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MULTI-AUTHOR REVIEW

Memory CD4$^+$ T Cells: fate determination, positive feedback and plasticity

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Abstract Naïve CD4$^+$ T cells undergo massive cell proliferation upon encountering their cognate ligand. This proliferation depends upon appropriate cues from the antigen-presenting cells that have processed the antigen and present the peptide to the T cells, and requires the establishment of a cytokine environment that can support such proliferation. Expansion of antigen-specific CD4$^+$ T cells needs to be coupled with differentiation into one of several effector/regulatory phenotypes if the priming event is to result in cells that can initially act to control the particular pathogen that elicited the response, and later to serve as memory cells to insure an appropriate response upon reintroduction of the pathogen. Here, we discuss the initiation of T helper lineage commitment, the positive feedback regulation by the cytokine environment to enhance and stabilize the differentiation into distinct T helper subsets, and the biological significance of CD4$^+$ T cell plasticity and long-term CD4$^+$ T cell memory.

Keywords T helper cell differentiation · Effector CD4$^+$ T cells · Memory CD4$^+$ T cells · T cell plasticity · T helper lineage-specific transcription factors · Positive feedback regulation by cytokines

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Multiple CD4$^+$ T cell fates

While it has been known since the early 1980s, and even before, that CD4$^+$ T cells can produce extremely potent factors that would regulate the immune/inflammatory response, it was only with the pathbreaking work of Mosmann and Coffman [1] and slightly later Bottomly and her colleagues [2] that it was recognized that production of distinct sets of effector and regulatory molecules is a property of distinct types of memory phenotype CD4$^+$ T cells. This initial work established a paradigm that dominated the field for more a decade and a half, the Th1/Th2 concept. What was demonstrated was that long-term CD4$^+$ T cell clones can be separated into two groups that produce IFNγ and IL-2 as their dominant cytokines or alternatively IL-4. The former cells are designated Th1 and the latter Th2 cells [1].

The cytokine-producing properties of these cell types are very stable in tissue culture. Long-term T cell lines retain their pattern of cytokine secretion apparently indefinitely. Indeed, later, when it was learned how to differentiate naïve CD4$^+$ T cells into Th1 or Th2 cells in vitro, these differentiated cells could be transferred into recipient mice where they would retain their properties for the life of the recipient ([3]; Jane Hu-Li and William E. Paul, unpublished observations), implying that a key element of the CD4 differentiated state is stability.

Quite soon after the demonstration that long-term T cell clones largely fall into the Th1 or Th2 differentiated states, several groups came to the conclusion that in vivo CD4$^+$ T cells can be polarized into one of these two alternative fates during particular types of infections, and that choosing the "wrong" fate could have disastrous consequences in animals infected with particular pathogens. Perhaps the most striking results came from the analysis of immune

responses to infection with parasites. It was shown by Locksley and his colleagues that most inbred mouse strains mount protective immune responses to *Leishmania major*. In these animals, the antigen-specific $CD4^+$ T cells are dominated by those that produce IFNγ [4]. However, some mouse strains, most notably BALB/c, succumb to *L. major* infection. These animals have made a vigorous immune response, but the responding $CD4^+$ T cells were largely IL-4-producers [5]. Indeed, IL-4 or IL-4 receptor knockout BALB/c mice control *L. major* infection substantially better than do wild-type BALB/c mice, establishing that an IL-4-dominated $CD4^+$ T cell response is "inappropriate" for *L. major* infection [6]. Further, it showed that naive $CD4^+$ T cells can differentiate in vivo into cells that dominantly produce IFNγ (Th1 cells) or cells that dominantly produce IL-4 (Th2 cells), that the host generally chooses a response that is most appropriate to the threat, and that failing to choose the most appropriate fate could have disastrous consequences [6].

Molecular basis of $CD4^+$ T cell fate determination

These results implied that the conditions established at the time of antigen encounter would have a major impact on the differentiated state that the expanding naïve $CD4^+$ T cells would choose and on the properties they would later display as effector and subsequently as memory cells. Accordingly, to understand the molecular basis of the differentiation process became an important goal. The first successes in determining the conditions important to the adoption of one of the alternative differentiated states was for in vitro polarization of naïve $CD4^+$ T cells into Th2 cells [7, 8]. This work showed that naïve $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28, and somewhat later with their cognate antigen [9, 10], would acquire the capacity to produce IL-4 and other Th2 cytokines if their initial T cell receptor (TCR) stimulation occurs in the presence of IL-4 and IL-2. The initial striking finding that the principal product of the differentiated cell is also a key factor in its differentiation is very provocative and raises the possibility that $CD4^+$ T cell differentiation may have an important positive feedback component.

When the analysis of Th1 differentiation in vitro was carried out sometime later [10], it was found that the key factor that needed to be added to the culture to foster such differentiation was IL-12, a product of dendritic cells (DC) and macrophages but not of $CD4^+$ T cells. Indeed, it was first shown that the addition of heat-killed *Listeria monocytogenes* (HKLM) to the differentiation culture led to Th1 differentiation. This was then shown to be due to the capacity of HKLM to cause macrophages in the culture to produce IL-12 [11].

The dominant role played by IL-12 appeared at odds with the positive feedback concept. Rather, it argued that different pathways of differentiation are determined by the particular STAT family member that is activated at the time of priming, with STAT6 activated by IL-4 favoring Th2 differentiation and STAT4 activated by IL-12 favoring Th1 differentiation. Indeed, $CD4^+$ T cells from STAT4 KO mice are deficient in Th1 differentiation in vitro and these mice are susceptible to *L. monocytogenes* [12, 13]. STAT6 KO mice fail to differentiate to Th2 cells in vitro [14–16] although they can, under certain conditions, undergo such differentiation both in vitro and in vivo [17, 18].

It was only somewhat later, with further analysis of the Th1 differentiation process, that such differentiation was recognized to be both more complex and more analogous to the Th2 process. Thus, a careful study revealed that neutralization of IFNγ during IL-12-driven Th1 differentiation partially diminishes polarization to the Th1 phenotype [19], and analysis of Th1 differentiation by T cells from IFNγ receptor 2 KO mice reveals major defects [20]. Further analysis indicated that there is a complex process underlying Th1 differentiation in which both STAT4 and IFNγ, acting through STAT1, promote activation of the "master regulator transcription factor" of Th1 cells, T-bet, whose activity feeds back to cause additional IFNγ production and more T-bet expression, thus promoting Th1 differentiation and the acquisition of the capacity for high-rate IFNγ production [21–23].

In parallel with the recognition of the greater complexity of Th1 differentiation involving a requirement for two cytokines, IL-12 and IFNγ, at the time of antigen-stimulation for optimal differentiation, came a reexamination of the role of IL-2 in Th2 priming. The very first paper describing in vitro Th2 priming had indicated a need for IL-2 [7], but the importance of IL-2 in Th2 differentiation was not fully appreciated because of its central role in T cell survival and proliferation. Careful reexamination revealed that IL-2 acting through STAT5 collaborates with the Th2 master regulatory transcription factor, GATA3, in the activation and transcription of the *Il4* gene [24, 25]. Indeed, mutations in STAT5 result in defective Th2 differentiation both in vitro and in vivo [26, 27].

The lessons learned from in vitro differentiation of Th1 and Th2 cells were relearned when the existence of additional fates was recognized and when in vitro differentiation of the naïve cells to these "new" fates was studied. Thus, Th17 cells can be induced in vitro from naïve cells when stimulated through their TCR in the presence of TGFβ and IL-6, a STAT3 activator [28–30]. The key master regulator in this case is RORγt [31]. Although the differentiating Th17 cells fail to produce IL-6, they produce another potent STAT3 activator, IL-21, which in turn acts on activated $CD4^+$ T cells to further

upregulate STAT3-driven IL-23 receptor expression [32–34]. Indeed, IL-23, also a STAT3 activator, produced by DC plays a crucial role in the maintenance of Th17 phenotype [32–34]. Thus, the possibility of a major feedback loop in Th17 differentiation and maintenance as an indirect process, through the intermediary first of IL-21 and later of IL-23, becomes a real possibility.

Having established that positive feedback is a possibility, in that differentiating cells can make one of the key cytokines that drives their differentiation, the regulation and dynamics of this process needs to be established. In a later section of this review, we consider in detail the molecular basis of endogenous IL-4 production in differentiating Th2 cells and how such "early" T cell-derived IL-4 contributes to Th2 differentiation.

In vivo differentiation

However, it is also important to determine to what degree the proliferating/differentiating T cell itself is the source of the "biasing" cytokine that can begin the fate determination process, and how physiologically important self-feedback is in this process.

There are many potential sources of IFNγ and IL-12 that could be important in Th1 differentiation. Perhaps most notable are DCs, in particular the subset of DCs expressing CD8α, which produce the Th1-polarizing cytokines in response to encounter with and detection of microbial and viral components [35, 36]. NK cells may also contribute to establishing the cytokine environment that influences the differentiation of CD4$^+$ T cells as they respond to their cognate antigen. NK cells are normally excluded from lymph nodes (LN), but are recruited to the draining LN upon immunization with antigen plus adjuvant. NK cells produce large amounts of IFNγ in response to IL-12 that has been produced by DC. Thus, NK cells may play an important role as an exogenous source of IFNγ driving toward Th1 differentiation [37].

The issue of what cell makes the IL-4 that induces Th2 differentiation has been grappled with ever since the importance of IL-4 in in vitro Th2 differentiation was established. In addition to CD4$^+$ T cells (as discussed later), IL-4 can be made in large amounts by NKT cells [38], basophils [39], and some γ/δ T cells [40]. Since these cells are generally not in the immediate priming environment of naïve CD4$^+$ T cells, their role has been a matter of uncertainty. However, this may need to be rethought in view of a report that the effect of IL-4 produced during a priming event may be detected throughout the whole lymph node and is not simply confined to the immediate vicinity of the IL-4-producer [41].

Even more provocative is the question of how important IL-4 is in vivo Th2 differentiation. Indeed, Th2 differentiation in response to infection with some, but not all, helminths can be achieved in the absence of IL-4, IL-4 receptor (IL-4R), or STAT6 [42]. However, GATA3 is still indispensable for Th2 differentiation elicited by helminth infection [43]. This may be either accounted for by an IL-4-independent pathway leading to GATA3 upregulation during in vivo Th2 induction or because GATA3 upregulation above the level found in naïve CD4$^+$ T cells may not be essential to initiate and sustain the Th2 state, although GATA3 is essential for Th2 differentiation. Therefore, understanding the regulation of GATA3 expression and its mechanism of action in in vivo Th2 differentiation is an issue of considerable importance.

Pathways regulating IL-4-independent GATA3 expression

Naïve CD4$^+$ T cells express a measurable amount of GATA3, presumably a relic of the important function GATA3 plays in CD4$^+$ T cell differentiation in the thymus [43, 44]. Its expression is upregulated when stimulated under Th2-polarizing conditions in vitro; fundamentally, such conditions are the provision of exogenous IL-4 and the neutralization of IFNγ and IL-12 [45]. Upregulation of GATA3 in this setting requires both the provision of IL-4 and the engagement of the TCR, implying the involvement of the pathways downstream of TCR in GATA3 upregulation. Indeed, several pathways have been proposed to mediate TCR-induced GATA3 upregulation independent of IL-4/STAT6. These include the NF-κB1/Bcl3 complex [46, 47], the Notch1/CSL pathway [48, 49], and the Wnt/β-catenin/TCF1 pathway [50]. In each of these situations, CD4$^+$ T cells from gene-targeted mice have been reported to be deficient in expressing GATA3 in response to TCR. A potential difficulty with these experiments is that the deleted genes play an important role in thymocyte development as well as in peripheral T cell activation. Therefore, the cells being studied may bear developmental abnormalities and/or activation defects that result in their failure to upregulate GATA3 expression and subsequent IL-4 production. Thus, more detailed study will be needed to determine whether these pathways can directly mediate GATA3 upregulation in naïve peripheral CD4 T cells, whether they do so independently of IL-4/STAT6, and whether they are indispensable for such upregulation.

In order to clarify the role of TCR signaling in IL-4-independent GATA3 upregulation, we made use of a TCR signal strength model in which weak and strong TCR signals have been reported to lead to Th2 and Th1 differentiation, respectively [51]. When naïve CD4$^+$ T cells

from 5C.C7 TCR-transgenic (Tg) mice are stimulated in vitro with low concentrations of their cognate antigen, a pigeon cytochrome c (PCC) peptide, they undergo substantial Th2 differentiation. In these weakly stimulated T cells, TCR-driven activation of extracellular signal-regulated kinases (ERK) is weak and transient, and GATA3 is upregulated beginning at 12 h of activation in an IL-4-independent manner by an as yet unknown mechanism. The upregulated GATA3 collaborates with STAT5 activated by endogenously produced IL-2 to mediate IL-4-independent production of IL-4. We refer to this phase of the Th2 differentiation process as the "induction phase". It is generally completed within the first 24 h of stimulation of naïve $CD4^+$ T cells with low concentrations of PCC peptide. This limited amount of TCR-induced endogenous IL-4 in turn acts through the IL-4 receptor/STAT6 pathway to further enhance GATA3 expression in activated $CD4^+$ T cells. This further enhanced GATA3, and the continued IL-2-driven STAT5 activation, jointly lead to production of a far larger amount of IL-4, providing a positive feedback loop that results in the completion and stabilization of Th2 phenotype. We refer to this IL-4-dependent phase of the Th2 differentiation process as the "polarization phase" [25].

By contrast, 5C.C7 TCR-Tg naïve $CD4^+$ T cells stimulated with high concentrations of PCC peptide fail to differentiate into Th2 cells. In these cells, TCR-dependent, IL-4-independent GATA3 upregulation does not occur, and IL-2-driven STAT5 activation is transiently blocked despite the production of a large amount of IL-2 by the "high dose-stimulated" T cells. It appears that the failure to upregulate GATA3 by cells stimulated with high concentrations of peptide is mediated by ERK. Indeed, stimulation with high concentrations of PCC peptide induces strong and prolonged TCR-driven ERK activation. Blockade of the ERK pathway with a pharmacological inhibitor allows T cells receiving a strong TCR signal to upregulate GATA3 and to activate STAT5 in response to endogenously produced IL-2, leading to the restoration of IL-4 production during the induction phase and the subsequent completion of the Th2 polarization process [25].

Controversy on the role of ERK pathway in regulating GATA3 expression

The role of ERK as the major negative regulator of GATA3 upregulation and IL-4 production has been called into question by studies using mice in which dominant-negative (dn) Lck or dn H-Ras is selectively overexpressed in T cells. Naïve $CD4^+$ T cells from these mice show diminished Th2 differentiation in vitro [52]. This result has been interpreted to indicate that TCR-driven Ras/ERK activation is required for Th2 differentiation, possibly through enhancement of IL-4-stimulated STAT6 activation [53] and/or through prevention of ubiquitin/proteasome-mediated degradation of GATA3 in the developing Th2 cells as a result of inhibiting the activity of Mdm2, an E3 ubiquitin ligase specific for GATA3 [54].

The opposing views on the role of ERK in Th2 differentiation need to be considered in terms of our two-phase model of Th2 polarization. Indeed, naïve $CD4^+$ T cells from dn Lck-Tg mice actually produce significantly more IL-4 than do those from the littermate controls over the first 24 h after stimulation with a cognate peptide [52], implying that the Lck/Ras/ERK pathway actually blocks IL-4 production during the induction phase. However, IL-2 production by stimulated $CD4^+$ T cells from dn Lck-Tg mice is significantly decreased in comparison to that by control cells [52]. Diminished IL-2 production could account for impaired Th2 differentiation since STAT5 activation is essential for both the induction and polarization phases of Th2 differentiation, and the degree of STAT5 activation may have fallen below a threshold level during the polarization phase. Indeed, we observed that blockade of the ERK pathway impairs Th2 differentiation of 5C.C7 TCR-Tg naïve $CD4^+$ T cells stimulated with low concentrations of PCC peptide because of a substantial diminution in IL-2 production and that exogenous IL-2 fully rescues Th2 differentiation in cells in which the ERK pathway is blocked [25].

Recently, Leonard and his colleagues showed that IL-2 suppresses Mdm2 mRNA expression in $CD4^+$ T cells in a STAT5-dependent manner [27]. Therefore, it is conceivable that the Ras/ERK cascade may indirectly stabilize GATA3 expression through its action on IL-2 production by activated $CD4^+$ T cells, which downregulates Mdm2 expression. Thus, the degree of ERK activation in response to TCR stimulation may coordinately control Th2 differentiation by suppressing GATA3 upregulation at a transcriptional level during the induction phase and by protecting GATA3 from proteasome-mediated degradation during the polarization phase.

Significance of $CD4^+$ T cell fate to memory

While early events in $CD4^+$ T cell differentiation have been analyzed in considerable detail and much is known about the timing and nature of the process, we are only now coming to understand their significance for long-term T cell memory. Early in this review, it was pointed out that long-term Th1 and Th2 $CD4^+$ T cell lines were very stable. It seemed reasonable that this should be the case. In general, the fate adopted during a primary $CD4^+$ T cell response to a given pathogen is optimized to eliminate or

control the pathogen. The consequences of developing a non-optimized response are often quite disastrous as illustrated by the inappropriate Th2 response mounted by BALB/c mice infected with *L. major* [5, 6]. It seemed reasonable therefore that CD4$^+$ T cells that had adopted a given fate as a result of priming by infection with a particular microbial pathogen would retain that differentiated state so that when challenged by the same pathogen, they would surely make a response of the correct phenotype. However, an analysis of chromatin accessibility of T-bet and GATA3, the master regulatory factors of Th1 and Th2 cells, respectively, indicated that these genes show association with both trimethylated lysine 4 histone H3 (H3K4me3) and H3K27me3 in the lineages other than those in which they are expressed [55]. This implies that these master regulatory factors are in a poised state, often associated with plastic genes. Indeed, an increasing number of instances of change in phenotypic state have now been observed, indicating that plasticity in gene expression and function may be important physiologic properties of differentiated T helper cells. Striking examples of plasticity include the capacity of Th17 cells to adopt Th1 characteristics [56] or of iTregs to become Th17 cells [57]. Lohning and colleagues have reported a particularly impressive example of Th2 cells changing their properties when confronting an infection with lymphocytic choriomeningitis virus [58]. In this case, rather than adopting a "full" Th1 phenotype, they acquired the capacity to produce IFNγ and expressed T-bet while retaining, although at lower levels, potential for IL-4 production and expression of GATA3 [58].

Plasticity can certainly occur in vitro and under experimental conditions in vivo. It is still far from clear how often such plasticity occurs in the course of normal responses to pathogens and what its importance is in protective immunity. However, it may be argued that plasticity could be of considerable importance when memory cells respond to pathogens different from those that elicited their priming. There is considerable evidence of T cell cross-reactivity. This has been shown most persuasively for virus-specific CD8 responses [59] but is probably a general phenomenon. Indeed, in aging individuals, the proportion of naïve CD4$^+$ T cells falls quite precipitously. It can be expected that, in such individuals, responses to newly introduced pathogens may depend to a greater or lesser degree on cross-reactivity of memory T cells that had been primed to different pathogens. Under these circumstances, the capacity of the differentiated CD4$^+$ T cells to alter the cytokines they produce and their phenotypic state could be of great importance since there is no assurance that the polarized phenotype adopted by cells responding to the priming pathogen would be appropriate to a response to the cross-reactive pathogen.

Concluding remarks

The differentiation of CD4$^+$ T cells has been studied in greater detail than any other inducible polarization process in the immune system and represents one of the most tractable systems to study differentiation in higher vertebrates. Here, we have shown that this process has, as a general rule, the potential for a potent feedback regulatory control. The positive feedback nature of this control should make possible a strong phenotype polarization even under conditions in which the initial biasing stimulation is relatively weak and thus should strongly aid in the development of a CD4$^+$ T cell response that is most appropriate for the nature of the infectious agent that elicited the response. Indeed, positive feedback should serve to ensure that cells adopt predictable differentiation patterns and that, when mixtures of stimulants are present, the outcome will be that some cells adopt one polarized phenotype and others an alternative polarized phenotype, rather than all the precursors developing into cells with intermediate properties. Much work is nonetheless needed to ascertain not only whether one can demonstrate such striking positive feedback regulation in vivo but also whether this mechanism is critical to the development of polarized CD4$^+$ T cells under conditions of in vivo infections and inflammatory responses.

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