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Chromatin-based regulatory mechanisms governing cytokine gene transcription

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On initial contact with antigen, naive T cells differentiate and acquire effector characteristics, including the ability to transcribe specific cytokine genes rapidly and at high levels on subsequent exposure to antigen. Several effector T-cell subsets showing distinct patterns of cytokine gene transcription have been described. The patterns of cytokine expression in response to pathogenic challenges have a significant impact on the outcome of immune and inflammatory reactions. Here we review recent studies suggesting that the ability of naive T cells to differentiate into specific cytokine-expressing cells is regulated by epigenetic changes in the accessibility and chromatin structure of cytokine genetic loci. Antigen and cytokine stimulation of naive T cells activates diverse intracellular signaling pathways, which result in chromatin remodeling and demethylation of cytokine genes. These changes are likely to increase, in a stable and heritable fashion, the accessibility of these genes to the basal transcriptional machinery. Chromatin-based regulatory mechanisms may explain several features of cytokine gene expression in effector versus naive T cells, including their monoallelic expression, coordinate regulation, and stable maintenance in memory T cells. The hypothesis of epigenetic changes occurring during T-cell differentiation provides a framework for a comprehensive understanding of cytokine expression by T cells. (J Allergy Clin Immunol 1999;103:990-9.)

Key words: *Chromatin remodeling, DNA methylation, T-cell differentiation, T-cell activation, IL-4*

Abbreviations used
AP-1: Activator protein-1
BAF: *Brahma*-associated factors
BCL: B-cell lymphoma
CpG: Cytosine-guanine
NFAT: Nuclear factor of activated T cells
NFκB: Nuclear factor-κB
PIP2: Phosphatidylinositol (4, 5) bisphosphate
STAT: Signal transducer and activator of transcription

Unlike the development of most nonimmune tissues, which occurs as a single continuous process, lymphocyte development occurs in 2 distinct stages and cellular compartments (Fig 1). The first stage, which occurs in the thymus and the bone marrow for T and B lymphocytes, respectively, culminates in the cell surface expression of a functional antigen receptor. However, the lymphocytes at this stage are "naive"; they have not yet encountered any nonself antigens, and they are not capable of significant effector function. Naive lymphocytes emerge from the bone marrow and thymus into the peripheral lymphoid organs, where they become activated if they recognize antigen in the correct molecular and costimulatory context.

The second stage of lymphocyte differentiation occurs in the peripheral lymphoid organs and is triggered by the first encounter of the naive cells with antigen (Fig 1). Certain correlates of activation are observed rapidly (within minutes to hours) after antigen stimulation of naive T cells (ie, activation of intracellular signaling pathways; association of *Brahma*-associated factor [BAF] complexes with chromatin; cell surface expression of the activation antigen CD69; cell surface expression of CD25, a subunit of the high-affinity IL-2 receptor; and transcription of the *IL-2* gene and perhaps certain other cytokine genes).[1, 2] However, recently activated naive T cells typically show little effector function. In particular, the development of cytolytic T cells requires several days and several cycles of cell division, as does production of the cytokine IL-4. During this time period, cytolytic T-cell precursors begin to express effector proteins, such as granzymes and perforins, whereas T helper cells become capable of high-level cytokine expression.

The antigen-driven differentiation of naive into effector lymphocytes is at the heart of the immune response. Here we review recent work indicating that the first con-

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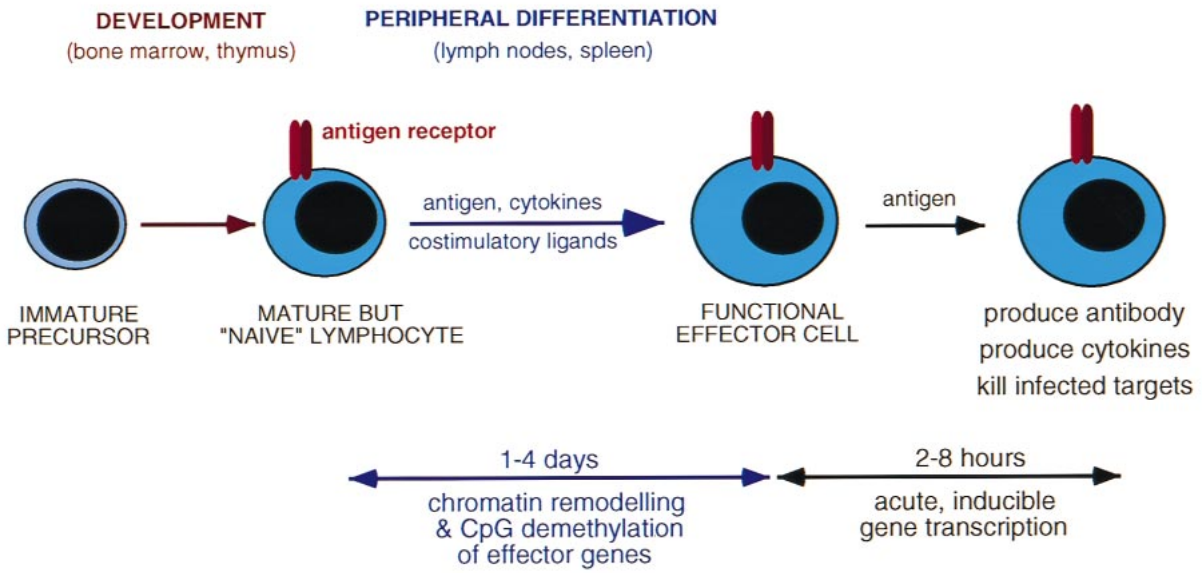

FIG 1. Two stages of lymphocyte differentiation. The first developmental stage occurs in the thymus or bone marrow and culminates in the cell surface expression of a functional antigen receptor. The second stage is triggered by signals through antigen and costimulatory and cytokine receptors, occurs in peripheral lymphoid organs, and results in the acquisition of mature effector functions, such as cytolysis and cytokine production. The circumstances of initial stimulation (ie, the strength of antigen and costimulatory signals and the identities and local concentrations of cytokines) determine both the qualitative nature and the magnitude of the effector response.

tact of naive T cells with antigen triggers epigenetic processes, such as chromatin remodeling and DNA demethylation, which influence the transcription of effector genes in the differentiated cells (Fig 1). Almost all the work discussed here relates to cytokine gene expression; however, very similar mechanisms may regulate the expression of other genes (eg, *FasL*, *CD40L*, *granzyme A*, and *perforin*), the products of which are also involved in determining effector function. To orient the reader, we have included a brief outline of T helper cell differentiation, a topic covered in more detail in several excellent reviews.[3-10]

CHROMATIN REMODELING, HISTONE ACETYLATION, DNA DEMETHYLATION, AND OTHER EPIGENETIC PROCESSES: A SHORT PRIMER

Cell differentiation may be defined as an ordered program of expression of tissue-specific genes, occurring in response to intrinsic (developmentally programmed) or extrinsic (local or environmental) stimuli. The expression of tissue-specific genes during cell differentiation is accompanied by chromatin "remodeling," a series of extensive changes in chromatin structure occurring over hundreds of kilobases of DNA. These changes include increased sensitivity to digestion by nucleases and restriction enzymes, increased histone acetylation of nucleosomal DNA, and decreased density of DNA methylation on cytosine-guanine (CpG) dinucleotides.[11-15] Although the changes often correlate with a low but detectable level of gene transcription in the precursor cell population, they occur before the high-level gene transcription characteristic of stably differentiated daughter cells.

Accessibility to DNAse I digestion

A crude but informative method of gauging gene "accessibility" in the chromatin context is to monitor the sensitivity of the relevant DNA sequences to digestion with DNAse I in intact nuclei.[16] Many kilobases of DNA within and around the gene of interest can be monitored by this method. In general, genes located in active chromatin that are actively transcribed or have the potential to be transcribed upon appropriate stimulation are more sensitive to DNAse I digestion than genes present in inactive or "closed" chromatin, which are not expressed in the cell type or developmental stage under investigation. Regions of strong DNAse I hypersensitivity often correlate with critical regulatory regions of the gene and may function as inducible or tissue-specific enhancers, locus control regions, matrix attachment regions, insulators/boundary elements, or sites of relief from transcriptional attenuation.[11] Once regions of strong DNAse I hypersensitivity have been identified, restriction enzymes may be used to obtain more quantitative information.[17] DNAse I hypersensitivity appears to reflect perturbations introduced by protein binding to nucleosomal DNA. When the hypersensitive sites have been closely mapped, binding sites for key transcription factors have been found within or immediately adjacent to the hypersensitive regions themselves.[18]

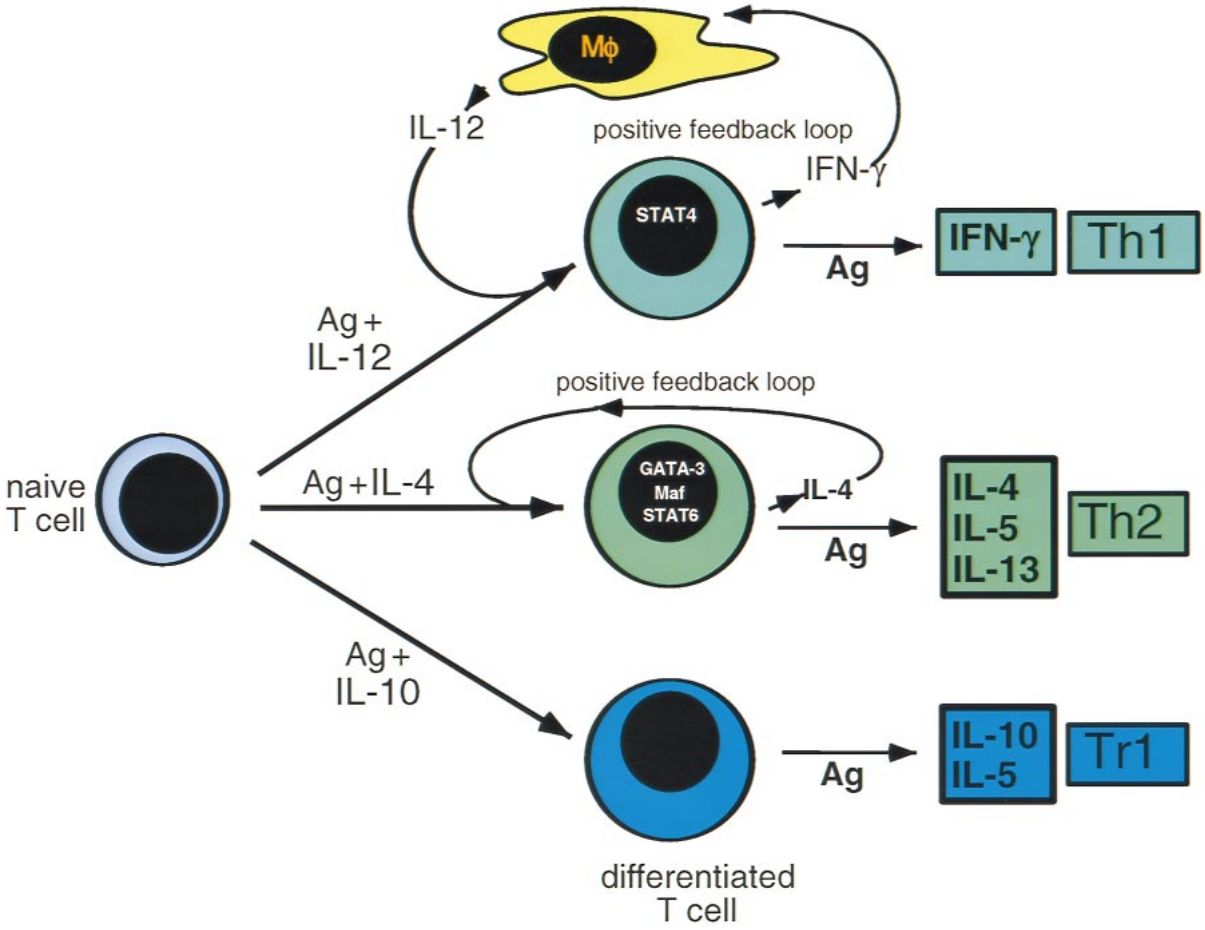

FIG 2. Differentiation of naive uncommitted T cells into IFN-γ–producing T_{H1} cells (*top*); IL-4, IL-5, and IL-13–producing T_{H2} T cells (*middle*); or IL-5– and IL-10–producing T_{R1} cells (*bottom*). The positive feedback loops involving IL-12/STAT4 and IL-4/STAT6 are depicted. T_{H2} cells constitutively express the transcription factors c-Maf (Maf) and GATA-3. *Ag,* Antigen.

DNA methylation and demethylation

CpG methylation of genetic loci strongly correlates with their transcriptional silencing in differentiated cells; conversely, undermethylated CpG islands correlate with regions of histone acetylation and nucleosome-free DNA.[12-15] Recent studies have provided an attractive mechanistic explanation. Methylated DNA is thought to recruit abundant methyl-CpG-binding proteins such as MeCP2, that in turn recruit corepressors, such as the SIN3 histone deacetylase complex.[19,20] In general, the methylation state of CpG dinucleotides is maintained through DNA replication, thus providing a plausible mechanism for how the differentiated state and the activity of specific genetic loci are maintained through successive cycles of cell division.[13]

The process of CpG demethylation during differentiation is less well understood. Developing embryos globally hypermethylate tissue-specific genetic loci on implantation; the methylation is constitutively inherited, except in the cell types in which the gene is scheduled to be expressed. Demethylation occurs on subsequent lineage commitment and correlates with gene activation and chromatin remodeling.[14] During cell differentiation, a cycle of DNA replication and nucleosome displacement may allow cell-specific transcription factors to bind to nearby enhancers, establishing a stable transcription complex that interferes with maintenance methylation and thereby inducing demethylation.[13]

Histone acetylation

It has recently become apparent that the access of transcription factors to DNA in nucleosomes is greatly facilitated by acetylation of the lysine-rich amino-terminal tails of the core histones in the nucleosomes.[12] The acetylation state of histones is dynamically regulated by the opposing actions of histone acetyltransferases and deacetylases. A large number of histone acetyltransferases have been identified, and many of these function as coactivators of transcription, making contact both with transcription factors and with the basal transcriptional machinery. Conversely, histone deacetylases interact

with transcriptional corepressors and with the core transcriptional apparatus.[12,13] A plausible speculation is that genes that are chronically repressed in differentiating cells may become targets for methyltransferases because such genes are characterized by dense CpG methylation in terminally differentiated cells.

T HELPER CELL SUBTYPES: CHARACTERISTIC PATTERNS OF CYTOKINE PRODUCTION

The T_{H1}/T_{H2} paradigm

The process of antigen-driven peripheral differentiation has been most extensively studied in CD4 T cells. Mature CD4 T cells have been categorized into different cytokine-producing subsets, depending on the particular pattern of cytokines that they produce (Fig 2).[3-7] The T_{H2} subtype of cytokine-producing cells is characterized by coordinate transcription of the *IL-4*, *IL-5*, and *IL-13* genes, which are closely linked on mouse chromosome 11 and human chromosome 5 (see below). Differentiation of naive T cells into T_{H2} cells is triggered by antigen and strongly potentiated by IL-4. IL-4 drives a powerful positive feedback loop that greatly increases its own synthesis. IL-4 activates the signal transducer and activator of transcription (STAT)6, and STAT6-deficient mice are severely compromised in their ability to differentiate toward the T_{H2} phenotype. The T_{H1} subtype of cytokine-producing cells characteristically transcribes the *IFN-γ* gene. This parallel differentiation pathway is elicited by antigen and IL-12, and requires the IL-12–activated transcription factor STAT4. This pathway also involves a strong positive feedback loop, mediated by IFN-γ–induced IL-12 release from macrophages. Most recently, it has been proposed that there exists yet another subtype of cytokine-producing cells, the T_{R1} subtype, which is characterized by production of high levels of IL-10 and IL-5 but low levels of IL-2 and IL-4.[7, 21] Like the differentiation of T_{H2} cells, which is potentiated in an autocrine fashion by IL-4, T_{R1} differentiation is triggered by antigen and strongly potentiated by IL-10 itself. However, less is known of the signaling pathways involved.

Positive and negative feedback effects

The strong polarizing effects of cytokines are initiated by the positive feedback effects outlined in Fig 2 and reinforced by powerful negative mechanisms that block transcription of the inappropriate cytokine genes. IL-4 downregulates IFN-γ expression in differentiating T cells by means of two independent mechanisms; it decreases the cell surface expression of the β_2-subunit of the IL-12 receptor, and it upregulates the expression of GATA-3 and Maf in differentiating T_{H2} cells.[8-10] Overexpression of GATA-3 or Maf, even in IL-4–deficient T cells, results in marked inhibition of IFN-γ production.[22,23] This was not due to direct repression of *IFN-γ* gene transcription because forced expression of GATA-3 in established T_{H1} clones had no effect.[22] Rather, the repressive effects appeared to be exerted at an early step of the differentiation process.[22,23]

The negative effect of IL-12 on IL-4 production is considerably less striking than the negative effect of IL-4 on IFN-γ production.[8] When IL-4 and IL-12 are both present in cultures of naive T cells at the time of antigen contact, the cells differentiate into IL-4–producing T_{H2} cells and lose IL-12R β_2-chain expression, suggesting that T_{H2} differentiation is dominated by the positive autocrine effect of IL-4 and is less susceptible to negative feedback from IL-12.

Deviations from the paradigm

Many established T-cell clones show nonoverlapping patterns of IL-4 and IFN-γ expression, thus completely fitting the T_{H1}/T_{H2} paradigm. Under physiologic conditions, however, the paradigm is better applied to populations than to single cells.[5] The strongest in vivo levels of T_{H1}/T_{H2} polarization are elicited by chronic antigen stimulation. With less chronic exposure to antigen, T_{H1} and T_{H2} patterns of cytokine expression may still be apparent at the population level, but individual T cells may show varied and complex patterns of cytokine expression. In this review we have avoided a rigid adherence to the T_{H1}/T_{H2} classification and instead have discussed all data in terms of how the transcription of individual cytokine genes might be regulated in single T cells.

At the single-cell level, stimulation of naive T cells yielded very limited numbers of cells that were detectably expressing any given cytokine mRNA other than IL-2, which was expressed rapidly and at high levels by 10% to 25% of the cells.[24] Nevertheless, a substantial fraction (20% to 40%) of IL-4–expressing cells also expressed IFN-γ mRNA at 20 hours after primary stimulation. With secondary stimulation, the population became more polarized because the fraction of cells inappropriately coexpressing IFN-γ and IL-4 declined.[24] To achieve strong ex vivo polarization within 48 hours, it was necessary to stimulate naive T cells in the presence of high concentrations of cytokines and neutralizing antibodies to the opposing cytokines.[25] These results are consistent with an earlier observation that in transgenic mice in which herpes simplex virus thymidine kinase was expressed under control of the IL-4 promoter, ablation of IL-4–producing precursor cells by inclusion of gancyclovir during the primary stimulation simultaneously eliminated IFN-γ–producing cells.[26] The implication is that antigen and costimulatory ligands are nonselective signals, eliciting moderate levels of transcription of both "T_{H1}" and "T_{H2}" cytokine genes, and that the major polarizing signals are provided by the cytokines IL-4 and IL-12.

The cytokine IFN-γ exerts a strong modulatory effect on T helper cell differentiation. Stimulation of naive T cells in the presence of both IL-4 and IFN-γ prevents IL-4–mediated downregulation of the IL-12R β_2-chain, thus promoting expression of the high-affinity IL-12 receptor and maintaining responsiveness to IL-12.[8] The resulting differentiated T cells show a mixed T_{H1}/T_{H2} phenotype because they are capable of expressing IFN-γ on subsequent stimulation in the presence of IL-12. Although this

unusual pattern of cytokine expression does not fit the T_{H1}/T_{H2} paradigm, T cells showing various degrees of IL-4/IFN-γ coexpression may in fact occur in the organism.

STAT-independent pathways

Experiments with B-cell lymphoma (BCL)-6–deficient mice suggest the existence of a STAT6-independent pathway of T_{H2} differentiation. T cells lacking the transcriptional repressor BCL-6 show a marked bias toward production of the T_{H2} cytokines IL-4, IL-5, and IL-13.[27] The same phenotype of T_{H2} bias was observed in T cells doubly deficient for STAT6 and BCL-6,[28] suggesting that BCL-6 inhibits a pathway of T_{H2} differentiation that does not involve IL-4 and STAT6.

IFN-γ gene transcription may also be regulated by STAT-dependent, as well as STAT-independent, pathways. Although $STAT4^{--}$ $STAT6^{++}$ T cells show a decrease in their ability to differentiate into IFN-γ–expressing T_{H1} cells,[8-10] T cells lacking both the STAT4 and STAT6 genes produce IFN-γ at appreciable levels.[29] This result is consistent with the negative feedback effect of IL-4, GATA-3, and Maf on *IFN-γ* gene transcription[8,22,23] and indicates that STAT6 inhibits a STAT4-independent pathway of *IFN-γ* gene expression.

Clinical significance

The question of how T cells differentiate down the T_{H1}, T_{H2}, or T_{R1} pathways is clinically quite significant because chronic disease states are often characterized by T_{H1}- or T_{H2}-dominant patterns of cytokine production.[5-7,21] T_{H2} responses involve production of IL-4, IL-5, IL-13, and eotaxin; are characterized by high IgE production and recruitment of mast cells and eosinophils; and are strongly associated with asthma and atopic disease. T_{H1} responses involve IFN-γ and TNF production, are characterized by the presence of activated macrophages and cytolytic T cells, and are associated with tissue injury, chronic inflammation, and autoimmune disease. T_{R1} responses are implicated in immune regulation and the development of tolerance. IL-10–producing T cells (T_{R1} cells, T_{H2} cells, and to some extent T_{H1} cells) exhibit striking immunoregulatory properties in various models of autoimmune and inflammatory disease.[6,7,21,30] IL-10, a powerful immunosuppressive and anti-inflammatory cytokine that downregulates the synthesis of a broad spectrum of proinflammatory cytokines by monocytes, macrophages, and neutrophils, has potential for treating diverse clinical conditions, including insulin-dependent diabetes, experimental autoimmune encephalomyelitis, allergic inflammation, transplant rejection, and tumor growth.

Historically, it has been difficult to demonstrate the existence of T_{H1} and T_{H2} cell subsets in humans. This most likely reflects the fact that the early experiments were performed with human peripheral blood cells, which contain a heterogeneous T-cell population. Many cells in adult peripheral blood have already been committed to particular patterns of cytokine expression through exposure to environmental antigens in vivo. However, it is now generally accepted that T-cell receptor and cytokine stimulation of naive human T cells under strongly polarizing conditions elicit T cells with the expected nonoverlapping patterns of cytokine expression.[3-5]

REGULATION OF CYTOKINE GENE EXPRESSION AT THE LEVEL OF CHROMATIN ACCESSIBILITY: A REVIEW OF THE AVAILABLE EVIDENCE

The *IL-4/IL-5/IL-13* locus

Several recent studies indicate that acquisition of the ability to transcribe the T_{H2} cytokines IL-4, IL-5, and IL-13 by naive T cells is regulated at the level of locus accessibility. Bird et al[31] reported that IL-4 production by differentiating T_{H2} cells required several cycles of cell proliferation, was accompanied by CpG demethylation of the *IL-4* and *IL-5* genes, was inhibited by cell cycle inhibitors, and was potentiated by inhibitors of histone deacetylases and DNA methyltransferases. These results are consistent with the hypothesis that a chromatin remodeling step is a prerequisite for *IL-4/IL-5/IL-13* gene transcription in T cells. DNA replication and concomitant nucleosome displacement during the cell cycle may be necessary to allow T_{H2}-specific transcription factors to bind to distal regulatory regions of the *IL-4/IL-5/IL-13* genetic locus. These factors may then recruit histone acetyltransferases, DNA demethylases, and chromatin remodeling enzymes, which stably establish an accessible chromatin configuration.

Agarwal and Rao[32] provided evidence, based on DNAse I hypersensitivity and methylation analysis, for chromatin remodeling of the *IL-4* and *IL-13* genes specifically during T_{H2} differentiation. Naive and T_{H1} cells displayed an inactive chromatin configuration on the *IL-4/IL-13* genetic locus, whereas T_{H2} cells acquired a chromatin configuration during differentiation that was consistent with an accessible locus. Remodeling of the *IL-4* locus was apparent within 48 hours of initial stimulation of naive T cells and was dependent on IL-4 and STAT6. The experiments were performed on resting cells that were not actively transcribing the cytokine genes, and therefore the results reflected differences in the transcriptional competence of the genetic loci rather than overt transcription of the genes.

Bix and Locksley[33] and Riviere et al[34] described a high frequency of monoallelic expression of the *IL-4* gene in individual IL-4–producing T cells, as previously also noted for expression of the *IL-2* gene.[35] The frequency of biallelic expression of IL-4 increased with increasing signal strength,[34] suggesting that gene expression involves a probabilistic or stochastic process that depends critically on the concentrations of one or more factors normally present at limiting levels but induced or activated by stimulation. Presumably, these factors are transcription factors or other nuclear proteins that effect locus opening and gene transcription.

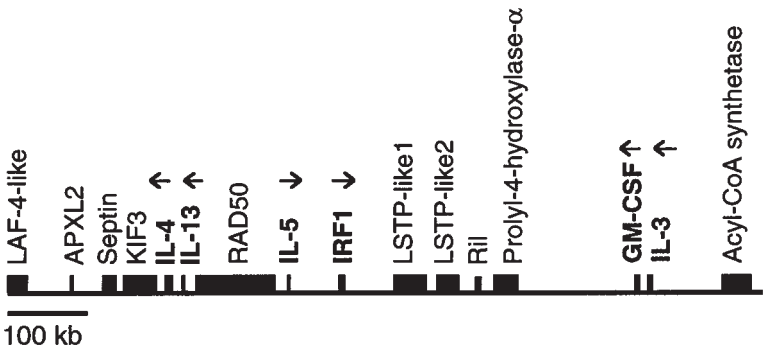

FIG 3. Organization of approximately 1000 kb of DNA from the cytokine gene cluster region of human chromosome 5q31 (syntenic to the cytokine gene cluster region on mouse chromosome 11). Sequence data are available for a total of 680 kb, including the region spanning the human *IL-4, IL-13, RAD50,* and *IL-5* genes. *Arrows* indicate the direction of transcription for selected genes.

The *IFN-γ* locus

A parallel program of locus remodeling may underlie acquisition of IFN-γ transcriptional capacity by T_{H1} cells. Naive cells make IFN-γ transcripts with delayed kinetics and at lower peak levels than T_{H1} cells. Bird et al[31] described cell-cycle dependence of IFN-γ transcription, although IFN-γ expression occurred after fewer replicative cycles than IL-4 expression. Agarwal and Rao[32] provided evidence for increased *IFN-γ* locus accessiblity in T_{H1} cells compared with naive and T_{H2} cells. Finally, several groups have shown that hypomethylation of the *IFN-γ* gene correlates with increased IFN-γ expression in T_{H1} cells.[36-38]

Inducible association of BAF complexes with chromatin

Primary stimulation of T cells was recently shown to elicit rapid activation of the BAF subset of chromatin-remodeling complexes.[2] The complexes bound strongly to chromatin in the nuclear matrix of activated, but not unactivated, cells. In vitro, association of the complexes with chromatin was enhanced by phosphatidyl inositol (4,5) bisphosphate (PIP2), a signaling intermediate the levels of which are potentially regulated by T-cell receptor signaling. The complexes contained both β-actin and an actin-related protein, BAF53. PIP2 is known to regulate actin function by displacing actin-binding proteins from actin, and therefore it is plausible that PIP2 regulates the targeting of BAF complexes to chromatin by modulating the function of a nuclear actin-binding protein.

Magnitude and kinetics of cytokine gene transcription by naive versus differentiated T cells

The strongest a priori argument for chromatin remodeling of cytokine genes in naive T cells is that these cells transcribe most cytokine genes at very modest levels and with very slow kinetics after activation. This has been well documented for the *IL-4* gene; detectable levels of IL-4 mRNA expression are not achieved until 16 to 24 hours after primary stimulation.[39,40] In contrast, differentiated T_{H2} cells express 100- to 1000-fold higher peak levels of IL-4 within 2 to 4 hours of stimulation. These data suggest that the process of T_{H2} differentiation is associated with increased accessibility of the *IL-4* genetic locus to RNA polymerase II, and that once "open," the locus remains accessible even in cells that have been rested for long periods in the absence of exposure to antigen. This feature of effective irreversibility is characteristic of differentiation processes in general and in the immune system is likely to underlie T-cell memory.

THE LINKED *IL-4, IL-5,* AND *IL-13* GENES: A COORDINATELY REGULATED CHROMOSOMAL LOCUS?

A particularly interesting feature of the T_{H2} pattern of cytokine production is that the *IL-4*, *IL-5*, and *IL-13* genes are located on a single chromosome in both humans and mice.[41-43] Of particular interest is the fact that genes that predispose to atopy and T_{H1}/T_{H2} development map to this region.[44,45] A physical map of this entire region (human chromosome 5q31) is available,[42] and it is syntenic to the corresponding region on mouse chromosome 11 (Fig 3). The region contains 2 cytokine gene clusters encoding the *IL-4*, *IL-5*, and *IL-13* genes and the *GM-CSF* and *IL-3* genes. In addition, the gene encoding interferon regulatory factor-1, which promotes T_{H1} development and is required for IL-12 secretion by activated macrophages, maps to this region.[9] The *IL-13* and *IL-4* genes are closely linked, approximately 12 kb apart, whereas the *IL-5* gene is more distant. It has the opposite orientation from the *IL-4* and *IL-13* genes and is separated from the *IL-4* gene by approximately 180 kb.[42,43] The *IL-5/IL-13* intergenic region contains the approximately 110-kb *RAD50* gene, which encodes a DNA repair protein that is expressed in disparate tissues.[42]

An attractive speculation is that the coordinate transcription of the *IL-4*, *IL-5*, and *IL-13* genes in T_{H2} cells and in mast cells results from this entire chromosomal locus becoming accessible to RNA polymerase II. Consistent with this hypothesis, T_{H2} differentiation was shown to be accompanied by chromatin remodeling of the *IL-4* and *IL-13* genes, as well as by increased consti-

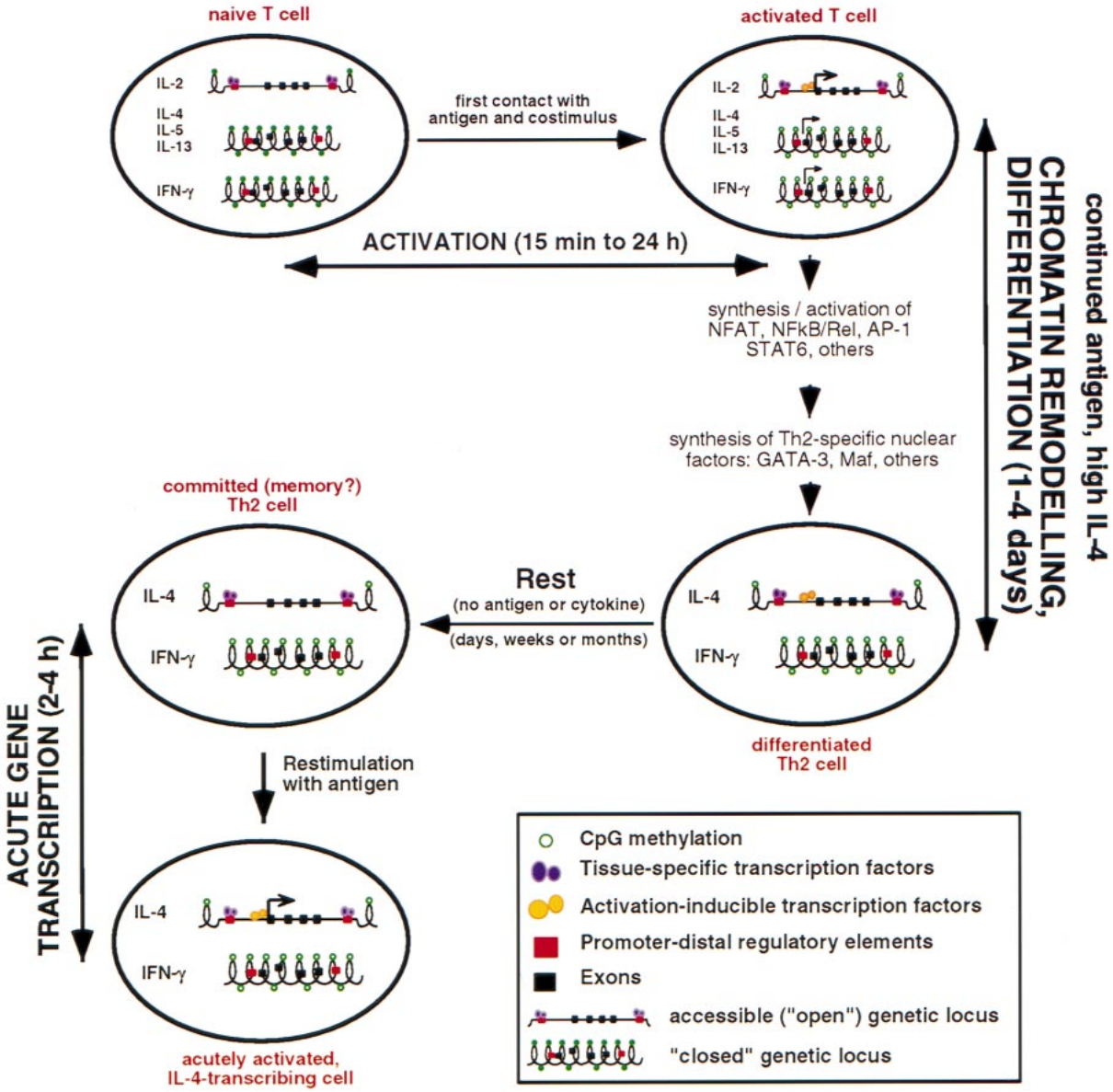

FIG 4. A multi-step model for cytokine gene transcription during T helper cell differentiation. Shown are changes in the chromatin structure and accessibility of the *IL-4* locus during T_{H2} differentiation. For details see text.

tutive expression of the *RAD50* gene.[32] Thus the entire approximately 180-kb region encompassing the *IL-13*, *IL-4*, *RAD50*, and *IL-5* genes may be subject to coordinate regulation in T_{H2} cells and in mast cells. T_{R1} cells constitute an apparent exception to this rule because they produce IL-5 but not IL-4.[21] It would be interesting to determine whether T_{R1} cells have remodeled the entire *IL-4/IL-5/IL-13* locus or only the *IL-5* gene. If the entire locus is remodeled, it would suggest that individual genes within this accessible locus are independently regulated and expressed, presumably at the level of acute transcription driven by promoter/enhancer regions.

A MULTI-STEP MODEL FOR CYTOKINE GENE EXPRESSION

These data suggest that effector gene expression by differentiated immune cells is preceded by a complex sequence of molecular events. The postulated stages, illustrated for the *IL-4* gene in Fig 4, are as follows.

Activation

Under this heading are grouped the early signaling events occurring within minutes after the first contact of naive T cells with antigen and costimulatory ligands.

They include calcium mobilization; increased turnover of phosphoinositides; activation of tyrosine and serine/threonine kinases and phosphatases; activation and synthesis of latent transcription factors, such as nuclear factor of activated T cells (NFAT), nuclear factor (NF)κB, Jun, and activation transcription factor-2; and association of BAF complexes with chromatin.[1,2] These early events lead within minutes or hours to new gene transcription, notably of immediate early genes, such as members of the activator protein-1 (AP-1; *Fos* and *Jun*) family, and to cell surface expression of activation antigens, such as CD69 and CD25 (IL-2R α-chain).

As outlined in a previous section, stimulation of naive T cells with antigen results in low-level transcription of multiple cytokine genes.[24] This phenomenon may be analogous to the germline transcription of antigen receptor loci that precedes V(D)J rearrangement or immunoglobulin class switching.[46] As such, it may be a marker for the early stages of chromatin remodeling.

Certain genes, such as the *IL-2* gene, are transcribed rapidly and at high levels, even by naive T cells. Presumably, this process requires an accessible genetic locus. Thus our model predicts that remodeling of the *IL-2* locus occurs during thymic development, preceding peripheral antigen-driven differentiation. Indeed, immature thymocytes lacking the T-cell receptor can produce IL-2 when stimulated pharmacologically with phorbol esters and calcium ionophores,[47] suggesting that the *IL-2* locus is accessible for transcription even at this very early stage of T-cell development.

Chromatin remodeling and differentiation

The key postulate of our model is that chromatin remodeling and CpG demethylation are critical steps of T-cell differentiation and are necessary for rapid and high-level transcription of effector genes by the differentiated T cells (Fig 4). Naive T cells display a repressed, transcriptionally incompetent chromatin structure over the *IFN-γ* and *IL-4/IL-5/IL-13* genetic loci that is likely to account for their slow kinetics and low levels of transcription. Our model proposes that differentiation signals derived from primary contact with antigen and polarizing cytokines elicit subset-specific remodeling of genes encoding cytokines and other effector proteins, which is necessary for mature effector function in the differentiated cells.

The process of chromatin remodeling may itself involve a sequence of multiple independent steps (Fig 4). Remodeling of the *IL4* locus requires both STAT6 and stimulation through the antigen receptor.[32] Although STAT proteins and antigen-induced transcription factors, such as NFAT and NFκB, may initiate locus remodeling, the nuclear localization of these inducible factors is rapidly terminated in the absence of continued stimulation.[32] Thus these factors are unlikely to be involved in maintaining an accessible locus in the differentiated cells. Rather, stimulation with antigen and cytokines must activate a differentiation-specific genetic program that results in the stable induction of nuclear factors expressed selectively in the appropriate T-cell subset. These subset-specific factors are presumed to bind to dispersed regulatory elements, thereby recruiting histone acetyltransferases and chromatin-remodeling enzymes to the appropriate genetic loci. The resulting accessible chromatin configurations are stably inherited and persist in differentiated cells in the absence of active transcription,[12,13] a molecular mechanism that may underlie T-cell memory.

The model predicts that STAT factors, and possibly other cytokine-induced transcription factors, act together with antigen-induced transcription factors, such as NFAT, NFκB, and AP-1, to induce subset-specific nuclear factors involved in chromatin remodeling of appropriate genetic loci. Consistent with this hypothesis, IL-4 and STAT6 are required for differentiation of naive T cells but are not essential for acute transcription of the *IL-4* gene by fully differentiated T_{H2} cells.[48] A silencer element at location 3′ of the *IL-4* gene has been identified in transient transfection assays and postulated to inactivate *IL-4* gene transcription in T_{H1} cells by means of the binding of ubiquitous nuclear factors.[49] T_{H2} cells were postulated to overcome silencing by means of binding of STAT6 to a site in the silencer element. However, this hypothesis cannot explain *IL-4* gene transcription by differentiated T_{H2} cells because STAT6 is not activated in resting T_{H2} cells stimulated with antigen alone.[40] Therefore it is likely that IL-4 and STAT6 play key regulatory roles only within a critical early window of T-cell differentiation and have more modest effects on gene transcription by differentiated effector T cells.[40,48]

The transcription factors GATA-3 and Maf are attractive candidates for target genes that are synergistically activated by antigen and STAT6 and play a role in *IL-4/IL-5/IL-13* locus opening during T_{H2} differentiation. Both proteins are expressed at low levels in naive T cells, remain at low levels in T_{H1} cells, and are upregulated in T_{H2} cells.[9,10] Members of the Maf and GATA families are known to control chromatin accessibility at a variety of other genetic loci.[50-52] Overexpression of Maf in M12 B cells, but not in T_{H1} cells, results in de novo expression of the endogenous IL-4 gene, whereas overexpression of GATA-3 in naive T cells or transgenic mice results in a bias toward T_{H2} differentiation.[9,10,22,23] As discussed above, these effects of GATA-3 and Maf are in part secondary to their strong negative influence on an early stage of *IFN-γ* gene expression.[22,23] It would be interesting to determine whether GATA-3 and Maf interfere with chromatin remodeling of the *IFN-γ* locus during T-cell differentiation.

Acute, inducible gene transcription by differentiated T cells

Restimulation of differentiated T cells with antigen recapitulates the early signaling events outlined for naive cells.[1,2] However, these cells, unlike naive T cells, have acquired the capacity to transcribe the appropriate cytokine genes rapidly and at high levels, presumably as a result of the chromatin remodeling that has occurred during differentiation (Fig 4). The acute transcriptional

response is most likely mediated by antigen-induced transcription factors that are activated and/or newly synthesized in response to antigen stimulation. These factors gain access to loci that are in the open configuration and promote rapid, high-level transcription of genes by binding to distal or promoter-proximal regulatory elements.[15] As discussed above, cytokine-induced transcription factors appear to play only a minor role in the acute transcription of cytokine genes by differentiated T cells.[40,49]

What nuclear factors control the acute transcription of cytokine genes? Many of the factors involved (eg, NFAT, NFκB, and AP-1) are likely to be antigen inducible and nonsubset specific, although different cytokine genes may preferentially utilize distinct NFAT and AP-1 family members.[9,10] Binding sites for NFAT proteins have been found in the promoters of the *IFN-γ*, *IL-4*, *IL-5*, and *IL-13* genes,[53] although there is some question as to whether the site in the IL-5 promoter is functional.[54] IFN-γ gene transcription in differentiated T_{H1} cells is modulated by the MAP kinase p38, presumably by means of its transcription factor targets ATF-2 and Jun.[55]

Subset-specific nuclear factors may also contribute to acute transcription of cytokine genes. The evidence derives largely from work on the *IL-4* gene.[9,10,22,23] A binding site for Maf-family proteins is immediately adjacent to one of the NFAT sites in the IL-4 promoter; coexpression of c-Maf and NFAT1 (NFATp) in M12 B cells results in activation of the IL-4 promoter and of the endogenous *IL-4* gene, an effect that is greatly potentiated by coexpression of the NFAT-interacting protein NIP45.[10,23] However, Maf overexpression does not directly influence expression of the *IL-5* and *IL-13* genes, suggesting that its effects may be specific for IL-4.[23] Similarly, mice transgenic for GATA-3 show a striking propensity, relative to normal mice, to express the T_{H2} cytokines IL-4, IL-5, IL-10, and IL-13,[9,10,22] and GATA-3 upregulates the activity of both the IL-4 and IL-5 promoters in transient transfection assays.[9,54]

Additional predictions and implications of the model

The model is consistent with several features of T-cell differentiation and can be used to make testable predictions. First, as noted above, differentiated T cells express most cytokines much more rapidly and at much higher levels compared with naive T cells, which is consistent with increased accessibility of the genetic loci in the differentiated cells. Second, naive T cells exhibit a much greater dependence on costimulation than differentiated T cells,[56] which is consistent with the possibility that nuclear factors upregulated by costimulation may be needed for chromatin remodeling rather than just for acute transcription. Third, T cells "remember" the context of their initial encounter with antigen in terms of their subsequent patterns of cytokine expression, which is consistent with the persistence and heritable propagation of the remodeled chromatin structures in the absence of antigenic stimulation. Fourth, naive T cells treated with azacytidine and trichostatin show more rapid kinetics of *IL-4* gene expression,[31] possibly because the global demethylation and histone acetylation observed with these drugs facilitates locus remodeling. Finally, The *IL-2* and *IL-4* genes tend to be monoallelically expressed, especially in the early stages of T-cell differentiation.[33-35] The stochastic nature of this process and its dependence on signal strength in the initial antigen stimulation suggests that it is governed by the availability of tissue-specific nuclear factors that regulate chromatin remodeling and locus demethylation. Presumably, these factors are present in limiting amounts in the naive T cell but are induced on activation. Monoallelic expression persisting after several stimulations may reflect late irreversible modification (eg, methylation) of the nontranscribed locus.

CONCLUSIONS AND PERSPECTIVES

In summary, work from several laboratories indicates that chromatin remodeling and changes in locus accessibility play a key role in regulating cytokine gene transcription in differentiating T cells. Although the complexity of this process is beginning to be appreciated, much additional work is needed to elucidate the molecular mechanisms events involved.

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REFERENCES

1. Alberola-Ila J, Takaki S, Kerner JD, Perlmutter RM. Differential signaling by lymphocyte antigen receptors. Annu Rev Immunol 1997;15:125-54.
2. Zhao K, Wang W, Rando OJ, Xue Y, Swiderek K, Kuo A, et al. Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. Cell 1998;25:625-36.
3. Mossman TR, Coffman RL. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145-73.
4. Carter LL, Dutton RW. Type 1 and Type 2: a fundamental dichotomy for all T-cell subsets. Curr Opin Immunol 1996;8:336-42.
5. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787-93.
6. O'Garra A. Cytokines induce the development of functionally heterogenous T helper cell subsets. Immunity 1998;8:275-83.
7. O'Garra A, Steinman L, Giijbels K. CD4+ T-cell subsets in autoimmunity. Curr Opin Immunol 1997;9:872-83.
8. Murphy KM. T lymphocyte differentiation in the periphery. Curr Opin Immunol 1998;10:226-32.
9. Rincon M, Flavell RA. T-cell subsets: transcriptional control in the Th1/Th2 decision. Curr Biol 1997;7:R729-32.
10. Szabo SJ, Glimcher LH, Ho IC. Genes that regulate interleukin-4 expression in T cells. Curr Opin Immunol 1998;9:776-81.
11. Ernst P, Smale ST. Combinatorial regulation of transcription I: general aspects of transcriptional control. Immunity 1995;2:311-9.
12. Wade PA, Pruss D, Wolfe AP. Histone acetylation: chromatin in action. Trends Biochem Sci 1997;4:128-32.
13. Kass SU, Pruss D, Wolffe AP. How does DNA methylation repress transcription? Trends Genet 1997;13:444-9.
14. Mostoslavsky R, Bergman Y. DNA methylation: regulation of gene expression and role in the immune system. Biochim Biophys Acta 1997;1:29-50.
15. Agarwal S, Rao A. Long-range transcriptional regulation of cytokine gene expression. Curr Opin Immunol 1998;10:345-52.

16. Gross DS. Nuclease hypersensitive sites in chromatin. Annu Rev Biochem 1988;57:159-97.
17. Boyes J, Felsenfeld G. Tissue-specific factors additively increase the probability of the all-or-none formation of a hypersensitive site. EMBO J 1996;15:2496-507.
18. Stamatoyannopoulos JA, Goodwin A, Joyce T, Lowrey CH. NF-E2 and GATA binding motifs are required for the formation of DNAse I hypersensitive site 4 of the human β-globin locus control region. EMBO J 1995;14:106-16.
19. Jones PI, Veenstra GJ, Wade PA, Verrmaak D, Kass SU, Landsberger N, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nature Genet 1998;19:187-91.
20. Nan X, Ng H, Johnson CA, Laherty CD, Turner BM, Eisenman RN, et al. Transcriptional repression by the methyl-CpG-binding protein MeCPs involves a histone deacetylase complex. Nature 1998;393:386-9.
21. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737-42.
22. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, et al. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 1998;9:745-55.
23. Ho IC, Lo D, Glimcher LH. c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and –independent mechanisms. J Exp Med 1998;188:1859-66.
24. Bucy RP, Karr L, Huang GQ, Li J, Carter D, Honjo K, et al. Single cell analysis of cytokine gene coexpression during $CD4^+$ T cell phenotype development. Proc Natl Acad Sci USA 1995;92:7545-69.
25. Nakamura T, Kamogawa Y, Bottomly K, Flavell RA. Polarization of IL-4- and IFN-γ-producing $CD4^+$ T cells. J Immunol 1997;158:1085-94.
26. Kamogawa Y, Minasi LE, Carding SR, Bottomly K, Flavell RA. The relationship of IL-4- and IFN-γ-producing T cells studied in lineage ablation of IL-4-producing cells. Cell 1993;75:985-95.
27. Dent Al, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science 1997;276:589-92.
28. Dent AL, Hu-Li J, Paul WE, Staudt LM. T helper type 2 inflammatory disease in the absence of interleukin 4 and transcription factor STAT6. Proc Natl Acad Sci USA 1998;95:12823-8.
29. Kaplan MH, Wurster AL, Grusby MJ. A signal transducer and activator of transcription (Stat)4-independent pathways for the development of T helper type 1 cells. J Exp Med 1998;188:1191-6.
30. Lalani I, Bhol K, Ahmed AR. Interleukin-10: biology, role in inflammation and autoimmunity. Ann Allergy Asthma Immunol 1997;79:469-83.
31. Bird JJ, Brown DR, Mullen AC, Moskowitz NH, Mahowald MA, Sider JR, et al. Helper T cell differentiation is controlled by the cell cycle. Immunity 1998;9:229-37.
32. Agarwal S, Rao A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 1998;9:765-75.
33. Bix M, Locksley RM. Independent and epigenetic regulation of the interleukin-4 alleles in $CD4^+$ T cells. Science 1998;281:1352-4.
34. Riviere I, Sunshine MJ, Littman DR. Regulation of IL-4 expression by activation of individual alleles. Immunity 1998;9:217-28.
35. Hollander GA, Zuklys S, Morel C, Mizoguchi E, Mobisson K, Simpson S, et al. Monoallelic expression of the interleukin-2 locus. Science 1998;279:2118-21.
36. Young HA, Ghosh P, Ye Jianping, Lederer J, Lichtman A, Gerard JF, et al. Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-γ gene. J Immunol 1994;153:3603-10.
37. Melvin AJ, McGurn ME, Bort SJ, Gibson C, Lewis DB. Hypomethylation of the interferon-γ gene correlates with its expression by primary T-lineage cells. Eur J Immunol 1995;25:426-30.
38. Fitzpatrick DR. Distinct methylation of the interferon gamma (IFN-gamma) and interleukin 3 (IL-3) genes in newly activated primary CD3+ T lymphocytes: regional IFN-gamma promoter demethylation and mRNA expression are heritable in CD44(high)CD8+ T cells. J Exp Med 1998;188:103-17.
39. Croft M, Swain SL. Recently activated naive CD4 T cells can help resting B cells, and can produce sufficient autocrine IL-4 to drive differentiation to secretion of T helper 2-type cytokines. J Immunol 1995;154:4269-82.
40. Lederer JA, Perez VL, DesRoches L, Kim SM, Abbas AK, Lichtman AH. Cytokine transcriptional events during helper T cell subset differentiation. J Exp Med 1996;184:397-406.
41. Marsh DG, Neely JD, Breazeale DR, Ghosh B, Freidhoff LR, Ehlich-Kautzky E, et al. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science 1994;264:1152-6.
42. Frazer KA, Ueda Y, Zhu Y, Gifford VR, Garofalo MR, Mohandas N, et al. Computational and biological analysis of 680 kb of DNA sequence from the human 5q31 cytokine gene cluster region. Genome Res 1997;7:495-512.
43. Koyano-Nakagawa N, Arai K. Specific versus cooperative regulatory mechanism of the cytokine genes that are clustered on the same chromosome. J Allergy Clin Immunol 1996;98:5174-82.
44. Postma DS, Bleecker ER, Amelung PJ, Holroyd KJ, Xu J, Panhuysen CI, et al. Genetic susceptibility to asthma-bronchial hyperresponsivenss coinherited with a major gene for atopy. N Engl J Med 1995;33:894-900.
45. Gorham JD, Güler ML, Steen RG, Mackey AJ, Daly MJ, Frederick K, et al. Genetic mapping of a murine locus controlling development of T helper 1/T helper 2 type responses. Proc Natl Acad Sci USA 1996;93:12467-74.
46. Sleckman BP, Gorman JR, Alt FW. Accessibility control of antigen-receptor variable-region gene assembly: role of cis-acting elements. Annu Rev Immunol 1996;14:459-81.
47. Rothenberg EV, Ward SB. A dynamic assembly of transcription factors integrates activation and cell-type information for interleukin 2 gene regulation. Proc Natl Acad Sci USA 1996;93:9358-65.
48. Huang H, Paul WE. Impaired interleukin 4 signaling in T helper Type 1 cells. J Exp Med 1998;187:1305-13.
49. Kubo M, Ransom J, Webb D, Hashimoto Y, Tada T, Nakayama T. T-cell subset-specific expression of the IL-4 gene is regulated by a silencer element and STAT6. EMBO J 1997;16:4007-20.
50. Blank V, Andrews NC. The Maf transcription factors: regulators of differentiation. Trends Biochem Sci 1997;22:437-41.
51. Motohashi H, Shavit JA, Igarashi K, Yamamoto M, Engel JD. The world according to Maf. Nucleic Acids Res 1997;25:2953-9.
52. Weiss MJ, Orkin SH. GATA transcription factors: Key regulators of hematopoiesis. Exp Hematol 1995;23:99-107.
53. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 1997;15:707-47.
54. Zhang D, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem 1997;272:21597-603.
55. Rincon M, Enslen H, Raingeaud J, Recht M, Zapton T, Su MS, et al. Interferon-γ expression by Th 1 effector T cells mediated by the p38 MAP kinase signaling pathway. EMBO J 1998;17:2817-29.
56. Bluestone JA. New perspectives of CD28-B7-mediated T cell costimulation. Immunity 1995;2:555-9.