NFAT pulls the strings during CD4+ T helper cell effector functions

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The Ca²⁺ dependent transcription factor family known as nuclear factor of activated T cells (NFAT) has been shown to be important in T-cell immune responses. Because NFAT proteins have a weak DNA-binding capacity, they cooperate with other transcription factors at composite sites within the promoters of target genes. Recently, NFAT was shown to be also important for the induction of specific genetic programs that guide the differentiation and effector or regulatory activities of CD4+ T helper subsets via the transcriptional regulation of their lineage-specific transcription factors, specifically T-bet (Th1), Gata3 (Th2), RORγt (Th17), and Foxp3 (iTregs). In addition, the NFAT family governs the transcription of several signature cytokines, including their cytokine receptors. Subsequently, the integration of these complex intracellular signal transduction cascades is considered to critically determine the crosstalk between the T-cell receptor and receptors that are activated by both the adaptive and innate immune systems to determine pathways of T helper cell differentiation and function. Here, we carefully review the critical role of the established transcriptional partners and functional outcomes of these NFAT interactions in regard to the effector responses of these clinically relevant CD4+ T helper subsets.

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Introduction

The Ca²⁺ -dependent transcription factor family, known as nuclear factor of activated T cells (NFATs), originally identified by Shaw et al., regulates not only T lymphocytes but also a large number of growth factors, cytokines, and cell-to-cell interaction molecules essential for the morphogenesis, development, and function of many cell types and organs. In T lymphocytes, NFAT proteins govern gene expression that regulates T-cell development, activation, differentiation, as well as the induction and maintenance of T-cell tolerance.

Briefly, the NFAT family consists of 5 family members that all share a highly conserved DNA-binding domain: NFAT1 (NFATc2; NFATp), NFAT2 (NFATc1; NFATc), NFAT3 (NFATc4); NFAT4 (NFATc3; NFATx), and NFAT5 (TonEBP; OREBP). In T lymphocytes, 2 or more splice forms of NFAT1, 2, and 4 are present, encoding the following domains: the NFAT homology region containing a transactivation (TAD-A) and regulatory domain (docking sites for calcineurin and the NFAT kinases), the Rel-homology region with the N-terminal DNA-binding domain that is essential for the morphogenesis, development, and function of many cell types and organs. In T lymphocytes, NFAT proteins govern gene expression that regulates T-cell development, activation, differentiation, as well as the induction and maintenance of T-cell tolerance.

Because NFAT proteins have a weak DNA-binding capacity, they cooperate with other transcription factors at composite sites within the promoters of target genes. Recently, NFAT was shown to be also important for the induction of specific genetic programs that guide the differentiation and effector or regulatory activities of CD4+ T helper subsets via the transcriptional regulation of their lineage-specific transcription factors, specifically T-bet (Th1), Gata3 (Th2), RORγt (Th17), and Foxp3 (iTregs). In addition, the NFAT family governs the transcription of several signature cytokines, including their cytokine receptors. Subsequently, the integration of these complex intracellular signal transduction cascades is considered to critically determine the crosstalk between the T-cell receptor and receptors that are activated by both the adaptive and innate immune systems to determine pathways of T helper cell differentiation and function. Here, we carefully review the critical role of the established transcriptional partners and functional outcomes of these NFAT interactions in regard to the effector responses of these clinically relevant CD4+ T helper subsets.

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transcriptional partners of NFAT have been well reviewed, but the transcriptional partners and different target genes, such as II2, Nfat, Cd25, Gitr, Il4, Tbx21, Twist, Il12Rb2, Il5, Il13, Il10, c-Maf, Gata3, Roryt, Il17, Il12, Il21, Il22, Il6, and Foxp3 (Table 1), have not been outlined in the context of the different CD4+ T helper subsets.

In addition, adding to the already well-established Th1 and Th2 subsets, the Th17 subset was identified in 2006, as well as the iTregs. From the medical viewpoint, the importance of the transcriptional partners of NFAT have been well reviewed, but the transcriptional partners important for CD4+ T cells is highly relevant for the outcome of the given immune response. Currently used immunosuppressive drugs, such as CsA and tacrolimus (both inhibiting the calcineurin/NFAT transactivation pathway) or rapamycin/sirolimus (inhibiting the mammalian target of rapamycin, pathway activities downstream of IL-2/phosphatidylinositol 3-kinase signaling) are efficient by reducing effector T-cell expansion and effector functions via different signaling cascades. Calcineurin inhibitors have been shown to inhibit Th0, Th1, Th2, and the Th17 subset in humans, albeit with slightly different efficiency. Sirolimus has been shown to be significantly more effective than CsA in inhibiting the Th1 subset responses, whereas the inhibition on the Th2 subset did not differ in kidney transplantation patients. The ideal immunosuppressive agent should not impair Tregs. CsA has been shown to inhibit Treg function. In contrast, it has also been suggested that CsA might even enhance the Treg subset in transplantation medicine.

A further advantage over current immunosuppressive regimens might be achieved by sotastaurin (AEB-071, NVP-AEB-071), an orally bioavailable compound that exerts its effects through the inhibition of protein kinase C, thereby inhibiting the antigen receptor signaling to nuclear factor-xB (NF-xB) and NFAT transactivation. The immunosuppressive effects of oral sotastaurin could be an effective novel treatment regimen for psoriasis. Because it represents an entirely new mechanism of drug action, it has the potential of an effective alternative and/or adjunct to calcineurin inhibitors for abrogating selective T-cell effector responses in future therapies.

T-cell effector functions are relevant in the pathogenesis of different diseases, including asthma, rheumatoid arthritis, and multiple sclerosis, and the role of NFAT has been well established in these diseases. Understanding subset-specific NFAT protein–protein interactions in the clinically relevant CD4+ T helper subsets, and in the long run pharmacologic intervention of specific CD4+ T-cell effector functions, may greatly facilitate medical treatment, either for immune suppression in autoimmune diseases or for immune augmentation in cancer.

Naive T cells: NFAT transcriptional partners and targets

Naive T cells, which develop in the thymus, are the common precursors of helper T cells that have not yet encountered antigen. In naive cells, full T-cell activation (Figure 1) requires costimulatory receptors, including CD4+ or CD8+, together with LFA1, CD28, and ICOS. All of these receptors additionally activate one of the major signaling pathways converging at the activation of the NFAT, AP-1, or NF-xB pathways, with the originally described transcription factors NF-xB, AP-1, EGR, and Oct, induce interleukin-2 (IL-2). In addition, the transcription of Il2Rx (Cd25) is up-regulated in an NFAT-dependent manner. IL-2 itself, via the interleukin-2 receptor (IL-2R), activates signal transducer and activator of transcription 5 (STAT5) and, subsequently, cell-cycle entry. In naive T cells, only low concentrations of NFATs are present, but in combination with the other aforementioned transcription factors, together with an autoregulatory loop, lead to sufficient Il2 and Cd25 promoter induction. Different NFAT proteins having selective roles in T helper cell differentiation...
Th1 helper cells: NFAT transcriptional partners and targets

Th1 cells are essential for clearing intracellular bacteria and viruses; their signature cytokine is interferon-gamma (IFN-γ). Briefly, Th1 differentiation (Figure 2) is induced by signals from the antigen-presenting cells: IL-12, which is mainly produced by monocytes and dendritic cells; IFN-γ, which is secreted by already differentiated Th1 cells and by natural killer (NK) and NKT cells; and IL-27, which is produced by NK cells. IFN-γ activates signal transducer and activator of transcription 1 (STAT1) via the IFN-γ receptor. Together with the TCR-induced transcription factors (NFAT, NF-kB, and AP-1), these signals activate the master transcription factor of the Th1 lineage, T-bet (Tbx21), while repressing IL12Rβ2 subunit gene expression in an NFAT-dependent manner.

Another order of complexity was recently added in the regulation of the NFAT-dependent cytokine gene expression. The committed Th1 cells are then reactivated by the TCR receptor pathway plus the positive feedback loop of the IFN-γ-STAT1 signaling pathway. One regulator of this positive amplification loop was reported recently, the basic helix-loop-helix transcription factor twist1, which is induced by NFAT and NF-kB as a result of IL-12/STAT4 signaling in Th1 cells and transiently expressed in repeatedly activated Th1 cells. The Notch3 signaling pathway via the recombination signal binding protein for immunoglobulin kappa J region (RBPJ) and mastermind-like (MAML1) is also mandatory during Th1 subset differentiation, and its key role was reviewed recently.
Th2 helper cells: NFAT transcriptional partners and targets

Th2 cells are essential in organizing the host defense against parasites and in inducing humoral responses in the form of helping B cells, which produce antibodies. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13.16,52,75 Briefly, in the presence of exogenous IL-4 (provided by mast cells, basophils, eosinophils, NKT cells, or previously differentiated Th2 cells), naive T cells, after TCR cross-linking, activate not only the TCR-dependent signaling machinery but also the IL-4 receptor (IL-4R) pathway (Figure 3). IL-4R, which is present on naive T cells, activates STAT6, which in turn, together with NFAT, AP-1, and NF-κB, drives IL4 transcription.19,76 Another downstream target of STAT6 is the master transcription factor in the Th2 subset, GATA3, which consequently induces the transcription of the long form of viral musculoaponeurotic fibrosarcoma oncogene homolog (c-MAF), which additionally helps to activate IL4 transcription. This activation results in a strong autocrine feedback loop that activates IL4, IL5, and IL13. Two STAT6-independent pathways have been discovered that promote Th2 differentiation: the Notch1/2 signaling pathway via RBPJ and MAML1 and the IL-2 receptor signaling pathway. The IL-2 receptor pathway boosts early IL4 transcription. Therefore, IL-12 can additionally activate STAT4, which together with STAT1, Hlx, Runx3, T-bet, AP-1, and NFAT, further induces IFN-γ transcription. In parallel, NFAT, together with STAT4 and NF-κB, binds to the promoter of the twist1 gene.
Th17 helper cells: NFAT transcriptional partners and targets

Th17 cells are important for the clearing of extracellular pathogens, especially at mucosal sites, but are also responsible for autoimmune diseases. Th17 cells have been extensively reviewed. Briefly, Th17 cells secrete the signature cytokines IL-17A, IL-17F, IL-21, and IL-22. NFAT is important in the induction of all of these cytokine genes (Figure 4). Furthermore, the crucial role of NFAT1 in Th17 differentiation has also been demonstrated by the analysis of NFAT1 knockout mice in an experimental colitis model because it plays a key regulatory role by controlling IL-6–dependent T-cell activation. Transforming growth factor-β (TGF-β), provided by dendritic and epithelial cells, inhibits Th1 and Th2 differentiation and favors Th17 and Treg differentiation, which is dependent on costimulatory signals and occurs in a concentration-dependent manner. If the cytokine milieu provides IL-6, which is mainly produced by monocytes, differentiation into the Th17 lineage is induced. The IL-6R activates STAT3, which subsequently induces the expression of IL-23, which is NFAT dependent, and several NFAT-binding sites are located on the Il23R locus. Il23R is a member of the IL-23 superfamily and is involved in the regulation of Th17 cell differentiation.

Th2 differentiation involves the cytokine genes (Figure 4). The crucial role of NFAT1 in Th17 differentiation has also been demonstrated by the analysis of NFAT1 knockout mice in an experimental colitis model because it plays a key regulatory role by controlling IL-6–dependent T-cell activation. Transforming growth factor-β (TGF-β), provided by dendritic and epithelial cells, inhibits Th1 and Th2 differentiation and favors Th17 and Treg differentiation, which is dependent on costimulatory signals and occurs in a concentration-dependent manner. If the cytokine milieu provides IL-6, which is mainly produced by monocytes, differentiation into the Th17 lineage is induced. The IL-6R activates STAT3, which subsequently induces the expression of IL-23, which is NFAT dependent, and several NFAT-binding sites are located on the Il23R locus. Il23R is a member of the IL-23 superfamily and is involved in the regulation of Th17 cell differentiation.
signaling is required for the expression of IL22. Once Th17 differentiation is initiated and IL-23R, which is not expressed on naive T cells, is expressed, IL-23 plus TGF-β are capable of driving IL17 and IL23R expression in an amplification loop. In vivo, IL-23, which is mainly released by dendritic cells and macrophages, is an essential component for the maintenance but not the induction of Th17 cells, has been demonstrated to be very important and, albeit still controversial, IL-22 also seems to be essential. Surprisingly, IL22-deficient mice are fully susceptible to experimental autoimmune encephalitis induction, which dismisses IL-22 as an essential and nonredundant pathogenic player in the development of autoimmune central nervous system inflammation.

Taken together, multiple proinflammatory cytokines might therefore be able to drive Th17 differentiation in combination with TGF-β in vivo, such as IL-1, IL-6, IL-21, or IL-22. The subsequently activated STAT3 pathway is required for RORγt and IL-17 induction. RORγt, together with Runx1, binds to the I17a promoter and inhibits Foxp3 expression. RORγt also directs RORγt to Foxp3; therefore, Foxp3 can inhibit its transcriptional activities. This inhibition is relieved by the presence of the proinflammatory cytokines that drive Th17 differentiation by a posttranslational mechanism. Another transcription factor essential for Th17 differentiation is IRF-4, which was already discussed in Th2 differentiation. IRF-4 regulates IL21 and IL23R expression and is inhibited by the IRF-4–binding protein. IRF-4 is up-regulated after TCR activation and is therefore not Th-dependent.33,44,46 suggesting that IL-21, which is produced by Th17 cells in an autocrine manner and involved in the positive feedback loop, is transcriptionally regulated by cooperation between IRF-4 and NFAT. The aryl hydrocarbon receptor (AhR), which is highly expressed in the Th17 subset, is necessary for IL22 and, to a lesser extent, IL17 expression. Zhou and Littman reported that AhR cooperates with RORγt to induce maximal expression of IL17 and IL22. Recently, ICOS and c-MAF were shown to be essential in Th17 cell differentiation; the induction of c-MAF by ICOS induces IL-21 production. In Th17 cells, differentiated and activated ex vivo, the loss of Nr2f6 results in amplified NFAT DNA-binding capabilities at the Il17 promoter and subsequently increases Il17 transcription. Nr2f6-deficient mice consistently have hyperreactive lymphocytes and develop a late-onset immune pathology. These mice are also more susceptible to the Th17-dependent model of experimental autoimmune encephalomyelitis.

In the presence of TGF-β and IL-2/IL-15, naive T cells induce STAT5, Smad, and non-Smad signaling after T-cell receptor stimulation. If exposed to low-affinity antigen without costimulation, however, the NFAT signaling cascade, but not the mitogen-activated protein kinase pathway (and subsequently AP-1), is activated. This combination of transcription factors induces the master transcription factor of inducible regulatory T cells, Foxp3, which consequently suppresses I-Smad7 expression. In the absence of AP-1, NFAT up-regulates Cbl-b further enhancing Foxp3 expression and resulting in a positive feedback loop for the expression of Foxp3. The Foxp3 promoter region has been analyzed in much detail to elucidate how TGF-β signaling can influence Foxp3 expression and Treg differentiation. Tone et al. revealed that, in addition to the Foxp3 promoter, which is activated by the combined binding of NFAT, AP-1, Sp1, and STAT5, additional enhancer regions exist (Figure 5). Enhancer region 1 is activated by NFAT/pSmad3 cooperation, whereas pSmad3 binding is important only during the early induction of TGF-β. NFAT binding is required throughout at least a 24-hour period. Another transcription factor discovered to be important in the differentiation of Tregs is Runx1 as it physically and functionally interacts with Foxp3. This interaction is different from the interaction between NFAT and Foxp3 because they interact through regions distinct from their DNA-binding domains on widely separated sites. In addition to its essential role in the induction of Foxp3 transcription, NFAT additionally cooperates with Foxp3 to up-regulate CTLA-4 and CD25, 2 highly expressed surface markers of Tregs. Chromatin-immune precipitations confirmed the binding of NFAT and Foxp3 to the promoters of Il2, Cita4, and Cd25. Foxp3 competes with NFAT1 to bind to the Nfat2 promoter to suppress the amplification loop of NFAT transcription, thereby potentially affecting the activation of the many NFAT-mediated cytokine genes downstream. In contrast to naive T cells where Runx1 cooperates with NFAT/AP-1 on the Il2 promoter to activate Il2 expression after TCR stimulation, Runx1 cooperates with the NFAT/Foxp3 complex in Tregs to suppress Il2 and activate Cita4, Cd25, and Gitr expression.

Two other regulatory T cells have been described, namely, the Th3 and Tr1 cells. The Th3 cells have been cloned from orally tolerized mice, which produce large amounts of TGF-β but small amounts of IL-10 and IL-4. In contrast, the Tr1 identified by Chen et al. arise in the periphery when naive CD4+ T cells are activated by tolerogenic antigen-presenting cells in the presence of IL-10. These resulting Tr1 cells regulate immune responses by secreting IL-10 and TGF-β and have the capacity to suppress both naive and memory T-cell responses in vivo and in vitro. The HOZOT cytotoxic Tregs are FOXP3+ CD4+ CD8+ CD25+ and produce high levels of IL-10 resulting from the activation of STAT5, NFAT, and NF-kB.

In conclusion, NFAT plays a key role in the regulation of immune effector functions, and understanding the underlying cellular mechanisms at the molecular level in the fast-evolving field of T helper subsets could be helpful in the treatment of immune diseases, the use of cancer immunotherapy, and increasing vaccination effectiveness. Here, we reviewed the recent progress in our understanding of the molecular nature and regulation of the Ca2+/NFAT signaling pathway in the determination of lymphocyte response. Not only is the NFAT pathway involved in the signaling response to antigenic stimulation, but it regulates in promoter context-dependent transcriptional complexes the activation of the different Th master/lineage-specific transcription factors, as well as signature cytokines and their cytokine receptors. Future challenges...
we face are (1) to establish the biologic relevance of this recently accumulated molecular and biochemical knowledge in the complex context of animal disease models and (2) to translate this fundamental knowledge into the design of sound therapeutic approaches for immune diseases, all the way to their application in clinically relevant situations.

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