Reduced T-Dependent Humoral Immunity in CD20-Deficient Mice

Deyaa El Deen Morsy, Ratna Sanyal, Anne K. Zaiss, Rucha Deo, Dan A. Muruve and Julie P. Deans

J Immunol published online 21 August 2013
http://www.jimmunol.org/content/early/2013/08/21/jimmunol.1202098

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/08/21/jimmunol.1202098.DC1

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Reduced T-Dependent Humoral Immunity in CD20-Deficient Mice

Deyaa El Deen Morsy,* Ratna Sanyal,* Anne K. Zaiss,† J. Rucha Deo,* Dan A. Muruve,† and Julie P. Deans* 

CD20 is a tetraspanning membrane protein expressed on B lymphocytes. CD20 deficiency in both mice and humans has recently been shown to have deleterious effects on Ab responses to T-independent Ags; however, no effect on T-dependent immunity has been reported. In this study, we used a Cd20−/− mouse line to evaluate Ab responses to adeno-associated virus and SRBCs. The neutralizing Ab response to adeno-associated virus was significantly reduced by CD20 deficiency; both primary (IgM) and secondary (IgG1 and IgG2b) responses to SRBC were also reduced in Cd20−/− mice, and this was associated with a reduction in the number of germinal center B cells. A successful humoral response requires the integration of intracellular signaling networks that critically rely on calcium mobilization. In this article, we confirm that BCR-mediated calcium mobilization is reduced in Cd20−/− murine B cells after BCR stimulation in vitro, and further show that the reduction is due to an effect on calcium influx rather than calcium release from intracellular stores. Calcium-dependent upregulation of CD69 was impaired in CD20-deficient B cells, as was upregulation of CD86. Altogether, this study demonstrates a role for CD20 in B cell activation and T-dependent humoral immunity. The Journal of Immunology, 2013, 191: 000–000.

Because CD20 is highly expressed on all mature B cells in both mice and humans, the lack of a demonstrated effect of CD20 deficiency on TD humoral immunity was surprising. We therefore sought to examine TD immune responses in Cd20−/− mice using the well-characterized complex Ags, adeno-associated virus (AAV) and SRBCs. We found significant reduction in humoral immunity to both Ags. The response to SRBCs was evaluated further and found to affect both primary (IgM) and secondary (IgG1 and IgG2b) Ab responses, and was associated with reduced germinal center (GC) B cell numbers. To our knowledge, this is the first report of an effect of CD20 deficiency on the humoral immune response to TD Ags.

The development of humoral immunity requires normal BCR signaling. Evidence from several laboratories, using cell lines expressing either endogenous or ectopic CD20, supported a role for CD20 in calcium signaling, potentially as a calcium channel linked to the BCR (6, 10, 11). However, previous studies examining BCR-mediated calcium mobilization in Cd20−/− primary murine B cells yielded inconsistent results (9, 12). We show in this article that calcium mobilization is indeed reduced in Cd20−/− B cells after BCR stimulation, and that this was due to reduced influx of extracellular calcium. Further, we found that calcium-dependent upregulation of CD69, as well as CD86, after BCR stimulation was impaired in CD20-deficient B cells, whereas CD69 upregulation was normal in CD3-activated T cells from the same mice. Altogether, the data reported in this article show that CD20 function is necessary for optimal BCR-mediated calcium entry, B cell activation, and TD humoral immunity.

Materials and Methods

Mice

Cd20−/− mice used in this study were previously described (12) and were backcrossed onto C57BL/6 background for at least seven generations. C57BL/6 wild-type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and crossed with Cd20−/− C57BL/6 mice. Heterozygous mice from the first generation were crossed and the resulting pups were genotyped to obtain homozygous Cd20−/− and WT C57BL/6 mice that were inbred to maintain colonies that were housed in a specific-
Injection of 1×10^11 particles/mouse, provided by Dr. J.S. Bartlett, as described previously (13). Injections were done via the femoral vein (i.v.) under general anesthesia in a total volume of 100 μl sucrose vehicle (3% sucrose, 150 mM NaCl, 10 mM Tris [pH 7.4], 1 mM MgCl2). All control animals received the vehicle alone. For immunization with SRBCs, fresh SRBCs (Cedarlane, Hornby, Ontario, Canada) were washed twice in sterile PBS, pH 7.2, before use. Mice were primed by i.p. injection of 1×10^9, 3×10^9, or 1×10^10 SRBCs in 0.5 ml PBS. Booster i.p. injections were conducted with the same dose for each experiment on day 21. Blood samples were collected by tail-vein puncture. Serum was obtained from blood by centrifugation at 1000 × g for 5 min and stored at −80°C.

Anti-AAV Ab titration

Neutralizing anti-AAV Abs were detected as described previously (13). In brief, mouse sera were serially diluted in serum-free DMEM tissue culture medium and incubated with 8×10^5 AAV-GFP particles for 10 min at 37°C in a total volume of 100 μl. The AAV-serum mix was then applied to wells of a 24-well plate (Thermo Scientific, Bremen, Germany) seeded with HEK293 cells that were ~90% confluent. Another 100-μl tissue culture medium containing 2×10^5 particles of WT Adeno-virus 2 was added to each well to enhance transgene expression of the AAV-transduced cells. Cells were collected after 24–48 h, washed in PBS, and resuspended in 2% paraformaldehyde. GFP expression was quantified by flow cytometry and plotted against the serum dilution. Titer of neutralizing Abs were estimated by determining the dilution of mouse serum that inhibited 50% of AAV transduction as measured by GFP-transgene expression.

Anti-SRBC ELISA

SRBC ghosts were prepared as described previously (14) and aliquots stored at −80°C until needed. Flat-bottom polystyrene microtiter plates (Nunc, Rochester, NY) were coated with 100 ng SRBC preparation in 100 μl PBS well and allowed to settle for 24 h at 4°C. Plates were washed twice with STT buffer (0.2 M NaCl, 0.04 M Tris [pH 9.0]) and blocked with 1% BSA in STT buffer for 24 h at 4°C. Plates were washed twice with STT buffer, and mouse sera (1:100) were added in a total volume of 100 μl PBS/well and incubated for 1.5 h at 37°C. Plates were washed twice with STT buffer and incubated with either rabbit anti-mouse IgG F(ab')2 goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) or F(ab')2 goat anti-mouse IgG (ImmunoResearch Laboratories, West Grove, PA). Thapsigargin (Calbiochem, EMD, Gibbstown, NJ) was used to induce passive calcium store release, and ionomycin (Molecular Probes) was used to access free calcium. Changes in fluorescence ratios of indo-1 emission at 405/485 nm were collected by flow cytometry using LSR II analyzer (BD). Data were analyzed using FlowJo software.

Flow cytometry

Purified B cells subjected to different conditions of stimulation were stained using aliphophycocyanin-conjugated hamster anti-mouse CD69 or PE-conjugated rat anti-mouse CD86 (eBioscience). Total splenocytes obtained from the same spleens were stimulated using purified rat anti-mouse CD3 (eBioscience), then stained with FITC-conjugated hamster anti-mouse CD3e (eBioscience) and aliphophycocyanin-conjugated hamster anti-mouse CD69. Some samples were treated with cyclosporin A (150 ng/ml; Sigma-Aldrich) immediately before cell stimulation. Data acquisition was performed using a Becton Dickinson FACScan cytometer (BD Biosciences). Data analysis was conducted using FlowJo software (Tree Star, San Carlos, CA).

Statistics

Data were statistically evaluated using the two-tailed paired Student t test. A p value <0.05 was considered statistically significant.

Results

Impaired humoral immunity in Cd20⁻/⁻ mice

i.v. administration of AAV is known to result in TD humoral immunity, with development of neutralizing anti-AAV Abs (15–17). We injected 1×10^11 AAV particles i.v. into five Cd20⁻/⁻ and five WT mice, and determined the levels of neutralizing Abs at days 14 and 21 posttransduction using transduction assays. The results showed that WT mice generated higher anti-AAV neutralizing Ab responses (mean ±1:11,000 on day 21 after AAV injection) compared with Cd20⁻/⁻ mice (mean ±1:6000 at the same time point; Fig. 1).

Intracellular calcium measurements

Splenic B cells were isolated by negative selection using the EasySep B cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada). A second cycle of enrichment was conducted for all B cell purities >96%. A total of 1×10^6 B cells was incubated with 2 μM indo-1 AM (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 at 37°C for 30 min. Cells were washed with RPMI 1640, stained using PE-conjugated rat anti-mouse CD19 (eBioscience, San Diego, CA), then loaded with either calcium buffer (1.5 mM CaCl2, 150 mM NaCl, 3 mM KCl, 20 mM Hepes, 10 mM d-glucose, 0.25 mM sulfinpyrazone) or EGTA buffer (25 mM EGTA, 150 mM NaCl, 3 mM KCl, 20 mM Hepes, 10 mM d-glucose, 0.25 mM sulfinpyrazone). Samples containing 2×10^5 B cells in 1 ml were incubated at 37°C, and basal levels of indo-1 fluorescence were acquired for 30 s before cell stimulation. Cells were then stimulated using F(ab')2 goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) or F(ab')2 goat anti-mouse IgG (ImmunoResearch Laboratories, West Grove, PA). Thapsigargin (Calbiochem, EMD, Gibbstown, NJ) was used to induce passive calcium store release, and ionomycin (Molecular Probes) was used to access free calcium. Changes in fluorescence ratios of indo-1 emission at 405/485 nm were collected by flow cytometry using LSR II analyzer (BD). Data were analyzed using FlowJo software.

FIGURE 1. Reduced neutralizing Ab response to AAV in Cd20⁻/⁻ mice. Neutralizing Ab titers were measured in C57BL/6 (WT) and Cd20⁻/⁻ mice. Five WT mice (solid boxes) and age- and sex-matched Cd20⁻/⁻ mice (solid circles) were immunized with 1×10^11 AAV particles i.v., and sera were collected at the time points indicated. Anti-AAV titers were determined by AAV transduction assays. Each mouse is represented by a box or circle. y-Axis represents the inverse of serum dilution (*p < 0.05).
SRBCs have been extensively used to assess TD humoral immunity in rodents. To further examine the effect of CD20 deficiency on Ab responses to a TD Ag, we primed $Cd20^{-/-}$ and WT mice with $1 \times 10^7$, $3 \times 10^7$, or $1 \times 10^8$ SRBCs on day 0 and boosted with similar doses on day 21. Serum IgM and IgG anti-SRBC were measured by ELISA on days 0, 4, 7, and 14, and IgG anti-SRBC were additionally measured on days 21, 25, 28, and 35. Typical time-course data showed that the peak IgM and IgG responses occurred 4 d after the initial or booster injections, respectively (Fig. 2A, 2B). At these time points, the primary IgM response was significantly reduced in $Cd20^{-/-}$ mice at all doses of SRBCs ($p < 0.05$; Fig. 2C), and the secondary IgG response was significantly reduced after injection with $3 \times 10^7$ SRBCs ($p < 0.01$). IgM anti-SRBC was reduced by $\sim$45% after immunization with $1 \times 10^7$ SRBCs, by 24% after immunization with $3 \times 10^7$ SRBCs, and by 21% after immunization with $1 \times 10^8$ SRBCs. IgG anti-SRBC was reduced by $\sim$47% in $Cd20^{-/-}$ mice on day 25 after injection with $3 \times 10^7$ SRBC (Fig. 2D). IgG anti-SRBC

**FIGURE 2.** Reduced primary and secondary immune responses to SRBCs in $Cd20^{-/-}$ mice. WT and $Cd20^{-/-}$ mice were immunized with SRBCs at day 0, boosted on day 21, and sera were collected at the indicated times for measurement of IgM and IgG by ELISA. (A) IgM anti-SRBC Abs were measured on days 4, 7, and 14 after immunization of eight WT and eight $Cd20^{-/-}$ mice with $1 \times 10^8$ SRBCs. (B) IgG anti-SRBC Abs were measured on days 4, 7, 14, 21, 25, 28, and 35 after immunization of six WT and six $Cd20^{-/-}$ mice with $3 \times 10^7$ SRBCs on days 0 and 21. (C) Mice were immunized with $1 \times 10^7$, $3 \times 10^7$, or $1 \times 10^8$ SRBCs, and IgM anti-SRBC Abs were measured on day 4. (D) Mice were immunized with $1 \times 10^7$, $3 \times 10^7$, or $1 \times 10^8$ SRBCs on days 0 and 21, and IgG anti-SRBC Abs were measured on day 25. (E) IgG1, IgG2a, IgG2b, and IgG3 anti-SRBC Abs were measured on day 25 after immunization with $3 \times 10^7$ SRBCs on days 0 and 21. *$p < 0.05$, **$p < 0.01$. (C-E) Each box or circle represents one mouse. Data are representative of the pattern of responses obtained from four experiments with a total number of 37 mice immunized with $1 \times 10^8$ SRBCs, two experiments with a total number of 12 mice immunized with $3 \times 10^7$ SRBCs; data for 5 mice immunized with $1 \times 10^7$ SRBC are from one experiment.
responses were mostly of the IgG1 and IgG2b isotypes, and both were significantly reduced in \( Cd20^{−/−} \) mice (Fig. 2E).

**Reduced GC B cell numbers in immunized \( Cd20^{−/−} \) mice**

GCs develop in response to TD Ags. During the primary immune response, GCs typically appear 2–4 d after immunization, become associated with a detectable reduction in GC B cells, four WT and four \( Cd20^{−/−} \) mice were immunized with \( 3 \times 10^7 \) SRBCs, and spleens were collected on day 7 postimmunization. Flow cytometric analysis performed using the GC markers PNA and GL7 demonstrated significant reduction in the numbers of PNA\(^+\) or GL7\(^+\) splenic B cells in \( Cd20^{−/−} \) mice (Fig. 3A, 3B). No significant change in the size or distribution of the red and white pulp (Fig. 3D), or in the distribution of T and B cells (Fig. 3C, left panels), was noted in \( Cd20^{−/−} \) mice spleens as compared with WT. However, splenic sections costained with PNA and anti-IgD, a marker for non-GC B cells, showed an obvious deficiency in the gross formation of GCs, as visualized by reduced PNA staining, in two of the four \( Cd20^{−/−} \) mice examined (Fig. 3C, right panels).

**Impaired BCR-activated calcium influx in \( Cd20^{−/−} \) mice**

Calcium signaling resulting from BCR ligation is one of the important mechanisms initiating B cell activation, and previous work has implicated CD20 in BCR-activated calcium mobilization. To examine calcium signaling in CD20-deficient B cells, we loaded splenic B cells from \( Cd20^{−/−} \) and WT mice with the calcium-sensitive ratiometric dye, indo-1. After acquiring basal levels of indo-1 fluorescence emission, we stimulated the cells using F(ab')\(^2\) anti-IgM. Calcium responses were found to be lower in \( Cd20^{−/−} \) B cells compared with WT B cells (Fig. 4A). This was a highly reproducible finding in \( >20 \) independently conducted experiments using a range of Ab concentrations from 1 to 40 \( \mu \)g, with the greatest differences seen at suboptimal concentrations (Supplemental Fig. 1). No difference in surface IgM expression was detected (Supplemental Fig. 2). Impaired BCR-activated calcium influx in \( Cd20^{−/−} \) mice BCR-activated calcium influx is clearly defective in CD20-deficient B cells. To test whether CD20 deficiency affects cellular activation, we examined upregulation of CD86 and CD69 in purified splenic B cells after stimulation with 1 \( \mu \)g F(ab')\(^2\) anti-IgM. As a control, we examined the expression of CD69 in T cells from the same splenic populations activated with 1 \( \mu \)g anti-CD3. Expression of CD69 and CD86 was similar in unstimulated WT and

**FIGURE 3.** Reduced GC B cells in spleens of immunized \( Cd20^{−/−} \) mice. Four WT and four \( Cd20^{−/−} \) mice were immunized with \( 3 \times 10^7 \) SRBCs. Spleens were collected on day 7 postimmunization. (A) Splenocytes were stained with anti-CD19-PE, PNA-FITC, and anti–GL7-Alexa 647. Gates were set on the brightest populations of PNA\(^+\) and GL7\(^+\) cells within the CD19\(^+\) gate. The percentage of cells in each quadrant is indicated in each histogram. (B) Bar graph representation of the data shown in (A). Data are presented as mean ± SEM, *p < 0.05. (C) Splenic sections were stained with antiCD3-Alexa 488 and antiB220-Alexa 647, to visualize T and B cells, respectively, or with PNA-FITC and anti–IgD-PE to visualize GC and naive B cells, respectively. The mean fluorescence intensity was measured for the green and red pixels within the yellow boxes indicated in each splenic section. The mean fluorescence ratio for CD3/B220 and PNA/IgD is indicated in the lower right corner of each section. Data for CD3/B220 are representative of sections from all four mice. Data for PNA/IgD are representative of sections from two of four mice. (D) To determine the size and distribution of red and white pulp, paraffin-embedded splenic sections from WT and \( Cd20^{−/−} \) mice were stained with H&E and visualized by light microscopy. (C and D) Original magnification \( \times 4 \).
CD20-deficient B cells. After activation, however, expression of both markers was reduced in CD20-deficient B cells (Fig. 6A, 6B, respectively), whereas there was no difference in CD69 upregulation in activated T cells from the same WT and Cd20<sup>−/−</sup> mice (Fig. 6C). Impaired upregulation of both CD69 and CD86 in CD20-deficient B cells was also observed in the presence and absence of anti-CD40 costimulation, and at a higher concentration of anti-IgM (Fig. 6D, 6E). In T cells, CD69 upregulation requires sustained high levels of intracellular calcium achieved with anti-CD3 cross-linking (18) or with ionomycin (19). Induction of CD69 expression in ionomycin-treated T cells was prevented by inhibition of calcineurin using cyclosporin A (19). To confirm that upregulation of CD69 was calcium dependent in B cells, we incubated WT and CD20-deficient B cells with anti-IgM in the presence or absence of cyclosporin A. We found that CD69 upregulation was completely inhibited under these conditions (Fig. 6F).

**FIGURE 4.** Reduced BCR-mediated calcium mobilization in Cd20<sup>−/−</sup> B cells. Calcium responses induced by cross-linking IgM or IgG in indo-1 loaded B cells from WT and Cd20<sup>−/−</sup> mice. (A) F(ab′)<sub>9</sub> goat anti-mouse IgM (5 µg) was added at 30 s (arrow) in the presence of calcium chloride or EGTA. (B) F(ab′)<sub>9</sub> goat anti-mouse IgG (5 µg) was added at 30 s (arrow) in the presence of calcium chloride. Results represent 10 (A) and 3 (B) independent experiments. (C) Ionomycin was added at 30 s (arrow) in the presence of calcium chloride. EGTA was added at 3 min. (D) Thapsigargin was added at 30 s (arrow) in the presence of EGTA. Results represent four independent experiments.

**Discussion**

To evaluate TD humoral immunity in Cd20<sup>−/−</sup> mice, we used two complex particulate Ags, AAV and SRBC, for immunization. Our results show that Cd20<sup>−/−</sup> mice had reduced levels of neutralizing Abs against AAV, and reduced primary and secondary responses to SRBCs. The secondary anti-SRBC response in both WT and Cd20<sup>−/−</sup> mice (Fig. 6C). Impaired upregulation of both CD69 and CD86 in CD20-deficient B cells was also observed in the presence and absence of anti-CD40 costimulation, and at a higher concentration of anti-IgM (Fig. 6D, 6E). In T cells, CD69 upregulation requires sustained high levels of intracellular calcium achieved with anti-CD3 cross-linking (18) or with ionomycin (19). Induction of CD69 expression in ionomycin-treated T cells was prevented by inhibition of calcineurin using cyclosporin A (19). To confirm that upregulation of CD69 was calcium dependent in B cells, we incubated WT and CD20-deficient B cells with anti-IgM in the presence or absence of cyclosporin A. We found that CD69 upregulation was completely inhibited under these conditions (Fig. 6F).

Th effect of CD20 deficiency in humans is more severe than in mice. Whereas basal levels of all serum Igs are normal in Cd20<sup>−/−</sup> mice, the case report of a Cd20<sup>−/−</sup> CVID patient described persistently low serum IgG levels over several years of testing (8). Serum IgM was at the high end of the normal range, suggesting a defect in isotype switching. Consistent with this, switched memory B cells were low in the circulation, and in vitro activation of the patient’s B cells with either TD or TI stimuli resulted in normal proliferation and IgM secretion, but absent or very reduced IgG. The response to vaccination with TI Ags was low. Although the response to one TD vaccine, tetanus toxoid, was normal, the persistently low serum IgG suggests decreased TD immune responses. These data, together with our own findings, indicate that in both mice and humans, CD20 expression is essential for optimal humoral immunity to both TI and TD Ags.
The effect of CD20 on humoral immunity is probably the result of its role in calcium mobilization. In numerous independent experiments conducted on murine Cd20−/− naive B cells, we found that calcium influx after BCR stimulation was consistently reduced. Uchida et al. (9) also reported lower calcium responses after anti-IgM stimulation, although in their study lower calcium flux was not entirely the result of reduced extracellular calcium levels. Lower expression of surface CD20 was also found that calcium influx after BCR stimulation was consistently reduced by around 50%. Similar experiments performed by Neuberger’s group (12) showed no significant reduction in calcium mobilization using F(ab′)2 anti-IgM at either 1 or 20 μg/ml. A possible explanation may lie in the source of anti-IgM used to stimulate the cells, or perhaps in the concentration of Ab, because we found the greatest differences at intermediate suboptimal concentrations of F(ab′)2 anti-IgM (5–10 μg/ml; Supplemental Fig. 1). Alternatively, other experimental conditions, such as indol-1 dye loading, may be responsible for inconsistent results between the two studies.

In human B cells, the involvement of CD20 in BCR-activated calcium influx has been demonstrated using Ramos cells in which CD20 was downregulated with siRNA (6). However, experiments on B cell lines derived from the Cd20−/− CVID patient showed no effect on calcium responses induced by either anti-IgM or anti-IgG (8). The manipulations required to generate these B cell lines undoubtedly altered the characteristics of the peripheral blood B cells from which they were derived (20), so it cannot be assumed that calcium signaling is not affected by CD20 deficiency in unmanipulated primary human B cells. Nevertheless, this finding shows that the requirement for CD20 in BCR-activated calcium mobilization is not absolute. The inability of Neuberger’s group to detect altered calcium mobilization in Cd20−/− murine B cells (12) also indicates that the role of CD20 is subtle and probably regulatory under certain conditions. Interestingly, we found enhanced calcium responses in Cd20−/− mice, and yet we detected no difference in the level of surface IgM on splenic B cells from the Cd20−/− mouse line generated by O’Keefe et al. (12), and we found BCR-activated calcium influx to be consistently reduced by ~50%. Similar experiments performed by Neuberger’s group (12) showed no significant reduction in calcium mobilization using F(ab′)2 anti-IgM at either 1 or 20 μg/ml. A possible explanation may lie in the source of anti-IgM used to stimulate the cells, or perhaps in the concentration of Ab, because we found the greatest differences at intermediate suboptimal concentrations of F(ab′)2 anti-IgM (5–10 μg/ml; Supplemental Fig. 1). Alternatively, other experimental conditions, such as indol-1 dye loading, may be responsible for inconsistent results between the two studies.

Activated calcium influx was also observed after BCR cross-linking in either the presence or absence of calcium chloride. Cells were stimulated (arrows) with pre-mixed biotinylated F(ab′)2 anti-IgM, biotinylated anti-CD19, and avidin at the following concentrations to derive the molar ratios shown in the figure: (A) 5 μg anti-IgM + 1 μg anti-CD19 + 1.5 μg avidin; (B) 5 μg anti-IgM + 5 μg anti-CD19 + 2.5 μg avidin; (C) 5 μg anti-IgM + 15 μg anti-CD19 + 5 μg avidin; (D) 3 μg anti-IgM + 21 μg anti-CD19 + 6 μg avidin. Results represent two independent experiments for each anti-IgM F(ab′)2-anti-CD19-avidin combination.

**FIGURE 5.** Enhanced BCR×CD19 calcium responses in Cd20−/− B cells. Calcium mobilization induced in indol-1 loaded B cells from WT (boxes) and Cd20−/− (circles) mice by cross-linking IgM and CD19 in the presence of calcium chloride. Cells were stimulated (arrows) with pre-mixed biotinylated F(ab′)2 anti-IgM, biotinylated anti-CD19, and avidin at the following concentrations to derive the molar ratios shown in the figure: (A) 5 μg anti-IgM + 1 μg anti-CD19 + 1.5 μg avidin; (B) 5 μg anti-IgM + 5 μg anti-CD19 + 2.5 μg avidin; (C) 5 μg anti-IgM + 15 μg anti-CD19 + 5 μg avidin; (D) 3 μg anti-IgM + 21 μg anti-CD19 + 6 μg avidin. Results represent two independent experiments for each anti-IgM F(ab′)2-anti-CD19-avidin combination.

**FIGURE 6.** Reduced activation marker expression in Cd20−/− B cells. Purified splenic2 B cells from WT (gray line) and Cd20−/− (black line) mice were stimulated with 1 μg F(ab′)2 anti-mouse IgM. After 16–18 h of incubation, cells were labeled with allopurinol-conjugated anti-mouse CD69 (A) or PE-conjugated anti-mouse CD86 (B). Total splenocytes from the same mice were incubated with 1 μg anti-mouse CD3 for 16–18 h, then labeled with FITC-conjugated anti-mouse CD3ε, and allophycocyanin-conjugated anti-mouse CD69. Purity-gated cells were examined for CD69 expression (C). Unstimulated WT and Cd20−/− cells are shaded in gray (A–C). (D and E) Purified splenic B cells were either left unstimulated (1), stimulated with 1 μg F(ab′)2 anti-IgM + 5 μg anti-mouse IgM + 5 μg anti-mouse CD40 (2), 10 μg F(ab′)2 anti-IgM + 10 μg F(ab′)2 anti-IgM + 5 μg anti-mouse CD40 (3), 10 μg F(ab′)2 anti-IgM + 5 μg anti-mouse CD40 (4), or 5 μg anti-mouse CD40 (5). Cells were examined for CD69 expression (D) and CD86 expression (E). (F) Total splenocytes from either WT or Cd20−/− mice were either treated with 150 ng/ml cyclosporin A (+) or left untreated (−). Cells were either unstimulated (thin lines) or stimulated using 10 μg F(ab′)2 anti-IgM (thick lines). After 12 h of incubation, cells were stained for CD69 and B cells were gated using PE-conjugated anti-mouse CD19.
absence of anti-CD40. However, it was not strictly dependent on BCR stimulation because some effect was observed even when CD40 alone was cross-linked. Physical and functional links between CD40 and CD20 have been previously reported (21, 22), but because anti-CD40 is not known to activate calcium responses, the basis of the effect of CD20 deficiency on B cell activation by anti-CD40 is not clear.

In summary, data reported in this article show for the first time, to our knowledge, reduced BCR/CD40-mediated B cell activation in vitro in CD20-deficient B cells, and reduced TD humoral immunity in Cd20−/− mice. Thus, although CD20 is a member of the membrane-spanning 4-domain A family of genes, several of which may be expressed in B cells (1, 2), its role in humoral immunity is not redundant and its absence results in decreased Ab responses in both humans and mice. The influence of CD20 is likely to be at the level of BCR-mediated signaling leading to calcium influx; however, its function is more likely to be modulatory than due to direct involvement as a calcium channel. It seems likely that reduced calcium responses and suboptimal cellular activation in Cd20−/− B cells leads to reduced humoral immunity; however, it is possible that other undiscovered effects of CD20 deficiency contribute to the phenotype observed.

Acknowledgments
We thank Dr. M. Neuberger (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.) for permission to import and use the strain of Cd20−/− mice generated in his laboratory, and Dr. Maria L. Palomba (Memorial Sloan-Kettering Cancer Center, New York, NY) for shipping breeding pairs to us. AAV-2 vectors were provided by Dr. Jeffrey Bartlett (Columbus, OH), Laurie Robertson and Laurie Kennedy (Flow Cytometry Facility in the Faculty of Medicine, University of Calgary) provided assistance with calcium assays. Microscopy was performed in the Snyder Live Cell Imaging Facility, University of Calgary, with expert advice from Dr. Pina Colarusso and Jen Amon.

Disclosures
The authors have no financial conflicts of interest.

References