcription factors and hallmark cytokines. An alternative view is that the Notch pathway does not directly instruct Th fate decisions but regulates the basal T-cell activation, T-cell expansion and survival, and cytokine production of differentiated Th cells upon secondary stimulation.

binding, in combination with TCR stimulation, can directly induce *Gata3* gene transcription in a STAT6-independent manner.

Notch activation has also been reported to regulate differentiation toward other Th phenotypes. Pretreatment of $CD4^+$ T cells with a γ-secretase inhibitor diminishes the frequency of $Foxp3^+$ cells induced by TGF-β1 due to a reduction in the N1ICD binding to the *Foxp3* promoter (94). γ-secretase inhibitor treatment also blocks the recruitment of SMAD proteins to the *Foxp3* promoter (94), consistent with a previous finding that N1ICD associates with SMAD3 to integrate Notch and TGF-β signals in myogenic cells (95). The interaction of Notch with Dll4 has been reported to cause a substantial increase in the expression of Th17-related genes in $CD4^+$ T cells stimulated under Th17-polarizing conditions (96). Binding of CSL to the *Rorc*(t) and *Il17a* promoter regions is greatly enhanced by Notch/Dll4 interaction, but is abrogated by treatment with γ-secretase inhibitor.

The Notch pathway appears to govern differentiation of naive $CD4^+$ T cells into each of the Th fates by directly controlling the expression of unique master regulatory transcription factors and hallmark cytokines. However, given the common machinery activated by Notch interaction with any Notch ligand, it is hard to provide a reasonable explanation for how different Notch ligands instruct naive $CD4^+$ T cells to undergo such a diverse set of Th differentiation outcomes. An alternative view is that the Notch pathway does not directly instruct Th fate decisions but regulates T-cell expansion during the priming period and cytokine production of differentiated Th cells upon secondary stimulation. Kopan, Murphy, and colleagues (97) reported that neither Dll1- nor Jag1-expressing artificial APCs instruct naive DO11.10 $CD4^+$ T cells to differentiate into Th1 or Th2 cells under non-polarizing conditions. Conditional deletion of CSL or of the Presenilins, components of the γ-secretase complex, in T cells did not affect T-bet or GATA-3 expression at day 6 of priming under Th1- and Th2-polarizing conditions, respectively. However, the loss of CSL or the Presenilins reduces the capacity of differentiated Th cells to secrete effector cytokines upon recall challenge. This reduced cytokine-secretion is associated with and may be due to decreased T-cell proliferation during the priming period (97). These results are consistent with those in earlier reports suggesting that Notch signaling controls global T-cell activation. Osborne and colleagues (88) showed that inhibition of Notch activation dramatically decreases TCR-driven cell division by both $CD4^+$ and $CD8^+$ T cells. Moreover, the inhibition of γ-secretase decreases IL-2 production and CD25 expression, resulting in diminished

proliferation of activated $CD4^+$ T cells (98). These data imply an important costimulatory role for the Notch pathway in controlling optimal T-cell activation, although it remains unclear how this pathway controls the basal activation of T cells.

Our unpublished observations indicate that the Notch pathway is required for the Th2 differentiation induced by weak TCR signal under non-polarizing conditions. However, in contrast to previous reports by Flavell's and Pear's groups (91–93), we found that early induction of both GATA-3 expression and IL-4 production by weak TCR signals during the induction phase was intact in naive $CD4^+$ T cells from the mice deficient in either CSL or the Presenilins, but IL-2 production was greatly impaired. Consistent with our previous reports demonstrating the central role of IL-2 in Th2 differentiation (6, 99), $CD4^+$ T cells lacking CSL or Presenilins failed to complete the polarization phase of Th2 differentiation since IL-4-dependent amplification of IL-4 production and GATA-3 expression during the polarization phase was diminished due to the reduced IL-2 production resulting in limited STAT5 activation. Exogenous IL-2 added to the culture after the induction phase restored IL-4 production and subsequent GATA-3 expression and thus corrected the impaired Th2 differentiation in $CD4^+$ T cells deficient in CSL or Presenilins. These results imply that during Th2 differentiation, TCR-dependent GATA-3 expression and IL-4 production do not require direct binding of the Notch/CSL complex to these gene loci. In the absence of Notch signaling, the failure to produce sufficient amounts of IL-2 to sustain IL-4 production during the polarization phase is responsible for defective Th2 differentiation. We also found that while Dll4 and Jag1 are expressed on splenic myeloid DCs ($CD11c^+CD49b^-DCIR2^+CD11b^+CD8\alpha^-$) used to stimulate naive $CD4^+$ T cells with a cognate peptide, only Dll4 was responsible for inducing Th2 differentiation initiated by weak TCR signals, arguing against the proposed model where Dll and Jag ligands play distinct roles in instructing naive $CD4^+$ T cells toward Th1 and Th2 phenotypes, respectively (91).

Concluding remarks

For the last decade or two, the advance of technology has allowed us to address the questions of how the differentiation of naive $CD4^+$ T cells toward distinct Th subsets is regulated. It has become clear that the interplay of the critical master regulators and of the stimuli that control their activation is at the center of these differentiation decisions. Here, we have emphasized the role of TCR signal strength in controlling these processes and in helping to shape the cytokine environment that is often deterministic in the polarization process. Such information will be of great value both in efforts to understand the basis of polarization in response to pathogen infection and to develop interventions that can control the 'direction' of polarization or alter the polarized state after it has been established.

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