

Transcription: Tantalizing Times for T Cells

Review

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The T helper lymphocyte is responsible for orchestrating an appropriate immune response to pathogens. To do so, it has evolved into two specialized subsets that direct type 1 and type 2 immunity. Here, we discuss the genetic programs that control lineage commitment of progenitor T helper cells along each of these pathways.

The versatility of the T lymphocyte has made it an attractive system in which to study cell differentiation and lineage commitment. The attention it has received from the immunologic community over the last several years has happily resulted in significant advances in this field. Several subset-specific transcription factors and surface molecules have been isolated, and novel signaling pathways identified. The generation and analysis of genetically engineered mice has not only allowed the confirmation of results of *in vitro* experiments, but has also yielded unexpected findings that led to new discoveries. Indeed, several reviews on this topic have been recently published, and readers are referred to these reviews for details (Glimcher and Murphy, 2000; Murphy et al., 2000; Dong and Flavell, 2000; O'Garra and Arai, 2000). Here, we will focus our discussion on events that are taking place in the nucleus of the T helper (Th) lymphocyte, in particular the transcription factors that control its differentiation from an uncommitted precursor cell to a specialized effector cell. Rather than reiterating previous reviews on the same topic, we will try to summarize recent discoveries in this field and emphasize the critical questions that remain to be answered.

Overview of T Cell Differentiation

An effective immune response against pathogens, be they microbial agents, allergens, tumor antigens or autoantigens, must be of both appropriate magnitude and type (Figure 1). Type 1 immunity relies on differentiation of one major subset of T lymphocytes, the T helper cell, bearing the surface receptor CD4, that induces both inflammatory and cytotoxic responses essential for destruction of intracellular pathogens such as *Mycobacterium tuberculosis* and *Leishmania Major*. The T helper lymphocyte activates macrophages and also activates a second major type of T cell, the CD8 cytotoxic T cell, which is critical for the effective handling of microbial agents such as the human immunodeficiency virus, and

bacteria such as *Listeria monocytogenes*. CD4 and CD8 cells arise from a common lymphoid progenitor cell in the thymus. In addition to this cell-mediated series of immune responses, T helper lymphocytes generate type 2 immunity, humoral immunity, which requires that they signal to B lymphocytes to produce antibodies. Type 2 immunity is particularly important to neutralize certain viruses and to ward off parasites. It is now clear that type 1 and type 2 immunity are actually directed by two distinct subsets of T helper lymphocytes as described below.

Overview of Transcriptional Regulation of Th Cell Differentiation

Two distinct functional subsets of CD4+Th cells were identified more than a decade ago (Mosmann et al., 1986; Mosmann and Coffman, 1989). Type 1 Th (Th1) cells secrete IFN- γ , TNF- α , and LT, which are responsible for delayed type hypersensitivity responses. Conversely, type 2 Th (Th2) cells produce IL-4, IL-5, IL-10, and IL-13, and are responsible for providing help to B cells, enhancing maturation and chemoattraction of eosinophils, and mounting allergic responses (Mosmann and Coffman, 1989; Paul and Seder, 1994). The hallmark cytokine of the Th1 cell is IFN- γ and of the Th2 cell, IL-4. In addition to distinct cytokine profiles, several surface markers have been shown to be differentially expressed in Th cells. For example, the IL-12 receptor (IL-12R) β 2 chain, chemokine receptors CXCR3 and CCR5, and IL-18 receptor are found mainly on Th1 cells, while T1/ST2, CCR3, CCR4 and ICOS molecules are enriched on the surface of Th2 cells (Bonecchi et al., 1998; D'Ambrosio et al., 1998; Lohning et al., 1998; McAdam et al., 2000; Sallusto et al., 1998; Szabo et al., 1997; Xu et al., 1998). The balance between the Th1/Th2 subsets determines the type of response, and ultimately, the outcome of that response for an individual organism to any given pathogen be it an infectious agent, tumor antigen, organ graft, allergen or autoantigen.

Despite functional and phenotypic differences, both subsets of Th cells are derived from the same precursor Th (Thp) cell (Kamogawa et al., 1993). The fate of differentiating Th cells can be influenced by multiple factors, which include cytokine milieu, type of antigen presenting cell, type and delivery route of antigens, and mode of costimulation (Figure 2) (Constant and Bottomly, 1997; Lane, 2000; Liu et al., 2001; O'Garra, 1998; Salomon and Bluestone, 2001; Sperling and Bluestone, 2001). While an increasing number of extracellular factors and signaling pathways have been discovered to affect the differentiation of Th cells, it is believed that it is the cytokine milieu itself that primarily determines this fate. The cytokine milieu is critical for both the initiation and the expansion of the Th1 and Th2 subsets. Mice that lack IFN- γ , IL-12 or their receptors, or the Stat signaling molecule Stat4 downstream of the IL-12 receptor, fail to develop a robust Th1 compartment while mice that lack IL-4, IL-4R or Stat6 have severely compromised Th2 development (Gessner and Rollinghoff, 2000; Magram et al.,

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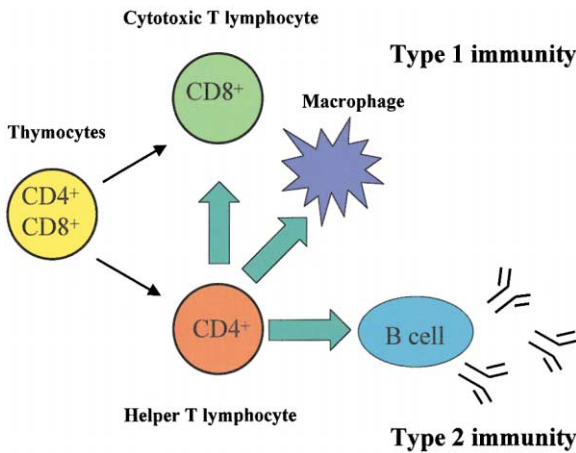


Figure 1. T Cell Differentiation

1996; Piccotti et al., 1998; Wurster et al., 2000; Zhang et al., 2001). Two other polypeptide mediators, Eta1 (osteopontin) and the chemokine MCP-1, also influence Th differentiation as demonstrated by the impairment in Th1 and Th2 compartments, respectively, observed in mice lacking these genes (Ashkar et al., 2000; Gu et al., 2000). The factors downstream of Eta-1 and MCP-1 that accomplish this, however, are unknown.

In contrast, recent studies demonstrate that the cytokines, IFN- γ , and IL-4, exert their effects through controlling the expression of subset-specific transcription factors in a bidirectional, positive feedback loop. For example, when a Thp cell is stimulated in the presence of IL-4 and antibodies against IL-12 or IFN- γ , the IL-4 signaling factor Stat6 is activated, translocates into the nucleus, and rapidly induces (either directly or indirectly) the expression of GATA-3 (Ouyang et al., 1998; Kurata et al., 1999), a Th2 cell-specific transcription factor that is a master regulator of the Th2 differentiation pathway (Ouyang et al., 1998; Zhang et al., 1997; Zheng and Flavell, 1997). The expression of GATA-3 is followed by the induction of the transcription factor c-Maf, also preferentially expressed in Th2 cells, that is a potent

and IL-4 gene-specific activator (Ho et al., 1996; Kim et al., 1999). In synergy with other transcription factors or coactivators, such as NFAT and NIP45, c-Maf and GATA-3 control the expression of IL-4, which further reinforces the IL-4R/Stat6 signal.

Conversely, if a Thp cell is stimulated in the presence of IL-12 and IFN- γ , and antibody against IL-4, IFN- γ signals through the IFN- γ receptor activate Stat1 which leads to upregulation of the expression of the Th1 cell-specific transcription factor, T-bet (Lighvani et al., 2001). T-bet is a potent transactivator of the IFN- γ gene and was recently demonstrated to be the master regulator of Th1 lineage commitment (Szabo et al., 2000, 2002). The expression of T-bet is followed by secretion of IFN- γ and upregulation of the IL-12R β 2 chain, which further strengthens the IFN- γ and IL-12 signals (Szabo et al., 1997; Mullen et al., 2001). Overall these experiments highlight the critical role of the cytokine milieu in determining lineage commitment, and support the idea that transcription factors that control the subset-specific cytokines IFN- γ and IL-4, will also control lineage commitment. This has indeed turned out to be the case for the tissue-specific factors that have been isolated to date.

This review will therefore focus primarily on what we have learned, and what remains to be learned, about the transcriptional control of Th cell differentiation. A brief summary of the three subset-specific, functionally important, transcription factors isolated to date is provided first.

T-bet

T-bet, a novel member of the T-box family of transcription factors, was originally cloned both by virtue of its ability to bind to the Th1-specific IL-2 promoter in a yeast one hybrid screen, and by its expression in Th1 but not Th2 cells (Szabo et al., 2000). Overexpression of T-bet by retroviral gene transduction in primary T cells forces uncommitted Thp cells, differentiating Th2 cells and most remarkably, terminally committed, fully polarized Th2 cells to display a Th1 cell phenotype, including production of IFN- γ and repression of the Th2 cytokines

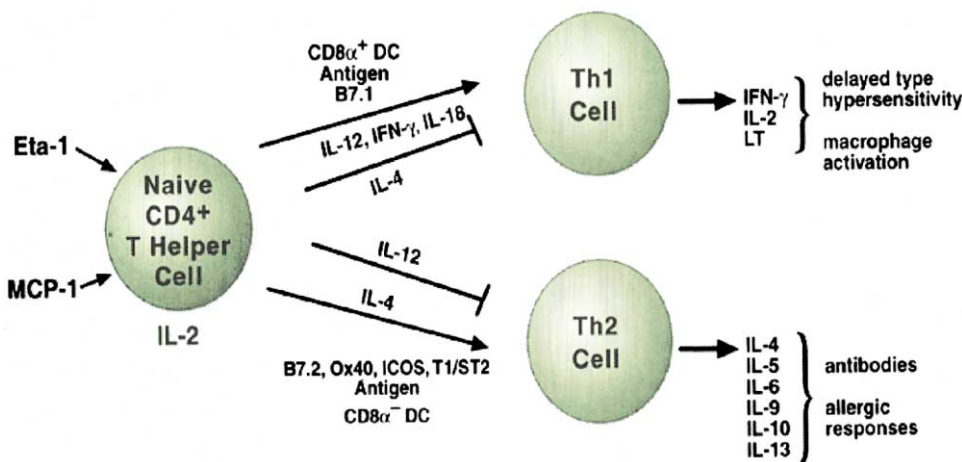


Figure 2. Signals that Influence Th Cell Differentiation

IL4 and IL-5. T-bet expression correlates with IFN- γ expression, and T-bet can transactivate the IFN- γ gene and induce both endogenous IFN- γ production, upregulation of IL-12R β 2 chain, and chromatin remodeling of individual IFN- γ alleles (Mullen et al., 2001; Szabo et al., 2000). Thus, T-bet likely activates Th1 genetic programs in part through directly controlling IFN- γ gene transcription. The in vitro functions of T-bet were confirmed by in vivo studies of genetically altered mice lacking T-bet. T-bet-deficient (T-bet^{-/-}) mice had normal lymphoid development but exhibited profound defects in mounting Th1 immune responses (Szabo et al., 2002). T-bet^{-/-} Th and NK cells produced severely reduced amounts of IFN- γ ; and T-bet deficiency rendered resistant C57BL/6 mice susceptible to *Leishmania major* infection. A corresponding increase in Th2 cytokines was present.

The balance between Th1 and Th2 subsets is critical in the immune response to pathogens, autoantigens, tumor antigens and allergens. Most autoimmune diseases arise from pathogenic Th1 cells while asthma and allergic responses reflect an overabundance of disease-causing Th2 cells. Given T-bet's role in controlling the Th1/Th2 balance, its function was assessed in several mouse models of human disease. Absence of T-bet was protective in inflammatory bowel disease mouse models (Neurath et al., 2002). Reciprocal effects of T-bet deficiency were seen in allergic asthma. The number of T-bet-expressing cells was substantially reduced in the airways of asthmatic patients (Finotto et al., 2002). T-bet^{-/-} mice developed spontaneous airway hyperresponsiveness, airway inflammation and airway remodeling, features of acute and chronic asthma that resembled human asthma, and these pathological changes could be adoptively transferred with T-bet^{-/-} Th cells (Finotto et al., 2002). The effects of T-bet may be explained both by an alteration of the Th1/Th2 balance, and by reciprocal regulation of IFN- γ and the immunosuppressive cytokine TGF- β by T-bet (Neurath et al., 2002). Taken together, these studies firmly establish T-bet as a transcription factor required for Th1 lineage commitment. While T-bet is the only Th1-specific transcription factor isolated to date, there are certainly additional transcription factors, most notably NF- κ B, that contribute to IFN- γ production as demonstrated most convincingly by the impairment in the production of this cytokine in mice expressing a dominant-negative I κ B transgene (Aronica et al., 1999)

GATA-3

GATA-3, a member of the GATA family of zinc finger proteins, was originally cloned as a T cell-specific transcription factor that bound to the enhancer of the T cell receptor (TCR) α gene (Ho et al., 1991). Indeed, GATA-3 is essential for the development of T cell lineage (Hendriks et al., 1999; Ting et al., 1996). By performing a representational difference analysis and studying the transcriptional regulation of IL-5, Zheng et al. and Ray and colleagues discovered that GATA-3 was almost exclusively expressed in mature Th2 cells, but not in mature Th1 cells (Zhang et al., 1997; Zheng and Flavell, 1997). Naive Th cells express negligible levels of GATA-3, which is rapidly induced in Th cells upon stimulation under Th2 skewing conditions. Once induced,

GATA-3 undergoes a process of autoactivation to augment its own expression in a Stat6-independent manner (Ouyang et al., 2000). Several in vitro studies have clearly demonstrated that GATA-3 is sufficient, although not very potent, in directing developing and polarized Th cells to produce Th2 cytokines (Lee et al., 2000; Ouyang et al., 1998).

Unfortunately, GATA-3 deficiency in the lymphoid system results in a complete block in the very early stages of T cell development, thus precluding a definitive test of its role in Th2 differentiation. However, strong support for its critical role here is provided by several recent reports. Despite the essential function of Stat6 in dictating the differentiation of Th2 cells in vitro, Th2 immune responses can still be elicited in Stat6-deficient (Stat6^{-/-}) animals whose Th2 cells continue to express high levels of GATA-3 (Finkelman et al., 2000; Jankovic et al., 2000; Ouyang et al., 2000). Transgenic mice overexpressing a dominant negative mutant of GATA-3, KRR, are more resistant to allergic asthma, a classical Th2 cell-mediated disease (Zhang et al., 1999). Th2 cells derived from these animals expressed significantly lower levels of Th2 cytokines upon rechallenge with antigens. More recently, Finotto et al. demonstrated that addition of GATA-3 antisense oligonucleotides during in vitro differentiation of Th2 cells significantly reduced the levels of GATA-3 protein and subsequent production of IL-4 (Finotto et al., 2002). In addition, local administration of GATA-3 antisense oligonucleotides markedly attenuated airway hyperresponsiveness, mucus production, and infiltration of eosinophils in an animal model of allergic asthma. These results strongly indicate that GATA-3 is essential for the development of Th2 cells and might also be essential to maintain Th2 phenotype and function. The mechanism by which GATA-3 controls Th2 cytokine gene expression is still unclear and may differ for each of the Th2 cytokines. Thus, GATA-3 can bind to and directly transactivate the IL-5 and IL-13 regulatory regions, but has only a minimal effect on the IL-4 promoter (Kishikawa et al., 2001; Lee et al., 1998; Siegel et al., 1995; Zhang et al., 1997).

c-Maf

c-Maf is a basic-leucine zipper protein that binds to a TRE/CRE-like sequence called MARE or C-MARE (Kataoka et al., 1994; Kerppola and Curran, 1994). c-Maf is preferentially expressed in Th2 cells and is also expressed at very low levels in naive Th cells (Ho et al., 1996). In contrast to the rapid induction of GATA-3 and T-bet by cytokines, the induction of c-Maf by TCR signaling occurs only after two days of initial stimulation under Th2 skewing conditions, and the levels of c-Maf can be further and rapidly induced in mature Th2 cells upon restimulation. Further, unlike T-bet and GATA-3, c-Maf expression is not regulated by cytokines, but rather by signals stemming from TCR. In vitro, c-Maf is an extremely potent transactivator of the IL-4 gene via its binding to a MARE site immediately 5' of the TATA box in the proximal IL-4 promoter, and can confer the ability to produce endogenous IL-4 upon nonproducer cells (Ho et al., 1996). The functions of c-Maf were confirmed by in vivo studies. c-Maf overexpressor transgenic mice had higher serum levels of IgE and IgG1, two IL-4-

dependent immunoglobulin isotypes, and Th cells derived from c-Maf transgenic mice spontaneously developed into Th2 cells in vitro (Ho et al., 1998). This shift in the Th1/Th2 balance toward the Th2 compartment afforded substantial protection from disease in two separate models of autoimmune diabetes (Pauza et al., 2001). Conversely, mice rendered deficient in c-Maf have defects in mounting Th2 immune responses in vivo with a spontaneous bias of Thp differentiation to the Th1 lineage (Kim et al., 1999). Interestingly, in the presence of exogenous IL-4, c-Maf-deficient (c-Maf^{-/-}) Th cells can develop into Th2 cells that produce normal levels of the Th2 cytokines IL-5, IL-10, and IL-13, although the production of IL-4 by c-Maf^{-/-} Th2 cells remains severely impaired. Taken together, these results demonstrate that c-Maf is an IL-4-gene-specific transactivator, and that c-Maf and GATA-3 promote the differentiation of Th2 cells by distinct but complementary mechanisms. We can conclude that expression of IL-4 requires both c-Maf and GATA-3, but that the cell-type specificity of other Th2 cytokines, such as IL-5 and IL-13, appears to mainly depend on GATA-3. However, the failure to fully redirect polarized Th1 cells into the Th2 lineage by the provision of both GATA-3 and c-Maf (Kishikawa et al., 2001), in contrast to the actions of T-bet in accomplishing the reverse task, raises the intriguing possibility that there are other yet-to-be-discovered factors necessary for complete Th2 lineage commitment.

While a tremendous amount of progress in understanding the transcriptional regulation of Th cell differentiation has occurred, there are several important questions that remain unanswered, and many new questions raised by the recent discoveries. We consider some of these below.

Selection/Stochastic versus Instructive—Which Is the Correct Model of Th Differentiation?

Our current understanding of the transcriptional regulation of Th cell differentiation favors the notion that lineage determination of Th cells follows an instructive model, but this is still very much an unsettled question that will require further work. In this model, signals downstream of IL-12/IFN- γ or IL-4 directly induce the expression of lineage-determining transcription factors, T-bet or GATA-3, and subsequently drive Th cell differentiation along the Th1 or Th2 pathway, respectively. This model is supported by several observations. (1) Newly committed Th1 cells can be forced to produce IL-4 when restimulated in the presence of exogenous IL-4 (Farrar et al., 2001). (2) GATA-3 is rapidly induced in naive Th cells when stimulated in the presence of IL-4 in vitro, but very little induction of GATA-3 is observed in Stat6^{-/-} Th cells (Ouyang et al., 1998). (3) Ectopic expression of GATA-3 allows developing Stat6^{-/-} Th1 cells to secrete Th2 cytokines (Ouyang et al., 2000). Similarly, overexpression of T-bet can force terminally committed Th2 cells to display a Th1 cell phenotype (Szabo et al., 2000). However, several papers have suggested that the induction of GATA-3 by IL-4 is actually due to an absence of IL-12 (Ouyang et al., 2000, 1998; Mullen et al., 2001).

The instructive model was recently challenged by Mullen et al., who presented data demonstrating that, within

hours after encountering antigen, developing Th cells stochastically expressed IFN- γ in a monoallelic fashion even in the absence of Stat4 signaling (Mullen et al., 2001). In addition, the initial induction of T-bet in naive Th cells upon stimulation is independent of the IL-12/Stat4 signal pathway, which actually provides a selective advantage for IFN- γ -producing cells by enhancing their proliferation and survival. Thus, the modest induction of T-bet by IL-12/Stat4 may simply reflect better survival of T-bet-expressing cells. While these data are consistent with a selection model, they do not exclude a concurrent instructive mechanism for Th1 differentiation. Of note, IFN- γ -receptor-deficient Th cells are unable to develop into IFN- γ -producing Th1 cells even under optimal conditions, suggesting that the IFN- γ signal is at least as important as the IL-12/Stat4 signal for Th1 cell development (Zhang et al., 2001). Indeed, IFN- γ is a very potent inducer of T-bet expression (Lighvani et al., 2001). Thus, the induction of T-bet by IFN- γ /Stat1 in developing Th1 cells is analogous to that of GATA-3 by IL-4/Stat6 in developing Th2 cells, and strongly supports an instructive model of Th differentiation. It will be important to test whether Stat1^{-/-} Thp cells retain the capacity to secrete IFN- γ , and if so, to delineate the signaling pathways that account for such expression.

What Factors Account for Stat6-Independent Th2 Cell Differentiation?

It has been convincingly demonstrated that Stat6 is essential for maximal Th2 differentiation in vitro (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). Stat6^{-/-} Th cells, generated by in vitro differentiation under Th2 skewing conditions, produced very small amounts of Th2 cytokines. In vivo Th2 immune responses, however, can still be elicited in Stat6^{-/-} animals, and Th2 cells derived from Stat6^{-/-} mice express high levels of GATA-3 and produce normal levels of Th2 cytokines (Finkelman et al., 2000; Jankovic et al., 2000; Ouyang et al., 2000). These results imply the presence of Stat6-independent, nonautonomous pathways for Th2 cell differentiation, although the nature and composition of these pathways remain unknown. One clue came from studies on Bcl-6-deficient (Bcl-6^{-/-}) mice. Bcl-6 is a zinc finger protein that belongs to the POZ family of transcription repressors, and mutation or translocation of Bcl-6 is commonly found in diffuse large cell B lymphoma (Staudt et al., 1999). Strikingly, Bcl-6^{-/-} mice spontaneously develop multiorgan inflammation characterized by marked infiltration of Th2 cells, eosinophils, and IgE-bearing B cells (Dent et al., 1997; Ye et al., 1997). Th cells isolated from Bcl-6^{-/-} mice produce very high levels of Th2 cytokines in a cell-autonomous fashion, however, the enhanced in vitro Th2 differentiation was completely reversed in the absence of Stat6 (Harris et al., 1999). Therefore, Bcl-6 negatively regulates the differentiation of Th2 cells at least partly via a Stat6-dependent mechanism. Surprisingly, Bcl-6^{-/-}Stat6^{-/-} mice still develop the lethal Th2 inflammatory response in vivo, and Th cells directly harvested from the inflamed organs continued to produce Th2 cytokines at levels comparable to those of Bcl-6^{-/-} animals (Dent et al., 1998). This surprising phenotype might well be explained by the Stat6-independent, nonautonomous Th2

pathway, which is also augmented by the absence of Bcl-6 *in vivo*.

These data raise an important and still unanswered question in this field. What is the factor and from what cell type does it come, that initiates differentiation along the Th2 pathway *in vivo*? Is this factor IL-4 itself working via the Stat6-dependent pathway, and if so, from what cell is it secreted? It has been suggested that NK T cells, Thp cells themselves, or a non-T, non-B cell may be the initial source of IL-4. The strongest evidence has been marshaled for the Thp itself being the source of IL-4, perhaps arising spontaneously as in the stochastic model of Th differentiation (Noben-Trauth et al., 2000). Another possibility is that some other factor, produced by non-Th cells, initiates the Th2 response by a Stat6-independent mechanism. Here, we return to clues provided by the phenotype of the Bcl-6^{-/-} mice. By generating a series of mixed chimeric animals, Toney et al. (2002) showed that the Th2 inflammatory responses seen in Bcl-6^{-/-} mice appeared to be mediated by non-B, non-T cells. Recently, it was discovered that the expression of the chemokine MCP-1 in macrophages was negatively regulated by Bcl-6 at the level of transcription (Toney et al., 2000). Earlier work demonstrated that the chemokine MCP-1 promoted Th2 differentiation as MCP-1-deficient mice displayed defects in Th2 immune responses *in vivo* (Gu et al., 2000). Taken together, these results suggest a model in which Bcl-6 attenuates the differentiation of Th2 cells by inhibiting the production of MCP-1 by non-B, non-T cells. This putative Stat6-independent pathway for Th2 cell differentiation remains to be confirmed.

How Is the Expression of GATA-3 and T-bet Regulated at the Transcriptional Level?

If GATA-3 is essential and sufficient for Th2 cell differentiation, then induction of GATA-3, either by a Stat6-dependent or a Stat6-independent mechanism, should be the lineage-determining event for Th2 cells. Unfortunately, very little is known about the transcriptional regulation of GATA-3. As mentioned above, GATA-3 is a T cell-specific transcription factor that is rapidly induced by IL-4/Stat6. Once induced, GATA-3 can undergo autoactivation, a Stat6-independent process, to augment its expression. The *cis*-acting elements that are required for T or Th2 cell-specific expression of the *gata-3* gene remain elusive. A YAC encompassing approximately 625 kb of the murine *gata-3* locus failed to support the expression of a reporter gene in thymi or lymphoid organs in transgenic animals, indicating that *cis*-acting elements outside the 625 kb YAC are required to achieve cell-type-specific expression of GATA-3 (Lakshmanan et al., 1999). Even less is known about the transcription factors, other than Stat6, that regulate the expression of GATA-3. Two recent reports provide some answers to this question.

The transcription factor NF- κ B regulates the expression of many cytokine genes. Interestingly, mice rendered deficient in the p50 subunit of NF- κ B were resistant to allergic airway inflammation, and were unable to mount normal Th2 immune responses in both *in vivo* and *in vitro* systems, whereas their Th1 responses were intact (Das et al., 2001). In addition, developing p50-

deficient Th cells expressed substantially lower levels of GATA-3 even under Th2 skewing conditions. Furthermore, inhibition of NF- κ B translocation by a synthetic peptide, containing a signal sequence of Kaposi's fibroblast growth factor and the nuclear localization sequence of the p50 subunit, during the differentiation of Th2 cells almost completely abolished the induction of GATA-3 and the production of Th2 cytokines. Somewhat unexpectedly, blockade of NF- κ B translocation had no effect on the expression of GATA-3 and the production of cytokines in mature effector Th2 cells. These results imply that GATA-3 might be a downstream target gene of p50 in developing Th2 cells and that the transcriptional regulation of GATA-3 might vary at different stages of Th cell development.

The Polycomb group of genes was originally identified in *Drosophila* as transcriptional repressors. Many Polycomb members have mammalian homologs, which are involved in the regulation of Hox gene expression (van Lohuizen, 1999). *mel-18* is one of the mammalian homologs. Unexpectedly, *mel-18*-deficient (*mel-18*^{-/-}) mice had moderate reductions in numbers of thymocytes and peripheral T cells, and exhibited a striking impairment in Th2 immune responses (Kimura et al., 2001). *mel-18*^{-/-} Th2 cells, generated by *in vitro* differentiation, produced significantly lower levels of Th2 cytokines. In addition, antigen-induced IgG1 production and Nippostrongylus-induced eosinophilia were attenuated in *mel-18*^{-/-} mice. Interestingly, *mel-18*^{-/-} Th2 cells expressed GATA-3 at much lower levels than wild-type Th2 cells despite normal IL-4/Stat6 signaling (Kimura et al., 2001). It remains unanswered whether the lower levels of GATA-3 in *mel-18*^{-/-} Th2 cells are the cause or the result of defective Th2 cell differentiation. If *mel-18* is essential for optimal GATA-3 induction, it will be important to know if overexpression of *mel-18*, either in the absence or the presence of IL-4/Stat6 signals, can induce the expression of GATA-3 and subsequently promote Th2 cell differentiation.

The expression of T-bet and GATA-3 is mutually exclusive during the priming of Th cells. This observation raises the question whether T-bet and GATA-3 counter-regulate each other. Addressing this question requires identification and cloning of cell-type-specific promoters of the *T-bet* and *gata-3* genes, which are currently not available. Furthermore, there are very limited data on the transcriptional regulation of T-bet. Thus far, in addition to TCR stimulation, IFN- γ is the most potent inducer of T-bet expression (Lighvani et al., 2001). It remains unclear if there are other signaling pathways or transcription factors, in addition to TCR and IFN- γ , that can induce the expression of T-bet.

Is There Posttranslational Modulation of GATA-3 and T-bet Activity?

The activity of the GATA-3 protein can also be modulated by posttranslational mechanisms. For example, it has been known for many years that an increase in intracellular cyclic AMP enhances cytokine production by Th2 cells but has opposite effects on Th1 cytokines (Munoz et al., 1990). This effect has recently been shown to be due to a specific activation of p38 MAP kinase in Th2 cells by increased intracellular cAMP that results

in phosphorylation of GATA-3 (Chen et al., 2000). In agreement with these results, p38-specific inhibitors diminish the transactivation capacity of GATA-3 *in vitro*. In addition to phosphorylation, mature GATA-3 proteins appear to be heavily acetylated (Yamagata et al., 2000). This may be the basis for the dominant-negative phenotype of the KRR mutant of GATA-3, where the substitution of the lysine-arginine-arginine (KRR) residues at amino acid 305–307 with alanines (AAA) significantly diminishes GATA-3 acetylation. Despite these observations, it remains unclear whether posttranslational modifications of GATA-3 are functionally relevant *in vivo*. In contrast to GATA-3, very little is known about the posttranslational modification of T-bet, although it is intriguing that T-bet contains several potential tyrosine phosphorylation sites (Szabo et al., 2000), a feature rarely found in transcription factors, the most notable exception being the Stat proteins.

In addition to posttranslational modification, the activities of GATA-3 can be modulated by physical interaction with other nuclear proteins. A cDNA encoding a lymphoid-specific GATA-3 interacting protein, ROG, was recently isolated and shown to attenuate the activities of GATA-3 and repress the production of cytokines by Th2 clones *in vitro* (Miaw et al., 2000). More recently, FOG-1, a GATA-interacting multitype zinc finger protein enriched in hematopoietic cells, was found to be critical for the development of the T cell lineage. Forced expression of FOG-1 significantly repressed the transcriptional activity of GATA-3, the production of Th2 cytokines, and the differentiation of Th2 cells *in vitro* (Zhou et al., 2001). However, the roles of ROG and FOG-1 in modulating the activity of GATA-3 and, subsequently, the differentiation of Th cells *in vivo* remain unclear. The generation and analysis of mice lacking ROG and FOG-1 in the lymphoid system should prove informative.

How Do Members of the NFAT Family of Transcription Factors Control Th Differentiation?

We have focused so far on the three factors, T-bet, GATA-3, and c-Maf, that are themselves expressed in a subset-selective manner. However, there are several other factors which, although not tissue specific, nevertheless play critical roles in Th differentiation. The most important of these are members of the Stat and NFAT family of transcription factors. We will leave it to a companion review in this issue to cover the functions of the Stat family in Th differentiation and will focus here on what is known about the NFATs.

Many questions still remain about the function of the nuclear factor of activated T cells (NFAT) family of transcription factors (NFATc1, NFATc2, NFATc3, and NFATc4) in regulating the Th1/Th2 balance. NFAT proteins share a conserved Rel homology domain distantly related to the NF- κ B family, but are otherwise distinct from each other in both sequence and tissue distribution, raising the possibility that they possess both unique and overlapping functions. These calcium-regulated proteins reside in the cytosol and upon stimulation through TCR undergo dephosphorylation by calcineurin, and translocate into the nucleus, a process inhibited by the immunosuppressant cyclosporin A (CsA) (Rao et al.,

1997). In the nucleus, NFATs activate the expression of multiple genes involved in the immune response to antigen, including those encoding cytokines and cell surface receptors. *In vitro*, all NFAT members are capable of binding to a conserved sequence, originally identified in the IL-2 promoter (Durand et al., 1988; Shaw et al., 1988). The global abolition of cytokine gene transcription that occurs in the presence of CsA indicates that NFAT proteins also play important roles in regulating the transcription of multiple other cytokines including IL-4, TNF α , IFN- γ , and GM-CSF. Indeed, specific sequences within the IL-4 proximal promoter that are bound by NFAT proteins are critical for both the CsA sensitivity and inducibility of IL-4 (Todd et al., 1993). *In vitro*, NFAT proteins clearly function as transcription activators.

The *in vivo* role of NFAT proteins as evidenced by the phenotype of mice that lack one or more NFAT family members, appears, however, to be very complex, and still not satisfactorily explained. For example, as described below, although NFATc1 (NFATc, NFAT2), NFATc2 (NFATp, NFAT1), and NFATc3 (NFAT4, NFATx) are present in both Th subsets, they nevertheless have profound effects on cytokine gene transcription that can be subset selective.

NFATc1-deficient (NFATc1^{-/-}) Th cells, generated by reconstitution of RAG-2-deficient mice, proliferated poorly in response to stimulation, and produced considerably lower levels of Th2, but not Th1 cytokines (Ranger et al., 1998a; Yoshida et al., 1998). Moreover, NFATc1 and c2 doubly-deficient chimeras had a profound and global defect in cytokine secretion including IL-2, IL-3, IL-4, IL-5, IFN- γ , TNF α , and GM-CSF (Peng et al., 2001). These results are in agreement with the expected functions of NFAT proteins as transcription activators deduced from *in vitro* experiments. However, other NFAT family members have more complicated functions *in vivo* that cannot be explained by their ability to control cytokine genes. Thus, NFATc2-deficient (NFATc2^{-/-}) animals unexpectedly developed a mild age-dependent lymphoproliferative disorder, accompanied by modest increases of Th2 cytokines upon repeated stimulation (Hodge et al., 1996b; Xanthoudakis et al., 1996). Further, NFATc2^{-/-} Th2 cells maintained the expression of IL-4 transcripts longer relative to wild-type. Although NFATc3-deficient (NFATc3^{-/-}) mice do not display defects in cytokine production (Oukka et al., 1998), mice lacking both NFATc2 and NFATc3 had a very striking phenotype characterized by excessive overproduction of IL-4 and other Th2 cytokines, highly elevated IgE and IgG1 titers and severe allergic and inflammatory disease in the skin and lung (Ranger et al., 1998b). In addition, the absence of these two NFAT members rendered activated Th cells resistant to activation-induced cell death. The combination of hyperproliferation and resistance to AICD resulted in a severe lymphoproliferative disorder, a phenotype that persisted even in the absence of IL-4 (Rengarajan et al., 2002). The increased proliferation and Th2 bias occurs very early in differentiation as naive NFATc2^{-/-} NFATc3^{-/-} Thp intrinsically differentiate into the Th2 lineage. This may be related to the recently described function of NFATc2 and NFATc3 in setting the threshold of responsiveness of naive Thp to signals transmitted via the TCR, by regulating their activation threshold and subsequent cell division profiles (Ren-

garajan et al., 2002). Interestingly, this hyperresponsiveness of naive Thp to TCR-mediated activation occurs without coengagement of CD28, raising the possibility that NFATs lie downstream of or parallel to signaling pathways stemming from coreceptor molecules in addition to their positioning downstream of the TCR.

These studies suggest that NFATc2 and NFATc3 have inhibitory functions *in vivo* and may regulate negative feedback mechanisms that ultimately control Th2 polarization. The NFAT target genes that account for this negative feedback remain unknown, although the repressive effect is probably achieved by a mechanism not involving IL-4/Stat6. It thus provides another example, in addition to Bcl-6, of IL-4/Stat6 independent mechanisms for Th2 cell differentiation. One means by which NFATc2 and NFATc3 may inhibit Th2 differentiation is by regulating the access of GATA-3 to the IL-4-IL-5-IL-13 locus, since both GATA-3 and NFATc2 bind to an enhancer region 3' of the IL-4 gene in Th2 but not in Th1 cells (Agarwal et al., 2000). In the absence of NFATc2 and NFATc3, GATA-3 may gain unrestricted access to this locus, leading to excessive transcription of Th2 cytokines. Alternatively, the role of NFATc2 and NFATc3 in regulating the "strength of signal" perceived from membrane receptors may intersect with as yet unidentified genes that control Th1/Th2 cytokine production.

Taken together, the analysis of NFAT-deficient mice clearly demonstrates that each NFAT member has both unique and redundant functions *in vivo*. We can conclude that NFATc1 is essential for normal Th2 responses, whereas NFATc2 and NFATc3 function as negative regulators of Th2 cell differentiation. It still remains unclear how the functional dichotomy of NFAT proteins is achieved given the fact that all NFAT members perform similarly *in vitro*. An attractive explanation is that NFATc2 and NFATc3, but not NFATc1, might induce one or a group of negative regulators for Th2 cell differentiation. Such negative regulators have yet to be identified.

An alternative explanation is that the functions of NFAT proteins might be modulated by proteins with which they physically interact. One such interacting protein is NIP45, which was originally cloned by its ability to interact with the Rel homology domain of NFAT proteins (Hodge et al., 1996a). NIP45 does not contain any known functional domains found in classical transcription factors and, by itself does not transactivate cytokine promoters. However, NIP45 substantially augments NFAT-dependent IL-4 gene transcription. Provision of NIP45, NFATc2, and c-Maf confers on non-Th2 cells the ability to produce IL-4 at levels comparable to those of Th2 cells, suggesting that NIP45 might act as a coactivator of NFAT for IL-4 gene transcription. However, NIP45 can also interact with TRAF2 to repress the production of IL-4, a finding consistent with the increased levels of Th2 cytokines found in mice carrying a dominant-negative TRAF2, which can not interact with NIP45 (Lieberson et al., 2001). These observations indicate that NIP45 can function both as a coactivator of NFAT and IL-4, and also as an inhibitor of NFAT-driven IL-4 production by a TRAF2-dependent mechanism. Thus, the function of NFATs in regulating cytokine production can be modulated in both positive and negative fashion by their interaction with other proteins by mechanisms yet to be elucidated.

Interplay of Th-Selective and Nonselective Factors

Transcription factors typically influence each other either directly via protein-protein interaction or indirectly via cross-regulation. The array of transcription factors present in Th cells makes assembling a comprehensible picture of this pathway a daunting task and one that remains very much to be determined. However, we offer the following version, shown schematically in Figure 3. We will assume that the two types of receptors on the naive Thp cell that are most critical for Th differentiation are the TCR, and the cytokine receptors, IFN- γ and IL-4. The encounter of a Thp cell with specialized subsets of dendritic cells that express an MHC/antigen complex will activate the TCR signaling complex, resulting in activation of the NFAT and c-Maf transcription factors. These transcription factors can bind to cytokine promoters leading to the production of multiple cytokines, perhaps especially IL-4. At the same time, small amounts of IFN- γ and IL-4 cytokines produced stochastically in the Thp cell or provoked by TCR-activated NFATs/c-maf will next induce, via Stat 1 or Stat6, the expression of T-bet and GATA-3 respectively. T-bet then drives the transcription of IFN- γ while GATA-3 drives the transcription of IL-4 in positive feedback loops. T-bet and GATA-3 simultaneously inhibit the expression of the cytokines of the opposing subset, thus initiating a negative feedback loop as well. IFN- γ also causes upregulation of the IL-12R β 2 chain (Szabo et al., 1997), thus making the naive Thp susceptible to IL-12, the potent Th1-differentiation factor secreted by dendritic cells.

Putting this all together leads us to the following schema (Figure 3). The expression of T-bet and GATA-3 during Th differentiation are striking mirror images, and these factors are similar in their ability to induce one lineage while simultaneously repressing the opposing lineage. Thus, it is highly likely that T-bet and GATA-3 are the master regulators of Th lineage commitment. T-bet lies downstream of IFN- γ /Stat1, the major inducer of Th1 differentiation, while GATA-3 is activated by IL-4/Stat6, the critical Th2-inducing cytokine. The NFATs and c-Maf are induced by TCR rather than cytokine signals, and the NFATs act both to directly drive the transcription of cytokines, and to set the threshold of TCR activation. This threshold controls the Th1/Th2 balance by controlling the "strength of signal." One question immediately comes to mind. What determines which factor, T-bet or GATA-3, wins the race in any given Thp? We do not have the answer to this question but can engage in some speculation. It is tempting to suggest that T-bet and GATA-3 regulate each other with T-bet blocking GATA-3 expression during Th1 differentiation and GATA-3 repressing T-bet expression during Th2 development. Indeed, cytokine gene expression may more generally reflect a balance between repressors and activators as demonstrated by the known functions of T-bet in repressing Th2 programs (Szabo et al., 2000), while GATA-3 and c-Maf act as Th1 repressors. (Ho et al., 1998; Ouyang et al., 1998). Another relevant example is the Stats. Mice lacking both Stat4 and Stat6 mount a Th1 response that exceeds that seen in Stat6^{-/-} mice, suggesting that Stat6 may act as a repressor in Th1 cells (Kaplan et al., 1998). The relative predominance of T-bet and GATA-3 elicited by any given pathogen may

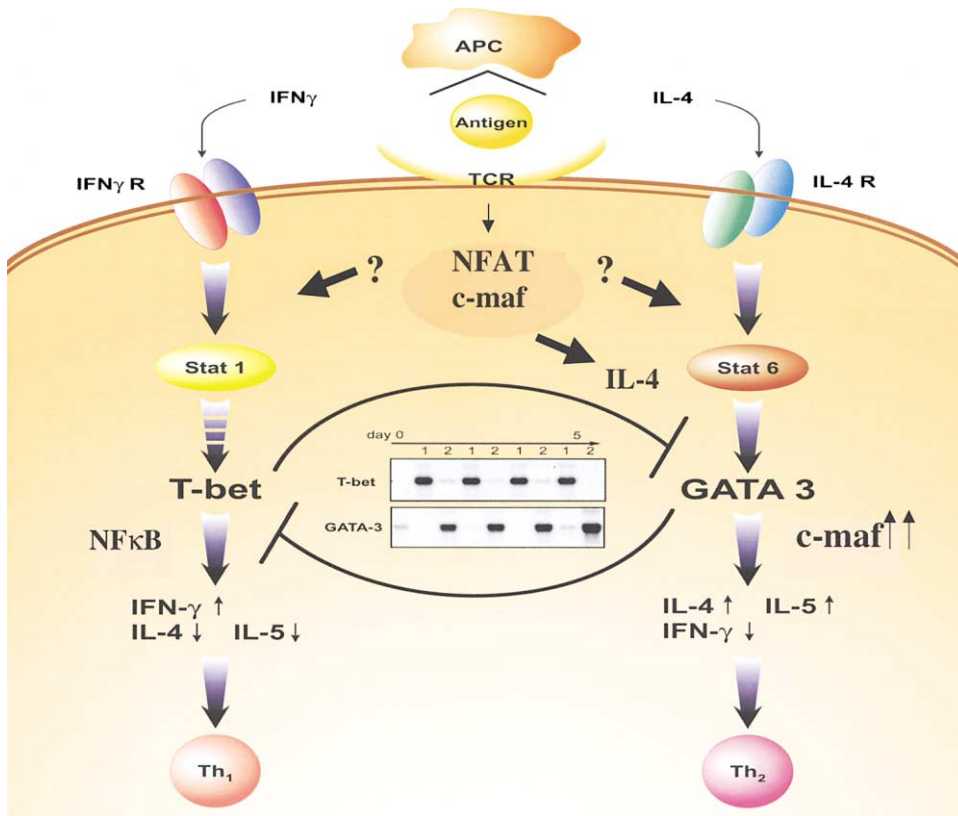


Figure 3. Schematic Diagram of Signaling and Transcriptional Events that Lead to the Differentiation of Th1 and Th2 Cells

thus ultimately determine the lineage choice of any given Thp. It is intriguing to speculate that the interaction of the pathogen/MHC complex with TCR acts through individual NFAT family members to control T-bet or GATA-3 expression, thus placing these factors both upstream/downstream and parallel to each other.

Is the Th2 Locus Coordinately Regulated?

Epigenetic Control of Cytokine Loci

In addition to regulation by transcription factors, it is increasingly apparent that expression of cytokine genes is also subject to epigenetic control. IL-4, IL-5, and IL-13 reside at the same chromosomal location in both man and mouse with the IL-4 gene only 10 kb away from the IL-13 gene. During Th2 differentiation, the Th2 cytokine locus undergoes a remodeling process which results in the appearance of several Th2 cell-specific DNaseI hypersensitivity sites (Agarwal and Rao, 1998). GATA-3 is sufficient to induce the remodeling of this locus in a Stat6-independent fashion (Ouyang et al., 2000). A similar remodeling process is present at the IFN- γ locus during the differentiation of Th1 cells (Agarwal and Rao, 1998; Young et al., 1994). The functional significance of epigenetic control was highlighted by a recent report. Both IFN- γ and IL-4 genes are positioned apart from heterochromatin in naive Th cells, which are capable of transcribing both genes simultaneously within hours after stimulation in a Stat4-/Stat6-independent manner. In contrast, the silenced cytokine alleles are repositioned to transcriptionally inactive heterochro-

matin in committed Th cells, presumably as a way to stabilize the effector Th cell phenotype (Grogan et al., 2001). An important role for epigenetic inheritance of the IL-4 gene has also been described. Thus, monoallelic IL-4 expression, reminiscent of that observed for immunoglobulin and T cell receptor genes, has been described in T cell clones derived from heterozygous mice (Bix and Locksley, 1998) and in mice deliberately made heterozygous by insertion of reporter genes into the IL-4 locus (Hu-Li et al., 2001; Riviere et al., 1998). A role for cytosine methylation in the regulation of the IL-4 gene has also been invoked, as hypomethylation of several CpGs in the second intron of the IL-4 gene only in Th2 but not Th1 clones was observed during the differentiative process (Bird et al., 1998).

Is There a Locus Control Region for the Th2 Cytokine Cluster?

Given the physical proximity and concordant remodeling of Th2 cytokine genes, it has been postulated that a locus control region might regulate the Th2 locus, although its nature and location remain elusive. By comparing mouse and human sequences of noncoding DNA in the Th2 cytokine locus, several homologous regions were identified. One of these stretches of DNA, called CNS-1, is located in the intergenic region between the IL-4 and the IL-13 genes (Loots et al., 2000). A 425 kb YAC transgenic construct containing the human Th2 cytokine locus, including CNS-1, was sufficient to support the expression of human IL-4, IL-5, and IL-13 in transgenic murine Th2 cells. In contrast, deletion of

CNS-1 from the transgenic construct substantially reduced the production of human Th2 cytokines. In another transgenic model, however, CNS-1 alone was not sufficient to dictate Th2 cell specificity. Instead, CNS-1 functioned as a subset nonspecific enhancer (Lee et al., 2001). Furthermore, deletion of CNS-1 by homologous recombination substantially reduced the production of Th2 cytokines but did not completely abrogate the differentiation of Th2 cells (Mohrs et al., 2001). Taken together, these results indicate that the concerted action of multiple *cis*-acting elements, including CNS-1, within the 425 kb YAC, rather than a single locus control region, is required to achieve coordinate expression of IL-4, IL-5, and IL-13 in a Th2 cell-specific manner. Of note, multiple potential GATA binding sites have been identified along the Th2 cytokine locus, and some of these sites appear to be functional *in vitro* (Ranganath et al., 1998). It remains unclear if the remodeling process precedes or is the result of the binding of GATA-3 to these sites, although GATA-3 would appear to be genetically upstream of the formation of Th2-specific hypersensitive sites (Lee et al., 2000 Ouyang et al., 2000).

What Factors Account for Cytokine Production by Cytotoxic T Cells?

The Th1/Th2 paradigm extends to immune system cells other than the helper T lymphocyte. The cytotoxic CD8 T cell, NK cell, and $\gamma\delta$ T cell can also be divided into two subsets called Tc1/Tc2, NK1/NK2, and $\gamma\delta 1/\gamma\delta 2$, which secrete type 1 or type 2 cytokines (Mosmann and Sad, 1996; Peritti et al., 1998). For example, Tc1 cells express T-bet and produce IFN- γ , whereas Tc2 cells secrete IL-4 and IL-13. One might have predicted that the molecular machinery that works so well for CD4 cells would be operative in other cell types. Indeed both NK cells and $\gamma\delta$ T cells require T-bet to produce optimal amounts of IFN- γ . However, the extension of this paradigm to all cells has recently been challenged. Although CD8 cells express T-bet and regulate its expression in a cytokine-inducible fashion, T-bet^{-/-} CD8⁺ cells continue to produce IFN- γ at a level comparable to that of wild-type CD8 cells and possess unaltered cytotoxic function (Szabo et al., 2002; Yin et al., 2002). Further, while Stat4 is clearly critical for the *in vitro* differentiation of and IFN- γ production by Th1 cells, the development of Tc1 cells and their expression of IFN- γ are independent of Stat4 (Aronica et al., 1999). Thus, the transcriptional regulation of IFN- γ in Th1, NK, and $\gamma\delta$ T cells differs from that in CD8 cells. This unexpected result raises several interesting questions. First, what then is the function of T-bet in CD8 cells? It would seem wasteful for this cell to express a transcription factor which is exquisitely regulated by receptor signaling, for no apparent reason. Second, why have such closely related cells, CD4 and CD8, which arise from a common progenitor in the thymus, evolved distinct mechanisms to control the production of a shared cytokine? Perhaps this divergence deliberately evolved to maximize the chances that a protective immune response could be marshaled against a diverse range of microorganisms, protozoans, and viruses. Finally, if T-bet and Stat4 do not control IFN- γ production in CD8 cells, what transcription factor does so?

Conclusion

We know a great deal now about the events that occur inside the nucleus of an uncommitted T helper precursor cell as it embarks on its journey toward differentiation and commitment. Like other stem cells, the Thp has choices to make which are governed by competing stimuli in its environment. These stimuli engage membrane receptors to activate signaling pathways, which in turn activate a limited number of both tissue-specific and non-tissue-specific transcription factors. While we have learned much about the mechanism of action of the known transcription factors, there is more still to learn. It is likely, too, that there are additional important regulatory proteins whose sequences lie peacefully undiscovered in the various genome databases. Furthermore, in contrast to our increasing knowledge about the genetic programs executed by transcription factors, we know exceedingly little about the complicated signals transmitted from membrane to nucleus. How does the Thp cell integrate and translate the plethora of signals stemming from membrane receptors into the activation of select transcription factors? The discovery of an "immunologic synapse" has led to the recent demonstration of distinct patterns of microdomain partitioning in differentiated Th1 and Th2 cells, an exciting first step (Balamuth et al., 2001). Examination of the membrane compartmentalization into lipid rafts in the naive Thp as it first encounters antigen-presenting cells may lead to a molecular definition of the earliest stages of lineage commitment. Lessons learned from the analysis of developmental and differentiative processes in other organ systems and in other organisms may also provide valuable clues.

Acknowledgments

Our thanks go to all members of the Ho and Glimcher laboratories, and also to our many wonderful colleagues in this field. We apologize for not including references to all of their excellent work because of space limitations. We especially thank Susanne Szabo, Adrian Erlebacher, Mohamed Oukka, and Michael Grusby for their thoughtful comments on the review, and Christine McCall for expert manuscript preparation.

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