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Selective Role of NFATc3 in Positive Selection of Thymocytes

Kirsten Canté-Barrett,* Monte M. Winslow,2† and Gerald R. Crabtree3*†

The four Ca2+-dependent NFATc proteins are both signal transducers and transcription factors that reside in the cytoplasm until dephosphorylation by calcineurin. Dephosphorylation exposes nuclear import sequences and sends NFATc proteins into the nucleus where they assemble with nuclear partners into NFAT transcription complexes. Recent genetic studies have indicated that calcineurin-NFAT signaling is a major determinant of vertebrate morphogenesis and development. Mice lacking calcineurin activity show a complete block in positive selection of CD4 and CD8 double-positive thymocytes, yet the role of the NFATc proteins in T cell development has been controversial. In this study, we address the requirement for NFATc3 in T cell development by generating NFATc3 conditional knockout mice. We show that specific deletion of NFATc3 in thymocytes causes a partial block at the double-negative stage 3 and also a partial block in positive selection. Furthermore, the defect does not become more pronounced when NFATc2 is also absent, consistent with the fact that NFATc2-null mice do not have a T cell developmental defect.

Expression of a nuclear (and constitutively active) NFATc1 even at subphysiological levels can rescue the transition of double-negative to double-positive thymocytes in RAG-null mice, but is unable to rescue development of CD4 and CD8 single-positive cells. In addition to NFATc3, this suggests a role for NFATc1 in T cell development. Our studies indicate that the signals that direct positive selection likely use both NFATc1 and NFATc3 downstream of calcineurin.

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Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; ES, embryonic stem cell; intracellular.
roles during pre-TCR-induced differentiation and during positive selection.

Materials and Methods

**Generation of NFATc3 conditional knockout mice**

A clone from a 129/SvEv embryonic stem (ES) cell genomic library was used to generate the NFATc3 targeting vector. Three loxP sites flank exon 3 of NFATc3, which includes the core residues of the DNA-binding domain. After transfection, ES cells positive for homologous recombination of the targeting vector were selected for injection into C57BL/6 blastocysts or for a secondary transfection with a plasmid expressing Cre recombinase (pMC-CreN; a gift from Dr. F. Alt, Harvard Medical School, Boston, MA) to remove the neomycin-resistance cassette. Chimeric mice were outcrossed and backcrossed to MeuCre40 mice (21), which express Cre recombinase in a mosaic, early embryonic, and ubiquitous manner. This created an allele lacking the neomycin cassette (NFATc3f) and one with complete deletion of exon 3 (NFATc3Δ). The null allele creates a frame shift in the transcript resulting in an early stop codon in exon 4. ES cell recombination and germline transmission were analyzed by PCR using the following oligos (arrows in Fig. 1A): 5'/H11032-CTGGTGATGGTAGTGTAC-3'/H11032, 5'/H11032-GCAAGAACAGCAAGTGTAC-3'/H11032, and 5'/H11032-TTGACCTCAACTGATTCTGGAG-3'/H11032.

**Mice**

MefCre40 mice (21) were a gift from Dr. M. Holzenberger (Hôpital Saint-Antoine, Paris, France). Lck-Cre mice (22) were a gift from Dr. C. Wilson (University of Washington, Seattle, WA) and Lck-Bcl-xL mice (23) were a gift from Dr. S. Korsmeyer (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA). RAG2-null mice (Taconic Farms), NFATc2-null mice (19), Bim-null mice (Ref. 24; The Jackson Laboratory), and NFATc1nuc mice (8) have been described. All mice were maintained in...
the animal facility of Stanford University in accordance with federal and institutional guidelines. In all experiments, sets of littermates were used between 4 and 12 wk of age. Ratios are: NFATc3β;Lck-Cre−/− to NFATc3β+ thymocytes (n = 21), lymph nodes (n = 11), and NFATc3β+/− to NFATc3β+ thymocytes (n = 4), lymph nodes (n = 3). B, Flow cytometry analysis of NFATc3β and NFATc3β;Lck-Cre− thymocytes (left) and DP thymocytes (right, histograms). C, Flow cytometry analysis of NFATc3β and NFATc3β;Lck-Cre− lineage negative (CD4−, CD8−, B220−, Ly-6G−, CD11b+, and TER119+) thymocytes. Development of DN thymocytes progresses as follows: CD44−/CD25− (DN1), CD44+CD25− (DN2), CD44−/CD25− (DN3), CD44+CD25− (DN4) (top plots) and from icTCRβ−CD25− to icTCRβ+CD25+ expression (bottom plots). D, Flow cytometry analysis of NFATc3β and NFATc3β;Lck-Cre− lymph node cells (top) and splenocytes (bottom).

Flow cytometry

Thymocytes, splenocytes, and lymph node cells were obtained by disaggregating the whole organ through a 70-μm nylon cell strainer. Abs for surface markers and flow cytometry analysis were obtained from BD Biosciences and staining was performed according to manufacturer’s recommendations. For intracellular staining, cells were fixed at room temperature with 4% formaldehyde in PBS. All subsequent steps were performed on ice and in 0.3% (permeabilization step) or 0.1% saponin (wash and incubation steps), 5% FBS, and 10 mM HEPES (pH 7.4) in PBS. Cells were permeabilized for 30 min followed by an incubation of 1 h with anti-Bim (StressGen Biotechnologies), anti-Bcl-xL (BD Transduction Laboratories), anti-Mcl-1 (Chemicon International), and FITC-conjugated anti-Bcl-2 (eBioscience), respectively, and a secondary incubation (except for Bcl-2) of 30 min with PE-conjugated anti-rabbit IgG (The Jackson Laboratory).

Western blotting

Total cell lysates were prepared on ice in radioimmunoprecipitation assay buffer and loaded on 4–12% Bis-Tris NuPage gels (Invitrogen Life Technologies). The Abs used were: mouse anti-NFATc1 (7A6), rabbit anti-NFATc3 (both generated in our laboratory), mouse anti-NFATc2 (Santa Cruz Biotechnology), and rabbit anti-actin (Sigma-Aldrich). Signal was detected with ECL followed by exposure to autoradiograph film.

RT-PCR

After reverse transcription of total RNA, the following oligos were used for PCR: 5’-TGTGCAGCTACACGGTTACTTGGA-3’ and 5’-AGTTATGGCAGACAGCACCATCT-3’ (NFATc1, 482-bp product); 5’-AACATGAGAGCCACCATCGACT-3’ and 5’-CTGTAGTCTTCTCCATGAACACAACC-3’ (NFATc2, 330-bp product); 5’-ACCTCATTGGGAGGCTGAAGGAAA-3’ and 5’-TATGCTGGCTGCACTTGACAAA-3’ (NFATc3, 342-bp product); 5’-GAAGCTACCCTCCGGTACAGAG-3’ and 5’-GCTTCATAGCTGGCTGTAGCC-3’ (NFATc4, 441-bp product).

Results

Creation of NFATc3 conditional knockout mice

To selectively delete NFATc3 in developing thymocytes, we generated conditional knockout mice in which exon 3 is flanked by
loxP sites (Fig. 1A). Exon 3 contains the core DNA-binding domain and was deleted in the germline by Meu-Cre40 and in developing thymocytes by Lck-Cre. Wild-type (NFATc3\(^{+/+}\)), “tri-lox” (NFATc3\(^{tri-lox}\)), floxed (NFATc3\(^{f/f}\)), and null (NFATc3\(^{+/−}\)) alleles are indicated. Correct recombination and genotypes were verified by Southern blot and PCR (Fig. 1, B and C). Total thymocytes from NFATc3\(^{+/+}\), NFATc3\(^{+/−}\), NFATc3\(^{f/f}\), and NFATc3\(^{+/−}\); Lck-Cre\(^{+/+}\) mice were analyzed for NFATc3 protein expression. First, the genomic deletion in NFATc3\(^{+/−}\) mice results in complete absence of NFATc3 protein from the thymus and all other organs (Fig. 1D and data not shown). Notably, heterozygous mice have less NFATc3 protein than wild-type mice, suggesting haploid insufficiency. Second, the conditional allele is functional because NFATc3 protein is specifically absent in thymocytes from NFATc3\(^{+/−}\); Lck-Cre\(^{+/+}\) mice (Fig. 1, D and E). We next analyzed the expression of other NFATc proteins and found that NFATc1 and NFATc2 protein levels are comparable between NFATc3\(^{+/+}\) and NFATc3\(^{+/−}\); Lck-Cre\(^{+/+}\) thymocytes (Fig. 1E). Furthermore, NFATc1 and NFATc2 expression in thymocytes is low relative to NFATc3 expression (25). It is therefore unlikely that increased protein expression of either NFATc1 or NFATc2 compensates in the absence of NFATc3. The fourth NFATc family member, NFATc4, is not expressed in the thymus (Fig. 1F).

Because NFATc3-null mice have been previously generated and analyzed (20), we focused on characterizing NFATc3\(^{+/−}\) mice in the presence or absence of the Lck-Cre transgene. It is noteworthy, though, that consistent with the previously reported germline deletion of NFATc3 (20), NFATc3\(^{Δ/Δ}\) mice are born at less than the expected Mendelian ratio of 25% from heterozygous parents. From 15 litters with 78 pups, 12 were NFATc3\(^{Δ/Δ}\) (15%), 43 NFATc3\(^{Δ+/−}\) (55%), and 23 NFATc3\(^{+/+}\) (30%). Knockout animals are viable, fertile, and have no obvious abnormalities, although their litter size

**FIGURE 3.** NFATc3\(^{+/−}\); Lck-Cre\(^{+/+}\) DP thymocytes have increased cell death but normal Bim, Bcl-xL, Mcl-1, and Bcl-2 levels. A, Annexin V flow cytometry analysis of NFATc3\(^{+/−}\) and NFATc3\(^{Δ/Δ}\); Lck-Cre\(^{+/+}\) DP thymocytes. B, Time course of NFATc3\(^{+/−}\) (circles) and NFATc3\(^{Δ/Δ}\); Lck-Cre\(^{+/+}\) (triangles) DP thymocyte viability, without (solid symbols) and with (open symbols) anti-CD3 plus anti-CD28 activation-induced cell death to mimic negative selection. Viability is measured by flow cytometry and expressed as the annexin V-negative percentage of DP-gated thymocytes. The experiment was done in quadruplicate and presented as mean ± SD. C, Intracellular staining of Bim, Bcl-xL, Mcl-1, and Bcl-2 and flow cytometry analysis of NFATc3\(^{+/−}\) and NFATc3\(^{Δ/Δ}\); Lck-Cre\(^{+/+}\) DP thymocytes. Each plot shows a histogram in light gray as a control for staining.
is generally smaller with an average of five pups per litter. In contrast, NFATc3f/f animals are indistinguishable from wild-type and breed well with normal litter sizes.

A partial block in positive selection of NFATc3f/f;Lck-Cre+ thymocytes

Initial characterization of the lymphoid organs in NFATc3f/f;Lck-Cre+ mice showed reduced thymus and lymph node cellularity when compared with NFATc3f/f littermates (Fig. 2A). Thymocytes from sets of littermates (NFATc3f/f;Lck-Cre+ and NFATc3f/f control) between 4 and 12 wk of age were counted (n = 21). NFATc3-deficient thymi have approximately half the number of total thymocytes of littermate controls. Similar results were obtained for total cell numbers of both inguinal lymph nodes (n = 11). Several NFATc3f/f mice were analyzed and although lymph node numbers were lower, thymocyte numbers were not different when compared with control littermates (Fig. 2A), indicating that acute deletion of NFATc3 from thymocytes has a more severe effect on total thymocyte numbers.

When we analyzed thymocyte development in NFATc3f/f;Lck-Cre+ mice, we first noticed a consistent decrease in CD4 SP (6.6 vs 10.6% in control) and CD8 SP (1.08 vs 2.06% in control) thymocytes (Fig. 2B, left panels). In addition, NFATc3-deficient thymocytes have reduced TCRβhigh DP cells (3.3 vs 5.1% in control) and CD69high DP cells (5.7 vs 10.9% in control, Fig. 2B, right histograms). These data indicate a T cell-intrinsic defect in positive selection.

A partial block at stage DN3 of NFATc3f/f;Lck-Cre+ thymocytes

In addition to the defect in positive selection, we observed higher DN thymocyte percentages in NFATc3f/f;Lck-Cre+ mice (Fig. 2B, left panels). Therefore, we examined DN cells in more detail and found that NFATc3-deficient thymocytes have a partial, but consistent DN development defect (Fig. 2C). DN thymocytes (CD4–, CD8–, B220+, Ly-6G+, CD11b–, and TER119−) from sets of littermates (Fig. 2, top row, Annexin V analysis of DP thymocytes from mice with genotypes as in the top row) were analyzed and although lymph node numbers were lower, thymocyte numbers were not different when compared with control littermates (Fig. 2, bottom panels) as well as spleen (bottom panels) compared with control mice.

NFATc3f/f;Lck-Cre+ DP thymocytes have increased cell death but normal Bim, Bcl-1x2, Mcl-1, and Bcl-2 levels

In addition to the partial blocks in DN development and positive selection, reduced viability of NFATc3-deficient thymocytes could also contribute to the reduction of total thymocyte numbers. Therefore, we analyzed thymocyte viability and found that NFATc3f/f;Lck-Cre+ mice have more DP thymocytes undergoing apoptosis in
null mice have been generated and it has been shown that NFATc2 less calcineurin has other, unknown targets. Two lines of NFATc2-B, other NFAT family members must also play a role un-

Together, these data are consistent with the fact that the calcineurin-

Because positive selection neither requires NFATc2 (Refs. 18 and 19) nor NFATc4 due to its absence in thymocytes (Fig. 4) nor NFATc3 because the observed defect in positive selection is not more pronounced than in NFATc3-deficient thymocytes as shown by CD4 and CD8 SP percentages (Fig. 4A, bottom row) and annexin V data not shown). This indicates that in contrast to NFATc3, NFATc2 contributes to thymocyte survival in culture.

Similar to the positive selection defect, the partial block at DN3 does not become more severe in NFATc2/c3 double-deficient thymocytes (Fig. 4B). However, double-deficient thymocytes have increased DN1 and subsequently more severely decreased DN4 populations that are not observed in either NFATc2- or NFATc3-deficient thymocytes. Therefore, NFATc2 plays a redundant role in DN thymocyte development that only comes to light when both NFATc2 and NFATc3 are absent, but NFATc2 does not contribute to positive selection. This is illustrated not only by the fact that NFATc2-null mice do not have a positive selection defect, but also by the fact that NFATc2 does not compensate for the lack of NFATc3 because the observed defect in positive selection is not more severe in double-deficient thymocytes than in NFATc3-deficient thymocytes.

Active NFATc1 drives the development of DN to DP thymocytes in the absence of pre-TCR signaling

Because positive selection neither requires NFATc2 (Refs. 18 and 19 and Fig. 4) nor NFATc4 due to its absence in thymocytes (Fig. 1F), we next focused on NFATc1. Even though NFATc1-wt-Rag2-wt chimeric mice (in which the lymphoid compartment completely consists of NFATc1-deficient cells) have no defect in

NFATc2 deficiency does not enhance the block in positive selection or the block at DN3

Because the defect in positive selection is complete in the absence of calcineurin B1 (5), but incomplete in the absence of NFATc3 (Fig. 2B), other NFAT family members must also play a role unless calcineurin has other, unknown targets. Two lines of NFATc2-null mice have been generated and it has been shown that NFATc2 is dispensable for thymocyte development (18, 19). However, it is possible that NFATc2 contributes to the signaling required for pre-TCR-induced DN developmental progression and positive selection. We therefore bred NFATc2-/- mice with our NFATc3-/- mice, again in the presence or absence of the Lck-Cre transgene. We did not observe any defect in thymocyte development in NFATc2-/- mice (Fig. 4, A and B, third plot). When both NFATc2 and NFATc3 are absent from thymocytes, the defect in positive selection is not more pronounced than in NFATc3-deficient thymocytes (Fig. 3, fourth plots). Although NFATc2 knockout thymocytes do not exhibit initial decreased viability at t = 0, the rate of viability loss in culture of NFATc2-deficient and NFATc2/c3 double-deficient thymocytes is slightly increased compared with NFATc3-deficient or control thymocytes (Fig. 4A, bottom row and data not shown). This indicates that in contrast to NFATc3, NFATc2 contributes to thymocyte survival in culture.

Next, we stimulated thymocytes with plate-bound anti-CD3 and anti-CD28 to mimic negative selection. Both NFATc3-deficient and control DP thymocytes die under negative selection conditions in vitro, as shown by an increase in cell death during the first 4 h in culture (Fig. 3B, open symbols). Furthermore, the level of the proapoptotic protein Bim, which is necessary for negative selection (26), is comparable in NFATc3-/-;Lck-Cre-/- and NFATc3-/- control thymocytes (Fig. 3C, top left) and increased but comparable between NFATc3-deficient and control thymocytes under anti-CD3/anti-CD28-stimulated conditions (data not shown). Therefore, we conclude that negative selection is unaffected in the absence of NFATc3. In contrast to what has been reported for mice with germline deletion of NFATc3 (20), we found no change in Bcl-2 levels between NFATc3-deficient and control DP or SP thymocytes (Fig. 3C, bottom right and data not shown). Prosurvival Bcl-2 family members Bcl-xL and Mcl-1 are also unaffected by NFATc3 deficiency (Fig. 3C, top right and bottom left histograms). Thus, we conclude that the increased apoptosis observed in NFATc3-deficient thymocytes is at least in part due to increased “death by neglect” as a result of the defect in positive selection and defect in TCR signaling and that the expression of Bcl-2 family members does not contribute to this cell death phenotype. Together, these data are consistent with the fact that the calcineurin-NFAT-signaling pathway is necessary for positive selection, but is not involved in negative selection (5, 27).

**FIGURE 5.** Active NFATc1 drives the development of DN to DP thymocytes from Rag2-/-, Rag2-/-; NFATc1<sup>1<sup>uc</sup></sup>, and Rag2-/-; NFATc1<sup>1<sup>uc</sup></sup> doxyccycline (DOX) suppressed mice. B, CD5 histograms of DN and DP thymocytes from mice in A.
positive selection, delayed lymphoid reconstitution and consequently lower numbers of thymocytes were observed (16, 17). Therefore, it is still unclear whether DN thymocyte development is affected in NFATc1-deficient thymocytes. In either case, NFATc1 and NFATc3 double-deficient thymocytes could very well cause the complete block in positive selection seen in calcineurin-deficient thymocytes (5). However, NFATc1-null mice are embryonic lethal due to cardiac valve defects (28, 29) and conditional alleles are not yet available. Instead, we made use of mice expressing a constitutively active, nuclear NFATc1 under the control of the tetracycline-responsive operator that were bred to Eμ-tTA mice. The tetracycline-responsive operator-driven expression of active NFATc1 can be suppressed with doxycycline. These mice have been described and are referred to as NFATc1nuc mice (8). Surprisingly, when NFATc1nuc mice were bred onto the Rag2−/− background, in which no TCR rearrangement, no TCR signaling, and consequently no thymocyte development takes place, ~4–30% DP thymocytes appeared (Fig. 5A, middle panel). These DP thymocytes express CD5 (Fig. 5B, middle panel) and CD90, but lack TCRβ (data not shown). The development of these cells is suppressed when the mice are treated with doxycycline to turn off NFATc1nuc expression (Fig. 5, A and B, third plot). The results suggest that active NFATc1 bypasses the need for pre-TCR signaling and rescues, albeit to a limited extent, the DN to DP thymocyte transition. The fact that nuclear NFATc1 is (at least partly) sufficient for the DN-to-DP transition points to the possibility of NFATc1 responding to (pre-)TCR signaling and contributing to DN development and potentially positive selection in the absence of NFATc3, thus explaining the incomplete thymocyte development block in NFATc3−/−;Lck-Cre+ mice.

Discussion

We have examined the role of the transcription factor NFATc3 in thymocyte development by specific deletion of NFATc3 from thymocytes. Given that the phosphatase calcineurin regulates NFATc nuclear translocation and is necessary for positive selection (5), it is likely that one or more NFATc transcription factors regulate thymocyte positive selection. Of the four Ca2+2/calcineurin-dependent NFATc family members, three (NFATc1, NFATc2, and NFATc3) are expressed in the immune system (Ref. 30 and Fig. 1F). Because NFATc3-deficient thymocytes only exhibit a partial defect in positive selection, we hypothesized that other NFATc family members play redundant roles and may compensate for the lack of NFATc3. Genetic deletion of NFATc2 does not cause a defect in thymocyte development (18, 19) and does not enhance the defect in NFATc3-deficient thymocytes (Fig. 4). This indicates that among NFAT family members, a variety of functional differences exist, even within the lymphoid lineage. For example, while NFATc2 is not involved in thymocyte development, it controls, together with NFATc1, immune responses in peripheral T and B cells (31). In contrast, NFATc3 is expressed at its highest level in DP thymocytes (20, 25) where it is involved in positive selection (Ref. 20 and Fig. 2). Analysis of previously generated NFATc2/c3 double knockout mice revealed, among many other things, massive lymphadenopathy and splenomegaly (32), not observed in our NFATc3−/−;Lck-Cre+;NFATc2−/− mice. The difference can be explained by the fact that in our mice, NFATc3 is absent only from the T cell lineage and implicates important roles for NFATc3 in other organs as well.

NFATc1-null mice have a modest defect in the transition from DN to DP thymocytes (16, 17) and therefore NFATc1 could be involved in a redundant manner with NFATc3. Our data show that a constitutively active form of NFATc1 can drive DN thymocyte development to the DP stage in the absence of pre-TCR signaling (Fig. 5). Additionally, pre-TCR signaling has been correlated with a rise in cytosolic Ca2+ concentration and NFAT activation (33). This suggests that NFATc1, together with NFATc3, regulates thymocyte development. Thymocyte-specific deletion of both NFATc1 and NFATc3 would decisively answer the question of whether positive selection is blocked as efficiently in NFATc1/c3 double-deficient thymocytes as in calcineurin-deficient thymocytes (5).

Our work shows that freshly isolated NFATc3-deficient thymocytes initially exhibit more apoptosis (Fig. 3, A and B, at 0 h), but we find that the rate of cell death in culture is the same between NFATc3-deficient and control DP thymocytes (Fig. 3B). This discrepancy can at least partly be explained by the fact that in vivo, DP thymocytes with a TCR-signaling defect fail to undergo positive selection and die by “death by neglect.” In vitro however, no positive selection takes place and all thymocytes undergo death by neglect at the same rate. If NFATc3 were a general survival factor, the NFATc3-deficient thymocytes would show an increased rate of cell death in culture. Interestingly, NFATc2-deficient thymocytes have a slightly increased rate of cell death in culture, but do not have increased cell death at t = 0 (Fig. 4A and data not shown). These observations indicate that NFATc2 plays a role in thymocyte survival in culture and are consistent with the fact that NFATc2 is not involved in positive selection.

Additionally, none of the Bcl-2 family members involved in general cell death/survival that were analyzed are differentially expressed between NFATc3-deficient and control DP thymocytes (Fig. 3C). In essence, thymocytes lacking NFATc3 are equivalent to cells without effective TCR gene recombination or effective TCR expression and hence do not receive an effective positive selection signal. We therefore conclude that decreased thymocyte viability in the absence of NFATc3 is a result of the defect in positive selection and not vice versa.

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Disclosures

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