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Immunology

Btk dosage determines sensitivity to B cell antigen receptor cross-linking

ANNE B. SATTERTHWAITE*, HILDE CHEROUTRE†‡, WASIF N. KHAN§, PASCALIS SIDERAS¶, AND OWEN N. WITTE*‖**

*Department of Microbiology and Molecular Genetics, ‖Howard Hughes Medical Institute, and †Department of Microbiology and Immunology, University of California, Los Angeles, CA 90095; §Howard Hughes Medical Institute, The Children's Hospital, Boston, MA 02115; and ¶Unit of Applied Cell and Molecular Biology, Umeå University, S-901 87 Umea, Sweden

Contributed by Owen N. Witte

ABSTRACT **Mutations in Btk result in the B cell immunodeficiencies X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice. Btk is a critical component of signaling pathways regulating B cell development and function. We used a genetic approach to determine whether Btk is also limiting for these processes. One allele of a murine Btk transgene expressed a dosage of Btk (25% of endogenous levels in splenic B cells) sufficient to restore normal numbers of phenotypically mature conventional B cells in xid mice. 2,4,6-trinitrophenyl–Ficoll response, anti-IgM-induced proliferation, B1 cell development, and serum IgM and IgG_3 levels remained significantly impaired in these animals. B cells from Btk −/− transgenic mice also responded poorly to anti-IgM, indicating that the xid mutation does not create a dominant negative form of Btk. Response to 2,4,6-trinitrophenyl–Ficoll and B cell receptor cross-linking were increased 3- to 4-fold in xid mice homozygous for the transgene. These results demonstrate that Btk is a limiting component of B cell antigen receptor signaling pathways and suggest that B cell development and response to antigen may require different levels of Btk activity.**

The outcome of B cell receptor (BCR)-mediated signals depends on both their strength and the context in which they are received (1). This complexity necessitates the identification of both essential B cell signaling components and those that determine signaling thresholds. Changes in the effective dosage of such proteins may result in qualitative differences in the response to BCR engagement.

Several proteins such as CD19 (2, 3), CD38 (4), CD22 (5–10), and SHP1 (11–13) modulate the degree of BCR cross-linking required for a given response. Mutations in these molecules dramatically alter the outcome of BCR signals. Low-avidity encounters with antigen lead to positive selection rather than anergy when autoreactive B cells are desensitized to BCR cross-linking by deletion of CD45 (14). The same events result in deletion of B cells that are hypersensitive to BCR signaling (11).

An essential component of several B cell signaling pathways is the nonreceptor tyrosine kinase Btk. Mutations in Btk result in X-linked agammaglobulinemia (XLA) in humans (15, 16) and X-linked immunodeficiency (xid) in mice (17, 18). XLA patients have a block in B cell development at the pre-B stage (19), resulting in a deficit of mature B cells and serum Ig (20). Loss of Btk expression and point mutations in all subdomains of Btk can cause XLA (21).

Xid mice, which have a point mutation in the Btk PH domain (17, 18), and Btk −/− mice (22–24) have a milder phenotype than most XLA patients. They have a 30–50% decrease in peripheral B cell numbers with the most pronounced loss in the mature $IgM^{lo}IgD^{hi}$ population (25). These cells respond abnormally to cross-linking of a variety of cell surface receptors, including BCR (26), interleukin (IL)-5R (27), IL-10R (28), and CD38 (29). Biochemical evidence also implicates Btk as a mediator of IL-6 (30) and FcεRI signals (31). Xid mice have reduced levels of serum IgM and IgG_3 (32), do not respond to type II T independent antigens (33), and lack peritoneal B1 cells (34). A competitive disadvantage of Btk −/− cells is not observed until the pre-B to immature B transition, despite expression of Btk from the pro-B stage onward (24).

To understand the minimal dosage and expression pattern required for Btk function, we generated transgenic mice expressing a murine Btk cDNA driven by the Ig heavy chain promoter and enhancer and crossed them to xid mice. Poor rescue of 2,4,6-trinitrophenyl (TNP)–Ficoll response (see *Results* below; ref. 35) was observed in several lines despite expression of transgene-derived RNA in B lineage cells at endogenous levels. This is in contrast to a recent report that a human Btk transgene driven by the major histocompatibility complex (MHC) class II locus control region (LCR) can rescue the Btk −/− phenotype (36). The MHC class II LCR-driven transgene expressed endogenous levels of Btk protein in splenocytes (36), whereas our best Ig enhancer/promoter-driven transgene produced 25% of endogenous Btk levels (see *Results* below). We compared the degree of phenotypic rescue in xid mice hemizygous (xid^{1xtg}) with that of homozygous (xid^{2xtg}) for a wild-type Btk transgene. Our results indicate that B cell development and function require different threshold levels of Btk activity and that Btk is limiting for B cell responses.

MATERIALS AND METHODS

Mice. Transgenic mice were generated on a C57B6 × C3H background with a murine Btk cDNA in the vector pIgTE/N (37) and genotyped as described (35). This vector contains the murine Ig enhancer, the human Ig promoter, and a simian virus 40 splice site and polyadenylation signal and directs expression in spleen, thymus, bone marrow, and lymph nodes (37). Transgene copy number was determined by calculating the ratio of transgene specific to endogenous Btk bands by using a Phosphoimager (Molecular Dynamics). Transgenic mice were backcrossed three generations onto a Balb/c (Jackson Laboratories) or Balb/xid (DNAX) background, then mated to each other to obtain nontransgenic, heterozygous, or

Abbreviations: xid^{nxtg} or wt^{nxtg}, xid or wild-type mice with *n* alleles of Btk transgene; BCR, B cell antigen receptor; IL, interleukin; XLA, X-linked agammaglobulinemia; xid, X-linked immunodeficiency; TNP, 2,4,6-trinitrophenyl; FACS, fluorescence-activated cell sorter; PMA, phorbol 12-myristate 13-acetate.

‡Current address: La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121.
**To whom reprint requests should be addressed at: 5-720 MacDonald Research Laboratories, 675 Circle Drive South, Los Angeles, CA 90095-1662. e-mail: owenw@microbio.ucla.edu.

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homozygous transgenic animals. Endogenous Btk alleles were identified as xid or wild type using a PCR strategy. The region surrounding the xid mutation was amplified from genomic DNA by using the following primers: 5′-TTCTGAAG-CGCTCCCAGC-3′ (exon 2) and 5′-TTCTCATTTGGGAAA-CTTAC-3′ (intron 2). Digestion of the PCR products with *Cfo*I results in two bands for the wild-type allele and one for the xid allele due to the destruction of a *Cfo*I site by the xid mutation. The transgene was crossed onto a Btk −/− background (23) for protein analysis. Wild-type and targeted endogenous Btk alleles were distinguished by Southern analysis as described (23).

B Cell Purification and Culture. Pre-B cell lines were generated by infecting bone marrow with retroviruses encoding v-abl as described (38). $B220^+$ spleen cells were isolated using the Minimacs magnetic bead system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Single-cell suspensions were depleted of red blood cells before incubation with magnetic beads. B cell-enriched populations were greater than 90% $B220^+$ by fluorescence-activated cell sorter (FACS) analysis. [^{3}H]thymidine labeling: $B220^+$ splenic B cells were seeded into 96-well plates at 10^6/ml in RPMI 1640 medium with 10% heat-inactivated FCS. Where indicated, cells were incubated for 60 hr with 2 or 20 μg/ml goat-anti-mouse IgM $F(ab')_2$ fragments (Jackson ImmunoResearch) or 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Calbiochem) and 1 μM ionomycin (Calbiochem). [^{3}H]thymidine (1 μCi; 1 Ci = 37 GBq) (Dupont/NEN) was added per well for the final 12–18 hr. Cells were harvested and counted on a scintillation counter. BrdUrd labeling: Total splenocytes were depleted of red blood cells and plated in RPMI 1640 medium with 10% heat-inactivated FCS at 10^6/ml. Where indicated, goat-anti-mouse IgM $F(ab')_2$ fragments (Jackson ImmunoResearch) were added at either 2 or 20 μg/ml. At 24 hr, BrdUrd (Sigma) was added to a final concentration of 10 μM. Cells were harvested at 48 hr, and FACS analysis was performed.

FACS. Single-cell suspensions from spleen or peritoneal wash were depleted of red blood cells and stained with combinations of the following antibodies (PharMingen): anti-B220 (RA2–6B2) phycoerythrin (PE), anti-IgM (R6–60.2) fluorescein isothiocyanate (FITC), anti-IgD^a (AMS 9.1) PE, anti-IgD^b (217–170) PE, anti-CD5 (53–7.3) FITC, anti-CD4 (H129.19) PE, and anti-CD8 (53–6.7) FITC. Data were acquired on a FACScan (Becton Dickinson) and analyzed using LYSIS II software. Live cells were gated based on forward and side scatter. A gate characteristic of lymphoid cells based on forward and side scatter was used for peritoneal cells. Analysis of BrdUrd incorporation: BrdUrd-labeled cells were stained with anti-BrdUrd FITC (Becton Dickinson) and anti-B220 (RA3–6B2) PE (PharMingen) as described (39) and analyzed as above.

ELISA. IgM and IgG_3: Plates were coated with 2 μg/ml goat anti-mouse Ig (Southern Biotechnology Associates) and blocked with 1% BSA in borate-buffered saline (BBS). Serum or Ig standards (mouse IgM and IgG_3, Sigma) were diluted serially into BBS and added to wells in duplicate. Plates were then incubated with secondary antibody (goat anti-mouse IgM-ALPH or goat anti-mouse IgG3-ALPH, Southern Biotechnology Associates) diluted 1:500 in BBS, 0.05% Tween-20, and 1% BSA and developed with an alkaline phosphatase substrate kit (Bio-Rad). OD_{405} was read on a V_{max} kinetic microplate reader (Molecular Devices). TNP: Mice were immunized intraperitoneally with 10 μg TNP–Ficoll (a gift of John Inman, National Institutes of Health, Bethesda, MD) and bled 6 days later. ELISA was performed as above with the following modifications. Plates were coated in 25 μg/ml TNP–BSA in PBS, and serum and secondary antibody (goat anti-mouse IgM, Southern Biotechnology Associates) were diluted into PBS, 0.1% BSA, and 0.05% Tween-20.

Western Blotting. Whole cell extracts from 2×10^6 $B220^+$ splenocytes, v-abl-transformed pre-B cells, and thymocytes were run on an 8% SDS/PAGE gel and transferred to nitrocellulose. The bottom portion of the gel was stained with Coomassie blue to ensure equal loading. Blots were probed with an affinity-purified anti-Btk polyclonal antibody (15) or anti-lyn polyclonal antibody as described (40) and developed using an ECL kit (Amersham).

Statistics. *P* values were determined using a two-tailed Student's *t* test with unequal variance.

RESULTS

We evaluated the ability of a wild-type Btk transgene driven by the Ig heavy chain enhancer and promoter (37) to rescue the xid phenotype. The level of Btk protein produced by this construct was determined by Western blot analysis of purified $B220^+$ spleen cells, v-abl-transformed pre-B cells, and thymus from Btk −/− mice carrying one transgenic allele (ko^{1xtg}). Several lines expressed endogenous levels of transgene-derived RNA but had little to no detectable Btk protein in B cells (data not shown). The line that produced the highest amount of Btk protein was chosen for further study. $B220^+$ splenocytes and v-abl-transformed pre-B cells from these mice expressed approximately 25% and 50–75% of endogenous Btk protein levels, respectively (Fig. 1). Expression in thymus was also observed (data not shown).

Btk Transgene Rescues Conventional but Not B-1 Cell Development. Because the phenotype of Btk deficiency has been described to vary according to genetic background (23, 41, 42), transgenic mice were backcrossed three generations onto the Balb/c background before being used in experiments. The progeny of several breeding pairs were analyzed with little variation observed among nontransgenic xid animals (see below). Mice were between 8 and 12 weeks of age at the time of analysis.

Xid and Btk −/− mice have two developmental defects in the B lineage. Peripheral B cells with a mature $IgM^{lo}IgD^{hi}$ phenotype are decreased in number (25), and $CD5^+$ B1 cells are absent (34). Both the frequency and number of mature splenic B cells were restored to normal in all xid (xid^{1xtg}) and Btk −/− (ko^{1xtg}) mice carrying one allele of the transgene (Fig. 2*A* and Table 1; data not shown). In contrast, the percentage of peritoneal $B220^+CD5^+$ cells was on average

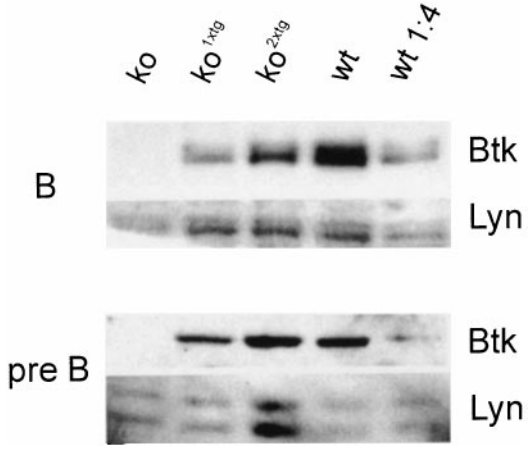

FIG. 1. Transgene expression in ko^{tg} mice. Total cell lysates from $B220^+$ spleen cells and v-abl-transformed pre-B cells were subjected to Western blot analysis and probed with an anti-Btk polyclonal antibody. Transgene-derived Btk protein was quantified by comparison with a 1:4 dilution of wild-type cell lysates into Btk-deficient cell lysates. Equal loading of samples was confirmed by Coomassie blue staining of the bottom portion of the gel and reprobing the blots with an anti-lyn polyclonal antibody.

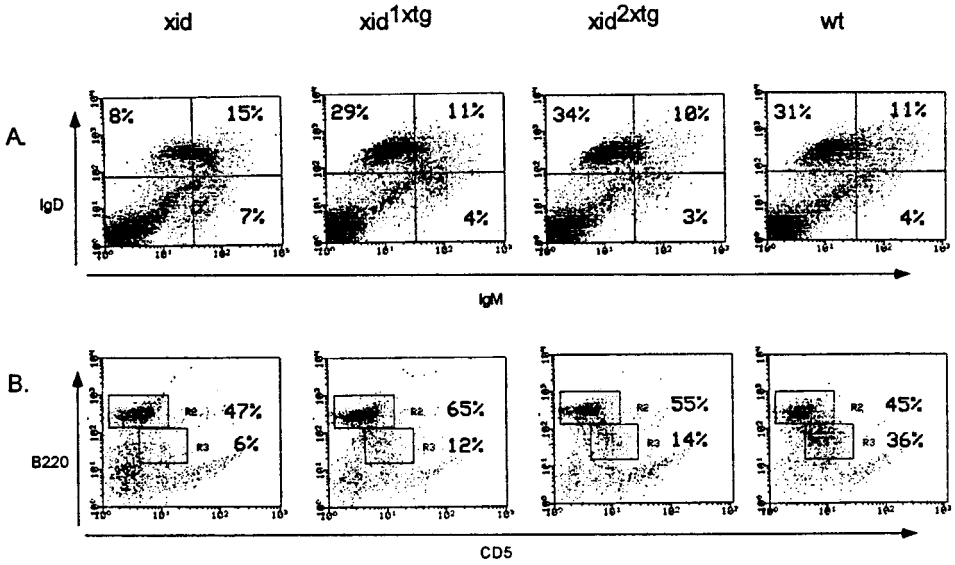

FIG. 2. Rescue of B cell development in xid^{tg} mice. (*A*) Spleen cells from 8- to 12-week-old mice were depleted of red blood cells and stained with anti-IgM FITC (*x* axis) and anti-IgD PE (*y* axis). Live cells were gated based on forward and side scatter. The percentage of cells in each quadrant is indicated. (*B*) Peritoneal cells were depleted of red blood cells and stained with anti-CD5 FITC (*x* axis) and anti-B220 PE (*y* axis). Lymphocytes gated based on forward and side scatter are shown. The percentage of B220$^+$CD5$^-$ and B220$^+$CD5$^+$ cells in this gate is indicated.

only 2-fold above xid mice and still significantly below wild-type controls (Fig. 2*B* and Table 1).

B Cell Functional Responses Are Partially Restored by Btk Transgene. Xid and Btk −/− mice fail to respond to TNP–Ficoll (33) and have low levels of IgM and IgG$_3$ (32). Although TNP–Ficoll response (Fig. 3) and serum IgM and IgG$_3$ levels (Fig. 4) were partially restored in xid^{1xtg} mice, all three titers remained lower than wild-type controls. This is surprising in light of the normal numbers of IgM^{lo}IgD^{hi} splenic B cells in these animals. Transgenic mice on a wild-type background (wt^{1xtg}) mounted a normal response to TNP–Ficoll and had normal levels of IgM and IgG$_3$ (data not shown), so the poor response by xid^{1xtg} mice was not due to an inhibitory effect of the transgene on immune function.

To test whether the defects in xid^{1xtg} mice are B cell-intrinsic, the *in vitro* proliferative response of purified splenic B220$^+$ cells to BCR cross-linking was assessed by [^{3}H]thymidine incorporation (Fig. 5*A*). Xid B cells did not respond in this assay. Xid^{1xtg} cells proliferated upon BCR cross-linking but were significantly less sensitive to anti-IgM than wild-type controls. Xid^{1xtg} B cells did not respond to a low dose of anti-IgM (2 μg/ml). They incorporated 5-fold less [^{3}H]thymidine than wild-type cells at 20 μg/ml anti-IgM. This was not a result of a general proliferative defect because cells from all mice responded equally well to PMA and ionomycin (data not shown). Decreased proliferation of xid^{1xtg} cells relative to wild-type controls was also observed when total splenocytes were incubated with anti-IgM and BrdUrd incorporation by B220$^+$ cells was measured by FACS (Fig. 5*B*). Ko^{1xtg} B cells were also hyposensitive to BCR cross-linking (data not shown), demonstrating that the xid mutation does not create a dominant negative form of Btk.

B Cell Functional Responses Depend on Btk Dosage. One possible reason for the poor rescue of B cell function and B1 cell development in xid^{1xtg} mice is that although the level of transgene expression is sufficient to support conventional B cell development, B cell functional responses require a higher level of Btk protein. To address this hypothesis, mice homozygous for the transgene on both xid (xid^{2xtg}) and Btk −/− (ko^{2xtg}) backgrounds were generated. Btk was expressed at approximately 50% and 100–150% of endogenous levels in splenic B cells and v-abl-transformed pre-B cells, respectively (Fig. 1). As in xid^{1xtg} animals, complete rescue of the IgM^{lo}IgD^{hi} population of splenic B cells was observed in xid^{2xtg} mice (Fig. 2*A* and Table 1).

Some aspects of the xid phenotype did not respond to increased transgene dosage. A less than 2-fold increase in IgM

Table 1. Spleen cells and peritoneal washes from 8- to 12-week-old mice were depleted of red blood cells and stained with the following markers: anti-B220 vs. anti-IgM; anti-IgM vs. anti-IgD; anti-CD4 vs. anti-CD8; anti-B220 vs. anti-CD5

Cell type	xid	xid^{1xtg}	xid^{2xtg}	wt	wt^{1xtg}	wt^{2xtg}
Spleen						
% B220$^+$	36 ± 6.5*	49 ± 6.2	48 ± 4.2	49 ± 5.4	51 ± 1.7	56 ± 4.3†
# B220$^+$ ($\times 10^6$)	26 ± 8.4*	48 ± 22	37 ± 10	40 ± 12	47 ± 18	48 ± 17
% IgM^{lo}IgD^{hi}	9.7 ± 2.7*	31 ± 5.0	31 ± 2.5	34 ± 4.3	36 ± 4.8	38 ± 4.4†
# IgM^{lo}IgD^{hi} ($\times 10^6$)	7.0 ± 2.6*	31 ± 17	24 ± 6.4	27 ± 9.0	34 ± 16	36 ± 13
% CD4$^+$	21 ± 3.6	21 ± 2.4	20 ± 2.0	20 ± 4.9	23 ± 1.5	21 ± 3.7
% CD8$^+$	12 ± 2.0†	9.7 ± 1.6	10 ± 1.9	8.9 ± 2.4	10 ± 1.3	8.0 ± 1.9
Peritoneum						
% CD5$^+$B220$^+$ (all cells)	1.6 ± 0.62*	3.2 ± 2.0*	5.7 ± 3.1*	20 ± 7.6	ND	ND
% CD5$^+$B220$^+$ (lymphoid cells)	7.0 ± 2.4*	11 ± 6.0*	18 ± 9.0*	39 ± 12	ND	ND

For spleen cells, live cells were gated based on forward and side scatter. For peritoneal cells, the gate was either set on live cells (all cells) or lymphoid cells (lymphoid cells) based on forward and side scatter. The percentage or number of cells having the indicated phenotype is presented as mean ± SD. Between 6 and 20 animals were analyzed per experiment. ND, not determined.

*Decreased relative to wild type ($P < 0.001$).

†Increased relative to wild type ($P < 0.05$).

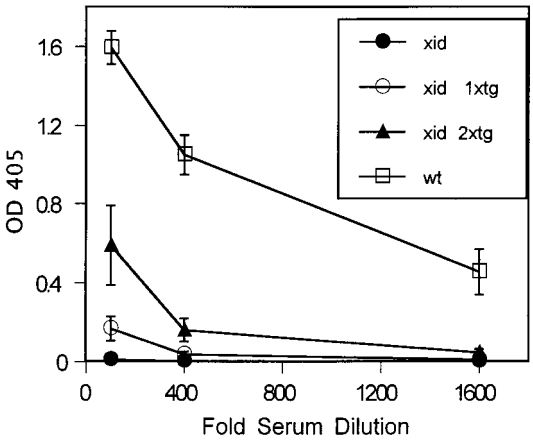

FIG. 3. TNP–Ficoll response depends on Btk dosage. Eight- to 12-week-old xid, xid^{1xtg}, xid^{2xtg}, and wild-type (wt) mice were immunized with 10 μg TNP–Ficoll and bled 6 days later. Serum was diluted 1:100, 1:400, and 1:1,600. Anti-TNP IgM was measured by ELISA. OD_{405} readings are presented as mean ± SD. The number of individual mice tested in each group is as follows: xid, 8; xid^{1xtg}, 8; xid^{2xtg}, 8; wt, 5. All results differed significantly from each other ($P < 0.001$).

and IgG_3 levels was observed in xid^{2xtg} animals compared with xid^{1xtg} mice (Fig. 4). Most xid^{2xtg} mice had similar numbers of $B220^+CD5^+$ peritoneal cells to xid^{1xtg} animals. However, 4 of 11 had frequencies of B1 cells (24–32% of lymphoid cells) in a near-normal range (Fig. 2*B* and Table 1). The variability in B1 cell levels (7–32% of lymphoid cells) may indicate that on certain genetic backgrounds the amount of Btk activity in xid^{2xtg} mice is sufficient to support B1 cell development. Consistent with this observation, genetic context can influence B1 cell numbers in wild-type mice (34).

A dependence on Btk dosage was clearly observed for BCR signaling both *in vivo* and *in vitro*. xid^{2xtg} mice consistently mounted a 4-fold better response to TNP–Ficoll than xid^{1xtg} animals (Fig. 3). Wild-type titers of anti-TNP were not attained, however. Purified xid^{2xtg} B cells proliferated three to four times as well as xid^{1xtg} B cells at 20 μg/ml anti-IgM (Fig. 5*A*). The sensitivity of xid^{2xtg} B cells to BCR cross-linking was similar to that of wild-type cells (Fig. 5 *A* and *B*). These combined results indicate that Btk is limiting for the transmission of mitogenic signals from the BCR.

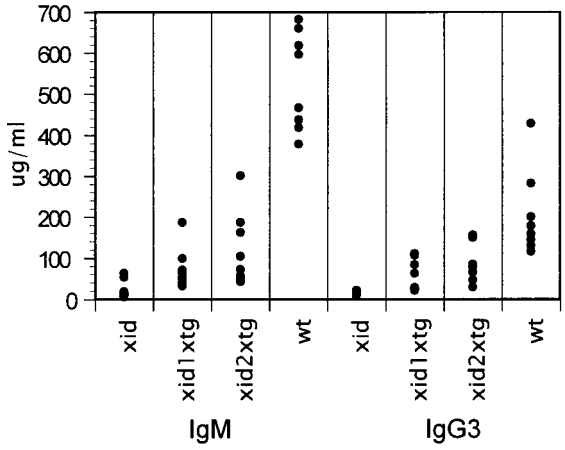

FIG. 4. IgM and IgG_3 levels are partially restored in xid^{tg} mice. Serum IgM and IgG_3 levels from 8- to 12-week-old xid, xid^{1xtg}, xid^{2xtg}, and wild-type (wt) mice were measured by ELISA. Each symbol represents an individual animal ($n = 8$). xid^{1xtg} and xid^{2xtg} are significantly different from both xid ($P < 0.05$) and wt ($P < 0.001$) but not each other.

To determine whether Btk overexpression results in hypersensitivity to BCR cross-linking, we examined the response of wt^{1xtg} and wt^{2xtg} mice to TNP–Ficoll (data not shown) and anti-IgM (Fig. 5*B*). No change in the BCR signaling threshold was observed, nor was there increased response to anti-IgM or TNP–Ficoll at a given dose. Intriguingly, the response of wt^{2xtg} mice in both assays was reduced 1.5- to 2-fold relative to control animals. This was not due to a decrease in mature B cell numbers (Table 1). These data suggest that efficient BCR signaling occurs within a narrow range of Btk expression levels.

DISCUSSION

Mature $IgM^{lo}IgD^{hi}$ splenic B cells develop normally in the presence of reduced Btk levels but respond poorly to BCR cross-linking. This suggests that the immunodeficiency in xid mice is not simply a result of poor B cell maturation. Increased Btk expression resulted in increased response to anti-IgM and TNP–Ficoll, demonstrating that Btk is limiting for these processes and plays a role in determining BCR signaling thresholds.

These data suggest a model in which two types of Btk-dependent signals regulate B lymphopoiesis. The first mediates the development and survival of mature B cells and requires a relatively low dosage of Btk. The amount of Btk expressed in xid^{1xtg} mice exceeds this threshold, allowing normal numbers of $IgM^{lo}IgD^{hi}$ B cells to accumulate. A higher level of Btk is necessary for signals directing optimal expansion of these cells in response to antigen. These signals could be initiated by different receptors or by the same receptor with alternative outcomes based on the strength of the Btk-mediated signal. The function of signal threshold modulators such as CD19 or CD38 may also depend on Btk dosage.

Several lines of evidence support this model. xid^{1xtg} mice have a strikingly similar phenotype to xid mice carrying a bcl-2 transgene (43), indicating that a Btk-dependent survival signal exists but is insufficient to allow normal B cell function. Second, a Btk transgene driven by the MHC class II LCR that is expressed at levels comparable to endogenous Btk from the pre-B stage onwards rescues all aspects of the Btk $-/-$ phenotype (36). Therefore, it is unlikely that lack of transgene expression at the earliest stages of B cell development explains the results obtained here. Finally, anti-CD3-induced secretion of IL-2 by $CD4^+$ T cells is more sensitive than $CD4^+$ T cell development to the dosage of Itk, a member of the Btk family of kinases (K. Q. Liu and L. J. Berg, personal communication).

Another possibility is that B cell development and functional responses require the same amount of Btk. The transgene may be expressed at higher levels at a stage where developmental decisions are impaired in xid mice, then down-regulated as the cells mature to a level too low to mediate efficient BCR signaling. Consistent with this hypothesis, pre-B cells expressed the transgene at a higher level relative to endogenous Btk than did splenic B cells (Fig. 1).

B1 cell development is more sensitive to Btk dosage than conventional B lymphopoiesis. Mutations in other B cell signaling molecules also affect B1 cells more than conventional B cells (44, 45). A correlation between sensitivity of conventional B cells to BCR cross-linking and B1 cell numbers in several mutant mice (44) implies that B1 cell survival requires BCR signaling (46). Diminished response to BCR cross-linking may contribute to low B1 cell numbers in xid^{1xtg} animals. The variability in the number of B1 cells in xid^{2xtg} mice indicates that additional factors also play a role in the maintenance of this population (34).

Mechanisms in addition to intrinsic signaling defects may contribute to the low titers of IgM, IgG_3, and anti-TNP in xid^{tg} mice. Because B1 cells produce a large fraction of serum IgM (47–49), the low level of IgM in xid^{tg} mice is likely a result of reduced B1 cell numbers. B1 cells do not participate in

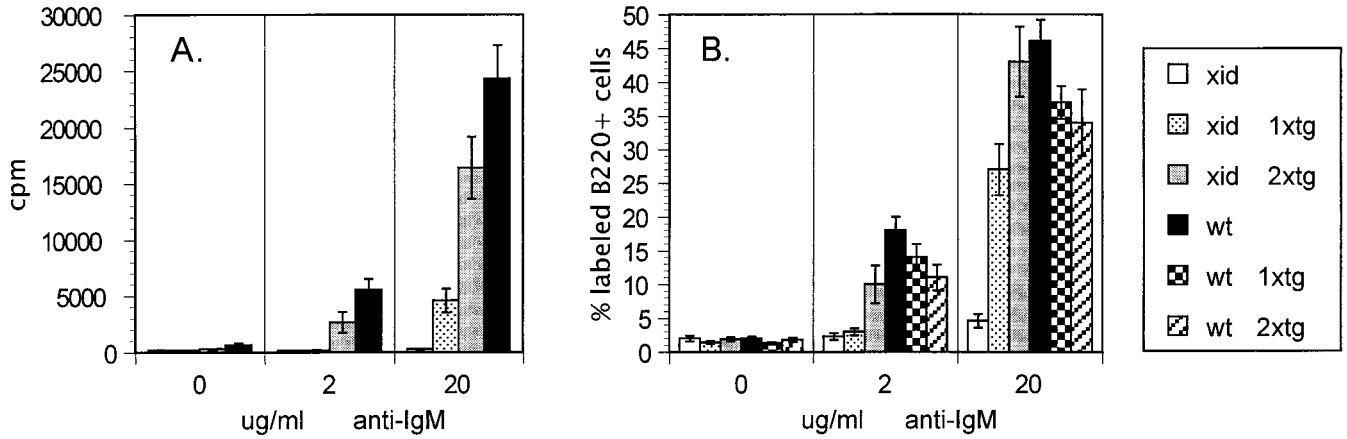

FIG. 5. Anti-IgM response depends on Btk dosage. (*A*) [^{3}H]thymidine incorporation. Purified B220$^+$ cells from spleen were incubated in triplicate for 48 hr with medium alone, 2 μg/ml anti-IgM, 20 μg/ml anti-IgM, or 10 ng/ml PMA and 1 μM ionomycin, then labeled overnight with [^{3}H]thymidine. Counts per minute (cpm) incorporated in untreated or anti-IgM treated cells were normalized to the PMA and ionomycin response, which was similar for all cell types. The number of mice tested per group is: medium alone, 9; 2 μg/ml anti-IgM, 3; 20 μg/ml anti-IgM, 9; PMA and ionomycin, 9. Data are plotted as mean ± SEM. xid^{1xtg} is significantly different from both wild type (wt) and xid^{2xtg} ($P < 0.005$). (*B*) BrdUrd incorporation. Total splenocytes were incubated for 48 hr with medium alone, 2 μg/ml or 20 μg/ml anti-IgM, and labeled with BrdUrd for the final 24 hr. Samples were stained with anti-B220 PE and anti-BrdUrd FITC. The percentage of B220$^+$ cells that incorporated BrdUrd is presented as mean ± SEM. The number of mice tested per group is: xid, 7; xid^{1xtg}, 7; xid^{2xtg}, 7; wt, 10; wt^{1xtg}, 5; wt^{2xtg}, 5. xid^{1xtg} is significantly different from both wt ($P < 0.005$) and xid^{2xtg} ($P < 0.05$). For wt vs. wt^{2xtg}, $0.05 < P < 0.1$.

TNP–Ficoll response, however (47). Because wild-type and xid splenic accessory cells present TNP–Ficoll with almost equal efficiency (50, 51), it is unlikely that this function is impaired in xid^{tg} mice. However, wt^{2xtg} mice have an approximately 2-fold reduction in response to TNP–Ficoll. This may result from an inhibitory effect of the transgene because of ectopic expression or insertional mutagenesis, which could be exacerbated at limiting Btk levels. This is not a result of impaired T cell development because transgenic mice have normal T cell frequencies in spleen (Table 1).

The sensitivity of BCR signaling to Btk dosage suggests that Btk activity is tightly regulated during normal physiological processes. Several molecules are involved in the activation of Btk, including Src family kinases (40, 52, 53), PI3 kinase (Z. Li, M. I. Wahl, L. R. Stephens, P. T. Hawkins, and O.N.W., unpublished data), and G$\beta\gamma$ subunits (54–56). Protein kinase C has a complex functional interaction with Btk. It decreases Btk kinase activity *in vitro* (57), but the xid-like phenotype of PKCβ-deficient mice suggests that PKC positively regulates or is downstream of Btk (45).

XLA gene therapy protocols must ensure that endogenous levels of Btk protein are expressed in the appropriate pattern to restore both B cell development and function. Their success may be thwarted by dominant negative effects of mutant Btk proteins. The slight reduction in B cell function observed in wt^{2xtg} mice suggests that activating mutations of Btk may also result in an XLA- or xid-like phenotype.

Btk may be an attractive therapeutic target for autoimmune diseases typified by excessive B cell function. Autoantibody production requires Btk (58–60, A.S., W.K., P.S., F. Alt, C. Lowell, and O.W., unpublished data). Hypersensitivity to BCR cross-linking correlates with autoimmunity in SHP1 $-/-$ (11) and lyn $-/-$ (61, 62) mice. B1 cells also play an important role in the pathogenesis of autoimmunity (47, 63). Because BCR signaling thresholds and B1 cell development are both sensitive to Btk dosage, a drug that even partially inhibits Btk function may be an effective treatment.

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