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B Lymphocyte Depletion by CD20 Monoclonal Antibody Prevents Diabetes in Nonobese Diabetic Mice despite Isotype-Specific Differences in FcγR Effector Functions

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NOD mice deficient for B lymphocytes from birth fail to develop autoimmune or type 1 diabetes. To assess whether B cell depletion influences type 1 diabetes in mice with an intact immune system, NOD female mice representing early and late preclinical stages of disease were treated with mouse anti-mouse CD20 mAbs. Short-term CD20 mAb treatment in 5-wk-old NOD female mice reduced B cell numbers by ~95%, decreased subsequent insulitis, and prevented diabetes in >60% of littermates. In addition, CD20 mAb treatment of 15-wk-old NOD female mice significantly delayed, but did not prevent, diabetes onset. Protection from diabetes did not result from altered T cell numbers or subset distributions, or regulatory/suppressor T cell generation. Rather, impaired CD4+ and CD8+ T cell activation in the lymph nodes of B cell-depleted NOD mice may delay diabetes onset. B cell depletion was achieved despite reduced sensitivity of NOD mice to CD20 mAbs compared with C57BL/6 mice. Decreased B cell depletion resulted from deficient FcγRI binding of IgG2a/c CD20 mAbs and 60% reduced spleen monocyte numbers, which in combination reduced Ab-dependent cellular cytotoxicity. With high-dose CD20 mAb treatment (250 µg) in NOD mice, FcγRIII and FcγRIV compensated for inadequate FcγRI function and mediated B cell depletion. Thereby, NOD mice provide a model for human FcγR polymorphisms that reduce therapeutic mAb efficacy in vivo. Moreover, this study defines a new, clinically relevant approach whereby B cell depletion early in the course of disease development may prevent diabetes or delay progression of disease. The Journal of Immunology, 2008, 180: 2863–2875.

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5 Abbreviations used in this paper: T1D, type 1 diabetes; ADCC, Ab-dependent cellular cytotoxicity; [Ca2+]i, intracellular calcium level.

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immune systems allowing for mechanistic studies, detailed tissue analysis, and genetic manipulation (23–28).

CD20 is a B cell-specific molecule that is first expressed on the cell surface during the pre-B to immature B cell transition, but is lost upon plasma cell differentiation (22, 29). In mice, CD20 mAbs deplete B cells rapidly by Ab-dependent cellular cytotoxicity (ADCC) through the engagement of FcγR on monocytes (23, 25, 30). Mouse effector cells express four different FcγR classes, FcγRI, FcγRII, FcγRIII, and FcγRIV, (31, 32), that contribute significantly to isotype-specific effectiveness of CD20 mAbs in vivo (25, 33). Therefore, NOD mice were treated with IgG1, IgG2a/c, and IgG2b CD20 mAbs in a dose-dependent manner to assess whether therapeutic B cell depletion in mature mice with intact immune systems could deplete B cells and influence T1D progression.

Materials and Methods

Mice
Wild-type C57BL/6J, NOD/Lt, and NOD/LtSz-scid (NOD.scid) mice were obtained from The Jackson Laboratory. FcγRI/III−/− C57BL/6 mice were generated in our laboratory by crossing FcγRI−/− and FcγRII−/− mice (25). All mice were housed in a specific pathogen-free barrier facility. These studies were approved by the Animal Care and Use Committee of Duke University (Durham, NC) or University of North Carolina (Chapel Hill, NC).

Antibodies
Mouse CD20-specific mouse mAbs MB20-11 (IgG2c), MB20-18 (IgG2b), and MB20-1 (IgG1) were as described (23). IgG2a and IgG1 isotype switch variants of the MB20-18 mAb were produced by transient cotransfection of 293T cells with expression plasmids containing engineered H chain and native mouse FcRRIIa Abs were subsequently purified from culture supernatants using protein A or G affinity chromatography columns as suggested by the manufacturer (Amersham). The 9G8.1 hamster anti-mouse FcγRIV mAb (provided by J. Ravetch, Rockefeller University, New York, NY) was as described (34). FITC-conjugated mAb reactive with L-selectin (CD62L; clone LAM1-116) was as described (35). Other mAbs used in this study were obtained from Duke University (Durham, NC) or University of North Carolina (Chapel Hill, NC).

Cell isolation and immunofluorescence analysis
Single-cell suspensions of bone marrow (bilateral femurs), spleen, and peritoneal lymph node (paired axillary and inguinal) lymphocytes were generated by i.p. injection of 1 ml of 3% w/v thioglycolate (Sigma-Aldrich). Peritoneal cavity leukocytes were generated by gentle dissection. To isolate peritoneal cavity leukocytes, 10 ml of culture medium containing appropriate mAbs (20 μg/ml) were added (106 in 200 μl) in 300 μl of culture medium before extensive washing of the cell monolayers with culture medium before horizontal rotation at 4°C. After 60 min, unbound A20 cells were removed by gentle washing three times with culture medium, mAb-coated A20 cells were quantified for106 cells) were stained at 4°C with flow cytometry analysis as described (23, 25). In some cases, 1 ml of 3% thiglycolate solution was injected i.p. into mice 1 day before mAb treatment. For B cell depletion analysis in NOD mice, 5-wk-old female NOD mice were treated with MB20-11 mAb (250 μg) three times at 2-wk intervals. For long-term disease incidence experiments, 5- or 15-wk-old female NOD mice were treated with three MB20-11 mAb (250 μg) injections at 2-wk intervals.

Adaptive transfer experiments
Unfractionated splenocyte suspensions from NOD and C57BL/6 mice were labeled with 0.1 and 1.0 μM Vybrant CFSE, respectively, according to the manufacturer’s instructions (Invitrogen Life Technologies). The relative frequency of B220+ cells among CFSE-labeled splenocytes was determined by immunofluorescence staining with flow cytometry analysis. Subsequent equal numbers of CFSE-labeled B220+ splenocytes (×107 total) were injected i.v. into NOD and C57BL/6 recipients 1 day before i.v. injection of either MB20-11 or control mAb (250 μg/mouse). After 1 day, spleen and lymph node cells were harvested with numbers of CFSE-labeled B220+ cells determined by immunofluorescence staining with flow cytometry analysis.

FcγR sequencing and constructs
Total RNA was isolated from spleens using the RNasy kit (Qiagen). Random hexamer primers (Promega) and SuperScript II RNase H- Reverse Transcriptase (Invitrogen Life Technologies) were used to generate cDNA as described (38). Primers were as follows: for FcγRI, forward 5′-aagcatc attagct aatcggagagg-3′, C57BL/6, reverse 5′-gggtctgtt catttttgctgg-3′; for FcγRII, forward 5′-acagtaaatc tctaccaccgccct-3′, reverse 5′-aagctagcag acacatgtcgcagctacagggttcatcaccctt-3′; for FcγRIII, forward 5′-aagactgcaag acactgctgttgaggctggttcgag-3′; for FcγRIV, forward 5′-atcctgctgctgctgtgcagagagctgtgtctgtgac-3′, reverse 5′-aagactcagc aggtagtttgctccttgggac-3′, for mouse FcγRI common γ-chain, (FcγR), forward 5′-aagctgctgctgctgtgcagagagctgtgtctgtgac-3′, reverse 5′-aagactcagc aggtagtttgctccttgggac-3′, for mouse FcγRII, FcγRII, FcγRIII, and FcγRIV, and FcγR RIV sequences were deposited into GenBank (EU050648). C57BL/6 FcγRII sequences were as published (GenBank accession number AK033874). NOD and C57BL/6 FcγRIII sequences were as published (GenBank accession numbers AY897426 and AY997419). Our NOD FcγRII sequences were deposited into GenBank (EU050648). C57BL/6 FcγRIII sequences were as published (40).

FcγR functional assays
COS cells were cultured in DMEM (Mediatech) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. COS cells at 90% confluency were transfected with 2 μg/ml collagenase P (Roche) and digested for 20 min at 37°C. Islets were purified from digested tissues using a Ficoll gradient and then handpicked. Purified islets were dissociated into a single-cell suspension using enzyme-free cell dissociation solution (Sigma-Aldrich), washed, and cultured overnight in RPMI 1640 complete medium (Medi-atech) containing 10% FBS (Sigma-Aldrich), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Mediatech), 55 μM 2-ME (Invitrogen Life Technologies), and 4 ng/ml recombinant murine IL-2 (PeproTech).

For FcγRII and FcγRI expression analysis, macrophages were elicited by i.p. injection of 1 ml of 3% w/v thiglycolate (Sigma-Aldrich). After 4 days, peritoneal cavity cells were collected by lavage or isolated from single-cell spleenocyte populations (34).
Calcium response measurements

Four days after thioglycolate injection, macrophages were purified from the peritoneal cavity by depleting CD19<sup>+</sup> cells using MACS system following the manufacturer’s instructions (Miltenyi Biotec). Cells were resuspended (1 × 10<sup>7</sup> cells/ml) in RPMI 1640 medium (Sigma-Aldrich) containing 5% FBS and 10 mM HEPES buffer (Invitrogen Life Technologies). The cells were loaded with 1 μM Indo-1 AM ester (Molecular Probes) for 30 min at 37°C, labeled for an additional 20 min with FITC-conjugated CD11b mAb, washed, and resuspended in warm tissue-culture medium (2 × 10<sup>6</sup> cells/ml) for flow cytometry analysis. Baseline emission fluorescence ratios (405:525 nm) of CD11b<sup>+</sup> gated cells were collected for 1 min before the addition of DNP-BSA/anti-DNP Ab immune complexes produced by incubating DNP-BSA (60 μg/ml; Biosearch Technologies) with rabbit anti-DNP IgG (90 μg/ml; Novus Biologicals) at 37°C for 2 h. Fluorescence ratios were plotted at 20-s intervals.

Diabetes and insulitis assessment

Diabetes was monitored weekly by measuring urine glucose levels with Diastix (Bayer). Mice were diagnosed as diabetic when urine glucose levels exceeded 250 mg/dl as determined by an Autokit Glucose Cl2 assay (Wako Chemicals). Insulitis was assessed by histology as described (41). Briefly, pancreas were embedded in OCT and 5 μm tissue sections were stained using H&E. Insulitis severity was scored as no infiltration, peri-insulitis (islets surrounded by a few lymphocytes), or intra-insulitis (lymphocytic infiltration into the interior of islets) as described (41).

Adoptive transfer of diabetogenic splenocytes into NOD.scid mice

Splenocytes (1 × 10<sup>7</sup>) isolated from recent diabetic NOD female mice (diabetogenic splenocytes) were mixed with equal numbers of splenocytes from untreated, CD20, or control Ab-treated (250 μg/mouse) mice in 200 μl of PBS. Cell mixtures were injected i.v. into 6- to 8-wk-old NOD.scid recipients. Mice were monitored weekly for urine glucose.

In vitro T cell proliferation assays

After 14 days of mAb treatment, CFSE-labeled (0.1 μM) splenocytes or lymph node lymphocytes were plated in 24-well plates (2 × 10<sup>6</sup> cells) in 2 ml of RPMI 1640 complete medium with soluble CD3 mAb (2 μg/ml; 145-2C11; BD Pharmingen) plus CD28 mAb (2 μg/ml; 37.51, BD Pharmingen). After 70 h of incubation, the cultured cells were stained with CD4 or CD8 mAb to identify CFSE-labeled CD4<sup>+</sup> and CD8<sup>+</sup> T cells by flow cytometry.

Statistical analysis

All data are shown as means ± SEM. The Student t test was used to determine the significance of differences between sample means. Disease incidence curves were compared using the log-rank test.

Results

Defective B cell depletion in NOD mice after CD20 mAb treatment

In contrast with efficient B cell depletion in C57BL/6 mice using the MB20-11 (IgG2c) CD20 mAb, initial studies in NOD mice revealed less efficient B cell depletion (data not shown). Therefore, three CD20 mAbs representative of each IgG isotype, MB20-1 (IgG1), MB20-18 (IgG2b), and MB20-11, were assessed for their ability to deplete mature bone marrow, blood, and tissue B cells in NOD and C57BL/6 mice over a range of mAb concentrations (2–250 μg/mouse). In both mouse strains, the CD20 mAbs displayed a dose-dependent hierarchy of B cell depletion efficiencies over 7 days of treatment, while isotype- and dose-matched control mAbs were without effect (Fig. 1A). At each mAb dose, the IgG2c MB20-11 mAb depleted B cells most efficiently, followed by the IgG2b MB20-18 mAb and the IgG2c MB20-11 mAb were able to deplete B cells equally...
CD20 mAb-MEDIATED B CELL DEPLETION IN NOD MICE

Decreased B cell depletion in NOD mice is not intrinsic to C57BL/6 mice, and this deficiency was not limited to individual spleen B cells. Likewise, the MB20-11 mAb given at 10 µg/mouse depleted ~98% of circulating and spleen B cells in C57BL/6 mice by day 7. By contrast, NOD mice required a 25-fold higher dose of mAb (250 µg/mouse) to remove only 90% of blood and 70% of spleen B cells. Likewise, the MB20-1 IgG1 and MB20-18 IgG2b mAbs depleted 80–90% of spleen B cells when used at 100 µg/mouse in C57BL/6 mice, whereas this mAb dose depleted only 30–60% of B cells in NOD mice. Thus, B cells in NOD mice were significantly more resistant to CD20 mAb treatment compared with C57BL/6 mice, and this deficiency was not limited to individual CD20 mAb isotypes.

Decreased B cell depletion in NOD mice is not intrinsic to B cells

Whether attenuated B cell depletion in NOD mice resulted from abnormal or low-density CD20 expression by unique B cell subsets was assessed. All blood and peripheral lymph node B220+ B cells expressed CD20, with comparable expression densities in NOD and C57BL/6 mice (Fig. 2A). Splenic marginal zone B cells (CD21hiCD1dhiB220hi), T1 cells (CD21−CD24hiB220hi), T2 cells (CD21hiCD24hiB220hi), and mature B cells (CD21−CD24+) expressed CD20 at similar higher densities in both NOD and C57BL/6 mice (Fig. 2B). CD20 was also expressed at comparable developmental stages and at similar levels by bone marrow immature IgM−B220low and mature IgM+ B220high B cells (Fig. 2C). Peritoneal cavity B1a (CD5−CD11b+B220low), B1b (CD5−CD11b+B220high), and B2 (CD5−B220high) cells in NOD and C57BL/6 mice also expressed CD20 similarly (Fig. 2D). Thus, altered CD20 expression did not explain decreased B cell depletion in NOD mice.

Whether CD20 mAb resistance was an intrinsic property of NOD B cells was assessed by labeling NOD and C57BL/6 splenocytes with CFSE at different intensities before transfer into NOD or C57BL/6 recipients. One day following CD20 or control mAb treatments of recipient mice, the relative frequencies of CFSE-labeled B220+ and B220− lymphocytes in the spleen and lymph nodes of recipient mice were quantified by flow cytometry analysis. B220− splenocytes served as internal controls for relative numbers of transferred NOD or C57BL/6 splenocytes and to control for any variability in numbers of i.v. injected lymphocytes. CD20 mAb treatment depleted NOD and C57BL/6 B cells equally, with equivalent ratios of CFSE-labeled B220+ and NOD or C57BL/6 splenocytes in C57BL/6 (Fig. 2E) or NOD recipients (Fig. 2F). Because the assay times were short and no differences were observed between C57BL/6 and NOD donor cells, histocompatibility Ag differences were unlikely to influence the results. Thus, intrinsic B cell abnormalities did not explain decreased B cell depletion in NOD mice.

Whether CD20 mAb resistance reflected an inability to deplete specific B cell subsets was assessed in NOD and C57BL/6 mice after CD20 mAb treatment. One week following CD20 or control
defective FcRn function in NOD mice

Because CD20 mAb-mediated B cell depletion is FcγR dependent (23), FcγR expression and amino acid sequences were examined in NOD mice. Thioglycollate-elicited peritoneal CD11b+ F4/80+ macrophages from NOD mice expressed FcγRII/III epitopes identified by the 2.4G2 mAb at 40% lower levels than macrophages from C57BL/6 mice as determined by immunofluorescence staining with flow cytometry analysis (Fig. 3A). A smaller but reproducible decrease in FcγRII/III expression by spleen monocytes from NOD mice was also observed relative to spleen monocytes from C57BL/6 mice. However, FcγRIII transcript levels in NOD and C57BL/6 peritoneal macrophages were similar when analyzed by semiquantitative RT-PCR analysis (data not shown). Thus, reduced 2.4G2 mAb staining in NOD mice was most likely due to reduced FcγRII expression, as described (43). By contrast, FcγRIV expression by peritoneal and spleen CD11b+ F4/80+ monocytes was similar in NOD and C57BL/6 mice, as determined using the 9G8.1 mAb (34). Thus, reduced ADCC in NOD mice was not due to reduced FcγRIII or FcγRIV expression.

FcγR polymorphisms were also examined in NOD mice. NOD FcγRI transcripts differed from C57BL/6 transcripts by 24 nucleotides within coding regions, 17 of which encoded different amino acids including a four amino acid insertion between domains 2 and 3, and a frame shift that lead to premature truncation of the cytoplasmic domain (data not shown), as described (44, 45). FcγRIII and FcγRIV polymorphisms were also observed in coding regions from NOD mice. Four amino acid substitutions were identified in the FcγRIII extracellular and cytoplasmic domains (Fig. 3B), as described (GenBank accession number: AY897426). One polymorphic residue was identified in FcγRIV, which led to an amino acid change in extracellular domain 1. FcγRI, FcγRIII, and FcγRIV require FcγR for assembly and ADCC effector mechanisms (32). However, NOD and C57BL/6 FcγR transcripts were identical (data not shown), despite the amino acid sequence differences found in FcγRI, FcγRIII, and FcγRIV.

To determine whether the FcγR sequence differences were functionally significant, COS cells transiently expressing FcγRs from mAb treatment of NOD mice, tissue B cell numbers were quantified by flow cytometry analysis. The majority of splenic B cells (B220+), marginal zone B cells (CD21highCD1d−B220+), T1 cells (CD21−CD24highB220+), T2 cells (CD21highCD24high B220+), and mature B cells (CD21−CD24−B220−) were depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I). Most peripheral lymph node B cells (B220+) were also depleted in both NOD and C57BL/6 mice (Table I).
NOD and C57BL/6 mice were analyzed for their ability to bind mouse A20 B lymphoma cells coated with CD20 mAbs in vitro. A20 cell binding to COS cells was visualized by light microscopy. COS cells expressed equivalent levels of NOD and C57BL/6 FcRI transcripts as verified by semiquantitative RT-PCR analysis (data not shown). Equivalent FcRIII and FcRIV transcription by transfected COS cells was verified by flow cytometry analysis, as each FcRI expression plasmid contained an internal ribosomal entry site-GFP expression cassette downstream of the FcRI cDNA insert. Specifically, 50–60% of the COS cells expressed GFP, with similar fluorescence intensities. COS cells expressing C57BL/6 FcRI bound IgG2a CD20 mAb-bearing A20 cells at significant levels (127 ± 13 cells/field), whereas cells expressing NOD FcRI did not bind IgG2a CD20 mAb (9 ± 1 cells/field; p < 0.001, Fig. 3, C and D). C57BL/6 FcRI did not bind IgG1 or IgG2b CD20 mAbs in vitro. By contrast, COS cells expressing FcRIII from either NOD or C57BL/6 mice bound A20 cells coated with IgG1, IgG2a, or IgG2b CD20 mAbs similarly. COS cells expressing NOD or C57BL/6 FcRI also bound IgG2a and IgG2b isotype CD20 mAbs equally, but did not bind IgG1 CD20 mAb. Thus, NOD FcRI did not bind IgG2a mAbs efficiently when compared with C57BL/6 FcRI, while NOD and C57BL/6 FcRIII and FcRIV were equivalent in their CD20 mAb-binding properties.

Although the pathways regulating macrophage FcγR signaling during ADCC are poorly defined, immune complex-mediated FcγR cross-linking induces a slow increase in intracellular calcium levels ([Ca2+]i) as described (32, 46). Therefore, DNA-BSA/anti-DNP IgG immune complex-induced changes in [Ca2+]i by peritoneal macrophages from NOD and C57BL/6 mice were compared to determine whether global defects in FcγR signaling explained reduced ADCC in NOD mice. However, induced [Ca2+]i levels were similar if not identical in both NOD and C57BL/6 mice, suggesting intact FcγR signaling in NOD mice (Fig. 3E).

**FIGURE 3.** FcγR expression and function in NOD mice. A, FcγRII/III and FcγRIV expression by spleen and thioglycolate-elicited peritoneal CD11b+ F4/80+ monocytes from NOD (thick line) and C57BL/6 (thin line) mice. Dashed lines indicate control mAb staining. Representative results from one of three experiments is shown. B, Predicted FcγRIII and FcγRIV protein sequences for NOD and C57BL/6 mice. Identical residues are denoted with a dash. Differences in amino acid sequences are boxed. Residue numbering is as published (GenBank accession numbers: AY897419, AY897426, NM_144559, and EC050668). Extracellular domain 1 (EC1) and the cytoplasmic (CYTO) domains are shown. C and D, FcγR function. Binding of CD20 mAb-coated or untreated (Medium) A20 cells to COS cells transiently cotransfected with FcγR (FcγRI, FcγRII, FcγRIV) and FcγR-subunit cDNAs. Values represent mean (± SEM) numbers of A20 cells bound to COS cells in at least 30 fields from three independent experiments. Significant differences between sample means for CD20 mAb-coated and untreated A20 cells are indicated (+, p < 0.05; **, p < 0.01). D, Representative A20 cell binding to COS cells transfected with FcγRI from NOD or C57BL/6 mice. E, IgG-immune complexes induce equal [Ca2+]i response in Indo-1-loaded macrophages from NOD (thick line) and C57BL/6 (thin line) macrophages. Changes in [Ca2+]i, were quantified by flow cytometry with immune complexes added at the time point indicated (arrow). These results are representative of three experiments.
**FIGURE 4.** FcγRI is required for IgG2a CD20 mAb-mediated B cell deletion in vivo. Blood and spleen B cell depletion in C57BL/6 FcγRI/III−/− (filled circles), C57BL/6 (open circles) and NOD (shaded circles) mice. FcγRI/III−/− and C57BL/6 mice were treated using the IgG2a MB20-18, IgG2b MB20-18, and IgG1 MB20-18 CD20 mAbs, while NOD mice were treated using the IgG2c MB20-11, IgG2b MB20-18, and IgG1 MB20-1 mAbs. Values (±SEM) indicate the percentage of B cells present in mAb-treated mice (n = 6 for 10 μg/mouse, n = 3 for other doses) relative to control (CTL) mAb-treated littermates at each mAb dose evaluated. Significant differences between sample means of FcγRI/III−/− and C57BL/6 mice (*, p < 0.05), or FcγRI/III−/− and NOD mice (†, p < 0.05) are indicated.

**FcγRI is required for optimal IgG2a CD20 mAb-mediated B cell depletion in vivo**

Using a panel of CD20 mAbs of different isotypes, previous studies demonstrated that IgG2a CD20 mAbs required FcγRI and potentially FcγRII for B cell depletion in vivo, while FcγRIII deficiency was without effect (23, 25). IgG2b CD20 mAbs preferentially used FcγRI, and IgG1 mAbs used FcγRIII exclusively. To rule out the influence of mAb binding or affinity/avidity differences, the MB20-18 mAb was used to generate IgG1, IgG2a, and IgG2b mAbs with identical V regions so that the influence of mAb isotype on FcγR use could be assessed directly. Under these conditions, the IgG2a MB20-18 mAb was most potent for depleting B cells in C57BL/6 mice, while the IgG1 MB20-18 mAb was more potent than the IgG2b MB20-18 mAb (Fig. 4).

FcγRI/III−/− double-deficient C57BL/6 mice were also generated and compared with wild-type mice for CD20 mAb-mediated B cell depletion using the IgG2a, IgG1, and IgG2b MB20-18 mAbs. Thereby, mice expressing only FcγRIV were compared directly with mice expressing FcγRI, FcγRIII, and FcγRIIV. For the IgG2a CD20 mAb at 250 μg/mouse, B cell depletion was complete in both wild-type and FcγRI/III−/− mice (Fig. 4). However, at lower IgG2a CD20 mAb doses, the IgG2a mAb depleted significantly more B cells in wild-type mice. For example, IgG2a CD20 mAb (10 μg/mouse) only reduced circulating and spleen B cell numbers by 67% and 52% in FcγRI/III−/− mice, respectively, while reducing B cell numbers by >95% in wild-type mice. Thus, CD20 mAb-mediated B cell depletion was significantly less efficient when only FcγRIV was expressed, demonstrating that FcγRI and/or FcγRIII were also required for optimal B cell depletion. Circulating and spleen B cells were depleted similarly by IgG2b CD20 mAb in both wild-type and FcγRI/III−/− mice, confirming that IgG2b CD20 mAbs can use FcγRIV efficiently in the absence of FcγRI. Spleen B cells were not depleted by the IgG1 CD20 mAb in FcγRI/III−/− mice, consistent with IgG1 mAbs using FcγRIII exclusively for CD20 mAb-mediated B cell depletion. However, the IgG1 CD20 mAb depleted 40–60% of circulating B cells in FcγRI/III−/− mice, suggesting that FcγR-dependent and -independent pathways contribute to circulating B cell clearance by CD20 mAb, as described (25). When blood and tissue B cell depletion in NOD and FcγRI/III−/− mice were compared over a range of IgG2a/c and IgG2b CD20 mAb doses, B cell depletion was similar at most mAb doses (Fig. 4). Thus, the absence of normal FcγRI function in NOD mice explains the significant in vivo defect in IgG2a CD20 mAb-mediated B cell depletion.

**Intrinsic ADCC defects in NOD mice**

Spleen monocyte numbers were significantly reduced (60 ± 3%, p < 0.01, n = 5) in NOD mice compared with age-matched C57BL/6 mice. To assess whether monocyte activation could compensate for reduced monocyte numbers and enhance B cell depletion in vivo, NOD and C57BL/6 mice were each treated i.p. with thioglycolate to induce systemic macrophage activation and migration into the peritoneal cavity over a 24- to 48-h period, as described (47). One day after thioglycolate treatment, mice were given suboptimal doses of MB20-11 (25 μg), MB20-18 (100 μg), and MB20-1 (250 μg) mAb, with blood and tissue B cell numbers analyzed 7 days later. In mice treated with an IgG2c CD20 mAb, thioglycolate treatment did not enhance blood, spleen, or lymph node depletion in NOD mice, while B cells were depleted efficiently in C57BL/6 mice without thioglycolate treatment (Fig. 5). Within the peritoneal cavity, thioglycolate treatment resulted in a 58% decrease in B cell numbers in NOD mice, with a 98% decrease in C57BL/6 mice relative to littermates that were not treated with thioglycolate. In mice treated with IgG1 and IgG2b CD20 mAbs, circulating B cells were depleted by 56% (IgG1) and 2% (IgG2b) without thioglycolate treatment, but 81% (IgG1) and 47% (IgG2b) with thioglycolate treatment at day 7. Thioglycolate treatment did enhance B cell depletion in lymph nodes of NOD mice, but most significantly reduced peritoneal cavity B cell numbers in both NOD and C57BL/6 mice. Thus, B cell depletion in both NOD and C57BL/6 mice was accelerated by monocyte activation regardless of CD20 mAb isotype, arguing that ADCC could be induced in NOD mice, although at reduced levels compared with C57BL/6 mice. Thus, reduced monocyte numbers and deficient FcγRI function are likely to explain reduced B cell depletion in NOD mice rather than defective monocyte activation.

**B cell depletion protects NOD mice against T1D**

Despite reduced ADCC in NOD mice, B cells were nonetheless effectively depleted by high-dose IgG2c CD20 mAb treatment (Fig. 1). Therefore, 5 wk-old female NOD mice were treated with CD20 mAb (250 μg) given biweekly over 6 wk (Fig. 6A). Circulating B cell numbers remained reduced by >95% for up to 12 wk of age, but began to normalize thereafter. Circulating B cell numbers did not change in untreated littermates or littermates treated with an isotype-matched control mAb. Likewise, a single CD20 mAb treatment at 5 wk of age depleted the majority of spleen, pancreatic lymph node, and mesenteric...
lymph node B cells in 8-wk-old mice, with >95% fewer B cells within the islets of these mice (Fig. 6B). Thus, B cell depletion was significant and durable in NOD mice following high-dose CD20 mAb treatment.

The impact of B cell depletion on diabetes progression was assessed using 5-wk-old NOD female mice that were untreated, or treated biweekly three times with high-dose CD20 or control mAb, with disease onset and incidence monitored for up to 53 wk. The majority of CD20 mAb treated NOD mice (7 of 11) remained diabetes free, while all of the untreated (n = 11) or control mAb-treated mice (n = 11) developed diabetes (Fig. 6C). Moreover, B cell depletion significantly reduced the frequency of intraisulitis when compared with untreated control mAb-treated littermates when assessed at 14 wk of age (p < 0.05; Fig. 6D). In CD20 mAb-treated mice, 32% of islets were free of insulitis with only 12% of the islets heavily infiltrated (e.g., >50% intraisulitis). By contrast, the majority (e.g., >50%) of islets were heavily infiltrated in untreated or control mAb-treated mice. In a cohort of mice treated similarly, the number of lymphocytes within islets of 17-wk-old mice was reduced by 65% after CD20 mAb treatment (Fig. 6E). However, the relative frequencies of CD19+ B cells and CD4+ and CD8+ T cells was similar in untreated, and CD20 or control mAb-treated mice (Fig. 6E). Thus, the return of circulating B cells at 13 wk in NOD mice (Fig. 1A) was likely to parallel the return of tissue B cells, as occurs in C57BL/6 mice (23, 24), with normalized B cell compartments by 17 wk. Nonetheless, the absence of B cells during the period of normal disease development in NOD mice significantly reduced the severity of insulitis and the frequency of lymphocytes migrating into islets, with most CD20 mAb-treated mice remaining disease free for up to 53 wk.

To test whether CD20 mAb treatment at a later preclinical stage of T1D was also effective, 15-wk-old female NOD mice were treated three times with CD20 mAb (weeks 15, 17, 19), with diabetes monitored for up to 40 wk. B cell depletion did not prevent 15-wk-old mice from developing diabetes, but diabetes onset was significantly delayed following CD20 mAb treatment when compared with control mAb-treated littermates (p < 0.05, Fig. 6F).

Collectively, B cell depletion either prevented or delayed the
progression of T1D in NOD mice, depending on the stage of β cell autoimmunity.

**B cell depletion reduces T cell proliferation, but does not induce regulatory effector cells**

Others have demonstrated generally impaired lymph node T cell activation in congenitally B cell-deficient NOD mice, suggesting a critical need for B cell costimulatory signals within these microenvironments (48). Therefore, the effects of B cell depletion on T cell numbers, phenotypes, and proliferative capacity was assessed in NOD mice given CD20 or control mAb at 5 wk of age. Two weeks after CD20 mAb treatment, B cell numbers were significantly reduced, but CD4+ and CD8+ T cell numbers were not changed in the spleen, or pancreatic and peripheral lymph nodes (Table I). Similarly, CD4+ and CD8+ T cell expression of cell surface markers indicative of activation and memory cell development were not changed following CD20 mAb treatment for 2 wk. This included CTLA-4, CD28, CD44, CD62L, CD69, CD154, and OX-40 expression within the spleen, and peripheral and pancreatic lymph nodes (Fig. 7A and data not shown). By contrast, the ability of NOD pancreatic and peripheral lymph node CD4+ and CD8+ T cells to proliferate in response to CD3 plus CD28 co-stimulation in vitro was significantly reduced by CD20 mAb treatment in vivo (Fig. 7B). Furthermore, splenic CD4+, but not CD8+ T cell, proliferation in response to CD3 plus CD28 co-stimulation was also affected by CD20 mAb treatment, but to a lesser extent. Thus, induced B cell depletion did not have global effects on the numbers or phenotypes of either CD4+ or CD8+ T cells, but significantly reduced the proliferative capacity of CD4+ and CD8+ T cells within the lymph nodes of NOD mice.

In lupus patients, B cell depletion is proposed to result in regulatory or suppressor T cell generation (49, 50). To assess this, splenocytes from diabetic NOD mice were mixed with splenocytes from CD20 or control mAb-treated NOD mice and coadopitively transferred into NOD.scid recipients. The adoptive transfer of splenocytes from diabetic and control mAb-treated NOD donors into NOD.scid mice resulted in median diabetes onset 5 wk post-cell transfer. The time of onset and the frequency of diabetes were not significantly affected, however, in NOD.scid mice receiving splenocytes from CD20 mAb-treated and diabetic NOD mice (Fig. 7C). Consistent with this, the numbers of spleen and lymph node CD25 FoxP3+ CD4+ regulatory T cells were not changed in NOD
B cell depletion effects on T cell phenotypes, mitogen responses, and suppressor cell function in NOD mice 14 days after CD20 or control mAb treatment. A, Spleen CD4$^+$ and CD8$^+$ T cell phenotypes after B cell depletion. Histograms represent T cell expression of activation molecules after CD20 (thick line) or control (thin line) mAb treatments. Dashed lines represent control mAb reactivity. B, CD4$^+$ and CD8$^+$ T cell proliferation after B cell depletion. Splenocytes, and peripheral (PLN) and pancreatic (PanLN) lymph node cells from CD20 or control mAb-treated mice were isolated 2 wk after mAb treatment, CFSE labeled, and stimulated in vitro with CD3 plus CD28 mAbs (thick line) or medium alone (dashed line) for 70 h. Diminished cell depletion. Splenocytes, and peripheral (PLN) and pancreatic (PanLN) lymph node cells from CD20 or control mAb-treated mice were isolated 2 wk after mAb treatment.

Discussion

B cells have multiple roles in the development and organization of the immune system and play key, albeit ill-defined, roles in driving $\beta$ cell autoimmunity. Several studies indicate that B cells can serve as APCs or prime $\beta$ cell-specific T cells (16–19). Consequently, targeting B cells offers a potential approach to modulate $\beta$ cell-specific autoimmunity. With this in mind, the current study was initiated to assess the efficacy of CD20 mAb treatment in depleting B cells and preventing diabetes in NOD female mice with intact immune systems. The data demonstrate that NOD mice are resistant to the depleting effects of different isotypes of CD20 mAbs relative to C57BL/6 mice, and that this resistance is in part due to altered FcγRII function and reduced monocyte numbers (Figs. 1 and 3). Despite this defect, high-dose CD20 mAb administration transiently depleted B cells and could prevent diabetes onset in 5-wk-old, but not 15-wk-old, NOD female mice (Fig. 6, C and F). Likewise, induced B cell depletion in vivo significantly inhibited lymph node CD4$^+$ and CD8$^+$ T cell activation in vitro (Fig. 7B).

Continuous B cell depletion in newborn NOD mice by anti-mu Ab or genetically induced B cell deficiency results in no or limited insulitis, and the absence of diabetes (10–12). By contrast, this study is the first to demonstrate that CD20 mAb depletion in NOD mice with intact immune systems also limits insulitis and diabetes (Fig. 6, C–E). Remarkably, B cell depletion in 5-wk-old NOD female mice, a time at which $\beta$ cell autoimmunity has been initiated, effectively prevented diabetes in $>60\%$ of treated littermates (Fig. 6C) despite the reappearance of B cells by 14 wk of age (Fig. 6A). Protection induced by B cell depletion also paralleled a significant delay in insulitis progression (Fig. 6D) and a significant reduction in B and T cell numbers within islets of 17-wk-old NOD mice (Fig. 6E). These results and the finding that CD20 mAb treatment inhibited CD4$^+$ and CD8$^+$ T cell activation suggests that B cells are most important for disease initiation, with B cell recovery after depletion potentially reinitiating disease progression as occurs in collagen-induced arthritis (28). A role for B cells in disease initiation explains the limited efficacy of B cell depletion in 15-wk-old NOD mice (Fig. 6F) that represent a late preclinical stage of T1D.

The mechanism by which B cell depletion alters $\beta$ cell autoimmunity is likely to involve multiple factors. Prominent among these mechanisms may be the finding that B cell depletion in vivo significantly attenuates foreign- and autoantigen-specific CD4$^+$ T cell proliferation in vivo in C57BL/6, DBA-1, and NOD mice (19). For instance, proliferation of transferred BCD2.5 transgenic CD4$^+$ T cells is significantly reduced in the pancreatic lymph nodes of NOD recipients depleted of B cells via CD20 mAb treatment (48). Similarly, induced B cell depletion attenuated lymph node CD4$^+$ and CD8$^+$ T cell proliferation in vitro (Fig. 7B). By contrast, B cell depletion does not induce a generalized state of T cell immunosuppression or attenuate T cell responses to Ags once induced, or affect naive or memory T cell, or immunoregulatory T cell numbers within the spleen or lymph nodes (19). Likewise, induced B cell deficiency in NOD mice did not affect CD4$^+$ or CD8$^+$ T cell numbers (Table 1), alter their cell surface activation markers, or...
naive and memory phenotypes (Fig. 7A). Furthermore, CD20 mAb treatment did not alter regulatory T cell numbers (Table I) or induce a sufficient frequency of immunoregulatory T cells to suppress the diabetogenic capacity of established effector T cells in coadptive transfer experiments (Fig. 7C). These collective data and the limited efficacy of B cell depletion in 15-wk-old NOD female mice (Fig. 6F) indicate that B cell depletion significantly attenuates β cell-specific T cell priming, with less of an effect on significant numbers of β cell-specific T effectors are established. However, B cells may also be required for the recruitment of T cell clones with additional specificities as autoimmunity progresses or for avidity maturation of key pathogenic T cell clonotypes. Avidity maturation of CD8+ T cell clones specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein 206–214 correlates with the progression of T1D in NOD mice (51). Thereby, in the absence of B cells, previously generated autoreactive T cells may continue to be stimulated and infiltrate the islets, but the repertoire of these effectors may be limited and unable to promote efficient β cell destruction. This scenario likely explains why B cell depletion in 15-wk-old NOD female mice was at least partially effective in delaying disease onset (Fig. 6F).

As in the current study, induced B cell depletion early during disease onset significantly delays or prevents disease in mouse models of rheumatoid arthritis and systemic sclerosis, but not after autoimmunity is established (26, 28). B cell depletion may also remove mature autoreactive B cells. Indeed, B cells expressing insulin-specific Ag receptors develop accelerated diabetes (52), while the expression of a fixed transgenic Ag receptor delays diabetes onset (53). B cells may also provide necessary costimulatory signals or cytokines, or generate the appropriate microenvironment for pathogenic T cell expansion at important checkpoints of disease progression. For instance, a qualitative change in insulin-limits occurs at 12 wk of age in NOD female mice that drives efficient β cell destruction (54). NOD mice treated with CD20 mAb at 5 wk of age are still B cell deficient at 12 wk of age, which may have delayed transition through this checkpoint. Nonetheless, CD20+ B cell depletion does not eliminate circulating Abs or autoantibodies, or long-lived plasma cells in mice (26, 28, 55) so it remains possible that autoantibodies also contribute to disease pathogenesis once established.

Circulating and tissue B220+ B cell depletion was reduced in NOD mice compared with CD20 mAb-mediated B cell depletion in C57BL/6 mice (Fig. 1). Resistance to CD20 mAb-mediated depletion was not due to reduced CD20 expression or an intrinsic feature of NOD B cells (Fig. 2), but reflected defects in NOD FcyRI and reduced macrophage numbers. The effectiveness of CD20 mAb-induced B cell depletion correlated closely with mAb isotype, with IgG2a/c->IgG1->IgG2b mAbs in both NOD and C57BL/6 mice (Fig. 1). This hierarchy was reinforced using isotype-switched IgG2a and IgG1 variants of an IgG2b CD20 mAb, where all mAbs shared Ag-binding V regions (Fig. 4). However, NOD mice expressed a unique FcyRI allele that differs significantly from C57BL/6 FcyRI, as described (39). Transfected COS cells expressing NOD-FcyRI failed to bind IgG2a, IgG2b, or IgG1 CD20 mAb-coated A20 cells at significant levels (Fig. 3, C and D). NOD blood CD11b+ cells also express cell surface FcyRI at lower levels than C57BL/10SnNm macrophages (44). Thus, decreased IgG2a CD20 mAb potency and ADCC in NOD mice could be attributed to functional defects in FcyRI, particularly when mAb doses were limiting in vivo (Fig. 1).

FcyRI is thought to play a minor role in most in vivo circumstances because administered mAbs must compete with intrinsic circulating Abs that are retained by this high-affinity receptor. However, the in vivo dependence of IgG2a mAbs on FcyRI function has been observed in other experimental systems (56–58), in addition to CD20 mAb-mediated B cell depletion (25). In C57BL/6 mice, high-affinity FcyRI preferentially binds IgG2a CD20 mAbs, whereas FcyRIV binds with intermediate affinity to IgG2a and IgG2b CD20 mAbs, while FcyRIII binds with low affinity to IgG2a, IgG1, and IgG2b CD20 mAbs in vitro (Fig. 3C, Ref. 33). However, FcyRIV expression alone in FcyRI/III−/−/FcyRIII mice was not sufficient for optimal IgG2a CD20 mAb-mediated B cell depletion (Fig. 4), arguing for FcyRI, FcyRIII, and FcyRIV binding of IgG2a CD20 mAbs in vivo. Moreover, the ability of IgG2a and IgG2b CD20 mAbs to bind FcyRIV equally, while IgG2b mAbs did not bind FcyRI (Fig. 4), provides an explanation for the modest depletion abilities of IgG2b CD20 mAbs when compared with IgG2a/c mAbs in either NOD or C57BL/6 mice. This result contrasts with a recent study where Ab-mediated tumor clearance and platelet depletion were unaltered in FcyRI−/− mice, while blocking FcyRIV function with a specific mAb significantly reduced these IgG2a-mediated activities (34). Regardless, in NOD or FcyRI/III−−/− mice, IgG2a CD20 mAbs remained more potent for B cell depletion than IgG2b CD20 mAbs (Figs. 1 and 4). However, IgG2b mAbs engage inhibitory FcyRII better than IgG2a mAbs (31, 59) and may therefore appear less potent in vivo because FcyRII can inhibit CD20 mAb-induced B cell depletion (25). Thus, IgG2a CD20 mAbs are intrinsically potent in mice due to their ability to bind FcyRI, FcyRIII, plus FcyRIV, with reduced potency in NOD mice due to the absence of FcyRI function.

The importance of FcyR-dependent ADCC in B cell depletion was reinforced in the current studies. Normally, IgG2b CD20 mAbs have minimal B cell-depleting activity in vivo (23, 25). However, switching an IgG2b CD20 mAb to the IgG2a and IgG1 isotypes resulted in mAbs that were as potent as other independent IgG2a and IgG1 CD20 mAbs (Figs. 1 and 4). This argues that mAb isotype, FcyR-mediated ADCC, or other Fc-mediated activities are of primary importance for B cell depletion compared with signals generated within B cells following mAb binding to CD20. As in NOD mice, mAbs interacting with FcyRs is an important factor influencing the efficacy of rituximab for human therapy. Polymorphisms in human FcyRIIa and FcyRIIIa correlate with the efficiency of B cell and tumor depletion during CD20 mAb therapy in lupus and lymphoma patients (60–62). FcyRIIa is predominantly expressed by human macrophages and DCs, while FcyRIIIa is predominantly expressed by macrophages and NK cells. FcyRIIIa polymorphisms are predictive of tumor clearance in follicular lymphomas (60, 62), where ADCC appears critical. In NOD mice, FcyRIV and FcyRIII compensated for inadequate FcyRI function and mediated B cell depletion with high-dose CD20 mAb treatment. The FcyRI polymorphism and defective effector cell function demonstrated by NOD mice may thereby provide a model for studying molecular mechanisms of resistance to mAb therapy in patients.

Spleen monocyte numbers were reduced by 60% in NOD mice when compared with age-matched C57BL/6 mice, as described (63), thereby limiting the ADCC effector cell capacity of NOD mice. Consistent with this, decreased monocyte numbers in C57BL/6 mice following clodronate treatment reduces CD20 mAb-mediated B cell depletion to only ~40% clearance by day 7 (23). In NOD mice, reduced monocyte numbers reflect a failure to fully differentiate in response to CSF-1 and IFN-γ exposure (64), reduced apoptotic cell phagocytosis (65), and impaired recruitment of leukocytes into sites of inflammation due to defects in CCL2-induced migration (66). Defective ADCC in NOD mice did not result from elevated serum IgG because serum IgM and IgG levels were comparable in the 4- to 8-wk-old NOD and C57BL/6 mice.
used for depletion studies (data not shown). In addition, CD20 mAb treatment does not reduce serum IgM or IgG levels, or pre-
fomed Ag-specific Ab levels (55). Despite reduced ADC in NOD mice, defective B cell depletion was overcome by admin-
istering higher doses of CD20 mAb, which resulted in effective B cell depletion (Figs. 1 and 6, A and B). In addition, thio
glycolate treatment induced effector cell recruitment and increased B cell depletion in NOD mice (Fig. 5). IgG-containing immune
complexes also induced comparable [Ca2+]i responses in NOD and C57BL/6 macrophages (Fig. 3E), suggesting that downstream
FcγR signaling was not significantly impaired in NOD macrophages. Thereby, increasing the relative concentration of therapeu-
tic mAbs relative to endogenous IgG appears to be a valid strategy for overcoming FcγR and ADCC defects in NOD mice.

In summary, these data demonstrate the effectiveness of pre-
emptive CD20 mAb-mediated B cell depletion in treating diabetes
in NOD mice with intact immune systems. This provides a ratio-
nale for applying similar approaches to the management of predi-
abetic patients and patients with recent onset disease. Moreover,
the finding that high-dose CD20 mAb treatment can overcome the
effects of defective FcγR function in vivo provides a rationale for
applying similar approaches for oncology and autoimmune disease
patient management.

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