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B Cell Hyperresponsiveness and Expansion of Mature Follicular B Cells but Not of Marginal Zone B Cells in NFATc2/c3 Double-Deficient Mice¹

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Marginal zone (MZ) B cells and peritoneal B-1 cells provide a first defense system of thymus-independent Ab responses against foreign pathogens and therefore share a number of functional properties. Recently, development of B-1a cells was shown to be controlled by the transcription factor NFATc1. We show here that mice deficient for NFATc2 and c3 display a distinct lower representation of MZ B cells, which is correlated with a reduced capturing of trinitrophenyl-Ficoll. In contrast, mature follicular B cells from NFATc2/c3^{-/-} mice are strongly increased in number. NFATc2/c3^{-/-} B cells exhibit a marked increase in BCR-induced intracellular Ca²⁺ mobilization and proliferation. However, trinitrophenyl-Ficoll-specific IgM and IgG3 responses of NFATc2/c3-deficient mice are intact, and chimeric mice reconstituted with NFATc2/3-deficient B cells show a normal number of MZ B cells and normal BCR responses. These observations suggest that the strongly elevated Th2 cytokine milieu in NFATc2/c3-deficient mice leads to a hyperactivation of mature, follicular B cells, whereas MZ B cells are less responsive to these signals. *The Journal of Immunology*, 2005, 174: 4797–4802.

Nuclear factor of activated T cell proteins represent a family of five transcription factors that share a highly conserved DNA-binding domain (1, 2). Among those, the four genuine NFATc proteins share several conserved phosphorylation motifs within their N-terminal regulatory domain. The Ca²⁺/calmodulin phosphatase calcineurin can bind to these motifs and control NFAT activity (3). In both T and B lymphocytes, an increase in intracellular-free Ca²⁺ and calcineurin activity stimulated by TCR or BCR signals leads to the rapid nuclear translocation and activation of NFATs (1–3).

NFATc1, c2, and c3 are expressed in lymphoid cells where they control the transcription of lymphokine genes, e.g., of *IL-2*, *IL-3*, *IL-4*, *IL-5*, *IFN-γ*, and *TNF-α* genes. In addition, several receptor and ligand genes, e.g., the *CD25*, *CD95*, and *CD154* genes, as well as other genes controlling T cell activation, apoptosis, and cell cycle, are regulated by NFATc proteins (1–3). Compared with the well-studied role of NFAT factors in T lymphocytes, much less is known about the function of NFATs in B lymphocytes, although NFATs have been shown to be expressed in B cells at levels similar to those in T cells (4). One NFAT target gene, which is ex-

pressed specifically in the subset of B-1a cells, is the *CD5* gene whose transcription is controlled by an upstream enhancer. The activity of this enhancer is controlled by the binding of NFAT factors to two sites, which are highly conserved between mouse and human (5–7). Subsequent studies showed that NFATc1 is more strongly expressed in B-1a cells than in B-2 cells and is essential for the generation of peritoneal and splenic B1-a cells (8).

B-1 cells represent a special subset of B lymphocytes, which are abundant in peritoneal and pleural cavities. During fetal development, B-1 cells are the main B cell population. It had been assumed that B-1 cells are derived exclusively from fetal precursor cells, whereas B-2 cells were thought to be generated from adult bone marrow (BM)⁵ cells only (9). However, experimental data published recently indicate that splenic B cells can also be precursors for B-1 cells (10). The BCR repertoire, the receptor density, and the type of Ag contact appear to drive B-1 cell development (11). B-1 cells share a number of properties with marginal zone (MZ) B cells, which reside in the region surrounding the marginal sinuses of splenic lymphoid follicles and therefore have immediate contact with blood-borne Ags (12). Both B-1 and MZ B cells have a restricted BCR repertoire, produce Abs in response to thymus-independent type 2 (TI-2) Ags, and neither of both proliferate in response to BCR cross-linking (11–13).

Studies using transgenic (tg) and gene-deficient animals have shown that B cell fate depends on the type and strength of the BCR signal. Disruption of BCR signaling affects maturation of splenic B cells and the peritoneal B1 population (reviewed in Ref. 14). In contrast, the MZ B cell population appears to be less sensitive to impaired BCR signaling. Rather, a reduction in MZ B cells is found in some gene-deficient mice, which show increased BCR signaling (15, 16). Other processes such as chemokine receptor triggering and expression of adhesion molecules also affect the MZ population (12, 17). These properties indicate that B-1 and MZ

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⁵ Abbreviations used in this paper: BM, bone marrow; MZ, marginal zone; TI-2, thymus-independent type 2; TNP, trinitrophenyl; tg, transgenic; T1, transitional type 1; T2, transitional type 2.

cells, although sharing a number of common functional properties, are individual sets of B lymphocytes, which are regulated by specific signals.

The identification of NFATc1 as an important regulator of development of murine B-1a cells (8) prompted us to investigate whether its relatives, NFATc2 and c3, which are also expressed in peripheral B lymphocytes, might have similar important functions on B cell development. We show here that in mice double-deficient for NFATc2/c3, MZ B cells are underrepresented, whereas mature follicular B cells are strongly increased in numbers. Upon BCR stimulation, B cells from NFATc2/c3^{-/-} mice show a distinct increase in Ca²⁺ mobilization and proliferation. However, chimeric mice reconstituted with NFATc2/c3^{-/-} B cells show a normal number of MZ B cells and a normal B cell responsiveness. These data suggest a differential response of MZ and follicular B cells to the elevated Th2 cytokine levels found in NFATc2/c3-deficient mice (18).

Materials and Methods

Mice

NFATc2/c3 double-deficient mice (BALB/c background) (18) or control mice (either wild-type or NFATc2/c3^{+/-} mice, always littermates or matched age from similar matings) of 7–10 wk of age were used in all experiments.

Flow cytometry

Single-cell suspensions of BM cells or splenocytes (after isotonic erythrocyte lysis) or directly isolated peritoneal lavage cells were incubated for 30 min at 4°C with different combinations of the following Abs: anti-B220-biotin, anti-B220-FITC, anti-IgD-FITC, anti-CD21-FITC, anti-IgM-FITC, anti-IgM-PE, anti-CD23-PE, and anti-CD5-PE (all from BD Pharmingen). To block the FcγRII receptor, stainings were performed in PBS containing 0.1% BSA (Roche Diagnostic Systems), 0.1% Na-azide, and saturating concentration of anti-CD16/CD32 (BD Pharmingen). Biotin-labeled Ab was revealed by streptavidin-cychrome (BD Pharmingen) in a second staining step. Cell surface marker expression was analyzed using a four-color flow cytometer (FACSCalibur) and CellQuest software (BD Biosciences).

Histology

Spleen sections were stained with the same Abs as published previously (16). For trinitrophenyl (TNP)-Ficoll-capturing assays, 9-wk-old control or NFATc2/c3^{-/-} mice were injected with 100 μg of TNP-Ficoll (Biosearch

Technologies) in 500 μl of PBS i.v. Mice were sacrificed 30 min later, and spleens were isolated.

Ca²⁺ measurements

Splenocytes were loaded with indo-1 as previously described (19) and stained extracellularly with anti-Mac1-FITC and anti-CD5-PE (BD Pharmingen). Ca²⁺ movement was measured upon stimulation of splenocytes with B7.6 anti-IgM Ab at 37°C. Increases in intracellular-free Ca²⁺ of B cells were measured in real time using FACSVantage equipment (BD Biosciences) by gating on Mac1- and CD5-negative B cells. The recorded files were transferred to FlowJo software, and the median of each sample was calculated and analyzed in overlays.

Proliferation assays

Splenocytes from animals indicated were T cell depleted by pretreating the cells with anti-CD4 and anti-CD8 mAbs (IgM isotypes) on ice followed by incubation with baby rabbit complement (Cedarlane Laboratories) for 45 min at 37°C afterward. For stimulation, 1 × 10⁵ B cells in RPMI 1640 medium containing 5% FCS were stimulated for 48 h with a different concentration of LPS (Calbiochem), anti-IgM F(ab')₂ (The Jackson Laboratory), B7-6 (monoclonal anti-IgM, our hybridoma collection), anti-CD40 (BD Pharmingen), or IL-4 (200 U/ml). Proliferation was measured by [³H]thymidine incorporation (1 μCi/well) during the last 8 h of culture.

TNP-Ficoll immunization and ELISA

Mice of 8–10 wk of age were immunized with 10 μg of TNP-Ficoll (Biosearch Technologies) and bled on days 0, 5, and 7. The TNP-specific Abs in sera were determined by ELISA with TNP-BSA-coated polysorb plates as described previously (20).

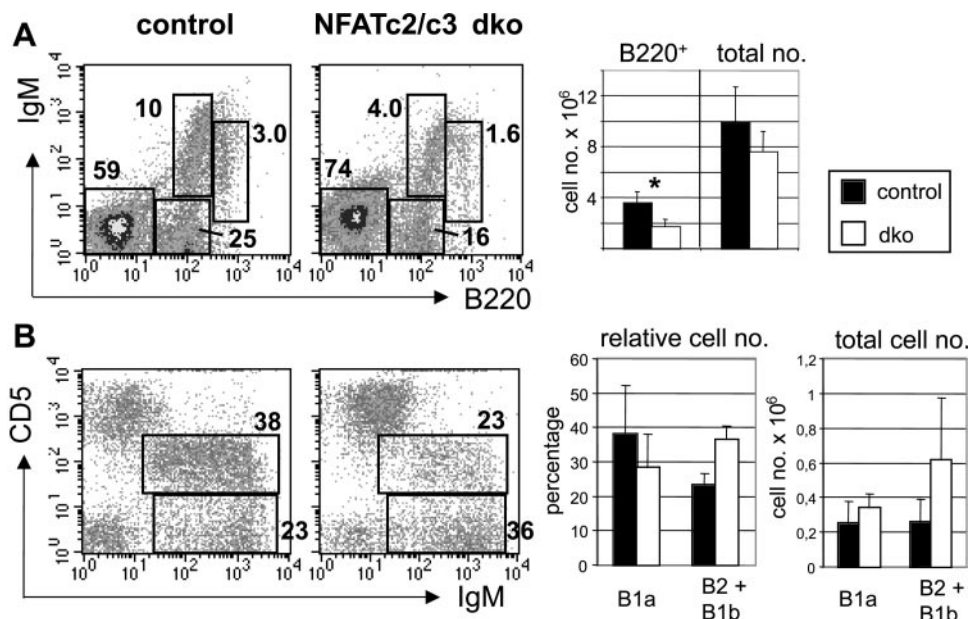
Adoptive transfer experiments

For BM reconstitution experiments, μMT recipient mice (on BALB/c background) (21) were irradiated sublethally twice with 3.5 Gy (with a 2-h interval between the two irradiations). After a rest for 24 h, 2 × 10⁶ BM cells of NFATc2/c3^{-/-} × DO11.10 tg and DO11.10 tg control donor mice (both on BALB/c background) were injected into each recipient i.v. (four mice per group). At day 32 after repopulation, the animals were sacrificed, and spleen cells were prepared for FACS, histology, and proliferation assays. The reconstitution of the B cell compartment was analyzed from blood cells with anti-IgM and anti-B220 Abs, and the contribution of transferred tg T cells to the recipient T cell compartment was determined using the Id-specific anti TCR KJ1–26 (22).

Results

To investigate whether NFATc2 and NFATc3 play a role in B cell differentiation, B cells from NFATc2/c3 double-deficient mice were analyzed by flow cytometry. In the BM, B cell development

FIGURE 1. NFATc2/3 double deficiency does not affect composition of B cell populations in BM and peritoneal cavity. **A**, Stainings of BM cells from control mice and NFATc2/c3 double-deficient (dko) mice with anti-IgM and anti-B220 mAb (total life cells) are shown on the left. On the right, total BM cell numbers and numbers of B220⁺ cells are presented. There is a reduction in B220⁺ cells and an increase in number of non-B lineage cells in BM. **B**, Peritoneal lavage cells are stained with anti-IgM and anti-CD5 Abs. No significant change is found in relative numbers of B-1a B cells (B1a) or conventional B-2 B cells (boxed together with B-1b B cells (B1b)). Significance was determined by Student's *t* test with four mice per group. *, *p* < 0.05.



appeared to be normal, i.e., pro-/pre-B cells and immature B cells, and mature B cells were found in normal relative proportions (Fig. 1A). However, because of the massive expansion of granulocytes and eosinophils in NFATc2/c3 double-deficient mice (18), a decrease was detected in the total number of B lineage cells (B220⁺) in BM (Fig. 1A). Additional stainings of CD43, BP-1, CD23, and IgD markers did not reveal any additional changes in BM B cell development (data not shown). Similarly, the population of B-1 B cells from peritoneal cavity, which is strongly reduced in NFATc1-deficient mice (8), appeared to be normal in NFATc2/c3 double-deficient mice. In FACS analyses, a slight but not significant relative decrease in B-1a cells (identified as CD5^{low}IgM⁺) from NFATc2/c3^{-/-} mice was detected, when compared with control mice, whereas the population of CD5⁻IgM⁺ B cells (mainly conventional B2 but also containing the minor population of B-1b cells) was slightly increased in relative numbers (Fig. 1B).

NFATc2/c3 double-deficient mice develop a massive splenomegaly (18). By analyzing the splenic B cell compartment from 6- to 8-wk-old mice, we observed 4-fold increased total numbers of B cells. Interestingly, this increase differently affected distinct B cell populations. Mature B cells, identified as CD21^{med}IgM^{low} cells, were ~4-fold and increased in number (Fig. 2A), whereas the immature populations, transitional type 1 (T1) and transitional type 2 (T2) B cells (23), remained unaffected. This resulted in a relative 2-fold decrease of T1 and T2 cells, as compared with control animals (Fig. 2A). The relative reduction in T1 and T2 cells was also confirmed in IgM/IgD stainings (data not shown).

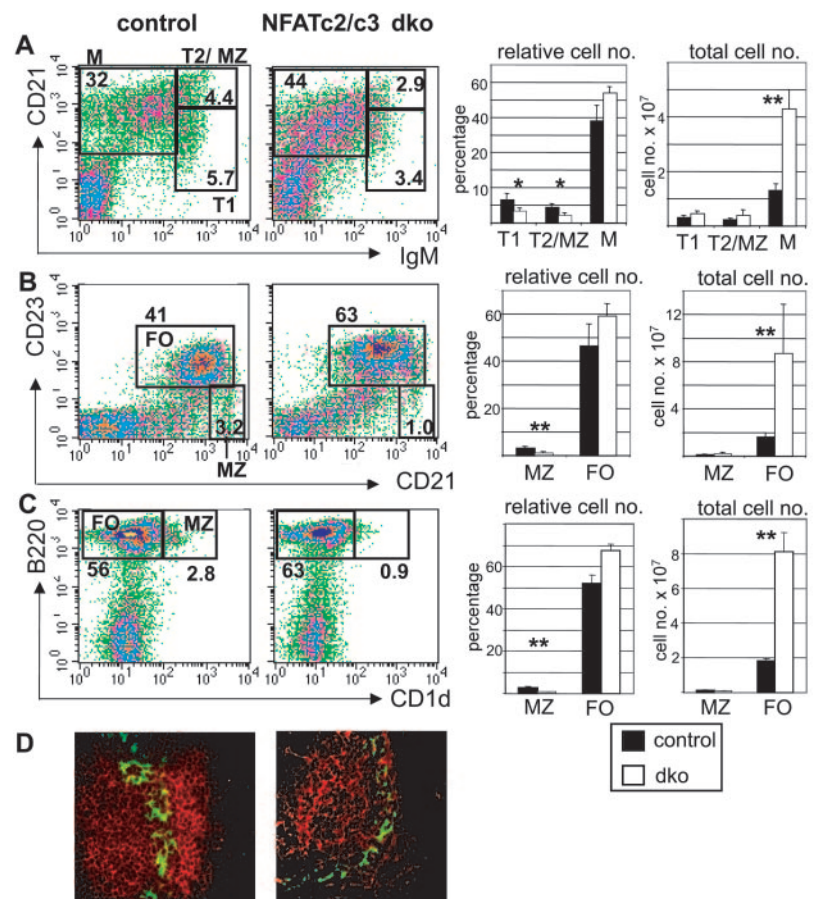
The population of CD21^{high}IgM^{high} cells also contains MZ B cells, and this B cell subpopulation was identified with mAbs against CD23 and CD21. CD21^{high}CD23^{lo-neg} MZ B cells were found to be 3-fold reduced in relative numbers but, due to a large

increase in total splenic B cells, were almost identical in total cell numbers (Fig. 2B). Also, when splenic B cells were stained for the MZ B cell marker CD1d, a ~3-fold underrepresentation was detected for MZ CD1d⁺ B cells in spleens from NFATc2/c3 double-deficient mice (Fig. 2C). The reduction in MZ B cells was also confirmed in histological stainings. Using anti-MOMA and anti-IgM Abs, fewer IgM⁺ cells were detected in the MZ outside of the marginal sinus in spleen sections from NFATc2/c3^{-/-} mice, compared with those from wild-type mice (Fig. 2D).

We next examined the responses of NFATc2/c3^{-/-} B cells after BCR triggering. Upon stimulation with 20 μ g/ml anti-IgM Ab, splenic B cells from NFATc2/c3^{-/-} mice showed a pronounced increase in Ca²⁺ mobilization, compared with B cells from control mice (Fig. 3A). This increase in Ca²⁺ levels was also detected after stimulation with lower anti-IgM Ab concentrations (data not shown). Likewise, isolated splenic B cells were stimulated with various mitotic stimuli, and the proliferative responses were measured. Strongly increased proliferative responses of NFATc2/c3 double-deficient B cells were found in response to anti-IgM Abs, as well as with other stimuli (Fig. 3B). More moderate but still significantly increased proliferation rates were also determined for B cells from NFATc2^{-/-} or NFATc3^{-/-} single-deficient mice, relative to wild-type control littermates (data not shown).

MZ B cells are known to participate in responses to TI-2 Ags. It has been shown that i.v.-injected TNP-ficoll is captured initially by MZ B cells but not by follicular B cells (24, 25). To study this, TNP-Ficoll was injected i.v., mice were sacrificed 30 min later, and spleen sections were stained with anti-TNP and anti-MOMA Abs. As shown in Fig. 4A, numerous MZ B cells from wild-type mice could efficiently capture TNP-Ficoll, whereas much less NFATc2/c3^{-/-} B cells were observed to capture TNP-Ficoll (Fig.

FIGURE 2. NFATc2/c3 double-deficient (dko) mice show a selective reduction in MZ B cells and in immature, transitional B cells in the spleen. **A**, Splenic cells were stained by anti-IgM/anti-CD21 Ab. A relative reduction in both T1 and T2/MZ B cells from NFATc2/c3 dko mice is detected, as shown in the *middle panel*. The total cell numbers for T1, T2/MZ, and mature (M) B cells are shown in the *right panel*. **B**, Stainings with anti-CD21/anti-CD23 Abs revealed a relative reduction in MZ B cells, whereas follicular (FO) B cells are unaffected (*left and middle panels*). The *right panel* shows total cell numbers. **C**, Staining using anti-B220 and anti-CD1d Abs indicates an underrepresentation of MZ B cells in NFATc2/c3 dko mice. **D**, Histological sections of spleens from NFATc2/c3 dko mice show a reduced population of IgM⁺ cells (stained in red with anti-IgM) in MZ. Staining in green was done with anti-MOMA-1 Ab, which is specific for metallophilic macrophages that separate the MZ from the follicle. Sections were viewed under $\times 400$ magnification. Significance was determined by Student's *t* test from three to five mice per group. *, $p < 0.05$; **, $p < 0.01$.



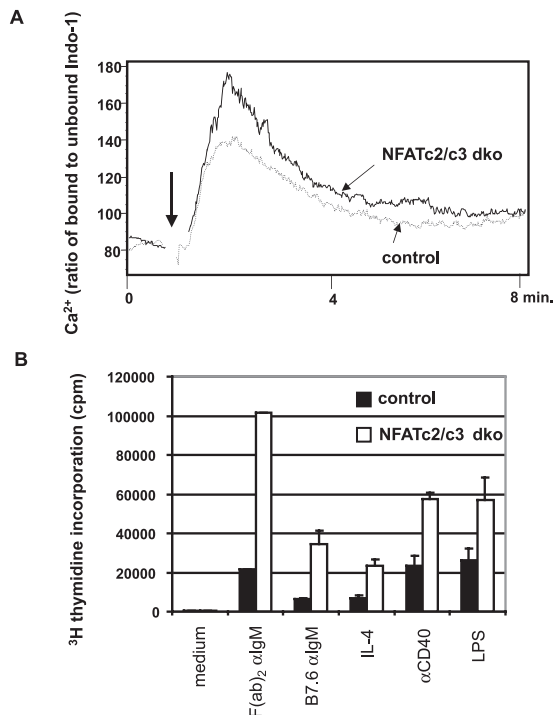


FIGURE 3. Enhanced Ca²⁺ mobilization and proliferation of NFATc2/c3^{-/-} B cells. *A*, Splenocytes from control mice (dotted line) and NFATc2/c3 double-deficient (dko) mice (bold line) were stimulated with 20 μg/ml B7-6 (anti-IgM) at the time point indicated by an arrow. *B* cells were gated (as CD5⁻, Mac-1⁻), and the ratio of bound to unbound indo-1 was determined. Intracellular Ca²⁺ concentration is proportional to the ratio of bound to unbound indo-1. *B*, Splenic B cells of mice indicated were cultured for 48 h with the stimuli shown, and [³H]thymidine incorporation was measured. Means of triplet measurements plus SD are shown. One of three typical assay experiments is shown.

4A). The rare NFATc2/c3^{-/-} B cells binding TNP-Ficoll were found both in the MZ and in the follicle (Fig. 4A). Subsequently, the Ig response to i.p.-injected TNP-Ficoll was measured in sera of both types of mice. Although NFATc2/c3^{-/-} mice showed increased TNP-specific Ig levels before immunization, they elicited a similar increase in levels of TNP-specific IgM and IgG3 at days 5 and 7 postimmunization, compared with control mice (Fig. 4B). This indicates that NFATc2/c3 double-deficient mice can mount normal TI-2 responses, although they show a relative reduction in MZ B cells per follicle.

To determine whether the reduction in MZ B cells is an intrinsic B cell defect, adoptive transfer experiments were performed. B cell-deficient μMT mice (21) were irradiated sublethally and reconstituted with BM cells from NFATc2/c3^{-/-} or control mice. For the identification of donor T cells generated from transferred BM, DO11.10 tg wild-type or DO11.10 tg NFATc2/c3^{-/-} mice were used as donors. Their genetic background allows the detection of transferred T cells with an idiotypic anti-TCR Ab. Before transfer (1), we confirmed that DO11.10 tg mice exhibit a similar defect in MZ B cells as NFATc2/c3^{-/-} mice (data not shown), and (2.) we tested conditions, i.e., the x-ray dose given to μMT recipient mice and the cell number used for i.v. injections, under which donor T cells contribute poorly to the T cell compartment of recipient mice. When mice were analyzed at day 32 after cell transfer, we found that <10% of T cells but all B cells were found to be donor derived (data not shown). However, when the B cell compartment of the reconstituted μMT mice was analyzed, we did

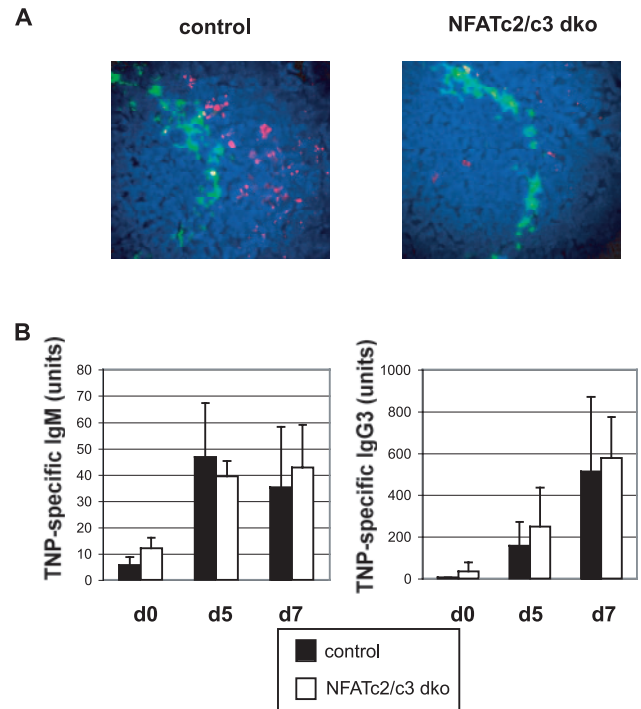


FIGURE 4. NFATc2/c3 double-deficient (dko) mice show a reduction in TNP-Ficoll-binding cells in the MZ but mount normal Ig responses to TNP-Ficoll immunization. *A*, Spleen sections from mice, 30 min after i.v. injection of TNP-Ficoll, were stained with biotin-labeled anti-TNP Abs (red) and FITC-labeled anti-MOMA-1 Abs (green). Biotinylated Ab was revealed with streptavidin-cyochrome. Sections were mounted with Moviol containing DNA-specific fluorescent dye 4',6'-diamidino-2-phenylindole (blue), which stains all cell nuclei. Sections were viewed under ×400 magnification. *B*, Control and NFATc2/c3 dko mice were immunized with TNP-Ficoll at day 0, and TNP-specific IgM and IgG3 responses were determined by ELISA at days 5 and 7 postimmunization. One of two (for *A*) and of three (for *B*) typical assay experiments is shown.

not detect any reduction in MZ B cell population. Both types of mice, either reconstituted with wild-type DO11.10 or with NFATc2/c3^{-/-} DO11.10 BM cells, showed a similar relative number of MZ B cells (Fig. 5, A–C) and of transitional and mature B cells (data not shown). Likewise, the proliferative responses of B cells from both types of reconstituted mice were identical (Fig. 5D). These results indicate that the B cell defects and changes in B cell responsiveness of NFATc2/c3 double-deficient mice are not cell-autonomous but must be due to extrinsic factors.

Discussion

The experimental data presented here and those of other authors (8) suggest that NFAT factors play a minor, if any, role in early B cell development in BM. However, distinct functional roles of NFAT factors have been identified for the generation and function of peripheral B cell populations. Although NFATc1 is critical for the development of B-1a cells (8), NFATc2/c3^{-/-} mice show an altered composition of the splenic B cell compartment, i.e., an underrepresentation of both MZ B cells and transitional B cells and a relative increase in mature follicular B cells. However, these changes are not B cell intrinsic, as we have shown by adoptive transfer experiments using μMT recipient mice.

Similarly to NFATc2/c3^{-/-} T cells, B cells from NFATc2/c3^{-/-} mice show a hyperresponsiveness to Ag receptor stimulation. This not only leads to increased proliferative responses but also to an increased Ca²⁺ mobilization. The hyperresponsiveness

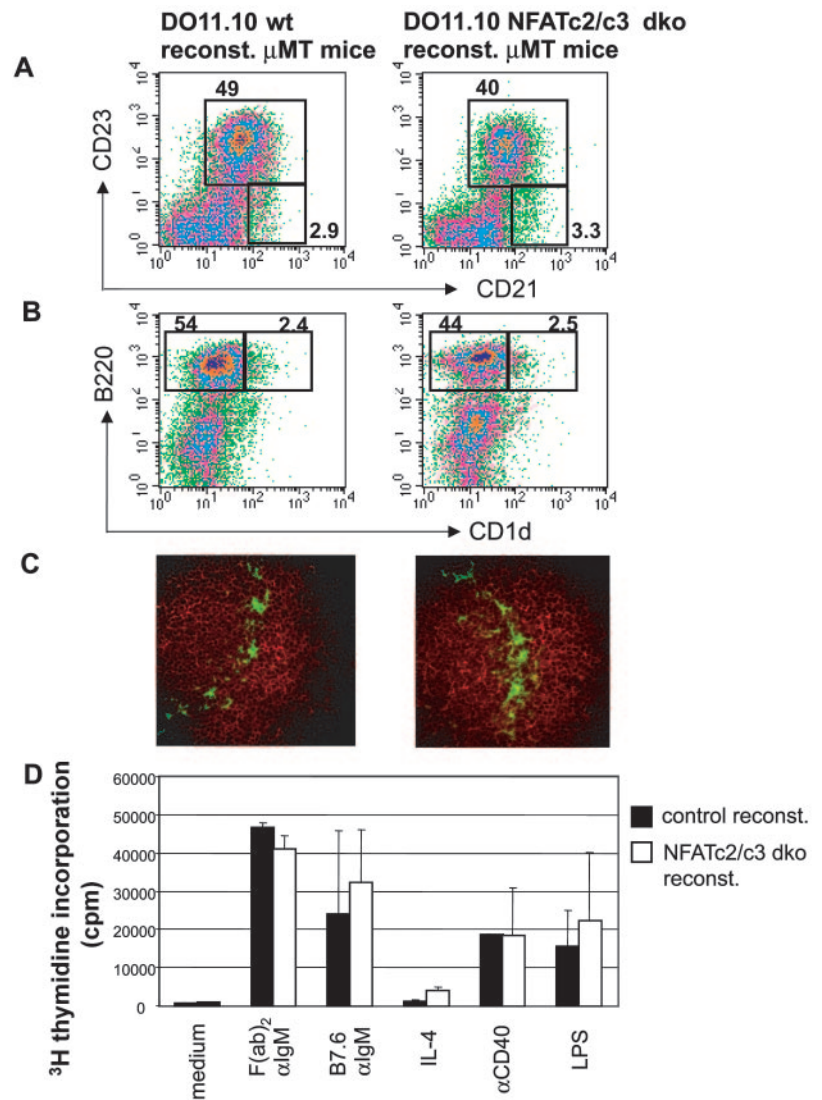


FIGURE 5. B cell-deficient mice reconstituted with BM cells from control or NFAT *c2/c3* double-deficient (dko) mice lack differences in MZ B cell compartment and in proliferative B cell responses. *A–C*, B cell-deficient μ MT mice were irradiated sublethally and reconstituted with BM cells from control DO11.10 tg or NFAT *c2/c3* dko/DO11.10 tg BM cells. The DO11.10 background of transferred T cells was identified to be <10% using KJ126 Ab. As shown in *A–C*, reconstituted mice with B cells derived from control or DO11.10 tg BM display a similar number of MZ B cells. Histological stainings were done as in Fig. 1. *D*, Proliferate responses of purified splenic B cells from both types of mice for various stimuli are shown (for details see Fig. 3*B*).

of B cells from NFATc2/c3^{-/-} mice is not cell-autonomous but affected by extrinsic factors. This was evident from the adoptive transfer experiments, in which B cells derived from NFATc2/c3-deficient donor BM showed a normal proliferative response in recipients with (mainly) wild-type T cells. Most likely the extrinsic factors in NFATc2/c3^{-/-} mice are strongly elevated levels of Th2-type cytokines, which lead to highly increased serum levels of IgG1 and IgE (18). This increased Th2 cytokine milieu not only affects class switching but also the BCR responsiveness of B cells. In accordance with this, activation markers, such as MHC class II or B7-2, were found to be up-regulated on NFATc2/c3^{-/-} B cells (Ref. 18 and our results). Alternatively, it cannot be excluded that cells other than, or in addition to, T cells are affected by the NFATc2/c3 deficiency and mediate the hyperresponsive phenotype of B lymphocytes.

Because B cells from NFATc2/c3^{-/-} mice are hyperresponsive in vitro, they probably also show an increased BCR signaling in vivo. The strength of BCR signals can determine the fate of peripheral B cells (14). This has been demonstrated recently in mice in which a BCR surrogate, e.g., the LMP2A protein from EBV, was found to determine the fate of B cells in different ways, depending on its surface expression level and the resulting signaling strength (26). In addition, mice deficient for CD22 (16) or the transcription factor Aiolos (15), which show increased B cell sig-

naling, exhibit a reduced number in MZ B cells and transitional B cells and a relative increase in mature B cells. A similar phenotype was found in NFATc2/c3 double-deficient mice. Therefore, the reduced population of MZ B cells in NFATc2/c3^{-/-} mice might be caused by the preactivated state and increased BCR signaling of their B cells.

This increased BCR signaling could affect MZ B cell development or MZ B cell proliferation directly. In vitro, MZ B cells do not show proliferative responses after BCR stimulation, in contrast to follicular B cells (27). Thus, the increased BCR signaling of NFATc2/c3^{-/-} mice could selectively expand mature follicular B cells in vivo. The hyperactivated state of B cells could also affect the responsiveness of B cells against chemokines and their migration to the MZ. This view is supported by the defects in the MZ B cell compartment observed upon inactivation of integrins LFA-1 and $\alpha_4\beta_1$ (17) and upon impairment of chemokine receptor signaling (25). Alternatively, the Th2 cytokine milieu or other factors may cause indirect affects on other cells of the MZ, which affect migration of B cells. Such cells could be MZ macrophages, which appear to control the trafficking and retention of B cells in the MZ (28). Because the critical chemokine for placement of B cells in the MZ is not known, these different possible mechanisms are hard to study experimentally.

NFATc2/c3-deficient mice mounted normal Ig responses to immunization with a TI-2 Ag. Therefore, the MZ B cells, which are present in NFATc2/c3^{-/-} mice, are functional. Although there is a reduction in functional MZ B cells per follicle, as indicated by reduced numbers of IgM⁺ MZ cells and reduced TNP-Ficoll-capturing cells in histological sections, the massive expansion of splenocytes probably leads to an increased number of follicles and therefore, overall, to a similar TI-2 response. The high number of follicular B cells could also lead to a stronger contribution of follicular B cells to TI-2 responses in NFATc2/c3^{-/-} mice than normally found.

Taken together, the data presented here on function of NFAT transcription factors in B cells indicate that NFATc2/c3 deficiency leads to B cell hyperresponsiveness toward BCR-induced signals by an indirect mechanism and to underrepresentation of MZ B cells. Thus, the data support the view that MZ B cells differ from conventional B cells in their reaction to strong extrinsic signals that drive normal B cells, but not MZ B cells, to proliferate.

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Disclosures

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