NFAT PROTEINS: KEY REGULATORS OF T-CELL DEVELOPMENT AND FUNCTION

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Abstract | Since the discovery of the first nuclear factor of activated T cells (NFAT) protein more than a decade ago, the NFAT family of transcription factors has grown to include five members. It has also become clear that NFAT proteins have crucial roles in the development and function of the immune system. In T cells, NFAT proteins not only regulate activation but also are involved in the control of thymocyte development, T-cell differentiation and self-tolerance. The functional versatility of NFAT proteins can be explained by their complex mechanism of regulation and their ability to integrate calcium signalling with other signalling pathways. This Review focuses on the recent advances in our understanding of the regulation, mechanism of action and functions of NFAT proteins in T cells.

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Nuclear factor of activated T cells (NFAT) was initially identified as an inducible nuclear factor that could bind the interleukin-2 (*IL-2*) promoter in activated T cells[1]. However, when all of the proteins of the NFAT family had been isolated and molecularly characterized, it became clear that their expression was not limited to T cells. At least one NFAT family member is expressed by almost every cell type that has been examined, including other cells of the immune system and non-immune cells[2–5]. Recent work has uncovered new regulatory roles for NFAT proteins in diverse organs, including the central nervous system, blood vessels, heart, kidney, bone, skeletal muscle and haematopoietic stem cells[3–6]; however, it is in immune cells that the function and regulation of NFAT proteins are best understood. In this Review, I provide an overview of recent advances in our understanding of the regulation, mechanism of action and functions of NFAT proteins in cells of the immune system, with a focus on T cells, in which most studies have been carried out.

The NFAT family

The NFAT family consists of five members: NFAT1 (also known as NFATp or NFATc2), NFAT2 (also known as NFATc or NFATc1), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATx or NFATc3) and NFAT5. Four of these proteins are regulated by calcium signalling (TABLE 1). All NFAT proteins have a highly conserved DNA-binding domain that is structurally related to the DNA-binding domain of the REL-FAMILY TRANSCRIPTION FACTORS. This REL-homology region (RHR) is the unifying characteristic of NFAT proteins and confers a common DNA-binding specificity. The only non-calcium-regulated NFAT protein, NFAT5 (REF. 7), was independently identified as the tonicity-responsive enhancer-binding protein (TonEBP; also known as OREBP)[8]. NFAT5 is the primordial member of the NFAT family, which emerged as early as *Drosophila* spp. by evolution from the REL family of transcription factors[7,9]. It is expressed by almost all cells and is activated in response to osmotic stress[8,10]. In lymphocytes, NFAT5 controls the osmotic stress-induced expression of several cytokines, including tumour-necrosis factor (TNF) and lymphotoxin-β[10]. NFAT5-deficient mice have impaired T-cell function under hyperosmotic conditions and decreased cellularity of the thymus and spleen[11].

REL-FAMILY TRANSCRIPTION FACTORS
Also known as the nuclear factor-κB (NF-κB) family of transcription factors. These factors share an amino-terminal REL-homology domain that contains sequences that are responsible for nuclear localization, dimerization and DNA binding. Homo- or heterodimers of NF-κB proteins modulate the expression of genes that control immune, inflammatory and acute-phase responses, as well as cell growth, apoptosis and oncogenesis. In vertebrates, this family includes NF-κB1 (also known as p50), NF-κB2 (also known as p52), REL (also known as cREL), REL-A (also known as p65) and REL-B.

TRANSACTIVATION DOMAIN
The domain of a transcription factor that binds the promoter region of a gene and induces its transcription.

CALMODULIN
A small calcium-binding protein. Calmodulin is the most important transducer of intracellular calcium signals. It interacts with, and regulates the activity of, a range of proteins that control many cellular processes, including protein phosphorylation and dephosphorylation, cyclic-nucleotide formation and breakdown, cytoskeletal rearrangement, gene transcription and membrane potential.

This Review focuses on the three calcium-regulated NFAT proteins that are expressed by T cells: NFAT1, NFAT2 and NFAT4. The role of these proteins in the regulation of T-cell function and development has been extensively characterized. Each protein has two or more alternatively spliced forms; splicing results in variation at the amino (N) and carboxyl (C) termini with the core region being conserved (FIG. 1a)[12–15]. The conserved core region of NFAT proteins consists of two tandem domains: a regulatory domain, which is also known as the NFAT-homology region (NHR); and the RHR, which binds DNA (FIG. 1b). The NHR is moderately conserved among NFAT proteins and contains a potent TRANSACTIVATION DOMAIN. The NHR contains many serine residues that are phosphorylated in resting T cells. It also includes the docking sites for calcineurin and the NFAT kinases, which regulate the activation of NFAT proteins by determining the phosphorylation status of the serines. The RHR domain shares structural homology with REL proteins and confers the DNA-binding specificity that characterizes NFAT family members[16]. A fourth family member, NFAT3, has a similar domain structure, but it is mainly expressed outside the immune system and will not be considered further here.

NFAT regulation

Regulation by calcium, calcineurin and NFAT kinases. The general aspects of the regulation of NFAT proteins by calcium and calcineurin have been thoroughly reviewed[3,17] and are only summarized here. Briefly, the activation of NFAT proteins is induced by the engagement of receptors that are coupled to the calcium-signalling pathway, such as the antigen receptors that are expressed by T and B cells, the Fcγ receptors that are expressed by monocytes and natural killer cells, or the Fcε receptors that are expressed by mast cells. Receptor ligation leads to the activation of phospholipase C-γ (PLC-γ), which hydrolyses phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ induces the release of calcium from intracellular stores, which triggers the opening of calcium-release-activated calcium channels in the plasma membrane such that the increased levels of intracellular calcium are maintained. Calcium binds CALMODULIN, which in turn activates the calmodulin-dependent phosphatase calcineurin. NFAT proteins are dephosphorylated by activated calcineurin, which leads to their nuclear translocation and the induction of NFAT-mediated gene transcription. During T-cell activation, it is the engagement of the T-cell receptor (TCR) that activates the calcium–calcineurin–NFAT pathway. As discussed later, NFAT proteins then cooperate with other transcriptional partners, which are activated in response to TCR and co-stimulatory receptor engagement, and thereby induce the expression of cytokines and many other T-cell-activation-induced proteins (FIG. 2).

Effective phosphate removal by calcineurin requires its docking on NFAT. Interactions between NFAT and calcineurin occur at a specific motif in the N terminus of NFAT, which has the consensus sequence PXIXIT, where X denotes any amino acid[18,19]. This motif is conserved among different NFAT family members and constitutes the main docking site for calcineurin on NFAT. A high-affinity version of this peptide (VIVIT) was shown to compete with NFAT proteins for calcineurin binding and to block NFAT dephosphorylation *in vitro*; when expressed in cells as a GREEN FLUORESCENT PROTEIN (GFP) FUSION PROTEIN, the peptide also selectively blocked NFAT-dependent transcription[20]. As expected, the docking site for the VIVIT peptide and, therefore, for NFAT proteins on calcineurin is distinct from the calcineurin catalytic site[21]. However, in addition to the PXIXIT sequence, other regions (such as the YLAVPQHP motif) that are located in the C terminus of the NFAT2 NHR, and are highly conserved in the other calcium-regulated NFAT proteins, contribute to their interaction with calcineurin and might have a role in facilitating the calcineurin-mediated dephosphorylation of certain NFAT family members[22–24].

The activity of calcineurin is controlled not only by calcium and calmodulin but also by several calcineurin inhibitors, which have been identified during the past few years. These include calcineurin-binding protein 1 (CABIN1; also known as CAIN), the A-kinase anchor protein AKAP79 (also known as AKAP5) and members of the Down's syndrome critical region (DSCR)/modulatory calcineurin-interacting protein (MCIP) family of calcineurin inhibitors, which are known as calcipressins[25–29]. Mice that express a truncated form of *Cabin1* show increased cytokine expression by T cells that are stimulated with CD3-specific antibodies[30]. Likewise, lower threshold levels for T-cell stimulation were described in mice that lack calcipressin-1, with increased expression of cytokines and CD95 ligand (CD95L; also known as FAS ligand) in response to TCR ligation[30,31], which emphasized the importance of CABIN1- and calcipressin-mediated regulation of T-cell activation and activation-induced cell death.

Of the 14 phosphorylation sites that have been mapped in NFAT1 and are conserved in all calcineurin-regulated NFAT proteins, all but one are dephosphorylated by calcineurin[32]. These sites are located in three different serine-rich motifs: the

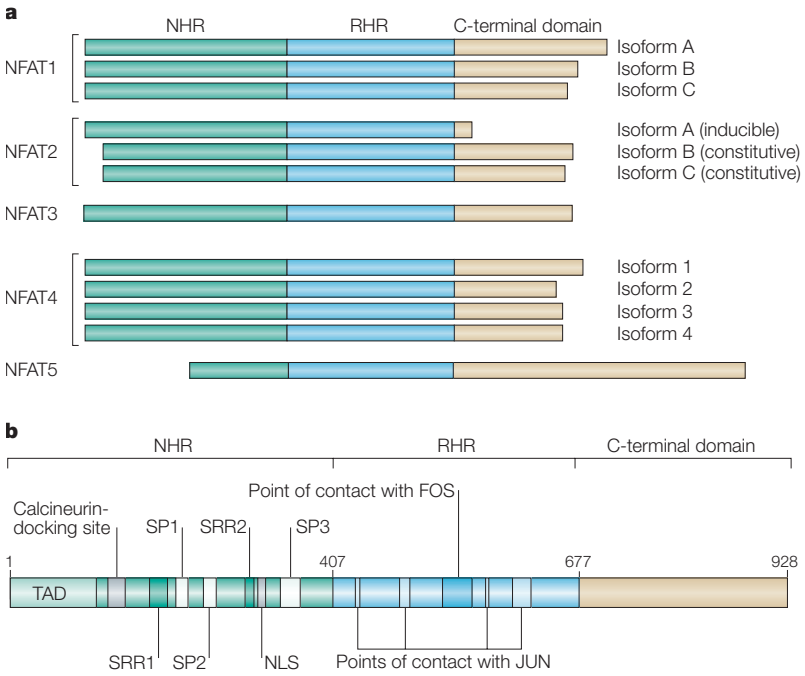
Table 1 | **NFAT family of transcription factors**

NFAT family member	Alternative names	Regulation	Expression in the immune system
NFAT1	NFATc2 and NFATp	Calcium–calcineurin	Yes
NFAT2	NFATc1 and NFATc	Calcium–calcineurin	Yes
NFAT3	NFATc4	Calcium–calcineurin	No
NFAT4	NFATc3 and NFATx	Calcium–calcineurin	Yes
NFAT5	TonEBP and OREBP	Osmotic stress	Yes

NFAT, nuclear factor of activated T cells; TonEBP, tonicity-responsive enhancer-binding protein.

GREEN FLUORESCENT PROTEIN FUSION PROTEIN (GFP fusion protein). A hybrid protein that is created by the fusion of the GFP from *Aequorea victoria* and another protein. This construct allows the tracking of the behaviour of the fused protein using the fluorescence that is emitted by GFP.

serine-rich region 1 (SRR1) motif, and the SPXX (where X denotes any amino acid) repeat motifs, SP2 and SP3 (FIG. 1b). Dephosphorylation of the serine residues in these motifs leads to exposure of the NFAT1 nuclear-localization signal and nuclear import, and it might also control the DNA-binding affinity of the RHR. Several kinases have been reported to phosphorylate NFAT proteins and control their nuclear shuttling, including glycogen-synthase kinase 3 (GSK3), casein kinase 1 (CK1), p38 and JUN N-terminal kinase (JNK)[33–39]. The present data point to a complex model, in which different kinases phosphorylate the various serine-rich motifs in NFAT proteins. It is also necessary to distinguish between maintenance kinases, which act in the cytosol to keep NFAT proteins in a fully phosphorylated state and prevent their translocation into the nucleus in resting cells, and export kinases, which rephosphorylate NFAT in the nucleus and promote its nuclear export, thereby stopping NFAT-mediated transcription after T-cell stimulation is withdrawn and calcineurin activity declines. CK1 docks at a conserved motif that is near the N terminus of NFAT proteins, and it functions as both a maintenance and an export kinase for SRR1 (REFS 36,38). GSK3 also functions as an export kinase for the SP2 motif of NFAT1 and for both the SP2 and SP3 motifs of NFAT2 (REFS 33,40). In NFAT2, substrate sites for GSK3 might only be created after previous 'priming' by the phosphorylation of those sites by cyclic-AMP-dependent protein kinase (PKA)[33]. Maintenance kinases that phosphorylate the SP2 and SP3 motifs, and the export kinase for the SP3 motif of NFAT1, remain to be discovered (FIG. 1b). Mitogen-activated protein kinases (MAPKs) differentially phosphorylate the first serine of SRR1 in the different NFAT proteins — p38 phosphorylates NFAT1, whereas JNK phosphorylates NFAT2 (REFS 35,37). This can potentiate the ability of CK1 to processively phosphorylate the remaining serines in SRR1. Therefore, depending on which MAPK pathways are active, a cell could express several NFAT proteins but have only one type that is actually located in the nucleus and that contributes to transcriptional activity. This might be the basis for certain reported isoform-specific functions of NFAT proteins.



Motif	Amino-acid sequence	Maintenance kinase	Export kinase
SRR1	SPASSGSSASFISD	CK1	CK1
SP2	SPRTSPIMSPRT	ND	GSK3
SP3	SPQRSRSPSPQPSP	ND	ND (GSK3 for NFAT2)

Figure 1 | **The NFAT family of transcription factors. a** | Schematic structure of the members of the nuclear factor of activated T cells (NFAT) family of transcription factors. Proteins are aligned by their REL-homology domain (RHR), which contains the DNA-binding motif. The NFAT-homology region (NHR) is also conserved among NFAT1–NFAT4. The three calcium–calcineurin-regulated NFAT proteins that are expressed by T cells — NFAT1, NFAT2 and NFAT4 — have several isoforms, which are generated by alternative splicing or the use of alternative promoters. The two alternative amino (N) termini of NFAT2 are represented in two constitutively expressed isoforms and an inducible isoform. **b** | A schematic representation of the location of the transactivation, regulatory, DNA-binding and carboxy (C)-terminal domains of NFAT1 is shown. Regulatory motifs in the NHR are highly conserved among the calcium–calcineurin-regulated NFAT proteins (NFAT1–NFAT4). This region includes the calcineurin-docking site, the nuclear-localization signal (NLS), the serine-rich regions (SRRs) and the SPXX-repeat motifs (SPs), where X denotes any amino acid. An inducible phosphorylation site has also been described in the N-terminal transactivation domain (TAD) of NFAT1. In addition to the DNA-binding domain, the RHR contains points of contact with FOS and JUN, which allows the formation of the synergistic NFAT–FOS–JUN–DNA quaternary complex. The phosphoserines that are targeted by calcineurin dephosphorylation (underlined) are located in the SP2, SP3 and SRR1 motifs. These serines are phosphorylated in the cytosol by maintenance kinases and in the nucleus by export kinases. CK1, casein kinase 1; GSK3, glycogen-synthase kinase 3; ND, not determined.

Autoregulation of NFAT2. Although the main mode of NFAT regulation is through calcium and calcineurin, NFAT2 is apparently unique in that it is also regulated at the transcriptional level through an autoregulatory loop. This mechanism is isoform specific — only one of the two NFAT2 N-terminal splice variants, NFAT2A, is under the control of an NFAT-dependent inducible promoter[41,42]. In T cells, NFAT1, which is the main isoform in naive cells[43], might act together with constitutively expressed NFAT2 and/or NFAT4 to turn on expression of the inducible isoform of NFAT2. Only when sufficiently high levels of NFAT2 expression are attained would the self-sustaining positive autoregulatory loop function to maintain high levels of NFAT2 expression and NFAT activity.

Regulation by cytokine receptors. More recently, several reports have shown that NFAT activation is mediated by cytokine signalling. Signalling through the receptors for IL-2 and IL-15 in human peripheral-blood mononuclear cells regulates the binding of NFAT proteins to the promoter of the gene that encodes CX_3C-chemokine receptor 1 (CX_3CR1). IL-2 promotes the binding of NFAT2 to the *CX_3CR1* promoter and induces its expression, whereas IL-15 represses *CX_3CR1* expression by inducing the preferential binding of NFAT1 (REF. 44). These effects can be blocked by the calcineurin inhibitor cyclosporin A (CsA) or the VIVIT peptide, which specifically blocks

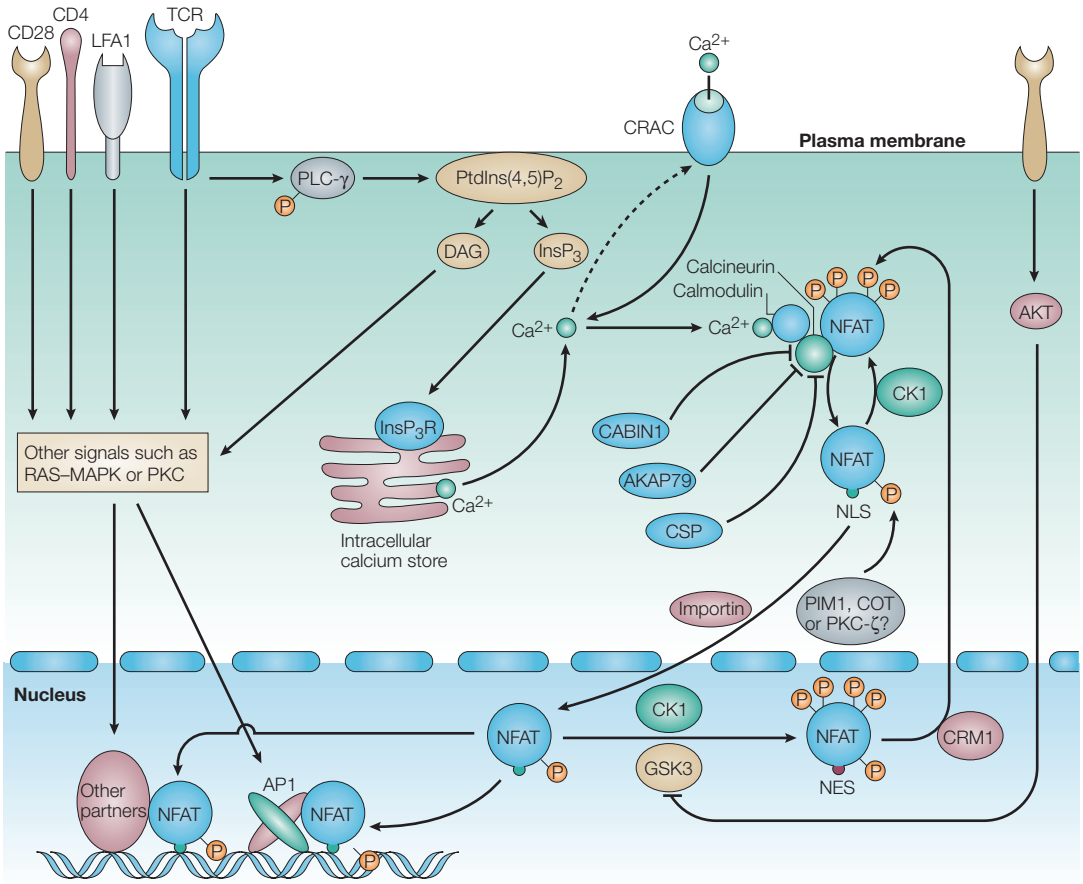


Figure 2 | **Regulation of NFAT activation.** Ligation of the T-cell receptor (TCR) triggers the activation of receptor-associated tyrosine kinases that lead to the activation of phospholipase C-γ (PLC-γ). Activated PLC-γ causes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), which generates inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ binds to its receptor ($InsP_3R$) and induces an increase in intracellular calcium levels that is caused by the depletion of intracellular stores. This increase triggers, through poorly characterized mechanisms, the opening of calcium-release-activated calcium (CRAC) channels in the plasma membrane, which leads to a sustained increase in intracellular calcium levels. Calcium binds calmodulin and activates calcineurin. Activated calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) proteins, which exposes their nuclear-localization signal (NLS) and induces their nuclear translocation. Other kinases, such as protein kinase C-ζ (PKC-ζ), PIM1 (proviral integration site 1) or COT (Cancer Osaka thyroid), might also phosphorylate NFAT and contribute to its activation[129–131]. The activity of calcineurin is also negatively regulated by calcineurin-binding protein 1 (CABIN1), calcipressins (CSPs) and A-kinase anchor protein 79 (AKAP79). After it has entered the nucleus, NFAT interacts with activator protein 1 (AP1) and other transcriptional partners to promote gene transcription. The activation of these partners during T-cell stimulation might be elicited by signals that are transmitted through different signalling pathways (such as the RAS–mitogen-activated protein kinase (MAPK) pathway or the PKC pathway), which are activated by the engagement of the TCR and/or other receptors. The activity of NFAT is also regulated by kinases, such as casein kinase 1 (CK1) and glycogen-synthase kinase 3 (GSK3), which help to maintain NFAT in a phosphorylated state in the cytosol (maintenance kinases) or induce the rephosphorylation of nuclear NFAT to expose a nuclear-export signal (NES) and translocate NFAT back to the cytosol (export kinases). GSK3 is negatively regulated by the kinase AKT, the activation of which is coupled to CD28 engagement. CRM1, chromosomal region maintenance 1; LFA1, lymphocyte function-associated antigen 1.

the calcineurin–NFAT interaction[44]. Similarly, signalling through the IL-6 receptor (IL-6R) results in the preferential induction of *NFAT1* transcripts. This leads to increased NFAT1 activity in response to T-cell stimulation, which results in increased IL-4 production[45].

SUMOYLATION has recently been identified as a new mechanism that regulates NFAT nuclear retention[46]. The sumoylation of NFAT1 is induced by increased intracellular calcium levels, and mutants that are unable to be tagged with small ubiquitin-like modifier (SUMO) molecules tend to leak out of the nucleus in stimulated cells. The sumoylation sites of NFAT1 are located in the C terminus and are not conserved in other NFAT family members, which indicates that this might be a specific regulatory mechanism that differentially controls NFAT1 nuclear export[46].

SUMOYLATION
The post-translational modification of proteins that involves the covalent attachment of small ubiquitin-like modifier (SUMO) and regulates the interactions of those proteins with other macromolecules.

Transcriptional partners of NFAT proteins

Because NFAT proteins can interact with different transcription-factor partners in the nucleus, they are important integrators of calcium signalling with many other signalling pathways in T cells. The structures of monomeric NFAT–DNA complexes emphasize the high flexibility of the linker region that is located between

NF-κB-BINDING MOTIF (Nuclear factor-κB-binding motif). A DNA-binding sequence that is recognized by NF-κB proteins. Nuclear factor of activated T cells (NFAT)-dimer complexes can form on motifs that are similar to these.

the N-terminal domain of the RHR, which contains the DNA-binding loop and confers base-specific recognition, and the C-terminal domain, which makes contact only with the phosphate backbone of DNA[47]. In principle, this flexibility allows many surfaces of both domains to interact with different transcription factors.

Activator protein 1. Activator protein 1 (AP1) proteins are the main transcriptional partners of NFAT during T-cell activation[48,49] (FIG. 2). Dimers of FOS and JUN form quaternary complexes with NFAT and DNA on NFAT–AP1 composite sites, which contain two adjacent binding motifs for both transcription factors and are present in many genes that are induced during T-cell activation[2,48]. These complexes have an extensive network of protein–protein contacts, which explains their stability and cooperative nature[16]. Cooperation between NFAT proteins and AP1 integrates two of the main signalling pathways that are induced in response to T-cell stimulation: calcium signalling, which is responsible for the activation of NFAT proteins; and the RAS–MAPK pathway, which induces the expression and activation of FOS and JUN[50]. NFAT–AP1 cooperation during T-cell activation is responsible for a specific pattern of gene expression, which induces the functional changes that characterize an activated T cell. In the absence of AP1, different sets of genes are activated by NFAT proteins, which might result in a completely different functional outcome[43,51].

The full activation of T cells requires the engagement not only of the TCR but also of various co-stimulatory receptors, including the co-receptors CD4 and CD8, the integrin lymphocyte function-associated antigen 1 (LFA1), and co-stimulatory molecules such as CD28 and inducible T-cell co-stimulator (ICOS). Some of these couple mainly to calcium influx and NFAT activation, whereas others couple to AP1 and nuclear factor-κB (NF-κB) activation, although most probably influence both pathways to varying extents. The CD4 and CD8 co-receptors bind LCK, which initiates a cascade of tyrosine phosphorylation that leads to PLC-γ activation and calcium signalling[52]. LFA1 increases calcium signalling[53] but also couples to AP1 activation[54]. CD28 has several effects, which are consistent with its role as a co-stimulatory receptor. It induces calcium signalling through phosphatidylinositol 3-kinase (PI3K) activation of TEC-family tyrosine kinases, which are required for the optimal phosphorylation and activation of PLC-γ in T cells[55]. CD28 also activates AKT through the PI3K pathway, one of the downstream targets of which is GSK3. Normally, the activation of GSK3 results in NFAT nuclear export; however, under conditions of AKT activation, GSK3 undergoes an inhibitory phosphorylation on a serine residue close to its N terminus, which diminishes NFAT nuclear export and prolongs the overall time of NFAT residence in the nucleus[56,57]. CD28 is also coupled to NF-κB and AP1 activation[58,59].

Although cooperation between NFAT and AP1 proteins is of crucial importance during T-cell activation, FOS and JUN proteins are not the only NFAT transcriptional partners. There are also many reports of functional synergy and protein–protein interactions between NFAT proteins and proteins belonging to several other families of transcription factors, including MAF and GATA-binding proteins (such as GATA3)[60–71] (TABLE 2). Binding to different transcription factors allows NFAT proteins to cooperate with them and allows the integration of different signalling pathways to activate specific programmes of gene expression in response to various stimuli.

Dimers of NFAT. As could be inferred from the fact that the DNA-binding domain of NFAT is structurally homologous to the REL domain, NFAT proteins can also function as dimeric transcription factors at quasipalindromic sites that resemble NF-κB-BINDING MOTIFS. Two structures of NFAT1 homodimers, which are bound to NF-κB-like NFAT sites in the *IL-8* promoter (two 4-base pair (bp) binding sites separated by 1-bp spacing) and the HIV-1 long terminal repeat (two 4-bp binding sites separated by 2-bp spacing) have recently been solved[72,73]. A site with 3-bp spacing has also been reported in the vascular endothelial growth factor (*VEGF*) promoter[74]. Dimerization involves the C-terminal domain of the RHR; depending on the spacing of the NF-κB-binding motifs, the N-terminal domains adopt different relative orientations, which might promote their interactions with different co-activator or co-repressor proteins. Therefore, because of the flexible linker region between the N- and C-terminal regions of the RHR domain, NFAT dimers are structurally more flexible

Table 2 | **NFAT transcriptional partners**

Transcription partner	Interaction site	Effect	References
AP1 (FOS, JUN)	Many cytokine-gene promoters	Positive synergy	2,16,48,49
C/EBP	*PPAR-γ* promoter	Positive synergy	69
MAF	*Il-4* promoter	Positive synergy	61,64
EGR1 and EGR4	*Tnf* promoter	Positive synergy	62
GATA3	*Il-4 3′* enhancer	Positive synergy	61
ICER	Many cytokine-gene promoters	Inhibition of NFAT activity	60
IRF4	*Il-4* promoter	Positive synergy	68
MEF2	*NUR77* promoter	Positive synergy	71
OCT	*IL-3* enhancer	Positive synergy	63
p21^{SNFT}	*IL-2* promoter	Inhibition of NFAT activity	65
PPAR-γ	*IL-2* promoter	Inhibition of NFAT activity	70
T-bet	*Ifn-γ 5′* enhancer	Positive synergy	66

AP1, activator protein 1; C/EBP, CCAAT/enhancer-binding protein; EGR, early growth response; GATA3, GATA-binding protein 3; ICER, inducible cyclic AMP early repressor; *Ifn-γ*, interferon-γ; *Il*, interleukin; IRF4, IFN-regulatory factor 4; MEF2, myocyte-enhancer factor 2; NFAT, nuclear factor of activated T cells; *NUR77*, orphan nuclear receptor 77; OCT, octamer-binding transcription factor; p21^{SNFT}, 21-kDa small nuclear factor isolated from T cells; *PPAR-γ*, peroxisome-proliferator-activated receptor-γ; *Tnf*, tumour-necrosis factor.

Table 3 | **NFAT-deficient mice**

NFAT protein	Phenotype in the immune system	References
NFAT1	Moderate hyperproliferation with splenomegaly. Moderately enhanced B- and T-cell responses, with bias towards T_H2-cell responses. Prolonged IL-4 expression and decreased IFN-γ production in response to TCR ligation. Delayed thymic involution. Enhanced germinal-centre formation.	80,81,96, 105,106
NFAT2	In the RAG2-deficient complementation system, reduced proliferative responses by T cells. Impaired repopulation of the thymus and lymphoid organs. Impaired T_H2-cell responses and IL-4 production.	82,112
NFAT1 and NFAT2	In fetal liver chimeras, grossly impaired T-cell effector functions, with profound defects in cytokine production and cytolytic activity. B-cell hyperactivity.	91
NFAT4	Impaired development of CD4 and CD8 single-positive cells, with increased apoptosis of double-positive thymocytes. Mild hyperactivation of peripheral T cells.	94
NFAT1 and NFAT4	TCR hyper-reactivity, with profound lymphoproliferative disorder. Notable increase in T_H2-cell responses. Allergic blepharitis and interstitial pneumonitis.	83,95
NFAT5	Impaired T-cell function under hyperosmotic conditions. Decreased cellularity of the thymus and spleen.	11

IFN-γ, interferon-γ; IL-4, interleukin-4; NFAT, nuclear factor of activated T cells; RAG, recombination-activating gene; TCR, T-cell receptor; T_H2 cell, T-helper-2 cell.

than those that are formed by REL proteins, or even NFAT5, which always acts as a homodimer[9,75]. Structural considerations indicate that it would be possible to form heterodimers between different NFAT family members; however, there is no experimental evidence, so far, to support this hypothesis. In cells that have been exposed only to stimuli that increase intracellular calcium levels, NFAT dimers would be expected to occupy their palindromic sites in gene regulatory regions and to turn on specific gene-expression programmes[43,51].

Redundancy in the NFAT family

The high degree of similarity among the RHRs of the different NFAT family members confers common DNA-binding specificities and partner interactions, which might explain the redundancy in some NFAT-regulated functions. NFAT1, NFAT2 and NFAT4 are all co-expressed in T cells and are activated in response to TCR engagement[76,77]. Only the short isoform of NFAT2 is also regulated through a positive-feedback loop by NFAT proteins[42,78]. Gene-knockout mice that lack individual NFAT proteins show only mild alterations in immune function, and it is only when more than one family member has been eliminated that severe changes in many cells and functions of the immune system become apparent[79–82] (TABLE 3). Although these data imply some degree of redundancy among NFAT family members, they also indicate that some functions might be controlled by regulating the balance of specific NFAT proteins that can occupy a given promoter at a specific time during T-cell activation or differentiation. Cell-specific differences in expression levels (for example, NFAT4 is the main NFAT protein that is expressed by thymocytes, but its expression by peripheral T cells is low compared with that of NFAT1 or NFAT2), distinct mechanisms of regulation (for example, positive feedback of *NFAT2A* expression) or the ability to interact with different co-activators through their less-conserved N- or C-terminal domains might explain why certain T-cell functions are specifically regulated by different NFAT family members, as indicated by the phenotypes of NFAT-deficient mice.

NFAT proteins in T-cell development

In the thymus, immature precursors that are generated in the bone marrow differentiate into mature T cells, which can develop into effector cells on antigen encounter. Double negative (DN) T-cell precursors that do not express CD4 or CD8 mature and first rearrange the β-chain of the TCR, which together with the invariant pre-TCR α-chain forms the pre-TCR, and then rearrange the α-chain of the TCR. DN thymocytes then become double positive (DP) cells that express both CD4 and CD8. $CD4^+CD8^+$ DP thymocytes undergo positive and negative selection to successfully generate $CD8^+$ or $CD4^+$ single positive (SP) T cells with an antigen receptor that can interact with self-MHC molecules (positively selected) but is unable to recognize self-antigens (negatively selected). Mature T cells that express either CD4 or CD8 are then released into the periphery[83,84].

Calcium and calcineurin signals are involved in the regulation of thymocyte proliferation and the development of immature DN thymocytes into mature SP cells. In addition, pre-TCR signalling induces an increase in intracellular calcium levels that results in the activation of both NFAT and NF-κB[85], although the exact targets of these transcription factors in immature thymocytes remain to be identified. The impairment of calcineurin activity has different effects on T-cell development, and these depend on the experimental method that is used. In early experiments, the inhibition of calcineurin activity with CsA led to defects in thymocyte development that included an impaired DP-to-SP transition and defects in negative selection[86,87]. However, in more recent experiments, mice with a selective gene knockout of the most common isoform of the catalytic subunit of calcineurin in T cells (calcineurin Aβ) showed a defect in peripheral T-cell activation that correlated with deficient activation of all NFAT proteins and a decreased number of SP thymocytes, indicating the involvement of calcineurin Aβ in positive selection[88]. This was confirmed by studies in which the regulatory subunit of calcineurin (calcineurin B) was specifically inactivated in DN thymocytes. These mice had a clear defect in positive selection, with reduced numbers of SP cells, but they seemed to undergo normal negative selection, which indicated that calcineurin-independent signalling pathways might be responsible for the regulation of the negative selection of thymocytes[89]. Conversely, the expression of an active form of calcineurin A resulted in increased positive selection, which was

possibly the result of increased calcium–calcineurin signalling in response to weak TCR interactions, and there was no notable alteration in negative selection[90]. Therefore, although other signalling pathways (such as those involving LCK or RAS–MAPK)[83] are likely to be involved in the decision of whether a DP thymocyte survives and becomes a mature T cell, it is evident that calcium–calcineurin signalling is also necessary for this process.

More direct evidence of the importance of NFAT proteins for thymocyte development has come from the analysis of NFAT-deficient mice. Defects in T-cell development in *Nfat4*$^{-/-}$ mice include a reduction in the number of SP thymocytes, which correlates with increased apoptosis of DP thymocytes, either as a result of increased TCR reactivity or because of defects in B-cell lymphoma 2 (BCL-2) expression by DP cells[91]. In any case, the data indicate that NFAT4 has a positive role in the control of thymocyte survival at the DP-to-SP transition stage. Consistent with this observation, the expression of a constitutively active form of NFAT4 in a DN cell line potentiates the transition to SP cells in response to phorbol 12-myristate 13-acetate (PMA) stimulation[92]. The activation of NFAT4 in response to TCR engagement could then be a crucial control in positive selection and SP cell survival. Other NFAT family members are unlikely to substitute for NFAT4 at this stage of thymocyte development, given their low levels of expression by DP thymocytes[91,93]. Surprisingly, mice that lack both NFAT1 and NFAT4 develop a hyperproliferative phenotype that might be a result of defects in apoptosis, owing to impaired CD95L expression or to hyperreactivity after TCR ligation[94]. Slight increases of TCR reactivity can also be detected in mice that lack either NFAT1 or NFAT4 (REFS 79,80,91). However, NFAT2-deficient cells are hyporesponsive[81,82]. It is therefore possible that NFAT1 and NFAT4, but not NFAT2, might directly or indirectly regulate the threshold for TCR activation[95]. It is not clear whether the defect in apoptosis can also affect negative selection in the thymus, although NFAT1-deficient mice have retarded THYMIC INVOLUTION[96].

NFAT and T-helper-cell differentiation

As for many transcription factors, NFAT proteins can regulate gene expression at two different levels. In a locus that is open and available for immediate transcription, such as the *IL-2* locus, NFAT proteins and other transcription factors bind and promote (or repress) transcription. By contrast, in a precursor cell type that is poised to differentiate along one or another pathway of cell-lineage specification, the loci that control genes of a distinct lineage are closed and require remodelling such that transcription factors can gain access to the promoter to drive gene expression. As such, inducible transcription factors, such as NFAT proteins, might be required to remodel the locus itself and also to induce the transcription of lineage-specific transcription factors that commit the cells to one or the other lineage.

A good example of this is the choice that is made by naive T helper (T_H) cells in the periphery to proceed along either the T_H1 or T_H2 pathway (FIG. 3). This is a process of antigen-driven differentiation that transforms these cells into two distinct populations with characteristic patterns of cytokine expression and, therefore, specific immune functions. T_H1 cells are characterized by the expression of interferon-γ (IFN-γ) and participate in the clearance of intracellular pathogens. (They also contribute pathologically to inflammation and autoimmune disease.) By contrast, T_H2 cells express IL-4, IL-5 and IL-13, constitute a defence against extracellular pathogens, and are important in atopy and asthma. The choice of differentiation into T_H1 or T_H2 cells is determined by the nature and intensity of the antigenic stimulus, the type of antigen-presenting cell that delivers it and the signals that are received from specific cytokines (IL-12 for T_H1 cells and IL-4 for T_H2 cells)[97].

Epigenetic changes that are induced by TCR signalling and cytokine-mediated signalling are responsible for the establishment of specific patterns of cytokine expression. The evidence supports a model in which NFAT proteins act together with signal transducer and activator of transcription (STAT) factors to determine the T_H1/T_H2 lineage choice (FIG. 3): STAT1 and STAT4 downstream of IL-12 and IFN-γ, respectively, for T_H1 cells; and STAT6 downstream of IL-4 for T_H2 cells. The synergistic action of these two families of acutely inducible transcription factors elicits the expression of lineage-specific T-BOX-FAMILY TRANSCRIPTION FACTORS (T-bet and eomesodermin) and GATA3, respectively, and at the same time leads to changes in chromatin structure that are induced by the recruitment of CHROMATIN-REMODELLING COMPLEXES to the regulatory regions of the *IFN-γ* and *IL-4* genes. These structural changes can be detected by the appearance of DNase-I-hypersensitivity sites, which identify regions in the chromatin with increased accessibility to DNase and, by implication, to transcription factors[98–100]. The appearance of new DNase-I-hypersensitivity sites in the *IFN-γ* and *IL-4* genes during T_H1- and T_H2-cell differentiation is blocked by CsA, which supports the involvement of NFAT proteins in restructuring these cytokine genes[101]. The net result is that, although NFAT proteins bind to the promoters of both *IFN-γ* and *IL-4* during the early stages of naive T-cell activation[102], after T-cell differentiation has been initiated in either the T_H1- or T_H2-cell direction, the inappropriate locus is progressively silenced so that NFAT proteins bind specifically to the promoters of the *IFN-γ* or *IL-4* genes in T_H1 or T_H2 cells, respectively. This leads to the selective expression of only the appropriate cytokine gene. This cell-type-specific binding is accompanied by changes in chromatin accessibility and histone acetylation[61,101,103].

Whether individual NFAT proteins have selective roles in T_H1/T_H2-cell differentiation has been controversial. The loss of NFAT1 diminishes T_H1-cell differentiation and promotes a mild bias towards T_H2-cell differentiation; this is probably because T cells from

THYMIC INVOLUTION
An age-dependent decrease of thymic epithelial volume that results in the decreased production of T cells.

T-BOX-FAMILY TRANSCRIPTION FACTORS
A family of transcription factors that contain a DNA-binding domain of 200 amino acids, which is known as the T box. These factors are usually involved in developmental programmes. The founding member of this family was Brachyury, and T-bet and eomesodermin are also members.

CHROMATIN-REMODELLING COMPLEXES
Enzymatic complexes that achieve the remodelling of DNA–nucleosomal architecture and determine transcriptional activity. The SWI–SNF (switching-defective–sucrose non-fermenting) ATPases are an example of complexes that remodel chromatin.

NFAT1-deficient mice maintain IL-4 production for longer[79,104]. Furthermore, NFAT1 deficiency also markedly impairs IFN-γ production by committed T_H1 cells[105]. Similarly, mice that are deficient in both NFAT1 and NFAT4 are characterized by exacerbated T_H2-cell responses, with increased expression of T_H2 cytokines, increased IgG1 and IgE titres, and severe interstitial pneumonitis[94,95]. By contrast, NFAT2-deficient T cells show impaired production of IL-4 and other T_H2 cytokines, and reduced titres of IgG1 and IgE[81,82]. These results have been interpreted as indicating that NFAT2 is required for T_H2-cell differentiation, whereas NFAT1 and NFAT4 promote T_H1-cell differentiation and act as negative regulators of T_H2-cell differentiation. However, when constitutively active forms of NFAT1 and NFAT2 are expressed by T_H1 and T_H2 cells, both proteins are equally able to induce the transcription of T_H1 and

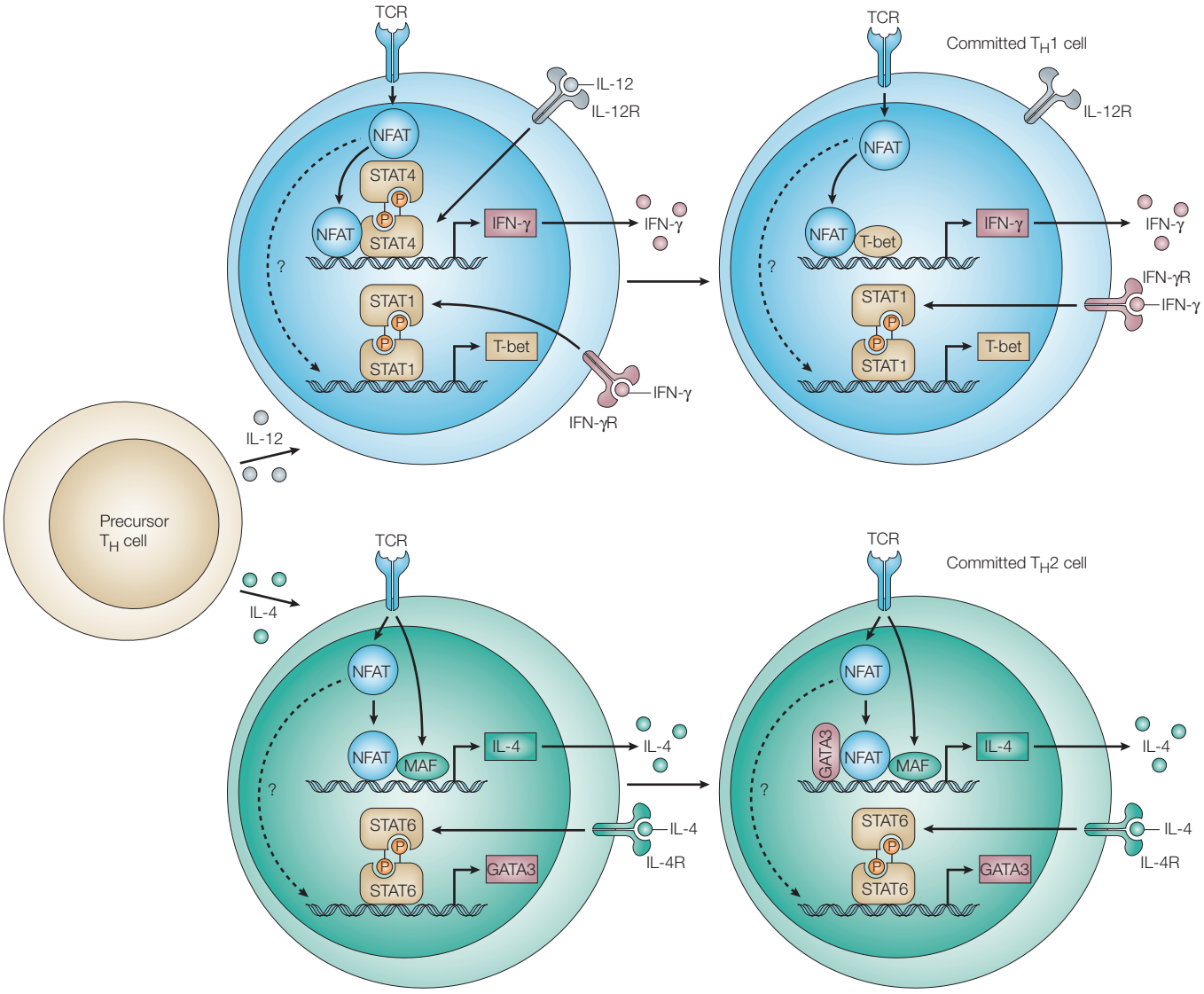


Figure 3 | **NFAT and T-helper-cell differentiation.** On encountering antigen-presenting cells presenting cognate peptide–MHC complexes that engage the T-cell receptor (TCR), naive T helper (T_H) cells differentiate into effector T_H1- or T_H2-cell populations. The outcome of this process depends on the nature of the stimulus (type of antigen, intensity of stimulation and type of cell that presents the antigen) and the signals that are received from specific cytokines. This process can be envisioned as occurring in two steps. In the first step, transcription factors that are activated by TCR engagement cooperate with signal transducer and activator of transcription (STAT) proteins to induce the expression of cytokine genes and lineage-specific transcription factors. In T_H1 cells, nuclear factor of activated T cells (NFAT) proteins cooperate with STAT4, which is activated in response to interleukin-12 receptor (IL-12R) engagement, to induce the expression of interferon-γ (IFN-γ). Signalling through the IFN-γ receptor (IFN-γR) induces STAT1 activation and translocation to the nucleus, where it induces the expression of T-bet. In T_H2 cells, MAF and NFAT proteins induce the expression of IL-4. IL-4R signalling causes the activation of STAT6, which promotes GATA-binding protein 3 (GATA3) expression. It is possible that NFAT proteins might also cooperate with STAT factors to induce the expression of T-bet and GATA3. In the second step, NFAT proteins cooperate with T-bet in T_H1 cells and GATA3 in T_H2 cells to maintain and commit to T_H-cell differentiation through the induction of IFN-γ or IL-4. Autoregulatory positive-feedback loops ensure the expression of T-bet and GATA3.

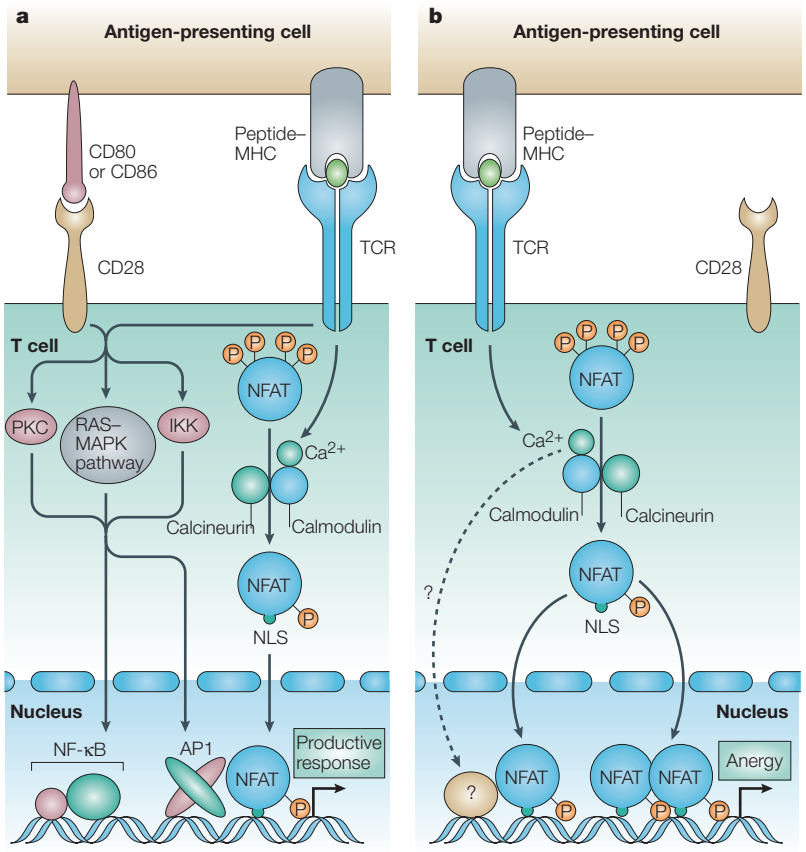


Figure 4 | **NFAT-activated programmes of gene expression: T-cell activation versus T-cell anergy. a** | Signals that are delivered by the engagement of the T-cell receptor (TCR; signal 1) and co-stimulatory molecules (such as CD28; signal 2) induce different signalling pathways that result in the activation of several transcription factors. In the nucleus, nuclear factor of activated T cells (NFAT) proteins cooperate with activator protein 1 (AP1) and other transcription factors to induce a programme of gene expression that is characteristic of a productive immune response. **b** | When TCR engagement (signal 1) occurs in the absence of co-stimulation, calcium-mediated signals induce the activation of NFAT proteins without concomitant AP1 activation. In the absence of cooperative binding to FOS and JUN, NFAT proteins, which might form dimer complexes or cooperate with other calcium-induced transcription factors, elicit the expression of a distinct set of anergy-inducing genes. The products of these genes inhibit T-cell function at different levels and induce a status of T-cell unresponsiveness. IKK, inhibitor of NF-κB (IκB) kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NLS, nuclear-localization signal; PKC, protein kinase C.

T_H2 cytokines[106,107]. Furthermore, the *in vivo* binding of NFAT1 has been shown at the promoters of both the *IFN-γ* and *IL-4* genes in activated T_H1 and T_H2 cells, respectively[61]. Overall, these data indicate that NFAT1 and NFAT2 are functionally redundant and have a role in both branches of T-cell differentiation, which contradicts the results from the analysis of mice that lack NFAT1 or NFAT2. Further studies are required to understand this paradox. Co-stimulatory molecules other than CD28 (for example, ICOS) might also influence differential NFAT activation and T_H-cell fate[108]. In addition, the observation that mice that are deficient in both NFAT1 and NFAT4 have a severe allergic phenotype is probably the result not only of the increase in T_H2 immunity but also of the hyper-responsive phenotype of T cells in these animals[95].

HISTONE DEACETYLASE
An enzyme that removes the acetyl groups from lysine residues located at the amino termini of histones. In general, decreased levels of histone acetylation are associated with the repression of gene expression. The balance of histone acetylation is maintained by the interplay between histone deacetylases and histone acetyltransferases.

T-cell activation

The simultaneous engagement of the TCR and co-stimulatory molecules (such as CD28) at the surface of T cells initiates a genetic programme of expression that leads to full T-cell activation[56,109,110]. The importance of NFAT proteins in T-cell activation is underscored by genetic data. In two human families, the inability to activate NFAT proteins because of a defect in store-operated calcium entry was associated with severe immunodeficiency[111]. In mice, deficiency in both NFAT1 and NFAT2 in T cells is associated with grossly impaired production of many cytokines, including IL-2, IL-4, IL-10, IFN-γ, granulocyte/macrophage colony-stimulating factor and TNF. IL-5 expression is also notably diminished, as well as the expression of CD40L and CD95L[112], which confirms that the activation of NFAT proteins is essential for T cells to carry out many of their effector functions. Indeed, the analysis of gene expression using DNA microarrays has clearly shown that the activation-induced expression of most genes in T cells is blocked by calcineurin inhibitors[43,56,113]. Furthermore, the specific inhibition of the activation of NFAT proteins using the VIVIT peptide results in the decreased expression of many cytokine genes[19]. The analysis of control elements in several cytokine-gene promoters has uncovered the presence of numerous consensus NFAT-binding sites that are responsible for the NFAT dependence of their expression[2,48].

Another important aspect of T-cell function that NFAT might regulate is cell-cycle control. NFAT1 downregulates cyclin-dependent kinase 4 (CDK4) expression by directly binding and repressing its transcription through the recruitment of HISTONE DEACETYLASES. CDK4 downregulation induces exit from the cell cycle and re-entry into G0 (REF. 114). Further evidence for this function of NFAT comes from the observation that T cells from NFAT1-deficient mice show defects in CDK4 downregulation and increased levels of several cyclins[114,115].

T-cell tolerance

For many years, NFAT has been considered to be a key regulator of T-cell activation through its interaction with proteins of the AP1 family of transcription factors. The discovery that NFAT proteins can also form transcriptional complexes with other partners, and can even be transcriptionally active by themselves, has introduced the possibility of defining new roles for NFAT proteins in T cells. In the classical two-signal model, the stimulation of T cells by engagement of their TCR (signal 1) and co-stimulatory molecules (such as CD28; signal 2) results in full productive T-cell activation, whereas signalling through the TCR in the absence of co-stimulation leads to ANERGY[116,117]. The absence of CD28–CD80/CD86 interactions leads to the stimulation of calcium-activated signalling pathways in the absence of the full induction of other pathways: for example, pathways that are regulated by the RAS–MAPK pathway, protein kinase C (PKC) or IKKs (inhibitor of NF-κB (IκB) kinases) (FIG. 4). This unbalanced activation ultimately results

ANERGY
A state of T cells that have been stimulated through their T-cell receptor in the absence of ligation of CD28. On restimulation, these T cells are unable to produce interleukin-2 or to proliferate, even in the presence of co-stimulatory signals.

E3 UBIQUITIN LIGASE
An enzyme that attaches the molecular tag ubiquitin to proteins. Depending on the position and number of ubiquitin molecules that are attached, the ubiquitin tag can target proteins for degradation by the proteasomal complex, sort them to specific subcellular compartments or modify their biological activity.

in the presence of different sets of transcription factors in the nucleus[43]. Under these circumstances, NFAT, in the absence of AP1 proteins and possibly other transcriptional partners, directs the transcription of a specific programme of gene expression that might be responsible for the block in TCR signalling that characterizes anergic T cells[43]. The transcription of these genes is clearly NFAT dependent: on receiving an anergizing stimulus, their expression is greatly reduced in *Nfat1*$^{-/-}$ T cells and is completely blocked by CsA, which correlates with the impaired induction of anergy in these cells[43]. It is possible that homo- or heterodimer complexes of NFAT proteins are responsible for the transcriptional activation of these genes[72,73], although interactions of NFAT with other calcium-activated transcription factors, such as myocyte enhancer factor 2 (MEF2)[71], might also be involved. So, because NFAT proteins control two opposing aspects of T-cell function — activation and anergy — it is probable that the availability of transcriptional partners in response to activating or anergizing stimuli determines which set of genes is activated. Furthermore, although CsA inhibits the induction of anergy, it still has a global immunosuppressive effect because of the crucial role of NFAT proteins in T-cell activation.

Among the proteins that are expressed by anergic T cells, a group of E3 UBIQUITIN LIGASES seems to have a crucial role in maintaining T cells in an unresponsive state[118]. Following sustained calcium–calcineurin signalling, several E3 ubiquitin ligases are upregulated at the mRNA and protein levels, including ITCH, the adaptor molecule CBL-B (Casitas B-lineage lymphoma B) and GRAIL (gene related to anergy in lymphocytes)[118–121]. The biochemical analysis of cells that are rendered anergic by sustained calcium signalling has shown that ITCH targets a limited number of signalling proteins (including PLC-γ1 and PKC-θ) for degradation, through a mechanism that requires cell–cell contact (probably between the T cell and the antigen-presenting cell) and involves monoubiquitylation and targeting for degradation in the lysosomal compartment[118]. However, the range of anergy-associated genes that are upregulated after sustained calcium signalling also includes tyrosine phosphatases, inhibitory cell-surface receptors, proteins that are potentially involved in intracellular trafficking and transcriptional repressors, which implies that the programme of anergy affects many cellular functions (TABLE 4).

Recently, an analysis of the function of CD4$^+$CD25$^+$ REGULATORY T (T_{REG}) CELLS in mice that are deficient in NFAT1 and NFAT4 showed that, even though these mice have functional T_{Reg} cells, their CD4$^+$CD25$^-$ cells are resistant to suppression by wild-type T_{Reg} cells[122]. These results imply that NFAT proteins might control not only T-cell inactivation that results from anergy induction but also the sensitivity of CD4$^+$CD25$^-$ cells to suppression by T_{Reg} cells. Common pathways might therefore be shared between these two mechanisms of peripheral T-cell tolerance.

Table 4 | **Calcium-dependent genes upregulated in anergic T cells**

Gene	Calcium–calcineurin dependent	NFAT dependent	References
E3 ubiquitin ligases			
Itch	Yes	ND	118
Cbl-b	Yes	ND	118,119
Grail	Yes	ND	120,121
Proteases			
Caspase-3	Yes	Yes	43
Transcriptional repressors			
Ikaros	Yes	Yes	43
Grg4	Yes	Yes	43
Protein tyrosine phosphatases			
Rptp-κ	Yes	Yes	43
Ptp1b	Yes	Yes	43
Others			
Diacylglycerol kinase	Yes	Yes	43
Gbp3	Yes	Yes	43
Cd98	Yes	Yes	43

Cbl-b, Casitas B-lineage lymphoma B; *Grail*, gene related to anergy in lymphocytes; *Gbp3*, guanylate-binding protein 3; *Grg4*, groucho-related gene 4; ND, not determined; NFAT, nuclear factor of activated T cells; *Ptp1b,* protein tyrosine phosphatase 1B; *Rptp-κ*, receptor-type protein tyrosine phosphatase-κ.

NFAT inhibitors: a new therapeutic approach

Given the important role of NFAT proteins in the control of T-cell activation, NFAT has always been considered to be an optimal target for therapeutic approaches that are aimed at regulating immune responses. Inhibitors of calcineurin, such as CsA and FK506, have been extensively used as immunosuppressive agents to improve graft survival and to treat autoimmune diseases[123–126]. These inhibitors act by blocking calcineurin enzymatic activity and are therefore not selective NFAT inhibitors[127]. In fact, the regulation of the activity of any other target that is dephosphorylated by calcineurin can be affected by these inhibitors, which might account for the nephro- and neurotoxicities that are associated with their clinical use[128,129]. The development of more specific inhibitors of NFAT function was made possible by the identification of the docking sites for the NFAT–calcineurin interaction[18,19]. A cell-permeable version of the VIVIT peptide, which is able to selectively inhibit calcineurin-mediated NFAT dephosphorylation[20], has been successfully used to notably prolong graft survival in an experimental system of islet-cell transplantation in mice[130]. These peptides, although they are much more selective than CsA, might still be able to inhibit the interaction of calcineurin with other substrates that use similar PXIXIT motifs, such as CABIN1 or AKAP79 (REF. 131). Recently, two different regions of calcineurin have been found to be necessary for NFAT–calcineurin interactions[131,132]. Mutations in one of these regions seem to selectively impair calcineurin binding to NFAT1 but not to other PXIXIT-containing proteins[132]. It is therefore possible

CD4$^+$CD25$^+$ REGULATORY T CELLS
(T_{Reg} cells). A specialized subset of CD4$^+$ T cells that can suppress the responses of other T cells. These cells provide a crucial mechanism for the maintenance of peripheral self-tolerance, and they are characterized by the expression of the α-chain of the interleukin-2 receptor (also known as CD25) and the transcription factor FOXP3 (forkhead box P3).

FLUORESCENCE-POLARIZATION ASSAY
A method that can be used to evaluate the strength of a protein–protein interaction. A fluorescent tag is attached to one of the protein partners. The formation of a complex is then deduced from an increase in fluorescence polarization, and the equilibrium dissociation constant of the complex can be determined.

that residues that flank the PXIXIT motif or are located in other regions of NFAT proteins can help to stabilize NFAT–calcineurin interactions and might constitute attractive targets for the design of even more specific inhibitors.

The therapeutic use of peptide inhibitors is still limited by problems that are associated with delivery and product stability. The use of small organic molecules could help to overcome these problems. Almost limitless structural changes can be designed to improve the specificity, stability, delivery and distribution of these molecules, which makes them an attractive alternative to peptides. Recently, this approach has yielded new specific NFAT inhibitors. Using a FLUORESCENCE-POLARIZATION ASSAY in which a library of compounds was screened for the ability to block the binding of labelled VIVIT peptide to calcineurin, several small organic molecules were identified that specifically blocked NFAT–calcineurin interactions and thereby blocked the activation of NFAT proteins and inhibited NFAT-dependent cytokine production by T cells[133]. A similar, but cell-based, approach, in which compounds were screened for their ability to block the nuclear translocation of an NFAT–GFP fusion protein, also produced small molecules that, in this case, seemed to target store-operated calcium channels and thereby inhibited calcium mobilization. These molecules act upstream of calcineurin, are efficient at blocking NFAT-dependent transcription and are able to potentiate CsA effects[134].

The ability of small molecules to selectively inhibit calcineurin and NFAT protein–protein interactions points to the possibility of using them to modulate specific NFAT-regulated functions. Differential interactions between different NFAT family members and specific transcriptional partners might underlie the ability of NFAT to integrate various signalling pathways and control diverse cellular programmes. If the protein–protein contact surfaces are specific for different interactions, molecules could be designed to block certain interactions without affecting others and, therefore, to inhibit only certain NFAT-regulated functions.

After the interactions that mediate specific cellular functions and the protein surfaces that maintain them have been fully characterized, molecules with the ability to interfere with such specific protein–protein interactions might be designed. These molecules will probably be therapeutically useful, with notably improved specificity and greatly reduced toxicity.

Concluding remarks

Evidence has accumulated in the past few years to show that the calcium-regulated NFAT transcription factors have key roles in the regulation of many aspects of T-cell function. During T-cell activation, most cytokine genes are regulated by NFAT proteins. However, it is now clear that NFAT targets also include many other genes that control alternative functions in activated T cells, such as cell-cycle progression and activation-induced cell death. The highly flexible structure of the NFAT DNA-binding domain allows several surfaces to be available for interactions with different transcriptional partners on DNA, thereby allowing NFAT to integrate many signalling pathways. Although AP1 proteins might be the most important transcriptional partners for NFAT during T-cell activation, cooperation with different transcription factors could be crucial for the regulation of other processes, including T_H-cell differentiation, T-cell tolerance and thymocyte development. Questions regarding the specificity of individual family members for distinct functions might be answered through the discovery of unique partner interactions. A better characterization of the regulation of NFAT activation might also shed some light on the mechanisms that control the functions of individual NFAT proteins (such as specific activating, priming, maintenance or export kinases). The identification of new transcriptional complexes and their target genes will lead to a full appreciation of the functions that are controlled by the NFAT transcription factors and the mechanisms that regulate them, not only in T cells but also in other cells of the immune system and in non-immune cells.

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Competing interests statement

The author declares no competing financial interests.

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