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Molecular Regulation of Cytokine Gene Expression During the Immune Response

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Cytokine expression by immune system cells plays an important role in the regulation of the immune response. On first encounter with antigen, naive $CD4^+$ T helper (Th) cells differentiate into cytokine-producing effector cells. Two types of effector cells characterized by their distinct expression of cytokine profiles have been described. Th1 cells produce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. In many pathological situations, the balance between Th1 and Th2 immune responses determines the outcome of diverse immunologically mediated clinical syndromes including infectious, autoimmune, and allergic diseases. However, the molecular basis for the tissue-specific expression of Th1/Th2-like cytokines has remained elusive. In this review we evaluate the possible *in vivo* role of different transcription factors and transcriptional mechanisms in T cell differentiation and the immune response.

KEY WORDS: Cytokine genes; immune response; molecular regulation.

INTRODUCTION

In response to antigen stimulation, naive $CD4^+$ T helper (Th) cells differentiate into cytokine-producing effector cells. Two classes of differentiated Th cells, characterized by distinct and mutually exclusive profiles of cytokine expression, have been described (1–3): Th1 cells preferentially produce IL-2 and IFN-γ, which promote cellular immune responses against intracellular pathogens, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, which promote humoral immunity by aiding in B cell growth and differentiation. T cell differentiation is highly sensitive to the detailed conditions prevailing within the microenvironment where the T cells recognize antigen. Key factors that influence differentiation are the dose and route of antigen administration, the engagement of costimulatory receptors, and most importantly, the local concentrations of cytokines. The cytokines IL-12 and IL-4 have been shown to be particularly important in facilitating differentiation of naive $CD4^+$ Th cells to the Th1 and Th2 phenotype, respectively (1, 2, 4).

The molecular basis for the cell-specific and mutually exclusive expression of Th1 and Th2 cytokines is not yet completely defined. An understanding of this phenomenon is fundamental to our comprehension of how the immune response is regulated, since in many pathological situations, the balance between Th1 and Th2 phenotype determines the outcome of different immunologically mediated clinical syndromes including infectious, autoimmune, and allergic diseases (2, 3). Over the past few years, several groups have made important contributions to the understanding of signalling and gene transcription in the immune system, by combining the powerful genetic techniques of *in vivo* gene disruption and production of transgenic mice, with biochemical analysis and studies of reporter function. This work has led to an appreciation of the specific roles of several transcription factors in the Th1/Th2 response, including STAT and NFAT proteins, GATA-3, Maf, and BCL-6. More recently, it has become apparent that cytokine production not only involves the acute transcription of cytokine genes by differentiated T cells, but also includes a prior phase of chromatin remodeling that occurs during the initial differentiation of naive cells. In this review we evaluate the role of selected transcription factors in regulating locus remodeling, cytokine production, and T cell differentiation during the *in vivo* immune response. The reader is also directed to several other recent and excellent reviews which cover other aspects of the topic (5–8).

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REGULATION OF CYTOKINE EXPRESSION AT THE LEVEL OF CHROMATIN ACCESSIBILITY

As discussed in the following section, genetic experiments with transgenic and gene-disrupted mice have implicated the transcription factors STAT4, STAT6, GATA-3, Maf, and NFAT in regulating T helper cell differentiation *in vivo*. At a mechanistic level, the effects of these transcription factors have been explained by their ability to regulate cytokine gene expression and promoter activity in fully differentiated cells. However, recent work suggests that these transcription factors may also function during the initial antigen-driven differentiation of naive T cells, to regulate, directly or indirectly, the chromatin accessibility of cytokine genes. In this section, we briefly summarize and discuss these recent results.

Generally, inactive genes are found in regions of condensed chromatin, characterized by dense CpG methylation of DNA, a preponderance of hypoacetylated histones, and a relative insensitivity to digestion by DNase I (9). Studies using a variety of cell types have shown that the first steps of cell differentiation involve chromatin remodeling and concomitant changes in locus accessibility, as documented for the β-globin and antigen receptor loci (10, 11). The mechanism of locus "opening" is associated with chromatin remodeling, and is accompanied by DNA demethylation, histone acetylation, and the appearance of DNase I hypersensitive sites (12, 13). In many cases, the hypersensitive sites have been shown to be *cis*-acting DNA sequence elements that bind trans-acting nuclear factors and function in the context of chromatin to regulate locus accessibility (14, 15).

Several early observations suggested that changes in locus accessibility might play a major role in directing T helper cell differentiation. First, whereas the initial stimulation of naive T cells with antigen resulted in barely detectable levels of cytokine production, differentiated T cells obtained after even a single round of antigen stimulation transcribed high levels of cytokine genes with very rapid kinetics (16–18), implying that the same genetic locus might be 100- to 1000-fold more accessible to RNA polymerase II in the differentiated cells. Second, whereas the naive T cells showed frequent coexpression of Th1 and Th2 cytokines, continued stimulation led to increasing polarisation of the response (19), suggesting that relatively slow epigenetic processes, such as histone acetylation/deacetylation and CpG methylation/demethylation, could be involved in enhancing the expression of certain cytokine genes while suppressing the expression of others. Third, the genes encoding the Th2 cytokines IL-4, IL-5, and IL-13 are closely linked in both the human and the mouse, being located within a 180-kb region in each case, thus their coexpression in Th2 cells and mast cells could reflect increased accessibility of a large chromosomal region.

The locus accessibility model has recently received specific experimental support. At least three groups have shown that the IFN-γ locus is hypomethylated in Th1 clones but methylated in Th2 clones which do not express IFN-γ (20–22). Bird *et al.* (23) reported that IL-4 production by differentiating Th2 cells required several cycles of cell proliferation, was accompanied by CpG demethylation of the IL-4 gene, and was potentiated by inhibitors of histone deacetylases and DNA methyltransferases, as expected if differentiation involved a chromatin remodelling step. Hollander *et al.* (24) and Riviere *et al.* (25) reported that many or most T cells showed monoallelic expression of the IL-2 and IL-4 genes, respectively; for the IL-4 gene, increasing the strength of TCR stimulation was shown to result in increased levels of biallelic expression, suggesting a stochastic process in which gene expression is regulated by the activation of each individual allele in response to TCR signaling. Finally, Agarwal and Rao (26) provided evidence, based on DNase I hypersensitivity analysis, for mutually exclusive remodeling of the IL-4/IL-5/IL-13 locus and the IFN-γ locus in differentiating Th2 and Th1 cells, respectively. Remodeling of the IL-4 locus was apparent within 48 hr of the initial stimulation of naive T cells and occurred much less efficiently in T cells lacking STAT6.

Based on these observations, a multi-step model for cytokine expression during Th differentiation has been proposed (9, 26). The model postulates that cytokine genes in naive T cells are contained within a transcriptionally-repressed chromatin structure that is likely to explain their poor expression upon initial stimulation. Differentiation of the naive cells toward the Th1 or Th2 phenotype is associated with subset-specific remodeling and increased accessibility of the IFN-γ or IL-4, IL-5, and IL-13 genes, respectively, explaining the greatly increased transcription of these cytokine genes during secondary stimulation with antigen. Thus the first step of cytokine gene expression is proposed to be mediated by the transient activation of antigen- and cytokine-induced transcription factors, including NFAT and STAT proteins, during the initial stimulation. These factors are proposed to induce a second generation of subset-specific nuclear factors such as GATA-3 and Maf, which are postulated to bind to DNase I hypersensitive regions of inducible genes and recruit chromatin-remodeling enzymes; indeed, other members of the GATA and Maf

families are strongly implicated in similar processes in both erythroid and nonerythroid cells (27–29). The resulting chromatin changes are stably inherited by the differentiated cells and form the basis for polarized gene expression in the secondary response. The final, acute step of gene expression by the differentiated cells is mediated by non-subset-specific transcription factors such as NFAT, NFκB and AP-1 that are activated in response to secondary antigen stimulation; these factors gain access to loci that are in the "open" configuration and promote rapid, high-level gene expression in the differentiated cells.

The model provides a framework of testable hypothesis with which to approach the current literature. As one illustration, naive T cells are known to require cytokines and costimulatory ligands for effective differentiation, whereas differentiated T cells produce robust levels of cytokines in response to antigen stimulation alone (30, 31); potentially, this difference could be explained by postulating that cytokine and costimulatory receptors played a critical role in locus remodeling as compared to acute transcription. Similarly, the model predicts that memory T cells would differ from naive or acutely activated T cells by stable epigenetic changes (such as methylation and changes in chromatin accessibility), selectively occurring on genes destined to be expressed in the secondary response; again, this prediction is experimentally testable.

GENETIC ASSESSMENT OF THE INVOLVEMENT OF TRANSCRIPTION FACTORS

A powerful way of assessing the role of specific transcription factors in Th differentiation is by genetic experiments in mice. Using a combination of biochemical experiments and transgenic mice, the transcription factors GATA-3 and c-Maf have been shown to be expressed selectively in the Th2 subset and to bias Th differentiation towards the Th2 phenotype when overexpressed. Conversely, gene disruption of STAT4 and STAT6 has indicated that these transcription factors play major roles in Th1 and Th2 differentiation respectively. Gene disruption experiments with NFAT proteins has yielded less clearcut results, perhaps because of the overlapping expression and potentially redundant functions of these proteins in T cells. In the following sections we introduce these families of transcription factors and review the data indicating that they play a role in Th differentiation. In the last section under this heading, we evaluate the general usefulness of the genetic approaches, using results obtained with the NFAT family as illustration.

STAT Proteins (Signal Transducers and Activators of Transcription)

STAT proteins are a family of cytokine-activated transcription factors which contain SH2 domains (32). Seven STAT proteins (STAT1–STAT6) have been identified, including STAT5A and 5B, the products of two closely linked genes (33, 34). The STAT proteins are activated through cytokine receptors which associate with JAK (Janus-family kinase) tyrosine kinases. Upon binding of cytokines to their receptors, receptor multimerisation is followed by cross-phosphorylation and activation of the associated JAK kinases, which then phosphorylate tyrosine residues on the receptor. These tyrosine-phosphorylated sequences function as docking sites for the SH2 domains of cytosolic STAT proteins, which are then in turn positioned for tyrosine phosphorylation by the activated JAKs (34). STAT phosphorylation results in their homo- or heterodimerization through reciprocal phosphotyrosine-SH2 domain interactions. The multimerized transcription factors translocate into the nucleus and bind to the promoter elements of various target genes, thereby activating their transcription (32, 34).

Gene disruption of the various STAT proteins indicated that STAT transcription factors may have more selective roles *in vivo* than one might expect from *in vitro* experiments. Targeted deletion of STAT1 yielded mutant mice that displayed marked unresponsiveness to IFN-α and IFN-γ and defects in antimicrobial defenses, without any other effects on cytokine signaling (35, 36), suggesting that STAT1 is a central mediator in innate immunity against intracellular pathogens, such as viral infection. Homozygous disruption of the STAT3 gene resulted in an embryonic lethal phenotype that attests to the pleiotropic role of cytokines in early development (37). Gene disruption of either STAT5A or STAT5B resulted in very limited immunological defects (38, 39). Interestingly, however, homozygous disruption of both STAT5A and STAT5B led to a complete loss of IL-2 responsiveness in peripheral T cells, despite normal regulation of the IL-2 receptor in response to T cell receptor engagement (40).

Gene disruption of two members of the STAT family, STAT4 and STAT6, indicated that they played critical roles in T cell differentiation and Th1/Th2 phenotype development (2). STAT4 is selectively activated by IL-12, a macrophage-derived cytokine (41), while STAT6 is selectively activated by IL-4 (42). Disruption of the STAT4 and STAT6 genes resulted in specific immunological defects that were very similar to the phenotypes presented by IL-12- and IL-4-deficient mice,

respectively (43–49). STAT4−/− mice showed impaired Th1 immune responses with abrogation of IFN-γ production in response to IL-12 and NK cell cytotoxicity (44, 48), while STAT6−/− mice showed impaired IL-4-mediated function including Th2 cell differentiation, expression of cell surface markers, and Ig class switching to IgE (45, 47, 49). Furthermore, the STAT6-deficient mice also showed abrogation of eosinophil-mediated allergic inflammation and airway hyperreactivity (50, 51). These findings demonstrate that STAT4 and STAT6 transcription factors are selectively activated by IL-12 and IL-4, respectively, and are fundamental for Th1/Th2 cell differentiation. Interestingly, however, T cells from mice doubly deficient for STAT4 and STAT6 fail to differentiate into IL-4-secreting Th2 cells but still produce significant amounts of IFN-γ when stimulated *in vitro* with IL-12 (52), suggesting that Th2 differentiation requires STAT6, whereas Th1 differentiation can occur by a STAT4-independent pathway.

The GATA Family of Transcription Factors

GATA transcription factors are zinc finger proteins that bind to the (A/T)GATA(A/G) DNA sequence (6, 53, 54). To date, six members of this family, GATA-1 to GATA-6, have been identified (54). Based on their differential expression profile, the GATA transcription factors may be grouped functionally as possessing hematopoietic (GATA-1 to GATA-3) or nonhematopoietic (GATA-4 to GATA-6) functions (54). Although GATA-1, -2, and -3 show overlapping expression in different hematopoietic cell types, their expression levels vary between different cells (55, 56). GATA-1 is expressed in erythroid cells, megakaryocytes, bone marrow-derived mast cells, eosinophils, and testis and plays a role in terminal differentiation in different myeloid cells (56–60). GATA-2 is expressed in various hematopoietic cells and in embryonic brain and liver, and GATA-3 is expressed in hematopoietic cells, such as T cells, and also in embryonic brain (54).

GATA proteins regulate distinct subsets of target genes. GATA-1 DNA binding sites are present in the promoter/enhancer regions of several erythroid-specific genes, including the α- and β-globin genes (61); indeed disruption of the GATA-1 gene demonstrated that this transcription factor plays an important role in the maturation of erythroid progenitors (62). GATA-2 plays a role in the proliferation of the erythrocyte lineage and in the development of the nervous system (63, 64). GATA-3 was first identified as a T cell-specific transcription factor that bound to the enhancers of the TCR-α, -β, and -δ genes (53, 65–68). Later, GATA-3 binding sites were identified in the CD8α promoter/enhancer, the CD4 enhancer, and the IFN-γ promoter (69–72). Homozygous disruption of the GATA-3 gene resulted in an embryonic lethal phenotype, with a failure of fetal hematopoiesis and defects in the central nervous system (73). By using RAG-2-deficient blastocyst complementation with homozygous GATA-3−/− mutant ES cell lines, this transcription factor was shown to be an essential component in the earliest steps of T cell development in the thymus (74). Taken together, these results indicate that nonoverlapping and nonredundant functions for the various GATA transcription factors, with a specific role for GATA-3 in T cells.

Recently, three groups demonstrated that the GATA-3 transcription factor controls Th2-specific cytokine expression and may play an important role in the balance of Th1/Th2 differentiation in the immune response (75–77). GATA-3 protein is expressed at high levels in Th2 cells but is down-regulated in Th1 cells (75–77). GATA-3 induction during Th2 differentiation requires IL-4 and STAT6; conversely, GATA-3 downregulation in Th1 cells depends on IL-12 signaling through STAT4 (77). GATA-3 overexpression was shown to transactivate the IL-4 promoter in a B cell line (76) and to activate the IL-5 promoter in Th1 cells (75). Furthermore, Th1 cells from GATA-3 transgenic mice showed aberrant expression of Th2-type cytokines, while antisense GATA-3 treatment inhibited the expression of several Th2 cytokine genes, including IL-4, IL-5, IL-10, and IL-13, in a Th2 cell clone (76). Although the IL-4 promoter contains two putative GATA-3 binding sites, the role of this transcription factor in direct transactivation of the IL-4 gene remains to be determined. Together these results suggested that GATA-3 might function as a general regulator of Th2 cytokine expression during the Th2 immune response.

In addition, GATA-3 appears to function as a negative regulator of Th1 development, by a mechanism that is not dependent on its ability to upregulate IL-4 (77). Expression of GATA-3 in differentiating Th1 cells strongly downregulated the production Th1 cytokines TNF-α, TNF-β, lymphotoxin-β, and IFN-γ by the differentiated cells upon restimulation and, also, caused the cells to lose expression of the IL-12 receptor β chain. In contrast, GATA-3 had no effect on IFN-γ production by fully differentiated Th1 cells or clones but was capable of downregulating IL-12 receptor β chain expression in these cells. These results indicated that GATA-3 downregulated Th1 differentiation at a early stage, perhaps by decreasing early IL-12 signaling via its effect on the IL-12 receptor. It remains to be tested whether GATA-3

might also have a negative effect on chromatin remodeling of Th1 cytokine genes.

Maf

The protooncogene c-Maf is more highly expressed in Th2 cells than in Th1 cells, and its expression is induced during *in vitro* Th2 differentiation (78). Transfection experiments demonstrated that c-Maf cooperated with NFAT1 to transactivate the IL-4 promoter (78), an effect enhanced by overexpression of the putative coactivator NIP-45 (NFAT interacting protein-45) (79). Although a B cell line stably expressing increased levels of c-Maf displayed increased expression of the endogenous IL-4 gene, Th1 cells expressing a c-maf transgene did not, suggesting that c-Maf and NFAT1 were not sufficient to activate IL-4 gene transcription in normal cells. Rather, c-Maf transgenic Th1 cells showed decreased production of IFN-γ, even when examined in an IL-4$-/-$ background, suggesting that c-Maf upregulated the production of some target gene that acted at an early stage of Th1 development to downregulate IFN-γ expression. Potentially, this c-Maf target gene could be GATA-3 (77), and the effect could be exerted at the level of IL-12 signaling or IFN-γ locus remodeling (see discussion above). Transgenic mice that overexpressed c-Maf displayed increased serum levels of the Th2-dependent immunoglobulins, IgG1 and IgE, and Th2 cells from these mice produced increased levels of the Th2 cytokines when differentiated under nonskewing conditions *in vitro* (80). The latter effect was dependent on increased production of IL-4 since it was not seen in c-Maf transgenics that had been backcrossed into an IL-4$-/-$ background. Interestingly, the effect of c-Maf was reported to be selective for IL-4 (80), unlike that of GATA-3 which was shown also to act on IL-5, IL-13, and IL-10 (75, 76).

BCL-6

Genetic alterations, such as translocations and mutations, in the Bcl-6 gene are frequently associated with diffuse large-cell lymphoma (81, 82). Bcl-6 is a potent transcriptional repressor (83, 84) that is highly expressed in germinal-center B lymphocytes (85, 86). Mice with Bcl-6 gene disruption developed myocarditis and pulmonary vasculits, and the cellular infiltrates in the hearts and lungs were composed of mononuclear and polymorphonuclear cells, with an important component of eosinophils (87). Interestingly, these mice had no germinal center formation in peripheral lymphoid organs and also showed an increased expression of Th2 cytokines, such as IL-4, IL-5, and IL-13 (87). Bcl-6 was shown to compete with STAT6 for binding to STAT6 sites *in vitro* and for transactivation at multimerized STAT6 sites in transient reporter assays; moreover overexpression of Bcl-6 inhibited basal and IL-4-induced endogenous CD23 expression in a B cell line, suggesting that the increased expression of Th2 cytokines in Bcl-6$-/-$ mice was due to relief of Bcl-6-mediated repression and therefore increased activity of unopposed STAT6 (87). Thus Bcl-6 and GATA-3 may function reciprocally as repressors of Th2 and Th1 gene transcription, respectively.

NFAT (Nuclear Factor of Activated T Cells)

The NFAT family of transcription factors plays a major role in the immune response and has also been implicated in gene transcription in several nonimmune cell types (88–91). Binding sites for NFAT proteins are present in the promoter/enhancer regions of diverse inducible genes, including those encoding the cytokines IL-2, IL-4, IL-5, IL-8, IL-13, IFN-γ, TNF-α, and GM-CSF, and the cell surface receptors CD40L, CTLA-4, and FasL (89, 92–96). The NFAT family encodes four distinct classes of proteins: NFAT1 (NFATp), NFAT2 (NFATc), NFAT3, and NFAT4 (NFATx) (89). At least three of these, NFAT1, NFAT2, and NFAT4, are expressed at the protein level in peripheral T cells (97, 98). NFAT1 is also expressed in mast cells, NK cells, monocytes, and macrophages (89, 97), NFAT1 and NFAT2 are expressed in B cells (97, 99, 100), and NFAT4 is expressed at high levels in the thymus (101, 102). While the distribution of NFAT3 protein has not been reported, NFAT3 mRNA is expressed mainly in tissues outside the immune system (91,103), and so this protein is not considered here.

The four NFAT proteins share several properties. First, they are normally activated by stimulation of receptors coupled to calcium mobilization, such as the antigen receptors on T and B cells, Fcϵ receptors on mast cells and basophils, Fcγ receptors on macrophages and NK cells, and receptors coupled to heterotrimeric G proteins (89). Second, they are all regulated by calcineurin, the calmodulin-activated serine phosphatase that is the direct target for the immunosuppressive agents cyclosporin A (CsA) and FK506 (104–109). Third, they all form cooperative complexes with AP-1 (Fos/Jun) on composite NFAT-AP-1 sites (103, 110, 111). Fourth, NFAT proteins are present in both Th1 and Th2 cells and can activate cytokine promoters in both cell types (112, 113). Finally, NFAT transcriptional activity appeared to be similarly regulated during Th1/Th2 development

(114), although it was enhanced in differentiated Th2 cells compared with differentiated Th1 cells (115).These similarities suggested that the four NFAT proteins were likely to overlap substantially, if not completely, in their ability to respond to environmental stimuli and activate NFAT-dependent genes. To test this hypothesis and elucidate the specific roles of individual NFAT proteins, "knockout" mice deficient in expression of the three proteins expressed in immune cells NFAT1, NFAT2, and NFAT4, were generated and analyzed. Each knockout mouse showed a different phenotype that is described in detail below, potentially suggesting distinct functions for the different NFAT proteins in the immune response.

NFAT1. Except for a moderate degree of splenomegaly, NFAT1 (NFATp)-deficient mice developed normally, did not exhibit any obvious behavioral deficiencies, and were immunocompetent (116, 117). When primary immune responses were assessed *in vitro*, by stimulation of spleen cells with anti-CD3 antibody or Con A, NFAT1-deficient mice showed no impairment of IL-2, IL-4, IFN-γ, and TNF-α production (117). However, when spleen cells were tested for primary responses 1 hr after *in vivo* injection with anti-CD3, NFAT1$-/-$ mice showed substantially lower levels of several inducible transcripts, including those encoding IL-4, IL-13, TNF-α, GM-CSF, CD40L, and FasL (116). Thus the early phenotype of NFAT1$-/-$ T cells was consistent with the idea that NFAT1 played a positive role in cytokine gene transcription.

In contrast to this early impairment, NFAT1-deficient mice consistently showed a marked increase in the secondary immune response using two different experimental models, suggesting that NFAT1 might also play a negative role (116, 117). First, cells from draining lymph nodes of mice that had been sensitized with ovalbumin hyperproliferated after a secondary *in vitro* stimulation with the same antigen (117); likewise, $CD4^+$ T cells and B cells hyperproliferated *in vitro* in response to anti-CD3 and anti-IgM antibodies, respectively (116). Second, in an *in vivo* model of the allergic/inflammatory response to antigen, mice that had been previously sensitized to ovalbumin were restimulated by intrapleural injection of antigen, and the accumulation of eosinophils in the pleural cavity was assessed. In this model, NFAT1-deficient mice showed a marked increased in the number of eosinophils in the pleural cavity and a corresponding increase in the level of serum IgE (117, 118); moreover, they displayed a significant increase, relative to wild-type mice, in the numbers of eosinophils in bone marrow and peripheral blood (118). Injection of neutralizing antibodies indicated that the increased development of the allergic eosinophilic response in these animals depended on overexpression of IL-4 and IL-5 (118). Consistent with these findings, NFAT1$-/-$ mice showed enhanced Th2 development at later time points in an *in vitro* model of T helper (Th) differentiation, as evidenced by increased levels of IL-4 production (116, 119). Furthermore, NFAT1$-/-$ mice showed increased expression of Th2 cytokines, such as IL-4, IL-5, and IL-13 (118, 119). This phenotype was traced to altered kinetics of IL-4 gene transcription. T cells from NFAT1$+/+$ and NFAT1$-/-$ mice were identical in the early phase of IL-4 gene expression; however, IL-4 gene expression was maintained at high levels at late times only in the NFAT1$-/-$ T cells (119). The resulting increase in IL-4 expression was responsible for the skewed differentiation toward the Th2 phenotype observed in NFAT1$-/-$ mice. These results suggested that NFAT1 normally initiated a negative feedback effect that selectively downregulated the late phase of IL-4 gene transcription. The nature of this feedback effect remains to be identified.

NFAT2. Unexpectedly, targeted disruption of NFAT2 (NFATc, NFATc1) resulted in an embryonic lethal phenotype as a result of defective cardiac valve development (120, 121). To investigate the role of NFAT2 in the immune system, two groups used the approach of complementing RAG-1- or RAG-2-deficient blastocysts with homozygous NFAT2$-/-$ mutant ES cell lines (122, 123). T and B cells from the resulting chimeric mice lack NFAT2; these mice showed impaired repopulation of both thymus and peripheral lymphoid organs and reduced proliferation of peripheral lymphocytes compared with wild-type mice (122, 123). In an *in vitro* model of Th differentiation, the chimeric mice displayed decreased production of IL-4 and IL-6 but normal production of IFN-γ and TNF-α, demonstrating an impaired Th2 response in mutant T cells (122, 123). These results suggest that NFAT2 is an important positive regulator of Th2 cytokine expression; as discussed below, it is also likely to lack the negative feedback effects attributed to NFAT1 and NFAT4.

NFAT4. NFAT4 proteins are preferentially expressed in thymocytes, specifically in double-positive cells (102, 124). Targeted disruption of the NFAT4 gene resulted in impaired development of $CD4^+$ and $CD8^+$ single-positive thymocytes and peripheral T cells (124). Interestingly, the thymic defect of NFAT4$-/-$ mice was correlated with increased apoptosis of double-positive thymocytes; furthermore, NFAT4-deficient mice showed decreased expression of Bcl-2 mRNA and protein in single-positive thymocytes (124). These results suggest that NFAT4 plays a role in T cell development and survival.

Both NFAT1 and NFAT4. Mice doubly deficient for NFAT1 and NFAT4 showed a highly exaggerated version of the phenotype of NFAT1-deficient mice, displaying a lymphoproliferative disorder with massive numbers of mast cells in the spleen, very high serum IgG1 and IgE levels, and a dramatic increase in Th2 cytokines including IL-4, IL-5, IL-6, and IL-10 (125). This observation suggests that both NFAT1 and NFAT4 can induce the postulated negative feedback mechanism that terminates IL-4 gene transcription (119, 125); thus their combined loss permits initiation of IL-4 gene transcription via the positive function of NFAT2, but transcription may not shut off unless the antigenic stimulus is withdrawn.

Overall Evaluation of Genetic Approaches

The phenotypic effects observed in gene-disrupted mice can often be interpreted in multiple ways, especially if they are complicated by differences in the patterns of tissue expression of different transcription factors in a family or by potential redundancies in their biological functions. In this section we have illustrated these caveats by considering the biologically complex phenotypes of NFAT-deficient mice.

When transcription factors of the same family are coexpressed in a given tissue (as are NFAT1, NFAT2, and NFAT4 in immune cells), elimination of individual members may have no apparent effect. Thus despite the fact that IL-2 is the best-characterized NFAT-dependent gene (88, 126, 127), there is no impairment or only modest impairment of IL-2 production in T cells deficient for NFAT1 or NFAT2 (116, 119, 122, 123). One explanation is that these two NFAT proteins play no role in IL-2 gene transcription. Another is that they are redundant and substitute completely for one another in this respect.

Some of the effects of gene disruption may be erroneously ascribed to a deficiency of a specific family member, when they are actually due to a decrease in the overall levels or kinetics of induction of the biological activity involved. Thus disruption of NFAT1 is not accompanied by compensatory upregulation of other the NFAT proteins (117); the consequence is an 80–90% decrease in overall NFAT activity in NFAT1−/− cells. Presumably, the double knockout of both NFAT1 and NFAT4 has even lower levels of overall NFAT activity, and this should be taken into consideration when studying the exaggerated phenotype of NFAT1−/− NFAT4−/− mice.

Finally, certain unique and nonredundant effects of transcription factors may merely reflect nonoverlapping patterns of tissue expression. Thus one explanation for the cardiac defects in NFAT2−/− mice is that NFAT2 activates unique target genes in the heart. However, because NFAT2 is the only NFAT protein expressed in embryonic endocardial cells, if another NFAT protein were "knocked-in" to the NFAT2 locus and hence expressed in the same cells at the same stage of development, it might be able to rescue the embryonic lethal phenotype of NFAT2-deficient mice. Similar results have been obtained for other pairs of transcription factors with conserved DNA-binding domains but nonoverlapping tissue expression. For instance, the neonatal lethality of Engrailed-1 knockout mice is rescued by inserting the Engrailed-2 gene into the Engrailed-1 locus (128), while the rib cage defect of Myf5-deficient mice is rescued by inserting the myogenin gene into the Myf5 locus (129).

FINAL REMARKS AND PERSPECTIVES

We have reviewed data on the specific roles of selected transcription factors in cytokine production and T helper cell differentiation, as judged by results of genetic experiments involving transgenic and gene-disrupted mice. These experiments provide the most clearcut and easily interpretable evidence when the transcription factors function uniquely in a single signaling pathway, as is the case for STAT4 and STAT6. In contrast, if different members of a family are expressed in the same cells and have potentially overlapping functions, interpretation can be difficult, as we have illustrated for NFAT-deficient mice. Similarly, the phenotypes of transgenic mice are highly dependent on the level of overexpression achieved, as illustrated in the case of c-Maf transgenic mice.

We have also reviewed the case for chromatin remodeling as a determinant of cytokine production during T helper cell differentiation. While this field is still in its infancy, predictions based on the model can readily be tested in different systems. In particular, it is likely that signaling pathways initiated through antigen, cytokine, and costimulatory receptors in naive T cells result in the induction of many as yet unidentified Th1- and Th2-specific nuclear factors. These factors, which include the transcription factors GATA-3 and c-Maf in Th2 cells, may be involved in chromatin remodeling of cytokine genes in naive T cells, acute transcription of cytokine genes in differentiated T cells, or both. Given the importance of the Th1/Th2 immune response in several clinical situations, it is of considerable interest to understand the mechanisms by which subset-specific transcription factors and other nuclear proteins exert their pro-

found effects on T helper cell differentiation and function.

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