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Review

Cytokine Signaling/Transcription Factor Cross-Talk in T Cell Activation and Th1-Th2 Differentiation

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Abstract. The secretion of interleukin 2 (IL-2) is a key event in T cell activation. IL-2 allows T cells to enter into the S phase of the cell cycle and divide. After the activation phase takes place, T lymphocytes proliferate and differentiate to generate effector T cells. Thereby, T helper (Th) precursor cells, which are functionally immature, may become Th1 or Th2 effector cells. These subsets are responsible for cell-mediated immunity and humoral responses, respectively. Both T cell activation and Th differentiation are processes that depend on changes in the pattern of gene expression. The expression and changes in the genes responsible for these events are regulated by transcription factors. This review will focus on both the transcription factors involved in the control of IL-2 as well as those that are key to T helper differentiation.

Key words: T cell differentiation; T cell activation; TCR; IL-2; GATA-3; T-bet.

Introduction

To achieve activation, T lymphocytes need two different types of signals. One signal is specific and requires the interaction of the T cell receptor (TCR) with the antigen or major histocompatibility complex (MHC). The other signal rises from the interaction of the accessory molecules (or cytokines) expressed by the T lymphocyte and the antigen-presenting cell (APC). Upon activation, T cells proliferate and differentiate to generate effector T cells. First, during the proliferation phase, there is an expansion of T lymphocytes that are specific to the antigen and an amplification of the specific clonal response. Second, lymphocytes evolve from cells with a basic function of recognition to cells that eliminate antigens or help to do so.

Interleukin 2 (IL-2) is a growth factor for antigen-stimulated T lymphocytes which is responsible for T cell clonal expansion. After clonal expansion occurs, cellular differentation takes place, which allows an adequate response to each different type of antigen. T lymphocytes differentiate from uncommitted precursor cells to sophisticated T helper (Th) and T cytotoxic effector cells. Th1 cells participate in phagocyte-mediated defence against intracelullar microbes and Th2 cells in humoral and eosinophil/mast cell-mediated immune reactions against helminthic and arthropod infections. The function of Th cells can be explained by the cytokines they secrete. The main Th1 effector cytokine is interferon γ (IFN-γ). This activates macrophages, enhancing their microbicidal actions and stimulates the production of immunoglobulin G (IgG) antibodies,

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which are involved in opsonization and phagocytosis. The hallmark cytokines of Th2 cells are IL-4 and IL-5. IL-4 induces the switch towards IgE production and therefore participates in IgE-dependent mast cell activities. IL-5, which activates eosinophils, participates in eosinophil responses to helminths. Moreover, IL-4 and IL-5 contribute to B cell proliferation and differentiation.

Both IL-2-dependent clonal expansion and Th1-Th2 differentation are processes that depend on changes in the pattern of gene expression and transcription factors (TF), which are key molecules in the transcriptional control of these target genes. Thus, TF function is essential as a link between the transient signaling events triggered by TCR/accessory molecule-mediated activation and the nuclear events responsible of sustained changes in cellular phenotype. Therefore, in this review we will focus on the TF-involved in the transcriptional control of IL-2, which is the key cytokine involved in T cell clonal expansion, as well as TF that drive Th1-Th2 differentiation.

Regulation of IL-2 Gene Transcription

Optimal IL-2 production and T cell activation requires the engagement of costimulatory receptors and the TCR. One of the most important costimulatory pathways[29] results from the engagement of CD28 with B7 expressed on APC[48, 50]. When B7 binds to the CD28 molecule, in T cells stimulated by ligands of the TCR, signals are generated that increase IL-2 gene transcription when compared with TCR stimulation alone (Fig. 1). CD28 exerts both nuclear and post-nuclear effects. The post-nuclear effects are more striking than the nuclear effects because CD28 costimulation causes a great increase in the stability of IL-2 mRNA[133].

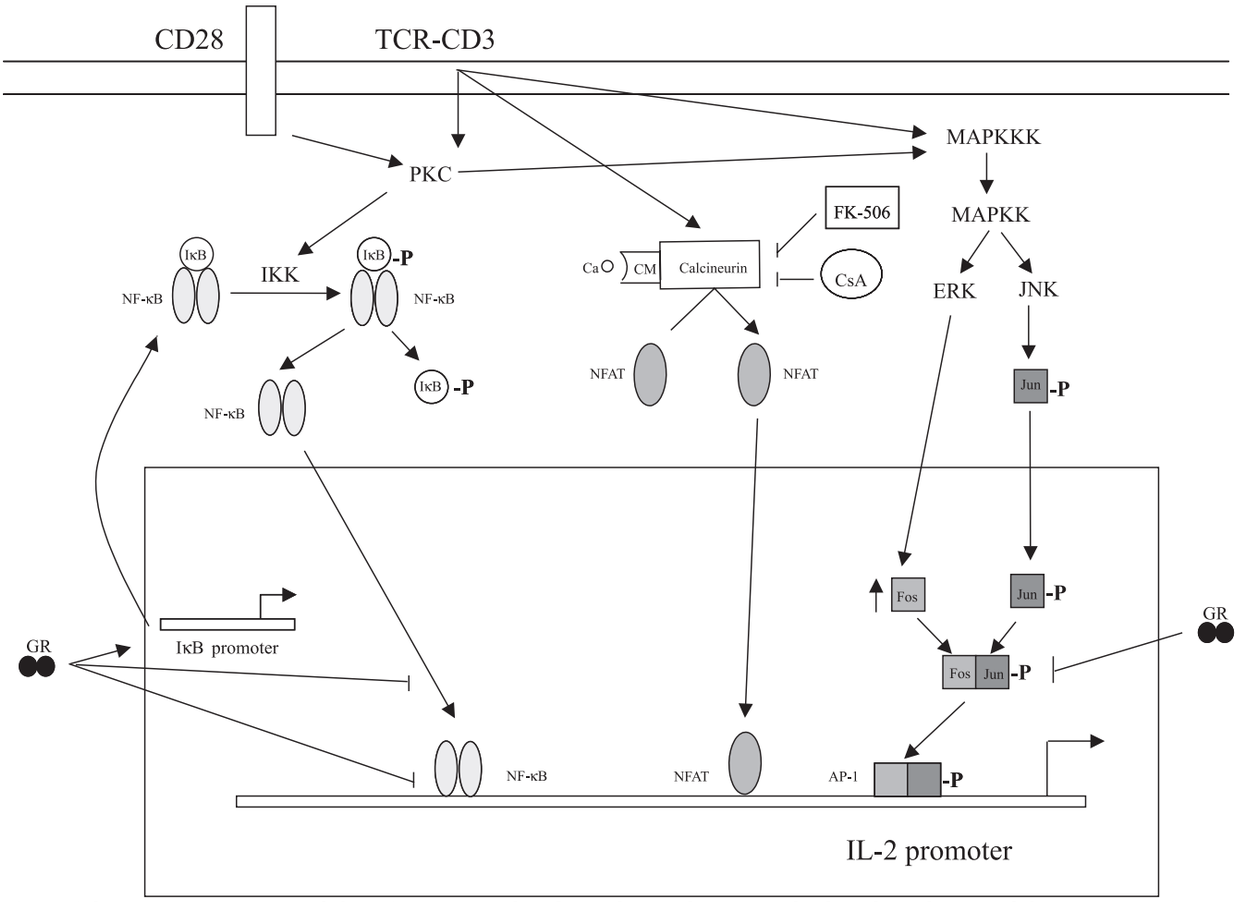

Fig. 1. Schematic representation of the TCR/CD28-mediated signaling pathways involved in the induction of the IL-2 gene. A more detailed analysis of specific transcription factor concensus sites on the promoter is shown in Fig. 2. Calcineurin has been used as a target for immunosuppressive drugs such as cyclosporin A (CsA) and FK-506. Glucocorticoid-induced inhibition by glucocorticoid receptor (GR)-mediated cross-talk is shown. Abbreviations: mitogen-activated protein kinase kinase kinase (MAPKKK), mitogen-activated protein kinase kinase (MAPKK), extracellular-regulated kinase (ERK), activating protein-1 (AP-1), nuclear factor of activated T cells (NFAT), inhibitor of NF-κB (IκB), IκB kinase (IKK)

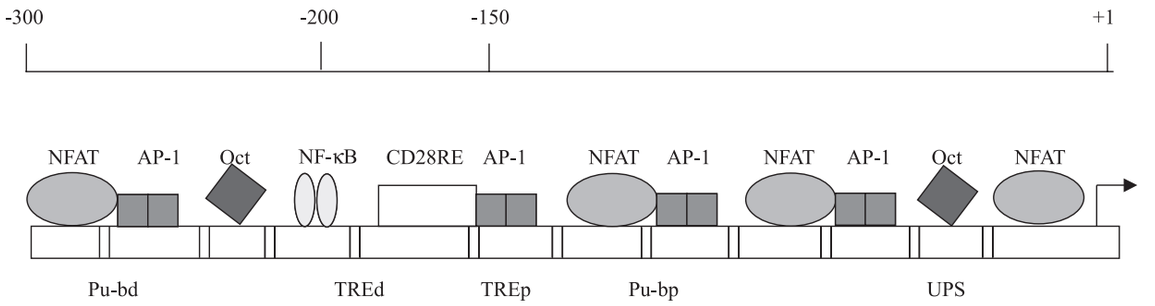

Fig. 2. Schematic representation of the IL-2 promoter with the binding sites of the principal regulation of the IL-2 gene. The CD28RE corresponds to an NF-κB/NFAT site. Abbreviations: upstream promoter site (UPS), proximal and distal purine box (Pu-bp and Pu-bd, respectively), proximal and distal TPA responsive element (TREp and TREd, respectively)

IL-2 gene expression is regulated mainly at the transcriptional level, where three different mechanisms are involved to guarantee T cell lineage-specific activation of IL-2 expression: cooperative assembly of transcription factors within the IL-2 gene, cell-type specific changes in chromatin architecture, and monoallelic transcription[38]. A minimal 300 bp promoter/enhancer region upstream from the transcriptional start site is sufficient for tissue-specific expression of the IL-2 gene[28] (Fig. 2). It contains sites for the binding of different transcription factors[109], including inducible proteins such as nuclear factor of activated T cells (NFAT), activating protein 1 (AP-1), nuclear factor κB (NF-κB) and constitutive factors such as Octamer proteins (Oct)[43, 104]. Point mutations or deletions at these sites cause severe alterations on IL-2 gene transcription. Thereby, the induction of the IL-2 promoter in response to activating stimuli depends on the binding of multiple distinct factors in a fine-tuned cooperative manner at multiple binding sites[34]. The most important of these TF (i.e. AP-1, NFAT, Oct and NF-κB) are discussed in detail below.

There are also several DNA segments outside the promoter that exhibit either a positive or negative control on the promoter activity. The region between –321 and –578 is responsible for an increase in promoter activity, whereas the sequences between –578 and –1219 have a negative effect on promoter activity[86]. There is also a fragment located between positions –413 and –502 of the IL-2 5' region which acts as a distal enhancer and contains a binding site for Oct and GATA factors and two binding sites for Ets-like factors (GABPα and β, which are targets of Raf/MEK/ERK cascade)[93, 128]. The negative regulation of IL-2 gene transcription may be important for the rapid shut-off of IL-2 transcription. A nucleotide sequence element named NRE-A was identified that negatively regulates IL-2 expression[138]. Binding of Zeb protein to the NRE-A element is implicated in the silencing of the IL-2 gene in Th2 cells[139], but this repressor is not T cell specific.

Studies on the chromatin structure by *in vivo* DNase I digestion of chromosomal DNA shows that IL-2 gene has a T cell lineage-specific architecture, which is altered upon activation. The IL-2 proximal promoter exists in a closed conformation in resting T and non-T cells. Within this region, there are no tissue-specific differences before stimulation. However, DNase I footprinting of the distal –600 to –300 bp region revealed multiple tissue-specific stimulation-independent DNase I hypersensitive residues. There is evidence for T cell-specific protein-DNA interactions within this region, such as TCF/LEF or HMG family and Oct family components. When stimulation takes place, new hypersensitive sites appear in the proximal and distal enhancer regions, implying a functional interaction between these two domains[135]. Thereby, the distal site may serve as a concentrator of tissue-specific factors and also as an initiator site for activation-dependent chromatin remodeling.

As mentioned above, there is a third regulatory mechanism in the control of IL-2 gene transcription: monoallelic expression[39]. Monoallelic expression is an epigenetic phenomenon in which only a single copy of a given gene is expressed. The exact molecular mechanisms regulating monoallelic IL-2 transcription are unknown. Basically, monoallelic expression can be regulated by two different mechanisms: a) in one model, a single allele of IL-2 is randomly marked (for example by methylation) and silenced; b) in a second model, transcription of IL-2 can be achieved by a limited concentration of specific transcription factors, which allows for the occupancy of only one of the two promoters of the IL-2 locus. As an effective mechanism for tight transcriptional control of the IL-2 gene, mono-

allelic expression may act to avoid a harmful deregulation of an immune response as a consequence of increased IL-2 production. More generally, single-allele expression of growth and differentiation factors may be important because cytokines mediate biological effects in a dose-dependent manner.

AP-1

AP-1 is a family of basic region leucin zipper of transcription factors composed of Jun isoforms (v-Jun, c-Jun, JunB, JunD) which can homo- or hetero-dimerize with Fos proteins (v-Fos, c-Fos, FosB, Fra1, Fra2) or subfamily members named activated transcription factor (ATF-2, TF-3, B-ATF). The DNA sites recognized by Jun homodimers or heterodimers with fos are phorbol 12-O-tetradecanoate-13-acetate (TPA)-responsive elements (TRE). When Jun interacts with ATF the target response elements are cyclic adenosine monophosphate (cAMP)-responsive elements (CRE). Nevertheless, AP-1 proteins can also participate at other regulatory sites in the IL-2 promoter.

Two sites in the IL-2 promoter share nucleotides with the TRE consensus sequence (Fig. 2): TREp (located at positions –153 to –147) and TREd (–187 to –181)[110]. TREp is an AP-1-binding site of low affinity[21, 47]. AP-1 was also found to bind to the upstream promoter site (UPS), where it is associated with Oct factors. Furthermore, AP-1 factors bind to the two NFAT binding sites of the IL-2 promoter. Members of the ATF-1/CREB family have been shown to transactivate the TREp site[12], representing a complex that may act to regulate the TREp site. Although Jun/Jun homodimers can transactivate AP-1 sites, Fos/Jun heterodimers are more potent transactivators[105]. The TREp site is a "low-affinity" site for Fos/Jun heterodimer[42], and may require a specific combination of Fos/Jun family members for activation. By contrast, the NFAT/AP-1 site and consensus AP-1 sites are "high--affinity" sites and can be transactivated with both Fos/Jun and Jun/Jun dimers[97]. Several Fos family members, such as Fra1, Fra2, and an alternatively spliced form of FosB, lack transcriptional activation domains[141]. These transcriptionally inactive forms might have preferential affinity for the TREp site while not efficiently binding and interfering with transactivation of the NFAT/AP-1, Oct/AP-1, or consensus AP-1 sites.

Overexpression of c-Fos resulted in a drastic increase of IL-2 synthesis in T cells[88], whereby c-Fos participates in the induction of the IL-2 promoter. However, c-Fos-deficient mice proliferate normally and secrete IL-2 in response to TCR stimulation[46], suggesting that other Fos family members substitute for c-Fos. This is also true for the substitution of c-Jun in T lymphocytes lacking a functional c-jun gene.

CD28 costimulation enhances transactivation mediated by the consensus AP-1 site in transgenic mice; the mechanism may involve c-Jun kinase (JNK)-family kinases[121]. The CD28RE works in conjunction with the adjacent AP-1 site[75]. AP-1, as described below for NF-κB, is a target for glucocorticoid inhibitory action[20].

NFAT

NFAT is a family of transcription factors implicated in the control of several cytokine syntheses, such as those of IL-2, IL-4, IL-5, granulocyte monocyte colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNF-α). They play an important role in activation and proliferation events dependent on inducible gene expression in the immune system.

In resting T cells, NFAT activity resides in the cytoplasm as phosphorylated forms. Upon activation through the TCR sustained increases in intracytoplasmatic calcium concentration activates calmodulin, which binds to calcineurin which modifies its autoinhibitory domain conformation and activates its own phosphatase activity. This desphosphorylates NFAT which is translocated into the nucleus. In the nucleus, NFAT associates with other transcription factors where it transactivates a variety of cytokines and other activation genes.

The IL-2 promoter contains two NFAT binding sites: the proximal and distal purine boxes, Pu-bp and Pu-bd[127]. NFAT is the target through which cyclosporine A (CsA) and FK-506 inhibit IL-2 promoter induction[23, 98]. The NFAT complex that binds to the distal NFAT site is a heterodimer: NFATn (the nuclear component, which is newly synthesized by TPA) and NFATc (the cytosolic component, CsA and FK-506 sensitive, which requires Ca^{2+} mobilization)[26]. NFATn corresponds to AP-1, suggested because a dominant negative mutant of c-Jun inhibits Pu-bd activity[97]. Thereby, NFAT proteins bind cooperatively with Fos- and Jun-family proteins to the distal IL-2 promoter[44, 74]. An alteration of the AP-1 site in the distal NFAT site eliminates transcription of the IL-2 promoter even though the proximal NFAT site is intact[145]. Moreover, the overexpression of Fos and Jun augments NFAT-driven transcription[45, 84]. The proto-oncogene product Vav enhances transcription of the IL-2 gene, and this is mediated by activation of the distal NFAT element in the IL-2 gene promoter. In NFAT-IL-2 activation by Vav,

a Rac1-dependent JNK/c-Jun/AP-1 pathway, plays a predominant role, rather than the Ca^{2+}/NFAT pathway[51]. Also, NFAT TF binds to the CD28RE[104].

Oct

The promoter of IL-2 contains two Oct-binding sites and is partially activated by the Oct proteins[52]. Point mutation within either site reduces the activity of IL-2 promoter, but mutation of both abolishes the promoter function[145].

The proximal Oct site binds Oct and AP-1 proteins[131, 132]. Mutation of both the Oct and AP-1 sites eliminates IL-2 promoter function even thought the distal Oct site is unchanged.

NF-κB

The NF-κB TF family includes NF-κB1 (p50/p105), NF-κB2 (p52/p100), Rel A (p65), cRel and RelB. The transcriptional regulation of target genes requires the binding of NF-κB dimers to κB DNA-binding sites. NF-κB proteins are retained in the cytoplasm of unstimulated cells in an inactive form via their interaction with inhibitor of NF-κB (IκB) proteins which prevents NF-κB nuclear translocation. In response to adequate stimuli, IκB proteins are phosphorylated and undergo polyubiquitination and degradation by the 26S proteasome pathway. Then, NF-κB dimers are translocated to the nucleus where they regulate gene expression. Finally, the NF-κB protein is resequestrated in the cytoplasm by newly synthesized IκB.

The IL-2 promoter contains binding site for NF-κB factors[40, 67, 109, 112]. The NF-κB site is located around position –200, and it is a low-affinity but important site for IL-2 promoter activity after T cell activation[40, 67]. Immediately after stimulation the heterodimer p50--p65[53, 63] binds to the IL-2 promoter. The p50/p50 homodimers, which lack a transactivation domain and are transcriptionally inactive, bind with high affinity to the NF-κB site, thereby they could be involved in the negative control of the promoter activity. These dimers are constitutively present in nuclear extracts of several T cell clones, and upon activation of these cells there is a decrease in the level of p50 dimers in the nucleus and an increase in the level of p50-p65 heterodimers by a mechanism which could involve the inhibitor Bcl-3[27], because Bcl-3 could be sequestrating the p50 homodimers[30, 68].

The CD28RE binds Rel-family proteins, and signaling through CD28 results in down-regulation of IκB and translocation of c-Rel into the nucleus[11]. T cells from mice deficient in p50 NF-κB show a decreased response to CD28 costimulation[111]. Glucocorticoids are very potent endogenous and pharmacological immunosuppressors acting on IL-2 and other cytokines. One of the most well-known mechanisms of this inhibition is the one exerted on NF-κB, by which glucocorticoids act by protein-protein interaction and by inducing IκB. This mechanism has been described to be important also in non-immune cells[7, 15, 16, 20, 76, 82, 106].

IL-2-Mediated Clonal Expansion and Activation

Once activated, IL-2 plays a central role in the clonal expansion of activated T lymphocytes. IL-2 has a suppresive effect on cell-cycle inhibitors (IL-2 eliminates Cdk inhibitor protein p27 Kip1), allowing T lymphocytes to enter the S phase and proliferate[25, 85]. Phosphorylation and inactivation of the forkhead TF Fox O3 by IL-2 is important for T cell proliferation and survival because Fox O3 can induce p27 Kip1 and the proapoptotic Bcl-2 family member BIM[117]. One of the essential events in IL-2-induced G_1 progression is the up-regulation of the cell cycle regulator E2F TF activity by phosphatidylinositol 3-kinase (PI3K)[10]. The increment in E2F activity in the mid- to late-G_1 phase allows the expression of E2F target genes and entry into the S phase[32].

IL-2 acts on the same cell to induce its own synthesis and therefore acts as an autocrine growth factor but it may also stimulate proliferation of adjacent T cells, thereby functioning as a paracrine factor. IL-2 also has effect on the differentiation of cells other than T lymphocytes. IL-2 stimulates the proliferation of natural killer (NK) cells and enhances their cytolytic function[114], on B cells IL-2 acts as a growth factor and stimulates antibody synthesis[78], and it promotes differentiation, proliferation and cytolytic capacity of macrophages[9].

IL-2 is a critical factor involved in programmed cell death[66]. Activation of T cells in the presence of IL-2 induces apoptosis of these cells mediated by Fas. Knockout mice that lack IL-2 or its receptor develop autoimmunity, reflecting a role of IL-2 in T cell apoptosis[4]. On the other hand, antiapoptotic actions for IL-2 have also been described. Activation of PI3K and its downstream effector, protein kinase B (PKB), is important for the survival functions mediated by IL-2[18]. There are many proapoptotic proteins which can be phosphorylated and inhibited by PKB, including BAD, human caspase 9, and the forkhead family of transcription factors. PKB can also cause stimulation of NF-κB, allow-

ing the transcription of genes involved in promoting survival, such as the bfl-1[147]. In addition, E2F-mediated transcription can also be activated. Moreover, overexpression of activated PKB has been shown *in vivo* to enhance the resistance of thymocytes and T cells to apoptotic stimuli and to promote survival following antigenic activation[49]. The transcription factors activated by PI3K and PKB are of great interest in the IL-2 response, as they regulate genes responsible for determining whether activated T cells survive, proliferate, or die. The c-Myb transcription factor is implicated in the regulation of proliferation, differentiation, and apoptosis[89]. During T cell activation, cell-cycle progression in response to IL-2 receptor (IL-2R) signaling is associated with induction of c-Myb, with the highest levels seen around late G_1[118]. c-myb is activated by PI3K and PKB following IL-2 stimulation. Activation of endogenous c-Myb can be blocked by chemical inhibitors of PI3K and NF-κB. Blocking Myb results in an increase in apoptosis which cannot be rescued by Bcl-2, while overexpressing v-Myb can protect activated cells from death[62].

Th Differentiation

When an immune response occurs, naive Th cells produce IL-2 and proliferate. After entering the cell cycle the progeny become competent to produce effector cytokines. Triggering of the TCR upon engaging with MHC peptide results in T cell proliferation and differentiation into different cell fates that determine the class of immune response. CD4 T cells can polarize into two distinct subsets characterized by their function and by the cytokines they secrete. These subsets are Th1 and Th2 cells, which produce IFN-γ, IL-2 and TNF-β, or IL-4, IL-5, IL-10 and IL-13, respectively.

There are many factors that may influence the fate of differentiating Th cells. To start with, there are different subsets of dendritic cell (DC) that can influence lineage commitment: $CD8\alpha^+$ DCs produce IL-12 and thereby favor Th1 differentiation, while the $CD8a^-$ dendritic subset stimulates Th2 differentiation, probably by a mechanism involving IL-6 secretion by APC[100]. Another possibility concerning the role of APC is that some chemokines may polarize Th cells, as shown in chemokine monocyte chemoattractant protein 1 (MCP-1)--deficient mice which are unable to mount Th2 responses[31]. Moreover, Eta-1 is a cytokine that may favor Th1 immune responses through differential regulation of macrophage IL-12 and IL-10 cytokine expression[5]. The antigen structure and dose might influence Th polarization and also the type of costimulatory molecules involved in the presentation[107].

However, the most important determinant of Th cell fate is the cytokine milieu and the presence of key transcription factors such as GATA-3 and T-bet. There are two critical cytokines that control Th differentiation[87]: IL-12 and IL-4 (Fig. 3). These cytokines promote the differentiation of their own subset and at the same time they inhibit the opposite subset[41, 73]. There are two models that may explain how cytokines act on Th differentiation[119]. The selection model implies that cytokines select cells that already have specific fates, given, for example, by GATA-3 or T-bet, thereby acting as growth factors, and the instructive model im-

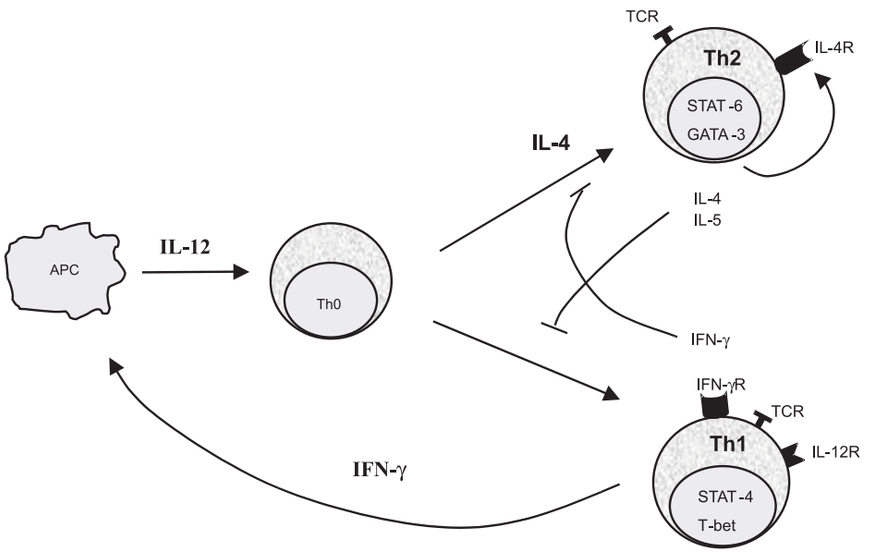

Fig. 3. Transcription factors and cytokines that control Th1--Th2 differentiation. Upon an antigenic stimulus, naive Th lymphocytes differentiate into two subsets: Th1 and Th2. IL-12 and IL-4 are two critical cytokines that control Th differentiation. In addition, GATA-3 and T-bet TF play an essential role in Th commitment. GATA-3 expression is dependent on TCR and IL-4/STAT-6 activation. T-bet is induced upon signaling through the TCR and IL-12R/STAT-4

plies that cytokines induce cells to assume specific fates, for example by targeting epigenetic remodeling or by inducing master TF such as T-bet or GATA-3. These two models may co-exist. Recently, a new model was proposed in which activated naive cells already express T-bet and GATA-3 before entering the cell--cycle. Then, they auto-induce themselves and target chromatin remodeling of specific cytokine genes (IFN-γ and IL-4) as the cells enter the S phase. IL-12 and IL-4 can repress the induction of GATA-3 and T-bet, respectively, but full repression of these master TF requires DNA replication so parent cells remain pluripotent when they enter the cell-cycle and these cytokines can only limit but not inhibit opposing fates. T-bet and GATA-3 are also likely to induce or repress cytokine and chemokine pathways.

As mentioned above, during Th differentiation there are dynamic changes in histone acetylation[8]. Stimulation of naive T cells results in a nonselective hyperacetylation at the IL-4 and IFN-γ genes. This process requires TCR signals and does not depend on polarizing cytokines. Later, during differentiation under polarizing conditions, the nonselective pattern of histone hyperacetylation gives way to selective histone hyperacetylation only at regulatory regions of these cytokine genes. This process depends on the presence of the appropriate polarizing cytokines and lineage-specific transcription factors (Fig. 3); early histone acetylation declines both in the absence of polarizing cytokine and in the presence of the inappropriate cytokine.

Th1 Differentiation

IL-12

During the innate immune response against a pathogen, IL-12 is produced by activated macrophages and DCs. IL-12 stimulates NK cells and αβ T cells as well as γδ T cells to produce IFN-γ. Macrophages are further activated by IFN-γ, resulting in the clearance of the microrganism. The adaptive immune response is directed towards a Th1 response by IL-12. T cell responsiveness to IL-12 depends on expression of the high-affinity IL-12R, which consists of a β1 and a β2 subunit. The β2 subunit is involved in downstream signaling which includes the activation of signal transducer and activator of transcription 4 (STAT-4). $STAT4^{-/-}$ deficient mice lack Th1 development[55, 125]. IL-12 is a key factor that up-regulates the TCR-induced expression of the IL-12Rβ2 chain[22]. During Th2 development, T cells are unresponsive to IL-12; thereby they do not produce IFN-γ nor phosphorylate STAT-4 in response to IL-12. IL-4 inhibits IL-12Rβ2 expression in human T cells. In the mouse, GATA-3 inhibits IL-12 signaling by repressing IL-12Rβ2 expression and the expression of this TF is increased by IL-4 and inhibited by IL-12[92]. The Th1-specific TF T-bet is up-regulated by IL-12. During T cell activation, T-bet expression is dependent on IFN-γ signaling and STAT-1 activation, but is independent of IL-12/STAT-4. T-bet mediates STAT-1-dependent processes of Th1 development by targeting chromatin remodeling of IFN-γ alleles and by inducing IL-12Rβ2 expression[1], and on the other hand, IL-12/STAT-4 serves as a growth signal, inducing survival and cell division, and as a trans-activator, prolonging IFN-γ synthesis through a genetic interaction with the CREB-binding protein[80]. On the other hand, fully polarized Th2 cells in the presence of IL-12 are able to shift towards Th1 phenotypes. In this condition there is a suppression of the expression of GATA-3, induction of T-bet and IL-12Rβ2 chain expression, and restored IL-12-inducible STAT-4 activation. This shift is stable, as restimulation in the presence of IL-4 is not able to undo the changes[115].

STAT-4

Activation of the STAT-4 is key to T cell differentiation. IL-12 induces tyrosine phosphorylation and activation of STAT-4[134]. CD4 and CD8 T cells require STAT-4 activation for IFN-γ induction by IL-12/IL-18 signaling, but only CD4 T cells require STAT-4 for IFN-γ induction via the TCR signaling pathway. CD8 T cells can produce IFN-γ independently of IL-12, but CD4 T cells require IL-12 and STAT-4 activation[13]. Mice deficient in IL-12 or STAT-4 show a strong defect in IFN-γ production by CD4 T cells. Moreover, mice lacking both STAT-4 and STAT-6 are able to mount an *in vivo* Th1 cell-mediated delayed-type hypersensitivity response and T cells from these mice can produce significant amounts of IFN-γ, thereby there is a STAT-4-independent pathway for the development of Th1 cells[56].

T-bet

T-bet is a member of the T-box family of transcription factors. It is expressed in Th1 cells and NK cells which produce IFN-γ[123]. T-bet is induced upon signaling through the TCR and IL-12R. The expression of T-bet correlates with the production of IFN-γ. Transient transfection assays in EL-4 cells cotransfected with a reporter construct of IFN-γ showed that T-bet can

transactivate the IFN-γ gene. Also, transfection of T-bet to these cells induced endogenous IFN-γ production. To examine the effect of T-bet in Th cell differentiation, uncommitted Th cells, developing Th2 cells, and fully polarized CD4 and CD8 T cells were transduced with a T-bet/GFP retroviral construct. In all cases, introduction of T-bet resulted in the generation of IFN-γ-producing Th1 cells that expressed Th1 markers, and simultaneously produced the inhibition of the Th2 cytokines IL-4 and IL-5. Mice homozygous for the T-bet deletion (T-bet$^{-/-}$) showed a decrease in IFN-γ production in CD4 T cells even in the presence of IL-12[124], demonstrating that T-bet is required for IFN-γ production. On the other hand, the same T-bet$^{-/-}$ CD4 T cells showed an increase in the production of the Th2 cytokines IL-4 and IL-5. Even when stimulated under Th1 conditions, T-bet$^{-/-}$ cells continued to produce very low levels of IFN-γ and were unable to suppress production of IL-4 and IL-5. Thus, T-bet may act to favor Th1 differentiation and simultaneously inhibit Th2 fate. Ectopic expression of T-bet induced IFN-γ production in STAT-4-deficient cells even when cultured in the presence of IL-4 and anti-IL-12 (Th2 polarizing conditions)[80]. Also, in the same culture conditions, in STAT--4-deficient cells T-bet can up-regulate IL-12Rβ2 chain expression, induce endogenous T-bet and target chromatin remodeling of the IFN-γ gene. Thus, T-bet appears to act before and independently of IL-12-induced STAT-4 activation. Then, the IL-12-associated increase in T-bet expression during Th1 differentation might reflect a survival advantage for cells that are already expressing T-bet[80].

T-bet function was assessed in several mouse models of human disease. The absence of T-bet was protective in mouse models of inflammatory bowel disease (T-bet-deficient T cells failed to induce colitis when used in adoptive transfer experiments)[83, 136]. T-bet-deficient mice developed spontaneous airway hyperresponsiveness, airway inflammation and airway remodeling, features of acute and chronic asthma in humans, and these features could be transferred with T-bet-deficient CD4 cells[24]. Recently, it has been shown that during T cell activation, T-bet expression is strongly dependent on IFN-γ signaling and STAT-1 activation, but is independent of STAT-4[1, 71]. Ectopic T-bet expression selectively induces expression of IL-12Rβ2 in wild-type and STAT-1-deficient Th2 cells, but does not extinguish the expression of GATA-3 and Th2 cytokines. Moreover, ectopic T-bet cannot induce the expression of endogenous T-bet independently of IFN-γ or STAT-1[1]. Thus, IFN-γ and T-bet are involved in an amplification loop.

Taking all these data into account, a model for the regulation of Th1 differentiation emerges[90]. Naive T cells express little STAT-4, IL-12Rβ2, and T-bet and are unresponsive to IL-12. Upon activation, TCR signaling may induce T-bet and IFN-γ synergies with TCR signaling to maximally induce T-bet in a STAT-1-dependent process but independent of IL-12 and STAT-4. Then, T-bet regulates IL-12 responsiveness via induction of IL-12Rβ2 expression. It seems that T-bet and IFN-γ are not sufficient to permit full Th1 development: activation of T cells also up-regulates IL-12R and STAT-4, which is essential for optimal Th1 differentiation. T-bet controls the expression of the IFN-γ gene in Th1 cells. Transient transfection experiments and deletion analysis of the IFN-γ promoter shows that the region between –445 and –415 bp is essential for the promoter activity induced by T-bet. In EMSA experiments using this region as probe, no Th1-specific protein-DNA complex or T-bet complex could be detected, suggesting that T-bet may act indirectly to regulate IFN-γ promoter activity[116]. To understand the role of T-bet in the maturation and maintenance of Th1 cells, a dominant negative form of T-bet was used in experimental approaches. In developing Th1 cells, T-bet was required for maintaining gene expression and chromatin remodeling of IFN-γ. Moreover, T-bet was essential for maintaining IL12Rβ2 expression in mature Th1 cells and descendants. Hlx is a Th1 restricted homeobox TF that is induced by T-bet. Transduction of cells with Hlx and T-bet showed that combination of both is necessary to achieve optimal expression of IFN-γ. Th1 differentiation was associated with demethylation of IFN-γ, which occurred at later time points and was a event separate from T-bet activity[81].

IFN-γ

Th1, CD8 and NK cells secret IFN-γ. Two conserved and essential regulatory elements between –108 and –40 bp which confer activation-specific expression in T cells, a distal element that contains a consensus GATA motif that binds GATA-3 and a proximal regulatory element which is a composite site that binds CREB/ATF (ATF-1 and ATF-2), c-Jun, and Oct-1 proteins, were identified in the IFN-γ promoter[95, 96]. Also, there are additional NFAT sites outside the proximal regulatory element[6, 122], and NF-κB and NFAT proteins bind to and transactivate regions in the first intron of the IFN-γ gene[58, 113]. Ying-Yang 1 (YY1) TF can suppress IFN-γ transcription by two mechanisms: 1) cooperation with a repressor protein and 2) competition for DNA-binding with AP-1[140]. On the other hand, YY1

may play a positive role because it was also shown to transactivate IFN-γ through binding to an element between two NFAT sites[122]. Using CD4 splenic T cells from transgenic mice containing a number of reporter constructs of various lengths of the IFN-γ promoter, a minimal (–565 to +64 bp) region containing a T-bet--responsive unit which confers Th1 selective expression was identified[116]. As mentioned above, the region between –445 and –415 bp is essential for the promoter activity induced by T-bet. In addition, the p38 MAP kinase signaling pathway, which also regulates IFN-γ gene transcription, targets the –70 to –40 bp promoter element.

Th2 Differentiation

IL-4

The cellular sources of IL-4 are CD4 and CD8 T cells, NK, γδ T cells, mast cells, basophils, and eosinophils. The IL-4 receptor signals through the JAK/STAT pathway, involving the insulin response substrate (IRS). IL-4 activates STAT-6, which is responsible in part for Th2 differentiation and IgE production by B cells. The activation of the IRS pathway accounts for IL-4-induced cellular proliferation. In STAT-6-deficient mice, IL-4R expression is completely inhibited and lymphocytes from these animals fail to proliferate in response to IL-4. Moreover, STAT-6--deficient lymphocytes fail to differentiate into Th2 cells in response to IL-4 or IL-13[54]. Studies using IL-4 promoter transgenics demonstrated that the region between –741 and +60 bp is selectively expressed in Th2 cells[137]. There are five NFAT sites in the proximal IL-4 promoter, two of which are composite NFAT/AP-1 sites[14, 130], each binding NFATc1 and NFATc2[129], and two also bind AP-1 factors in a NFAT-dependent manner[37, 102, 103]. The transcription factor cMaf also binds to the IL-4 promoter and activates transcription synergistically with NFATp[35]. Jun B binds directly to the IL-4 promoter and synergizes with cMaf to activate an IL-4 luciferase reporter[69]. Members of the TNF receptor-associated factor (TRAF) family proteins repress IL-4 gene transcription through their interaction with NIP45 (NFAT-interacting protein[70]). There is a hypersensitivity site between the IL-4 and the IL-13 genes which contains a conserved noncoding region 1 (CNS-1) which acts as an IL-4 enhancer, while the intronic and 3' distal enhancers confer Th2 specificity[72]. GATA-3 TF binds to the 3' distal enhancer and this is associated with recruitment of NFAT-1 to IL-4 promoter and the 3' enhancer[2]. On the other hand, the IL-4 promoter has three binding sites for the IRF-1 and IRF-2 factors which are induced by IFN-γ and function as transcriptional repressors, providing an important mechanism through which IFN-γ attenuates Th2 responses.

IL-5

IL-5 is produced by eosinophils, mast cells, epithelial cells and Th2 cells. IL-5 stimulates the growth and differentiation of eosinophils and activates mature eosinophils, which are able to kill helmints. Eosinophils have receptors for IgE, which coats microbes. On the other hand, IL-4 stimulates B lymphocytes to produce IgE which, in turn, opsonizes helminthes and binds eosionphils. In this way IL-4 and IL-5 act together to destroy extracellular microorganisms. IL-5 also stimulates B cell proliferation and the production of IgA. There are four cis regulatory elements in the mouse IL-5 promoter: IL-5A (–948 to –933), IL-5P (–117 to –92), IL-5C (–74 to –56), and IL-5CLE0 (–55 to –38). IL-5P has homology with the NFAT-binding site. An NFAT-related factor is involved in the regulation of this gene[65]. There are three regions in the human IL-5 promoter which are involved in the regulation of IL-5 expression: REI (–79 to –45), REII (–123 to –92) and REIII (–170 to –130). The REI acts constitutively. This site includes the conserved CLE0 element (conserved lymphokine element 0), which acts constitutively in stimulated and unstimulated T cells. It binds the AP-1 members JunD and Fos-related antigen 2 as well as the Oct-1 and Oct-2 factors[79, 126]. The REII site is critical for inducible IL-5 promoter activity, it is inhibited by CsA and mutants in this site show reduced reporter gene activity, and members of the NFAT-1 and AP-1 families of TF bind to this site[19, 120]. There are also two repressor elements, hPRE1 and hPRE2, in the proximal and distal promoters, respectively. There are three binding sites for GATA-3 in the human IL-5 promoter: at –70, –152 and –400. The –70 and –152 sites, positively regulate IL-5 transcription, but the site located at –400 acts as a repressor of IL-5 transcription. In unstimulated human T cell lines, Oct-1 binds to CLE0, whereas stimulation induces *de novo* synthesis of the AP-1 members JunD and Fra-2, which bind to CLE0. The amount of IL-5 produced correlates with the production of the AP-1 complex. Although the formation of the AP-1 complex is essential, the rate-limiting step is the synthesis of AP-1, especially Fra-2. Specific activation of human IL-5 depends on *de novo* synthesis of an AP-1 complex[108]. IL-5 is induced by cAMP, which inhibits other T cell cytokines, and is sensitive to protein

synthesis inhibitors, which have no effect on other cytokines.

STAT-6

IL-4 engagement with its receptor leads to phosphorylation of STAT-6 by JAK1 and JAK3 kinases, leading to STAT-6 dimerization and nuclear translocation. STAT-6 binds to a sequence in the IL-4 promoter and this STAT-6-binding site can support IL-4-dependent transcription of a heterologous promoter[17]. Prolonged activation of STAT-6 is characteristic of populations undergoing Th2 differentiation. Moreover, STAT-6 is activated in an autocrine manner when Th2 cells are stimulated by antigen receptor ligation[64]. STAT-6 activates IL-4 target genes such as IL-4R and GATA-3[92]. In STAT-6-deficient mice, IL-4 induces an increase in expression of MHC class II antigens and IL-4R is completely inhibited, lymphocytes fail to proliferate in response to IL-4, and T lymphocytes fail to differentiate into Th2 cells, showing that STAT-6 is essential for mediating IL-4 responses in lymphocytes[54]. Moreover, ectopic expression of STAT-6 into developing Th1 cells induces Th2-specific cytokines and suppresses IFN-γ production; it also induced GATA-3 and cMaf expression and down-regulated IL-12Rβ2 chain expression[61]. However, there is also a STAT-6-independent GATA-3 autoactivation pathway that directs IL-4-independent Th2 differentiation. Recently it was shown that maintenance of STAT-6 activation in the presence of IL-4 requires ongoing phosphorylation of latent cytoplasmic STAT-6 molecules. Cells treated with an inhibitor of nuclear export were unable to maintain STAT-6 activation thereby, the maintenance of STAT-6 activation requires a constant cycle of activation, deactivation, nuclear export, and reactivation[3].

GATA-3

GATA-3 was identified in the chicken as a member of a family of three Zn-finger domain-containing proteins which bind to a consensus site, (A/T) GATA(G/A). Targeted disruption of the GATA-3 gene causes massive internal bleeding, growth retardation, deformities of the brain and spinal cord, and aberrations in the fetal liver[94]. Mice lacking GATA-3 in the lymphoid system do not generate T cells because GATA-3 is required for the development of the earliest T cell progenitors and in the absence of this TF, thymocytes arrest at the double negative stage[33]. GATA-3 gene expression is up-regulated in Th2 cells and is down-regulated in Th1 cells[142, 146]. Moreover, expression of a dominant-negative mutant of GATA-3 in mice leads to a reduction in the levels of the Th2 cytokines IL-4, IL-5, and IL-13 and, in addition, attenuates airway eosinophilia, mucus production, and IgE synthesis, all features of asthma[143]. The expression of antisense GATA-3 in Th2 cells causes the inhibition of Th2 cytokine expression[146]. Ectopic expression of GATA-3 is sufficient to drive IL-5 but not IL-4 gene expression[144]. For full IL-4 expression, GATA-3 acts through elements surrounding the IL-13/IL-4 gene locus[99]. GATA-3 inhibits Th1 development by a mechanism that is not dependent on IL-4 and that may involve inhibition of IL-12 signaling by repression of IL-12Rβ2 expression[92]. Moreover, there is an IL-4/STAT-6 auto-activation mechanism for GATA-3 induction which creates a feedback pathway stabilizing Th2 differentiation, indicating that GATA-3 is a master switch in Th2 development[91]. Several factors interact with GATA-3 to regulate transcription. Recently, in a yeast two-hibrid screening using a Th2 cell-specific library, a factor called “friend of GATA” (FOG) was identified. It was shown that FOG functions in naive T cells as a repressor of GATA-3, and that down-regulation of FOG induces Th2 cell commitment by releasing GATA-3 from repression[60]. Moreover, a repressor of GATA-3, called ROG, which interacts with this GATA-3, was also identified. ROG overexpression represses GATA--3-dependent transcription of reporters and inhibits the production of Th cell cytokines[77]. CD28 activation also contributes to GATA-3 regulation, providing a pathway for GATA-3 induction independent of the IL-4/ /STAT-6 pathway[101].

cMaf

cMaf is a basic leucine zipper protein that binds to a TRE/CRE-like sequence called MARE[57]. cMaf is expressed only in Th2 clones and is induced along the Th2 but not the Th1 differentiation. The response element, MARE, is present in the proximal IL-4 promoter adjacent to a site footprinted by extracts from Th2 but not Th1 clones. Ectopic expression of cMaf transactivates the IL-4 promoter in Th1 cells, B cells, and non--lymphoid cells[35]. Overexpression of cMaf *in vivo* produces a switch towards the Th2 pathway in an IL-4-dependent way. Ectopic expression of cMaf in mature Th1 cells did not confer on them the ability to produce IL-4, but did decrease the production of IFN-γ by a mechanism that was independent of IL-4[36]. Mice deficient in cMaf have deficient IL-4 production, but in the presence of exogenous IL-4, T cells produce normal levels of other Th2 cytokines. Therefore it seems that

IL-4 production requires both GATA-3 and cMaf transcription factors, but other Th2 cytokines depend, mainly, on the presence of GATA-3[59].

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