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B Cell Selection Defects Underlie the Development of Diabetogenic APCs in Nonobese Diabetic Mice

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One mechanism whereby B cells contribute to type 1 diabetes in nonobese diabetic (NOD) mice is as a subset of APCs that preferentially presents MHC class II-bound pancreatic β cell Ags to autoreactive CD4 T cells. This results from their ability to use cell surface Ig to specifically capture β cell Ags. Hence, we postulated a diabetogenic role for defects in the tolerance mechanisms normally blocking the maturation and/or activation of B cells expressing autoreactive Ig receptors. We compared B cell tolerance mechanisms in NOD mice with nonautoimmune strains by using the IgHEL and Ig3-83 transgenic systems, in which the majority of B cells recognize one defined Ag. NOD- and nonautoimmune-prone mice did not differ in ability to delete or receptor edit B cells recognizing membrane-bound self Ags. However, in contrast to the nonautoimmune-prone background, B cells recognizing soluble self Ags in NOD mice did not undergo partial deletion and were also not efficiently anergized. The defective induction of B cell tolerance to soluble autoantigens is most likely responsible for the generation of diabetogenic APC in NOD mice. The Journal of Immunology, 2004, 172: 5086–5094.
used a previously described (26) N21 backcross stock of T1D-resistant NOD satellite markers delineating all known Idd Ig3-83 congenic stock is backcross generation were detected by PCR using the primers 5'-CAGCTvidin linked to FITC, PE, or APC Ig3-83 molecules. Biotinylated reagents were detected by means of streptavidin. The clonotype-specific Ab 54.1 was used to detect B cells expressing CD23, and the biotinylated M1-69 Ab specific for CD24 (BD Biosciences). Ab specific for splenic B cell populations were distinguished using the PE-conjugated 7G6 Ab (BD Biosciences) or the FITC-conjugated R5-240 Ab, a PE-conjugated B220-specific mAb (RA36B2). A streptavidin-FITC conjugate was used to detect the biotinylated anti-HEL Abs. Increasing concentrations of the HEL standards could be detected as increases of mean fluorescence intensity (MFI) in the FITC channel on B220⁺ B cells and were graphed as a standard curve. The concentration of HEL within the serum samples was calculated by plotting the MFI they yielded against the standard curve.

**In vitro proliferative responses of stimulated B cells**

B cells were purified from the spleens of the indicated mice using a previously described (7) magnetic bead system (Miltenyi Biotec, Auburn, CA). Subsequent FACS analysis indicated B cell purity was >95%. Triplicate aliquots of 5 × 10⁶ B cells were seeded into flat-bottom 96-well culture plates (Corning Glass, Corning, NY) in a final volume of 200 µl of complete RPMI 1640 medium (27) containing either 10 µg/ml LPS or 5 µg/ml denatured HEL (CD40-3; BD Biosciences) in combination with 20 µg/ml AffiniPure goat anti-mouse IgG + IgM (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA) or the IgD⁺-specific mAb H6-1 (kindly supplied by F. Finkelman, Cincinnati Veterans Affairs Medical Center, Cincinnati, OH). Control wells contained no stimulatory agents. The cultures were pulsed with 1 µCi/well [³H]thymidine for the final 24 h of a 72-h incubation period at 37°C. The cultures were then harvested, and [³H]thymidine incorporation was determined using an LKB Betaplate 1205 system (LKB Instruments, Gaithersburg, MD). B cell proliferative responses are presented as mean cpm ± SEM.

**ELISA quantitation of Abs**

Triplicate wells with 5 × 10⁶ purified B cells from the indicated strains were cultured in complete RPMI 1640 with or without anti-IgD⁺ and anti-CD40, as described above. After 96 h of incubation, supernatants from triplicate wells were pooled. The concentration of IgM in the supernatants was determined by ELISA. Briefly, 96-well ELISA plates high binding polystyrene plates (Corning Glass) were coated at 4°C overnight with a polyclonal goat anti-mouse IgM (Southern Biotechnology Associates) in 0.1 M carbonate buffer (pH 9.6) and blocked the next day with 10% BSA in PBS at room temperature for 1 h. The experimental supernatants were diluted 2-fold and added to the anti-IgM-coated wells for 1 h at 37°C. After washing with PBS containing 0.02% Tween 20 and 1% BSA, a biotinylated IgM⁺-specific Ab (DS-1) was added as a secondary detection reagent to each well, and the plate was incubated for 1 h at 37°C. Following another wash, streptavidin-conjugated alkaline phosphatase (Southern Biotech- Technology Associates) was added to each well, and the plate was incubated for another hour at 37°C. Each well was then developed by the addition of para-nitrophenyl phosphate substrate (Sigma-Aldrich) for 15 min and assessed for OD at 405 nm using a SPECTRAMax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentration of IgM⁺ in each of the supernatants was calculated from a standard curve generated with an isotype/allotype control Ab (TEPC 183; Sigma-Aldrich). IgM⁺ Ab concentrations in sera were determined in the same fashion. To induce the production of anti-HEL Abs, BM from B6 or B6.Igh(-/-) mice as well as non-tg littermates were immunized i.p. with 50 µg/ml HEL in CFA. HEL-specific Abs in sera obtained 21 days later were measured by ELISA, as described above, except: 1) plates were coated with 1 µg/ml HEL, and 2) serum anti-HEL Abs were detected with an IgG 1 chain-specific Ab conjugated to alkaline phosphatase. No HEL-specific mAbs were commercially available to serve as a control, and therefore serum levels are reported as the OD₅₆₀.

**Tolerance induction assays of immature and T1 B cell populations**

Immature B cell tolerance assays were performed using BM from NOD.Igh⁻/- and B6.Igh⁻/- female mice. BM cell suspensions were analyzed by flow cytometry to calculate the proportions of immature B cells (IgM⁺ IgD⁻) in the indicated strains of congenic mice. Immature B cells were incubated in complete RPMI 1640 medium with the indicated concentrations of soluble HEL for 24 h at 37°C. The remaining viable (pro-pidium iodide-negative) immature B cells in each sample were then determined by flow cytometry. For the T1 B cell tolerance assays, total B cells were initially purified from the spleens of NOD or B6 mice using the magnetic beadsaxis system described above. Purified B cells were subsequently stained with an Ab mixture containing RA36B2 PerCP (anti-B220), M1-69 PE (anti-CD24), and 7G6 FITC (anti-CD21; BD Biosciences). A Vantage flow cytometer (BD Biosciences) was used to sort and collect T1 B cells, defined by a B220⁺ CD21low CD24high phenotype. Aliquots of 2 × 10⁶ T1 B cells were seeded in complete RPMI 1640 into 24-well plates previously coated with 0, 10, or 50 µg/ml AffiniPure goat anti-mouse IgG + IgM (H + L). Cells were incubated for either 0 or 24 h, and then
Results

B cell tolerance mechanisms against membrane-bound autoantigens are intact in NOD mice

We initially compared the ability of B cells from NOD mice and a nonautoimmune-prone strain to undergo deletion following exposure to a membrane-bound self Ag. Hemyzygous NOD.mHEL and B6.mHEL mice were respectively crossed with homozygous NOD.IgHEL and B6.IgHEL mice (previously described in Refs. 10 and 16) to yield IgHEL/mHEL double-tg and control IgHEL single-tg progeny. All (IgHEL) and tg (IgM+ HEL) B cells were measured in the spleens of 6-wk-old IgHEL/mHEL vs IgHEL mice of both genetic backgrounds (Fig. 1A). Consistent with previous reports (10, 16), allelic exclusion resulted in greater than 98% of the splenic B cells in both NOD.IgHEL and B6.IgHEL mice expressing tg IgM/ IgD molecules, while 94% retained the capacity to bind HEL biotin (Fig. 1B). In contrast, very few IgM+ HEL B cells were detected in the spleens of IgHEL/mHEL double-tg B6 or NOD mice (5.9 and 1.1% compared with IgHEL single-tg controls, respectively; Fig. 1A), and essentially none of these were capable of binding HEL (≤0.2% of B220+ B cells in all mice; Fig. 1B). Coexpression of IgHEL and mHEL transgenes on either background was not associated with an increase in B cells expressing endogenous b-allotypic IgM molecules. Thus, NOD mice did not differ from nonautoimmune-prone B6 mice in their ability to eliminate B cells recognizing a multivalent membrane-bound protein.

The small numbers of non-HEL-binding IgM+ HEL B cells in the spleens of IgHEL/mHEL double-tg NOD and B6 mice presumably escaped deletion by undergoing receptor editing of their L chain after encountering autoantigen. In two separate experiments, slightly higher numbers of non-HEL-binding IgM+ HEL B cells were detected in the spleens of B6 vs NOD IgHEL/mHEL double-tg mice (Fig. 1A) consistent with a greater success rate of receptor editing in self-reactive B cells. However, <2.5% of non-HEL-binding IgM+ HEL B cells remaining in the spleens of B6 or NOD IgHEL/mHEL double-tg mice expressed endogenous λ L chains (data not shown), whose presence would indicate receptor editing. Therefore, the non-HEL-binding IgM+ HEL B cells detected in the spleens of IgHEL/mHEL double-tg mice may have arisen either as a result of exclusive receptor editing by endogenous Igκ L chains or due to the enrichment of cells in which the tg L chain failed to
elicit allelic exclusion. Supporting the latter explanation are the findings of Fang et al. (28), who detected very low overall levels of receptor editing in B cells from B6 IgHEL/mHEL double-tg mice.

Although most B cells expressing the Ig3-83 transgene on the B10.D2 genetic background are also deleted upon encountering their cognate membrane-bound H-2Kb Ag (14), in contrast to the IgHEL/mHEL system, they demonstrate a greater capacity to use receptor editing as an alternate tolerance mechanism (15). We used the Ig3-83 tg system to compare the ability of NOD and B6 B cells to use receptor editing as an alternate tolerance mechanism when encountering membrane-bound self Ags. NOD Ig3-83 mice were crossed with H-2Kb-expressing NOD.H2<sup>ns</sup> mice to produce progeny for studying B cell tolerance. Nonautoimmune-prone controls consisted of (B10.D2.Ig3-83 × B10(H-2Kb))<Fi> progeny. NOD.Ig3-83 and B10.D2.Ig3-83 mice were used as non-tolerance-inducing controls. As expected, a high percentage of tg B cells was found in the splenels of NOD.Ig3-83 and B10.D2.Ig3-83 mice (Fig. 2A). Although lower than the total number of B cells detected using the IgM<sup>+</sup>-specific Ab (DS-1; Fig. 2B), >8% of the B cells from mice of both backgrounds stained with the Ig3-83 clonotype-specific Ab 54.1, indicative of fairly stringent allelic exclusion mediated by the tg H and L chains. Compared with what was observed when using the IgHEL/mHEL double-tg system, a greater number of IgM<sup>+</sup> B cells was detected in the spleenels of NOD.Ig3-83 × NOD.H2<sup>ns</sup>) and (B10.D2.Ig3-83 × B10) F<sub>1</sub> mice (Fig. 2A). Nevertheless, very few of the remaining splenic IgM<sup>+</sup> B cells (~4%) from either background stained with the Ig3-83 clonotype-specific 54.1 Ab. This suggested they had changed their autoreactive Ig specificity by pairing tg H chains with endogenous L chains. Although <0.3% of IgM<sup>+</sup> B cells in the spleenels of NOD.Ig3-83 and B10.D2.Ig3-83 expressed an endogenous A L chain, ~60% of the remaining 54.1 IgM<sup>+</sup> B cells in the spleenels of (NOD.Ig3-83 × NOD.H2<sup>ns</sup>) and (B10.D2.Ig3-83 × B10) F<sub>1</sub> mice did so (Fig. 2B), implying a high proportion of these cells had undergone receptor editing. However, there were no significant differences in the numbers of IgM<sup>+</sup> 54.1<sup>+</sup> B cells remaining in the spleenels of (NOD.Ig3-83 × NOD.H2<sup>ns</sup>) and (B10.D2.Ig3-83 × B10) F<sub>1</sub> mice (Fig. 2A), nor in their levels of endogenous Ig<sub>lambda</sub> chain usage (Fig. 2B). Hence, we conclude that the induction of the signaling pathways mediating deletional tolerance or receptor editing in B cells that engage strong Ig cross-linking membrane-bound autoantigens remains intact in NOD mice.

**NOD mice are defective in their ability to delete B cells recognizing soluble autoantigens**

Given that they are presented through the MHC class II pathway, β cell autoantigens recognized by diabetogenic CD4 T cells in NOD mice are likely to be soluble in nature. Ig-mediated capture allows B cells to be the subset of APC that most efficiently presents soluble β cell autoantigens to diabetogenic CD4 T cells in NOD mice (10). Therefore, even though B cell tolerance to membrane-bound Ags appears to be intact in NOD mice, this might not be the case for soluble self Ags. To test this possibility, hemizygous NOD.sHEL and B6.sHEL mice were respectively crossed with homozygous NOD.IgHEL and B6.IgHEL mice to produce IgHEL/ sHEL double-tg and control IgHEL single-tg progeny. Compared with B6.IgHEL controls, total numbers of splenic IgM<sup>+</sup> B cells were decreased by ~30% in B6.IgHEL/sHEL mice (Fig. 3A). In contrast, there was no reduction in the total numbers of splenic IgM<sup>+</sup> B cells in NOD.IgHEL/sHEL mice compared with controls (Fig. 3A). This suggested NOD mice are indeed defective in a mechanism leading to the partial deletion of B cells recognizing soluble self Ags. However, it was important to ensure that the different pattern of autoreactive B cell development observed in B6.IgHEL/sHEL and NOD.IgHEL/sHEL mice was not due to lowered levels of soluble HEL in the latter stock. As shown in Fig. 3B, this was not the case because serum HEL concentrations were actually marginally, but statistically higher in NOD.sHEL than B6.sHEL mice. This difference was less pronounced in the NOD vs B6 IgHEL/sHEL double-tg mice. The lower serum HEL levels observed in both strains of IgHEL/sHEL double-tg vs sHEL single-tg mice were probably due to the presence of residual nondeleted HEL-binding B cells in the former set of mice. Therefore, the reduction in IgHEL B cell deletion observed in NOD vs B6 IgHEL/sHEL double-tg mice could not be explained in terms of different levels of Ag expression. Collectively, these results indicate an intrinsic defect in the ability of NOD B cells to be deleted upon recognizing soluble self Ag.

**FIGURE 3.** Partial deletion of B cells specific for soluble HEL is impaired in NOD mice, despite higher production of the autoantigen. A, The left panel depicts mean total numbers (±SEM) of all subsets (Ig<sub>gamma</sub>), tg (IgM<sup>+</sup>), and non-tg (IgM<sup>-</sup>) B cells in the spleens of 6- to 7-wk-old NOD.IgHEL (■; n = 5), NOD.IgHEL/sHEL ( ▲; n = 4), B6.IgHEL ( □; n = 9), and B6.IgHEL/sHEL ( ■; n = 13) female mice. •, Signifies expression of soluble Ag results in a significant reduction in tg B cells in B6 mice (p < 0.05, Student’s t test). The right panel depicts the proportion of IgHEL-expressing B cells in the spleens of IgHEL/sHEL double-tg NOD ( ▲) and B6 ( □) mice relative to the levels in IgHEL single-tg mice with the same genetic background. B, Mean concentration (±SEM) of tg soluble HEL in the sera of three female mice of each of the indicated strains. Significance of difference was determined using Student’s t tests.
Defective induction of anergy in NOD B cells recognizing a soluble self Ag

Previous reports have demonstrated the majority of IgHEL B cells in B6.IgHEL/sHEL double-tg mice are rendered functionally anergic rather than undergoing deletion (16, 17). Thus, it remained possible that while NOD B cells recognizing a soluble self Ag during their development do not undergo a normal level of deletion, they could still be functionally anergized. This possibility was initially examined by comparing surface Ig expression on splenic B cells that had escaped deletion in the NOD and B6 IgHEL/sHEL double-tg stocks as a surrogate marker of anergy. Splenic B cells from both NOD and B6 IgHEL/sHEL double-tg mice displayed an 11- to 14-fold reduction in surface IgM expression compared with those from IgHEL single-tg controls (Fig. 4A). Although surface IgD expression was up-regulated 1.7-fold in the B6.IgHEL/sHEL splenic B cells compared with levels in B6.IgHEL controls, this was not observed in the NOD background stocks (p < 0.05, Student’s t test; Fig. 4A). The relevance of this modest difference in surface IgD expression is not known.

Anergy was examined by quantitating the number of HEL-binding B cells and serum levels of HEL-specific Abs in B6 and NOD IgHEL/sHEL double-tg mice. The vast majority (87–95%) of B cells remaining in the periphery of both lines of double-tg mice retained HEL-binding capacity (Fig. 1B), and thus had not undergone receptor editing. However, unlike in IgHEL single-tg controls, basal serum levels of IgM or HEL-binding Abs were undetectable in the NOD or B6 IgHEL/sHEL double-tg stocks (Fig. 4B). Thus, despite a large number of HEL-specific B cells in the periphery of both NOD and B6 IgHEL/sHEL double-tg mice, neither strain spontaneously produced HEL-specific autoantibodies, indicating some level of anergy induction in both backgrounds.

We next evaluated whether anergy was reversible in peripheral B cells from NOD or B6 IgHEL/sHEL double-tg mice. Splenic B cells were purified from the double-tg stocks and IgHEL single-tg controls and stimulated in vitro through Ag receptor-specific (anti-IgM/G + anti-CD40 or anti-IgD + anti-CD40) or nonspecific pathways (LPS). Anti-IgM/G, anti-IgD, or anti-CD40 stimulation on their own only produced minimal responses in all tested B cell populations (data not shown). Conversely, costimulation through the B cell receptor (BCR) and CD40 induced an optimum response by purified B cells from both NOD and B6 IgHEL single-tg mice (Fig. 5A). Consistent with the preservation of anergy, the proliferative responses of B6.IgHEL/sHEL B cells to anti-IgM/G + anti-CD40 or anti-IgD + anti-CD40 costimulation were markedly reduced compared with B6.IgHEL control B cells (49 and 21%, respectively). The greater degree of proliferation in anti-IgM/G + anti-CD40-stimulated B6.IgHEL/sHEL B cells compared with anti-IgD + anti-CD40 costimulation was probably due to activation of the small proportion of nonanergic B cells expressing endogenous Ig chains in these mice. In contrast, the reduction in proliferation of NOD.IgHEL/sHEL B cells stimulated with anti-IgM/G + anti-CD40 or anti-IgD + anti-CD40 was much less marked (86 and 70% of the NOD.IgHEL control response, respectively). A similar trend was observed when IgM Ab was measured. Thus, compared with the IgHEL single-tg controls, Ab production following BCR and CD40 costimulation was less suppressed in NOD than B6 IgHEL/sHEL double-tg mice (2.6- and 4.5-fold lower, respectively). The difference in LPS-induced proliferation between B cells from NOD and B6 IgHEL/sHEL double-tg mice was not as pronounced as that seen when using Ag receptor stimulation. Nevertheless, in comparison with IgHEL single-tg controls, the ability to proliferate in response to LPS was suppressed to a greater extent in B cells from B6 than non-tg IgHEL/sHEL double-tg mice (Fig. 5A).

An additional in vivo study was performed to compare the extent to which B cells in NOD- and nonautoimmune-prone mice become anergic after encountering soluble self Ag during their development. NOD and (B6 × CBA)F1 mice, both carrying the sHEL transgene, were immunized with HEL in CFA, and their sera were tested 21 days later for Ag-specific Ab levels (Fig. 5C). Non-tg NOD and (B6 × CBA)F1 mice served as controls. (B6 × CBA)F1 hybrids were used as non-autoimmune-prone controls because homozygous expression of the H2b MHC haplotype limits the ability of the B6 strain (sHEL transgene donor) to generate Th cell responses to HEL (29, 30). The H2b haplotype provided by the CBA strain overcomes this difficulty. Levels of HEL-specific Abs in the sera of NOD vs (B6 × CBA)F1 sHEL tg mice were 60 and 20%, respectively, of that detected in non-tg mice with the same genetic background. These results, coupled with the in vitro studies described above, indicate that while NOD B cells are initially anergized when encountering soluble self Ag during their development (accounting for the lack of circulating HEL-specific Abs in the NOD IgHEL/sHEL double-tg stock), unlike the case in non-autoimmune-prone B6 mice, this anergic state can be readily reversed if a subsequent antigenic encounter is accompanied by CD40-mediated costimulation. Certainly, in vivo, the defects in
were immunized i.p. with 50 non-tg littermates of the same genetic background.

IgG/M or the IgDa-specific (HM40-3) in combination with goat anti-mouse
c mAb

Experiments, both with similar results, is shown.

Indicate a significant decrease (p < 0.05, Student’s t

test) in responsiveness of B cells from IgHEL/sHEL

double-tg mice compared with IgHEL single-tg mice of the same background. B. Trplicate wells of 5 × 10^6 purified B cells from the indicated strains were cultured for 96 h with or without anti-Ig/D/anti-CD40 Abs. Supernatants from triplicate wells for each strain were pooled and assessed for IgM* concentrations by ELISA. C. Three (CBA × B6.sHEL)F1 and NOD,sHEL mice as well as three non-tg littermates of the same genetic background were immunized i.p. with 50 μg/ml HEL in CFA. HEL-specific Ab levels in sera obtained 21 days later were measured by ELISA. Levels of anti-HEL Abs are reported as mean OD_{405 nm} ± SEM.

Tolerance induction underlying the development of autoreactive diabetogenic T cells in NOD mice set up a scenario whereby they could provide the required help through CD40, and perhaps other receptors, that abrogates the weak anergic state induced in B cells by engagement of a soluble β cell autoantigen during their development. Such B cells could then serve as APCs that efficiently amplify diabetogenic CD4 T cell responses.

**NOD B cell tolerance defects occur at two developmental stages**

B cells recognizing self Ag can be tolerated either when at an immature state in the BM or at the T1 stage in the spleen. Hence, we assessed at which of these development points autoreactive B cells from NOD mice fail to become tolerant. For this purpose, an in vitro assay was performed in which BM suspensions from NOD.IgHEL and B6.IgHEL mice (normalized for IgM* IgD* immature B cell numbers) were exposed to different concentrations of soluble HEL ranging from 0.01 to 5 ng/ml. The extent to which immature B cells were deleted after 24 h of exposure to HEL was determined by FACS and compared with cells not encountering Ag. Under these in vitro conditions, the immature B cells from B6.IgHEL mice were eliminated at lower concentrations of HEL than those from NOD.IgHEL mice (Fig. 6). It should be noted that in the predisease stage, the circulating concentration of insulin, which is the antigenic target of an important population of diabetogenic B cells in NOD mice (11), is in the range of 1–2 ng/ml. Thus, the ~0.5–2 ng/ml Ag dose range that differentially induced the deletion of immature BM-derived B cells from NOD.IgHEL and B6.IgHEL mice is likely to be physiologically relevant.

We subsequently compared the susceptibility of B6 and NOD splenic T1 (B220+ CD21^{hi} CD23^{hi} CD24^{hi}) B cells to undergo deletion upon BCR cross-linking. Interestingly, FACS analyses of spleens demonstrated a significantly reduced population of T1 B cells in NOD compared with B6 mice, whereas NOD mice had a larger proportion of splenic T2 (B220+ CD21^{hi} CD23^{hi} CD24^{hi}), marginal zone (MZ), and mature (follicular) B cells (representative FACS profiles in Fig. 7A, and statistical summary in Table I). These changes in the different B cell populations were not always reflected in the total cell numbers due to the larger overall numbers of splenic B cells in B6 than NOD mice (Table I). Similar differences were seen when B cell populations in the spleens of NOD were compared with another diabetes-resistant strain, BALB/c (data not shown). T2 B cells are more mature that those at the T1 stage, and are believed to be direct precursors of follicular and MZ B cells (31, 32). T2 B cells are less susceptible to deletion when encountering Ag than T1 B cells due to their increased expression of antiapoptotic products (such as Bcl-xL, A1Bf-1, and Akt) and reduced expression of proapoptotic factors (such as Bak and Btk (23, 33)). The skewed proportions of T1 and T2 B cells in NOD
To compare their susceptibility to apoptosis-induced death, purified NOD and B6 T1 B cells were exposed to 0, 10, and 50 μg/ml anti-IgM in vitro (Fig. 7B). T1 B cells from NOD mice underwent lower levels of apoptosis compared with those from B6 mice at all levels of Ig stimulation tested. Thus, NOD B cells are impaired in their ability to be deleted following Ag receptor engagement both at the immature stage in the BM and at the T1 stage in the spleen.

**Discussion**

T1D in both humans and NOD mice results from T cell-mediated autoimmune destruction of pancreatic β cells. Thus, it is not surprising that there have been numerous reports describing disruptions in both central and peripheral T cell tolerance induction mechanisms in NOD mice, which ultimately lead to the production of both CD4 and CD8 autoreactive effectors (34–39). By using Ig tg models, we have demonstrated in the current study that NOD mice are also defective in their ability to both delete and sustain anergy in B cells that recognize soluble self Ags. These defects are likely to underlie the development of B cells expressing Ig molecules specific for pancreatic β cell autoantigens. The ability of such B cells to take up autoantigens by an Ig-mediated capture mechanism allows them to be the APC subpopulation that most efficiently expands diabetogenic CD4 T cell responses in NOD mice (10) and that also contributes to disease through the secretion of maternally transmitted autoantibodies (6).

Defects leading to impaired deletion of immature B cells in NOD mice were manifest at both the immature developmental stage in BM, as well as the later T1 stage in the spleen. The survival or deletion of B cells at various developmental stages is largely regulated by the relative ratio between pro- and ant apoptopic molecules, such as those of the Bcl-2 or TNFR families (reviewed in Refs. 40 and 41). Therefore, it is possible that the impaired deletion of B cells in the NOD strain may be due to an imbalance in the expression of these proteins. It is also interesting to note that exposure to soluble self Ag in the ng/ml range primarily caused immature B cells of NOD and B6 origin to undergo differential levels of deletion in vitro (Figs. 6 and 7), while the...
acquisition of functional anergy appeared to be a more predominant mode of tolerance induction in vivo (Figs. 3 and 4). This may be due to the in vivo environment providing survival factors that are not present or as accessible when the cells are dispersed in the in vitro cultures, thus allowing B cells to be energized, but not eliminated at high levels through deletional processes. Alternatively, the impaired ability of developing B cells in NOD mice to be deleted and/or anergized may be caused by a decrease in the strength of signaling through the BCR upon encountering weakly cross-linking soluble self Ags. This possibility is consistent with the enlarged total numbers of MZ B cells and slightly decreased follicular B cells in the spleens of NOD mice (Table I), a profile that has been observed in various mice with targeted mutations or transgenes that attenuate BCR signaling, e.g., Btk and CD21 knockout mice (32, 42).

Mechanistic studies to determine whether BM-derived immature and splenic T1 B cells from NOD mice and control strains are characterized either by differing balances of various pro- and antiapoptotic molecules and/or a disparity in BCR signaling after exposure to putatively tolerogenic Ags will be the subject of future studies. It is also of interest to note that abnormal balances in apoptotic regulatory molecules and impaired intrinsic signaling defects have also been reported to be a key factor in the development of autoreactive diabetogenic T cells in NOD mice (43–45). Hence, it is certainly possible that the impaired ability of both autoreactive T and B cells in NOD mice to be deleted or anergized when encountering soluble self Ags during their development is under common or largely overlapping genetic control, with both defects being necessary for T1D development.

It is tempting to speculate that autoreactive B cells also contribute to T1D in humans, and that they arise due to the same kind of tolerance induction defects characterizing NOD mice. There has been a report of a single human T1D patient who also has X-linked agammaglobulinemia, a disease characterized by markedly decreased numbers of functional B cells (46). However, without appropriate epidemiological studies, it is unknown whether the patient examined was characterized by a normal or exceptional pathogenic basis of T1D development. Indeed, we have previously reported that while most B cell-deficient NOD.Igμnull mice are T1D resistant, there are occasional exceptions (10). Moreover, in

NOD mice, the preferential role of B cells in expanding diabetogenic T cell responses appears to be due to developmental defects in other APC subtypes (reviewed in Ref. 3). Similarly, defects in APC subtypes other than B lymphocytes also characterize many, but not all, humans with T1D (reviewed in Ref. 3). Perhaps the single T1D patient with X-linked agammaglobulinemia represented one of the minority cases in which other APC populations differentiated normally, and hence bypassed a pathogenic need for B cells. Hence, the possibility is still open that B cells may contribute to T1D in most humans in the same way as in NOD mice. Because of this, gaining an increased understanding of the B cell tolerance induction defects in NOD mice may ultimately aid in developing protocols that might block progression to overt T1D in otherwise susceptible individuals.

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