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Involvement of NFAT1 in B Cell Self-Tolerance

Robert A. Barrington,* Madhuri Borde,* Anjana Rao,* and Michael C. Carroll2*†

B cells from anti-lysozyme Ig/soluble lysozyme double-transgenic mice are chronically exposed to self-Ag in the periphery, resulting in an anergic phenotype. Chronic exposure to self-Ag leads to nuclear translocation of NFAT1 and NFAT2, suggesting that they are involved in anergy. To directly test a role for NFAT1 in B cell anergy, NFAT1-deficient mice were crossed with anti-lysozyme Ig transgenic mice. As expected, B cell anergy was evident in the presence of self-Ag based on reduced serum anti-lysozyme levels, percentage and number of mature B cells, and reduced B cell responsiveness. By contrast, B cell anergy was relieved in NFAT1−/− mice expressing soluble self-Ag. Bone marrow development was equivalent in NFAT1-sufficient and -deficient mice, suggesting that loss of anergy in the latter is due to selection later in development. Taken together, these studies provide direct evidence that the transcription factor NFAT1 is involved in B cell anergy. The Journal of Immunology, 2006, 177: 1510–1515.

Signaling through lymphocyte Ag receptors initiates either proliferation and differentiation, or functional unresponsiveness. The former is required for defense against foreign Ags, whereas the latter, termed anergy, is one mechanism mediating self-tolerance. Autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis are characterized by breaks in B cell self-tolerance, as evidenced by autoantibody production that contributes to clinical symptoms (1).

The MD4/soluble hen egg lysozyme (sHEL) transgenic model system, in which B cells express the Ig receptor specific for HEL in mice expressing sHEL, has enabled several important factors influencing B cell anergy induction and maintenance to be identified (2–5). Several mechanisms may account for the balance between B cell tolerance and activation (6): 1) the strength of cytoplasmic signaling may differ between stimulated anergic and naive B cells (signal quantity); 2) different signaling pathways may be activated (signal quality); and/or 3) changes in transcription factor expression or chromatin structure (nuclear quality). Tolerant B cells from double transgenic (dTg; MD4 × sHEL) mice have chronic calcium oscillations and nuclear accumulation of NFAT1, NFAT2, as well as activation of the calcium-independent ERK pathway (6–8), indicating differential activation of particular signaling pathways in anergic and naive B cells. Although NFAT is basally activated in anergic B cells, a direct role for NFAT in inducing and maintaining B cell anergy has yet to be described.

The transcription factor NFAT1 is regulated by calcium signaling via the calcium-dependent phosphatase calcineurin (9–11). Calcium activates calcineurin, which subsequently dephosphorylates NFAT1, exposing its nuclear localization sequence and allowing nuclear entry. Low levels of sustained calcium flux, such as those observed in chronically stimulated anergic B cells (8), are sufficient to induce its nuclear translocation. NFAT proteins control expression of several immunoregulatory genes, including IL-4 (12), TNF-α (13), CD154 (14), and CD95L (15). The regulatory role of NFAT1 in many cases is coupled to AP-1 (16), leading to a model whereby temporal tissue-specific transcriptional regulation is achieved by controlling both calcium and protein kinase C pathways (17). Gene disruption studies suggest that NFAT1 has a repressive effect on B cell proliferation and differentiation. Although NFAT1−/− mice exhibit modest splenomegaly as well as B and T cell hyperproliferation (15, 18, 19), NFAT1/NFAT2 double knockout (DKO) mice generated by NFAT1−/−/NFAT2−/− mice have a more profound B cell phenotype (20). DKO mice have plasma cell infiltrates of lung, kidney, and liver and significantly increased levels of IgG1 and IgE serum Ab, the latter of which occurs even upon administration of anti-IL-4 Ab in vivo (20). In vitro, B cells from DKO mice spontaneously secrete Ab in addition to producing exaggerated Ab titers upon stimulation with IL-4, anti-IgM, and anti-CD40 (20). Therefore, the lack of NFAT1 and NFAT2 has an intrinsic effect on B cells.

NFAT1 has an important role in regulating T cell anergy, as NFAT1-deficient T cells are unable to be anergized following in vitro stimulation in the absence of proper costimulation, i.e., with anti-CD3 or ionomycin (16). In B cells, NFAT2 is present in nuclear extracts of unstimulated anergic B cells by Western blot (8). Further, EMSAs showed that the relative nuclear activity of NFAT1 and NFAT2 was increased in B cells from dTg mice relative to B cells from single transgenic (sTg) mice (21). Additional stimulation of B cells from dTg mice did not cause a substantial increase in NFAT1 and NFAT2 translocation. To address whether NFAT1 regulates anergy in B cells, MD4 mice were bred onto the NFAT1−/− background and B cell tolerance was examined in vivo following reconstitution of irradiated wild-type (WT) and sHEL transgenic mice with bone marrow (BM) from MD4 or NFAT1−/−/MD4 mice. B cells from NFAT1-deficient dTg chimeric mice were more responsive compared with B cells from NFAT1-sufficient dTg BM chimeric mice in vivo, as the loss of NFAT1 led to an increased autoantibody production and an increase in mature MD4 B cells. These results demonstrate that the transcription factor...
NFAT1 has a role in regulating B cell tolerance. Further, the involvement of NFAT1 in regulating B cell tolerance suggests that B cell anergy is dependent on calcium signaling pathway(s) and on active gene expression.

Materials and Methods
Mice and BM chimeras
Mice were housed at Harvard Medical School in a specific pathogen-free facility. Animal protocols used were reviewed and approved by the Animal Care and Use Committees at the CBR Institute for Biomedical Research and at Harvard Medical School. HEL Ig transgenic (MD4) mice were maintained on a C57BL/6 background, either with or without (NFAT1−/−) an intact NFAT1 locus. Mice secreting HEL (sHEL; ML5 strain) were maintained on a C57BL/6 background and were used as recipients with C57BL/6 mice. The serum (HEL) in ML5 mice is ~17 ng/ml, or an amount sufficient to occupy ~45% of HEL-specific BCRs (22). BM chimeric mice were generated also as described (23). Chimeric mice were maintained on acidified water in filtered cages for 6 wk to prevent opportunistic infections.

Flow cytometric analysis
Splenic mononuclear cells (MNCs) were isolated by density gradient centrifugation using Lympholyte M (Cedarlane Laboratories). To detect MD4 B cells, MNCs were stained with HEL-Cy5, and counterstained with FITC-conjugated anti-IgDε (BD Pharmingen), PerCP-conjugated anti-B220 (BD Pharmingen), and PE-conjugated anti-IgMa or anti-CD23 (BD Pharmingen). The allotype of MD4 BCR is IgMa/IgDa, while B cells from C57BL/6 mice are IgMa/IgDa. An additional allotypic marker, CD45.1, was also used to differentiate donor cells from endogenous cells. Transferred cells were identified as being HEL IgMaε or, alternatively IgDaε. The absolute number of cells was determined by multiplying the frequency by the cell count.

ELISA
Levels of serum IgMaε-anti-HEL were determined by ELISA. Ninety-six-well plates (Immulon 1B) were coated with 5 μg/well HEL overnight at 4°C (2). Plates were blocked by addition of 5% dry milk (Carnation) in PBS (blotto). HEL-specific IgMaε serum Ab in serially diluted samples was detected by addition of biotinylated anti-IgMaε, followed by streptavidin-conjugated alkaline phosphatase (Caltag Laboratories). PBS containing 0.1% Tween 20 was used in wash steps. Color was developed using p-nitrophenyl phosphate (Sigma Aldrich) as substrate and absorbance was measured at 405 nm using Softmax software package. Ab concentration was determined by extrapolation from standard curves generated from purified HyHEL-5 Ab (24). Samples were run in duplicate, and assays were repeated.

Bm12 adoptive transfer protocol
Preparation of splenocytes from bml2 and MD4 mice was performed as described (25). A total of 1 × 107 B cells purified from BM chimeric mice were transferred i.v. with 5 × 107 splenocytes from bml2 mice into lethally irradiated (650 rad) C57BL/6 and ML5 recipient mice. Frequency and number of MD4 B cells and Ab-secreting cells (ASCs) in recipient spleen were determined by FACS and ELISPOT assay 5 days after cell transfer.

ELISA for HEL-specific ASCs
Frequencies of HEL-specific ASCs were quantitated as previously described (25). In brief, 24-well polystyrene plates (Costar) were coated with 15 μg/well. After extensive washing, plates were blocked with 1% BSA in PBS for 2 h. Serially diluted splenic MNCs (106 to 102 cells/well) were added in DMEM medium with 2% FBS and incubated overnight at 37°C. Each dilution of cells was assayed at least in duplicate. Plates were washed with PBS containing 0.1% Tween 20. MD4-derived ASCs were detected with biotinylated anti-IgMaε followed by streptavidin-conjugated alkaline phosphatase. Plates were developed using 5-bromo-4-chloro-3-indolyl phosphate, and spots were counted using microscopy to determine ASC frequency. As controls, each sample was also plated in wells coated with BSA. No IgMaε BSA-specific ASCs were detected.

Results
To determine whether NFAT1 is important for B cell tolerance, the well-established HEL Ig (MD4)/sHEL dTg system was used (22). Because NFAT1 is widely expressed (10), a BM chimeric approach was taken to restrict the NFAT1 deficiency to hemopoietic cells. Chimeric WT and sHEL transgenic mice were created by reconstitution with BM from NFAT1-sufficient or -deficient MD4 mice. For simplicity, the resultant chimeric mice are referred to as sTg (MD4 BM into WT recipients) and dTg (MD4 BM into sHEL recipients). B cell maturation was assessed in BM, spleen, and lymph nodes. In addition, functional activity of MD4 B cells in the periphery (spleen and lymph nodes) was examined by measuring serum HEL-specific IgMaε levels and through activation assays.

Autoantibodies in BM chimeric mice
Previous studies using the lysozyme dTg model demonstrated an inverse correlation between production of HEL-specific IgMaε Ig and the amount of HEL present in the serum (22). Consistent with these previous findings, analysis of serum in sTg mice showed that MD4 B cells produced a mean level (±SD) of 580 ± 300 μg/ml HEL specific IgMaε in the absence of self Ag whereas the mean serum titer from dTg mice was 2.0 ± 2.0 μg/ml (p < 8.6 × 10−6) (Fig. 1). Therefore, HEL-specific B cells developing in the presence of specific Ag produce ~300-fold less Ab compared with their maturation in the absence of self-Ag.

Mean HEL-specific serum IgMaε titers were elevated in the absence of NFAT1. For sTg mice, the lack of NFAT1 resulted in an ~1.5-fold increase in Ab production. In dTg mice, more significant elevation in Ab titers was observed in the absence of NFAT1 (70 ± 122 vs 2 ± 2 μg/ml, p < 0.04) (Fig. 1). Therefore, the loss of NFAT1 permits increased production and/or secretion of autoantibodies.

B cell development/maturation in BM, spleen, and lymph nodes
To determine whether increased serum autoantibody in NFAT1−/− dTg mice may result from abnormal B cell maturation, B cell development and homeostasis was assessed in BM, spleen, and lymph nodes. As reported previously (4), developing B cells in the BM were apparently unaffected by the presence of soluble self Ag. Further, the absence of NFAT1 did not appear to alter B cell development in the BM (data not shown).

The frequency and number of splenic B cells in NFAT1-sufficient sTg and dTg mice were equivalent (Table I, 118.6 ± 6.0 vs 30.5 ± 7.3% B220+; 15.4 ± 5.1 × 106 vs 17.6 ± 3.2 × 106 B220+ cells), presumably due to the continuous influx of cells from the BM. Cell surface expression of IgDaε was used to limit active gene expression.

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Table I. Frequency and number of MD4 B cells in spleens of bone marrow chimeric mice

<table>
<thead>
<tr>
<th>BM Chimeric Mice</th>
<th>% B220&lt;sup&gt;+&lt;/sup&gt;</th>
<th>% IgD&lt;sup&gt;+&lt;/sup&gt; (B220 gated)</th>
<th>% CD23 (B220 gated)</th>
<th>IgM&lt;sup&gt;a&lt;/sup&gt; MFI</th>
<th>No. B220&lt;sup&gt;+&lt;/sup&gt; (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>No. IgD&lt;sup&gt;+&lt;/sup&gt; (B220, IgD&lt;sup&gt;a&lt;/sup&gt; gated)</th>
<th>No. CD23&lt;sup&gt;+&lt;/sup&gt; (B220, IgD&lt;sup&gt;a&lt;/sup&gt; gated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTg (n = 16)</td>
<td>28.6 ± 6.0</td>
<td>86.5 ± 6.6</td>
<td>90.0 ± 5.3</td>
<td>1351 ± 300</td>
<td>15.4 ± 5.1</td>
<td>16.3 ± 2.1</td>
<td>11.4 ± 4.3</td>
</tr>
<tr>
<td>dTg (n = 18)</td>
<td>30.5 ± 7.3</td>
<td>89.3 ± 9.1</td>
<td>80.2 ± 10.2</td>
<td>156 ± 80</td>
<td>17.6 ± 3.2</td>
<td>16.6 ± 3.3</td>
<td>10.2 ± 5.3</td>
</tr>
<tr>
<td>NFAT1&lt;sup&gt;−/−&lt;/sup&gt; sTg (n = 20)</td>
<td>32.2 ± 6.2</td>
<td>89.6 ± 3.6</td>
<td>89.6 ± 7.3</td>
<td>1357 ± 335</td>
<td>27.1 ± 11.3</td>
<td>25.6 ± 9.9</td>
<td>26.6 ± 15.1</td>
</tr>
<tr>
<td>NFAT1&lt;sup&gt;−/−&lt;/sup&gt; dTg (n = 21)</td>
<td>40.9 ± 4.9</td>
<td>89.6 ± 7.2</td>
<td>92.7 ± 5.4</td>
<td>211 ± 91</td>
<td>29.7 ± 14.5</td>
<td>29.8 ± 13.2</td>
<td>28.9 ± 10.6</td>
</tr>
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</table>

analysis to cells derived from the donor MD4 BM, as surface IgM levels are consistently reduced in dTg mice. Consistent with data from previous studies (2, 22), there are comparable percentages of IgD<sup>+</sup>-splenic B cells in sTg and dTg mice (86.5 ± 6.6 vs 89.3 ± 9.1% IgD<sup>+</sup>) (Fig. 2, a–d, and Table I). Inclusion of CD23, used to further delineate IgD<sup>+</sup> B cells as mature, demonstrated comparable percentages and numbers of positive cells in sTg and dTg mice (90.0 ± 5.3 vs 80.2 ± 10.2; 11.4 ± 4.3 vs 10.2 ± 5.3) (Table I).

As observed in NFAT1<sup>−/−</sup> mice, NFAT1<sup>−/−</sup>-MD4 chimeras developed mild splenomegaly (15, 18). Therefore, though the frequency of IgD<sup>+</sup>-B cells was equivalent between NFAT1-sufficient and -deficient sTg mice (Table I), the actual number of B220<sup>+</sup> splenocytes, as well as mature CD23<sup>+</sup>-B cells, was increased in NFAT1<sup>−/−</sup>-sTg mice (27.1 ± 11.3 × 10<sup>6</sup> vs 15.4 ± 5.1, p < 0.01; 26.6 ± 15.1 vs 11.4 ± 4.3, p < 0.02). An increase in both frequency and number of B220<sup>+</sup> splenocytes was observed in NFAT1<sup>−/−</sup>-dTg mice, relative to that found NFAT1-sufficient sTg and dTg mice (40.9 ± 4.9% B220<sup>+</sup>, p < 2.8 × 10<sup>−7</sup> and p < 1.8 × 10<sup>−5</sup>; 29.7 ± 14.5 × 10<sup>6</sup> B220<sup>+</sup>, p < 0.01) (Table I). Importantly, the number of mature, splenic CD23<sup>+</sup>-MD4 B cells was elevated in NFAT1<sup>−/−</sup>-dTg relative to dTg mice (28.9 ± 10.6 vs 10.2 ± 5.3, p < 0.02). Comparison of mice lacking NFAT1 in cells of hemopoietic origin showed a similar number of NFAT1<sup>−/−</sup>-MD4 B220<sup>+</sup> cells (27.1 ± 11.3 × 10<sup>6</sup> vs 29.7 ± 14.5 × 10<sup>6</sup>) (Table I). Therefore, the absence of transcription factor NFAT1 led to an accumulation in mature B220<sup>+</sup> cells in the spleen, independent of self-Ag.

IgM down-regulation is a characteristic feature of B cells chronically exposed to Ag, and it correlates with the anergic phenotype of MD4 B cells in sHEL mice (26). As expected, IgM<sup>a</sup> levels were down-regulated on the cell surface of B cells from dTg mice compared with sTg chimeras (Fig. 2e). Interestingly, the mean fluorescent intensities (MFI) of surface IgM<sup>a</sup> were equivalent in both NFAT1-sufficient and -deficient dTg (Table I, Fig. 2e). Taken together, these data suggest that differential down-regulation of surface IgM is not responsible for the break of tolerance in NFAT1-deficient chimeras. FACS analysis using CD23<sup>+</sup> as a marker of mature B cells confirmed that IgM<sup>a</sup> was similarly down-regulated on B cells from NFAT1-sufficient and -deficient dTg mice (data not shown).

Previous studies demonstrated a defect in the mature B cell compartment within lymph nodes of dTg mice (2). Consistent with these observations, the percentage of B220<sup>+</sup> cells was reduced in dTg relative to sTg mice (mean ± SD, 14.5 ± 4.2 vs 21.9 ± 10.3% B220<sup>+</sup>, p < 0.02) (Fig. 3, Table II). Importantly, most of the B cells within lymph nodes were CD23<sup>+</sup>, confirming their mature phenotype. As observed in the spleen, NFAT1<sup>−/−</sup>-sTg mice had an
increased frequency of circulating B220+ cells relative to NFAT-sufficient sTg mice (31.3 ± 12.6 vs 21.9 ± 10.3 × 10^5 in sTg, p < 0.02). NFAT1-deficient dTg mice have an ~2-fold higher frequency of B220+ cells relative to NFAT1-sufficient dTg mice (34.1 ± 10.5 vs 14.5 ± 4.2, p < 8.1 × 10^-3). Therefore, the loss of NFAT1 permits the maturation of self-reactive B cells.

Consistent with the reduced frequency of B220+ lymph node cells, dTg mice had a 3-fold reduction in numbers of peripheral IgD+ B cells compared with sTg chimeras (0.8 ± 0.7 × 10^6 vs 2.9 ± 1.0 × 10^6, p < 3.4 × 10^-5) (Table II). The reduction in numbers of CD23+ peripheral IgD+ B cells between sTg and dTg chimeras was even more pronounced (2.4 ± 0.5 vs 0.3 ± 0.3, p < 7.5 × 10^-9). NFAT1-deficient sTg mice had equal numbers of circulating IgD+ B cells compared with NFAT1-sufficient chimeras (2.8 ± 2.1 × 10^6 vs 2.9 ± 1.0 × 10^6). Unlike in NFAT1-sufficient chimeras, the presence of HEL self-Ag did not affect the IgD+ population, as NFAT1-deficient dTg mice had a 3-fold increase in IgD+ cells compared with NFAT1-deficient sTg mice (2.5 ± 1.7 × 10^6 vs 0.8 ± 0.7 × 10^6, p < 0.002) (Table II). Thus, NFAT1 deficiency not only permits maturation of self-reactive B cells, it also promotes their accumulation in the periphery.

Functional analysis of B cells from MD4 and NFAT1−/− MD4 chimeras

B cell anergy is the inability to differentiate into ASCs upon re-stimulation with Ag. To investigate the responsiveness of self-reactive B cells in an in vivo context, splenic B220+ cells from the BM chimeras were purified and transferred along with alloreactive bm12 splenocytes into B6 or sHEL-irradiated recipients (27). Due to minor differences in I-A^b MHC molecules, T cells from bm12 mice are activated upon interaction with class II I-A^b on B6 B cells, thereby providing B cells with T cell-dependent signals necessary to differentiate (25). Five days after cell transfer, spleens of secondary chimeras were isolated and assayed by ELISPOT for HEL-specific ASCs (Fig. 4). As expected, sHEL mice receiving self-reactive B cells from dTg mice failed to produce significant numbers of ASCs compared with B cells from sTg mice (419 ± 141 vs 9299 ± 2554, p < 0.01). In contrast, self-reactive B cells from NFAT1-deficient dTg mice produced elevated numbers of HEL-specific ASCs relative to NFAT1-sufficient controls, albeit less than in the absence of soluble self-Ag (2985 vs 419 ASCs/10^5 splenocytes, p < 7.4 × 10^-5). Accordingly, despite the similar reduction in surface IgM+ levels on splenic B cells from NFAT1-sufficient and -deficient dTg, the NFAT1−/− B cells were more responsive.

**Discussion**

Earlier studies have suggested an intrinsic role for NFAT in B cell regulation. To examine directly whether NFAT1 is involved in regulation of self-reactive B cells, we used the well-established MD4/sHEL model. We found that NFAT1 has a role in regulating B cell tolerance based on 1) increased levels of serum HEL-specific IgM+ autoantibodies, 2) increased numbers of splenic B cells in the presence of sHEL, 3) increased numbers of mature B220+ IgD+ cells within lymph nodes, and 4) increased responsiveness to Ag restimulation in the presence of allo-T cell help.

Mice expressing self-Ag secreted ~300-fold less IgM+ than no-Ag controls, as expected (Fig. 2). By comparison, Ab levels in NFAT1−/− dTg chimeras decreased only 14-fold relative to NFAT1−/− sTg controls. This phenotype may be partly explained by increased numbers of mature B220+ IgD+ cells in the spleen and lymph nodes of NFAT1-deficient relative to NFAT1-sufficient dTg mice (Table I). Notably, the level of surface IgM+ expressed by B cells from NFAT1-sufficient and -deficient dTg mice remained similar (Fig. 3). Previous reports showed that the extent of surface IgM+ down-regulation correlated with serum HEL concentrations (22). Therefore, similar IgM+ down-regulation suggests that the concentration of sHEL is saturating and that receptor proximal signaling events are comparable in both sets of chimeras.

Anergic B cells in dTg mice have a short half-life leading to a reduction in peripheral B cells (28, 29). The increased frequency and number of mature (CD23+) B220+ IgD+ cells in the periphery of NFAT1-deficient dTg mice (Tables I, II) relative to dTg mice, suggests the NFAT1−/− B cells have an accelerated rate of maturation, are aberrantly positively selected, and/or have a defect in cell death in the presence of self Ag. No increase in immature B cells in BM of NFAT1−/− dTg mice was observed, compared with other chimeras. Therefore, it appears that the mechanism(s) that contribute to the enhanced selection of circulating B220+ IgD+ cells in NFAT1−/− dTg mice occurs during or after maturation in the spleen. Consistent with this hypothesis, we observed an increase in the number of splenic IgD+ B cells in NFAT1−/− dTg chimeras compared with dTg chimeras (Table I). The transition of immature to mature B cells in the spleen is a major checkpoint for B cell tolerance (30–35), and therefore may be involved in the current model. Preliminary phenotypic analysis of sTg and dTg chimeras suggests that the T1/2 to T3 transition is also affected in the MD4 model, but clearly defining the checkpoint in this model is difficult because nearly all B cells express the
MD4 transgene. Interestingly, preliminary results suggest that Baff receptor signalings are increased in splenic B cells from NFAT1-deficient chimeras (relative to MD4 chimeras), consistent with a role for NFAT1 in the T1/2 to T3 transition (36).

Despite the fact that B cells from NFAT1-sufficient and -deficient dTg mice have equivalent IgM+ down-regulation (Fig. 2e), the latter produced 7-fold more Ab upon restimulation with allo-T cell help and Ag (Fig. 4). Though B cells from the two groups are not phenotypically distinguishable, the absence of NFAT1 alters responsiveness. Moreover, they are more responsive in vitro following stimulation with HEL and either bm12 splenocytes, anti-CD40, or IL-4 (our unpublished observations), further supporting that NFAT1 can play a negative role in B cell activation.

In our chimeric model, NFAT1 deficiency is limited to BM-derived cells. Because T cells are also affected by the deficiency, this raises the question whether the defect in B cell tolerance is intrinsic. The adoptive transfer experiment demonstrated that splenic B cells have partially escaped tolerance and are capable of secreting autoantibody in the presence of WT T cells. However, it is possible that the loss of anergy in the donor is due to multiple factors that are altered in the NFAT1−/− chimeras. Several immunoregulatory genes such as IL-4 (12), CD95L (15), and CD154 (14) are regulated by NFAT1. IL-4 was shown to block CD95-mediated apoptosis (37), a predominating mechanism limiting the lifespan of MD4 B cells in sHEL mice (5, 38). IL-4 mRNA expression appears unaffected between splenic T cells from NFAT-sufficient and -deficient dTg mice, though we cannot rule out differences in local IL-4 production.

While this manuscript was under review, Winslow et al. (39) reported that specific deletion of calcineurin b1 in B cells did not affect B cell anergy in the same HEL/anti-HEL model system. They observed that B cells from BM chimeras in which sHEL mice were reconstituted with CD19Cre+/− Cnb1f/− MD4 BM were phenotypically anergic (as measured by IgM down-regulation) and did not develop anti-HEL serum autoantibodies. These data were interpreted as showing that the calcineurin/NFAT pathway is not critical for B cell tolerance. Our experiments cannot be compared directly: although we also observed that B cells from NFAT1−/− dTg mice down-regulated their BCR in the presence of self Ag, loss of NFAT1 was associated with a significant increase in the number and percentage of mature B220+IgD+CD23+ B cells in the lymph nodes of NFAT1−/− dTg mice relative to dTg mice. Winslow et al. (39) did not examine the B cell populations in the lymph node, nor did they directly examine the functional responses of B cells from calcineurin-deficient dTg mice. Moreover, Cnb1-deficient B cells are hyperresponsive to stimulation with Ag or anti-IgM, and it is not clear how development of anergy can be evaluated in cells whose responses to Ag receptor cross-linking are intrinsically lower than those of WT cells.

The hyperresponsive phenotype of Cnb1-deficient B cells is in marked contrast to the hyperresponsive phenotype of B cells lacking the two major calcineurin substrates, NFAT1 and NFAT2 (20). In this study, as well as the work presented here, both T and B cells lacked NFAT, whereas in the study of Winslow et al. (39), only B cells lacked calcineurin, the upstream regulator of NFAT. Overall, therefore, Cnb1 deficiency does not lead to the same B cell phenotype as single or double NFAT deficiency, suggesting that calcineurin has functions beyond the regulation of NFAT. One possibility is that hyperactivated NFAT-deficient T cells contribute to the hyperresponsiveness and loss of tolerance of NFAT-deficient B cells observed by us and by Peng et al. (20). Arguing against a role for T cells in our system, HEL is not efficiently presented to T cells by I-Aα MHC (40). Moreover, HEL-specific T cells should be very infrequent because the BM chimeras developed should have a nominal T cell repertoire. Clearly, further studies are needed to reconcile these apparent inconsistencies and elucidate the role of the calcineurin/NFAT pathway in B cell tolerance.

The observation that serum IgM+ HEL-specific Abs are elevated in NFAT1−/− sTg mice (Fig. 1), as well as the observed increase in splenic IgD+ B cells (Table I) compared with sTg mice, is consistent with previous observations linking NFAT1 to B hyperresponsiveness (15, 19). Further, our results demonstrate that, on a transgenic BCR background, the loss of NFAT1 alone is sufficient to cause dysregulated serum Ig production and maturation of B cells. However, the partial loss of B cell anergy in the absence of NFAT1 is not likely explained by slight hyperresponsiveness alone as the increases in serum autoantibodies, and in number and responsiveness of mature self-reactive B cells, is disproportionately increased in the presence of soluble self Ag.

In summary, we report that NFAT1 is important in maintaining B cell anergy. It was proposed that calcium signaling in concert with protein kinase C signaling causes anergy (21). In the absence of NFAT1, the calcium pathway is compromised, leading to the loss of B cell tolerance. The ability of NFAT1 to regulate expression of several genes important in immunoregulation suggests that active transcription or suppression of transcription of these genes is necessary to protect against autoimmunity. Efforts to control NFAT1 activity may therefore prove important in controlling autoimmunity (41, 42).

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Disclosures

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