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Manipulating Immune Responses with Immunosuppressive Agents that Target NFAT

Review

Alexander Kiani,[†] Anjana Rao,[‡§] and Jose Aramburu[‡‖]
[†]Medizinische Klinik I
Universitätsklinikum Carl Gustav Carus
Technische Universität Dresden
01307 Dresden
Germany
[‡]Department of Pathology
Harvard Medical School and
The Center for Blood Research
Boston, Massachusetts 02115

The immune system is a complex network of cellular and humoral factors that ensures the integrity of the organism by attacking potential pathogens. Sometimes this normally protective system overreacts and directs its forces at the organism itself. The clinical syndrome of septic shock results from overactive host responses to gram-negative bacterial infections and can be fatal because of an overwhelming production of proinflammatory cytokines. Transplantation of solid organs is confounded by the immunologic problem of graft rejection, while graft-versus-host-disease is one of the major complications of transplantation with allogeneic bone marrow or peripheral blood stem cells, severely limiting the application of these potentially life-saving therapeutic options. Other examples of inappropriate immune responses include allergic asthma and chronic autoimmune diseases such as rheumatoid arthritis. A major challenge for clinicians is how to suppress deleterious immune responses in these diverse clinical settings.

The fungal metabolites cyclosporin A (CsA) and tacrolimus (FK506) are among the most potent immunosuppressive drugs available today. CsA revolutionized the field of organ transplantation following its discovery in 1976 (Borel et al., 1976); it was approved for clinical use in 1983. Tacrolimus was described soon after (Kino et al., 1987) and introduced into clinical trials in 1989. Despite their lack of structural similarity, the modes of action of these two drugs are very similar: at the low concentrations relevant to their immunosuppressive effects, they inhibit the calcium-dependent serine/threonine phosphatase calcineurin and its substrate, the transcription factor NFAT (Schreiber and Crabtree, 1992). The drugs do not interact directly with calcineurin but rather form complexes with intracellular receptors known as immunophilins. The immunophilin receptors for cyclosporin A are the cyclophilins, while those for FK506 are the FK506-binding proteins (FKBPs). Both classes of immunophilin receptors are widely expressed, abundant enzymes possessing peptidyl-prolyl isomerase activity, which function as protein chaperones within the cell (Schreiber and Crabtree, 1992). It is the drug-immunophilin complexes that bind calcineurin and inhibit its phosphatase activity toward peptide substrates, thus exhibiting a remarkable gain of function relative to the drugs themselves (Schreiber and Crabtree, 1992).

In the immune system, calcineurin and NFAT, the major targets of CsA and FK506, regulate the expression of a large number of inducible genes encoding cytokines and cell-surface receptors that are essential for a productive immune response (Rao et al., 1997). Some of the immunosuppressive effects of CsA may depend on its ability to induce TGFβ, an immunosuppressive cytokine, in diverse cells and tissues (Suthanthiran et al., 1996). CsA or FK506 are frequently used in combination with less specific immunosuppressants such as prednisone, azathioprine, methotrexate, and mycophenolate mofetil to achieve synergistic immunosuppressive effects (Suthanthiran et al., 1996; Gummert et al., 1999). The main drawback of these immunosuppressive regimens, however, is that they produce major toxic side effects and therefore are rarely employed except in serious clinical situations.

Our thesis in this review is that since NFAT is a validated target for two clinically important immunosuppressive drugs, interference with NFAT regulation might be expected to yield additional avenues for immunosuppression. Moreover, since NFAT is only one of many calcineurin substrates, compounds that interfere selectively with NFAT regulation might be expected to have fewer side effects than CsA and FK506. Unfortunately, this simple premise is complicated by the existence of multiple NFAT proteins, our incomplete knowledge of their regulation and function in immune and nonimmune cells, and our lack of information about the mechanisms underlying the toxic effects of CsA and FK506.

Here we review our current understanding of these points. We consider what features of NFAT regulation might be exploited to develop selective inhibitors of NFAT, whether such drugs would be as immunosuppressive as CsA or FK506, whether they would lack the toxic effects of CsA and FK506, and whether they might have unexpected side effects in immune and nonimmune organs. An informed understanding of these issues might spur the development of better, less toxic immunosuppressive agents that target the NFAT pathway.

What Features of NFAT Regulation Might Be Exploited to Develop Selective Inhibitors of NFAT?

The NFAT family, consisting of the "classical" members NFAT1 (p, c2), NFAT2 (c, c1), NFAT3 (c4), and NFAT4 (x, c3), has recently been extended by a fifth member, NFAT5/TonEBP (Table 1) (Lopez-Rodriguez et al., 1999; Miyakawa et al., 1999). NFAT5 differs from the other NFAT proteins in being regulated by osmotic shock rather than calcineurin. Here we consider only the calcineurin-regulated family members NFAT1–4. For simplicity, we use the term NFAT to refer to these four proteins, unless specific distinctions are being drawn. In the following sections, we describe different aspects of NFAT regulation, emphasizing basic features common to all the proteins and indicating the possibility of

[§]To whom correspondence should be addressed (e-mail: arao@cbr.med.harvard.edu).
[‖]Present address: Facultat de Ciències de la Salut i la Vida, Universitat Pompeu Fabra, 08003 Barcelona, Spain.

Table 1. The NFAT Family

NFAT Protein	Present in Immune Cells?	Other Features	Phenotype of Knockout Mice
NFAT1 (p, c2)	Yes	80%–90% of total NFAT in resting immune cells	Increased IL-4 production, Th2 bias (delayed shutoff of IL-4 transcription)
NFAT2 (c, c1)	Yes	One isoform induced upon activation	Embryonic lethality (heart valve defect) Lymphocytes produce less IL-4
NFAT3 (c4)	No	Implicated in cardiac hypertrophy	Viable and fertile Detailed phenotype not reported yet
NFAT4 (x, c3)	Yes	Highly expressed in thymocytes	Defect in positive selection of thymocytes
NFAT5 (TonEBP)	Yes	Nuclear, not cytoplasmic Not calcineurin-regulated Does not cooperate with Fos/Jun	Knockouts not available yet NFAT5 is not implicated in the regulation of conventional NFAT target genes

subtype-specific regulation where appropriate (Figures 1 and 2).

Overview

The general features of NFAT regulation have recently been reviewed (Crabtree, 1999). NFAT proteins are regulated by a reversible cycle of dephosphorylation and rephosphorylation (Figure 1). NFAT becomes activated when cells are stimulated through receptors coupled to phospholipase C. The resulting rise in intracellular calcium levels triggers the activation of calcineurin and other calmodulin-dependent enzymes. NFAT activation can be considered in several steps: dephosphorylation by calcineurin; translocation to the nucleus; potentially, activation by posttranslational modifications in the nucleus; binding to regulatory elements of NFAT target genes; and interactions with nuclear proteins. Together, these steps are necessary for the incorporation of NFAT into a transcriptionally active enhanceosome complex at the promoter/enhancer regions of NFAT-dependent genes. Sustained calcineurin activity is needed to maintain NFAT in an activated state; when calcium levels drop or when calcineurin is inactivated, for instance by removing the stimulus, inhibiting capacitative calcium entry, or treating cells with CsA or FK506, NFAT is rapidly

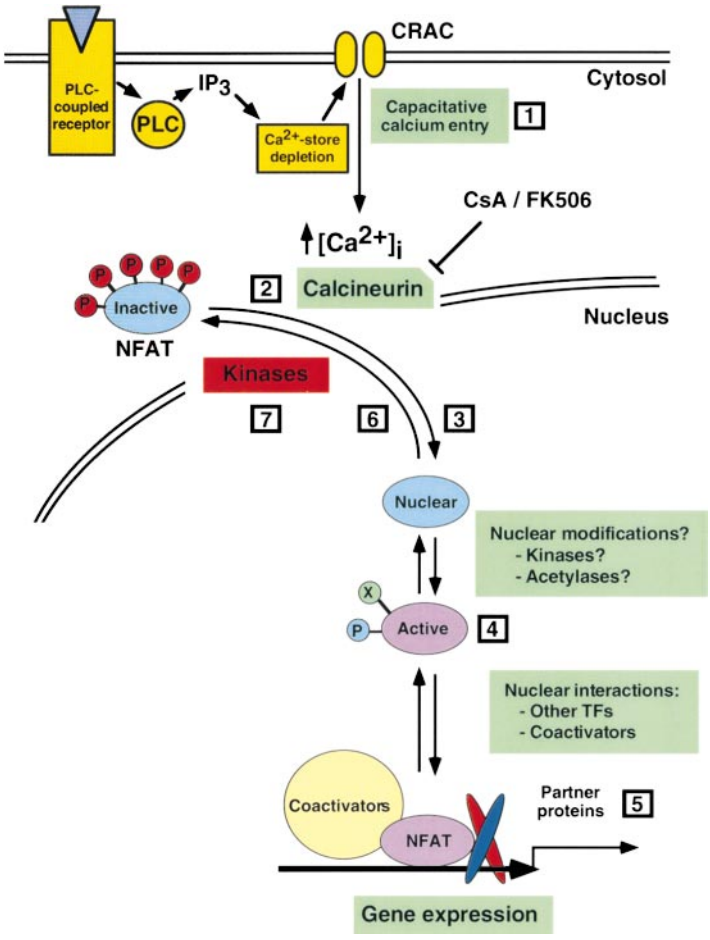


Figure 1. The NFAT Activation Cycle

In resting cells, NFAT resides in the cytoplasm, where it is maintained in a phosphorylated state by the action of constitutive kinases. Stimulation of cell surface receptors coupled to phospholipase C (PLC) results in calcium mobilization and activation of the phosphatase calcineurin. Calcineurin dephosphorylates NFAT, triggering its nuclear translocation and enhancing its DNA binding and transcriptional activity. In the nucleus, NFAT interacts with transcription factors (TFs) and coactivators, stimulating gene expression. When calcium levels drop, calcineurin becomes inactive, and NFAT is rephosphorylated by constitutive or inducible kinases and exported back to the cytoplasm. The immunosuppressive drugs cyclosporin A (CsA) and FK506 inhibit calcineurin and prevent the expression of NFAT target genes. The green boxes indicate the enzymes and processes that maintain NFAT in a transcriptionally active state. Numbers indicate points of potential intervention in the NFAT pathway.

rephosphorylated and exported from the nucleus (Loh et al., 1996a, 1996b; Shibasaki et al., 1996; Timmerman et al., 1996). At least one isoform of NFAT2 is regulated, in addition, by a calcium- and calcineurin-dependent induction of its mRNA in activated T cells (Northrop et al., 1994; Chuvpilo et al., 1999). Hence, NFAT's activation state is the overall result of a balance between multiple regulatory processes, which could all be potential targets of therapeutic intervention (Figure 1). These processes are identified by numbers in Figure 1 and cross-referenced to the sections below.

Capacitative Calcium Entry (1)

Sustained calcium mobilization appears essential for productive NFAT activation. Release of calcium from intracellular stores may trigger some early dephosphorylation and nuclear entry of NFAT, but this is not sustained in the absence of capacitative (store-operated) calcium entry through CRAC channels (Negelscu et al., 1994; Dolmetsch et al., 1997). Jurkat T cells selected for impaired NFAT activation showed varying levels of impaired calcium mobilization as their major signaling defect (Fanger et al., 1995). NFAT also monitors the frequency of calcium oscillations in cells (Dolmetsch et al., 1998; Li et al., 1998). Oscillations of physiological frequency are much more effective than lower frequency oscillations, consistent with rapid deactivation of both calcineurin and NFAT upon cessation of the calcium stimulus.

At least one case of severe immunodeficiency was traced to a defect in capacitative calcium entry that was apparent in T and B cells as well as granulocytes, platelets, and fibroblasts (Le Deist et al., 1995). T cells from this patient showed a severe reduction in NFAT activation and pronounced defects in cytokine production. The lack of reported cardiac dysfunction in this patient is noteworthy, given that calcium-dependent activation of NFAT2 has been implicated in morphogenesis of cardiac valves (de la Pompa et al., 1998; Ranger et al., 1998a). This phenotype might be due to a quantitative defect in capacitative calcium entry, which permitted normal valve development but was insufficient for immune function; alternatively, the defect might lie in a cell type–specific component of store-operated calcium entry. The latter possibility is particularly interesting for immunosuppression.

Interaction of NFAT with Calcineurin (2)

Calcineurin binds to the NFAT regulatory domain, which is located immediately N-terminal to the highly conserved DNA binding domain (Figure 2). This regulatory domain is absent in NFAT5, consistent with its distinct mode of regulation (Lopez-Rodriguez et al., 1999; Miyakawa et al., 1999). A major contributor to calcineurin binding is a conserved sequence motif (PxIxIT) located at the N terminus of the NFAT regulatory domain (Aramburu et al., 1998, 1999; Zhu et al., 1998). Peptides modeled on the PxIxIT motif inhibited the NFAT-calcineurin interaction and were markedly immunosuppressive when expressed in Jurkat T cells, inhibiting NFAT dephosphorylation, NFAT nuclear translocation, and PMA/ionomycin-induced expression of IL-2, IL-3, IL-13, TNFα, GM-CSF, and MIP-1α mRNAs (Aramburu et al., 1998, 1999). However PxIxIT-based peptides did not inhibit calcineurin phosphatase activity and therefore showed greater selectivity than CsA: for instance, they did not block the CsA-sensitive induction of the TNFβ and LTβ genes (Aramburu et al., 1998, 1999).

These results are promising in terms of developing pharmacological inhibitors selective for NFAT. They imply that it should be possible to identify small molecule inhibitors of the NFAT-calcineurin interaction that do not interfere with calcineurin phosphatase activity and therefore would target selectively the ability of calcineurin to dephosphorylate NFAT. Such selective NFAT inhibitors would be expected to have fewer side effects than generalized inhibitors of calcineurin and would also be expected to lack any calcineurin-independent toxic effects of CsA or FK506. Potential calcineurin-independent effects include, for instance, those arising from the ability of CsA and FK506 to inhibit the peptidyl-prolyl isomerase activities, and therefore the chaperone functions, of their respective immunophilin receptors (Schreiber and Crabtree, 1992).

A caveat of PxIxIT-based inhibitors, however, is that they might inhibit dephosphorylation or function of other calcineurin binding proteins that used a similar docking sequence or bound the same calcineurin surface as NFAT. So far there is only one known mammalian protein that contains a clear PxIxIT-related sequence (PEITVT) in its calcineurin binding domain: the nuclear calcineurin-interacting protein Cabin-1/Cain-1 (Sun et al., 1998; Lai et al., 1998). While this protein does not appear to be a substrate for calcineurin, it binds calcineurin and inhibits its phosphatase activity. Thus PxIxIT-based inhibitors might alter cellular balance by releasing active calcineurin from Cabin-1/Cain-1, presumably augmenting other calcineurin-dependent pathways while inhibiting NFAT. This possibility needs to be evaluated.

The PxIxIT motif may be part of a more extensive calcineurin-interacting surface of NFAT (Figure 2). Deletion analysis of NFAT4 has suggested the existence of additional calcineurin binding sites located at its N terminus (Zhu and McKeon, 1999) and at the C terminus of its regulatory domain (Liu et al., 1999). These regions are only partially conserved in the NFAT family. Further work is necessary to establish whether they are part of an extended calcineurin binding common to all NFATs, or whether they constitute regions of interaction unique to individual family members that might be targeted for subtype-specific inhibition.

Dephosphorylation and Nuclear Import (3)

In addition to the PxIxIT motif, the NFAT regulatory domain contains an extended serine-rich region (SRR), three serine- and proline-rich ("SP") sequence motifs with the characteristic sequence SPxxSPxxSPxx[SPxx]xxx[D/E][D/E], and a conserved nuclear localization signal (NLS) located between the second and third "SP motifs" (Figure 2). The regulatory domain is phosphorylated on serine residues in resting cells and dephosphorylated in a calcineurin-dependent manner upon stimulation (Loh et al., 1996b; Luo et al., 1996; Shibasaki et al., 1996). Dephosphorylation exposes the NFAT NLS, rendering it accessible to the machinery of nuclear import. The import receptors involved have not been identified but may include the commonly utilized karyopherin (importin) α/β family that recognizes basic NLSs (Torgerson et al., 1998).

We have little specific information about the NFAT dephosphorylation/rephosphorylation cycle. Neither the serine residues that are phosphorylated on endogenous

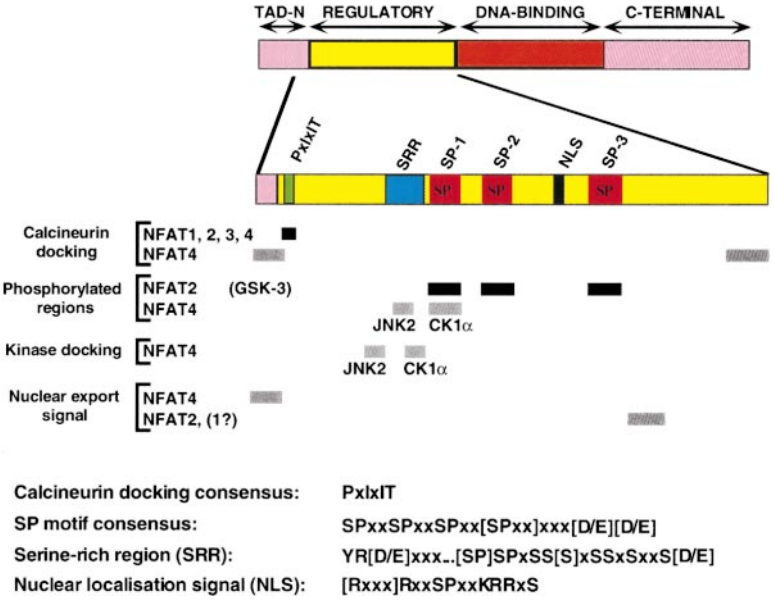


Figure 2. The NFAT Regulatory Domain

The conserved sequence motifs in the NFAT family are shown as colored boxes and their consensus sequences are indicated at the bottom of the figure. Regions involved in regulating NFAT functions are indicated by thick bars. Black bars indicate functional regions that are conserved in multiple NFAT proteins, while gray bars indicate regions whose functions have been tested only in individual family members or whose sequences are poorly conserved in the NFAT family.

NFAT proteins in resting cells nor those that are lost upon activation have been identified. While the presence of conserved sequence motifs in the regulatory domain (Figure 2) suggests regulatory mechanisms common to all four NFATs, the low level of sequence identity (~30%) suggests the existence of unique (perhaps cell type–specific) regulatory mechanisms as well.

In an attempt to identify phosphoserine residues whose dephosphorylation might control nuclear import, NFAT2 and NFAT4 proteins with deletions and serine-to-alanine mutations in the NFAT regulatory domain have been tested. These experiments have yielded divergent results (see Figure 2). In NFAT2, mutations in all four conserved motifs (the serine-rich region and the SP motifs) were shown to cause nuclear localization (Beals et al., 1997a, 1997b). For NFAT4, the results were less consistent: in one study, mutation of two residues in the serine-rich region of NFAT4 resulted in nuclear localization (Chow et al., 1997), while in another report, deletion of the entire serine-rich region rendered the protein hypersensitive to calcium ionophore, a result interpreted by the authors as the effect of deleting the docking site for CK1α, a proposed NFAT kinase (Zhu et al., 1998; see below). The latter study located the phosphoserine residues responsible for nuclear import in the first SP motif of NFAT4 (Zhu et al., 1998). While, overall, the results implicate both the serine-rich region and the SP motifs in regulating nuclear import, the specific discrepancies are puzzling given the high degree of sequence conservation in the NFAT regulatory domain and the fact that all four NFATs are regulated by calcineurin. These discrepancies will need to be resolved before pharmacological strategies targeting dephosphorylation-regulated nuclear import can be devised.

DNA Binding and Transcriptional Activity (4)

In addition to nuclear import, calcineurin regulates the DNA binding affinity of NFAT (Park et al., 1995; Shaw et al., 1995). However, the precise mechanism involved has not yet been elucidated. For NFAT2, dephosphorylation has been suggested to release a potentially inhibitory interaction between a serine-rich region in the regulatory domain and a basic sequence in the DNA binding domain (Beals et al., 1997a). Although this interaction was interpreted as controlling NFAT2 nuclear import, the basic sequence does not function as a required NLS in all NFAT proteins (Luo et al., 1996). Thus, it is equally possible that the interaction regulates DNA binding. If so, inhibitors targeting this interaction might be useful, since both interacting regions are conserved in the NFAT family and this mode of regulation may be common to all four NFATs.

Calcineurin activity is continuously needed to maintain NFAT in the nucleus in a form with high affinity for DNA (Loh et al., 1996a, 1996b; Shibasaki et al., 1996; Timmerman et al., 1996). Two possible scenarios may be envisioned to explain this result; neither has yet been tested experimentally. Calcineurin might be needed as an integral part of the NFAT transcriptional complex to maintain NFAT in an active and dephosphorylated form, particularly if DNA-bound NFAT present in active transcriptional complexes is still capable of being inactivated by rephosphorylation. Alternatively, the DNA-bound, transcriptionally active fraction of NFAT might not be accessible to calcineurin or nuclear kinases; if so, the ongoing requirement for calcineurin indicates that this fraction of NFAT would be in continuous equilibrium with free nuclear NFAT. These possibilities are not mutually exclusive: the detailed structure of NFAT-containing transcriptional complexes is likely to vary depending on the regulatory region involved, and hence the accessibility of NFAT to modifying enzymes could be different in each case.

Paradoxically, it has been suggested that calcineurin activity is not necessary for transcription mediated by NFAT2 and NFAT4 that have been artificially localized to the nucleus (Timmerman et al., 1996; Zhu and McKeon, 1999). In both cases, constitutively nuclear mutant proteins were used. The first report showed that constitutively nuclear NFAT2 could be activated with PMA alone

and concluded on this basis that calcineurin was required only for nuclear entry (Timmerman et al., 1996). In direct contrast, the second report showed that constitutively nuclear NFAT4 was not transcriptionally active in PMA-stimulated cells but could be rendered active by coexpression of either wild-type, constitutively active calcineurin or a phosphatase-inactive version (Zhu and McKeon, 1999). This study also showed that calcineurin competed with the nuclear export receptor Crm1 for binding to a nuclear export signal (NES) of NFAT4 and suggested on this basis that calcineurin promoted the transcriptional function of NFAT4 by interfering with "futile shuttling." This function of calcineurin did not require calcineurin phosphatase activity. These disparate findings need to be resolved before the role of calcineurin in regulating NFAT-mediated transcription can be understood and targeted pharmacologically.

How might dephosphorylation regulate these multiple functions of NFAT? As shown for the yeast transcription factor Pho4 (Komeili and O'Shea 1999), the phosphorylation status of distinct residues (or clusters of residues) may control distinct functions of NFAT; alternatively, as shown for retinoblastoma (Harbour et al., 1999), progressive changes in phosphorylation status may induce progressive changes in the overall conformation of NFAT. A final possibility is that the phosphorylated form of the NFAT regulatory domain possesses a repressive (masking) function, which is relieved by calcineurin-mediated dephosphorylation. By analogy with the estrogen receptor and other steroid hormone receptors, which repress the accessibility or function of adjacent protein domains in their unliganded form but lose this repressive activity following ligand binding, the postulated changes in the repressive function of the NFAT regulatory domain may be mediated by differential interactions of its phosphorylated and dephosphorylated forms with chaperones (Rutherford and Zuker, 1994).

Do posttranslational modifications of NFAT contribute to transcriptional activation? The transcriptional activity of NFAT is enhanced by binding to the nuclear coactivators p300 and CBP (Garcia-Rodriguez and Rao, 1998; Avots et al., 1999). The fraction of NFAT that is bound to p300 in the nucleus shows an altered mobility in SDS gels (Garcia-Rodriguez and Rao, 1998), suggesting that it might be posttranslationally modified by phosphorylation or acetylation. It is not known whether these (or other) modifications are required for transcriptional function. If so, inhibition of the relevant nuclear modifying enzymes would be another way of suppressing NFAT activity, particularly if the enzymes were specific for NFAT. By analogy with calcineurin, however, even nonselective enzymes might be good targets for inhibition if they possessed a sufficiently limited range of potential substrates.

DNA Binding and Interactions with Partner Proteins (5)

In the nucleus, NFAT binds to regulatory regions of its target genes. Optimal binding and transcriptional activation requires its incorporation into functional enhanceosome complexes, a process facilitated by cooperative binding with other transcription factors to DNA. A well-characterized cooperative interaction is that between NFAT and AP-1 (Fos/Jun) (Chen et al., 1998a). This interaction occurs on composite DNA sites of specific structure and spacing, and the residues involved in the interaction are conserved in all NFATs. The highly conserved E′F loop of NFAT, which makes major contacts with Jun and DNA, is supported by a small hydrophobic patch that contains Phe473. Compounds that target the E′F loop or the hydrophobic pocket may selectively inhibit gene expression controlled by NFAT/Fos/Jun complexes.

GATA proteins constitute another common category of nuclear partners for NFAT. GATA2-NFAT2 interaction was suggested based on coimmunoprecipitation of these two proteins and their coexpression in nuclei of differentiating myoblasts (Musaro et al., 1999), whereas GATA4-NFAT3 interaction was observed in a yeast two-hybrid assay (Molkentin et al., 1998). Interactions involving GATA3 and NFAT have not yet been demonstrated, but GATA3 and NFAT are both implicated in IL-5 gene transcription (Rao et al., 1997; Zhang et al., 1997, 1999), and targeting this interaction may be an effective way of selectively inhibiting eosinophil function in atopy. It is not clear whether NFAT-GATA interactions, like the NFAT-AP-1 interaction, involve conserved regions in NFAT and GATA proteins, such that any NFAT protein could potentially interact with any GATA protein if expressed in the same cell type at the same developmental stage. Alternatively, NFAT-GATA interactions could be constrained to individual members of the NFAT and GATA families, offering the possibility of selectively inhibiting particular classes of target genes.

Nuclear Export (6)

Rephosphorylation of NFAT is accompanied by its nuclear export. Again, studies with NFAT2 and NFAT4 have yielded somewhat discordant results. A nuclear export signal (NES) identified near the C terminus of the NFAT2 regulatory domain is fairly well conserved in NFAT1 but poorly conserved in NFAT3 and NFAT4; likewise, an NES localized at the N terminus of NFAT4 is poorly conserved in NFAT1, 2, and 3 (Figure 2). The export receptor for NFAT has been identified as Crm1 (Kehlenbach et al., 1998; Zhu and McKeon, 1999) based in part on the ability of the Crm1-binding compound leptomycin B to inhibit NFAT nuclear export. Since Crm1 is involved in the nuclear export of multiple substrates (Pemberton et al., 1998), it may not be useful to devise pharmacological strategies that target this export receptor. It should be borne in mind, however, that the import and export receptors used in vivo may differ from those identified experimentally. The interferon-induced transcription factor Stat1 uses a specialized import receptor in an unusual mode of nuclear import (Sekimoto et al., 1997); likewise, the yeast transcription factor Pho4, which specifically regulates phosphate metabolism, uses dedicated import and export receptors (Komeili and O'Shea, 1999).

NFAT Kinases: Inactivation by Rephosphorylation (7)

It is not yet clear whether NFAT rephosphorylation is mediated by the same constitutively active kinases that maintain it in its inactive state in resting cells. The subcellular distribution of such kinases is an important point: they would need to be located in the nucleus to inactivate previously activated NFAT, and in the cytoplasm to maintain NFAT in its inactive form. Alternatively,

NFAT may be inactivated by inducible kinases that become activated and are present in the nucleus in stimulated cells. These possibilities are not mutually exclusive.

A total of four candidate NFAT kinases—two constitutive and two inducible—have been identified to date (see Figure 2). (i) Glycogen synthase kinase-3 (GSK-3), which is constitutively active in differentiated cells but is the target of the Wnt signaling pathway during development (Wodarz and Nusse, 1998), phosphorylates the conserved SP motifs of NFAT2 and promotes its nuclear export when overexpressed (Beals et al., 1997b). GSK-3 has also been shown to phosphorylate NFAT3 and accelerate its nuclear export (Graef et al., 1999). For both NFAT2 and NFAT3, the extent of potentiation of nuclear export is approximately 3- to 4-fold. GSK-3 requires a priming kinase, and protein kinase A (which is not constitutively active) can fulfill this function in vitro, although it does not appear to be the priming kinase in vivo. Lithium, which inhibits several cellular enzymes including GSK3, slowed by 2- to 3-fold the nuclear export of NFAT2 (Klemm et al., 1997; Kehlenbach et al., 1998), apparently by inhibiting the release of NFAT2 from DNA (Kehlenbach et al., 1998). (ii) Casein kinase 1α (CK1α) has been reported to be a major regulatory kinase for NFAT4 (Zhu et al., 1998). Like GSK-3, CK1α is constitutively active in resting cells. Overexpressed CK1α slows, while kinase-inactive CK1α promotes, the nuclear import of NFAT4. CK1α binds to a site in the serine-rich region of NFAT4 that is not fully conserved in the other NFATs but phosphorylates conserved residues in the adjacent first SP motif. Thus, it remains to be determined whether CK1α uniquely regulates NFAT4 or is a common kinase for all members of the NFAT family. (iii) The MAP kinase kinase kinase MEKK1 has been proposed to mediate the suppressive effects of serum on NFAT dephosphorylation and nuclear translocation in cultured cells (Zhu et al., 1998). MEKK1 does not phosphorylate NFAT4 directly but rather enhances its association with CK1α in stimulated cells, perhaps by acting via an intermediate kinase. (iv) The Jun N-terminal kinase JNK2, a stress-activated MAP kinase that is activated in cells exposed to UV or osmotic shock, has been reported to inhibit the nuclear accumulation of NFAT4 but not of other NFAT proteins (Chow et al., 1997). This result contradicts a second report that found no effect for the very similar enzyme JNK1 (Zhu et al., 1998). However, T cells from JNK1-deficient mice showed increased nuclear accumulation of NFAT2 (but not NFAT1) after stimulation, suggesting a selective role—direct or indirect—for JNK1 in regulating NFAT2 (Dong et al., 1998). These discrepancies need to be resolved.

In light of these data, it will be important to establish whether NFAT rephosphorylation and inactivation is primarily regulated by a common kinase capable of phosphorylating all NFATs or by specific kinases that act on individual NFATs. Unfortunately, the candidate kinases have in most cases been tested only with single NFAT proteins under conditions of significant overexpression of both the kinase and NFAT. Compounds that target NFAT kinases are likely to be useful immunomodulatory agents once the questions of inducible versus constitutive kinases and specific versus common mechanisms of rephosphorylation are resolved. Activation of an inducible kinase would render NFAT inactive by releasing it from DNA and favoring its nuclear export, while suppression of either constitutive or inducible kinases would prolong NFAT activation by potentiating the effect of calcineurin.

Summary

In summary, several features of NFAT regulation are promising in terms of immunosuppressive strategies. (i) Although capacitative calcium entry is a mechanism common to all cells, it may be regulated in part by cell type–specific mechanisms. Inhibitors that target such mechanisms could be useful given the immunodeficiency phenotype discussed above. (ii) In light of the potent inhibitory effects of PxIxIT-based peptides in cultured cells, the NFAT-calcineurin interaction is a valid and attractive target for immunosuppressive drugs. (iii) Hyperactivation of NFAT kinases required for nuclear export would be expected to depress the immune response. Unfortunately, the known candidates have biological activities ranging well beyond NFAT and may not be suitable pharmacological targets: dedicated NFAT kinases or nonselective kinases with a more limited range of substrates are preferable if they exist. Also, more information is needed as to whether NFAT inactivation is mediated by kinases that target all NFATs in common or are specialized to act on individual NFATs. (iv) It seems worthwhile to explore whether NFAT is activated by posttranslational modifications in the nucleus, since the enzymes involved would be highly attractive targets, especially if they were specific for one or more NFATs. (v) Targeting the interaction of NFAT with nuclear partner proteins such as Fos/Jun and GATA and nuclear coactivators such as p300 and CBP is also an interesting option, since specialized regions of interaction will be used.

Would NFAT Inhibitors Be as Immunosuppressive as CsA or FK506?

On the plus side of this equation, NFAT is known to regulate a large number of genes required for immune function, and impairment of NFAT activation correlates strongly with severe immune deficiency. On the minus side, calcineurin regulates several transcription factors other than NFAT, which undoubtedly contribute to the immune response, and certain cellular effects of CsA and FK506 do not involve either calcineurin or NFAT. Finally, the cytokine TGFβ has been suggested to mediate both immunosuppressive and other effects of CsA and FK506. These aspects are discussed individually below (Figure 3).

NFAT Regulates a Plethora of Genes Essential for Immune Function

NFAT is thought to regulate a large number of target genes expressed by activated immune cells, including the cytokine genes IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, GM-CSF, and IFNγ; the cell surface receptors CD40L, FasL, and IL-2Rα (CD25) and the transcription factor EGR3 in T cells; CD5 and Igκ in B cells; IL-4, IL-5, and TNFα in mast cells; and TNFα and GM-CSF in NK cells (reviewed in Rao et al., 1997; for recent work see Latinis et al., 1997; Berland and Wortis, 1998; Mages et al., 1998; Meyer and Ireland, 1998; Schuh et al., 1998a; Cron et al., 1999). Expression of these genes meets three or more of the following criteria for involvement of NFAT: (i) the genes are induced by signals that include calcium

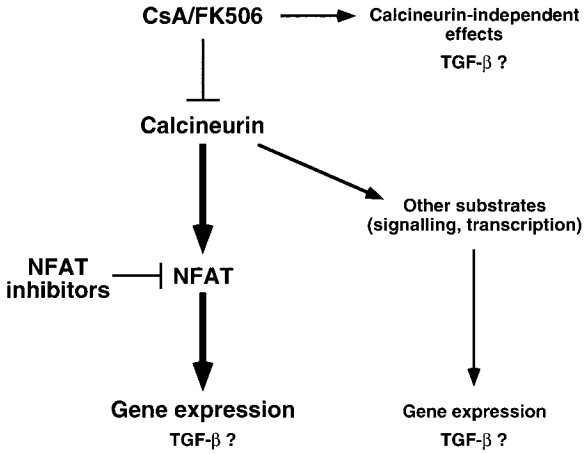


Figure 3. Multiple Pathways May Contribute to Immunosuppression Mediated by CsA and FK506

A major component of the immunosuppressive effects of CsA and FK506 is the inhibition of calcineurin and its substrate NFAT (heavy arrows). However, immunosuppression may also be effected through inhibition of other calcineurin substrates or through increases in TGFβ levels. It is not yet known whether TGFβ production in response to CsA/FK506 is mediated through calcineurin or NFAT.

entry; (ii) their expression by activated cells is inhibited by CsA; (iii) their promoter/enhancer regions contain specific binding sites for NFAT; (iv) in transient transfection assays, NFAT augments reporter activity driven by these promoter/enhancer regions; and (v) when introduced into the reporter plasmid, mutations in the NFAT binding sites of the promoter/enhancer regions abolish (or significantly diminish) reporter activity. The expression of many of these same genes (IL-2, IL-3, IL-13, GM-CSF, TNFα, MIP-1α) in T cells was inhibited by expression of a selective NFAT inhibitor, a high-affinity PxIxIT peptide (Aramburu et al., 1999). In contrast, the PxIxIT peptide did not affect the induction of other CsA-sensitive genes such as lymphotoxin-β or TNFβ, which do not fit the other criteria for involvement of NFAT.

Impairment of NFAT Activation Correlates with Immune Deficiency

At least four cases of severe immunodeficiency have been correlated with a marked impairment in the activation of NFAT (Castigli et al., 1993; Le Deist et al., 1995; Feske et al., 1996). The patients, who originated in three independent families, presented as infants with repeated infections and failure to thrive. In each case their phenotype was traced to a syndrome of multiple cytokine deficiency. T cells from these patients were unable to generate functional NFAT complexes but activated other transcription factors normally, and cytokine expression was profoundly impaired. The affected genes (IL-2, IL-3, IL-4, IL-5, TNFα, and IFNγ) are all potential NFAT target genes based on the criteria discussed above.

Calcineurin Regulates Many Processes and Transcription Factors in Addition to NFAT

As befits a highly conserved and ubiquitous enzyme, calcineurin participates in many calcium-dependent processes and has many substrates other than NFAT (Aramburu et al., 2000). Some of these involve signaling enzymes and transcription factors that are likely to contribute to gene expression in activated immune cells (Figure 3). (i) Calcineurin directly dephosphorylates and inactivates the transcription factor Elk-1, a target of MAP kinase pathways that regulates c-Fos gene transcription (Sugimoto et al., 1997, 1998). (ii) Calcineurin regulates NFκB activity in T cells, where it is implicated in IκB kinase (IKK) activation, IκB degradation, and nuclear appearance of NFκB p50, c-Rel, and RelA (Marienfeld et al., 1997; Trushin et al., 1999). (iii) Calcineurin indirectly modulates the activity of the transcription factor CREB (Shaywitz and Greenberg, 1999). (iv) Calcineurin cooperates with PKC-θ to activate JNK, which phosphorylates and activates the transcription factors c-Jun and ATF-2 (Werlen et al., 1998). (v) Calcineurin and JNK kinases synergistically stablize IL-2 and IL-3 mRNAs in T cells and mast cells respectively; CsA inhibits this stabilization, a feature that may contribute to its immunosuppressive effects (Chen et al., 1998b; Ming et al., 1998). NFAT-based inhibitors would not be expected to target these diverse processes in immune cells and so might be less immunosuppressive than CsA and FK506.

Certain Cellular Effects of CsA and FK506 May Not Involve Calcineurin or NFAT

CsA and/or FK506 influence the activity of certain transcription factors in a manner independent of calcineurin (Aramburu et al., 2000). The cyclophilin Cyp-40 binds c-Myb and inhibits its DNA binding; this effect requires the peptidyl-prolyl isomerase activity of Cyp-40 and is blocked by CsA (Leverson and Ness, 1998). In transient reporter assays, CsA and FK506 also markedly potentiated transcription driven by ligand-activated glucocorticoid receptors (Renoir et al., 1995). When analogs of CsA and FK506 were tested, potentiation did not correlate either with calcineurin inhibition or with inhibition of the peptidyl-prolyl isomerase activities of cyclophilins and FK506-binding proteins (FKBPs). Rather, the compounds were suggested to influence the function of glucocorticoid receptor-containing complexes indirectly via binding to cyclophilin-40 and FKBP59 chaperone proteins present in the complexes (Rutherford and Zuker, 1994; Renoir et al., 1995). Again, NFAT-based inhibitors would not be expected to exhibit these functions in immune cells.

CsA and FK506 Augment TGFβ Production

Some of the immunosuppressive effects of CsA and FK506 have been attributed to their ability to induce TGFβ, a potent immunosuppressive cytokine, in various cell types (Suthanthiran et al., 1996). The effects are modest and somewhat variable but likely to be biologically significant. Stimulation of human peripheral blood T cells resulted in 1.5- to 4-fold increases in expression of TGFβ mRNA and protein; supplementation of the cultures with CsA or FK506 augmented this increase by a further 1.5- to 5-fold (Li et al., 1991; Khanna et al., 1999b). Increased TGFβ levels were also reported in CsA-treated patients awaiting renal transplantation (Shin et al., 1998), although these results have been questioned (Hughes et al., 1999).

It remains to be determined whether the effects of CsA on TGFβ production are mediated through calcineurin and/or NFAT. Treatment of T cells or non-lymphoid cell lines with drug alone, in the absence of stimulation, caused a significant increase in TGFβ production, suggesting a calcineurin-independent mechanism operating in resting cells (Li et al., 1991; Khanna et al.,

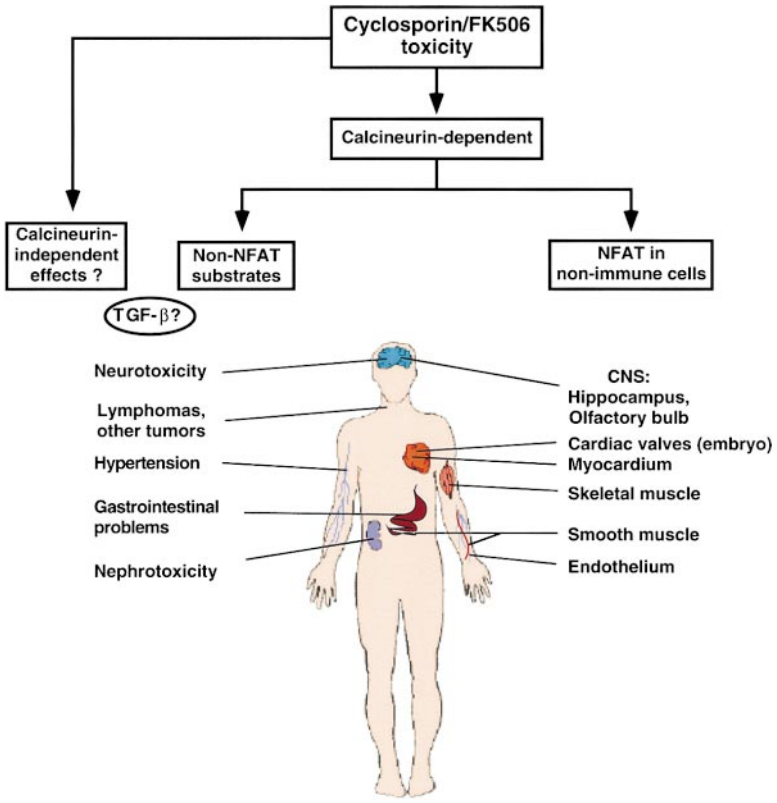


Figure 4. Comparison of the Toxic Effects of CsA and FK506 with the Expression of NFAT in Nonimmune Cells

On the left are shown the major side effects of CsA and FK506, which may be independent of calcineurin or due to inhibition of calcineurin substrates other than NFAT. On the right are shown some of the nonimmune tissues reported to express NFAT. The comparison suggests that, overall, NFAT inhibitors will have fewer side effects than CsA and FK506. Note that the information on the left is based on studies of CsA/FK506 toxicity in humans, while most data on NFAT expression and function (right) are derived from studies in the mouse.

1999b). However, these results do not exclude additional calcineurin-dependent mechanisms in stimulated cells whereby CsA and FK506 counteract potential negative effects of calcineurin and/or NFAT on TGFβ expression or gene transcription. Clearly, further experiments are needed. Note that TGFβ has also been implicated in some severe side effects of CsA and FK506—nephrotoxicity, hypertension, fibrosis, and tumor invasiveness (Pascual et al., 1998; Hojo et al., 1999; Khanna et al., 1999a, 1999b).

Summary

In summary, NFAT seems likely to be a major target of CsA/FK506-induced immunosuppression given the severe phenotype of immunodeficiency patients impaired for NFAT activation and the large number of immune genes that NFAT controls. However, some of the immunosuppression produced by CsA and FK506 could be mediated through TGFβ, through calcineurin substrates other than NFAT, or through various calcineurin-independent processes. To resolve this issue, it will be necessary to compare directly the immunosuppressive effects of selective NFAT inhibitors (e.g., PxIxIT-based peptides) with those of CsA and FK506 in animal models of immunosuppression.

Would NFAT Inhibitors Lack the Toxic Side Effects of CsA and FK506?

The most common side effects of CsA and FK506 (Figure 4) are nephrotoxicity, neurotoxicity (usually manifest as seizures), tremor, insomnia, and headache; impaired glucose metabolism (hyperglycemia, diabetes); infections; gastrointestinal disturbances; and hypertension (U.S. Multicenter FK506 study group, 1994; Mayer et al., 1997). The nephrotoxicity occurs in two phases: an early phase characterized by vasoconstriction and decreased glomerular filtration rate, which progresses to interstitial tubular fibrosis, glomerular sclerosis, widespread vascular damage, and loss of functional nephrons (Myers, 1986; Pascual et al., 1998). CsA or FK506 treatment is also associated with an increased incidence of lymphomas and other malignancies, although this may be a general feature of long-term immunosuppression (London et al., 1995; Penn, 1987). TGFβ produced in response to CsA/FK506 treatment is reported to cause increased tumor invasiveness (Hojo et al., 1999). The molecular mechanisms underlying these diverse side effects are poorly understood. While some of them are likely due to calcineurin inhibition, reflecting the ubiquitous tissue distribution of calcineurin and its wide range of substrates, others have been suggested to be independent of calcineurin. Below we outline the arguments for involvement or noninvolvement of calcineurin in CsA/FK506-induced toxicity, bearing in mind that these two scenarios are not mutually exclusive.

Calcineurin-Dependent Toxicity

The best evidence in favor of calcineurin involvement is that CsA and FK506 produce essentially the same spectrum of toxic effects (US Multicenter FK506 study group, 1994; Mayer et al., 1997). This observation points strongly to their common target calcineurin rather than to their immunophilin receptors, which are distinct. Indeed, early studies with a panel of CsA and FK506 analogs indicated that immunosuppression and nephrotoxicity could not readily be separated: both effects

correlated with inhibition of calcineurin but not peptidyl-prolyl isomerase activity, and all the nonimmunosuppressive analogs tested lacked nephrotoxic effects in rodent and canine models (Sigal et al., 1991; Dumont et al., 1992).

Calcineurin, with its wealth of substrates, and NFAT, with its many potential target genes in nonimmune cells, could both play a role in CsA/FK506-induced toxicity (Figure 4). Calcineurin maintains cell function in multiple ways: it modulates Ca^{2+} responses by regulating inositol trisphosphate (IP3) receptors, ryanodine receptors, NMDA receptors, and L-type Ca^{2+} channels; regulates neuronal, vascular, and smooth muscle function through effects on nitric oxide (NO) synthases; regulates cellular ion homeostasis through effects on Na^+/K^+ channels; affects vesicle recycling and neurotransmitter release through amphiphysin and dynamin; and regulates cytoskeletal functions through the microtubule-associated protein τ (Drewes et al., 1993; Christopherson and Bredt, 1997; Snyder et al., 1998; Aramburu et al., 2000). It is plausible that long-term interference with these activities would have an impact on the proper functioning of organs such as the kidney, which plays a preeminent role in ion homeostasis, or brain and muscle, which are tightly regulated by Ca^{2+} and membrane polarization (Figure 4). Likewise, NFAT and other calcineurin-dependent transcription factors could regulate the expression of proteins important for the normal function of those organs in which toxicity is observed. Indeed, as suggested for both NFAT2 and for the IP3 receptor, certain proteins that are posttranslationally regulated by calcineurin could at the same time be transcriptional targets for NFAT (Northrop et al., 1994; Snyder et al., 1998; Graef et al., 1999).

Calcineurin-Independent Toxicity

A large number of the toxic effects of CsA and FK506 have been suggested to be independent of calcineurin. However, this may simply reflect the complex nature of the damage, which even in the case of a single organ may be due to many underlying processes. Thus, dysregulation of calcineurin substrates may initiate but may not be involved in maintaining the sequence of events that leads to clinically apparent organ damage. It should also be borne in mind that the molecular processes thought to underlie toxicity have typically been studied in cultured cells, and their relevance to the damage occurring in vivo is not always apparent.

To illustrate, the early renal vasoconstriction associated with CsA treatment has been related to the ability of this drug to potentiate Ca^{2+} influx in cultured vascular smooth cells stimulated with vasoconstrictor hormones such as angiotensin II (Lo Russo et al., 1996; Avdonin et al., 1999). This response was not immediate but required 20 hr of treatment with high doses of CsA. In cell culture, the effect was at least partially calcineurin independent, since CsA analogs that do not inhibit calcineurin, such as CsH, had similar but lesser effects. However the cell culture findings did not correlate with nephrotoxic potential in vivo, since FK520, a potent immunosuppressant that is at least as toxic as CsA, did not have the same effects. Thus, while calcineurin-independent potentiation of calcium influx might contribute to the renal damage induced by CsA in vivo, it does not account for the very similar damage induced by FK506.

The later phases of vascular damage in the kidney, characterized by fibrosis, microangiopathy, and thickening of vessel walls, have been attributed to TGFβ, a cytokine believed to play a major role in CsA- and FK506-induced nephrotoxicity (Myers, 1986; Pascual et al., 1998). Mice treated with CsA showed increased TGFβ levels in the serum; increased renal expression of TGFβ, collagen and fibronectin mRNAs; and early histopathological changes in the kidney (Khanna et al., 1999a). These effects were all diminished by concurrent administration of anti-TGFβ antibody. As discussed in a previous section, it is unclear whether TGFβ production is mediated via calcineurin or NFAT.

Summary

In summary, it is unclear exactly what fraction of the toxic effects of CsA and FK506 are mediated through NFAT. However, NFAT-based immunosuppressants might be predicted to have fewer and less severe side effects than CsA or FK506. This prediction could be tested experimentally in animal models by comparing the toxic side effects of selective NFAT inhibitors (such as PxIxIT-based peptides) with those of CsA/FK506.

Would NFAT Inhibitors Have Unexpected Side Effects in Immune or Nonimmune Cells?

In this section, we consider the possibility that NFAT inhibitors would have side effects that could not have been predicted from our knowledge of CsA/FK506 toxicity. We briefly review the available information, obtained from targeted gene disruption, on the specific roles of individual NFAT proteins in immune and nonimmune cells (for a more extensive review see Viola and Rao, 1999). It has been suggested that nonimmune cells are relatively more resistant to CsA and FK506 than cells of the immune system because they express higher levels of calcineurin (Crabtree, 1999); if so, compounds that directly targeted NFAT would be more effective than the calcineurin inhibitors CsA and FK506 at inhibiting NFAT functions in nonimmune cells. If this were the case, unexpected side effects might be observed.

Immune Phenotypes of NFAT-Deficient Mice

To date, the phenotypes of knockout mice lacking NFAT1, NFAT2, NFAT4, and a double mutant lacking NFAT1 and NFAT4 have been reported. Mutant mice lacking individual NFAT family members show remarkably mild immune phenotypes, possibly because of their redundant expression in immune cell types (Table 1). Specifically, T cells deficient in NFAT2 show variable (2- to 10-fold) impairment of IL-4 production, while T cells lacking NFAT1 tend to hyperproduce IL-4 (Hodge et al., 1996; Kiani et al., 1997; Ranger et al., 1998b; Yoshida et al., 1998). As a result, NFAT1$^{-/-}$ mice display enhanced allergic reactions and increased susceptibility to Leishmania infection in vivo (Hodge et al., 1996; Xanthoudakis et al., 1996; Kiani et al., 1997; Viola et al., 1998). NFAT4-deficient mice show a minor defect in thymocyte selection and no abnormality in cytokine production (Oukka et al., 1998). Surprisingly, however, the mild Th2 bias of NFAT1$^{-/-}$ mice is dramatically enhanced in mice lacking both NFAT1 and NFAT4, which suffer from severe spontaneous allergic reactions manifested as allergic blepharitis, interstitial pneumonitis, and massive infiltration of lymphoid organs with mast cells and eosinophils (Ranger et al., 1998c).

T cells lacking NFAT2 also display mild defects in activation and proliferation (Ranger et al., 1998b; Yoshida et al., 1998), whereas T cells lacking NFAT1 or NFAT4 (especially those obtained from older mice) appear preactivated or hyperactivated compared to wild-type T cells (Oukka et al., 1998; Schuh et al., 1998b). Again, this phenotype is exacerbated in mice doubly deficient for NFAT1 and NFAT4: T cells from these mice show massive hyperactivation, and the mice spontaneously develop severe splenomegaly and lymphadenopathy (Ranger et al., 1998c).

While the mechanism underlying these effects is still not understood at a molecular level, it is likely that all three NFAT proteins activate T cell proliferation and IL-4 expression, but only NFAT1 and NFAT4 are able to induce negative-regulatory feedback mechanisms that blunt proliferation and the late phase of IL-4 gene transcription (Kiani et al., 1997). According to this model, cells lacking NFAT1 and NFAT4 have completely lost the feedback inhibitory mechanism but retain the positive function of NFAT2. As a result, these cells become massively hyperactivated and show an unusually prolonged production of IL-4. In contrast, cells lacking NFAT2 are mildly suppressed with respect to proliferation and IL-4 production, presumably because the positive effects of NFAT1 and NFAT4 are counteracted by the relative increase in their combined negative feedback effects.

The nature of this postulated negative feedback mechanism remains to be understood, since the inhibitory gene products have not been identified. Conceivably, these gene products could interfere with any step of the signaling pathway leading to proliferation/IL-4 gene expression, including any of the steps of NFAT activation itself (e.g., capacitative calcium entry, calcineurin activity, nuclear localization, etc.). In an extreme scenario, inhibition could be due to a late modification of NFAT1/NFAT4 by an inducible enzyme, a process which might convert these proteins from transcriptional activators in the early stages to DNA-bound transcriptional repressors in the late stage of the response. In any case, agents that interfere with NFAT function would be expected to inhibit not only the positive but also any negative effects of NFAT proteins and so might have complex effects on the immune response.

Functions of NFAT in Nonimmune Cells

There is much evidence for NFAT expression and function in cells outside the immune system. NFAT2-deficient mice die in utero because of defects in the morphogenesis of cardiac valves, thus demonstrating a vital role for this protein in a limited subset of cells during a very limited period of heart development (de la Pompa et al., 1998; Ranger et al., 1998a). Overexpression of either a constitutively nuclear form of the nonlymphoid family member NFAT3 or an activated form of calcineurin resulted in the development of cardiac hypertrophy in transgenic mice (Molkentin et al., 1998). The "classical" criteria noted previously have been used to implicate NFAT in expression of the b-natriuretic peptide gene in cardiomyocytes; the aP2 gene in adipocytes; the IL-3, GM-CSF, and tissue factor genes in endothelial cells; the glucagon gene in pancreatic islet cells; and the vasoactive intestinal peptide gene in neuroblastoma cells (Cockerill et al., 1995; Ho et al., 1998; Molkentin et al., 1998; Symes et al., 1998; Armesilla et al., 1999; Furstenau et al., 1999). NFAT1 is expressed in the olfactory bulb and shows limited expression in other areas of the nervous system (Ho et al., 1994), while NFAT3 is expressed in hippocampal neurons, where it can mediate transactivation of an NFAT-dependent reporter gene in response to depolarization (Graef et al., 1999). Regulated expression and activation of NFAT proteins has also been reported in skeletal muscle cells, vascular smooth muscle cells, and a microglial cell line (Abbott et al., 1998; Boss et al., 1998; Chin et al., 1998; Ferrari et al., 1999; Musaro et al., 1999; Semsarian et al., 1999).

Summary

NFAT is clearly important in many cell types outside the immune system, and its crucial role in cardiac valve development would restrict the use of NFAT inhibitors during pregnancy. In adults, however, the role of NFAT may be modulatory rather than essential, and chronic administration of NFAT inhibitors may be relatively innocuous in a variety of conditions that call for immunosuppression. Again, these predictions need to be tested in appropriate animal models.

Looking Forward: Will NFAT-Based Immunosuppression Play a Role in the Future?

The most compelling argument in favor of NFAT-based immunosuppression is that the clinically valuable immunosuppressants CsA and FK506 are already known to interfere with NFAT activation. As outlined at the end of the section on NFAT regulation, several features of NFAT regulation are promising in terms of immunosuppressive strategies. A good immunosuppressive agent will combine efficacy with specificity, but specificity does not necessarily require the existence of regulatory proteins, which are unique for NFAT. As exemplified by PxIxIT-based peptides for the NFAT-calcineurin interaction, even abundant proteins (such as kinases, components of the nuclear import and export machinery, or nuclear partner proteins and cofactors) may be targeted, as long as the interaction is relatively selective for NFAT and amenable to pharmacological interference.

What Is the Likelihood that NFAT Inhibition Would Have Undesired Side Effects?

This is an area in dire need of more specific information. As discussed in previous sections, the contribution of NFAT to CsA and FK506 toxicity is not known. However, patients treated with CsA or FK506 do not develop muscle dysfunction as a result of NFAT2 inhibition nor memory loss as a result of hippocampal NFAT3 inhibition. Likewise, the immunodeficiency patients in which all NFAT activation is impaired do not display the phenotypes of knockout mice lacking individual or multiple NFAT proteins, nor do they show features associated with CsA or FK506 toxicity. This is encouraging, because it suggests that the side effects of CsA and FK506 are unrelated to NFAT inhibition in nonimmune cells.

There are two possibilities to explain these findings. One is that CsA and FK506 simply do not inhibit NFAT effectively in nonimmune cells, perhaps because nonimmune cells express higher levels of calcineurin than cells of the immune system. If so, one would have to take seriously the possibility that direct inhibitors of NFAT

function would have a different spectrum of side effects from those of the calcineurin inhibitors CsA and FK506. In contrast if CsA and FK506 inhibit NFAT function as effectively in nonimmune cells as in immune cells, the implication would be that NFAT inhibition does not contribute in any major way to the side effects of these drugs. This would in turn imply that NFAT plays a modulatory rather than a critical role in non-immune cell types. Clearly, these possibilities could be resolved by directly comparing the extent of NFAT inhibition in immune and nonimmune cells of CsA/FK506-treated animals.

Although PxIxIT-based peptides are not cell permeant and therefore cannot be used directly as pharmacological agents in vivo, they are likely to be useful tools with which to evaluate both the utility and the potential toxicity of selective NFAT inhibition. Transgenic mice expressing these peptides in different tissues under inducible control may be used to test whether NFAT inhibitors are sufficiently immunosuppressive in vivo, whether they lack the toxicity of CsA and FK506, and whether they have unexpected side expects of their own. Indeed, a transgenic mouse has been generated that expresses a "dominant-negative" NFAT, whose effectiveness is at least partly due to inhibition of NFAT-calcineurin binding (Chow et al., 1999). This mouse expresses 2-fold lower levels of IL-2 in response to T cell stimulation but has not been reported to show other behavioral or functional deficits.

How Do the Predicted Features of NFAT Inhibitors Compare with Those of "Ideal" Immunosuppressive Drugs?

Prospectively, inhibitors specific for NFAT may be envisioned as powerful immunosuppressants with the potential to replace drugs such as CsA or FK506 in clinical practice. However, like CsA and FK506, NFAT inhibitors will suffer from certain intrinsic limitations. For example, NFAT inhibitors will most likely resemble CsA and FK506 in having only minimal effects on expression of proinflammatory cytokines by monocytes and macrophages; thus, they will still need to be combined with glucocorticoids (which suppress AP-1- and NFκB-dependent pathways [Auphan et al., 1995; Scheinman et al., 1995]); or other anti-inflammatory drugs in many clinical situations.

"Classical" immunosuppressive agents such as glucocorticoids, CsA, or FK506 have two other important drawbacks. First, they cannot distinguish between "good" (protective) and "bad" (destructive) immune responses directed against infectious pathogens or tumor cells versus self-antigens, respectively. In consequence, patients who are chronically treated with classical immunosuppressive agents tend to develop opportunistic infections and secondary tumors. Second, classical immunosuppressants do not generate "memory," which means that they have to be administered for long, sometimes lifelong, periods. In attempts to overcome these limitations, new immunosuppressive strategies involving blockade of costimulatory pathways and induction of anergy in antigen-specific lymphocytes are being tested (Gudmundsdottir and Turka, 1999) and are expected to complement classical immunosuppressive regimens in the future.

Might It Be Useful to Develop Inhibitors of Individual NFAT Proteins?

While the idea of targeting individual NFAT proteins is attractive, current data suggest that this approach might be more deleterious in terms of immunosuppression than simply targeting all NFAT proteins. Balanced elimination of all NFAT function (as in the immunodeficient patients) correlates with severe suppression of immune function, while elimination of individual NFAT proteins produces biologically significant biases in the immune response (Table 1). The surprising inference from these experiments is that individual NFATs in immune cells have largely redundant positive functions but their feedback effects are nonredundant. Nevertheless, "skewing" or "redirecting" of the immune response rather than simple "immunosuppression" is a fascinating potential extension of an NFAT-based targeting approach, as is targeting of individual NFAT proteins in nonimmune cells. Clinical situations where inhibition of an individual family member may be useful include allergic asthma (NFAT2) and cardiac hypertrophy (NFAT3). The development of such specific inhibitors will require a greater appreciation of shared versus unique regulatory mechanisms, and shared versus unique functions, of individual NFAT proteins within and outside the immune system.

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