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REVIEW

Heterogeneity and plasticity of T helper cells

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CD4 T helper (Th) cells play critical roles in adaptive immune responses. They recruit and activate other immune cells including B cells, CD8 T cells, macrophages, mast cells, neutrophils, eosinophils and basophils. Based on their functions, their pattern of cytokine secretion and their expression of specific transcription factors, Th cells, differentiated from naïve CD4 T cells, are classified into four major lineages, Th1, Th2, Th17 and T regulatory (Treg) cells, although other Th lineages may exist. Subsets of the same lineage may express different effector cytokines, reside at different locations or give rise to cells with different fates, whereas cells from different lineages may secrete common cytokines, such as IL-2, IL-9 and IL-10, resulting in massive heterogeneity of the Th cell population. In addition, the pattern of cytokine secretion may switch from that of one lineage toward another under certain circumstances, suggesting that Th cells are plastic. Tregs are also more heterogeneous and plastic than were originally thought. In this review, we summarize recent reports on heterogeneity and plasticity of Th cells, and discuss potential mechanisms and implications of such features that Th cells display.

Keywords**:** CD4, Tregs, T cell differentiation, transcription factors, cytokines

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Introduction of T helper (Th) cell types and their functions

In response to an infection, a variety of cells of the innate and adaptive immune systems become activated and collaborate in the effort to control and eliminate invading pathogens. CD4 T cells (also known as Th cells) play critical roles during adaptive immune responses [1]. They help B cells to produce antibody and to undergo class switching and affinity maturation; they recruit and activate CD8 T cells, macrophages, neutrophils, eosinophils, basophils and other effector cells. They also directly act on many tissue cells, including epithelial cells and mucosal cells, during the process of pathogen clearance. The diverse functions of CD4 T cells are determined by their cytokine secretion patterns and their tissue locations.

Beginning in the 1980s, immunologists came to believe that different types of Th cells were involved in humoral and cell-mediated immune responses. In 1986, Coffman and Mosmann showed the existence of Th1 and Th2 clones differing from each other in the cytokines they produced [2]. Th1 cells mainly produce IFNγ, which is important for macrophage activation and clearance of intracellular pathogens, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13, later shown to be critical for IgE production, eosinophil recruitment and clearance of extracellular parasites [3, 4]. Th1 and Th2 cells are also involved in many diseases. Th1 cells were thought to cause many organ-specific autoimmune diseases, whereas Th2 cells are responsible for asthma and other allergic reactions.

With the discovery of IL-23, which shares the subunit p40 with IL-12, it became clear that many autoimmune diseases, earlier attributed to Th1 cells, are indeed induced by other Th cells that are IL-23 responsive [5]. Soon, this lineage of Th cells was identified as Th17 cells, which produce many cytokines including IL-17a, IL-17f, IL-22 and IL-21 [6, 7]. In addition to their involvement in autoimmune diseases, Th17 cells also play critical roles during immune responses against extracellular bacteria and fungi [8].

Naturally occurring regulatory T cells (nTregs) develop in the thymus [9]. IL-2 and TGFβ signaling, as well as CD28 co-stimulation and self-recognition seem to be important for nTreg generation. At essentially the same

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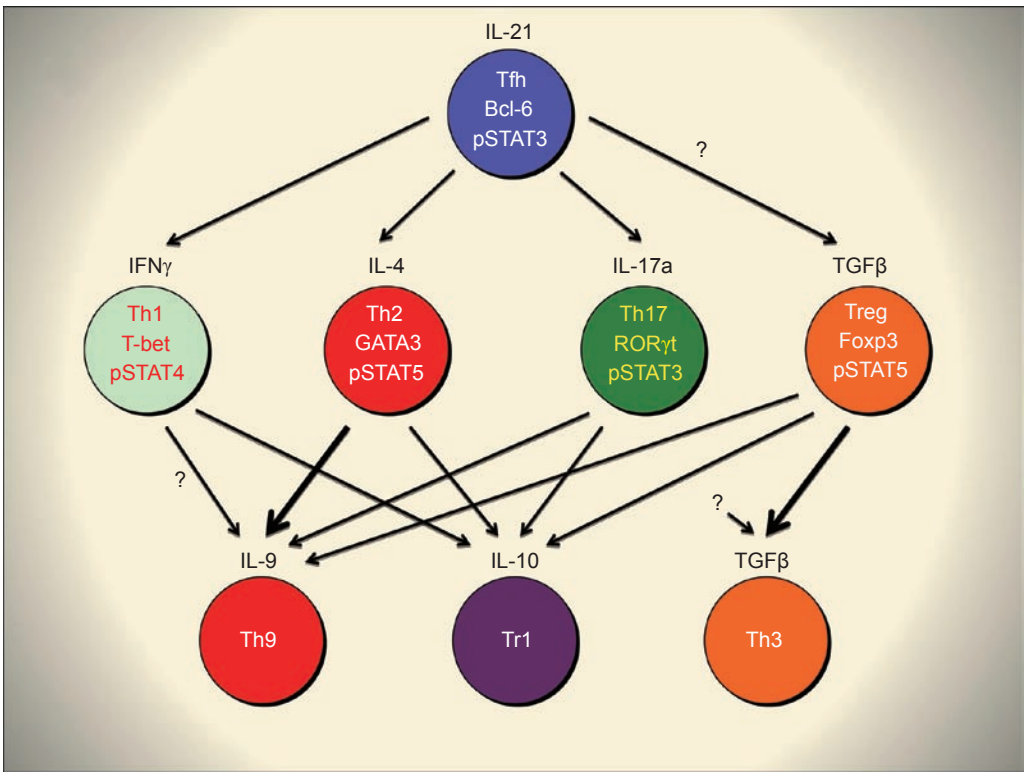

Figure 1 T helper lineages and subsets. CD4 T helper cells can be divided into at least four lineages, namely, Th1, Th2, Th17 and Treg. These cells express master transcription factors (shown inside the cells, pSTATs stands for phosphorylated active STATs) and secrete signature cytokines (shown on the top of the cells). Other 'lineages', including Th3, Tr1, Tfh and Th9 cells, have been proposed. However, different Tfh cells also produce Th1, Th2, Th17 or Treg cytokines, whereas some Th1, Th2, Th17 and Treg subsets secrete Th9, Tr1 or Th3 'signature' cytokines,IL-9, IL-10 or TGFβ, respectively, as shown by arrows. Therefore, Tfh, Th9, Tr1 and Th3 cells may represent subsets of Th1, Th2, Th17 and Treg lineages.

time that Th17 cells were discovered, it was shown that treatment of naïve peripheral CD4 T cells with a TCR stimulant and with TGFβ plus IL-2 caused the appearance of inducible regulatory T cells (iTregs) [10]. The Treg population found *in vivo* consists of both nTregs and iTregs, both of which express transcription factor Foxp3. Like nTregs, iTregs may also be involved in self-tolerance, immune modulation and promoting immune responses under certain circumstances [11].

Thus far, four CD4 Th cell lineages are generally recognized, namely, Th1, Th2, Th17 and Treg cells [1]. The cytokine environment during priming and the consequent activation of specific transcription factors are two key elements controlling Th cell differentiation from naïve CD4 T cells. A distinct set of cytokines promotes the differentiation processes for each lineage: IL-12/IFNγ for Th1; IL-4/(IL-2, IL-7, TSLP) for Th2; TGFβ/(IL-6, IL-21, IL-23) for Th17 and TGFβ/IL-2 for Tregs. The transcription factors that govern the differentiation of these cells are also well defined: T-bet/Stat4 for Th1, GATA3/Stat5 for Th2, RORγt/Stat3 for Th17 and Foxp3/Stat5 for Tregs.

Other potential Th cell 'lineages' have been proposed including Th3 cells [12, 13] (TGFβ-producing CD4 T cells), Tr1 cells [14] (IL-10-producing CD4 T cells), Th9 cells [15, 16] (IL-9-producing CD4 T cells) and Tfh cells [17-19] (T follicular helper (Tfh) cells located in the follicular regions of lymph nodes and spleen). Since the 'signature' cytokines produced by these Th cells are also the products of Th1/Th2/Th17/Treg cells and the transcription factors they express are not unique, whether these cells represent lineages separate from the known four lineages is uncertain and needs further investigation. The relationships between Th1/Th2/Th17/Treg and Th3, Tr1, Th9, Tfh cells are shown in Figure 1.

Th 'lineages' other than Th1/Th2/Th17/Treg?

Th3 cells or TGFβ-producing cells

Oral tolerance induces TGFβ-producing cells, which have been designated as Th3 cells [13]. However, TGFβ can be produced by most activated Tregs. The membrane

bound form of TGFβ, associated with latency-associated peptide (LAP), can be detected on the surface of many Tregs [20, 21]. LAP has been found on the surface of some $CD4^+CD25^-$ cells, which are also capable of suppressing colitis [22]; ~40% of such cells express Foxp3 [21], implying that they are also Tregs. It now has been proven that oral tolerance induces $CD4^+CD25^+$ cells that express CTLA-4, TGFβ and IL-10 [23], as well as Foxp3 [24]. Therefore, the population of cells designated as Th3 contains a large proportion of iTregs.

Some Th3 clones also produce IL-4 and IL-10; however, those clones that produce the highest levels of TGFβ fail to produce IL-4 or IL-10 [12]. Since ~60% of $CD4^+CD25^-LAP^+$ cells do not express Foxp3 [21], this suggests that Th cells, other than Tregs, can produce TGFβ. However, the function of TGFβ-producing Foxp3-negative cells has not been studied. TGFβ, in combination with IL-2, has been shown to induce B cells to switch to expression and production of IgA [25]. Whether the TGFβ that is produced by CD4 T cells is important for IgA switching is unknown. If that were to be the case, then the TGFβ-expressing CD4 T cells that are responsible for IgA switching could be designated as 'real' Th3 cells.

Tr1 cells or IL-10-producing cells

IL-10 was initially recognized as a Th2 cytokine [2]. It was then reported that some cells with regulatory functions express IL-10 and such cells were designated Tr1 cells [14]. However, some 'conventional' Tregs also produce IL-10, and IL-10 production by these cells is important for controlling inflammation in the gut [26]. Nonetheless, it appears that Tr1 cells are different from $Foxp3^+$ IL-10-producing cells [27]. However, the situation is complicated by the finding that IL-10 production is also found in subsets of Th17 cells and Th1 cells [28-30], and that IL-10 production by Th17 and Th1 cells negatively regulates their function. Indeed, in such cells, strong TCR stimulation, which leads to sustained Erk activation, results in IL-10 production [31], suggesting that IL-10 serves as a critical negative feedback regulator to control the magnitude of many types of immune responses and to prevent tissue damage. Therefore, Tr1 cells may very well not be a separate lineage, but include both IL-10-producing Trges, as well as those subsets of Th1, Th2 and Th17 cells that are capable of expressing IL-10.

Th9 cells or IL-9-producing cells

IL-9 was first recognized as a Th2 cytokine [32]; introduction of a GATA3-encoding retrovirus or a retrovirus encoding a constitutively active form of STAT5 can induce cells to be competent to produce IL-9 [33]. However, IL-9 expression is also inducible by TGFβ stimulation [34]. Indeed, TGFβ can induce competence to produce IL-9 by differentiated Th2 cells, while the expression of IL-4 is suppressed in such cells [15, 16]. TCR stimulation of naïve CD4 T cells in the presence of TGFβ plus IL-4 can also induce cells competent to produce IL-9. It has been proposed that such IL-9-producing cells be designated as Th9 cells. However, IL-9 production is not unique to such cells. Tregs and Th17 cells also express IL-9 [35-37]. In addition, just as IL-10 production in Th2 cells depends on GATA3, our unpublished data suggest that induction of IL-9 competence by TGFβ and IL-4 requires GATA3 expression. Therefore, IL-9-producing cells may represent subsets of known Th lineages including Th2, iTreg and Th17 cells. It is unknown whether Th1 cells can express IL-9 under certain circumstances.

Tfh cells or follicular Th1/Th2/Th17 cells

Tfh cells were initially proposed as a separate lineage based on their failure to express Th1/Th2/Th17 cytokines and lineage-specific transcription factors [17-19]. However, recent reports showed that Tfh cells developed *in vivo* during Th1, Th2 or Th17 immune responses may express IFNγ, IL-4 or IL-17a [38-41]. Therefore, Tfh cells seem to be heterogeneous and to have a close relationship to Th1, Th2 or Th17 cells. Although Tfh cells express high levels of Bcl-6 and Bcl-6 is critical for Tfh cell development [42-44], the involvement of other lineage-specific transcription factors, including T-bet and GATA-3, in such cytokine-secreting Tfh cells has not been determined. However, based on the data from GATA3 conditional knockout mice, GATA3 is absolutely required for IL-4 production and IgE synthesis *in vivo* [45], suggesting that the IL-4-secreting Tfh cells also depend on GATA3 and that GATA3 is expressed in such cells at least at basal levels, which may be sufficient to induce IL-4 production. Therefore, Tfh cells may represent a particular state of Th1, Th2 or Th17 cells, particularly those Th1/Th2/Th17 cells that migrate to the B-cell follicle. However, the alternative possibility is that naïve CD4 T cells differentiate into Tfh cells on certain forms of stimulation and then they acquire IL-4-, IFNγ- or IL-17-producing capacity. Interestingly, in the gut, Tfh cells mainly originate from Tregs [46]. Further careful analyses on the subsets of Tfh cells and their patterns of gene expression and epigenetic modification may allow deeper insights into understanding the relationship between Tfh and Th1/Th2/Th17/Treg cells.

Memory-like CD4 cells

After the peak of an immune response, memory Th

cells start to be generated following massive death of effector cells. In C57BL/6 mice, CD44 is a marker for memory T cells. Most CD8 memory T cells express high levels of IL-2Rβ and rely on IL-15 for their survival and expansion [47]. In the lymph nodes of naïve mice, the CD8 memory phenotype cells are largely CD62L-expressing cells, which is similar to that of authentic antigen-specific central CD8 memory cells. By contrast, only a minority of lymph node CD4 memory ($CD44^{bright}$) cells expresses CD62L and IL-2Rβ. It has been reported that those antigen-specific antiviral CD4 memory cells that have a slow turnover rate require IL-15 for their survival and proliferation [48], suggesting that the more rapidly turning over $CD4^{+}CD44^{hi}IL\text{-}2R\beta^{low}$ cells represent a special population different from the *bona fide* memory cells. The functions of these memory-like CD4 T cells have not been determined.

Heterogeneity among Th1/Th2/Th17 cells

Each Th lineage is able to produce more than one cytokine. However, individual cells within same lineage may display different patterns of cytokine production. Indeed, most of the stimulated cells express one, two or three but rarely all of the cytokines that the lineage can produce. For example, Th1 cells are able to express IFNγ, LTα, IL-2 and TNFα, but only a limited proportion of Th1 cells are able to express all these cytokines at the same time [49]. As mentioned above, some Th1 cells can acquire IL-10-producing capability, while IFNγ production is maintained. Similarly, Th2 cytokines, IL-4, IL-5, IL-13 and possibly IL-25 are expressed differently at single-cell level and an individual Th17 cell may express any combination of Th17 cytokines, including IL-17a, IL-17f, IL-21 and IL-22. In some cases, the differential pattern of cytokine production by individual cells may be determined by stochastic formation of a transcriptional complex of NFAT with other factors at a particular cytokine locus, as well as by the differential states of epigenetic modification of the cytokine loci [50].

Differential expression of some transcription factors may also be responsible for the heterogeneity among a certain lineage. For example, PU.1 expression is enriched in those Th2 cells that fail to produce IL-4 [51]. It is also possible that the amounts of GATA3 expression in individual cells influence their ability to preferentially express either IL-4 or IL-13. Th17 cells express high levels of both RORγt and the aryl hydrocarbon receptor AhR [52]; however, whether these two molecules are co-expressed in every Th17 cell or AhR expression is limited to Th17 cells capable of producing IL-22 is not known. As discussed above, the combined expression of Bcl-6 with the 'master' Th1/Th2/Th17 transcription factors, T-bet, GATA3 and RORγt, may determine the ability of Tfh cell to produce IFNγ, IL-4 or IL-17.

Heterogeneity of regulatory T cells

Tregs consist of nTregs (developed in the thymus) and iTregs (induced from naïve CD4 T cells in the periphery). So far, no reliable marker has been shown to distinguish iTregs from nTregs. A total of 20-30% of the Tregs in a normal animal express CD103 [53, 54]. These $CD103^{+}$ Tregs display an activated phenotype and have higher suppressive activity. Most iTregs induced *in vitro* and those Tregs found in inflamed brain express CD103 [55]. Whether CD103 is a marker for iTregs *in vivo* is not clear.

The master transcription factors T-bet, GATA3 and RORγt, are also expressed by subsets of regulatory T cells, suggesting that Tregs are more complex than were originally thought. In studying the relationship between Th17 cells and iTregs, it was initially found that many differentiating cells co-express RORγt and Foxp3, and such cells were considered to be the progenitors of either Th17 or iTregs [56]. However, IL-6-stimulated nTregs express RORγt and IL-17 [57, 58], suggesting that some RORγt-expressing Tregs found *in vivo* may have originated from nTregs. T-bet-expressing Tregs, which express CXCR3, exist in normal mice. T-bet-deficient Tregs proliferate less well in a Th1 inflammatory environment and fail to suppress Th1-related autoimmune diseases [59]. Our unpublished data indicate that GATA3 is expressed in both nTregs and iTregs, although its function in Tregs needs to be further characterized.

Plasticity of Th1/Th2 cells

Th2 cells can be induced by IL-12 to produce IFNγ. On the other hand, differentiated Th1 cells fail to express IL-4 even when they are re-stimulated under Th2 culture conditions. The plasticity of Th1/Th2 cells seems to depend on their differentiation state [60]. The further the cells differentiate, the harder it is to alter their cytokine production profile. Therefore, although fully differentiated Th1 cells cannot turn on IL-4 expression, Th1 cells primed *in vitro* for one round (that is, ~1 week) were able to produce IL-4 when they were switched to Th2 culture conditions [45]. The existence of $IFN\gamma^{+}IL\text{-}4^{+}$ cells in such cultures implies that partially differentiated Th1 cells retain their capability to become IL-4-producing cells. Similarly, despite the report that Th2 cells could gain the capability to produce IFNγ when they are stimulated through IL-12, our unpublished data suggest that

most fully differentiated Th2 cells, that is, those obtained after 3-4 rounds of *in vitro* Th2 priming, fail to produce IFNγ, which correlated with the failure of T-bet induction in these cells. A small proportion of these Th2 cells that have been primed for 3-4 rounds do upregulate T-bet and IFNγ; however, such cells may represent partially differentiated Th2 cells.

The expression of two Th2 transcription factors, GATA3 and Gfi-1, is required to maintain Th2 phenotype. *Gata3* deletion in Th2 cells results in IFNγ production [45] and the induction of IFNγ in these cells is due to a T-bet-independent activation of the Runx3-Eomes pathway (our unpublished data), which is predominantly used by CD8 T cells for IFNγ production. Active epigenetic modifications at *Rorc*, *Il23r* and *Cd103* loci are detected in Gfi-1-deficient Th2 cells, suggesting that Gfi-1 is not only important for Th2 cell expansion but also for suppressing Th17 and iTreg differentiation [55].

It is very difficult to redirect Th1 and Th2 cells to become either Th17 or Treg cells, consistent with suppressive genomic modifications at *Rorc* and *Foxp3* loci in Th1 and Th2 cells [61]. However, when TGFβ is given to Th2 cells, IL-4 production is suppressed, while IL-9 is induced [15, 16]. Our preliminary data suggest that GATA3 and Gfi-1 may play important roles in such transition of cytokine production.

Plasticity of Th17/Treg cells

IL-17-producing cells are relatively unstable with regard to their cytokine-producing phenotype compared to Th1 and Th2 cells. On transfer, purified IL-17-producing cells acquire the capacity to make IFNγ in a STAT4- and T-bet-dependent manner [62]. Our unpublished data showed that even after 5-6 rounds of Th17 priming, when more than 90% of such cells could produce IL-17, IL-12 induces these cells to produce IFNγ and IL-4 induces them to be competent to produce IL-4, while they maintain IL-17 expression. Thus, Th17 cells are plastic throughout their entire differentiation stage. It has been well documented that IFNγ$^+$IL-17$^+$ cells exist *in vivo*. The existence of IL-4$^+$IL-17$^+$ cells has not been reported; however, it is likely that such cells exist. The importance of the Th17 cells switching to Th1 or Th2 phenotype has not been determined. It is interesting to note that many Th1 and Th2 responses, including those mediating autoimmune diseases and allergic reactions, also require Th17 cells, partly due to their role in neutrophil recruitment as well as tissue inflammation.

Some Th17 memory cells have been shown to be stable *in vivo* [63]. Therefore, it remains possible that some subsets of Th17 cells may become terminally differentiated.

When cultured under Th1 conditions [61] or in a Th1-biased inflammatory environment such as that induced by *T. gondii* infection, Foxp3-expressing Tregs can also produce IFNγ [61, 64]. The expression of IFNγ correlated with the upregulation of T-bet in these cells. Therefore, although IFNγ is the Th1 signature cytokine, each of the other lineages retains the capacity to produce it. The plasticity of non-Th1 cells in producing IFNγ may be explained by the bivalent H3K4 and H3K27 modification of the *Tbx21* gene in Th cells other than Th1, including Th17 and Tregs [61]. In addition, the chromatin accessibility of the *Ifng* locus at CNS-22 in non-Th1 cells may also contribute to such flexibility [65, 66].

Dong and his colleagues [57] have reported that Tregs can become IL-17-producing cells when they are cultured with IL-6 and this correlates with their upregulation of RORγt. Strober's group [58] also reported that Tregs can be self-induced to become IL-17-producing cells in the absence of TGFβ when IL-6 is present. Similarly, other studies showed the existence of IL-17-producing Foxp3$^+$ cells, both in mice and humans [67, 68]. Transferring Tregs into a lymphopenic host also results in downregulation of Foxp3 accompanied by the production of IL-17 and IFNγ [69].

Reduced expression of Foxp3 in Tregs by genetic means results in the acquisition of Th2 phenotype [70]. In addition, Tregs may become Tfh cells in the gut, as discussed earlier [46]. By using Foxp3-Cre to mark cells expressing or having expressed Foxp3, Bluestone and his colleagues [71] reported that many effector cells have previously expressed Foxp3, and thus are derived from Tregs, although a transient expression of Foxp3 during effector cell differentiation may also contribute to this finding.

Relationship between heterogeneity and plasticity

Th cells are heterogeneous and plastic. Heterogeneity comes not only from distinct Th lineages but also from many subsets within a same lineage and cells at different development stages. Plasticity reflects the cells' capability to switch from one lineage to another or to a mixed phenotype.

Cell plasticity may be relevant to cell heterogeneity. Even within a 'pure' differentiating population, individual cells respond differently. Therefore, some cells may represent fully differentiated cells that have lost plasticity, whereas others may retain the flexibility to switch due to their partial differentiation state. This is especially applicable to Th1 and Th2 cells (Figure 2).

It seems that most of the Th17 cells remain plastic,

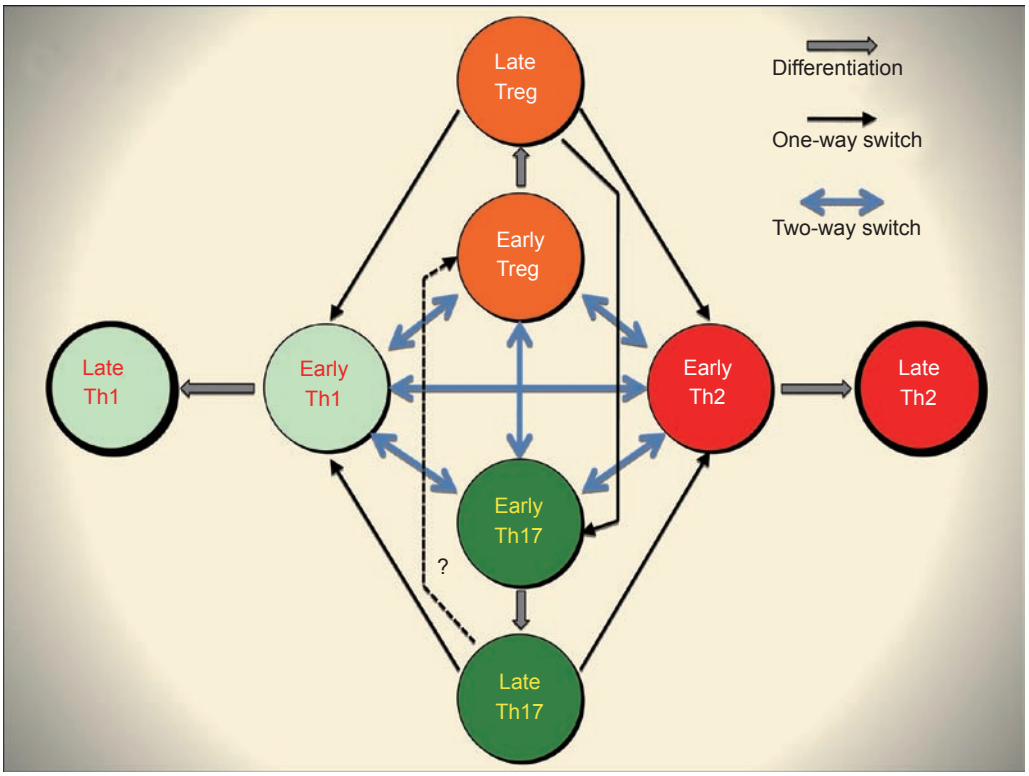

Figure 2 Plasticity of T helper cells. CD4 T helper cell plasticity depends on the differentiation state and cell types. At early stages of Th differentiation, each lineage can be easily redirected to other directions. However, at later stages, most Th1 and Th2 cells are terminally differentiated and cannot be switched, whereas the majority of Th17 and Treg cells remain plastic throughout their differentiation process.

since majority of the 'fully differentiated' Th17 cells are able to upregulate the master regulators of other lineages, including T-bet and GATA3, and to co-express IL-17/IFNγ or IL-17/IL-4.

IFNγ and IL-17 can also be induced in Tregs correlated with upregualtion of T-bet and RORγt, suggesting that a large proportion of Tregs are plastic. However, since there is no marker to distinguish nTregs from iTregs, it is difficult to determine whether both are plastic. It has been shown that iTregs, but not nTregs, lose Foxp3 when TGFβ is removed during restimulation [72] and human $CD45RA^{+}Foxp3^{+}$ cells are more stable than $CD45RA^{-}Foxp3^{+}$ cells in maintaining Foxp3 expression [73]. On the other hand, another report showed that iTregs generated by TGFβ plus IL-2 stimulation are resistant to IL-6-mediated Th17 conversion [74].

What heterogeneity and plasticity mean to immune modulation

In conclusion, Th cells are heterogeneous and somewhat plastic. Many Tregs, just like naïve CD4 T cells, can be differentiated into various types of Ths. Th17 cells are more plastic than Th1/Th2 cells, suggesting that Th17 cells may represent cells that are not terminally differentiated. Alternatively, Th17 cells may continue to express important molecule(s) in determining cell plasticity, which presumably have been repressed in fully differentiated Th1/Th2 cells. At early stages of Th cell differentiation or within certain subsets that are partially differentiated, CD4 T cells can be reprogramed into different lineages on receiving appropriate stimulus. Understanding the mechanisms, through which reprograming one Th cell type to another and then stabilizing the resulting phenotype can be achieved, has enormous implications for immune intervention.

An alternative strategy may be reprograming terminally differentiated antigen-specific CD4 T cells with certain phenotype into earlier progenitors, such as hematopoietic stem cells, naïve CD4 T cells or partially differentiated cells with plasticity, and re-differentiating such cells to a desirable Th effector population. As little as four transcription factors, namely, Oct4/Klf4/Sox2/Myc, are able to reprogram terminally differentiated fibroblasts into

inducible pluripotent stem cells [75, 76]. Klf4 is highly expressed in naïve T cells, but is downregulated during T-cell activation [77]. Finding other critical transcription factors that are highly expressed in naïve cells but are downregulated during T-cell differentiation, may allow the construction of combinations of transcription factors that could reprogram the differentiated Th cells back to naïve stage. Research in such dedifferentiation and redifferentiation holds great promise for future treatment of multiple immune-related human diseases, including autoimmunity, asthma and other allergic diseases, chronic infections and cancer.

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