Early Arrest in B Cell Development in Transgenic Mice That Express the E41K Bruton's Tyrosine Kinase Mutant Under the Control of the CD19 Promoter Region

Alex Maas, Gemma M. Dingjan, Frank Grosveld and Rudolf W. Hendriks

*J Immunol* 1999; 162:6526-6533; ;
http://www.jimmunol.org/content/162/11/6526

**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

*average

**References**

This article cites 42 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/162/11/6526.full#ref-list-1

**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

The *Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 1999 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Early Arrest in B Cell Development in Transgenic Mice That Express the E41K Bruton’s Tyrosine Kinase Mutant Under the Control of the CD19 Promoter Region

Alex Maas,* Gemma M. Dingjan,*† Frank Grosveld,* and Rudolf W. Hendriks**

Bruton’s tyrosine kinase (Btk) is a nonreceptor protein kinase that is defective in X-linked agammaglobulinemia in humans and in X-linked immunodeficiency in mice. To study the effect of Btk activation in early B cell development in vivo, we have created transgenic mouse strains expressing Btk under the control of the human CD19 promoter region. The transgenic expression of wild-type human Btk corrected all X-linked immunodeficiency features in mice carrying a targeted disruption of the Btk gene. In contrast, expression of an activated form of Btk, the E41K mutant, resulted in an almost complete arrest of B cell development in the immature IgM\textsuperscript{IgD\textsuperscript{−}} B cell stage in the bone marrow, irrespective of the presence of the endogenous intact Btk gene. Immature B cells were arrested at the progression from IgM\textsuperscript{low} into IgM\textsuperscript{high} cells, which reflects the first immune tolerance checkpoint at which autoreactive B cells become susceptible to apoptosis. As the constitutive activation of Btk is likely to mimic B cell receptor occupancy by autoantigens in the bone marrow, our findings are consistent with a role for Btk as a mediator of B cell receptor-induced apoptotic signals in the immature B cell stage. Whereas the peripheral mature B cell pool was reduced to <1% of the normal size, significant numbers of IgM-secreting plasma cells were present in the spleen. Serum IgM levels were substantial and increased with age, but specific Ab responses in vivo were lacking. We conclude that the residual peripheral B cells were efficiently driven into IgM\textsuperscript{+} plasma cell differentiation, apparently without functional selection. The Journal of Immunology, 1999, 162: 6526–6533.

B

ruton’s tyrosine kinase (Btk) is a Tec family nonreceptor tyrosine kinase that is critically involved in B cell receptor (BCR) signaling and thereby directs B cell development (reviewed in Refs. 1 and 2). Defects in Btk result in X-linked agammaglobulinemia (XLA) in humans and in X-linked immunodeficiency (Xid) in mice. XLA patients manifest recurrent bacterial infections due to a profound reduction of serum Ig of all classes. They have very low B cell numbers in the peripheral blood, and those few B cells present exhibit an immature IgM\textsuperscript{high} surface phenotype (3). Because the numbers of pre-B cells in the bone marrow (BM) are not significantly reduced, XLA reflects impaired developmental progression or increased cell death at the pre-B to B cell transition (1–3).

The xid phenotype, which is present both in CBA/N mice carrying an Arg\textsuperscript{28} mutation and in mice with targeted disruptions of Btk in their germline, is distinct from XLA (4–6). Compared with normal mice, Btk-deficient mice have ~50% fewer B cells in the periphery, with an overrepresentation of immature IgM\textsuperscript{IgD\textsuperscript{−}} cells. The CD5\textsuperscript{+} B-1 B cell population is absent, and the levels of IgM and IgG3 in the serum are reduced. Although Btk-deficient mice do not make Abs to a subset of T cell-independent (TI) type II Ags, they are able to respond to most T cell-dependent (TD) Ags. Btk-deficient B cells do not enter S phase after BCR triggering (4, 5). Although Xid is characterized by a defect in peripheral B cell maturation and function, there is no substantial block in early B cell development, as in XLA. Nevertheless, by analysis of competition in vivo between wild-type (WT) and Btk-deficient cells, it was shown that the first selective disadvantage of Btk-deficient cells in the mouse is also at the transition from pre-B to immature B cells (6).

Btk is a 659-aa protein that contains a single C-terminal catalytic domain, the Src homology domains 2 and 3, and a unique pleckstrin homology (PH) domain at the amino terminus with an adjacent proline- and cysteine-rich Tec homology domain (1, 2). Btk is expressed throughout B cell development, but not in plasma cells (1, 6, 7).

It has been shown that Btk tyrosine phosphorylation and in vitro kinase activity increase upon BCR stimulation (8–10). BCR engagement leads to activation of phosphatidylinositol 3-kinase, which generates the second messenger, phosphatidylinositol-(3,4,5)-triphosphate (PIP\textsubscript{3}). In concert with Src family kinases, PIP\textsubscript{3} initiates Btk activation by targeting the kinase to the plasma membrane through interactions with the Btk PH domain (11–15). These interactions are critical to the activity of Btk and result in phospholipase C-γ tyrosine phosphorylation, inositol triphosphate production, and calcium mobilization. This pathway is inhibited by engagement of the Fc receptor γIIB through the activity of the Src homology 2-containing inositol polyphosphatase SHIP, which regulates the association of Btk with the membrane by reducing the

Departments of *Cell Biology and Genetics and †Immunology, Faculty of Medicine, Erasmus University, Rotterdam, The Netherlands

Received for publication November 23, 1998. Accepted for publication March 12, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grant NWO 901-07-224 from the Netherlands Organization for Scientific Research (to A.M.) as well as by the Royal Academy of Arts and Sciences (to R.W.H.).

2 Address correspondence and reprint requests to Dr. Rudolf W. Hendriks, Department of Immunology, Faculty of Medicine, Erasmus University, Dr. Molewaterplein 50, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail address: hendriks@immu.fgg.eur.nl

3 Abbreviations used in this paper: Btk, Bruton’s tyrosine kinase; BCR, B cell receptor; XLA, X-linked agammaglobulinemia; Xid, X-linked immunodeficiency; BM, bone marrow; TI, T cell independent; TD, T cell dependent; WT, wild type; PH, pleckstrin homology; PIP\textsubscript{3}, phosphatidylinositol-(3,4,5)-triphosphate; HSA, heat stable Ag; SA, streptavidin; TNP, trinitrophenol; KLH, keyhole limpet hemocyanin; PNA, peanut agglutinin; H chain, heavy chain; L chain, light chain; LMP-2A, latent membrane protein-2A.

Copyright © 1999 by The American Association of Immunologists

0022-1767/99/$02.00
level of \( \text{PIP}_3 \) (14–15). Btk-deficient B cells manifest a reduced inositol trisphosphate peak level and Ca\(^{2+} \) flux following BCR cross-linking (16). Conversely, a constitutively active form of Btk, E41K, a Glu-to-Lys mutant at position 41 in the PH domain (close to the predicted inositol phosphate binding site) (17), is associated with increased membrane localization and tyrosine phosphorylation of Btk (18). Expression of the E41K Btk mutant was shown to stimulate the growth of NIH-3T3 fibroblasts in soft agar (18) and with increased membrane localization and tyrosine phosphorylation (16). Conversely, a constitutively active form of Btk, BM. Crosses onto a Btk
\[^{2}\]\( \text{E41K} \) mice, we did not observe any defects in developing B cells in the BM. However, this finding does not imply that constitutive activation of Btk does not affect early B cell development, as the expression level of the MHC class II-h\( ^{\text{E41K}} \) transgene may not have reached a critical value.

To study the effect of constitutive Btk activation in early B cell development, we have now placed Btk expression under the control of the human CD19 promoter, which was shown to provide expression throughout the B cell lineage (20, 21). Mice in which expression of the E41K Btk mutant was driven by the CD19 promoter manifested an almost complete arrest in B cell development at the transition of IgM\(^{\text{low}} \) to IgM\(^{\text{high}} \) immature B cells in the BM. Crosses onto a Btk\(^{-} \) background demonstrated that this developmental block was independent of the presence or absence of the endogenous intact Btk gene.

Materials and Methods

Generation of CD19-h\( ^{\text{WT}} \) and CD19-h\( ^{\text{E41K}} \) transgenic mice

The cosmid clone containing the human CD19 promoter region (kindly provided by M. Busslinger, Research Institute of Molecular Pathology, University of Vienna, Vienna, Austria) was isolated from a human genomic cosmid library by screening with a homologous murine CD19 cDNA probe (20). A 16-kb BamHI fragment containing 5' flanking DNA and a 317-bp Xbal-MspI (blunted) fragment (positions 1072–1389 of the human CD19 gene; Ref. 20) were cloned into cosmid vector pTIL5 (22) using a BglII-MluI-BamHI-Sacl-NolI-BgII/I fragment. A unique Eagl site in the 16-kb BamHI fragment was used to isolate a 6.3-kb Eagl (blunted)-Pvull fragment and to ligate it to the 27.1-kb NolI-PvuII fragment containing 400 bp (Btk exons 1–3) of the WT or E41K-mutated hBtk cDNA, a 27-\( \text{kb} \) genomic DNA fragment (Btk exons 3–19), and a 109-bp LoxP fragment (19).

The 34-kb Mid-NolI inserts of the CD19-h\( ^{\text{WT}} \) and CD19-h\( ^{\text{E41K}} \) constructs were excised from the vector and gel-purified. DNA (1–2 ng/\( \mu \)l) was injected into the pronuclei of FVB \( \times \) FVB fertilized oocytes, which were subsequently implanted into pseudopregnant foster mice. To determine the genotype of the founder mice and the subsequent generations generated by crosses with Btk\(^{-}\)l/lacZ mice of a mixed 129Sv \( \times \) C57BL/6 background (6), tail DNA was analyzed by Southern blotting of BamHI digests and subsequent hybridization to a partial btk cDNA probe (btk position 133-1135). Southern and Western blotting techniques have been described previously (6).

Flow cytometric analyses

The preparation of single-cell suspensions and three- or four-color flow cytometry have been described previously (6, 19). The following mAbs were obtained from PharMingen (San Diego, CA): FITC-conjugated anti-B220/RA3-6B2, anti-IgM/R5-240, anti-heat stable Ag (IgS/MA/1/6), anti-CD3, and anti-CD4, anti-CD19, and anti-CD11b/Mac-1; CyChrome-conjugated anti-B220/RA3-6B2 and anti-CD8; and biotinylated anti-HSA/M1/6, anti-IgA/ R26-46, and anti-IgM. PE-conjugated anti-IgD was purchased from Southern Biotechnology Associates (Birmingham, AL). Affinity-purified polyclonal rabbit-anti-Btk (PharMingen) was used for intracellular flow cytometric detection of cytoplasmic Btk protein, as described previously (19). The secondary Abs used were tricolor- or PE-conjugated streptavidin (SA) (Caltag Laboratories, Burlingame, CA), SA-APC (PharMingen), or FITC-conjugated goat-anti-rabbit Ig (Nordic, Capistrano Beach, CA).

Serum Ig detection, in vitro immunizations, and immunohistochemistry

Total or nitrophenyl-specific levels of serum Ig were measured by subclass-specific sandwich ELISA as described previously (19). In these assays, nitrophenyl-specific standards were used for IgG1, IgG2a, and IgG2b, whereas values were calculated as arbitrary units for IgM and IgG3 using a reference serum sample. Immunizations with TD and TI type II Ags were essentially performed as described previously (23). Booster doses were administered after 4 wk.

For immunohistochemical analyses, tissue samples were embedded in optimal cutting temperature compound; frozen 5-\( \mu \)m cryostat sections were acetone-fixed, and single and double labelings were performed using standard procedures (24). The mAbs anti-B220/RSA3-6B2, anti-CD3/KT3, anti-CD11c/N418 (25), and MOMA-1 (26) were applied as hybridoma culture supernatants; biotinylated anti-IgM was obtained from PharMingen, biotinylated peanut agglutinin (PNA) was supplied by Sigma (St. Louis, MI), and anti-IgD was obtained from Southern Biotechnology Associates. The second-step reagents used have been described previously (19).

FIGURE 1. Comparison of transgenic h\( ^{\text{E41K}} \) expression driven by the CD19 promoter or the MHC class II locus control region. Intracellular Btk expression in the BM (A) and spleens (B) from 4-mo-old nontransgenic Btk\(^{-}\) (non-Tg) or Btk\(^{+}\) mice and CD19-h\( ^{\text{WT}} \) WT, CD19-h\( ^{\text{E41K}} \) E41K, and MHC class II-h\( ^{\text{E41K}} \) transgenic mice on the Btk\(^{-}\) background. Cell suspensions were stained for surface markers and subsequently for intracellular Btk. The indicated B-lineage subpopulations were gated and analyzed for Btk expression. The results are displayed as histograms of the indicated mice (bold lines), together with those of Btk\(^{-}\) mice, which served as background stainings (thin lines). Data are from 1 to 5 \( \times \) 10\(^5\) total events (B cell numbers were significantly lower in the CD19-h\( ^{\text{E41K}} \) transgenic mice, see Table 1). Pre-B cells are B220\(^{+}\)CD43\(^{+}\)IgM\(^{-}\); pre-B cells are B220\(^{+}\)CD43\(^{-}\) IgM\(^{-}\); immature B cells are B220\(^{+}\)CD43\(^{-}\) IgD\(^{-}\). The IgM\(^{+}\)IgD\(^{+}\)IgG\(^{+}\) and IgM\(^{+}\)IgD\(^{-}\)B cell subpopulations in the spleen correspond to fractions III, II, and I, respectively (42).
The pattern differed noticeably from the pattern observed in the previous studies (20) and 5’flanking DNA was used to express the hBtkWT and hBtkE41K transgenes. To construct the transgenes, two previously described (19) 27.4-kb hBtk exons 1–3 together with a genomic DNA fragment with Btk exons 1–2 were used. These segments were ligated to an exon 1 of the human CD19 gene, located 18 bp 5’ of the critical B cell-specific activator protein/pax-5 site (20) and 5’ of the 3’ ATG site in the Btk promoter (6). The offspring of these mice contained either WT or the E41K-mutated human Btk cDNA expression in the BM (Table I). In mice that expressed the CD19-hBtkE41K transgene on the Btk background, expression of CD19-hBtkE41K transgene on the Btk background was analyzed by flow cytometry (Table I and Fig. 2). As described previously (6), the CD19-hBtkE41K transgenic mice manifested a significant overexpression compared with nontransgenic mice, the CD19-hBtkE41K transgenic mice a specific deficiency of 3% of normal) in the spleen, lymph node, peripheral blood, and BM (BM). In addition, we observed a specific deficiency of mature IgM+IgD+ B cells in the spleen (Fig. 2B) and CD5+ B-1 B cells in the peritoneal cavity (Fig. 2C). In contrast, the CD19-hBtkE41K transgene resulted in a block of B cell development at the immature B cell stage. To examine the effect of CD19-hBtkE41K expression on B cell development, total cell suspensions from various lymphoid tissues from transgenic and nontransgenic mice, either on the Btk+ or the Btk- background, were analyzed by flow cytometry (Table I and Fig. 2). As described previously (6), the Btk- mice expressed BtkWT or the BtkE41K transgenic mice. In mice that expressed the CD19-hBtkE41K transgene on the Btk- background, a complete correction of Btk-deficient mice was observed in all lymphoid tissues analyzed. On the Btk- background, expression of CD19-hBtkE41K did not appear to have any effect on the sizes of B cell subpopulations (Table I; Fig. 2). In contrast, expression of the CD19-hBtkE41K transgene resulted in a block of B cell development at the immature (BM220+IgM+IgD-) B cell stage in the BM, both on the Btk+ and the Btk- background. The sizes of the pro-B and pre-B cell populations in the CD19-hBtkE41K transgenic mice were similar to those in the Btk-deficient mice (Table I). Compared with WT or Btk- mice, the size of the immature B cell population was reduced by a factor of two to three and lacked IgM+b cells (Table I; Fig. 2). In the IgM+b IgD- population present, the surface IgM heavy (H) chains were associated with conventional light (L) chains and not with the surrogate L chain 55 and 55 pre-B gene products (reviewed in Ref. 27), as assayed by flow cytometry using IgH and IgL chain-specific mAbs (data not shown). The almost complete absence of the B220+IgM+ population of mature recirculating B cells in the BM and the peripheral lymphoid compartments analyzed indicated a reduction of the mature B cell pool to <1% of the normal size (Table I and Fig. 2). In addition to the defect in conventional B cells, the CD19-hBtkE41K transgenic mice also lacked CD5+ B-1 B cells in the peritoneal cavity.

The six groups of mice analyzed did not manifest significant differences in pro-B cell fractions in the BM, as assayed by expression of the B220, CD43, HSA, and BP-1 surface markers (Ref. 28 and data not shown). The CD19-hBtkE41K transgenic mice

---

### Table I. Frequencies of lymphocyte populations in CD19-hBtkWT and CD19-hBtkE41K transgenic mice

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Cell Population</th>
<th>Nontransgenic</th>
<th>CD19-hBtkWT</th>
<th>CD19-hBtkE41K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Btk+</td>
<td>Btk-</td>
<td>Btk+</td>
<td>Btk-</td>
</tr>
<tr>
<td>Splenic</td>
<td>Nucleated cells (×10^6)</td>
<td>114 ± 29</td>
<td>58 ± 13</td>
<td>103 ± 17</td>
</tr>
<tr>
<td></td>
<td>B220+ cells (%)</td>
<td>42 ± 5</td>
<td>22 ± 3</td>
<td>38 ± 7</td>
</tr>
<tr>
<td></td>
<td>CD3+ T cells (%)</td>
<td>27 ± 3</td>
<td>29 ± 2</td>
<td>23 ± 1</td>
</tr>
<tr>
<td></td>
<td>CD3+ T cells (%)</td>
<td>14 ± 3</td>
<td>19 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Lymph Node</td>
<td>B220+ cells (%)</td>
<td>18 ± 2</td>
<td>6 ± 0.4</td>
<td>20 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>CD3+ T cells (%)</td>
<td>50 ± 4</td>
<td>56 ± 1</td>
<td>47 ± 2</td>
</tr>
<tr>
<td></td>
<td>CD3+ T cells (%)</td>
<td>25 ± 3</td>
<td>32 ± 2</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>Blood</td>
<td>B220+ cells (%)</td>
<td>52 ± 2</td>
<td>27 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>Peritoneum</td>
<td>CD5+ IgM+ B cells (%)</td>
<td>24 ± 8</td>
<td>0.5 ± 0.2</td>
<td>22 ± 14</td>
</tr>
<tr>
<td></td>
<td>CD5- IgM- B cells (%)</td>
<td>34 ± 7</td>
<td>19 ± 4</td>
<td>24 ± 1</td>
</tr>
<tr>
<td></td>
<td>CD5+ IgM+ T cells (%)</td>
<td>30 ± 6</td>
<td>48 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td></td>
<td>CD43+ IgM+ pro-B cells (%)</td>
<td>48 ± 5</td>
<td>35 ± 8</td>
<td>46 ± 8</td>
</tr>
<tr>
<td></td>
<td>CD43- IgM- pre-B cells (%)</td>
<td>7 ± 2</td>
<td>10 ± 2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>IgM+ IgD+ B cells (%)</td>
<td>23 ± 5</td>
<td>15 ± 3</td>
<td>27 ± 7</td>
</tr>
<tr>
<td></td>
<td>IgM+ IgD+ B cells (%)</td>
<td>10 ± 2</td>
<td>9 ± 2</td>
<td>9 ± 2</td>
</tr>
<tr>
<td></td>
<td>IgM+ IgD+ B cells (%)</td>
<td>8 ± 3</td>
<td>3 ± 1</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

*The phenotypes of the lymphocyte populations were determined by flow cytometry; dead cells and high side scatter cells were excluded by gating.

**Btk**+ males were Btk+/Y males or Btk- females; Btk- mice were Btk+/Y females.

**Mice** were 6–9 wk old. Data are mean values ± SDs from five to ten mice analyzed per group, except for blood, where values are from two to four mice per group.

### Results

**Transgenic expression of hBtkWT and hBtkE41K under the control of the CD19 promoter**

A 6.3-kb genomic fragment with the CD19 promoter containing the critical B cell-specific B cell-specific activator protein/pax-5 site (20) and 5’flanking DNA was used to express the hBtkWT and hBtkE41K transgenes. To construct the transgenes, two previously described (19) 27.4-kb hBtk cDNA-genomic DNA fusion segments containing either WT or E41K-mutated human Btk cDNA exons 1–3 together with a genomic DNA fragment with Btk exons 3–19 were used. These segments were ligated to an MspI site in exon 1 of the human CD19 gene, located 18 bp 5’ of the ATG translation start. After microinjection into fertilized oocytes, two CD19-hBtkWT and four CD19-hBtkE41K founder mice were obtained. These founders were mated onto a Btk-deficient background, in which the Btk gene was inactivated by a targeted in-frame insertion of a lacZ reporter (6). The offspring of these founders did not exhibit developmental defects or any increased susceptibility to malignancies until they were >9 mo of age.

In the BM and spleens of the transgenic mice, expression of Btk was detected by Western blotting analyses (data not shown) and by intracellular flow cytometry using a polyclonal rabbit antiserum specific for Btk (Fig. 1). Expression of transgenic Btk was restricted to B220+ cells; T cells, granulocytes, monocytes, and macrophages did not express detectable amounts of transgenic Btk (data not shown). Compared with nontransgenic mice, the CD19-hBtkE41K transgenic mice manifested a significant overexpression of Btk protein throughout B cell development, including early pro-B and pre-B cell stages in the BM (Fig. 1). This expression pattern differed noticeably from the pattern observed in the previously reported MHC class II-hBtkE41K mice (19), for which Btk expression was not detectable by flow cytometry in the BM but was found to increase significantly as B cells matured to IgMlow IgD+ IgD+ cells in the spleen (Fig. 1).

The experiments described below were mainly performed on one of the CD19-hBtkE41K transgenic lines, whereas a CD19-hBtkWT transgenic line that manifested comparable Btk expression levels served as a control (shown for the spleen in Fig. 1B). In these experiments, we observed a dose dependency of the effect of hBtkE41K expression in the four independent CD19-hBtkE41K transgenic lines, which carried different transgene copy numbers.
showed a relative increase of the proportions of T cells in the spleen, lymph node, and peritoneal cavity, but their absolute numbers were in the same ranges as those for the nontransgenic or CD19-hBtk<sup>WT</sup> transgenic mice (Table I); no differences in thymocyte subpopulations were observed.

In summary, the expression of constitutively active Btk resulted in an almost complete arrest of B cell development within the immature B cell stage in the BM, irrespective of the presence or absence of endogenous intact murine Btk.

The residual splenic B cell population in CD19-hBtk<sup>E41K</sup> mice has an aberrant surface phenotype

The residual splenic B cells that were present in the CD19-hBtk<sup>E41K</sup> transgenic mice were further characterized by three- and four-color flow cytometry. As shown in Fig. 3, those B220<sup>+</sup> B cells present manifested close to normal IgM expression on the membrane, but the level of cell surface IgD was decreased compared with CD19-hBtk<sup>WT</sup> transgenic B cells. The B cells manifested a normal forward scatter profile and did not appear to belong to the B-1 lineage, as CD5 or Mac-1 were not present on the cell surface (data not shown). The B cells had a B220<sup>low</sup>HSA<sup>high</sup> phenotype, reminiscent of recent emigrants from the BM that have not yet differentiated into mature B220<sup>high</sup>HSA<sup>low</sup> cells of the long-lived B cell pool (29). The lack of B220<sup>high</sup>HSA<sup>low</sup> cells (Fig. 3) indicated that in CD19-hBtk<sup>E41K</sup> transgenic mice, those few immature B cells that have left the BM failed to mature in the spleen into long-lived recirculating cells.

IgM is present but other Ig subclasses are severely reduced in the serum of CD19-hBtk<sup>E41K</sup> transgenic mice

To evaluate the capacities of the residual B cell population present in the CD19-hBtk<sup>E41K</sup> mice, serum Ig levels were determined in 2-mo-old CD19-hBtk<sup>WT</sup> and CD19-hBtk<sup>E41K</sup> transgenic mice, whereas nontransgenic Btk<sup>+</sup> and Btk<sup>−</sup> background cells of the long-lived B cell pool (29). This defect was corrected by transgenic CD19-hBtk<sup>WT</sup> expression: IgM levels were somewhat elevated and IgG3 levels were in the normal range.

By contrast, the serum levels of IgM were low in CD19-hBtk<sup>E41K</sup> transgenic mice, with values between those for Btk-deficient and control mice. Notably, the serum levels of all other subclasses were strongly reduced, whether on the Btk<sup>+</sup> or Btk<sup>−</sup> background (Fig. 4A). In the CD19-hBtk<sup>E41K</sup> transgenic mice, the serum IgM levels increased with age: when the mice were 6–7 mo old, the IgM concentration in the serum was elevated compared with normal or CD19-hBtk<sup>WT</sup> transgenic mice (Fig. 4B). At this age, IgG1 and IgG2a remained undetectable, IgG2b and IgG3 were in the range of Btk<sup>−</sup> mice, and IgA was variable.

The finding of substantial IgM levels in CD19-hBtk<sup>E41K</sup> transgenic mice implied that, despite the reduction of the mature B cell pool to <1% of the normal size, significant numbers of IgM-secreting plasma cells were present.

Ag responses are defective in CD19-hBtk<sup>E41K</sup> transgenic mice

To investigate whether functional Abs could be produced when the CD19-hBtk<sup>E41K</sup> transgene was expressed, we analyzed the immune responses to a TI type II Ag, DNP-Ficoll, and a TD Ag, trinitrophenol (TNP)-keyhole limpet hemocyanin (KLH).

Consistent with the reported findings (4, 5, 30), the Btk<sup>−</sup> mice were unresponsive to DNP-Ficoll: the detected NP-specific IgM or
IgG3 levels at day 7 after i.p. injection did not differ from the values of unimmunized animals. Whereas expression of the CD19-hBtk\textsuperscript{WT} transgene restored the TI type II response in Btk\textsuperscript{−/−} mice, expression of the CD19-hBtk\textsuperscript{E41K} transgene abolished this response, irrespective of the Btk\textsuperscript{−/−} or Btk\textsuperscript{+/−} background (Fig. 5A).

The primary IgM response at day 7 or the secondary IgG1, IgG2a, or IgG2b responses at day 7 after booster injection with TNP-KLH were not significantly different between Btk\textsuperscript{−/−}, Btk\textsuperscript{+/−}, or CD19-hBtk\textsuperscript{WT} transgenic mice. However, the CD19-hBtk\textsuperscript{E41K} transgenic mice were unable to mount detectable primary or secondary humoral immune responses against the TD Ag TNP-KLH (Fig. 5B).

Taken together, these data demonstrate that expression of the CD19-hBtk\textsuperscript{WT} transgene corrected the in vivo B cell response to TI type II Ags in Btk\textsuperscript{−/−} mice, and that, by contrast, CD19-hBtk\textsuperscript{E41K} B cells did not respond productively to TD or TI type II Ags.

Follicular and marginal zone B cells are absent but plasma cells are present in CD19-hBtk\textsuperscript{E41K} mice

At 7 days after booster injection with the TD Ag TNP-KLH, the spleens from 3-mo-old nontransgenic mice or CD19-hBtk\textsuperscript{WT} and CD19-hBtk\textsuperscript{E41K} transgenic mice, each on the Btk\textsuperscript{+/−} or Btk\textsuperscript{−/−} background, were examined by immunohistology. Double labelings of serial spleen sections with mAbs specific for B cells (anti-IgM, anti-IgD, and anti-B220), T cells (anti-CD3), metallophilic macrophages (MOMA-1), or interdigitating dendritic cells (anti-CD11c/N418), as well as PNA are shown in Fig. 6. The spleens of nontransgenic or CD19-hBtk\textsuperscript{WT} mice, either on the Btk\textsuperscript{−/−} or Btk\textsuperscript{+/−} background, demonstrated a characteristic histological organization in terms of separate T and B cell areas in the white pulp, with T cells surrounding central arterioles (Fig. 6, a–c) and B cells in follicles (Fig. 6, a–c, e–g, and i–k) with PNA\textsuperscript{+} germinal centers (Fig. 6, e–g). In these mice, the marginal zones at the outer boundaries of the white pulp contained IgD\textsuperscript{low} B cells as well as MOMA-1\textsuperscript{+} macrophages interrupted with nests of strongly N418-expressing dendritic cells that formed bridging channels into the red pulp (24, 25).

By contrast, only few B cells were detected in the CD19-hBtk\textsuperscript{E41K} transgenic mice. These cells were present partly as isolated cells in the T cell areas and partly in small clusters close to the N418\textsuperscript{high} dendritic cells at the periphery of the white pulp nodule (Fig. 6, d, h, and i). There was no evidence for the presence of a distinct marginal zone, as the outer boundary of the white pulp did not contain B220\textsuperscript{+} cells; MOMA-1\textsuperscript{+} metallophilic macrophages were largely absent, except for the incidental presence of some MOMA-1\textsuperscript{+} cells close to the small B cell clusters (Fig. 6p).

In the red pulp, IgM\textsuperscript{+} plasma cells were present in apparently normal numbers (Fig. 6d). Consistent with the absence of TD immune responses in vivo, PNA\textsuperscript{+} germinal center B cells were not detectable (Fig. 6h).

In summary, these findings show that in CD19-hBtk\textsuperscript{E41K} transgenic mice, those few B cells that emerged from the BM did not develop into follicular or marginal zone B cells, but were present in T cell areas and were efficiently driven into IgM\textsuperscript{+} plasma cell differentiation.

Discussion

We have generated transgenic mice in which the expression of WT human Btk or the E41K gain-of-function mutant is under the control of the promoter and 5’ flanking region of the CD19 gene. The
CD19 promoter has been shown to contain a critical high-affinity binding site for the B cell-specific transcription factor B cell-specific activator protein/pax-5 (20), whereas the expression of a human CD19 transgene was reported to be completely restricted to the B cell lineage and to appear early in B cell development (21). High-level Btk expression was obtained specifically in B cells, but because the transgene constructs used contained ~27 kb of genomic DNA from the Btk gene itself, it is very possible that Btk endogenous regulatory elements contributed to the expression pattern of the transgene. When the CD19-hBtkWT mice were mated onto a Btk<sup>−/−</sup> background, the appropriate expression of transgenic human Btk resulted in a complete correction of the Xid features. This observation paralleled our earlier findings in transgenic mice, in which Btk expression was driven by either the MHC class II Ea gene locus control region or endogenous regulatory regions in a 340-kb yeast artificial chromosome (19, 23, 30).

In contrast, expression of the E41K Btk mutant under the control of the CD19 promoter resulted in an almost complete arrest of B cell development in the immature B cell stage in the BM. The phenotype of CD19-hBtk<sup>E41K</sup> transgenic mice differed markedly from our previously reported MHC class II-hBtk<sup>E41K</sup> transgenic mice, which did not exhibit any detectable defects in developing B cells in the BM, but manifested a deficiency of recirculating B cells (19). From our flow cytometric analyses showing the amount of hBtk<sup>E41K</sup> expressed in vivo from the two transgene constructs, we conclude that the differences between the two mouse strains most likely reflect the earlier expression during B cell development of the transgene driven by the CD19 promoter region.

Expression of the CD19-hBtk<sup>E41K</sup> transgene did not appear to affect the pre-B to immature B cell transition, which is defective in XLA and partially blocked in Xid (1, 2, 6), because IgM<sub>low</sub> immature B cells that expressed significant levels of the E41K Btk mutant were still present. Instead, B cell development was blocked at the progression from IgM<sub>low</sub> into IgM<sub>high</sub> B cells. It was recently shown that this transition is accompanied by a differential response to autoantigen recognition (31). With productive L chain gene rearrangement and the assembly of surface IgM, immature B cells acquire Ag specificity and pass through a tolerance-susceptible stage (27, 32). Autoantigen binding in newly generated IgM<sub>low</sub>-IgD<sub>2</sub> immature B cells results in continued Ig L chain rearrangement (i.e., receptor editing) (31, 33). In contrast, immature B cells that have advanced to the IgM<sub>high</sub> stage lose this ability and concomitantly acquire sensitivity to Ag-mediated apoptosis (31, 33). Thus, our findings show that in CD19-hBtk<sup>E41K</sup> transgenic mice, B

---

**FIGURE 6.** Histology of the spleens from CD19-hBtk<sup>WT</sup> and CD19-hBtk<sup>E41K</sup> transgenic mice. Immunohistochemical analyses of 5-μm splenic frozen sections from Btk<sup>+</sup> mice, Btk<sup>−</sup> mice, as well as CD19-hBtk<sup>WT</sup> and CD19-hBtk<sup>E41K</sup> transgenic mice on the Btk<sup>−</sup> background are shown. Sections were stained with anti-CD3 (blue, a–d) for T cells; with anti-IgM (brown, a–d), anti-IgD (blue, e–h), or anti-B220 (blue, i–l) for B cells; with PNA (brown, e–h) for germinal center B cells; with anti-CD11c/N418 (brown, i–p) for dendritic cells; and with MOMA-1 (blue, m–p) for metallophilic marginal zone macrophages. Open arrows indicate the presence of PNA<sup>−</sup> germinal centers in e–g. Closed arrows indicate the location of small clusters of B cells (d, h, and l) or MOMA-1<sup>−</sup> cells (p) in CD19-hBtk<sup>E41K</sup> transgenic mice. Original magnifications were ×16.
cell development is arrested at the first immune tolerance checkpoint at which autoreactive B cells become susceptible to apoptosis. Assuming that the constitutive activation of Btk mimics B cell occupancy by self Ags, the finding of developmental arrest would be consistent with a role for Btk in the transduction of BCR-linked apoptotic signals in immature B cells. This hypothesis is supported by the observation that Btk regulates the apoptosis induced by IL-3 withdrawal in cultured mast cells (34), and that BCR-engagement triggers apoptosis in WT but not in Btk-deficient DT-40 chicken lymphoma B cells (35). The alternative explanation, which is that the developmental arrest in the CD19-hBtkE41K transgenic mice merely resulted from a direct inhibition of the basal BCR signal that is thought to be required for the survival of B cells (36, 37), seems less likely. As inferred from the phenotype of mice that lack essential signaling components of the BCR complex, such as the CD79α (Iγ) cytoplasmic tail or the tyrosine kinase syk, a basic BCR signaling defect results in an arrest of B cell development at a slightly later stage. In contrast to CD19-hBtkE41K transgenic mice, IgG-deficient or syk−/− mice do have IgMhigh immature B cells in the BM; however, due to defective BCR signaling, the recruitment of these cells into the circulating B cell pool is hampered (38, 39).

The nature of the signals provided by BtkE41K is obviously different from that of the EBV-encoded latent membrane protein-2A (LMP-2A), which is the only other protein that reportedly possesses constitutive BCR-linked signaling activity (40). When expressed in progenitor mouse B lymphocytes in vivo transgenic mice, LMP-2A can provide signals that mimic those initiating from a functional Ig H chain in the context of the pre-BCR, allowing IgG cells to colonize peripheral lymphoid organs (40). Apparently, the LMP-2A-derived signals obviate the necessity for basal BCR-mediated survival signals and, as a result, B-lineage cells are maintained in the absence of a competent BCR (40). In contrast to the Btk E41K mutant, LMP-2A signaling does not appear to mimic BCR signals in immature B cells that trigger a checkpoint that physically eliminates autoreactive B cells in the BM (27, 32). This finding may reflect the complicated nature of the constitutive signaling activity of LMP-2A, which forms tyrosine-phosphorylated aggregates in the plasma membrane, has the capacity to associate with the BCR-linked kinases syk and lyn, and prevents normal calcium fluxes and the accumulation of tyrosine-phosphorylated proteins following BCR cross-linking (40, 41). In CD19-hBtkE41K transgenic mice, the developmental block at the IgMhigh immature B cell stage was leaky, allowing very small numbers of B cells to populate the peripheral immune system. Such cells were almost exclusively found in the spleen as B220lowHSAhigh cells, which normally represent immature B cells that have just left the BM. In parallel with our findings in the spleens of MHC class II-hBtkE41K transgenic mice (19), the expression of BtkE41K further impeded the follicular entry of these cells, as B220highHSAlow cells were absent in the spleen; recirculating B cells in the BM or lymph nodes were completely lacking.

Consistent with the severe reduction of the mature B cell pool to <1% of the normal size and the absence of germinal center formation, serum concentrations of IgA and IgG subclasses were very low, and IgG responses to TD and TI type II Ags were lacking in the CD19-hBtkE41K mice. However, significant numbers of IgM-secreting plasma cells were present in the splenic red pulp. Despite the substantial serum IgM levels, which increased with age to levels that were elevated compared with those in normal mice, specific IgM responses to TD and TI type II Ags were absent. Therefore, we conclude that the constitutive activation of Btk drives the residual B cells that were able to emerge from the BM into IgM− plasma cell differentiation, apparently without functional selection.

Taken together, the phenotypes of Btk-deficient and MHC class II-hBtkE41K or CD19-hBtkE41K transgenic mice indicate that Btk is essential in the multiple signaling pathways that govern the maturation of peripheral B cells, such as B cell follicular entry, maturation of B cells, and plasma cell differentiation (4–6, 19). Regarding the role for Btk in early B cell development, evidence for a role for Btk in pre-BCR signaling is lacking. In the absence of Btk, signaling through the pre-BCR complex still mediates its normal checkpoint function (27, 32) by effecting Ig H chain allelic exclusion, IL-7 driven proliferative expansion, and progression to the resting small pre-B cell stage. However, it is obvious that Btk transduces BCR signals at several checkpoints in B cell development in the BM. The phenotype of XLA patients as well as the selective disadvantage of Btk− cells during the pre-B to immature B cell transition (1, 2, 6) indicates that Btk-mediated BCR signaling is required for the survival of immature B cells that have performed a successful Ig L chain locus rearrangement. The present study shows that activation of Btk blocks the progression of IgMlowIgD− immature B cells into the subsequent stage of IgMhighIgD− cells. This finding would be consistent with a role for Btk in a BCR-linked signaling pathway that eliminates autoreactive B cells in the BM. Additional experiments are needed to directly demonstrate the involvement of Btk as a mediator of apoptosis of autoreactive B cells in the BM.

Acknowledgments

We thank A. Boonenstra, L. Braam, M. Busslinger, W. van Ewijk, M. Kuit, and J. Voerman for assistance at various stages of the project.

References


