NFAT5 induction by the pre-T-cell receptor serves as a selective survival signal in T-lymphocyte development

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The Rel-like transcription factors nuclear factor kappa B (NF- κ B) and the calcineurin-dependent nuclear factor of activated T cells (NFATc) control specific points of thymocyte maturation. Thymocytes also express a distinct member of the Rel family, the calcineurin-independent, osmotic stress response regulator NFAT5. Here we show that IKK β regulates the expression of NFAT5 in thymocytes, which in turn contributes to the survival of T-cell receptor $\alpha\beta$ thymocytes and the transition from the β -selection checkpoint to the double-positive stage in an osmotic stress-independent manner. NFAT5-deficient thymocytes had normal expression and proximal signaling of the pre-T-cell receptor but exhibited a partial defect in β -chain allelic exclusion and increased apoptosis. Further analysis showed that NFAT5 regulated the expression of the prosurvival factors A1 and Bcl2 and attenuated the proapoptotic p53/Noxa axis. These findings position NFAT5 as a target of the IKK β /NF- κ B pathway in thymocytes and as a downstream effector of the prosurvival role of the pre-T-cell receptor.

T-cell development | gene expression

Early thymocyte differentiation proceeds through double-negative (DN; CD4⁻ and CD8⁻) steps (1, 2) from the DN1 to the early DN3 (E-DN3) stages until cells express a recombined β allele of the T-cell receptor (TCR), which, together with the nonvariant pre-T α and the CD3 complex, constitute a functional pre-TCR (3). The pre-TCR marks the commitment to the $\alpha\beta$ T-cell lineage and controls the β -selection checkpoint in a ligand-independent manner by regulating allelic exclusion of the TCR β locus, proliferation, survival, and differentiation of thymocytes, all of which are required for progression of DN3 to double-positive (DP; CD4⁺ and CD8⁺) cells (4–6). At the DP stage, thymocytes recombine the TCR α and express the mature TCR $\alpha\beta$ complex, which interacts with self-peptide–MHC complexes of thymic antigen-presenting cells (7). This interaction determines the positive and negative selection of DP thymocytes from which CD4⁺ or CD8⁺ single-positive (SP) cells emerge. SP cells then migrate to peripheral organs to populate them with mature T lymphocytes.

Despite knowledge accumulated on signaling pathways activated by the pre-TCR, less is known about the specialization of transcription factors downstream from this receptor and the genes they control (8). The Rel-like transcription factors NF- κ B and the calcineurin-dependent NFATc proteins regulate thymocyte development downstream of the pre-TCR (9–16). Thymocytes also express the calcineurin-independent NFAT protein NFAT5, which has hybrid features of both NF- κ B and NFATc proteins (17, 18). NFAT5 protects cells from osmotic stress (19), and NFAT5-deficient mice present severe atrophy of the renal medulla, systemic hypernatremia, and a reduced thymocyte compartment and mature T-cell lymphopenia (20, 21). Whereas the thymocyte and T-lymphocyte deficiency of NFAT5-null mice can be explained by their systemic hypernatremia (21, 22), mice expressing a T-lymphocyte-restricted dominant negative NFAT5 transgene were shown to have reduced thymic cellularity and peripheral T lymphopenia (23). Because the tonicity of the

thymus is not high enough to activate NFAT5 (21, 24, 25), this suggests an osmotic stress-independent role of NFAT5 in thymocyte development. Here we describe that NFAT5 expression is regulated by the IKK β kinase β (IKK β) pathway in thymocytes and acts as a survival factor downstream from the pre-TCR, independent of its osmoprotective function.

Results

Reduced Number of Thymocytes and Peripheral T Cells in *Nfat5*^{fl/fl}, Lck-Cre Mice. To gain insight into the role of NFAT5 in thymocyte development, conditional deletion of *Nfat5* at early (DN2) or later (DP) thymocyte stages was induced by crossing mice with LoxP sites flanking both *Nfat5* alleles (*Nfat5*^{fl/fl}) with mice expressing Cre at DN2 (Lck-Cre) or DP (CD4-Cre). *Nfat5*^{fl/fl}, Lck-Cre mice, but not *Nfat5*^{fl/fl}, CD4-Cre mice, had smaller thymi than their wild-type (WT) littermates (*Nfat5*^{wt/wt}, Lck-Cre or *Nfat5*^{fl/fl} mice) (Fig. 1A and B) and decreased cellularity in lymphoid organs (Fig. 1C), with fewer mature CD4 and CD8 cells in lymph nodes and spleen (Fig. 1D and E and ref. 21), but normal numbers of B cells (B220⁺, IgM⁺), macrophages (CD11b), and dendritic cells (CD11c) (Fig. 1E and F). These results indicated that loss of NFAT5 in DN cells affected thymocyte development.

NFAT5 Deficiency Causes a Thymocyte-Intrinsic Defect in Development. *Nfat5*^{fl/fl}, Lck-Cre mice had significantly fewer DP and SP thymocytes

Significance

The pre-T-cell receptor (pre-TCR) marks a functional checkpoint in thymocyte development that controls functions such as survival, proliferation, and differentiation. Despite knowledge accumulated on signaling pathways activated by the pre-TCR, less is known about the specialization of transcription factors downstream from this receptor and the genes they control. The Relish (Rel)-like transcription factors NF- κ B and NFATc were previously known to control specific points of thymocyte maturation. Our work now positions the Rel-like protein NFAT5 as a target of the IKK β pathway in thymocytes and as a downstream effector of the prosurvival role of the pre-TCR through its ability to regulate the expression of the survival-promoting proteins A1 and Bcl2 and attenuate the proapoptotic axis comprised by p53 and Noxa proteins.

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Fig. 1. Reduced number of thymocytes and peripheral T cells in *Nfat5*^{fl/fl}, Lck-Cre mice. (A) Thymus size in control (WT), *Nfat5*^{fl/fl}, Lck-Cre (Upper), or *Nfat5*^{fl/fl}, CD4-Cre (Lower) mice. (Scale bar, 1 cm.) (B) Body, thymus, and spleen weight in WT and *Nfat5*^{fl/fl}, Lck-Cre mice. (C) Cell numbers in inguinal lymph nodes, thymus, and spleen in WT and *Nfat5*^{fl/fl}, Lck-Cre mice. Each circle in B and C represents a single mouse, and the horizontal lines show the mean. (D) Numbers of CD3, CD4, and CD8 cells in lymph nodes of WT and *Nfat5*^{fl/fl}, Lck-Cre mice (Left) and WT and *Nfat5*^{fl/fl}, CD4-Cre mice (Right) (mean $\pm$ SEM); $n \geq 6$. Numbers of B220-positive, CD3-positive, CD4-positive, and CD8-positive (E) and IgM-positive, CD11b-positive, and CD11c-positive (F) splenocytes in WT and *Nfat5*^{fl/fl}, Lck-Cre mice (mean $\pm$ SEM); $n = 8$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

than WT littermates and *Nfat5*^{fl/fl}, CD4-Cre mice (Fig. 2A and B). They also had an altered ratio of DN3 (CD44⁺ and CD25⁺) to DN4 thymocytes (CD44⁺ and CD25⁺) (Fig. 2A–C), with a greater proportion of large, late DN3 cells (L-DN3) (Fig. 2D) and fewer $\alpha\beta$ thymocytes, but normal numbers of $\gamma\delta$ precursors (Fig. 2E). We did not observe obvious defects in markers of the DN to DP transition, such as expression of surface CD3, intracellular TCR β in DN cells, and mRNA levels of *Ptcr*, *Tcr*, *Lef1*, and *Tcf7* (SI Appendix, Fig. S1). DN cells of *Nfat5*^{fl/fl}, Lck-Cre mice also displayed normal levels of activated PDK1 and Erk1/2 (SI Appendix, Fig. S2), indicating a normal function of proximal pre-TCR signaling.

We tested whether NFAT5 had a thymocyte-intrinsic role by analyzing the differentiation of isolated precursors cocultured with stromal cells expressing the Notch ligand Delta-like 1 (OP9-DL1) (26). Thymic precursors from *Nfat5*^{fl/fl}, Lck-Cre mice exhibited poorer differentiation capacity than WT ones, producing fewer DP and SP thymocytes and showing defective progression from DN3 to DN4 (Fig. 2F and G). These results reproduce the defects observed in vivo and support a cell-autonomous role for NFAT5 in thymocyte development.

NFAT5 Has Different Effects on Thymocyte Proliferation and Survival.

It has been suggested that the slightly hypertonic milieu of the thymus could be harmful for NFAT5-deficient thymocytes (24), but it is unclear whether thymic tonicity can activate NFAT5 (25). *Nfat5*^{fl/fl}, Lck-Cre mice had normally isotonic plasma, and their DN3, DN4, and DP thymocytes expressed normal levels of

the osmotic stress-inducible, NFAT5-dependent genes *Akr1b3* and *Slc5a3* (SI Appendix, Fig. S3A and B). This result indicated that thymocytes are not undergoing an osmotic stress response and that NFAT5 regulates their development independent of its osmosensitive function. This view is consistent with the in vitro differentiation experiments in Fig. 2F and G, done in controlled isotonic medium.

Because our results were consistent with a role for NFAT5 in thymocyte survival or proliferation, we examined both functions. NFAT5-deficient DP and CD8 thymocytes showed a mild increase in proliferation rate in vivo and a slightly elevated proportion of cells in S and G2/M cell cycle phases (Fig. 3A and SI Appendix, Fig. S4A and B). Nonetheless, *Nfat5*^{fl/fl}, Lck-Cre thymocytes expressed normal levels of the pre-TCR- or NFAT-regulated cyclins D3 or A2 (22, 27, 28) during their DN3 to DP transition (SI Appendix, Fig. S4C). In contrast with the moderate change in proliferation, freshly isolated thymocytes from *Nfat5*^{fl/fl}, Lck-Cre mice displayed a higher proportion of apoptotic cells in the DN, DP, and SP compartments (Fig. 3B), suggesting that the overall effect of lacking NFAT5 in thymocytes was a reduced survival capacity. Consistent with this, enforced induction of apoptosis in vivo through CD3/TCR stimulation (29) caused a greater depletion of DP thymocytes in *Nfat5*^{fl/fl}, Lck-Cre mice than in WT ones (Fig. 3C vs. Fig. 2A). Altogether, these data indicate that pre-TCR-driven thymocyte survival required NFAT5.

Inefficient TCR β Allelic Exclusion in *Nfat5*^{fl/fl}, Lck-Cre Thymocytes. Although pre-TCR signaling appeared to be normal in *Nfat5*^{fl/fl}, Lck-Cre mice, defects in other pre-TCR functions were suggested by their having more L-DN3 thymocytes, which is the earliest stage at which the pre-TCR is expressed, and increased apoptosis since DN cells. In addition to promoting thymocyte survival and proliferation, the pre-TCR controls TCR β allelic exclusion by preventing further rearrangements at the TCR β locus (30). We observed that *Nfat5*^{fl/fl}, Lck-Cre mice had normal levels of intracellular TCR β in DN thymocytes (SI Appendix, Fig. S1) and a largely normal representation of TCR V β chain species in mature T cells (SI Appendix, Fig. S5A) but showed a higher percentage of thymocytes coexpressing V β 8 together with V β 5, V β 7, or V β 9 (SI Appendix, Fig. S5B), suggestive of anomalous TCR β allelic exclusion. Although some degree of faulty allelic exclusion occurs in normal mice (30, 31), this defect was more pronounced in NFAT5-deficient cells. The increased allelic inclusion of *Nfat5*^{fl/fl}, Lck-Cre thymocytes was not linked to defects in the expression of the transcription factors Ets-1 or E47, two known regulators of this process (31, 32) (SI Appendix, Fig. S5C and D). In addition, the Ets-1 target gene *Runx3* (33) was normally expressed in DN3 to DP thymocytes of *Nfat5*^{fl/fl}, Lck-Cre mice (SI Appendix, Fig. S5E).

Thymocyte markers expressed beyond the β -selection checkpoint (CD62L, CD44, IL-7R α , CD24, and TCR β), as well as positive selection markers, were normal in NFAT5-deficient cells (SI Appendix, Fig. S6A–C). However, analysis of their progression through the DP steps of large preselection, small preselection, and small postselection cells (34) showed that they accumulated as large CD69⁺ preselection cells and had a mild reduction in CD69 expression in cells with high intensity of TCR β (SI Appendix, Fig. S6D and E). To exclude the possibility that impaired survival of DP thymocytes could be a result of defective positive selection, we crossed *Nfat5*^{fl/fl}, Lck-Cre mice with Marilyn mice and assessed positive and negative selection of CD4 cells, respectively, in their female and male progeny (35). We observed that positive and negative selection proceeded similarly in Marilyn mice regardless of NFAT5 (SI Appendix, Fig. S7).

Expression of NFAT5 in Thymocytes Is Enhanced by the Pre-TCR and Regulated by IKK β . NFAT5 expression was higher in DP thymocytes than in DN and SP CD4 or CD8 cells (WT; Fig. 4A).

Fig. 2. *Nfat5^{fl/fl}, Lck-Cre* thymocytes have a cell-intrinsic defect in development. (A) Cell numbers for thymocyte subsets in WT, *Nfat5^{fl/fl}, Lck-Cre*, and *Nfat5^{fl/fl}, CD4-Cre* mice (mean ± SEM); $n \geq 6$. (B) Representative flow cytometry analysis of CD4 versus CD8 in total thymocytes, and CD44 versus CD25 gated in DN cells of WT and *Nfat5^{fl/fl}, Lck-Cre* mice (Left) and WT and *Nfat5^{fl/fl}, CD4-Cre* mice (Right). Numbers indicate percentages. (C) Ratio of DN4 to DN3 thymocytes in WT and *Nfat5^{fl/fl}, Lck-Cre* mice (mean ± SEM); $n = 8$. (D) Percentage of L-DN3 cells in WT and *Nfat5^{fl/fl}, Lck-Cre* mice, determined by cell size (forward light scatter height parameter, FSC-H). One representative experiment is shown out of six independently performed. (E) Number of TCR $\gamma\delta^+$ or TCR β^+ thymocytes in WT and *Nfat5^{fl/fl}, Lck-Cre* mice (mean ± SEM); $n \geq 8$. (F) Relative numbers of thymocyte subsets after in vitro differentiation with OP9-DL1 cells for 2, 4, and 6 d (mean ± SEM); $n = 3$. (G) Representative flow cytometry analysis of CD4 versus CD8 (Upper) in total thymocytes, and CD44 versus CD25 (Lower) in gated DN cells, from thymocytes cocultured with OP9 control or OP9-DL1 cells. Percentages are indicated in the quadrants. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Likewise, its mRNA increased in the DN to DP transition (WT, Fig. 4B). *Nfat5^{fl/fl}, Lck-Cre* mice had reduced NFAT5 expression in DN cells and lost it completely in DP and SP thymocytes (Fig. 4A and B). The deficiency in NFAT5 was maintained in peripheral mature T cells (Fig. 4C). In contrast, *Nfat5^{fl/fl}, CD4-Cre* mice had NFAT5 in DN cells and still expressed moderate levels of it in DP thymocytes (Fig. 4A and B). Residual expression of NFAT5 in CD8⁺ thymocytes of *Nfat5^{fl/fl}, CD4-Cre* mice (Fig. 4A) likely is a result of immature CD8⁺ CD4⁻ SP cells that do not express Cre. These findings indicated that the thymic hypocellularity and peripheral T-cell lymphopenia of *Nfat5^{fl/fl}, Lck-Cre* mice were caused by the loss of NFAT5 between late DN and early DP thymocytes.

Because the pre-TCR is a major player in the transition from DN to DP thymocytes, and NFAT5 expression was higher in DP than in DN cells, we tested whether this increase could be induced by stimulating the pre-TCR. *Rag2^{-/-}* mice were injected with the anti-CD3 antibody 2C11 to enforce pre-TCR-induced DN to DP progression in developmentally arrested thymocytes in a manner independent of a functional mature $\alpha\beta$ TCR (36). Anti-CD3 treatment increased the expression of NFAT5 protein in *Rag2^{-/-}* thymocytes before they presented the surface markers CD4 and CD8 (Fig. 4D and E). Given that the IKK β /NF- κ B pathway regulates NFAT5 expression in macrophages (37) and that this pathway promotes thymocyte survival downstream from the pre-TCR (12), we tested its involvement in the induction of NFAT5 in thymocytes. IKK β -deficient thymocytes had substantially fewer DP and SP cells than control mice, but normal DN cell numbers (SI Appendix, Fig. S8). Nonetheless, both total thymocytes and isolated DN cells from these mice showed reduced expression of NFAT5 (Fig. 4F and G). Altogether, these results indicated that

NFAT5 is present in different thymocyte populations and that pre-TCR-activated IKK β could further up-regulate its expression.

Altered Expression of Genes That Control the Balance Between Cell Death and Survival in Thymocytes of *Nfat5^{fl/fl}, Lck-Cre* Mice. Analysis of the expression of genes that regulate cell death and survival (38) showed that NFAT5-deficient thymocytes had reduced mRNA levels of the antiapoptotic genes *Bcl2a1* (Bcl2-related protein A1, or A1) and *Bcl2* (B-cell leukemia/lymphoma 2, Bcl2), increased expression of the proapoptotic gene *Pmaip1* (Noxa), and normal expression of other cell survival [*Bcl2l1* (Bcl-x_L)] or apoptosis [*Bcl2l1* (Bim), *Bbc3* (Puma)] regulators (Fig. 5A). Moreover, although enforced CD3 signaling in vivo reduced the number of NFAT5-deficient thymocytes, it did not reveal additional NFAT5 targets in control of cell survival (including *Mcl1*, *Bax*, and *Bad*; SI Appendix, Fig. S9). The ratio of A1 or Bcl2 to Noxa was around three times lower in NFAT5-deficient thymocytes, in line with their higher proportion of apoptotic cells. In addition to reduced levels of Bcl2 mRNA and protein, NFAT5-deficient DP thymocytes also expressed a lower-mobility form of Bcl2 associated with increased cell death (39) (Fig. 5B). We could not analyze A1 protein because of the lack of available antibodies. The elevated level of Noxa mRNA suggested that p53, its upstream regulator, could be more active in NFAT5-deficient DP cells. We detected a moderate increase in p53 expression and phosphorylation in NFAT5-deficient thymocytes (Fig. 5B and C) without increased γ H2AX (Fig. 5C), a marker of persistent double-strand DNA breaks.

The 3-kb upstream regions of the genes encoding for A1 (*Bcl2a1*, isoforms b and d) and Bcl2 (*Bcl2*) contain consensus NFAT5 binding sites (ref. 17; 5'-TGGAAA-3'). Chromatin

Fig. 3. NFAT5 has different effects on thymocyte proliferation and survival. (A) Relative numbers of BrdU-positive thymocyte populations 5 h after BrdU injection in WT and *Nfat5*^{fl/fl}, Lck-Cre mice (mean $\pm$ SEM); $n = 4$. (B) Percentage of annexin V-positive cells in thymocytes freshly isolated from WT and *Nfat5*^{fl/fl}, Lck-Cre mice (mean $\pm$ SEM); $n > 8$. Histograms in bottom panels show one representative experiment. (C) Number of thymocytes in each subset in WT and *Nfat5*^{fl/fl}, Lck-Cre mice 48 h after anti-CD3 injection (mean $\pm$ SEM); $n \geq 6$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

immunoprecipitation assays showed specific association of NFAT5 with the regulatory regions of *Bcl2a1* and *Bcl2* (−1120) in thymocytes, above background levels observed for a non-NFAT5 target region proximal to the *Bcl2* gene (−2100), in NFAT5-deficient thymocytes, or with the preimmune serum (Fig. 5D).

We then analyzed whether overexpression of Bcl2 or A1, or deletion of p53, could rescue the survival defect of NFAT5-deficient thymocytes. Bcl2 had an overall protective effect in NFAT5-deficient thymocytes, as it reduced the proportion of annexin V-positive cells throughout different stages of maturation (Fig. 5E) and rescued the numbers of SP CD4 and CD8 cells (SI Appendix, Fig. S10A). Bcl2 overexpression also reduced the number of DP cells in WT thymi, attenuating the cellularity difference between WT and NFAT5-deficient DP thymocytes (SI Appendix, Fig. S10A). Overexpression of A1 had a mild effect on the survival of NFAT5-deficient thymocytes, which was more noticeable as cells progressed through development (Fig. 5F) but did not rescue their hypocellularity (SI Appendix, Fig. S10B). Finally, lack of p53 in NFAT5-deficient thymocytes only caused a subtle decrease in the percentage of annexin V-positive DN cells (Fig. 5G and SI Appendix, Fig. S10C). These findings support the idea that NFAT5-induced up-regulation of Bcl2 could be relevant in the prosurvival function of the pre-TCR. Altogether, our work shows that NFAT5 is induced by the pre-TCR in an IKK β -regulated manner and controls a survival checkpoint during thymocyte development by balancing the expression of

specific pro- and antiapoptotic effectors that act downstream from the pre-TCR.

Discussion

This work indicates that NFAT5 acts as an IKK β -regulated survival factor downstream of the pre-TCR to facilitate the transition between DN and DP thymocytes. NFAT5-deficient thymocytes accumulated at the late DN3 stage, which marks the activation of the pre-TCR, had increased apoptosis and defective β -chain allelic exclusion. NFAT5 regulated the balance between specific antiapoptotic Bcl2 family members and the p53/Noxa axis in thymocyte differentiation. Our findings show that NFAT5 has specific functions during T-cell development independent of its role as an osmoprotective transcription factor, and place it as a regulator of distinct pre-TCR-induced outcomes relevant for thymocyte survival.

Although the participation of NF- κ B and NFATc transcription factors downstream from the pre-TCR is well recognized (8, 40, 41), knowledge of their targets in this process and their specialization in particular pre-TCR functions is still quite limited. In this regard, the prosurvival function of NFAT5 more resembles the role of the conventional NF- κ B pathway than that of the NFATc pathway (9–16). Moreover, the finding that NFAT5 expression downstream of the pre-TCR is under the control of IKK β suggests that part of the prosurvival function of the NF- κ B pathway at this point could be channelled via NFAT5.

NFAT5 may promote thymocyte viability, at least in part, by inducing the expression of A1 and Bcl2, as well as by reducing the p53/Noxa axis. Expression of A1 and repression of p53 are well recognized to control cell survival downstream from the pre-TCR (42–45). However, in addition to the role of NF- κ B on A1 expression (45), not much was known about other regulators of A1 and p53 downstream from the pre-TCR (8). In this regard, our work identifies NFAT5 as a factor that controls A1, Bcl2, and p53 at this checkpoint of thymocyte development. Although overexpression of Bcl2 reduced the proportion of apoptotic NFAT5-deficient thymocytes, defects of NFAT5-deficient thymocytes were not rescued by overexpressing A1 or deleting p53. This could suggest that A1 and p53 do not play major roles downstream from NFAT5 or that changes in both need to occur in a coordinated manner, or in combination with other factors, to rescue NFAT5-deficient thymocytes. This possibility seems consistent with the notion that defects in NFAT5-deficient thymocytes could result from the cumulative effect of moderate changes in various genes.

Defective pre-TCR signals leading to inefficient allelic exclusion could facilitate persistent TCR β rearrangements and, therefore, increase p53 activation and cause cell death of NFAT5-deficient thymocytes. However, as no functional connection has been established between A1 expression and β -chain allelic exclusion, it is likely that NFAT5 might modulate more than one pre-TCR-induced function. In contrast, NFAT5-deficient thymocytes did not show signs of impaired proliferation or differentiation, indicating that only certain functions of the pre-TCR take place in an NFAT5-regulated manner. Therefore, the specific participation of NFAT5 downstream from the pre-TCR also lends support to the notion that this receptor controls distinct outcomes that are, for the most part, functionally independent.

Materials and Methods

Mouse Models. *Nfat5*^{fl/fl}, CD4-Cre mice were previously described (22). *Nfat5*^{fl/fl}, Lck-Cre and littermate *Nfat5*^{wt/wt}, Lck-Cre or *Nfat5*^{fl/fl} control mice (WT) were obtained after consecutive crosses of *Nfat5*^{fl/fl} mice with mice expressing Cre under the control of the mouse Lck proximal promoter (46) (Jackson Laboratory). p53-deficient mice were from the Jackson Laboratory (Trp53^{tm1Tyv}). Marilyn and Rag2^{−/−} mice were provided by Olivier Lantz (Institut Curie, Paris) (35). Transgenic mice expressing Bcl2 (47) or A1 (48) were provided by Ramón Merino (Universidad de Cantabria, Santander, Spain). IKK β knockout mice in thymocytes

Fig. 4. NFAT5 expression is up-regulated by the pre-TCR and IKK β . (A) Western blot for NFAT5 in thymocyte subpopulations (DN, DP, CD4, CD8) from WT and *Nfat5*^{fl/fl}, Lck-Cre mice (Upper), and WT and *Nfat5*^{fl/fl}, CD4-Cre mice (Lower). n.s., nonspecific band. (B) *Nfat5* mRNA levels in thymocyte subpopulations from WT and *Nfat5*^{fl/fl}, Lck-Cre mice (Left) or *Nfat5*^{fl/fl}, CD4-Cre mice (Right). Graphs show the mean $\pm$ SEM after normalization to L32 mRNA; $n \geq 4$, *** $P < 0.001$. (C) NFAT5 expression in cultured T- and B-cell blasts from WT and *Nfat5*^{fl/fl}, Lck-Cre mice. (D) Western blot of NFAT5 in thymocytes from *Rag2*^{-/-} mice in basal conditions (0) and 6, 18, 48, or 72 h after anti-CD3 injection. (E) Surface expression of CD25 versus CD4 plus CD8 in thymocytes from *Rag2*^{-/-} mice treated as in D. Western blot of NFAT5 in isolated DN cells (F) or total thymocytes (G) from IKK β -deficient (*Ikbbp*^{fl/fl}, Mx-Cre, IKK β ^{-/-}) or control (*Ikbbp*^{wt/wt}, IKK β ^{+/+}, *Ikbbp*^{wt/fl}, Mx-Cre, IKK β ^{+/+}) mice. Pyruvate kinase was used as loading control, and one (A, D, E, and G) or two (F) representative experiments are shown of three independently performed.

were generated by crossing *Ikbbp*^{fl/fl} mice with Mx-Cre mice (49, 50) (from Marc Schmidt-Suppran, Max Planck Institute of Biochemistry, Munich). *Ikbbp* deletion was induced by injecting mice with double-strand poly(I):poly(C) (Amersham Biosciences). NFAT5-deficient mice (21) were used as a source of thymocytes for chromatin immunoprecipitation. Mice were maintained on

a pure C57BL/6 background in specific pathogen-free conditions and handled according to guidelines by the institutional ethical committee (Barcelona Biomedical Research Park Animal Care and Use Committee). Injection of mice with anti-CD3 antibody or Bromo-2'-deoxyuridine (BrdU) are described in SI Appendix, SI Materials and Methods.

Fig. 5. Altered expression of anti- and proapoptotic genes in *Nfat5*^{fl/fl}, Lck-Cre thymocytes. (A) mRNA level of antiapoptotic (*Bcl2l1*, *Bcl2*, *Bcl2l1b/d*) and proapoptotic (*Bcl2l11*, *Pmaip1*, *Bbc3*) Bcl2 family genes in WT and *Nfat5*^{fl/fl}, Lck-Cre DP thymocytes. Values normalized to each L32 mRNA are represented relative to WT cells (value of 1) (mean $\pm$ SEM); $n \geq 3$, *** $P < 0.001$. (B) Western blot of p53 and Bcl2 in DP thymocytes of WT and *Nfat5*^{fl/fl}, Lck-Cre mice. Pyruvate kinase was used as loading control. One representative experiment is shown of three independently performed. (C) Percentage of phospho-p53-positive (Ser15; Left) and γ H2AX-positive (Right) thymocytes in WT and *Nfat5*^{fl/fl}, Lck-Cre mice. Data are representative of three independent experiments. (D) Chromatin immunoprecipitation analysis of NFAT5 binding to *Bcl2* and *Bcl2l1* regulatory regions in total thymocytes. Values show the enrichment in chromatin immunoprecipitated by the NFAT5-specific antibodies in the indicated genomic regions relative to the preimmune serum (value of 1) after correction for the input signal and are shown as mean $\pm$ SEM ($n = 3$, * $P < 0.05$). (E–G) Percentage of annexin V-positive thymocyte subsets in control and *Nfat5*^{fl/fl}, Lck-Cre mice overexpressing Bcl2 (E) or A1 (F), or lacking p53 (G). Data are the mean $\pm$ SEM from three or more independent experiments including four or more individual mice of each genotype (* $P < 0.05$; ** $P < 0.01$).

OP9 and OP9-DL1 Cocultures. OP9-DL1 cells (26) were used to differentiate thymocyte precursors (CD4⁺ CD8⁺ CD25⁺) in the presence of 10 ng/mL recombinant murine IL-7 (ImmunoTools) and 5 ng/mL recombinant human Flt3-ligand (ImmunoTools).

Isolation of Thymocyte Subpopulations and Analysis of Cell Death. Procedures for magnetic or flow cytometry isolation of thymocyte subsets and detection of apoptotic cells are described in *SI Appendix, SI Materials and Methods*.

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