Engine mass detector controlled by a Chemstation HPUX. Analyses were performed after electron impact (EI-GC-MS, 2 bars He, 70 eV) with a program of scanned masses of mass-to-charge ratio (*m/z*) from 45 to 600 and after chemical ionization (CI-CH_4^+, 1.5 bar He, 150 eV), scanning from 100 to 600 *m/z*. The capillary column and temperature program were the same as those used for GC. Injection was performed in the splitless mode for 1 min. Analysis was done either by total ion chromatogram or selected ion chromatogram.

10. Peak 11, which often appears in Fig. 1, was chosen as a reference to illustrate step by step the complexity of the qualitative and quantitative changes that occur in the chemical signature of *P. atrimandibularis*.
11. In *P. biglumis bimaculatus*, peak 11 contains only 2-methylhexacosane and accounts for 0.76% of the signature mixture (relative proportion). Conversely, in *P. atrimandibularis* peak 11 is made up mainly of a combination of heptacosene isomers and the 2-methylhexacosane and accounts for 31% of the mixture. The proportion of the 2-methylhexacosane is low.
12. At this time peak 11 accounts for 1.1% of the cuticular mixture in *P. biglumis bimaculatus* foundresses and 6.46% in the *P. atrimandibularis* queen and is composed only of 2-methylhexacosane in both species.
13. The *P. biglumis bimaculatus* descendants (workers and reproductives) cannot distinguish between their mother and the heterospecific parasite. They work for the parasite and contribute to her reproductive success. In contrast, they recognize a conspecific parasite and refuse to cooperate with her.
14. PCA displays different normalized variables (vectors) on a single plane, accounting for maximum dispersion. This unbiased representation allows one to distinguish or associate individuals (wasps) according to their degree of similarity using the relative proportions of the different quantitative variables (cuticular hydrocarbons).
15. It is known that cuticular mixtures from a colony, or part of a colony, change continuously over time. These changes are controlled by a dynamic system of regulation that induces a chemical drift in the whole group of insects and probably contributes to establish the colonial odor of social insects [E. Provost, G. Rivière, M. Roux, E. D. Morgan, A.-G. Bagnères, *Insect Biochem. Molec. Biol.* **23**, 945 (1993)].
16. At the time of chemical usurpation, peak 11 is still composed only of 2-methylhexacosane and accounts for a similar relative proportion in the two species, that is, 0.58% in *P. biglumis bimaculatus* and 1.13% in *P. atrimandibularis*.
17. Peak 11 follows the same pattern: It returns to the same proportions as in June, that is, 1.06% for *P. biglumis bimaculatus* and 6.4% for *P. atrimandibularis* (including both the 2-methylhexacosane and the heptacosenes).
18. Comparable observations have been made in termites in which soldiers are fed by workers despite differences in caste signature [A.-G. Bagnères, thesis, Paris 6 University (1989)], and in slave-making ants in which the latter are fed by slaves taken at a larval stage, despite differences in specific signatures [C. Habersetzer and A. Bonavita-Cougourdan, *Physiol. Entomol.* **18**, 160 (1993)].
19. Peak 11 accounts for 1.02% of the 2-methylhexacosane in *P. biglumis bimaculatus* female descendants, 6.13% of a mixture of 2-methylC26 and heptacosenes in *P. atrimandibularis* female descendants, and 30.3% of the heptacosenes in the *P. atrimandibularis* queen.
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21. In the present discussion the term cuticle means integument that is the cuticle plus associated epidermal cells and tissue including lipophorin (transport proteins). Oenocytes that are usually associated with the epidermal layer are currently thought to be the most likely site of biosynthesis of cuticular hydrocarbons carried to the epicuticular layer through pore and wax canals [D. R. Nelson *et al.*, in *Insect Lipids: Chemistry, Biochemistry and Biology*, D. W. Stanley-Samuelson and D. R. Nelson, Eds. (Univ. Nebraska Press, Lincoln, NE, and London, 1993), pp. 271–315; G. Blomquist *et al.*, *ibid.*, pp. 317–351; X. Gu, D. Quilici, P. Juarez, G. J. Blomquist, C. Schal, *J. Insect Physiol.* **41**, 257 (1995)].
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An Enhanced Immune Response in Mice Lacking the Transcription Factor NFAT1

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Transcription factors of the NFAT family are thought to play a major role in regulating the expression of cytokine genes and other inducible genes during the immune response. The role of NFAT1 was investigated by targeted disruption of the NFAT1 gene. Unexpectedly, cells from NFAT1$^{-/-}$ mice showed increased primary responses to *Leishmania major* and mounted increased secondary responses to ovalbumin in vitro. In an in vivo model of allergic inflammation, the accumulation of eosinophils and levels of serum immunoglobulin E were increased in NFAT1$^{-/-}$ mice. These results suggest that NFAT1 exerts a negative regulatory influence on the immune response.

The response of the immune system to antigen is coordinated by an interacting network of transcription factors that dictate expression of effector proteins such as cell surface receptors and cytokines (*1*, *2*). The recently identified NFAT family of transcription factors is thought to play a critical role in this process (*3*). NFAT DNA binding activity has been detected in nuclear extracts of antigen-stimulated T cells, B cells, mast cells, and natural killer (NK) cells (*4*, *5*), and NFAT binding sites have been identified in the promoter and enhancer regions of many genes encoding immunoregulatory proteins (*4*, *6*). The NFAT family comprises several structurally related proteins that are encoded by at least four distinct genes (*7*–*9*). The best characterized member of the NFAT family, NFAT1 (formerly NFATp) (*7*, *8*), is expressed constitutively as a cytoplasmic phosphoprotein in resting immune system cells (*10*, *11*); upon stimulation, it is rapidly dephosphorylated and translocated to the nucleus by way of a calcium-calcineurin–dependent pathway (*11*, *12*). Individual NFAT proteins may be able to substitute for one another in regulating expression of certain target genes, because each can bind cooperatively with Fos and Jun to DNA and activate transcription of the interleukin-2 (IL-2) promoter in transient transfection assays (*8*, *9*). However, the differential expression of NFAT proteins in tissues and the differences in their binding preferences for recognition sites in cytokine genes (*9*) suggest that each NFAT protein may control the expression of a distinct set of genes in vivo.

To study the unique functions of NFAT1 in vivo, we generated mutant mice carrying a disrupted NFAT1 allele (*13*). The targeting vector was designed to delete most of an exon encoding amino acids near the NH_2-terminus of the DNA binding domain (Fig. 1, A and B). A short segment of conserved amino acids encoded by this exon has been shown to be critical for DNA binding activity (*14*). Reverse transcriptase polymerase chain reaction (RT-PCR) anal-

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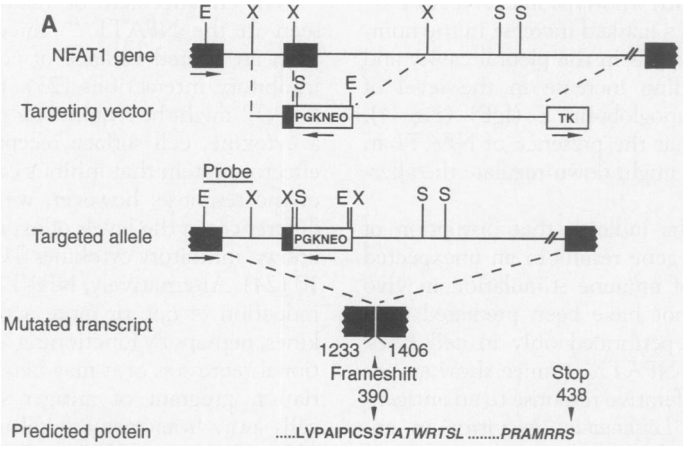
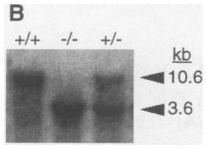
ysis indicated that sequences contained within the targeted exon were absent, as expected, in NFAT1 transcripts from the mutant mice. The deletion results in an aberrant splice between the flanking exons, which generates an out-of-frame mutation at amino acid 389 and a predicted stop codon at amino acid 438 [for numbering, see (8)]. However, this predicted mutant fragment (molecular size, ~50 kD) is either not made or is rapidly degraded, because three different antibodies to NFAT1 failed to detect any NFAT1 protein expression in T cells from mutant mice (Fig. 1C). The same antibodies detected both the phosphorylated and the dephosphorylated forms of NFAT1 in T cells from wild-type mice, confirming that the homozygous mutant mice possessed a null phenotype for NFAT1.

We examined cytosolic extracts from NFAT1$^{-/-}$ and wild-type T cells (*15*) for NFAT DNA binding activity (Fig. 2). The specificity of the NFAT DNA-protein complexes was established by competition with excess unlabeled NFAT oligonucleotides. NFAT DNA binding activity was markedly reduced in the NFAT1$^{-/-}$ extracts compared with that in the wild-type extracts. The residual activity observed in the NFAT1$^{-/-}$ extracts was due to the presence of NFATc, as determined by sensitivity to an antibody to NFATc (anti-NFATc); however, there was no compensatory increase in the level of NFATc in NFAT1$^{-/-}$ mice. Identical results were obtained with nuclear extracts from T cells stimulated with anti-CD3.

Preliminary analysis of NFAT1$^{-/-}$ mice did not reveal any gross anatomical abnormalities. The animals grew to normal size, were fertile, and did not exhibit obvious behavioral deficiencies. Flow cytometric analysis of splenocytes and thymocytes from 4-week-old mice did not indicate any alteration in the numbers or ratios of T and B cells or CD4$^+$ and CD8$^+$ populations, suggesting that NFAT1 does not play a major role in lymphocyte development. In older mice (7 to 12 weeks), however, there was a small but consistent increase in the number of T cells in the spleens of NFAT1$^{-/-}$ mice compared with the number in wild-type mice; the ratio of T cells (CD3$^+$) to B cells (B220$^+$) was 0.42 ± 0.08 (n = 5) for wild-type mice compared with 0.70 ± 0.17 (n = 6) for NFAT1$^{-/-}$ mice ($P < 0.01$). At 11 to 12 weeks, the spleens of NFAT1$^{-/-}$ mice were roughly 18% larger by weight than the spleens of their wild-type counterparts (n = 10, $P < 0.05$), although histological analysis did not detect differences in spleen architecture or in the ratio of white to red pulp. A complete blood count and differential leukocyte analysis did not reveal





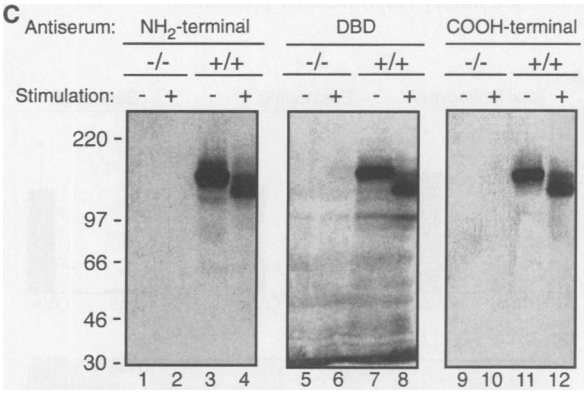


Fig. 1. Generation of NFAT1 mutant mice by gene targeting. (**A**) Schematic diagram of the NFAT1 locus, the targeting vector, and the targeted allele. The mutated NFAT1 transcript and its predicted protein product are diagrammed below the targeted allele. As a result of an out-of-frame mutation, the protein predicted to be encoded by the targeted allele has an altered sequence (shown in italics) beyond amino acid 390 and terminates at amino acid 438. The solid boxes and straight lines denote exon and intron sequences, respectively. Restriction enzyme sites are indicated (E, Eco RI; X, Xho I; and S, Spe I). Arrows indicate the relative orientations of the NFAT, NEO, and TK sequences. (**B**) Southern (DNA) analysis, used to assess homologous recombination. Genomic DNA isolated from tails of transgenic mice was digested with Eco RI and Spe I, resolved on a 0.7% agarose gel, transferred to nylon membranes, and hybridized with a 1.8-kb Eco RI–Xho I fragment of the NFAT1 gene [indicated in (A) by a bar above the depiction of the targeted allele]. The 10.6-kb and 3.6-kb fragments are diagnostic for the presence of a wild-type (+/+) or targeted (−/−) allele, respectively. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; I, Ile; L, Leu; M, Met; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; and W, Trp. (**C**) Detection of NFAT1 by immunoblotting of total proteins from cultured T cells of NFAT1$^{-/-}$ and wild-type (+/+) mice. Cells were left unstimulated (−) or stimulated with 1 μM ionomycin for 5 min at 37°C. SDS lysates of 2 × 10^6 T cells were separated on a 7.5% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with three different antibodies to NFAT1. The antibodies used are described in (*10*) and are specific for the 67.1 peptide near the NH_2-terminus of all isoforms of NFAT1 (NH_2-terminal), a bacterially expressed fragment (NFATpXS) that includes the DNA binding domain (DBD), and a peptide at the COOH-terminus of the NFAT1-C isoform (*8*) (COOH-terminal). Stimulation results in the appearance of a dephosphorylated form of NFAT1 that migrates with higher mobility on SDS gels (*11, 12*). Molecular sizes are indicated on the left (in kilodaltons).

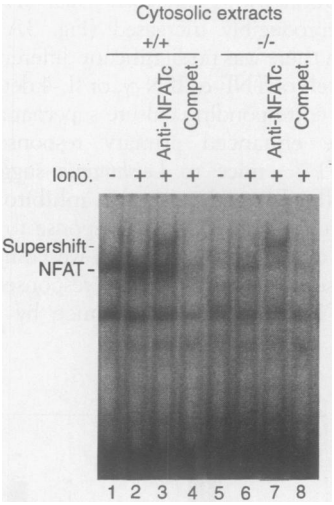


Fig. 2. NFAT DNA binding activity in cultured T cells from NFAT1$^{-/-}$ and wild-type (+/+) mice. Cytosolic extracts from unstimulated (−) or ionomycin-stimulated (+) cells were incubated with a ^{32}P-labeled NFAT oligonucleotide and analyzed by EMSA (*15*). Where indicated, a monoclonal antibody (7A6) to NFATc (Anti-NFATc, lanes 3 and 7) or a 200-fold excess of unlabeled NFAT oligonucleotide (Compet., lanes 4 and 8) were included in the binding reactions. Comparison of lanes 3 and 7 indicates that wild-type and NFAT1$^{-/-}$ T cells express similar levels of NFATc. Comparison of lanes 5 and 6 indicates that the DNA binding activity of NFATc, like that of NFAT1 (*11*), is increased in ionomycin-stimulated cells.

any differences in the blood composition of 20-week-old NFAT1$^{-/-}$ and NFAT1$^{+/+}$ mice (n = 6).

Because NFAT1 has been proposed to play an important role in immune cell function, we assessed the primary immune responses of NFAT1$^{-/-}$ mice both in vitro and in vivo. Spleen cells from NFAT1$^{-/-}$ and wild-type mice displayed similar levels of proliferation in response to primary in vitro stimulation with concanavalin A (Con A), anti-CD3, phorbol myristate acetate (PMA), IL-2, PMA plus IL-2, PMA plus ionomycin, or an allogeneic stimulus (irradiated BALB/c spleen cells). Cyclosporin A (1 μM) completely inhibited the responses of both NFAT1$^{-/-}$ and wild-type cells to anti-CD3, PMA plus ionomycin, and irradiated allogeneic cells, establishing that NFAT1 is not the only target of this immunosuppressive drug. There was no difference in the production of tumor necrosis factor–α (TNF-α), interferon-γ (IFN-γ), IL-2, and IL-4 in response to Con A or anti-CD3 or in the time course of TNF-α production in response to Con A. The basal levels of IL-4, IL-10, TNF-α, IFN-γ, and transforming growth factor–β (TGF-β) mRNAs in spleen cells of wild-type and NFAT1$^{-/-}$ mice were comparable, as was the early (1 hour) induction of these mRNAs in spleen cells of mice that had been injected intravenously with anti-CD3 (*16*). In contrast, proliferation of NFAT1$^{-/-}$ spleen cells in response to primary in vitro stimulation with *Leishmania major* (*17*, *18*) was reproducibly increased (Fig. 3A), although there was no significant difference in the levels of TNF-α, IFN-γ, or IL-4 detected in the corresponding culture supernatants.

The enhanced primary responses of NFAT1$^{-/-}$ mice to *Leishmania* suggested that NFAT1 might exert an inhibitory effect on the normal T cell response to antigen. To test this hypothesis, we compared the secondary immune responses of NFAT1$^{-/-}$ and wild-type mice by using two experimental systems (*19*, *20*). In the first system, the secondary stimulation was performed in vitro. Cells were harvested from draining lymph nodes of mice that had been actively sensitized by injection with ovalbumin, and their proliferation in response to antigen was assessed (*19*). Cells from NFAT1$^{-/-}$ mice consistently showed a marked increase in proliferation in this assay compared with cells from wild-type mice (Fig. 3B). In the second experimental system, an allergic (inflammatory) response to antigen was assessed in vivo. Mice that had been previously sensitized to ovalbumin (*19*) were restimulated by intrapleural injection of antigen, and the accumulation of eosinophils and other inflammatory cells in the pleural cavity was assessed (*20*, *21*). Compared with wild-type mice, NFAT1$^{-/-}$ mice showed a marked increase in the number of eosinophils in the pleural cavity and a corresponding increase in the level of serum immunoglobulin E (IgE) (Fig. 4), suggesting that the presence of NFAT1 in normal mice might down-regulate the allergic response.

Our results indicate that disruption of the NFAT1 gene results in an unexpected phenotype of immune stimulation in vivo that could not have been predicted from experiments performed only in cell lines. Specifically, NFAT1$^{-/-}$ mice show an enhanced proliferative response to an antigenic stimulus, *Leishmania*, in vitro; an enhanced secondary response to a protein antigen, ovalbumin; and increased eosinophil accumulation in an in vivo model of allergic inflammation. Although eosinophil accumulation in this and other models is dependent on secretion of the T helper 2 (T_H2) cytokines IL-5 and IL-4 by sensitized mast cells and CD4$^+$ T cells (*22*), we have not observed that NFAT1 selectively directs differentiation of T cells toward either the T_H1 or the T_H2 pathway, nor have we observed any characteristic bias (*1*) in the cytokines produced by NFAT1$^{-/-}$ spleen cells exposed to T cell stimuli in vitro or in vivo. Likewise, previous work has shown that the level of proliferation in the primary in vitro response to *Leishmania* does not correlate with either a T_H1 (protective) or a T_H2 (nonprotective) response (*17*).

The enhancement of immune responses seen in the NFAT1$^{-/-}$ mice may result from an altered balance of activating and inhibitory interactions (*23*). For example, NFAT1 might be required for production of a cytokine, cell surface receptor, or other effector protein that inhibits certain aspects of the response; however, we detected no differences in the levels of expression of the known inhibitory cytokines TGF-β and IL-10 (*24*). Alternatively, NFAT1 may repress induction of one or more activating cytokines, perhaps by functioning as a transcriptional repressor, or it may bias the differentiation program of antigen-stimulated T cells away from memory cell development (*25*). Northern (RNA) analysis of total

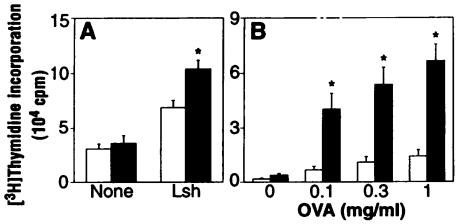


Fig. 3. Immune responses of NFAT1$^{-/-}$ and wild-type (+/+) mice. (**A**) Primary immune response to *L. major* (Lsh). (**B**) Secondary immune responses of NFAT1$^{-/-}$ and wild-type mice to ovalbumin (OVA). Wild-type and NFAT1$^{-/-}$ mice are represented by the open and solid bars, respectively. Data are expressed as the mean ± SD of values from three mice in (A) and 9 to 11 mice in (B). An asterisk (*) indicates data significantly greater than in wild-type mice ($P < 0.05$, Student's t test).

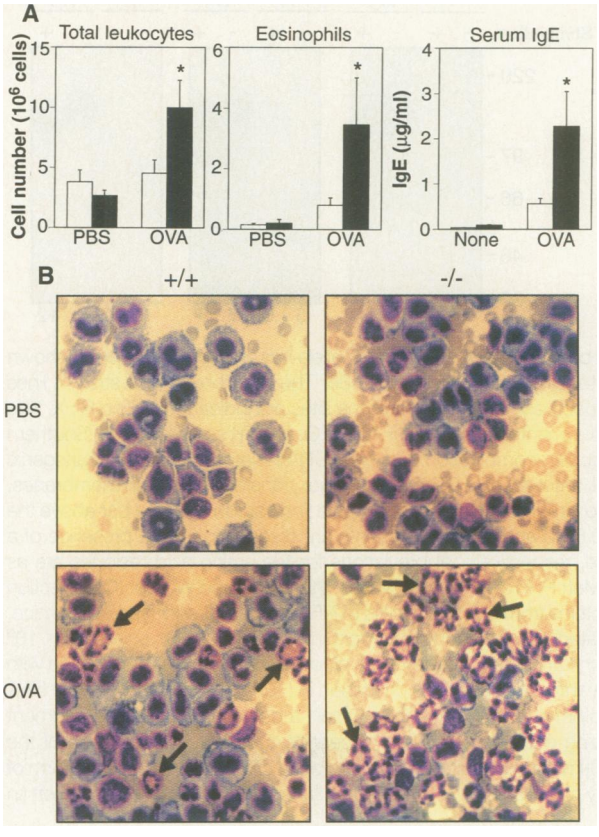


Fig. 4. Antigen-induced allergic pleurisy in NFAT1$^{-/-}$ and wild-type mice. (**A**) Total number of leukocytes, eosinophils in the pleural cavity, and IgE levels in the sera of ovalbumin-sensitized mice injected intrathoracically with ovalbumin (OVA) or PBS. Wild-type and NFAT1$^{-/-}$ mice are represented by the open and solid bars, respectively. Data are expressed as the mean ± SD of values from six mice. An asterisk (*) indicates significantly greater than wild-type mice ($P <$ 0.05, Student's t test). (**B**) Photomicrography of cytospin preparations from pleural fluid obtained as in (A). Large numbers of eosinophils (arrows) are present in the pleural fluid of NFAT1$^{-/-}$, compared with that of wild-type (+/+) mice.

RNA from spleens of mice injected 1 hour previously with anti-CD3 showed a two- to threefold increase in the induced level of CTLA-4 mRNA in NFAT1$^{-/-}$ mice compared with that in wild-type mice, indicating that the enhanced immune response of NFAT1$^{-/-}$ mice is not due to a decrease in CTLA-4 expression. Given that CTLA-4 is thought to down-regulate immune responses (*26*), the increase in CTLA-4 may represent a compensating mechanism that limits the total enhancement of immune function in NFAT1$^{-/-}$ mice.

Our results do not exclude the possibility that NFAT1 also functions as a positive regulator of the immune response. The immunocompetence of NFAT1$^{-/-}$ mice may be rationalized on the basis of the potential redundancy in NFAT activity. The four known members of the NFAT family display overlapping DNA binding and transcriptional specificities and may be coexpressed in mature lymphocytes (*7–9*). Thus, marked deficiencies in immune responsiveness may not become apparent unless mice lacking two or more members of the NFAT family are obtained. A precedent is provided by IL-2$^{-/-}$ IL-4$^{-/-}$ mice, which are also immunocompetent with a phenotype of enhanced basal proliferation (*27*). Older IL-2– and IL-2/IL-4–deficient mice suffer from dysregulation of the immune response, because they are prone to inflammatory bowel disease and other pathological changes of complex etiology (*28*). These or other pathologies may become apparent in older NFAT1$^{-/-}$ mice.

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13. NFAT1 gene fragments were cloned from a genomic library derived from mouse strain 129/Sv (Stratagene). A 4.5-kb region of the NFAT1 gene was deleted by Xho I digestion and replaced with a neomycin-resistance cassette (PGKNEO). The thymidine kinase (TK) gene was inserted at the 3′ end of the targeting construct. The relative orientations of the NFAT1, NEO, and TK sequences are indicated in Fig. 1A by arrows below the boxes representing these sequences. The targeting plasmid was introduced into embryonic stem (ES) cells, and transfectants resistant to G418 and FIAU were isolated and expanded. Four of ~500 drug-resistant ES cell clones analyzed were positive by genomic DNA analysis for the targeted allele. After injection into blastocysts, one of the targeted clones generated chimeric mice that were capable of transmitting the targeted allele through the germ-line. Heterozygous mice were established from several independent chimeras and bred to homozygosity.
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18. Spleen cells (10^6) from 6- to 10-week-old NFAT$^{-/-}$ or wild-type mice were grown in 96-well microtiter plates in the presence of 10^5 infective *L. major* promastigotes and assessed for [^{3}H]thymidine incorporation during a 4- to 16-hour pulse after 7 days. Cytokine production was quantified by enzyme-linked immunosorbent assay (Genzyme) in culture supernatants (taken at 7 days) of 10^7 spleen cells incubated in 2 ml of medium with 10^6 infective *Leishmania* promastigotes.
19. Six- to 8-week-old NFAT1$^{-/-}$ and wild-type mice were sensitized by injections of 0.1 ml of ovalbumin (200 μg) emulsified in complete Freund's adjuvant. Fifteen days later, draining lymph nodes were removed, and cells (2 × 10^5) were grown with the indicated concentrations of ovalbumin and assessed for [^{3}H]thymidine incorporation during a 4- to 16-hour pulse 4 days later.
20. Allergic pleurisy was induced in ovalbumin-sensitized mice by intrathoracic injection of ovalbumin (0.1 ml, 12 μg/cavity) 14 days after sensitization. Animals were killed 24 hours later, and the thoracic cavity was rinsed with 1 ml of sterile phosphate-buffered saline (PBS) containing heparin (10 U/ml). The pleural washes were collected and their volume measured, and total leukocyte counts were obtained after dilution of the pleural fluid in Turk solution (2% acetic acid). Differential analysis of leukocytes removed from pleural fluid was done under an oil immersion objective, in cytocentrifuged smears stained with Diff-Quik (Baxter, FL).
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