I-A^{g7}-Mediated Antigen Presentation by B Lymphocytes Is Critical in Overcoming a Checkpoint in T Cell Tolerance to Islet β Cells of Nonobese Diabetic Mice[1]

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B cell-deficient nonobese diabetic (NOD) mice are protected from the development of spontaneous autoimmune diabetes, suggesting a requisite role for Ag presentation by B lymphocytes for the activation of a diabetogenic T cell repertoire. This study specifically examines the importance of B cell-mediated MHC class II Ag presentation as a regulator of peripheral T cell tolerance to islet β cells. We describe the construction of NOD mice with an I-A^{g7} deficiency confined to the B cell compartment. Analysis of these mice, termed NOD B^{CIID}, revealed the presence of functionally competent non-B cell APCs (macrophages/dendritic cells) with normal I-A^{g7} expression and capable of activating Ag-reactive T cells. In addition, the secondary lymphoid organs of these mice harbored phenotypically normal CD4$^+$ and CD8$^+$ T cell compartments. Interestingly, whereas control NOD mice harboring I-A^{g7}-sufficient B cells developed diabetes spontaneously, NOD B^{CIID} mice were resistant to the development of autoimmune diabetes. Despite their diabetes resistance, histologic examination of pancreata from NOD B^{CIID} mice revealed foci of noninvasive peri-insulitis that could be intentionally converted into a destructive process upon treatment with cyclophosphamide. We conclude that I-A^{g7}-mediated Ag presentation by B cells serves to overcome a checkpoint in T cell tolerance to islet β cells after their initial targeting has occurred. Overall, this work indicates that the full expression of the autoimmune potential of anti-islet T cells in NOD mice is intimately regulated by B cell-mediated MHC class II Ag presentation. ***The Journal of Immunology*****, 1999, 163: 743–750.**

Autoimmune diabetes is an important paradigm of spontaneous organ-specific T cell-mediated disease. The nonobese diabetic (NOD)[3] mouse serves as an excellent model for study of the immune pathogenesis of insulin-dependent diabetes mellitus (1–4). Diabetes in the NOD mouse is the result of a selective destruction of insulin-producing pancreatic β cells by autoreactive T lymphocytes. Indeed, the critical role of islet-reactive CD4$^+$ T cells in NOD diabetogenesis has been well established (5–9). Furthermore, the frequency of diabetogenic I-A^{g7}-restricted CD4$^+$ T cells in the peripheral repertoire as well as diversification of islet-specific CD4$^+$ T cell responses are strongly linked to the development of autoimmune diabetes (10–13). These findings have led to the notion that recruitment of a threshold frequency of diabetogenic T cells into the islet milieu may be required for progression to β cell destruction. However, the nature of Ag presentation responsible for the initial activation of anti-islet T cells and subsequent diversification of the diabetogenic T cell response remains unclear. In this regard, B lymphocytes, by virtue of their ability for uptake and presentation of specific Ags, are logical candidate APCs capable of driving an islet-destructive inflammatory process (14–17). In fact, several recent studies have conclusively demonstrated that B cell-deficient NOD mice are protected from autoimmune diabetes (18–21). B lymphocytes have also been implicated as critical APCs for the in vivo priming of potentially diabetogenic CD4$^+$ T cells (19, 21–25). However, direct evidence for a requisite role of B cell-mediated Ag presentation via I-A^{g7} in the evolution of a diabetogenic T cell response in NOD mice is lacking. To address this issue, we have examined the impact, on diabetogenesis, of selectively rendering the B cell compartment incapable of I-A^{g7}-mediated Ag presentation. In the present study, NOD mice with a B cell-restricted deficiency of I-A^{g7} expression (NOD B^{CIID}) were generated and monitored for susceptibility to anti-islet autoimmunity.

Materials and Methods

Mice

NOD/LtJ, NOD/*Scid*, and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free barrier conditions. I-A^{g7}(−/−) NOD mice were developed as previously described (26). μMT(−/−) NOD mice were generated by backcrossing the mutant μMT(−) allele from C57BL/6 mice onto the NOD background. The transgene status of the backcross progeny mice was determined by PCR screening performed on DNA extracted from tail tissue using previously described conditions and primers (27). At the seventh backcross, a founder μMT(+/−) NOD mouse was selected using PCR product length polymorphisms to identify an individual homozygous for the following microsatellite markers linked to all identified Idd loci in NOD mice: D17 Mit34, D9 Mit25, D3 Mit206, D3 Mit95, D3 Mit21, D3Nds6, D11 Mit115, D11Nds16, D11 Mit320, D1 Mit5, D1 Mit46, D1 Mit18, D6 Mit52, D6 Mit339, D7 Mit20, D14 Mit11, D14 Mit222, D14 Mit110, D4 Mit59, D3 Mit103, D3Nds11, D3Nds8, D2 Mit395, D2 Mit17, D13 Mit61, D5 Mit48, D5 Mit69. After selection of a founder mouse, we

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Received for publication February 22, 1999. Accepted for publication April 27, 1999.

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[1] This work was supported by Grants DK34878 and DK54215 from the National Institutes of Health and Juvenile Diabetes Foundation International. H.N. was supported by Training Grant NEI T32 from the National Eye Institute.

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[3] Abbreviations used in this paper: NOD, nonobese diabetic; AF, aldehyde fuchsin; CFSE, 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; GAD, glutamic acid decarboxylase; H&E, hematoxylin and eosin; BM, bone marrow.

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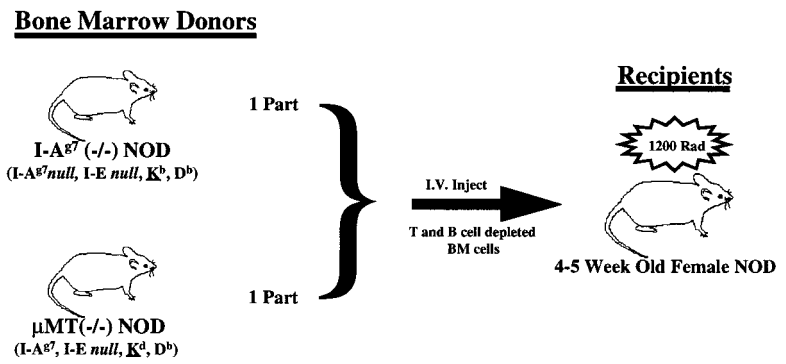

FIGURE 1. Strategy for the generation and expected reconstitution profile of chimeric NOD B^{CIID} mice. The MHC haplotype of the bone marrow donors is noted in parentheses. The expected contribution of each bone marrow donor to the reconstituting immune system of the NOD B^{CIID} mice is presented.

Donor Contribution to the Reconstituting Immune System of NOD B^{CIID} Mice

Origin of T cells	*Origin of B cells*	*Origin of Non-B Cell APCs*
I-A^{g7}(-/-) NOD (K^b)	I-A^{g7}(-/-) NOD (K^b)	I-A^{g7}(-/-) NOD (K^b)
μMT (-/-) NOD (K^d)		μMT (-/-) NOD (K^d)

continued backcrossing up to the tenth generation, intercrossed μMT(+/−) progeny, and selected B cell-deficient (μMT(−/−)) F_2 mice to expand the B cell-deficient NOD colony. All mice on the NOD genetic background were monitored weekly for the development of spontaneous diabetes. Blood glucose measurements were made using CHEMSTRIP bG (Boehringer Mannheim, Indianapolis, IN). Three consecutive daily nonfasting glucose measurements with values >250 mg/dl constituted a diagnosis of diabetes.

Induction of diabetes by cyclophosphamide

Mice were treated i.p. with 200 mg/kg of cyclophosphamide (Sigma, St. Louis, MO) dissolved in PBS. Two weeks following the initial treatment, remaining nondiabetic mice were treated a second time. Mice were followed weekly for the development of diabetes, as described above.

Generation of chimeric NOD mice

Five-week-old female NOD recipient mice were lethally irradiated (1200 rad), and within 10–24 h following irradiation were reconstituted with 5–10 × 10^6 T and B cell-depleted bone marrow cells from donor mice. NOD B^{CIID} mice were generated using a mixture (in a 1:1 ratio) of bone marrow cells from female I-A^{g7}(−/−) and female B cell-deficient (μMT(−/−)) NOD donor mice. Control chimeric mice were generated using a 1:1 mixture of bone marrow cells from female wild-type I-A^{g7} (+/− or +/+) and female B cell-deficient (μMT(−/−)) NOD donor mice. NOD intermediate B^{CIID} mice were generated using a 1:1 mixture of bone marrow cells from female wild-type I-A^{g7}(+/+) and I-A^{g7}(−/−) NOD mice. All mice were monitored up to 30 wk following reconstitution for the development of spontaneous diabetes, as described above.

Flow cytometry

A total of 1 × 10^6 cells was surface stained according to a previously described protocol (28). The following Abs were used: GK1.5-FITC (anti-CD4), 53-6.7-PE (anti-CD8a), RA3-6B2-biotin (anti-B220), 7G6-FITC (anti-CD21/35), Cy34.1-FITC (anti-CD22), 3/23-FITC (anti-CD40), IM7-FITC (anti-CD44), 10-3.6-FITC (anti-I-A^{g7}), 28-8-6 biotin (anti-H-2K^b/H-2D^b), SF1-1.1 biotin (anti-H-2K^d), 1B1 FITC (anti-CD1), 1D3-FITC (anti-CD19), 2.4G2-FITC (anti-CD16/32) (PharMingen, San Diego, CA), polyclonal anti-IgM-PE (Southern Biotechnology Associates, Birmingham, AL), and streptavidin-RED670 (Life Technologies, Gaithersburg, MD). All samples were analyzed on FACScan (Becton Dickinson, Mountain View, CA) using Cellquest software. Twenty thousand events were collected within a live lymphoid gate set based on forward and side scatter.

Histochemistry

Spleens were suspended in OCT, frozen in 2-methylbutane cooled with liquid nitrogen, sectioned, and fixed with acetone. The sections were blocked using PBS/5% normal goat serum/0.1% Tween 20, and then incubated with FITC-conjugated anti-I-A^{g7} (10-3.6) and biotin-conjugated anti-B220 (RA3-6B2) (PharMingen). Sections were incubated with streptavidin conjugated to HRP (Southern Biotechnology Associates) and alkaline phosphatase-conjugated anti-FITC (Sigma). HRP and alkaline phosphatase were then developed using the substrate 3-amino-9-ethylcarbazole and Fast-Blue BB base (Sigma), respectively. H&E and AF staining was performed on pancreata, as previously described (29), to identify islets and infiltrating lymphocytes.

ELISA

To determine the serum titers of total Ig, IgM, total IgG, IgG1, IgG2a, IgG2b, and IgG3, unlabeled goat anti-mouse total Ig Ab (Southern Biotechnology Associates) was used as the capture Ab, incubated with serum, and then developed with alkaline phosphatase-conjugated goat anti-mouse total Ig, IgM, IgG, or the IgG subclasses (Southern Biotechnology Associates). Serum titers of each isotype were determined by interpolating to 1/(serum dilution) at which an absorbance reading of 0.5 was achieved at the wavelength of 405λ used to detect the *p*-nitrophenyl phosphate substrate.

In vivo stimulation of I-A^{g7}-reactive CD4$^+$ T lymphocytes

Allogeneic CD4$^+$ T cells from BALB/c (H-2d) mice were utilized as I-A^{g7}-reactive T cells. To purify CD4$^+$ T cells, pooled splenic and lymph node cells were incubated with anti-CD8 and anti-B220 MACS beads (Miltenyi Biotech, Auburn, CA) and passed through a VarioMACS column. This procedure consistently yielded 90–95% purified CD4$^+$ T cells. Cells were then labeled with the fluorescent dye 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), as previously described (30, 31). A total of 20–30 × 10^6 CFSE-labeled CD4$^+$ T cells was then i.v. injected into stimulator mice. These stimulator mice had been lethally irradiated with 1500 rad 10–24 h before injection of responder CD4$^+$ T cells. After a 70-h stimulation period, splenocytes from the stimulator mice were isolated and counterstained with anti-CD4 PE to allow for the identification of the transferred CFSE-labeled, CD4$^+$ responder T cells using flow cytometry.

Results and Discussion

Generation of NOD mice with an I-A^{g7} deficiency confined to the B cell compartment

To establish whether B cells act as APCs necessary for the activation of diabetogenic CD4$^+$ T cells, we sought to abrogate the capacity of B cells to present Ags via I-A^{g7}. This goal was accomplished by generating NOD mice with a B cell-specific deficiency in I-A^{g7} expression, but with normal I-A^{g7} expression on non-B cell APCs. The strategy for the development of such NOD mice, termed NOD B^{CIID}, relied on the construction of mixed bone marrow (BM) chimeras, utilizing bone marrow stem cells from

female I-A^{g7}(−/−) and B cell-deficient (μMT−/−) NOD mice (Fig. 1). We reasoned that reconstitution of lethally irradiated female NOD recipients with stem cells from I-A^{g7}(−/−) NOD donors would lead to the development of an I-A^{g7}-deficient B cell compartment; whereas stem cells from μMT(−/−) NOD mice, although developmentally blocked in their ability to generate B cells, would give rise to I-A^{g7}-sufficient non-B cell APCs such as macrophages and DCs. Conveniently, cells originating from the I-A^{g7}(−/−) NOD donor bone marrow were phenotypically distinguishable from those derived from μMT(−/−) NOD (I-A^{g7}(+/+)) donor bone marrow. Lymphoid cells derived from μMT(−/−) stem cells express the H-2K^{d} MHC class I haplotype. On the other hand, those originating from I-A^{g7}(−/−) stem cells do not express H-2K^{d}, but instead express H-2K^{b}. This H-2K disparity between the bone marrow donors allowed the determination of the relative contribution of each donor bone marrow to the reconstituting immune system of NOD B^{CIID} mice. As will be discussed below, this H-2K disparity exists because the I-A^{g7}(−/−) NOD mice are congenic NOD mice whose MHC locus was derived from I-Aβ^{b}(−/−) mice.

A group of control chimeric female NOD mice with an I-A^{g7}-sufficient B cell compartment and harboring I-A^{g7}-sufficient non-B cell APCs was generated for comparison. Lethally irradiated female NOD recipients in this group were reconstituted with a mixed bone marrow cell inoculum from I-A^{g7}-sufficient (+/+ and +/−) NOD and B cell-deficient (μMT−/−) NOD mice. Another group of NOD mice that harbored a partially I-A^{g7}-deficient B cell compartment was also generated. These chimeras, termed NOD intermediate B^{CIID}, were constructed by reconstituting lethally irradiated female NOD recipients with a mixed bone marrow inoculum from I-A^{g7}-sufficient and I-A^{g7}(−/−) NOD mice at a 1:1 ratio.

Characterization of I-A^{g7}-deficient and B cell-deficient bone marrow donor NOD mice

I-A^{g7}(−/−) NOD mice. Congenic I-A^{g7}(−/−) NOD mice were generated by 11 backcrosses (genetically 99.95% NOD) of the I-Aβ^{b}(−) mutation from the H-2^{b} genetic background onto the NOD background (26). Female I-A^{g7}(−/−) NOD mice are completely protected from autoimmune diabetes as compared with their I-A^{g7}-sufficient (+/− and +/+) female littermates that exhibited a diabetes incidence of 60–70% by 25 wk of age (data not shown). In the present work, BM stem cells derived from I-A^{g7}(−/−) NOD mice were used as part of a donor cell inoculum to generate the I-A^{g7}-deficient B cell compartment in NOD B^{CIID} mice. As expected, I-A^{g7}(−/−) NOD mice are deficient in peripheral CD4^{+} T cells due to a failure of positive selection (32–34). Despite their CD4^{+} T cell and MHC class II deficiency, I-A^{g7}(−/−) NOD mice have a mature peripheral B cell compartment, albeit lacking MHC class II expression (data not shown). Thus, B cell development proceeds independently of CD4^{+} T cells and MHC class II expression by B cell precursors. Given the demonstrated importance of both I-A^{g7} (35–38) and CD4^{+} T cells (5–9) in NOD diabetogenesis, it is not surprising that female I-A^{g7}(−/−) NOD mice are protected from autoimmune diabetes as compared with their I-A^{g7}-sufficient female littermates.

μMT(−/−) NOD mice. Genetically B cell-deficient (μMT−/−) NOD mice at the tenth backcross (genetically 99.90% NOD) were utilized as a source of donor BM stem cells used, in part, to generate I-A^{g7}-sufficient non-B cell APCs in NOD B^{CIID} mice. μMT(−/−) NOD mice are blocked in their ability to generate a mature IgM^{+}/B220^{+} B cell compartment. Importantly, it was shown that B cell-deficient NOD mice develop normal CD4^{+} and CD8^{+} T cell compartments (18, 19). Furthermore, T lymphocytes from B cell-deficient NOD mice are functionally competent (19, 39). Several reports have demonstrated that genetically B cell-deficient NOD mice are resistant to the development of insulitis and autoimmune diabetes (18, 20, 21). Fig. 5*a* demonstrates that μMT(−/−) NOD mice in our colony are, indeed, protected from spontaneous diabetes as compared with their B cell-sufficient littermates. Thus, given the functional competency of T cells in B cell-deficient mice, the protection from autoimmune diabetes observed in these mice is most likely due to inefficient activation of islet Ag-specific T lymphocytes rather than an intrinsic T cell defect. In support of this possibility, two recent studies demonstrate inefficient activation of GAD-reactive CD4^{+} T cells in the B cell-deficient NOD mice (22, 23). Furthermore, GAD-reactive CD4^{+} T cells were shown to be efficiently primed in B cell-deficient NOD mice reconstituted with B cells (22), indicating an important role for B lymphocytes in priming the anti-GAD T cells.

NOD B^{CIID} mice have an I-A^{g7}-deficient B cell compartment and a phenotypically normal T cell compartment

NOD B^{CIID} mice were allowed to reconstitute for up to 30 wk. Representative mice were analyzed at several time points after reconstitution (starting at 12 wk). As shown in Fig. 2*a*, B220^{+}/IgM^{+} splenic B cells in NOD B^{CIID} mice were deficient in I-A^{g7} expression as compared with unmanipulated wild-type NOD mice. In contrast, B220^{+}/IgM^{+} splenic B cells from control chimeric NOD mice expressed I-A^{g7} at a level comparable with that seen in unmanipulated wild-type mice (Fig. 2*b*). In addition, Fig. 3*c* shows that NOD intermediate B^{CIID} mice reconstituted with a B cell compartment composed of ~50% I-A^{g7}-deficient and 50% I-A^{g7}-sufficient B cells. Importantly, splenic B cells in all groups of chimeras were proportionally and numerically comparable with unmanipulated age-matched NOD mice (Table I). I-A^{g7}-deficient B cells of NOD B^{CIID} mice did not express H-2K^{d} (Fig. 2*a*), as dictated by their origin from the I-A^{g7}(−/−) (H-2K^{b}) donors. Thus, the reconstitution of an I-A^{g7}-deficient B cell compartment in NOD B^{CIID} mice occurred, as was anticipated.

It was important to determine whether the B cell compartment of NOD B^{CIID} mice was functionally competent and phenotypically mature. Therefore, the cell surface phenotype of I-A^{g7}-deficient B cells in NOD B^{CIID} mice was characterized with respect to the expression of several markers of B cell development, including CD1, CD19, CD21/35, CD22, CD32, CD40, and CD44 (Fig. 2*c*). The expression of these markers on splenic B cells of NOD B^{CIID} mice was comparable with that seen in unmanipulated wild-type NOD mice (Fig. 2*c*), indicating that I-A^{g7}-deficient B cells in NOD B^{CIID} mice are developmentally mature. Functionally, B cells from NOD B^{CIID} mice proliferated normally in response to anti-IgM and LPS stimulation in vitro (data not shown). Collectively, these data indicate that peripheral B cells in NOD B^{CIID} mice, despite being I-A^{g7} deficient, are otherwise phenotypically and functionally normal.

To further characterize the competence of the B cell compartment in NOD B^{CIID} mice, serum from these mice was examined for the presence of the various Ig isotypes. ELISA quantification of total serum Ig, IgM, and IgG did not reveal any significant difference between NOD B^{CIID} mice and the control chimeric NOD mice at 25 wk after reconstitution (Fig. 2*d*). Furthermore, no appreciable differences were detectable between serum levels of IgG1, IgG2a, or IgG2b. On the other hand, an impressive 100-fold elevated titer of the IgG3 isotype was detected in serum from NOD B^{CIID} mice compared with that of the control chimeric NOD mice. Interestingly, serum titers of IgG3 were also elevated in I-A^{g7}(−/−) NOD mice (data not shown). The elevated titer of serum IgG3 in NOD B^{CIID} mice was not reflected in the total IgG titer due to the relatively small contribution of this isotype to the

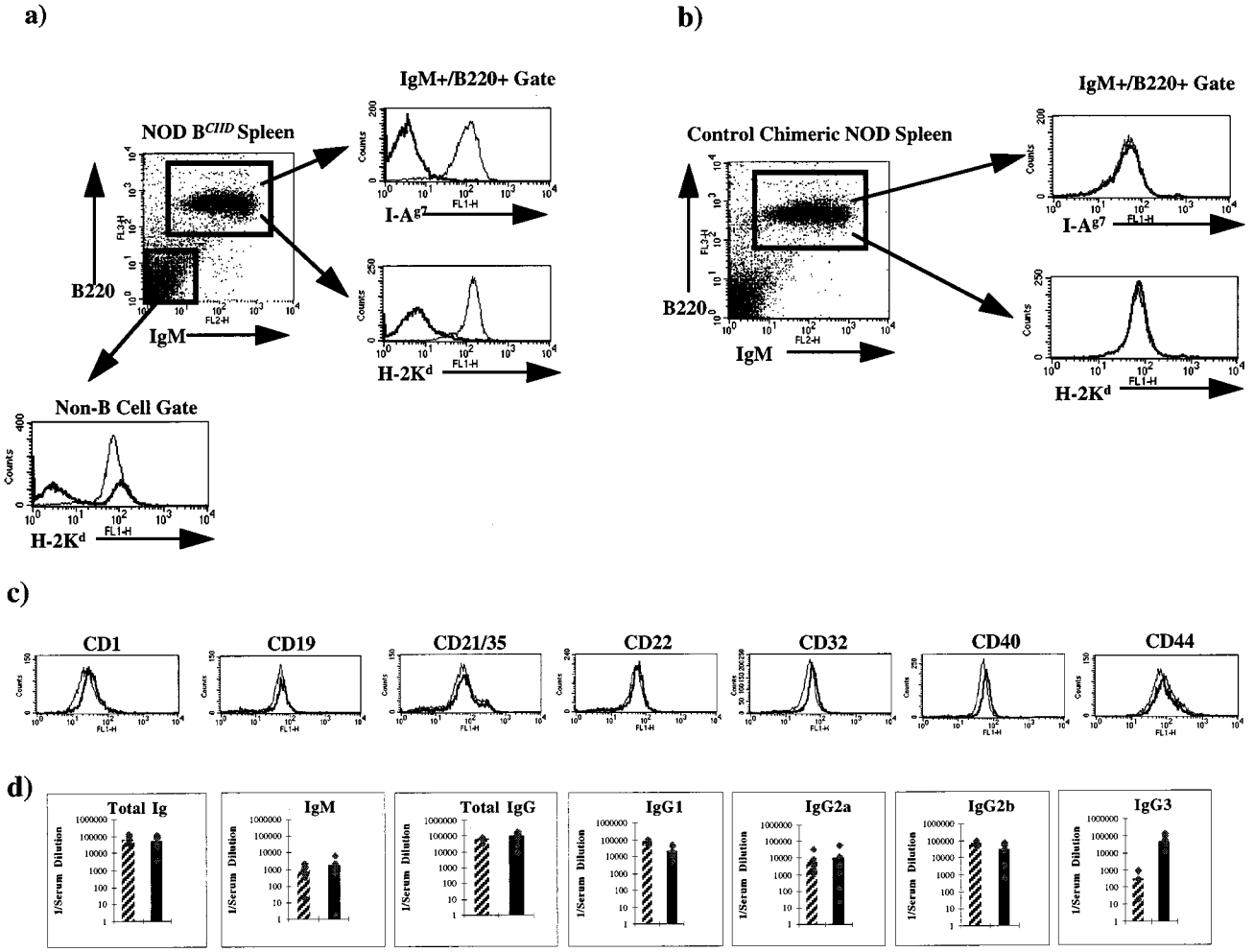

FIGURE 2. *a*, Flow-cytometric analysis of the splenocytes from NOD B^{CIID} mice revealed the presence of IgM$^+$/B220$^+$ B lymphocytes. IgM$^+$/B220$^+$-gated splenocytes from NOD B^{CIID} (bold line), and wild-type NOD control (solid line) mice were stained with anti-I-A^{g7} and anti-H-2K^d. Non-B cell-gated splenocytes from NOD B^{CIID} (bold line) and wild-type NOD (solid line) mice were stained with anti-H-2K^d. *b*, IgM$^+$/B220$^+$ B lymphocytes were present in spleens of control chimeric NOD mice. IgM$^+$/B220$^+$-gated splenocytes from control chimeric NOD (bold line) and wild-type NOD control (solid line) mice were stained with anti-I-A^{g7} and anti-H-2K^d. *c*, Flow-cytometric analysis and comparison of IgM$^+$/B220$^+$-gated splenic lymphocytes from NOD B^{CIID} (bold line) and wild-type NOD (solid line) mice with respect to the expression pattern of the indicated panel of B cell developmental/activation markers. *d*, ELISA assay for the quantification of the titers of all indicated Ig isotypes in serum from NOD B^{CIID} (solid bars) and control chimeric NOD (hatched bars) mice at 25 wk after reconstitution. Serum titers are standardized and expressed as (1/serum dilution) at which an absorbance reading of 0.5 was achieved. Diamonds represent values achieved from each individual mouse examined at 25 wk after reconstitution.

pool of serum IgG isotypes. The IgG3 isotype has been shown to be secreted preferentially in response to immunization with T cell-independent type 2 Ags (40, 41). That IgG3 was elevated in serum from both NOD B^{CIID} and I-A^{g7}(−/−) NOD mice is taken as evidence that the serum titer of this IgG isotype in wild-type NOD mice is regulated specifically by MHC class II-mediated cognate B cell interactions, presumably with CD4$^+$ T cells.

We next considered the functional integrity of the T cell compartment in NOD B^{CIID} mice. Interestingly, a recent study by the Benoist and Mathis laboratory (42) indicated that nonautoimmune chimeric mice with an MHC class II deficiency restricted to the B cell compartment (similar to the NOD B^{CIID} mice described herein) retain their ability to effectively prime a T cell response upon immunization with foreign Ag. This finding supports our contention that the T cell compartment of NOD B^{CIID} mice is not globally defective in its ability to initiate an immune response against inciting immunogens. In fact, we found that both CD4$^+$ and CD8$^+$ T cells were present in spleens and lymph nodes of NOD B^{CIID} and control chimeric mice as early as 6 wk postreconstitution (data not shown). Splenic (Table I) and lymph node (data not shown) CD4$^+$ and CD8$^+$ T cells in NOD B^{CIID} and control chimeric NOD mice were proportionally and numerically normal by 8 wk following reconstitution. It is known that both intra- and extrathymic APCs expressing MHC class II are required to drive the differentiation and survival of a normal peripheral CD4$^+$ T cell compartment (32–34, 43). Mice deficient in either extrathymic or intrathymic MHC class II-expressing APCs/stromal cells are defective in their ability to develop CD4$^+$ T cells. Therefore, the existence of a phenotypically normal CD4$^+$ T cell compartment in NOD B^{CIID} mice implied that sufficient MHC class II-expressing non-B cell APCs must have been present to drive the differentiation and survival of these T cells. In fact, examination of non-B cells in NOD B^{CIID} mice revealed that an equal proportion of these cells was derived from both H-2K^d-expressing (derived from μMT(−/−), I-A^{g7}-sufficient, H-2K^d donor) and non-H-2K^d-expressing (derived from I-A^{g7}(−/−), H-2K^b donor) NOD stem cells (Fig. 2*a*). This equal proportion of H-2K^d to non-H-2K^d-expressing non-B cells in NOD B^{CIID} mice conformed with the

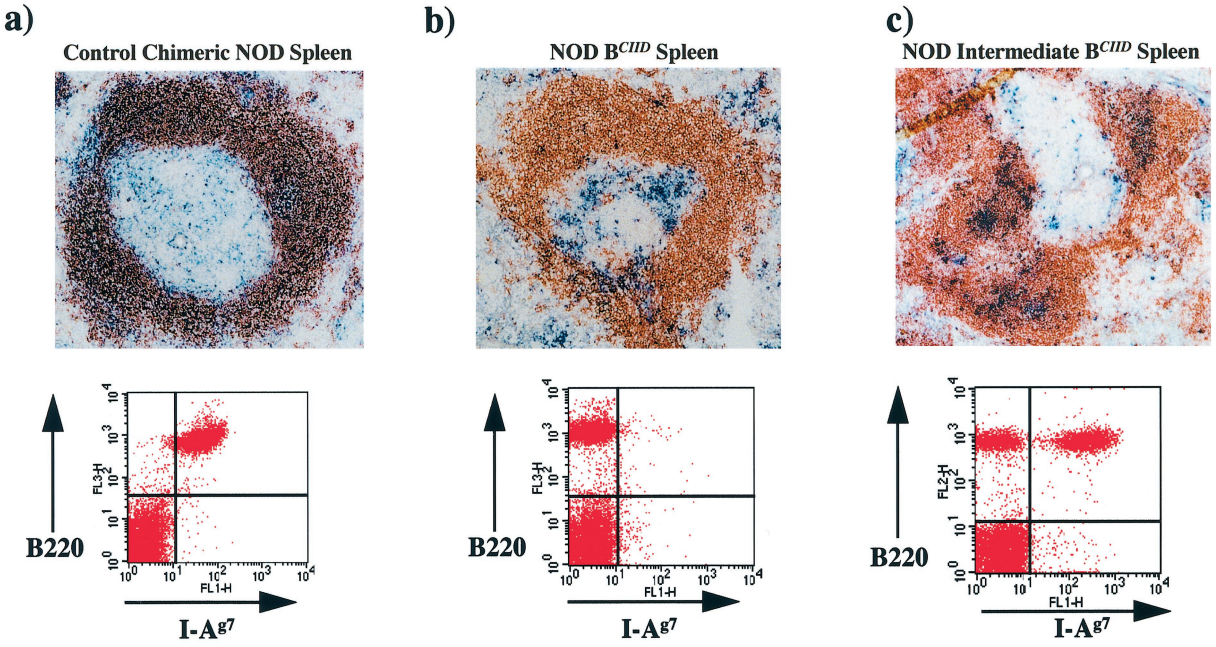

FIGURE 3. *a*, Immunohistochemical double staining of a splenic section from a representative control chimeric NOD mouse stained with anti-I-A^{g7} (blue stain) and anti-B220 (orange stain). Double-stained, I-A^{g7+}/$B220^+$ B cell follicles (dark brown) are found in control chimeric NOD chimeras. Flow-cytometric analysis reveals the presence of I-A^{g7+}/$B220^+$ double-stained splenic lymphocytes in control chimeric NOD mice. *b*, Immunohistochemical double staining of a splenic section from a representative NOD B^{CIID} mice stained with anti-I-A^{g7} (blue stain) and anti-B220 (orange stain). Flow-cytometric analysis reveals the presence of I-A^{g7-}/$B220^+$ single-stained splenic lymphocytes in NOD B^{CIID} mice. *c*, Immunohistochemical double staining of a splenic section from a representative NOD intermediate B^{CIID} mice stained with anti-I-A^{g7} (blue stain) and anti-B220 (orange stain). Flow-cytometric analysis reveals the presence of both I-A^{g7-}/$B220^+$ and I-A^{g7+}/$B220^+$ splenic lymphocytes in NOD intermediate B^{CIID} mice.

original 1:1 ratio of the mixed reconstituting donor BM stem cells. The expression of H-$2K^d$ by a portion of non-B cells suggested that I-A^{g7}-sufficient APCs (macrophages/DC), derived from I-A^{g7}-sufficient μMT(−/−) NOD BM, are present in NOD B^{CIID} mice. Next, we directly assessed NOD B^{CIID} mice for the presence and functional competence of non-B cell APCs.

I-A^{g7}-sufficient non-B cell APCs are present and functionally competent in NOD B^{CIID} mice

Immunohistochemical analysis of tissue sections from spleens of NOD B^{CIID} and control chimeric NOD mice allowed direct identification of MHC class II-expressing cells within the splenic white pulp of these mice. As expected, Fig. 3*a* demonstrates that the B cell follicles of control chimeric NOD mice express I-A^{g7}. This is consistent with the I-A^{g7}-sufficient phenotype of $B220^+$ splenic lymphocytes in these animals determined by flow cytometry (Fig. 3*a*). On the other hand, Fig. 3*b* shows that the B cell follicles of NOD B^{CIID} mice are virtually devoid of I-A^{g7} expression, consistent with the I-A^{g7}-deficient status of $B220^+$ splenocytes in these mice (Fig. 3*b*). Interestingly, both NOD B^{CIID} and control chimeric NOD mice contain I-A^{g7}-expressing non-B cell APCs in the inner-PALS/T cell zone. MHC class II-expressing cells confined to the T cell zone of the splenic white pulp are known to be of the macrophage/DC lineage. Thus, immunohistological analysis directly indicated that I-A^{g7}-sufficient non-B cell APCs are present in the peripheral lymphoid organs of NOD B^{CIID} mice.

To determine the functional competency of the I-A^{g7}-sufficient non-B cell APCs present in NOD B^{CIID} mice, we assessed the ability of these cells to directly activate I-A^{g7}-reactive $CD4^+$ T cells. To accomplish this task, we utilized an in vivo approach for stimulating I-A^{g7}-reactive $CD4^+$ T cells derived from allogeneic BALB/c mice (44). CFSE-labeled BALB/c responder $CD4^+$ T cells were adoptively transferred into irradiated stimulator mice to trace the division history of the transferred T cells over the course of a 70-h stimulation period. The division of $CD4^+$, I-A^{g7}-reactive T cells was used as an indicator of their activation status and the ability of APCs in the stimulator mice to prime such $CD4^+$ T cells. Fig. 4*a* shows that CFSE-labeled $CD4^+$ responder cells transferred into control chimeric NOD mice divide up to six generations during the stimulation period. That this division is specifically directed against I-A^{g7} is proven by the absence of such division upon transfer of responder cells into I-A^{g7}(−/−) (Fig. 4*b*) or syngeneic BALB/c stimulator mice. Importantly, as shown in Fig. 4*c*, transfer of responder cells into stimulator NOD B^{CIID} mice resulted in the efficient activation of I-A^{g7}-reactive $CD4^+$ T cells, as evidenced by up to six rounds of division similar to that seen in control

Table I. *Proportions and absolute numbers of $B220^+$, $CD4^+$, and $CD8^+$ Splenic lymphocytes in control chimeric NOD, NOD B^{CIID}, and wild-type NOD mice*

	Control Chimeric NOD (n = 6)		NOD B^{CIID} (n = 7)		Wild-Type NOD (n = 11)	
Lymphocyte Gate	Proportion (%)	Total no. ($\times 10^{-6}$)	Proportion (%)	Total no. ($\times 10^{-6}$)	Proportion (%)	Total no. ($\times 10^{-6}$)
$B220^+/IgM^+$	35 ± 5.3	31.5 ± 4.8	36.1 ± 4.7	30.7 ± 4.0	34.3 ± 5.3	25.0 ± 3.9
$CD4^+$	31 ± 2.5	27.9 ± 2.2	29.3 ± 4.2	28.4 ± 4.1	35 ± 6	30.1 ± 5.1
$CD8^+$	7.4 ± 1	7.0 ± 0.9	8.1 ± 1	7.2 ± 0.8	7.8 ± 1	6.6 ± 0.9

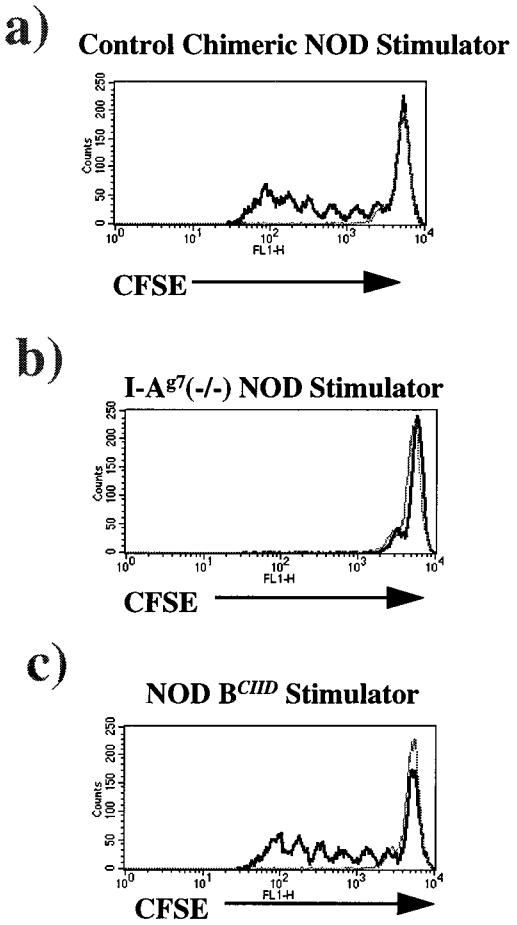

FIGURE 4. In vivo stimulation of I-A^{g7}-reactive BALB/c CD4$^+$ responder T lymphocytes. CFSE-labeled responder CD4$^+$ cells were transferred into lethally irradiated stimulator mice. Cell division was used as a measure of T cell activation. The bold line in each histogram maps the division history of reactive CD4$^+$ T cells in an irradiated stimulator of the genotype indicated above each histogram during the course of a 70-h stimulation period. The thin line in each histogram represents a control syngeneic BALB/c stimulator into which CFSE-labeled CD4$^+$ responder cells were adoptively transferred. The presented data are representative of three experiments. *a*, CD4$^+$ responder T cells transferred into control chimeric NOD (bold line) or syngeneic BALB/c (thin solid line) stimulator mice. *b*, CD4$^+$ responder T cells transferred into I-A^{g7}(−/−) NOD (bold line) or syngeneic BALB/c (thin solid line) stimulator mice. *c*, CD4$^+$ responder T cells transferred into NOD B^{CIID} (bold line) or syngeneic BALB/c (thin solid line) stimulator mice.

chimeric NOD stimulators (Fig. 4*a*). Therefore, despite the I-A^{g7}-deficient status of the B cell compartment in NOD B^{CIID} mice, I-A^{g7}-sufficient non-B cell APCs are present and functionally capable of activating I-A^{g7}-reactive CD4$^+$ T cells.

NOD B^{CIID} mice are resistant to the development of spontaneous autoimmune diabetes

NOD B^{CIID}, NOD intermediate B^{CIID}, and control chimeric NOD mice were monitored for the development of spontaneous autoimmune diabetes for up to 30 wk following their reconstitution. Strikingly, as shown in Fig. 5*b*, NOD B^{CIID} mice were protected from the onset of diabetes with only 1 of 24 NOD B^{CIID} mice spontaneously developing autoimmune diabetes. This was in contrast to 19 of 23 mice in the control chimeric NOD cohort and 5 of 9 NOD intermediate B^{CIID} mice that became diabetic at various time points between 12 and 30 wk following reconstitution. In concordance with these findings, examination of pancreata from a cohort of NOD B^{CIID} mice at 25 wk after reconstitution showed rare foci of peri-insulitis to be present (Fig. 6*a–d*). This finding indicates that the initial targeting of islets in NOD mice occurs independently of I-A^{g7}-mediated Ag presentation by B lymphocytes. On the other hand, as expected, invasive insulitis was present in pancreata from diabetes-prone control chimeric NOD mice (Fig. 6, *e* and *f*), but not the NOD B^{CIID} mice. The benign nature of insulitis in NOD B^{CIID} mice, together with their diabetes-resistant phenotype, argues in favor of the importance of I-A^{g7}-mediated Ag presentation by B cells for the conversion to invasive insulitis and progression to diabetes. A potential limitation of this interpretation is that the H-2^b-derived MHC loci expressed by cells derived from the congenic I-A^{g7}(−/−) NOD BM donor mice might have exerted a diabetes-resistant effect in NOD B^{CIID} mice. In this regard, given that the NOD intermediate B^{CIID} mice were found to be diabetes susceptible despite having been partially reconstituted with bone marrow from I-A^{g7}(−/−) mice strongly suggests that H-2^b-linked loci derived from the I-A^{g7}(−/−) congenic NOD donors do not contribute a dominant diabetes-resistant effect. Thus, the lack of I-A^{g7}-mediated Ag presentation by B lymphocytes rather than a diabetes-resistant effect of the I-A^{g7}(−/−) BM donor most likely accounts for the diabetes-resistant phenotype of NOD B^{CIID} mice. Overall, these findings suggest that the initial targeting of islets proceeds efficiently in the absence of MHC class II-mediated Ag presentation by B cells. However, conversion of this process into a diabetogenic response is dependent upon B cell-mediated I-A^{g7} Ag presentation. To test this latter hypothesis, we next sought to intentionally overcome the benign nature of insulitis targeting the islets of NOD B^{CIID} mice by provoking its conversion to a diabetogenic process.

Nondestructive insulitis in NOD B^{CIID} mice can be converted into a β islet cell-destructive process

To determine whether the benign insulitis observed in NOD B^{CIID} mice could be converted into a pathogenic process, a cohort of these mice was treated with cyclophosphamide at 12 wk after reconstitution. Fig. 5*c* demonstrates that cyclophosphamide treatment of nondiabetic control chimeric NOD and NOD B^{CIID} mice led to the development of diabetes in both groups. Cyclophosphamide-induced diabetes did not occur in insulitis-free BALB/c or NOD/*Scid* mice, indicating its specific dependence on the presence of the inflamed islet milieu of NOD mice. Interestingly, cyclophosphamide treatment of a cohort of nondiabetic female B cell-deficient NOD mice at ~35 wk of age led to diabetes in 20% of these mice within 2 wk (Fig. 5*c*).

Cyclophosphamide treatment is known to overcome a checkpoint in pathogenicity of diabetogenic T cells after the initial targeting of islets has occurred. In fact, induction of cyclophosphamide-induced diabetes requires preexisting insulitis in treated animals and is dependent on the severity of islet inflammation (45). Therefore, that cyclophosphamide can overcome diabetes resistance in insulitis-prone NOD B^{CIID} mice and a number of older nondiabetic B cell-deficient NOD mice indicates that islet β cells in these mice are targeted by potentially diabetogenic lymphocytes that, unless provoked, are unable to mediate destruction in the absence of I-A^{g7}-mediated Ag presentation by B cells or B cell deficiency, respectively. A model for NOD diabetogenesis proposes that progression to fulminant diabetes is dependent upon bypassing two temporally distinct checkpoints: initial targeting of islet β cells (checkpoint 1) and later conversion of nondestructive insulitis into a diabetogenic inflammatory process (checkpoint 2) (46). As is evident from the presence of noninvasive insulitis in

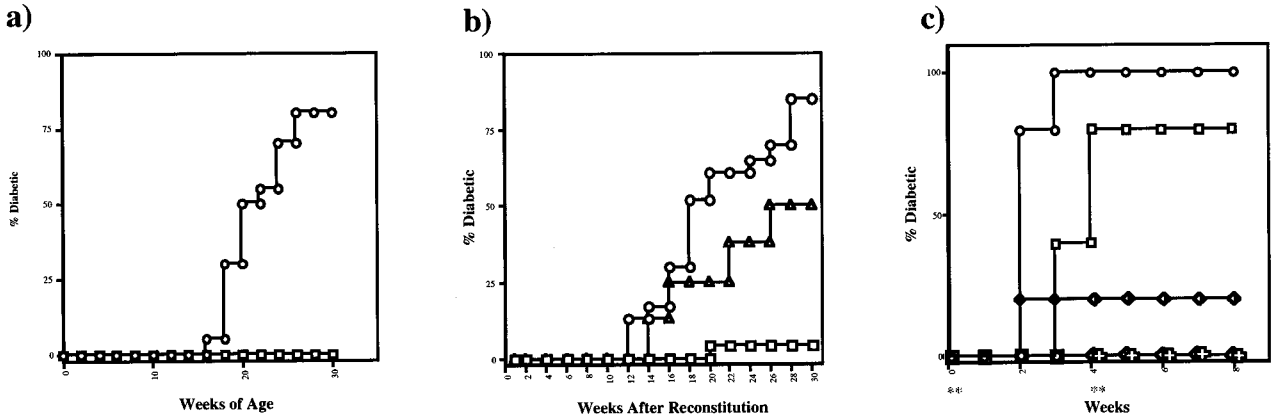

FIGURE 5. *a*, Incidence of spontaneous diabetes in a cohort of B cell-deficient female NOD (□; $n = 30$) and wild-type female NOD (○; $n = 30$) mice. *b*, Incidence of spontaneous diabetes in a cohort of female NOD B^{CIID} (□; $n = 24$), control chimeric NOD (○; $n = 23$), and NOD intermediate B^{CIID} (△; $n = 9$) mice. *c*, Cyclophosphamide-induced diabetes in a group of NOD B^{CIID} (□; $n = 10$), control chimeric NOD (○; $n = 8$), BALB/c (◇; $n = 5$), NOD/*Scid* (✣; $n = 10$), and female μMT(−/−) NOD (◆; $n = 10$) mice. NOD B^{CIID} and control chimeric NOD mice were treated with cyclophosphamide at 12 wk after reconstitution. μMT(−/−) NOD mice were free of spontaneous diabetes up to ~35 wk of age, at which time they were first treated with cyclophosphamide. Asterisks indicate the time of treatment with cyclophosphamide.

NOD B^{CIID} mice, checkpoint 1 appears to be spontaneously overcome in the absence of I-A^{g7} Ag presentation by B cells, and is, therefore, independent of B lymphocytes. However, overcoming checkpoint 2 requires MHC class II-mediated Ag presentation by B cells and is, thus, not easily overcome spontaneously in NOD B^{CIID} mice with I-A^{g7}-deficient B cells, unless their benign insulitis is potentiated by cyclophosphamide treatment.

Collectively, using the NOD model as an example of a spontaneous T cell-mediated autoimmune disease, the importance of B cell MHC class II Ag presentation as a regulator of peripheral T cell tolerance was examined. Specifically, the requirement of I-A^{g7}-mediated Ag presentation by B cells for diabetogenesis in NOD mice was determined. We generated NOD B^{CIID} mice with an I-A^{g7} deficiency confined to the B cell compartment. Despite the presence of noninvasive insulitis, NOD B^{CIID} mice were found to be resistant to the spontaneous development of autoimmune diabetes. This observation highlights the critical requirement for I-A^{g7}-mediated cognate T/B interactions for the progression of insulitis from its benign form to a β cell-destructive inflammatory process. It has been suggested that the mIg specificity of B cells

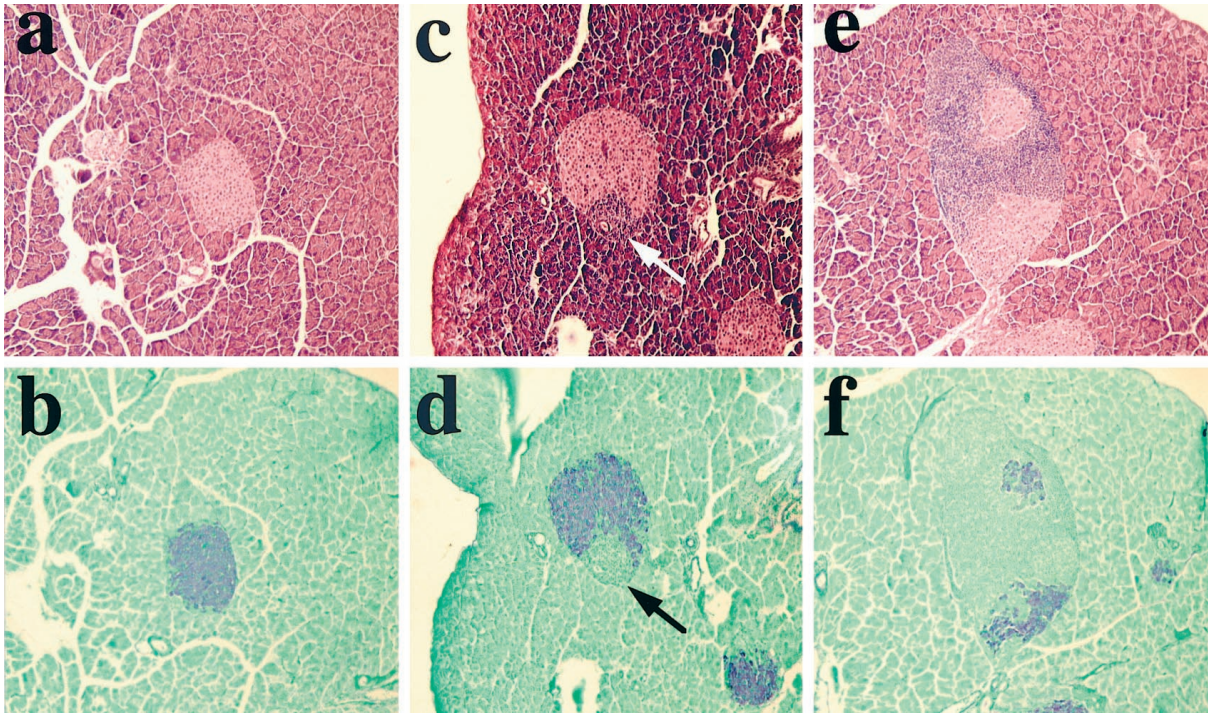

FIGURE 6. *a–d*, Representative H&E- and AF-stained serial sections of pancreas from NOD B^{CIID} mice. The majority of islets in NOD B^{CIID} mice are healthy and noninsulitic, as shown in *a*. AF staining of a serial section of the same pancreas in *b* shows insulin-containing islet β cells staining dark blue. Rare sections of pancreas from NOD B^{CIID} mice showed a mild peri-insulitis. H&E staining in *c* shows the presence of a noninvasive pole (see arrows) of mononuclear cells adjacent to the islet. AF staining of a serial pancreatic section in *d* shows insulin-containing islet β cells stained dark blue. *e* and *f*, H&E- and AF-stained serial sections of pancreas from control chimeric NOD mice showing typical invasive insulitis. H&E staining in *e* shows the presence of invasive mononuclear cell infiltration of the islet. AF staining of a serial pancreatic section in *f* shows the remaining islet β cells stained dark blue.

imposes a selective uptake of Ags leading to the presentation of a limited array of Ags at high density by individual B lymphocytes (47). Thus, the dependence of NOD diabetogenesis on the expression of I-A^{g7} by B lymphocytes may stem from the unique capacity of B lymphocytes for specific uptake and efficient MHC class II-mediated presentation of islet autoantigens.

Finally, using the NOD model as a naturally occurring paradigm, the present study establishes B cell-mediated MHC class II Ag presentation as a critical regulator of peripheral T cell tolerance. We speculate that the full expression of the autoreactive potential of the T cell repertoire is intimately regulated by B cell Ag presentation that, in disease-susceptible individuals, could serve to overcome checkpoints in the maintenance of peripheral T cell tolerance to organ-specific targets.

Acknowledgments

We thank Amy J. Reed for invaluable discussion and comments on the manuscript, Dr. Jan Erikson and Anh Bui for expert technical advice regarding immunohistochemistry, and Brigitte Koeberlein for preparation of H&E and AF pancreatic sections.

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