B Lymphocytes Are Critical Antigen-Presenting Cells for the Initiation of T Cell-Mediated Autoimmune Diabetes in Nonobese Diabetic Mice

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Nonobese diabetic (NOD) mice genetically deficient in B lymphocytes (NOD.*Igμ^{null}*) are resistant to T cell-mediated autoimmune insulin-dependent diabetes mellitus (IDDM). Ig infusions from diabetic NOD donors did not abrogate IDDM resistance in NOD.*Igμ^{null}* mice. However, T cell responses to the candidate pancreatic β cell autoantigen glutamic acid decarboxylase (GAD), but not the control Ag keyhole limpet hemocyanin, were eliminated in NOD.*Igμ^{null}* mice. To initially test whether they contribute to IDDM as APC, NOD B lymphocytes were transferred into NOD.*Igμ^{null}* recipients. B lymphocytes transferred into unmanipulated NOD.*Igμ^{null}* recipients were rejected by MHC class I-restricted T cells. Stable T and B lymphocyte repopulation was achieved in irradiated NOD.*Igμ^{null}* mice reconstituted with syngeneic bone marrow admixed with NOD B lymphocytes. IDDM susceptibility was restored in NOD.*Igμ^{null}* mice reconstituted with syngeneic marrow plus B lymphocytes, but not with syngeneic marrow only. T cell responses to GAD were restored only in NOD.*Igμ^{null}* mice reconstituted with syngeneic marrow plus B lymphocytes. Hence, B lymphocytes appear to contribute to IDDM in NOD mice as APC with a preferential ability to present certain β cell Ags such as GAD to autoreactive T cells. ***The Journal of Immunology*****, 1998, 161: 3912–3918.**

In both humans and nonobese diabetic (NOD)[2] mice, insulin-dependent diabetes mellitus (IDDM) results from autoimmune destruction of pancreatic β cells by T lymphocytes (1, 2). The primary diabetes susceptibility (*Idd*) genes in both humans and NOD mice are provided by multiple alleles within specific MHC haplotypes (3–5). However, the development of IDDM in both genera also requires contributions from multiple non-MHC *Idd* genes (3–5). In NOD mice, it appears that interactions between *Idd* susceptibility genes both within and outside of the unusual *H2^{g7}* MHC haplotype (K^d, $I\text{-}A^{g7}$, $I\text{-}E^{null}$, D^b) diminishes the ability of hematopoietically derived APC to activate various immunoregulatory functions that would normally block the development and/or function of T lymphocytes autoreactive against pancreatic β cells (6).

Subpopulations of hematopoietically derived APC include B lymphocytes, macrophages, and dendritic cells (DC). To partially address which of these APC subpopulations could exert immunotolerogenic defects underlying the development of IDDM, we produced a stock of NOD mice made deficient in B lymphocytes by congenic transfer of an *Igμ* gene functionally disrupted by homologous recombination (formally designated as *Igh6^{tmiCgn}* and, here, as *Igμ^{null}*) (7). It had been reported that B lymphocytes have a greater capacity to induce various immunotolerogenic functions than other APC populations (8–10). Thus, it was anticipated that the elimination of B lymphocytes would not alter, or might even accelerate, IDDM development in NOD mice. However, we were surprised to find that B lymphocyte-deficient NOD.*Igμ^{null}* mice are IDDM resistant (7). Similar results were obtained by two other groups that produced B lymphocyte-deficient NOD mice by either congenic transfer of an *Igμ^{null}* allele or treatment with a μ chain-specific Ab (11, 12). The finding that IDDM is inhibited rather than accelerated in NOD.*Igμ^{null}* mice indicated that B lymphocytes played a newly identified diabetogenic role that is distinct, but not mutually exclusive, from the APC-controlled tolerogenic defects underlying the original development of β cell-autoreactive T cells in NOD mice.

This newly identified diabetogenic role for B lymphocytes in NOD mice could be as APC with an unique ability to process and present certain β cell Ags to autoreactive T cells that have been generated as a consequence of the tolerogenic defects described above. Alternatively, B lymphocytes may contribute to IDDM in NOD mice through their ability to secrete autoantibodies that bind to pancreatic β cells and subsequently trigger autoreactive T cells through an Ab-dependent cell-mediated cytotoxicity response. The present study was conducted to determine whether B lymphocytes contribute to the development of T cell-mediated autoimmune IDDM in NOD mice through either of these two mechanisms.

Materials and Methods

Mice

NOD/Lt mice are maintained at The Jackson Laboratory (Bar Harbor, ME) by brother-sister mating. Currently, IDDM develops in 90% of female and 63% of male NOD/Lt mice by one year of age. Derivation of a "speed congenic" N7 backcross stock of B lymphocyte-deficient NOD.*Igμ^{null}* mice fixed to homozygosity for linkage markers delineating all previously identified *Idd* loci of NOD origin has been described previously (7). From >900 NOD.*Igμ^{null}* mice generated to date, only 4 female breeders of >30 wk of age have spontaneously developed IDDM. The previously described stock of T and B lymphocyte-deficient NOD-*scid* (official designation NOD-*Prkdc^{scid}*) mice is maintained at the N11 backcross generation (13, 14). Similarly, the previously described congenic stock of MHC class I and $CD8^+$ T cell-deficient NOD.*β2m^{null}* (official designation NOD.*β2m^{tm1Unc}*) mice is also maintained at the N11 backcross generation (15). These latter two strains served as progenitors for the previously described stock of

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[2] Abbreviations used in this paper: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; DC, dendritic cells; MIS, mean insulitis score; GAD, glutamic acid decarboxylase; KLH, keyhole limpet hemocyanin; LN, lymph nodes.

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NOD mice homozygous for both the *scid* and $\beta 2m^{null}$ mutations (designated NOD-*scid*.$\beta 2m^{null}$) (16, 17). Mice housed at The Jackson Laboratory were maintained under specific pathogen-free conditions and allowed free access to food (National Institutes of Health diet 31A, Purina, Richmond, IN) and acidified drinking water. In addition, all stocks of *scid* mice were treated for 3 days/week with trimethoprim-sulfamethoxazole (Sulfatrim, Barre National, Baltimore, MD) in the drinking water. Some experiments used NOD/Lt and NOD.$Ig\mu^{null}$ mice housed in the animal facility of the Department of Microbiology and Immunology at the University of North Carolina at Chapel Hill. Mice in this facility were maintained under viral pathogen-free conditions and allowed access to National Institutes of Health diet 31A (Purina) and acidified drinking water.

Assessment of diabetes and insulitis development

The indicated mice were monitored for development of glycosuria with Ames Diastix (kindly supplied by Miles Diagnostics, Elkhart, IN). Glycosuric values of ≥ 3 were considered diagnostic of diabetes onset. Pancreases from mice assessed for insulitis development were fixed in Bouin's solution and sectioned at three nonoverlapping levels. Granulated β cells were stained with aldehyde fuchsin and leukocytes with a hematoxylin and eosin counterstain. Islets (at least 25/mouse) were individually scored as follows: 0, no lesions; 1, peri-insular leukocytic aggregates, usually periductal infiltrates; 2, $<25\%$ islet destruction; 3, $>25\%$ islet destruction; and 4, complete islet destruction. An insulitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined. Data are presented as mean insulitis score (MIS) $\pm$ SEM for the indicated experimental group.

Ag preparations

The cloning and preparation of the 65-kDa isoform of the candidate murine β cell autoantigen glutamic acid decarboxylase (GAD) has been described previously (18). Briefly, the cDNA was engineered to encode six histidine residues at the COOH terminus of the protein. Recombinant murine GAD65 was generated in SF21 cells using a *Baculovirus* expression system and purified using a Ni^{2+}-conjugated resin (Invitrogen, San Diego, CA). The GAD65 was further purified by preparative SDS-PAGE. The protein was then electroeluted and extensively dialyzed against PBS. An SF21 cell extract purified in an identical manner to the recombinant GAD65 protein is not antigenic to T cells. Keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, MO) was used as a control Ag.

Assessment of Ag-primed T cell responses

The indicated number of NOD or NOD.$Ig\mu^{null}$ mice were immunized in a hind foot pad with a 50-μl emulsion of CFA containing 20 μg of GAD65. After 10 days, T cells were purified by the previously described panning technique (14) from pooled draining lymph nodes (LN) of mice in each group. Triplicate aliquots of 2.5×10^5 T cells were seeded into flat-bottom 96-well microtiter plates in a final volume of 200 μl of the previously described culture medium (19) containing the indicated concentration of GAD, plus 2.5×10^5 irradiated (2000 rad) splenic leukocytes from NOD or NOD.$Ig\mu^{null}$ mice as a source of APC. Following a 72-h incubation at 37°C in a 95% air/5% CO_2-humidified atmosphere, the cultures were pulsed with 1 μCi/well of [^{3}H]thymidine for an additional 16 h. The cultures were then harvested, and [^{3}H]thymidine incorporation was determined using an LKB Betaplate 1205 system (LKB Instruments, Gaithersburg, MD). Data are presented as mean cpm $\pm$ SEM.

In other experiments, the indicated mice were immunized in a hind foot pad with a 50-μl emulsion of CFA containing 20 μg of GAD65 or KLH. At 10 days after priming, single-cell suspensions were prepared from pooled draining LN of two to three mice in each experimental group. Triplicate aliquots of 5×10^5 LN cells were seeded into flat-bottom 96-well microtiter plates in a final volume of 200 μl of medium with or without 10 μg/ml of GAD or KLH plus 5×10^5 irradiated (2000 rad) NOD or NOD.$Ig\mu^{null}$ splenocytes as an additional source of APC. Following a 72-h incubation, the cultures were pulsed with 1 μCi/well of [^{3}H]thymidine for an additional 16 h. Data are presented as mean Δcpm $\pm$ SEM (calculated from the mean Ag stimulated minus unstimulated responses).

Assessment of APC requirements for preactivated GAD-autoreactive T cell clones from NOD mice

GAD65-specific T cell clones were established by culturing 5×10^6 splenocytes from 4-wk-old unimmunized NOD female mice in a 24-well plate in 1.5 ml of RPMI 1640, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, 1$\times$ nonessential amino acids, 1 mM glutamine, 1.0% NOD serum, and 10 μg/ml of intact murine GAD65 for 7 days. T cells (1×10^6) harvested on a Lympholyte M gradient (Cedarlane Laboratories, Hornby, Ontario, Canada) were cultured with 5×10^6 irradiated (3000 rad) NOD splenocytes in 1.5 ml of the above medium and 10 μg/ml GAD65 in a 24-well plate. After 3 days, the cultures were supplemented with medium containing 20 U/ml murine IL-2 (PharMingen, San Diego, CA) and maintained for an additional 3 days, at which time $CD4^+$ T cells were purified using magnetic bead separation (Miltenyi Biotec, Auburn, CA). GAD65-specific $CD4^+$ T cell clones were established via limiting dilution.

To assess the capacity of APC from NOD vs NOD.$Ig\mu^{null}$ mice to activate GAD65-specific T cell clones, 2.5×10^4 cells from the GAD65-specific 6E12 T cell clone were cultured with 2.5×10^5 irradiated (3000 rad) splenocytes from NOD or NOD.$Ig\mu^{null}$ mice in 0.1 ml of the above medium containing 10% FBS with or without 10 μg/ml of GAD65 for 72 h in a 96-well plate. T cell proliferation was assessed by measuring the amount of [^{3}H]thymidine incorporation following a 16-h pulse (1 μCi/well). Data are presented as mean Δcpm $\pm$ SEM of triplicate cultures.

Purification of B lymphocytes

B lymphocytes were purified from NOD splenic leukocyte preparations using a streptavidin-conjugated magnetic bead system (Miltenyi Biotec) to deplete T cells and macrophages/granulocytes that had been prestained with biotinylated mAbs specific for CD3 (145-2C11) or Mac-1 (M1/70), respectively. Subsequent FACS analysis using an FITC-conjugated goat polyclonal antiserum specific for mouse Ig (Southern Biotechnology Associates, Birmingham, AL) indicated that $>95\%$ of the resulting cell preparation consisted of B lymphocytes.

*Effect of Ig reconstitution on IDDM development in NOD.*Igμ^{null} *mice*

Total Ig was precipitated from pooled serum of overtly diabetic NOD females by 40% saturation with $(NH_4)_2SO_4$. The precipitated Ig was then dialyzed extensively against PBS and quantified by ELISA as described previously (16). Female NOD.$Ig\mu^{null}$ mice were injected i.p. with 70 μg of this Ig preparation twice weekly between 8 and 20 wk of age and simultaneously monitored for diabetes development. Controls consisted of NOD.$Ig\mu^{null}$ injected on the same schedule with the PBS vehicle alone. At 20 wk of age, circulating serum Ig levels in both groups were determined by ELISA. Insulitis development was also assessed at this time.

*Effect of NOD.*Igμ^{null} *T cells on NOD B lymphocyte repopulation*

In an initial experiment, a group of 6-wk-old NOD.$Ig\mu^{null}$ female mice were injected i.v. with 3×10^6 purified B lymphocytes from standard NOD donors. Controls consisted of NOD-*scid* females injected with the same preparation of B lymphocytes. At the indicated timepoints, the proportion of donor B lymphocytes among PBL in the NOD.$Ig\mu^{null}$ and NOD-*scid* recipients were assessed by FACS using the FITC-conjugated polyclonal antiserum specific for mouse Ig described above.

In subsequent experiments, 3×10^6 purified B lymphocytes from NOD or NOD.$\beta 2m^{null}$ female donors, respectively, were injected i.v. into 6-wk-old NOD-*scid* and NOD-*scid*.$\beta 2m^{null}$ female mice. At the indicated timepoints, the proportion of donor B lymphocytes among PBL in the NOD-*scid* and NOD-*scid*.$\beta 2m^{null}$ recipients was assessed by FACS. Total PBL counts were also determined to calculate the total number of donor B lymphocytes per ml of peripheral blood in the NOD-*scid* and NOD-*scid*.$\beta 2m^{null}$ recipients. After stable B lymphocyte repopulation was achieved, a subset of the NOD-*scid* and NOD-*scid*.$\beta 2m^{null}$ recipients were injected i.v. with 1×10^7 T cells purified from the spleens of female NOD.$Ig\mu^{null}$ donors as described previously (14). At weekly intervals thereafter, total numbers of donor B lymphocytes per ml of peripheral blood were compared in NOD-*scid* and NOD-*scid*.$\beta 2m^{null}$ recipients that had or had not been subsequently injected with NOD.$Ig\mu^{null}$ T cells.

Generation of mixed bone marrow/B lymphocyte chimeras

Female NOD.$Ig\mu^{null}$ mice were lethally irradiated (1200 rad) at 4 wk of age and then reconstituted as described previously (20) with 5×10^6 T cell-depleted syngeneic bone marrow cells that had been mixed with 3×10^6 purified NOD B lymphocytes. Control chimeras consisted of NOD.$Ig\mu^{null}$ females reconstituted with syngeneic bone marrow only. Bone marrow chimeras were then monitored at 21 wk postreconstitution for diabetes development. Pancreases from mice that remained normoglycemic through 21 wk postreconstitution were assessed for insulitis development. In addition, upon diabetes onset or at 21 wk postreconstitution, splenic leukocytes from the bone marrow chimeras were typed by FACS for the presence of B lymphocytes as described above and for $CD4^+$ and $CD8^+$ T lymphocytes with the mAbs GK1.5 and 53-6.72, respectively. Furthermore, at 8 wk postreconstitution, a subset of NOD.$Ig\mu^{null}$ mice reconstituted with syngeneic bone marrow in the presence

Table I. *Ig-reconstituted NOD.* Igμ^{null} *mice remain IDDM-resistant*[a]

NOD. *Ig*μ^{null} Treatment Group	Mean Serum Ig Concentration at 20 Weeks of Age	IDDM Incidence at 20 Weeks of Age	MIS at 20 Weeks of Age
Diabetic NOD Ig (n = 8)	69.2 ± 4.9 μg/ml	0/8	0.85 ± 0.40
Vehicle control (n = 8)	0.9 ± 0.2 μg/ml	0/8	1.29 ± 0.36

[a] IDDM development was monitored in female NOD. *Ig*μ^{null} mice injected twice weekly between 8 to 20 wk with either 70 μg of Ig isolated from overtly diabetic NOD donors or the PBS vehicle alone. At 20 wk of age, circulating serum levels of Ig and insulitis development were assessed in both groups as described in *Materials and Methods*.

or absence of NOD B lymphocytes were assessed for presence of GAD-primed T cell responses as described above.

Results

*Ig-reconstituted NOD.*Igμ^{null} *mice remain IDDM resistant*

We hypothesized that, if the production of autoantibodies represents the primary mechanism by which B lymphocytes contribute to the development of IDDM, disease resistance would be abrogated in NOD.*Ig*μ^{null} mice infused with Ig isolated from overtly diabetic NOD donors. Two groups of NOD.*Ig*μ^{null} females were injected twice weekly from 8 to 20 wk of age with either 70 μg of Ig isolated from overtly diabetic NOD donors or with the PBS vehicle control. By 20 wk of age, ELISA measurements confirmed the presence of circulating Ig in sera of the experimental (69.2 ± 4.9 μg/ml), but not the control group (0.9 ± 0.2 μg/ml), of NOD.*Ig*μ^{null} mice (Table I). However, IDDM failed to develop in any of the Ig-reconstituted (0/8) or control (0/8) NOD.*Ig*μ^{null} female mice. Furthermore, the level of insulitis in Ig-reconstituted NOD.*Ig*^{null} mice (MIS = 0.85 ± 0.40) was not significantly different from the low levels observed in PBS-treated controls (MIS = 1.29 ± 0.36). Thus, the levels of Ig reconstitution achieved in this experiment failed to accelerate autoimmune destruction of pancreatic β cells in NOD.*Ig*^{null} mice. This indicates that the production of autoantibodies is unlikely to represent the primary mechanism by which B lymphocytes contribute to the development of T cell-mediated autoimmune IDDM in standard NOD mice.

B lymphocytes are necessary APC for initiating T cell responses to the candidate β cell autoantigen GAD in NOD mice

Given that Ig infusions did not abrogate IDDM resistance or promote insulitis development in NOD.*Ig*μ^{null} mice, we hypothesized that the pathogenic role for B lymphocytes in this disease may be as APC with a unique ability to process and present certain pancreatic β cell Ags to autoreactive T cells. As an initial test of this hypothesis, we determined whether NOD and NOD.*Ig*μ^{null} mice differed in ability to generate T cell responses to the candidate pancreatic β cell autoantigen GAD. While clearly many different β cell proteins are recognized by autoreactive T cells in IDDM, we chose GAD as a model autoantigen for our studies based on reports that it is among the earliest targets of diabetogenic T cells in NOD mice (18, 21). NOD.*Ig*μ^{null} mice were characterized by an absence of spontaneous T cell responses to GAD (data not shown). However, it was possible that GAD-reactive T cells were still present but not efficiently activated in NOD.*Ig*μ^{null} mice because of a quantitative loss in APC resulting from the absence of B lymphocytes. We addressed this issue through use of an in vivo priming protocol that would amplify any GAD-reactive T cells present in either standard NOD or NOD.*Ig*μ^{null} mice. As expected, T cells from GAD65-primed NOD mice proliferated when restimulated with this Ag in vitro in the presence of standard B lymphocyte-positive NOD APC and, to a lesser extent, in the presence of B lymphocyte-deficient APC from NOD.*Ig*μ^{null} mice (Fig. 1). In contrast, T cells from GAD65-primed NOD.*Ig*μ^{null} mice failed to respond upon antigenic restimulation in vitro in the presence of either B lymphocyte intact or deficient APC. These results indicated that the initial in vivo priming of GAD-reactive T cell responses in NOD mice requires the presence of Ag-presenting B lymphocytes. However, other APC populations such as macrophages and DC appear to be able to process and present GAD65, albeit less efficiently than B lymphocytes, to NOD T cells previously primed against this Ag.

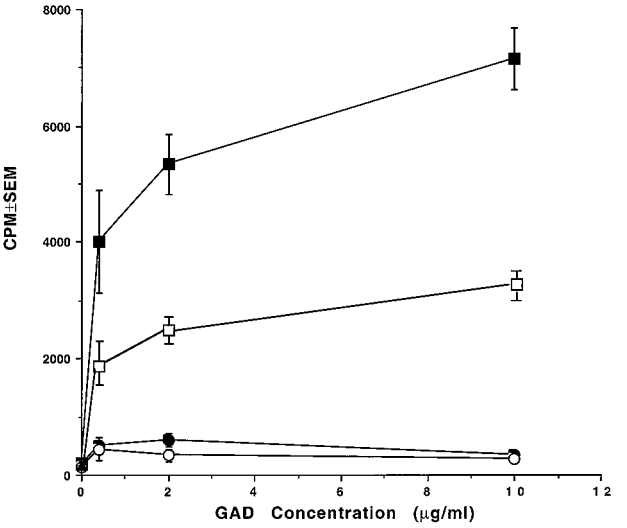

FIGURE 1. B lymphocytes are necessary APC for the initial priming of GAD-reactive T cell responses in NOD mice. Ten days after Ag priming in CFA, 2.5×10^5 T cells purified from pooled draining LN of 4 NOD (squares) or NOD.*Ig*μ^{null} (circles) mice were restimulated in vitro with the indicated concentration of GAD65 along with irradiated NOD (closed symbols) or NOD.*Ig*μ^{null} splenocytes (open symbols) as a source of APC. After an initial 72-h incubation period, the cultures were pulsed with [^{3}H]thymidine for an additional 16 h. Data represent mean cpm ± SEM of triplicate cultures.

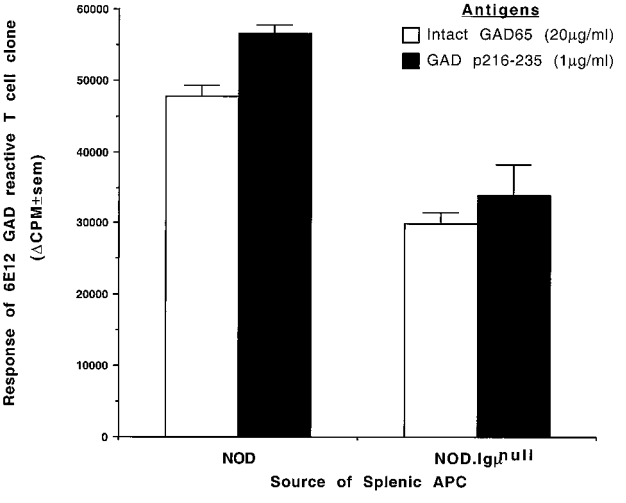

FIGURE 2. The response of preactivated GAD-reactive T cells from NOD mice can be maintained by APC other than B lymphocytes. Triplicate aliquots of 2.5×10^4 6E12 T cells were cultured for 72 h with 2.5×10^5 irradiated splenocytes from NOD or NOD.*Ig*μ^{null} mice as a source of APC with or without the indicated concentration of intact GAD or p216–235. The cultures were then labeled with [^{3}H]thymidine for an additional 16 h. Data represent the mean Δcpm ± SEM of triplicate cultures. Mean baseline responses of 6E12 T cells cultured with NOD or NOD.*Ig*μ^{null} APC in the absence of Ag were 2290 and 976 cpm, respectively.

While B lymphocytes most likely make direct contributions as APC to the initiation of GAD-reactive T cell responses in NOD mice, we recognized that they could also indirectly regulate such reactions by affecting the development of other leukocyte populations. We had previously found that both T cells and macrophages, which represent an important subpopulation of APC, develop normally in B lymphocyte deficient NOD.*Ig*μ^{null} mice (7). However, it was not known whether DC, which represent another important APC subpopulation, also develop normally in NOD.*Ig*μ^{null} mice. FACS analysis demonstrated the proportion of splenic DC, defined by coexpression of the cell surface markers Mac-1 and that detected by the previously described mAb 33D1 (22), were equivalent in standard NOD (0.91 ± 0.22%, $n = 3$) and B lymphocyte-deficient NOD.*Ig*μ^{null} mice (1.16 ± 0.33%, $n = 3$). Thus, these results support the conclusion that B lymphocytes directly serve as necessary APC for the initiation of GAD-reactive T cell responses in NOD mice.

The results described above indicated that once activated in a B lymphocyte-dependent fashion, the response of GAD-reactive T cells from standard NOD mice can be maintained by other APC subpopulations. Assuming that this conclusion was correct, we reasoned that B lymphocyte-deficient APC should stimulate a response by the GAD-reactive T cell clone 6E12, which was originally propagated from a standard NOD mouse. As shown in Figure 2, B lymphocyte-deficient APC from NOD.*Ig*μ^{null} mice were able to process and present antigenic peptides from intact GAD65 to the 6E12 T cell clone, albeit at lower levels than B lymphocyte-intact APC from standard NOD mice. Similar results were obtained using a peptide comprising amino acids 216 to 235 from GAD65, which is the antigenic target of the 6E12 T cell clone (R. M. T., personal observation). Thus, while B lymphocytes are necessary APC for initiating GAD-autoreactive T cell responses in NOD mice, once such effectors are triggered their activities can be maintained by other APC subpopulations.

*Only selected T cell responses are eliminated in B lymphocyte-deficient NOD.*Igμ^{null} *mice*

The failure of NOD.*Ig*μ^{null} mice to generate a T cell response to GAD suggested the diabetogenic role of B lymphocytes may be as APC with a preferential ability to process and present certain MHC class II-restricted β cell autoantigens. However, IDDM resistance in NOD.*Ig*μ^{null} mice could also be explained if the absence of B lymphocytes results in a generic inability to generate a T cell response to all, rather than a specific subset, MHC class II-restricted Ags. In mouse strains other than NOD, KLH has been reported to be an MHC class II-restricted Ag that does not require B lymphocytes for presentation to T cells (23). T cells within LN from both standard NOD and NOD.*Ig*μ^{null} mice primed with KLH responded equivalently when restimulated with this Ag in the presence of B lymphocyte-deficient APC (Fig. 3). However, the recall response of T cells within LN from both KLH-primed NOD and NOD.*Ig*μ^{null} mice was greater in the presence of B lymphocyte-intact than B lymphocyte-deficient APC. Thus, B lymphocytes are not required for the initial priming of KLH-reactive T cells in NOD mice but do contribute to the amplification of such responses. These data demonstrate that the absence of B lymphocytes in the NOD.*Ig*μ^{null} stock has only eliminated their ability to generate T cell responses to certain MHC class II-restricted Ags.

*NOD.*Igμ^{null} *mice harbor T cells that mediate the destruction of transplanted B lymphocytes in an MHC class I-restricted fashion*

The fact that NOD.*Ig*μ^{null} mice cannot generate a T cell response against GAD, but can do so to KLH, suggests that the diabetogenic role of B lymphocytes is as APC with a preferential ability to process and present certain MHC class II-restricted β cell autoantigens. As an initial test of this hypothesis, we wished to determine whether NOD.*Ig*μ^{null} mice repopulated with B lymphocytes were rendered IDDM susceptible and whether this was associated with a restored ability to generate a T cell response to GAD. However, purified NOD B lymphocytes only transiently repopulated unmanipulated NOD.*Ig*μ^{null} recipients (Fig. 4). These same B lymphocytes permanently repopulated NOD-*scid* recipients, which lack endogenous T as well as B lymphocytes. Thus, we hypothesized

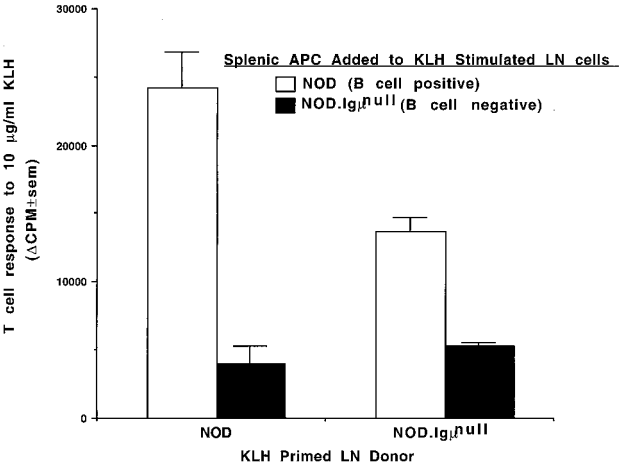

FIGURE 3. B lymphocyte-deficient NOD.*Ig*μ^{null} mice are not characterized by a generic inability to generate T cell responses to MHC class II-restricted Ags. NOD and NOD.*Ig*μ^{null} mice were primed with KLH suspended in CFA. Ten days later, draining LN cells pooled from three mice of each strain were restimulated in vitro in the presence or absence of 10 μg/ml KLH along with irradiated splenocytes from NOD (B lymphocyte intact) or NOD.*Ig*μ^{null} mice (B lymphocyte deficient) as a source of additional APC. After an initial 72-h incubation period, the cultures were pulsed with [^{3}H]thymidine for an additional 16 h. Data represent the mean Δcpm ± SEM of triplicate cultures. Mean NOD and NOD.*Ig*μ^{null} baseline responses in the absence of Ag were 2549 and 280 cpm, respectively.

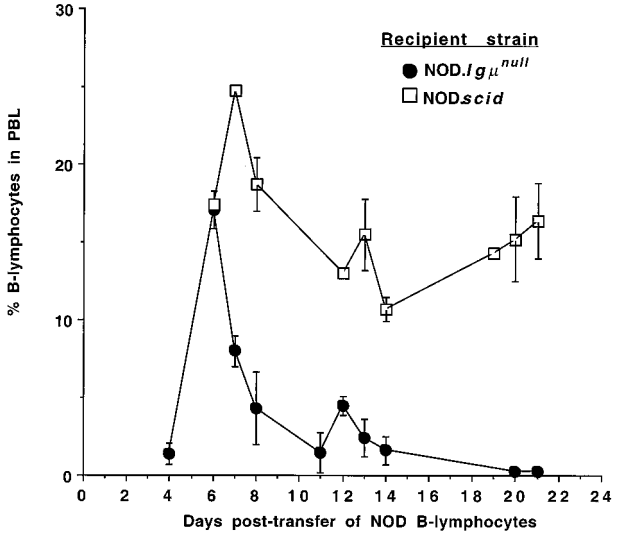

FIGURE 4. B lymphocytes permanently repopulate NOD-*scid*, but not NOD.*Ig*μ^{null}, mice. A total of 12 NOD.*Ig*μ^{null} and 5 NOD-*scid* female mice were repopulated at 5 to 8 wk of age with 3×10^6 B lymphocytes purified from spleens of standard NOD donors. Data represent the mean percentage of donor B lymphocytes ± SEM in PBL of recipients at the indicated timepoints postengraftment.

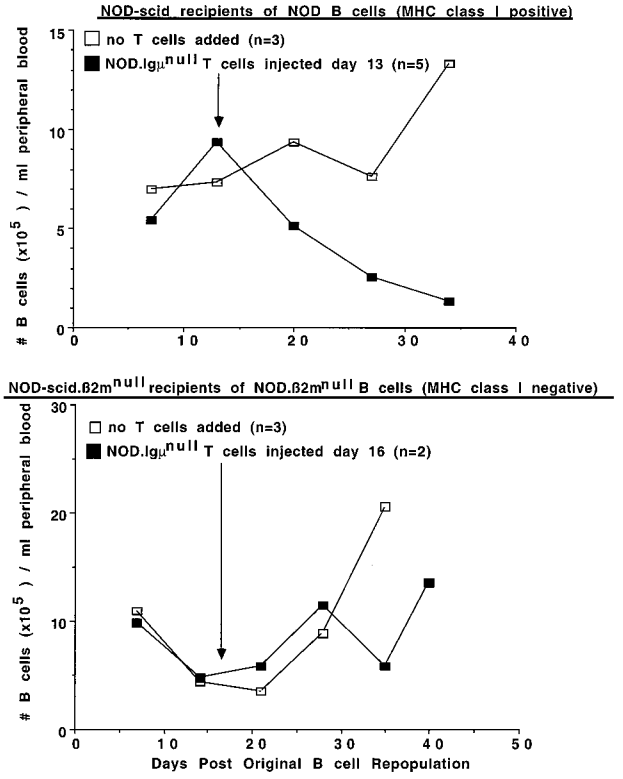

FIGURE 5. NOD.*Igμ*^{null} mice harbor T cells that mediate MHC class I-restricted cytotoxic responses against standard NOD B lymphocytes. Purified B lymphocytes (3×10^6) from standard NOD (MHC class I-positive) or NOD.*β2m*^{null} (MHC class I-negative) donors were injected i.v. into NOD-*scid* and NOD-*scid.β2m*^{null} recipients, respectively. At the indicated timepoints, a subset of the NOD-*scid* and NOD-*scid.β2m*^{null} recipients were injected i.v. with 10^7 NOD.*Igμ*^{null} T cells. Data represent the mean number of B lymphocytes/ml peripheral blood in recipients before and after injection of NOD.*Igμ*^{null} T cells.

that a population of MHC class I-restricted cytotoxic T cells present in NOD.*Igμ*^{null}, but not NOD-*scid*, recipients mediates an immunologic rejection of transplanted B lymphocytes. We were able to test this hypothesis because of the availability of both standard NOD and NOD-*scid* mice made deficient in MHC class I expression by the presence of a *β2m*^{null} allele (15–17). Standard MHC class I-positive NOD B lymphocytes previously engrafted into NOD-*scid* recipients were rapidly eliminated following the subsequent infusion of T cells from NOD.*Igμ*^{null} donors (Fig. 5). In contrast, infusion of these same T cells did not mediate the elimination of MHC class I-negative B lymphocytes from NOD.*β2m*^{null} donors that had been previously engrafted into NOD-*scid.β2m*^{null} recipients. Thus, NOD.*Igμ*^{null} mice harbor a population of T cells that can mediate a MHC class I-restricted cytotoxic response against standard NOD B lymphocytes. This is most likely due to the fact that during the course of their differentiation, T cells in NOD.*Igμ*^{null} mice fail to encounter B lymphocytes and thus are not rendered tolerant to them. It should also be noted that even following in vivo priming, NOD.*Igμ*^{null} mice failed to generate T cell responses to Ig isolated from standard NOD donors (data not shown). Thus, Ig does not appear to represent a B lymphocyte protein to which NOD.*Igμ*^{null} T cells fail to establish tolerance. This was not unexpected, since NOD.*Igμ*^{null} T cells reject transplanted NOD B lymphocytes in an MHC class I-restricted fashion, while any T cell response engendered against soluble Ig would most likely be MHC class II restricted.

Table II. *IDDM resistance is abrogated in NOD.* Igμ^{null} *mice following chimeric repopulation with both T and B lymphocytes*[a]

	Cell Types Used to Repopulate Irradiated NOD *Igμ*^{null} Recipients	
	Syngeneic marrow + NOD B lymphocytes	Syngeneic marrow only
% Splenic CD4$^+$ T cells ± SEM[b]	36.8 ± 1.5 (n = 17)	50.3 ± 2.8 (n = 11)
% Splenic CD8$^+$ T cells ± SEM[b]	14.6 ± 1.1 (n = 17)	19.1 ± 4.9 (n = 11)
% Splenic B lymphocytes ± SEM[b]	37.9 ± 2.3 (n = 17)	0.7 ± 0.1 (n = 11)
No. diabetic by 21 wk postrepopulation	15/23 (65.2%)[c]	1/15 (6.7%)
MIS ± SEM of nondiabetic mice at 21 wk postrepopulation	2.06 ± 0.41 (n = 7)	1.32 ± 0.33 (n = 11)

[a] Female NOD, *Igμ*^{null} mice were lethally irradiated at 4 wk of age and reconstituted with syngeneic bone marrow ± purified NOD B lymphocytes. All reconstituted mice were then monitored for IDDM development over a 21-wk period.

[b] Splenic leukocyte subsets were assessed by FACS at IDDM onset or at 21 wk postreconstitution with syngeneic marrow ± purified NOD B lymphocytes.

[c] IDDM incidence significantly higher ($p < 0.001$, χ^2 analysis) than in NOD.*Igμ*^{null} females reconstituted with syngeneic marrow alone.

*Restoration of B lymphocyte-mediated APC function through a bone marrow chimera approach abrogates IDDM resistance in NOD.*Igμ^{null} *mice*

We hypothesized that T cells from NOD.*Igμ*^{null} mice could be rendered tolerant to B lymphocytes if forced to mature in their presence. If correct, such an approach would make it possible to determine whether restoring the presence of B lymphocytes reconstitutes an APC function that abrogates IDDM resistance in NOD.*Igμ*^{null} mice. To address these issues, NOD.*Igμ*^{null} mice were lethally irradiated to ablate pre-existing immunologic effectors and then reconstituted with purified NOD B lymphocytes admixed with NOD.*Igμ*^{null} bone marrow as a source of T cell precursors. Controls consisted of lethally irradiated NOD.*Igμ*^{null} mice reconstituted with syngeneic bone marrow only, as well as NOD-*scid* recipients repopulated with the purified B lymphocytes alone.

As expected, spleens from NOD.*Igμ*^{null} mice reconstituted with syngeneic bone marrow alone contained both CD4$^+$ and CD8$^+$ T cells but no B lymphocytes (Table II). IDDM developed in only 1/15 of NOD.*Igμ*^{null} mice reconstituted with syngeneic marrow alone. Insulitis development was also quite limited (MIS = 1.32 ± 0.33) in NOD.*Igμ*^{null} mice that remained free of overt IDDM following reconstitution with syngeneic bone marrow alone, indicating minimal activation of diabetogenic effectors. In contrast, permanent B lymphocyte as well as CD4$^+$ and CD8$^+$ T cell repopulation was detected in spleens of NOD.*Igμ*^{null} mice reconstituted with syngeneic marrow admixed with purified NOD B lymphocytes. This indicated that T cells derived from NOD.*Igμ*^{null} marrow are rendered tolerant to B lymphocytes when forced to mature in their presence. IDDM developed in 15/23 of NOD.*Igμ*^{null} mice characterized by chimeric restoration of both T and B lymphocyte populations. In addition, significant levels of insulitis were present (MIS = 2.06 ± 0.41) in the few T and B lymphocyte-repopulated NOD.*Igμ*^{null} mice that remained free of overt IDDM. IDDM failed to develop in any (0/5) NOD-*scid* females repopulated with the purified B lymphocytes alone. GAD-reactive T cell responses were also restored in NOD.*Igμ*^{null} mice that had been rendered diabetes susceptible following reconstitution with syngeneic bone marrow plus NOD B lymphocytes (Fig.

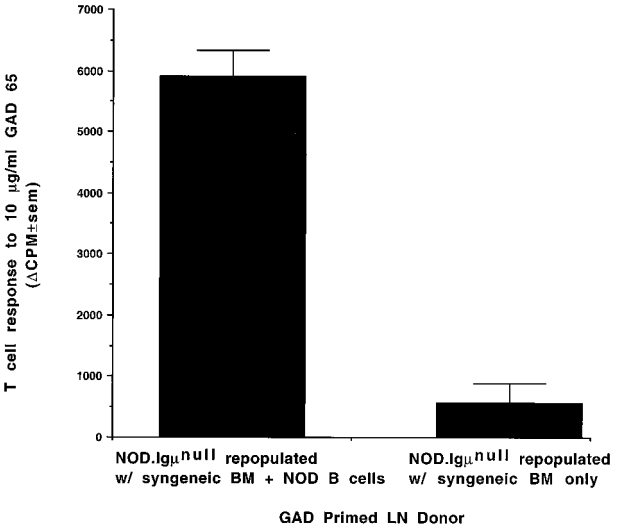

FIGURE 6. Repopulation with B lymphocytes restores the ability of NOD.*Igμ*^{null} mice to initiate GAD-reactive T cell responses. Four-week-old NOD.*Igμ*^{null} female mice were lethally irradiated and reconstituted with 5×10^6 syngeneic bone cells with or without 3×10^6 NOD B lymphocytes. At 8 weeks postreconstitution, two mice from each group were primed with GAD in CFA. Ten days after priming, aliquots of 5×10^5 draining LN cells pooled from two mice in each treatment group were restimulated in vitro in the presence or absence of 10 μg/ml GAD65. After an initial 72-h incubation period, the cultures were pulsed with [^{3}H]thymidine for an additional 16 h. Data represent the mean Δcpm $\pm$ SEM of triplicate cultures. Mean baseline responses of NOD.*Igμ*^{null} mice repopulated with syngeneic bone marrow in the presence or absence of B lymphocytes were 1248 and 189 cpm, respectively.

6). Such GAD-reactive T cell responses remained absent in diabetes-resistant NOD.*Igμ*^{null} mice that had been reconstituted with syngeneic marrow only. Thus, the presence of B lymphocytes appears to restore IDDM susceptibility to NOD.*Igμ*^{null} mice by reconstituting a normally absent APC function.

Discussion

We and others have previously demonstrated that NOD mice made deficient in B lymphocytes by congenic transfer of an *Igμ*^{null} allele or treatment with a μ chain-specific Ab are rendered resistant to T cell-mediated autoimmune IDDM (7, 11, 12). Our NOD.*Igμ*^{null} congenic stock is fixed to homozygosity for linkage markers delineating all previously identified *Idd* loci of NOD origin. However, as suggested by another group (24), it was possible that the inhibition of IDDM in our NOD.*Igμ*^{null} congenic stock did not result from the absence of B lymphocytes, but rather to the presence of a previously unidentified *Idd* resistance gene that was inadvertently cotransferred from the original donor strain of the *Igμ*^{null} allele. The current study indicates that the presence of a previously unidentified *Idd* resistance allele cannot account for the inhibition of IDDM development in our NOD.*Igμ*^{null} stock, since their disease susceptibility is restored upon repopulation with NOD B lymphocytes.

Unlike reconstitution with B lymphocytes, infusion of Ig isolated from overtly diabetic NOD donors did not abrogate IDDM resistance or enhance insulitis development in NOD.*Igμ*^{null} mice. This suggested that autoantibody production does not represent the primary diabetogenic role of B lymphocytes in NOD mice. It should be noted that the reconstituted levels of circulating Ig achieved in NOD.*Igμ*^{null} mice (~70 μg/ml) were approximately 10-fold less than that of standard NOD mice (~700–1000 μg/ml). Thus, we cannot exclude the possibility that higher levels of Ig reconstitution might have a diabetogenic effect in NOD.*Igμ*^{null} mice. However, findings from a recent report suggest that if autoantibodies do play a primary diabetogenic role, the levels of Ig reconstitution we achieved in NOD.*Igμ*^{null} mice should have been sufficient to trigger an observable pathogenic effect (25). This study found that the Sjogren's syndrome-like pathology that characterizes standard NOD mice does not develop in the same B lymphocyte-deficient NOD.*Igμ*^{null} stock used in this study. Interestingly, a Sjogren's pathology was restored in NOD.*Igμ*^{null} mice that received a single 100 μg injection of Ig isolated from either standard NOD mice or human patients with this disease. Given these results, it seems likely that if autoantibodies also play a primary role in IDDM development, their pathogenic effect would have been observed in NOD.*Igμ*^{null} mice injected twice weekly from 8 to 20 wk of age with 70 μg of Ig isolated from standard NOD donors. Thus, while contributions from autoantibody production cannot be completely excluded, our data support the conclusion that the primary diabetogenic role for B lymphocytes in NOD mice is as a subpopulation of APC essential to the initiation of certain β cell-autoreactive T cell responses.

B lymphocytes represent an essential subpopulation of APC for generating MHC class II-restricted T cell responses to certain Ags (23). It has been reported that an MHC class II-restricted T cell response against the putative β cell Ag GAD may have a critical early role in triggering a cascade of other autoreactive T cell responses, which ultimately leads to the development of IDDM in NOD mice (18, 21, 26). Thus, GAD was used as a model to test the hypothesis that the diabetogenic role for B lymphocytes in NOD mice is as a subpopulation of APC with a preferential ability to process and present certain MHC class II-restricted β cell Ags to autoreactive T cells. Unlike standard NOD, B lymphocyte-deficient NOD.*Igμ*^{null} mice were found to be incapable of generating primed T cell responses against GAD. However, NOD.*Igμ*^{null} mice could still generate primed T cell responses against the control Ag KLH. Thus, B lymphocytes represent a critical subpopulation of APC for triggering certain, but not all, T cell responses in NOD mice. The fact that GAD-specific T cell responses are among those eliminated in the NOD.*Igμ*^{null} stock indicates that the diabetogenic role of B lymphocytes in standard NOD mice is as a subpopulation of APC with a preferential ability to initiate T cell reactivity against certain key β cell autoantigens. Support for this conclusion was provided by the finding that GAD-reactive T cell responses were restored in NOD.*Igμ*^{null} mice that had been rendered IDDM susceptible by B lymphocyte repopulation. The fact that they are among the earliest cells to infiltrate the pancreatic islets of NOD mice (27, 28) also supports our conclusion that B lymphocytes represent a critical subpopulation of APC for initiating the development of T cell-mediated autoimmune IDDM.

Interestingly, only the initiation of GAD-reactive T cell responses in NOD mice requires B lymphocytes as APC. Once such GAD-reactive T cells have been initially activated in NOD mice, our data indicate that their responses can be maintained by APC other than B lymphocytes. It is also possible that B lymphocytes are necessary APC only at the initiative phases of other MHC class II-restricted β cell-autoreactive T cell responses. If preactivated β cell-autoreactive T cells do not require B lymphocytes as APC, this could explain the finding that T cells from overtly diabetic NOD donors transferred disease to young prediabetic recipients depleted of B lymphocytes by treatment with a μ chain-specific Ab (29).

One factor that complicated our ability to assess the diabetogenic function of B lymphocytes was the rejection of these cells upon transfer into unmanipulated NOD.*Igμ*^{null} recipients. This rejection of B lymphocytes in NOD.*Igμ*^{null} recipients was mediated

by an MHC class I-restricted cytotoxic T cell response. The presence of such effectors appears to result from the fact that during their differentiation, T cells in NOD.$Ig\mu^{null}$ mice fail to encounter B lymphocytes and thus are not rendered tolerant to them. This was demonstrated by our finding that T cells differentiating from precursors in NOD.$Ig\mu^{null}$ bone marrow were rendered tolerant to B lymphocytes when forced to mature in their presence. Full IDDM susceptibility was restored to NOD.$Ig\mu^{null}$ mice that had been permanently repopulated with both T and B lymphocytes by this chimerization technique.

The fact that T cells from unmanipulated NOD.$Ig\mu^{null}$ are not tolerant to B lymphocytes does not result from the generalized autoimmune proclivity of the NOD strain (30). We base this conclusion on the finding that T cells developing from NOD.$Ig\mu^{null}$ marrow could be rendered tolerant to B lymphocytes when forced to mature in their presence. This indicates that the induction of tolerance to B lymphocytes is a normal feature in development of the T cell repertoire. The thymic site of T cell differentiation contains very few B lymphocytes. Thus, it seems most likely that T cells that are potentially autoreactive against syngeneic B lymphocytes are deleted or inactivated shortly after their emigration from the thymus. If this supposition is correct, it would also suggest that an immediate encounter with their cognate Ag results in tolerogenic responses, rather than the functional activation of T cells that have recently emigrated from the thymus. Conversely, it has been reported that the small numbers of B lymphocytes that do reside within the thymus may play an important role in the normal induction of T cell tolerance to endogenous Ags (31, 32). Thus, the normal induction of T cell tolerance to B lymphocytes could occur intrathymically. Interestingly, NOD mice are reported to be characterized by increased numbers of intrathymic B lymphocytes (33). However, regardless of whether NOD B lymphocytes induce tolerance to themselves intrathymically or in the periphery, they are clearly unable to block the development or function of pancreatic β cell-autoreactive T cells.

In conclusion, this study indicates that B lymphocytes play a diabetogenic role in NOD mice as APC essential to the initiation of effector T cell responses against certain pancreatic β cell autoantigens such as GAD. It remains to be determined whether the initiation of T cell responses to any candidate β cell autoantigens other than GAD also requires B lymphocytes as APC. However, regardless of their identity or number, our study indicates that any β cell Ags which initially require B lymphocytes as APC will be among the most pathogenically relevant targets of MHC class II-restricted diabetogenic T cell responses.

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