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Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase

Gemma M.Dingjan[1,2], Alex Maas[1], Martijn C.Nawijn[2], Linda Smit[3], Jane S.A.Voerman[2], Frank Grosveld[1] and Rudolf W.Hendriks[1,4]

[1]Department of Cell Biology and Genetics and [2]Department of Immunology, Faculty of Medicine, Erasmus University Rotterdam, Dr Molewaterplein 50, P.O. Box 1738, 3000 DR Rotterdam, and [3]Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

[4]Corresponding author
e-mail: hendriks@ch1.fgg.eur.nl

To identify B-cell signaling pathways activated by Bruton's tyrosine kinase (Btk) *in vivo*, we generated transgenic mice in which Btk expression is driven by the MHC class II Ea gene locus control region. Btk overexpression did not have significant adverse effects on B cell function, and essentially corrected the *X-linked immunodeficiency (xid)* phenotype in Btk$^-$ mice. In contrast, expression of a constitutively activated form of Btk carrying the E41K gain-of-function mutation resulted in a B cell defect that was more severe than *xid*. The mice showed a marked reduction of the B cell compartment in spleen, lymph nodes, peripheral blood and peritoneal cavity. The levels in the serum of most immunoglobulin subclasses decreased with age, and B cell responses to both T cell-independent type II and T cell-dependent antigens were essentially absent. Expression of the E41K Btk mutant enhanced blast formation of splenic B cells *in vitro* in response to anti-IgM stimulation. Furthermore, the mice manifested a disorganization of B cell areas and marginal zones in the spleen. Our findings demonstrate that expression of constitutively activated Btk blocks the development of follicular recirculating B cells.

Keywords: B cell antigen receptor/B lymphocytes/Btk/xid/XLA

Introduction

Bruton's tyrosine kinase (Btk) is a non-receptor protein tyrosine kinase that is crucial for B lymphocyte development and function. Mutations in the *Btk* gene are the genetic basis for X-linked agammaglobulinemia (XLA) in man and X-linked immunodeficiency disease (*xid*) in the mouse (Rawlings *et al.*, 1993; Thomas *et al.*, 1993; Tsukada *et al.*, 1993; Vetrie *et al.*, 1993). *Btk* encodes a 659 amino acid protein that contains, in addition to the *Src* homology domains SH2 and SH3 and a single catalytic domain, a unique pleckstrin homology (PH) domain at the N-terminus and an adjacent proline- and cysteine-rich Tec homology (TH) domain (for review see Sideras and Smith, 1995; Desiderio, 1997).

XLA patients displaying a large variety of mutations in the *Btk* gene (Vihinen *et al.*, 1998) are very susceptible to bacterial infections. XLA is characterized by an almost complete block in B cell development at the pre-B cell stage, resulting in a severe decrease of circulating B cells. Plasma cells are virtually absent and serum levels of all Ig classes are very low. The B cell defects in the CBA/N *xid* mice which carry an R28C mutation in the *Btk* PH domain are less severe (Wicker and Scher, 1986). These mice have ~50% fewer B cells in the periphery and the residual cells exhibit an unusual $IgM^{high}IgD^{low}$ profile. They lack the $CD5^+$ B-1 B cell population and have low levels of IgM and IgG3. Although *xid* mice are generally able to respond to T cell-dependent (TD) antigens, they cannot make antibodies to thymus-independent type 2 (TI-II) antigens. Detection of a similar PH domain mutation, R28H, in a patient with classical XLA suggested that the distinction between the two phenotypes did not result from an allelic difference (DeWeers *et al.*, 1994a). This was confirmed by the construction of null mutations in the mouse *Btk* gene, which also resulted in *xid* phenotypes (Kahn *et al.*, 1995; Kerner *et al.*, 1995; Hendriks *et al.*, 1996). By analysis of competition *in vivo* between wild-type and Btk$^-$ 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 cell (Hendriks *et al.*, 1996).

Btk is expressed throughout B cell development, from the earliest pro-B cell stage up to mature B cells, and expression is downregulated in plasma cells (DeWeers *et al.*, 1993; Sideras and Smith *et al.*, 1995; Hendriks *et al.*, 1996). Btk is also expressed in cells of the myeloid lineage, but not in T cells. Btk has been implicated as a mediator of signals from various receptors, including the antigen receptor, interleukin 5 receptor (IL-5R), IL-6R, and CD38 in B lymphocytes, and the FcεRI in myeloid cells (reviewed in Desiderio, 1997). Btk activity is regulated by *Src* family kinases, phosphatidylinositol (PI) 3-kinase-γ and the α-subunit of the G_q class of G proteins (Rawlings *et al.*, 1996; Bence *et al.*, 1997; Li *et al.*, 1997). After stimulation of the antigen receptor or IL-5R in B cells and the FcεRI in mast cells, *Src* family kinases rapidly induce phosphorylation of Y551 in the Btk kinase domain, followed by Btk autophosphorylation at Y223 in the SH3 domain (Wahl *et al.*, 1997). These concerted phosphorylation events were shown to be enhanced by a Glu-to-Lys mutation, E41K, in the PH domain of Btk (Park *et al.*, 1996). The E41K mutant, which was isolated using a retroviral random mutagenesis scheme, was shown to induce transformation of NIH 3T3 fibroblasts in soft agar cultures and factor-independent growth of the IL-5-dependent pro-B cell line Y16 (Li *et al.*, 1995). The transforming activity of the E41K mutation is associated with increased membrane localization and tyrosine

phosphorylation of Btk in transfected NIH 3T3 fibroblast cells. PH domains recruit signaling molecules to the cell surface through specific interactions with phospholipids and proteins (reviewed in Lemmon *et al.*, 1996). Binding of the Btk PH domain to various (phosphatidyl)-inositol phosphates, βγ-subunits of heterotrimeric G proteins and protein kinase C isoforms has been described (Tsukada *et al.*, 1994; Yao *et al.*, 1994; Fukada *et al.*, 1996; Salim *et al.*, 1996). The activating nature of the E41K mutation might be explained by its close proximity to the predicted inositol-phosphate binding site, as was indicated by X-ray crystallography studies (Hyvönen and Saraste, 1997). In this context, the E41K mutant binds inositol 1,2,3,4,5,6-hexakiphosphate with a 2× higher affinity than wild-type Btk (Fukada *et al.*, 1996).

We have previously described the generation of transgenic mice that express human *Btk* (h*Btk*) under the control of the class II major histocompatibility complex (MHCII) Ea gene locus control region, which provides gene expression in myeloid cells and in B-lineage cells from the pre-B cell stage onwards (Drabek *et al.*, 1997). When the MHCII-h*Btk* mice were mated onto a Btk$^-$ background, Btk protein expression was restored to apparently normal levels in the spleen and the Btk$^-$ phenotype was corrected. B cells now differentiated to mature IgM^{low}IgD^{high} stages, peritoneal CD5$^+$ B cells were present and serum Ig levels and *in vivo* responses to TI-II antigens were in the normal ranges (Drabek *et al.*, 1997). These results indicated that in this system the h*Btk* gene was appropriately targeted to both conventional and CD5$^+$ B-1 B cells.

The activation of Btk by B cell antigen receptor-mediated phosphorylation (Aoki *et al.*, 1994; DeWeers *et al.*, 1994b; Saouaf *et al.*, 1994; Wahl *et al.*, 1997) raises the question about the nature of specific events that are controlled by Btk in developing B cells. To be able to identify signaling pathways that are activated by Btk *in vivo*, we have now modified the MHCII-h*Btk* transgene construct and generated two different types of transgenic mice, which either overexpress wild-type h*Btk*, or express various levels of the E41K gain-of-function *Btk* mutant. These transgenic models would indicate whether overexpression or constitutive activation of Btk leads to proliferation of cells in the B cell lineage, immunodeficiency caused by elimination of B cells from the circulation, or induction of B cell anergy.

In this report we show that overexpression of h*Btk* had only minor effects on B cell development and function. In contrast, E41K h*Btk* mutant mice manifested an immunodeficient phenotype that is more severe than *xid* and is characterized by very low numbers of circulating B cells, an almost complete absence of B cell responses *in vivo* and a disruption of the cellular architecture of the spleen.

Results

Generation of WT-hBtk and E41K-hBtk transgenic mice

The constructs used in this study containing either the wild-type (WT-h*Btk*) or the E41K mutant (E41K-h*Btk*) *Btk* gene, as well as the MHCII-h*Btk* construct previously used to obtain transgenic Btk expression (Drabek *et al.*, 1997), are shown in Figure 1A. The E41K mutation was

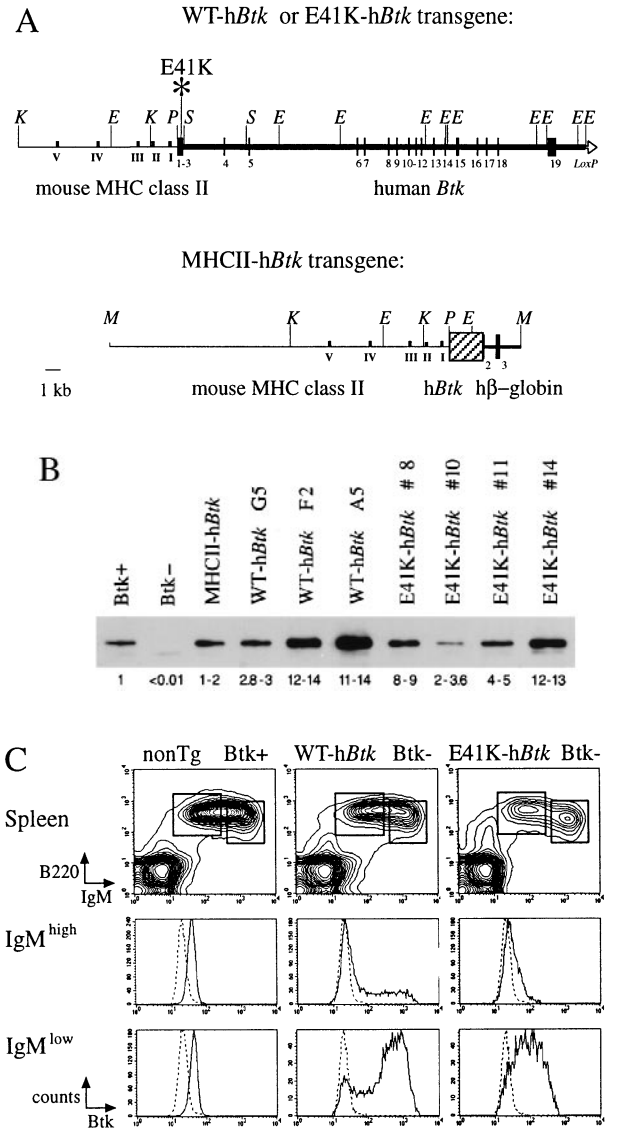

Fig. 1. Structure and protein expression of the h*Btk* transgenes. (**A**) Map of the transgene constructs, showing the locations of the five DNase I hypersensitivity sites present in the 10.6 *Kpn*I–*Pvu*I mouse MHC class II upstream Ea fragment. The WT-h*Btk* and E41K-h*Btk* transgenes contain a 27.4 kb h*Btk* cDNA–genomic DNA fusion segment with exons 1–19, as well as a *loxP* sequence. The MHCII-h*Btk* transgene contains a 2.1 kb h*Btk* cDNA fragment (hatched box) and a 2.8 kb human β-globin fragment with part of exon 2, exon 3 and the 3′ untranslated region. E, *Eco*RI; K, *Kpn*I; M, *Mlu*I; P, *Pvu*I; S, *Swa*I. (**B**) Western blot analysis of Btk protein expression in total spleen cell lysates (2×10^5 cells/lane) from the indicated mice. Transgenic mice were on the Btk$^-$ background. A polyclonal rabbit antiserum was used, which was raised against fusion proteins of gluthatione *S*-transferase (GST) and amino acids 163–218 of hBtk and also recognized E41K hBtk or murine Btk. Ranges of the relative densities of the 77 kDa Btk signals as compared with the control Btk$^+$ mice are given at the bottom. Values were corrected for differences in the proportion of B cells in the spleen, which were determined by flow cytometry. (**C**) Intracellular Btk expression during B cell maturation in the spleen. Single splenic cell suspensions from 3-month-old non-transgenic Btk$^+$ or Btk$^-$ mice, as well as WT-h*Btk* or E41K-h*Btk* transgenic mice on the Btk$^-$ background were stained for surface B220 and IgM and subsequently for intracellular Btk. Data are shown as 5% probability B220/IgM contour plots of total lymphocytes, which were gated by forward and side scatter characteristics (top). The indicated IgM^{high} and IgM^{low} B220$^+$ B cell populations were gated and analyzed for Btk expression (bottom). The results are displayed as histograms of the indicated mice (solid lines), together with the background staining as determined in Btk$^-$ mice (broken lines).

introduced by a G to A replacement at position 257, using *in vitro* site-directed mutagenesis. Since only high copy number MHCII-h*Btk* transgenic mice (two out of five lines) expressed h*Btk* levels similar to those found in normal mice (Drabek *et al.*, 1997), we attempted to increase h*Btk* expression levels by including more h*Btk* genomic DNA, as well as the endogenous 3′ untranslated region, in the transgene construct. The transgenes contained a 10.6 kb MHC class II genomic DNA fragment, a 0.3 kb fragment with the first three exons of h*Btk* as a cDNA sequence, as well as a 27.1 kb genomic DNA fragment, encompassing the h*Btk* exons 3–19 (Figure 1A). The transgene constructs were microinjected into fertilized oocytes and four independent E41K-h*Btk* transgenic lines (#8, #10, #11 and #14) and three WT-h*Btk* transgenic lines (G5, F2 and A5) were obtained. Founder mice were mated to *Btk*$^{-}$*/lacZ* mice, in which the *Btk* gene is inactivated by a targeted in-frame insertion of a *lacZ* reporter in exon 8 (Hendriks *et al.*, 1996).

Expression levels of the E41K and WT hBtk proteins

Btk protein expression was evaluated in transgenic mice on the Btk^{-} background by Western blotting of total spleen cell lysates (Figure 1B). The mice exhibited a wide range of transgenic Btk expression levels in the spleen, which were directly correlated with the transgene copy number as estimated by genomic Southern blotting analyses. To estimate the Btk expression levels of the individual transgenic mouse lines, the densities of the Western blot Btk protein signals were quantified and corrected for the proportion of B cells in the spleen (which were significantly lower in the E41K-h*Btk* transgenic mice; see below). In contrast to the MHCII-h*Btk* transgenic mice which showed approximately endogenous Btk levels, the WT- and E41K-h*Btk* mice manifested up to 14× overexpression of hBtk in their splenocytes (Figure 1B). The experiments described below were mainly performed on WT-h*Btk* line A5, and on E41K-h*Btk* line #8. Except where specifically indicated, no differences were detected between independent lines in the performed analyses, either for the WT-h*Btk* or the E41K-h*Btk* transgenic mice.

Using intracellular flow cytometry, we compared the expression levels of transgenic WT and E41K hBtk with the endogenous murine Btk during B cell differentiation. The individual subpopulations of developing B cells in the bone marrow or spleen showed equivalent expression levels of the endogenous Btk (shown for spleen in Figure 1C). In constrast, a significant increase in WT or E41K transgenic hBtk protein was found as B cells maturated from IgM^{high} to IgM^{low} cells in the spleen (Figure 1C). In the bone marrow, transgenic Btk was only detected in recirculating IgM^{+}IgD^{+} cells. Additional flow cytometric analyses demonstrated that transgenic Btk was also expressed in peritoneal B-1 B cells, in <10% of the Mac-1^{+} myeloid cells in the spleen and peritoneum, but not in T cells or NK cells (data not shown).

When transfected into NIH 3T3 fibroblasts, the E41K Btk mutant manifested enhanced auto-phosphorylation and increased membrane targeting, while the *in vitro* kinase activity was similar to wild-type Btk (Li *et al.*, 1995). However, when we analyzed unstimulated splenocytes, whether from normal mice, WT-h*Btk* or E41K-h*Btk* transgenic mice, the majority of Btk protein was found in the cytosolic fraction. Also, *in vivo* tyrosine phosphorylation or *in vitro* autokinase activity of the hBtk protein in these cells did not appear to be enhanced by the E41K mutation (data not shown).

Depletion of peripheral B cells in E41K-hBtk transgenic mice

The B cell populations in bone marrow, peripheral blood, spleen, mesenteric lymph nodes and peritoneal cavity from E41K-h*Btk* and WT-h*Btk* mice on the Btk^{+} or Btk^{-} background were examined by flow cytometry in 6- to 8-week-old mice (Table I; Figures 2 and 3). Cells from non-transgenic Btk^{+} and Btk^{-} littermates served as controls, showing that the Btk-deficient mice had fewer mature B cells (~30–50% of normal) in peripheral blood, spleen, mesenteric lymph node (Figure 2A) and bone marrow (IgM^{+}IgD^{+} fraction), and a specific deficiency of mature surface IgM^{low}IgD^{high} B cells (Figure 3A) as previously described (Hendriks *et al.*, 1996). In the peritoneal cavity of Btk^{-} mice, the numbers of conventional B cells were reduced and CD5^{+} cells were lacking (Table I; Figure 2B).

Correction of the *xid* B cell deficiency, although not complete, was obtained by WT-h*Btk* transgene expression on the Btk^{-} background. In the peripheral blood, spleen, mesenteric lymph node and bone marrow the B cell numbers only reached values similar to those of Btk^{-} mice, but the numbers of peritoneal CD5^{+} cells were in the normal ranges, and the peripheral B cells exhibited a normal surface IgM/IgD profile (Table I; Figures 2 and 3A). The effect of Btk overexpression on the Btk^{+} background was limited: the numbers of B220^{+} cells were slightly reduced in peripheral blood, but were in the normal ranges in the other organs analyzed (Table I).

In contrast, when E41K-h*Btk* mice on the Btk^{-} background were compared with Btk^{-} mice, a further depletion of B cells was observed in all lymphoid tissues analyzed (Table I; Figure 2). Also in the mesenteric lymph nodes from the three other independent E41K-h*Btk* transgenic lines, the proportion of B cells was 1–5%. Expression of the E41K-h*Btk* transgene on the Btk^{+} background resulted in an analogous reduction in the numbers of circulating B cells, although the effect was less severe than on the Btk^{-} background (Table I). The reduction of the proportions of circulating B cells in the E41K-h*Btk* transgenic mice was accompanied by a relative increase of the percentages of CD4^{+} and CD8^{+} T cells (Table I). The six groups of mice did not manifest significant differences in the numbers of Mac-1^{low}DX5^{+} NK cells or Mac-1^{high} myeloid cells in the spleen cell suspensions (data not shown). In the bone marrow of E41K-h*Btk* mice, pro-B, pre-B and immature B cells were present in normal proportions, whereas mature recirculating IgM^{+}IgD^{+} B cells were virtually absent (Table I). Additional analysis of the three pro-B cell subfractions, as defined by expression of surface markers B220, heat-stable antigen (HSA) and BP-1 (Hardy *et al.*, 1991) in E41K-h*Btk* mice revealed no detectable alterations from the distribution in normal or WT-h*Btk* mice (data not shown).

In strong contrast to the restored IgM/IgD expression profile found on peripheral B cells from WT-h*Btk* transgenic Btk^{-} mice, B cells in spleen, mesenteric lymph node and peritoneal cavity from E41K-h*Btk* transgenic Btk^{-}

Table I. Frequencies of lymphocyte populations in WT-h*Btk* and E41K-h*Btk* transgenic mice

Compartment	Cell population	Non-transgenic		WT-h*Btk*		E41K-h*Btk*	
		[a]Btk$^+$	Btk$^-$	Btk$^+$	Btk$^-$	Btk$^+$	Btk$^-$
Spleen[b]	Nucleated cells ($\times 10^{-6}$)	190 ± 50	84 ± 22	163 ± 34	112 ± 17	194 ± 20	191 ± 38
	B220$^+$ cells (%)	38 ± 9	15 ± 5	34 ± 10	22 ± 5	20 ± 6	10 ± 3
	CD3$^+$CD4$^+$ (%)	20 ± 3	22 ± 5	26 ± 4	23 ± 8	26 ± 7	27 ± 5
	CD3$^+$CD8$^+$ (%)	11 ± 2	12 ± 3	12 ± 3	12 ± 3	15 ± 4	15 ± 4
Lymph node	B220$^+$ cells (%)	24 ± 4	8 ± 2	21 ± 5	7 ± 2	5 ± 0.4	2 ± 1
	CD3$^+$CD4$^+$ (%)	46 ± 5	53 ± 2	50 ± 4	56 ± 1	60 ± 5	62 ± 3
	CD3$^+$CD8$^+$ (%)	22 ± 1	31 ± 2	25 ± 2	27 ± 3	28 ± 4	29 ± 3
Blood	B220$^+$ cells (%)	34 ± 9	12 ± 4	19 ± 6	10 ± 4	7 ± 2	4 ± 1
	of which B220^{low}IgD^{low} (%)	10 ± 4	41 ± 16	10 ± 5	23 ± 7	25 ± 14	51 ± 14
	of which B220^{high}IgD^{high} (%)	71 ± 3	36 ± 15	66 ± 5	46 ± 5	37 ± 12	17 ± 10
Peritoneum	CD5$^+$IgM$^+$ B cells (%)	16 ± 8	0.4 ± 0.3	28 ± 16	14 ± 7	3 ± 2	2 ± 1
	CD5$^-$IgM$^+$ B cells (%)	24 ± 10	10 ± 4	24 ± 15	8 ± 1	4 ± 3	2 ± 1
	CD5$^+$IgM$^-$ T cells (%)	31 ± 3	46 ± 8	29 ± 5	47 ± 11	65 ± 4	62 ± 8
Bone marrow	B220$^+$ cells (%)	37 ± 3	36 ± 1	39 ± 10	36 ± 7	37 ± 5	31 ± 2
	CD43$^+$IgM$^-$ pro-B cells[c] (%)	6 ± 0.3	8 ± 1	6 ± 3	8 ± 2	7 ± 3	6 ± 0.4
	CD43$^-$IgM$^-$ pre-B cells (%)	15 ± 2	16 ± 1	14 ± 5	12 ± 6	13 ± 5	12 ± 3
	IgM$^+$IgD$^-$ B cells (%)	7 ± 2	8 ± 0.3	9 ± 2	8 ± 2	8 ± 0.3	7 ± 1
	IgM$^+$IgD$^+$ B cells (%)	6 ± 2	2 ± 0.5	5 ± 1	2 ± 0.3	1 ± 0.3	0.4 ± 0.2

[a]Btk$^+$ mice were *Btk*$^+$/Y males or *Btk*$^{+/+}$ females; *Btk*$^-$ mice (Hendriks *et al.*, 1996) were either *Btk*$^-$/Y males or *Btk*$^{-/-}$ females. [b]Mice were 6–8 weeks old. Data are mean values ± standard deviations from three mice analyzed, except for spleen where values are from 5–20 mice per group. The phenotype of lymphocyte populations was determined by flow cytometry; dead cells and high side scatter cells were excluded by gating.
[c]Classification of pro-B and pre-B cells was according to Hardy *et al.* (1991).

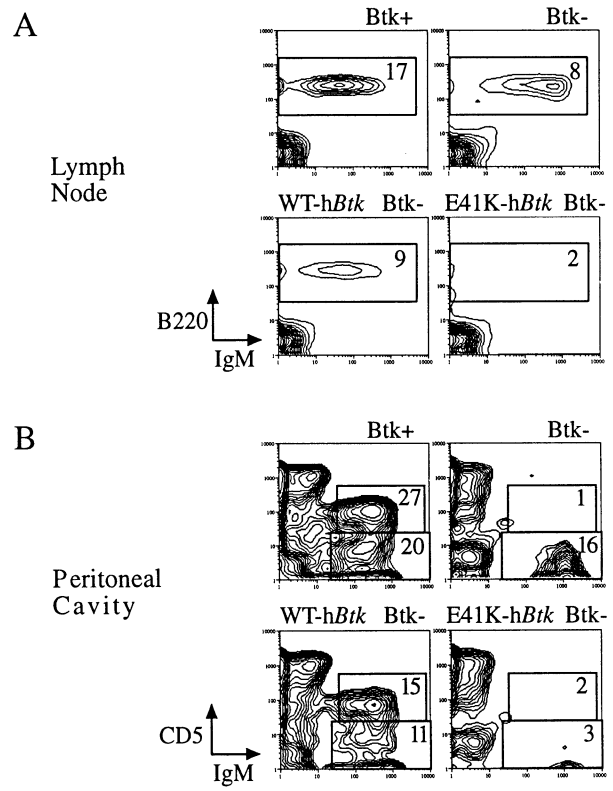

Fig. 2. Depletion of peripheral B cells in E41K-h*Btk* mice. Flow cytometric analysis of (**A**) mesenteric lymph node and (**B**) peritoneal cavity from 7-week-old mice of non-transgenic Btk$^+$ or Btk$^-$ mice, and WT-h*Btk* or E41K-h*Btk* transgenic mice on the Btk$^-$ background. Single-cell suspensions were stained with biotinylated anti-IgM and streptavidin-TriColor, and either FITC-conjugated anti-B220 or PE-conjugated anti-CD5. Data are displayed as 5% probability contour plots of total lymphocytes, which were gated by forward and side scatter characteristics. Percentages of total lymphocytes within the indicated gates are given. Data shown are representative of the mice examined (Table I).

mice manifested an IgM^{high}IgD^{low} phenotype, reminiscent of the B cell population found in non-transgenic Btk$^-$ mice (shown for spleen in Figure 3A). The peripheral blood contained mainly newly-generated B cells that had just left the bone marrow (B220^{low}IgD^{low} cells), rather than recirculating cells migrating between follicles (B220^{high}IgD^{high} cells: only 17 ± 10% of B cells in E41K-h*Btk* transgenic mice and 71 ± 3% in normal Btk$^+$ mice). As these observations suggested a maturational defect in the peripheral B cell compartment, we investigated the expression levels of B220 and HSA: B cells that are B220^{low}HSA^{high} have recently left the bone marrow and further differentiate into mature B220^{high}HSA^{low} cells of the long-lived B cell pool (Allman *et al.*, 1993). While the spleen of non-transgenic Btk$^+$ or Btk$^-$ mice contained ~60% mature B220^{high}HSA^{low} cells, a small reduction in this population was observed in WT-h*Btk* transgenic mice and a 3- to 4-fold reduction in E41K-h*Btk* transgenic mice (Figure 3B). These results indicated that recent emigrants from the bone marrow failed to mature in the spleen into long-lived B220^{high}HSA^{low} B cells.

Serum immunoglobulin levels in E41K-hBtk and WT-hBtk mice

Serum Ig levels were determined by ELISA in 2-month-old non-transgenic Btk$^+$ and Btk$^-$ mice, as well as E41K-h*Btk* and WT-h*Btk* transgenic mice (Figure 4). The Btk$^-$ mice had severely decreased levels of IgM and IgG3, variable levels of IgG1 and somewhat decreased levels of IgG2a as compared with control Btk$^+$ littermates (Drabek *et al.*, 1997). When the WT-h*Btk* transgene was expressed on the Btk$^-$ background, IgM levels were elevated and all other Ig subclasses were restored to normal levels, similar to the correction previously observed as a result of MHCII-h*Btk* transgene expression (Drabek *et al.*, 1997). In the WT-h*Btk* transgenic mice on the Btk$^+$ background, serum Ig subclass levels were in the same ranges. In the E41K-

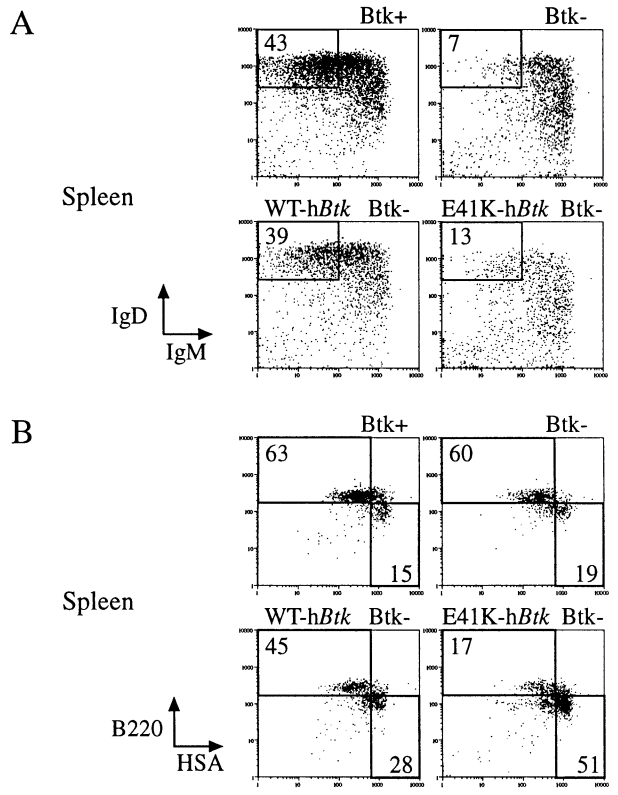

Fig. 3. Expression of E41K Btk induces a dominant maturational defect in peripheral B cells. (**A**) Surface IgM-IgD profiles of splenic B cells. Percentages of $B220^+$ cells that are $IgM^{low}IgD^{high}$ are given. These cells are mature B cells, whereas $IgM^{high}IgD^{low}$ cells are more immature (Hardy *et al.*, 1982). Data are displayed as dot plots of all gated viable $B220^+$ cells from 3×10^4 total events. (**B**) Surface B220-HSA profiles of splenic B cells. Percentages of IgM^+ cells that are immature ($B220^{low}HSA^{high}$) or mature ($B220^{high}HSA^{low}$) are indicated. Data are displayed as dot plots of all gated viable IgM^+ cells from 10^4 total events (or 3×10^4 total events for E41K-h*Btk* transgenic mice on the Btk^- background). Spleen-cell suspensions from 7-week-old mice of the indicated genotypes were incubated with biotinylated anti-IgM and streptavidin-TriColor, FITC-conjugated anti-B220 and either PE-conjugated anti-IgD or anti-HSA and analyzed by three-color flow cytometry. Data shown are representative of the mice examined; lymphocytes were gated on the basis of forward and side scatter.

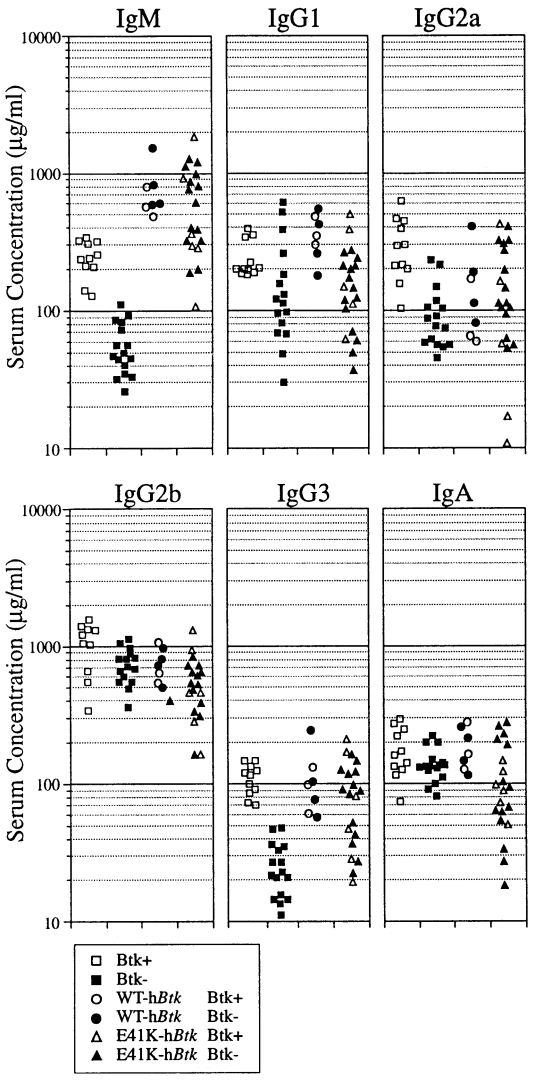

Fig. 4. Effects of transgenic WT-h*Btk* and E41K-h*Btk* expression on serum Ig levels. Serum concentrations of the indicated Ig subclasses in non-transgenic mice (Btk^+, $n = 11$; Btk^-, $n = 16$), as well as WT-h*Btk* ($n = 7$) and E41K-h*Btk* ($n = 20$) transgenic mice on the Btk^- background, whereby each symbol indicates an individual animal. Mice were 2 months old and Ig levels were determined by ELISA.

h*Btk* mice serum IgM was restored to normal or elevated levels, IgG1 was similar to the levels in Btk^- littermates, while serum IgG3 was corrected to normal values for 12 out of 20 animals analyzed (Figure 4). The concentrations of IgG2a and Ig2b were generally in the normal ranges, whereas IgA was quite variable but on average reduced compared with the other three groups of mice. No influence of the Btk^+ or Btk^- background was detected.

Except for IgM and IgG2b, the serum Ig concentrations of the E41K-h*Btk* mice decreased significantly with age. In 6-month-old E41K-h*Btk* mice, the levels of IgG1, IgG2a, IgG3 were only 37 ± 10, 38 ± 20 and 21 ± 15 μg/ml, respectively ($n = 3$). In age-matched WT-h*Btk* mice these levels were 970 ± 170, 470 ± 140 and 280 ± 100 μg/ml ($n = 4$).

Defective in vivo responses in E41K-hBtk transgenic mice

The absence of a dramatic decrease of serum Ig in E41K-h*Btk* transgenic mice at the age of 2 months indicated that despite the observed maturation defect of peripheral B cells, significant numbers of B cells were induced to differentiate into Ig-producing plasma cells. However, the possibility remained that these B cells could not mount specific antibody responses. Therefore, we tested the responses of 2-month-old non-transgenic, WT-h*Btk* and E41K-h*Btk* transgenic mice on the Btk^+ or Btk^- backgrounds to TI-II and TD antigenic challenges *in vivo*.

The responsiveness to the TI-II antigen dinitrophenol (DNP)-ficoll was measured seven days after intraperitoneal (i.p.) injection by enzyme-linked immunosorbent assay (ELISA; Figure 5A). Consistent with previous findings in Btk-deficient mice (Wicker and Scher, 1986; Kahn *et al.*, 1995; Drabek *et al.*, 1997), DNP-specific IgM or IgG3 was completely absent in Btk^- mice, as the absorbence measured did not differ from the values of unimmunized animals. On the Btk^+ background, the TI-II response of WT-h*Btk* mice was comparable with the response of normal mice, while on the Btk^- background, expression of the WT-h*Btk* transgene could only partially restore the

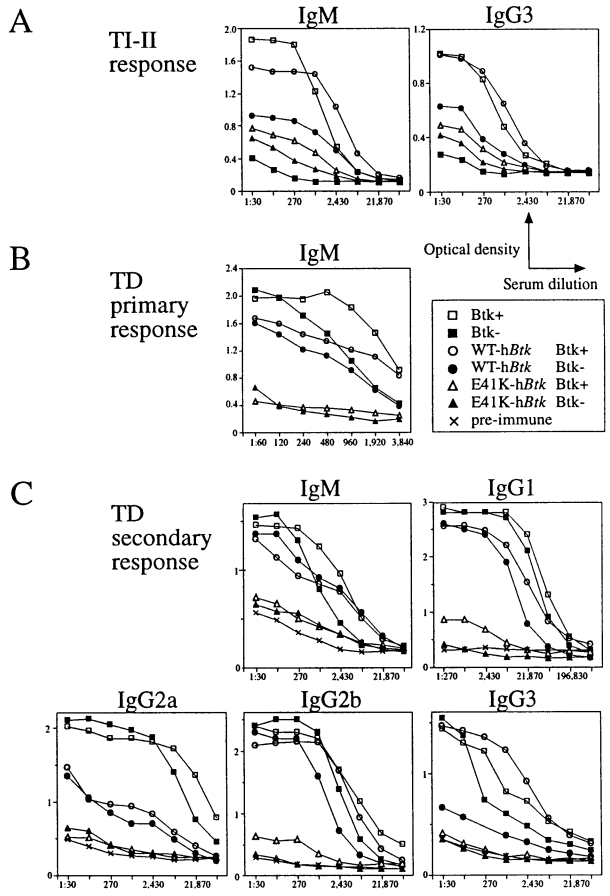

Fig. 5. Defective *in vivo* responses in E41K-h*Btk* mice. (**A**) IgM and IgG3 responses to the TI-II antigen DNP-ficoll. (**B**) Primary IgM responses to the TD antigen TNP-KLH, determined 7 days after i.p. injection. (**C**) Secondary TD responses to TNP-KLH, determined 7 days after i.p. injections performed 5–8 weeks after the primary immunization. Serial serum dilutions were analyzed for DNP/TNP-specific antibodies of the specific subclasses and optical densities are shown as mean values from 4–12 mice in each group. The optical densities in the pre-immune sera did not vary significantly between the six groups and are shown in (C).

TI-II response. The TI-II antibody response in E41K-h*Btk* mice was very low but detectable, whether on the Btk^+ or the Btk^- background.

To investigate TD antibody responses, mice were injected i.p. with trinitrophenol-keyhole limpet haemocyanin (TNP-KLH). After 7 days, TNP-specific IgM was measured by ELISA (Figure 5B). Btk^- mice mounted a moderate primary IgM response compared with wild-type littermates, as previously found in *xid* mice (Wicker and Scher, 1986). Whereas Btk overexpression in the WT-h*Btk* transgenic mice appeared to have a minor inhibitory effect on the levels of TNP-specific IgM and IgG1, the TD primary response was completely absent in E41K-h*Btk* transgenic mice, both on the Btk^+ and on the Btk^- background (shown for IgM in Figure 5B).

When the mice were 3–4 months old, TNP-KLH booster injections were given and secondary TD responses were measured at day 7. TNP-specific levels of IgM, IgG1 and IgG2b were not significantly different between the non-transgenic or WT-h*Btk* transgenic Btk^+ or Btk^- groups of mice (Figure 5C). TNP-specific IgG2a was decreased in WT-h*Btk* transgenic mice and TNP-specific IgG3 was low in WT-h*Btk* mice on the Btk^- background. In strong contrast to these limited adverse effects of the WT-h*Btk* transgene, we found that secondary TD responses were very low or lacking in E41K-h*Btk* mice, irrespective of their Btk^+ or Btk^- background (Figure 5C). Because Btk-deficient mice show a normal secondary response to TD antigens (Wicker and Scher, 1986; Kahn *et al.*, 1995; Drabek *et al.*, 1997; Figure 5C), the finding that TD responses are essentially absent in E41K-h*Btk* transgenic mice was striking.

Aberrant in vitro responses of E41K-hBtk transgenic B cells

To examine the capacity of B cells to respond to mitogenic signals *in vitro*, B cell-enriched spleen cell suspensions were stimulated with LPS, anti-CD40 and IL-4, and different concentrations of a goat-antiserum to mouse IgM.

Proliferative responses to LPS, determined by quantitating [^{3}H]thymidine incorporation after 60 h of culture, were low in Btk^- B cells compared with control B cells (Figure 6A). Expression of the WT-h*Btk* transgene completely restored the proliferative capacity, while expression of E41K-h*Btk* had a minor effect. These differences in proliferative responses to LPS were paralleled by the *in vitro* Ig production profiles in LPS-stimulated cell cultures as measured by ELISA at day 7 (Figure 6B). The defective IgM, IgG1 and IgG3 production in Btk^- B cells was corrected by transgenic WT-h*Btk* expression, but the effect of E41K-hBtk expression was moderate for IgM, and negligible for IgG1 and IgG3.

Consistent with previous reports (Kahn *et al.*, 1995; Anderson *et al.*, 1996; Ridderstad *et al.*, 1996) Btk^- B cells showed normal proliferation induced via CD40 in the presence of IL-4. Btk overexpression in the WT-h*Btk* transgenic mice was accompanied by a small but significant increase in proliferative responses. Transgenic expression of the E41K mutant did not have a detectable effect on proliferation in response to anti-CD40 and IL-4 (Figure 6B), nor in response to anti-CD40 alone or to anti-CD40 and INF-γ (data not shown). When the production of IgM, IgG1 and IgG3 in anti-CD40- and IL-4-stimulated cell cultures was evaluated, a marginally lower production was observed in Btk^- and E41K-h*Btk* B cells as compared with normal Btk^+ or WT-h*Btk* B cells.

One of the hallmarks of Btk^- B cells is that they do not enter S phase when triggered through their surface IgM receptor (Wicker and Scher, 1986; Kahn *et al.*, 1995; Anderson *et al.*, 1996). We observed that the absence of proliferative response to anti-IgM antibodies was corrected by transgenic expression of both wild-type and E41K mutated hBtk (Figure 6C). Moreover, when the E41K-h*Btk* transgene was expressed, B cells enlarged more rapidly in response to anti-IgM stimulation (Figure 6D). After 24 h of culture, the E41K-h*Btk* $B220^+$ cells showed a significantly higher proportion of large blast cells when compared with non-transgenic Btk^+ and Btk^- or WT-h*Btk* transgenic $B220^+$ cells. Blast formation was already manifest in the absence of anti-IgM, but became more pronounced in the presence of anti-IgM up to 1 μg/ml. At high anti-IgM concentrations also WT-h*Btk* transgenic B cells showed enhanced blastogenesis when compared with B cells from non-transgenic mice. Increased blast formation of E41K-h*Btk* transgenic B cells was also

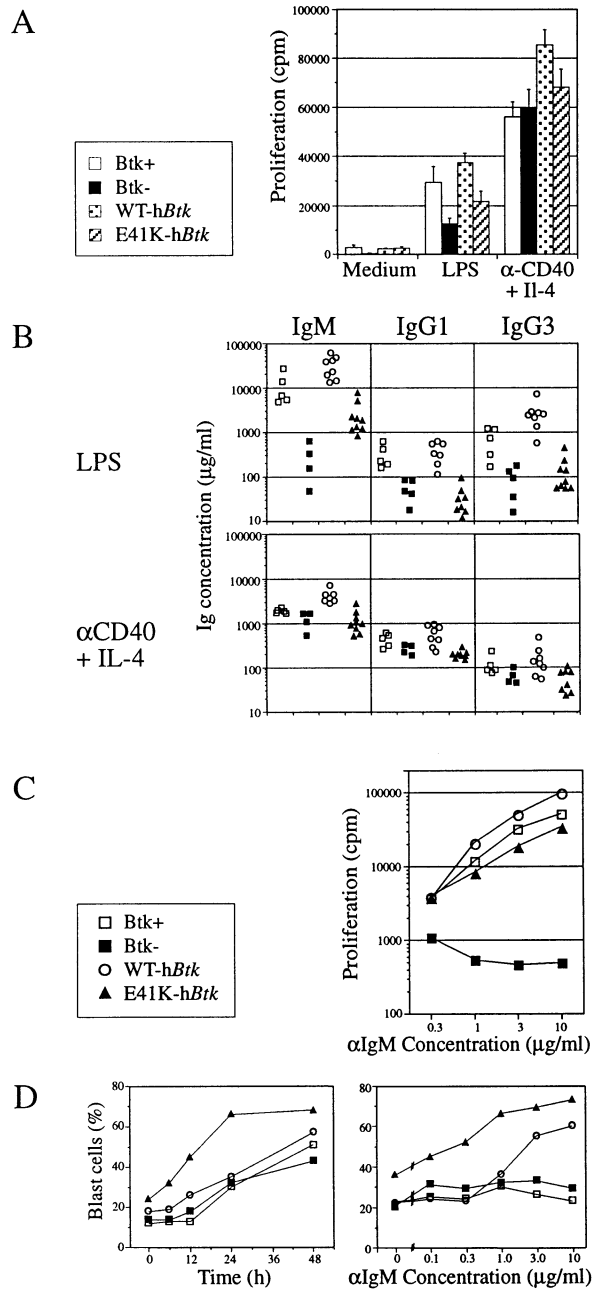

Fig. 6. Transgenic E41K-h*Btk* B cells show aberrant responses to mitogenic signals *in vitro*. (**A**) Proliferation, determined by [^{3}H]thymidine incorporation, of cells cultured in medium alone, in the presence of LPS, or anti-CD40 antibodies and IL-4. (**B**) Ig levels of supernatants of 7-day cultures in the presence of LPS or anti-CD40 and IL-4. (**C**) Proliferation, determined by [^{3}H]thymidine incorporation, in response to various concentrations of goat anti-mouse IgM. (A–C) Enriched splenic B cell fractions Btk$^+$, Btk$^-$, WT-h*Btk* transgenic and E41K-h*Btk* transgenic mice were cultured *in vitro* with various mitogens. The groups of transgenic mice contained animals on Btk$^+$ or Btk$^-$ background; no differences were detected between mice from these two backgrounds. Data are given as mean values ± SE with 4–9 animals in each group (A), as symbols that indicate the values in cultures of individual animals (B) or as mean values from 4–9 mice in each group (C). (**D**) Total splenocytes from Btk$^+$, Btk$^-$, WT-h*Btk* transgenic Btk$^-$ and E41K-h*Btk* transgenic Btk$^-$ mice were cultured with 1 μg/ml goat-anti-mouse IgM for the indicated time points (left), or in the presence of the indicated αIgM concentrations for 24 h (right). The proportion of blast cells was determined by flow-cytometric analysis of forward light scatter of viable B220$^+$ cells. Data are given as mean values from 2–4 mice in each group.

observed in response to stimulation with 50 μg/ml LPS (data not shown).

The impairment of TD responses in the E41K-h*Btk* did not appear to be due to defective induction of MHC class II or the co-stimulatory molecule B7.2 (CD86) on activated B cells, as these activation markers were upregulated after stimulation with goat anti-mouse IgM, LPS, or anti-CD40 and IL-4; culture with anti-CD40 and IL-4 also induced upregulation of CD23 on E41K-h*Btk* B cells (data not shown).

Disrupted splenic architecture in E41K-hBtk mice

By immunohistochemical analyses, the spleens of 2-month-old unimmunized Btk$^+$, Btk$^-$ or WT-h*Btk* transgenic mice demonstrated a characteristic organization, in terms of segregation of T and B cells in the white pulp, with T cells clustered in the periarteriolar lymphocyte sheath (PALS) and surrounded by B cell-rich areas containing follicles, and the presence of marginal zones at the outer boundaries of the white pulp. In contrast, E41K-h*Btk* mice derived from all separate founder lines showed a specific effect of the E41K Btk mutation on the architecture of the spleen. When expressed on the Btk$^-$ background, a disruption of splenic architecture was observed, characterized by a reduction in the number and size of B cell areas (consistent with our flow cytometric analyses), loss of the strict compartmentalization of B and T lymphocytes and loss of a distinct marginal zone.

Figure 7 shows double-labeling of serial spleen sections with either anti-B220, anti-CD3 or MOMA-1, a monoclonal antibody specific for the metallophilic macrophages, which constitute a major component of the marginal zone (Kraal, 1992), together with the N418 antibody specific for the CD11c integrin on dendritic cells (Steinman *et al.*, 1997). In the E41K-h*Btk* mice, B220$^+$ cells were present in B-cell areas neighboring the T cell zones (Figure 7A, B and C), but B cell numbers were reduced. These B cell areas contained significantly more T cells than the B cell follicles in control Btk$^+$ or Wt-h*Btk* transgenic mice (compare Figure 7D, E and F), and contained also CD11c$^+$ interdigitating dendritic cells, which normally do not extend into B cell follicles (Steinman *et al.*, 1997) (compare Figure 7G, H and I). No distinct marginal zones were present; the outer boundary of the white pulp did not contain B220$^+$ cells and metallophilic macrophages were only incidently present as a small rim of MOMA-1$^+$ cells adjacent to the B cell areas (Figure 7H). In this area, an expansion was manifest of CD11c$^+$ marginal dendritic cells, which normally form bridging channels into the red pulp (Steinman *et al.*, 1997) (compare Figure 7G, H and I). The numbers of ER-TR9$^+$ marginal zone macrophages (Kraal, 1992) were severely reduced, and the reticular fibroblast network, characteristic for marginal zones, as revealed by ER-TR7 staining (VanVliet *et al.*, 1986) appeared to be absent (data not shown). We observed a dose-dependency of the effect of E41K-h*Btk* expression on the disruption of splenic architecture, because in line #14, which showed a higher transgene expression level than line #8 (Figure 1B), MOMA-1$^+$ cells were essentially absent (Figure 7K and L). Nevertheless, in line #10 with low-level transgene expression (Figure 1B) the MOMA-1$^+$ cells were reduced in number, CD11c$^+$ cells extended into the B cell areas and the strict separation of B and T cell

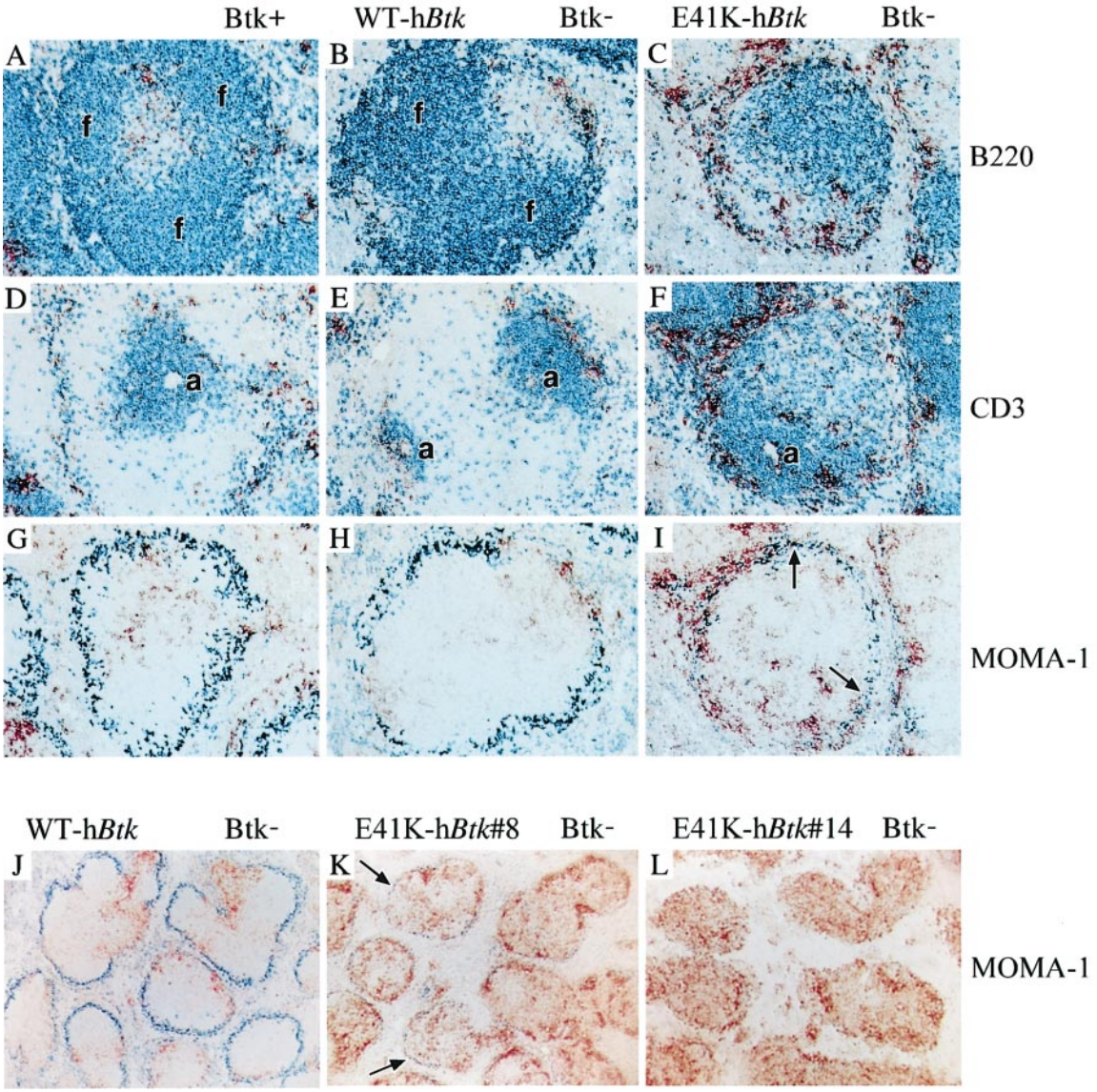

Fig. 7. Disrupted splenic architecture in E41K-h*Btk* transgenic mice. Immunohistochemical analysis of 5 μm splenic frozen sections from Btk$^+$ mice, WT-h*Btk* Btk$^-$ mice and E41K-h*Btk* transgenic mice [(C), (F), (I) and (K), line #8, and (L), line #14] on the Btk$^-$ background. Sections were stained with anti-B220 [blue, (**A**–**C**)] for B cells, anti-CD3 [blue, (**D**–**F**)] for T cells or MOMA-1 [blue, (**G**–**L**)] for metallophilic marginal zone macrophages, together with anti-CD11c/N418 (brown) to detect dendritic cells. Arrows [sections (I) and (K)] indicate the presence of small rims of MOMA-1$^+$ cells in the marginal zone. a, central arteriole; f, B cell follicle. Original magnifications: ×50 (A–I) and ×16 (J–L).

areas was lost (data not shown). In WT-h*Btk* mice, MOMA-1$^+$ cells were not affected and B cell areas did not contain CD11c$^+$ interdigitating cells (Figure 7H and J). The splenic abnormalities of E41K-h*Btk* transgenic mice on the Btk$^+$ background were variable, but became more severe with age. When spleens from 4- to 5-month-old mice were analyzed, the segregation of B and T cell areas was lost and the numbers of MOMA-1$^+$ and ER-TR9$^+$ cells were severely reduced.

Germinal center formation was evaluated by immunohistochemical analysis of the spleen, both in 4- to 5-month-old unimmunized mice and in mice 7 days after booster injection with TNP-KLH adsorbed to alum. In contrast to the Btk$^+$ mice, Btk$^-$ and WT-h*Btk* transgenic Btk$^-$ mice, the E41K-h*Btk* transgenic Btk$^-$ mice did not develop germinal centers, as B220$^+$IgD$^-$ cells that showed binding of peanut agglutinin (PNA) were completely absent (data not shown).

Analogous to the disruption of splenic architecture, also in mesenteric lymph nodes from E41K-h*Btk* mice on the Btk$^-$ background B cell distribution defects were observed. B220$^+$ cells expressing IgM and IgD were almost absent in the cortical B cell areas, whereas IgM$^+$IgD$^+$ B cells were present in low numbers as clusters in the paracortical T cell zones (data not shown).

Discussion

To date, numerous interactions of the individual domains of Btk with various protein or lipid molecules have been reported (Fukada *et al.*, 1996; Bence *et al.*, 1997; Li *et al.*, 1997; Wahl *et al.*, 1997; reviewed in Mattsson *et al.*, 1996). To be able to study the impact of these particular interactions on B cell differentiation and function *in vivo*, we have developed mouse models in which wild-type or mutated *Btk* can be appropriately expressed as transgenes.

We previously showed that expression of hBtk driven by the class II MHC Ea locus LCR resulted in physiological Btk protein levels in the spleen, which completely corrected the features of the *xid* phenotype (Drabek *et al.*, 1997).

Limited effects of overexpression of Btk

By the introduction of 16 out of 18 intron segments of the *Btk* gene, together with the endogenous 3′ untranslated region, high-level Btk expression was obtained. It seems likely that these modified transgenes contained endogenous regulatory elements of the *Btk* gene, since *Btk* intron sequences were shown to contain multiple clusters of extensive conservation between mouse and man (Oeltjen *et al.*, 1997), one of which, a 229 bp region in the fifth intron with 90% identity, showed co-localization with a DNase I hypersensitive site present in B cells but not in T cells from human tonsils (G.M.Dingjan, unpublished results). Thus, the transgenic Btk expression pattern may reflect a combination of the effects of MHC class II Ea gene LCR and endogenous *Btk* regulatory sequences.

On the Btk$^-$ background, overexpression of hBtk corrected most of the *xid* defects, as indicated by the appearance of normal proportions of mature IgM^{low}IgD^{high} circulating B cells, CD5$^+$ B cells in the peritoneum, restored serum IgM and IgG3 levels and *in vitro* responses to anti-IgM or LPS stimulation. However, in contrast with our findings in the MHCII-h*Btk* mice, TI-II responses were only partially restored and primary TD IgM responses were somewhat reduced.

Although WT-hBtk overexpression on the Btk$^+$ background was found to be associated with partially enhanced blastogenesis and proliferation of B cells in response to anti-IgM stimulation *in vitro* (Figure 6C and D), this did not appear to result in adverse effects on B cell development or function: B cell numbers were largely in the normal ranges, and B cell responses to TI-II or TD antigens *in vivo* or LPS *in vitro* were not notably affected. Therefore, we conclude that Btk overexpression *per se* does not lead to significant activation of downstream signaling pathways.

E41K-hBtk represents an activated form of Btk

In contrast, expression of the Btk E41K gain-of-function mutation on the Btk$^-$ background blocked maturation of peripheral B cells, leading to a B cell deficiency that was more severe than the *xid* phenotype. Although E41K-h*Btk* transgenic mice produced normal numbers of immature B cells in the bone marrow that began to express IgD, they failed to become mature recirculating follicular B cells in the periphery. A severe reduction of B cell numbers was observed in all peripheral tissues and those B cells present in the periphery were mainly B220^{low} and HSA^{high}. It is most probable that these cells represented newly-generated virgin B cells, which generally have a lifespan of only 3 days (Fulcher and Basten, 1997). Total serum Ig levels were not seriously affected at the age of 2 months, but they decreased significantly with age. The *in vivo* B cell response to TI-II antigen was very low and only marginally restored compared with Btk$^-$ mice, while B cell responses to TD antigen were lacking. The finding that E41K-h*Btk* transgene expression resulted in a decrease of circulating B cell numbers and a loss of B cell functions, even in the presence of intact endogenous murine Btk, substantiated the dominant nature of the E41K mutation.

When expressed in NIH 3T3 cells *in vitro*, the gain-of-function activity of the E41K Btk mutant was associated with increased membrane localization and was shown to require kinase activity (Li *et al.*, 1995). Our biochemical analyses in unstimulated B cells demonstrated that the E41K mutant had *in vitro* kinase activity, but did not reveal a dramatic increase in membrane targeting. Nevertheless, we cannot exclude that the E41K mutation was associated with relatively small changes in subcellular localization of Btk, which are difficult to detect in biochemical assays. In this context, it was shown that subtle variations in antigen concentration or expression of CD19, a co-receptor that lowers the threshold for antigen receptor stimulation, resulted in dramatic changes of the fate of B cells *in vivo* (Cook *et al.*, 1997; Tedder *et al.*, 1997). Nevertheless, two of our findings indicated that the E41K mutation represented an activated form of Btk. (i) Expression of E41K enhanced *in vitro* blast formation of splenic B cells in culture, either with or without mitogens (Figure 6D). (ii) Splenic E41K-h*Btk* B cells showed proliferation upon stimulation with anti-IgM antibodies (Figure 6C), despite the unusually high proportion of immature B220^{low}HSA^{high} cells in this population. Normally, such B220^{low}HSA^{high} virgin B cells are refractory to anti-IgM stimulation (Allman *et al.*, 1992).

Expression of E41K-hBtk impedes follicular entry and disrupts marginal zone microarchitecture

Our findings in the E41K-h*Btk* transgenic mice indicate that constitutive activation of Btk blocks the development of follicular B cells. The numbers of recirculating B cells were severely reduced, specifically in peripheral blood and lymph nodes. In the spleen, B cell areas typically contained CD11c$^+$ interdigitating dendritic cells, which normally do not extend into B cell follicles, as well as unusually high numbers of T cells. Signals that induce B cells to become recirculating follicular B cells are mediated by the B-cell antigen receptor, as most peripheral B cells are ligand-selected (Gu *et al.*, 1991) and mutations of the CD79α/Igα or Syk signaling components of the B cell antigen receptor block the entry of B cells into follicles (Torres *et al.*, 1996; Turner *et al.*, 1997). These findings demonstrated that low-level stimulation of the B cell antigen receptor provides a signal that is required for follicular entry. Our results show that in the presence of E1K-hBtk expression such signals were not transduced. Together with the reported abnormal maturation into IgM^{low}IgD^{high} follicular B cells in Btk-deficient mice (Wicker and Scher, 1986; Kahn *et al.*, 1995; Hendriks *et al.*, 1996), this argues for a crucial role of Btk in the recruitment of B cells in the long-lived recirculating B-cell pool.

Although the possibility that constitutive activation of Btk leads to a general defect that impedes the survival or affects the migration of B cells in the periphery cannot be excluded, it is attractive to hypothesize that expression of the E41K Btk mutant mimics B cell antigen receptor engagement. In the E41K-h*Btk* mice those cells that were present in the spleen were IgM^{high}HSA^{high}B220^{low} immature B cells that have just left the bone marrow. Normally, immature B cells in the bone marrow are

susceptible to negative selection (Goodnow *et al.*, 1995) and immature HSA^{high} splenic B cells are refractory to stimulation with anti-IgM or phorbol ester and calcium ionophore (Allman *et al.*, 1992). Therefore, the immature status of the E41K-h*Btk* activated B cells may hamper their subsequent expansion or differentiation and induce their elimination instead. In this context, E41K-h*Btk* B cells may resemble auto-reactive B cells which have received a stimulatory signal through their antigen receptor, resulting in arrest in the outer PALS where their lifespan is reduced to 3–4 days in the absence of T cell help (Fulcher and Basten, 1994; Goodnow *et al.*, 1995). Additional parallels with auto-reactive B cells include the rapid increase in size of auto-reactive B cells upon self-antigen recognition immediately before their disappearance (Fulcher *et al.*, 1996; Rathmell *et al.*, 1996) and their absence in the splenic marginal zones (Mason *et al*., 1992).

The lack of splenic marginal zone B cells in E41K-h*Btk* mice could be a direct result of the impeded follicular entry of B cells, as marginal zone B cells are derived from follicular B cells (Kumararatne and MacLennan, 1981). The B cell abnormalities in E41K-h*Btk* mice may also hamper the normal development of marginal zone macrophage populations. Further studies will be required to clarify whether the B cell or the macrophage population of the marginal zone is intrinsically affected in E41K-h*Btk* mice, or whether the defects in both populations are secondary to accessory cell dysfunction. Since we have observed a similar disruption of splenic marginal zone architecture in mice that express the E41K-h*Btk* mutant under the control of the B cell specific CD19 promoter (A.Maas, unpublished results), it seems less probable that macrophage development is intrinsically affected by the expression of the E41K Btk mutant. Clues about the relationship between these cell populations may come from experiments in mutant mice that present with closely related phenotypes, such as mice deficient in members of the nuclear factor-κB family of transcription factors (Franzoso *et al.*, 1997, 1998; Caamaño *et al.*, 1998) or tumor necrosis factor ligand and receptor family members (Matsumoto *et al.*, 1997), which present with defects in humoral responses, germinal center formation and marginal zone macrophage subpopulations.

The role of Btk in B cell development

Studies in transgenic mice carrying rearranged Ig genes have established that strong antigen receptor signals (e.g. transmitted by autoantigens) can result in antigen receptor editing or elimination of immature B cells in the bone marrow (reviewed in Goodnow *et al*., 1995; Melamed *et al.*, 1998). The absence of any defects in developing B cells in the bone marrow of E41K-h*Btk* mice (except in the recirculating mature cells) does not imply that constitutive activation of Btk would not have any effect in the bone marrow. Our intracellular flow-cytometric analyses indicate that the expression level of the E41K-h*Btk* transgene may not have reached a critical threshold value in the bone marrow to affect B cell development. In fact, the first defects in the B cell lineage only became apparent in the spleen, where we identified a significant increase in expression of the hBtk transgenes during maturation from IgM^{high} to IgM^{low} B cells.

In summary, we conclude that Btk is essential for the transduction of signals that govern the development of recirculating follicular B cells. The absence of Btk leads to abnormal maturation into IgM^{low}IgD^{high} follicular B cells (Wicker and Scher, 1986; Kahn *et al.*, 1995; Hendriks *et al.*, 1996). We have shown that transgenic expression of the E41K Btk mutant blocks the development of recirculating follicular B cells, indicating that constitutive activation of Btk induces the elimination of virgin peripheral B cells. Further experiments are required to investigate whether this elimination is caused by the absence of a basal antigen receptor signal that is thought to direct developmental progression of B cells, or by the presence of a signal that mimics B cell receptor occupancy by self-antigens.

Materials and methods

In vitro mutagenesis

The E41K mutation was created by a G to A replacement at position 257 in the h*Btk* cDNA clone ph*Btk*2.55 in pBlueScript (Drabek *et al.*, 1997). Double-stranded site-directed mutagenesis (Stratagene, La Jolla, CA) was performed with the E41K mutagenic primer 5′-GCACA AACTC TCCTA CTATA AGTAT GACTT TGAAC GTGGG-3′ and a 39 bp *Kpn*I→*Bgl*II selection primer, and the obtained mutant plasmids were sequenced, using standard methods. From the original wild-type h*Btk* and the E41K mutated cDNA clone, 303 bp *Pvu*I–*Nla*IV fragments, encompassing the first two exons and part of exon 3, were used in the construction of the transgenes.

Construction of WT-hBtk and E41K-hBtk transgenes

The two transgene constructs WT-h*Btk* and E41K-h*Btk* are shown in Figure 1A. From the MHC class II Ea gene cosmid 32.1 (Carson and Wiles, 1993) a 4.0 kb *Kpn*I fragment, containing DNase I hypersensitivity sites (HSS) I and II and a unique *Pvu*I site at position +14 in the Ea gene, was cloned into pBlueScript, using a *Not*I–*Sma*I–*Kpn*I–*Swa*I–*Xho*I–*Not*I polylinker. The resulting plasmid was partially digested with *Asp*718 in the presence of ethidium bromide to introduce a 9.4 kb *Asp*718 fragment from cosmid 32.1, encompassing HSS III to V. Subsequently, a 13.4 kb *Not*I fragment from this plasmid was cloned into a *Not*I-digested cosmid vector pTL5 (Lund *et al.*, 1982), in which the same polylinker as described above was introduced at a unique *Bgl*II site. In a next step two fragments were cloned into this plasmid, using the unique *Pvu*I site in the Ea gene and the unique *Swa*I site within the polylinker: (1) a 303 bp *Pvu*I–*Nla*IV WT or E41K-mutated h*Btk* cDNA fragment (as described above) and a 97 bp *Nla*IV–*Swa*I fragment obtained by PCR amplification using primers at positions 46846 and 47029 of the h*Btk* gene (Oeltjen *et al.*, 1997) and subsequent digestion with *Nla*IV and *Swa*I. In the two resulting plasmids with the WT or E41K-mutated first three *Btk* exons, a 109 bp *Sal*I–*Xho*I fragment containing *loxP* sequences (obtained by insertion of a *loxP* oligonucleotide, 5′-ATAAC TTCGT ATAGC ATACA TTATA CGAAG TTAT-3′ into the *Asp*718 site of the pPolyIII vector) was cloned into the *Xho*I site within the *Not*I–*Sma*I–*Kpn*I–*Swa*I–*Xho*I–*Not*I polylinker. Subsequently, a 23.1 kb *Swa*I–*Sal*I fragment, encompassing *Btk* exons 5–19, was introduced using unique *Swa*I and *Xho*I sites within the polylinker. This 23.1 kb *Swa*I–*Sal*I fragment was from a 32.9 kb cosmid which was isolated from a mini-library constructed from a 340 kb YAC clone containing the h*Btk* gene (DeWeers *et al*., 1997). Finally, a ~4 kb *Swa*I fragment from the same cosmid clone, which contained *Btk* exon 4, was introduced into the unique *Swa*I site.

Generation of transgenic mice

The WT-h*Btk* and E41K-h*Btk* constructs were digested with *Not*I to release the ~38.2 kb DNA fragments shown in Figure 1. Gel-purified DNA was injected into pronuclei of FVB×FVB fertilized oocytes at a concentration of ~2 ng/μl and implanted into pseudopregnant female mice. Tail DNA was analyzed by Southern blotting using a partial h*Btk* cDNA probe (bp 133–1153) to determine the genotype of the founder mice and mice generated in subsequent crosses with *Btk*$^{-}$/*lacZ* mice of mixed 129/Sv×C57BL/6 background (Hendriks *et al.*, 1996).

Flow cytometric analyses

Preparation of single-cell suspensions and three- or four-color flow cytometry have been described (Hendriks *et al.*, 1996). Intracellular flow

cytometric detection of cytoplasmic Btk protein was performed on cells that were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin. Events ($3–5\times10^4$) were scored using a FACScan or FACSCalibur flow cytometer and analyzed by CellQuest software (Becton Dickinson, Sunnyvale, CA). The following monoclonal antibodies were obtained from Pharmingen (San Diego, CA): FITC-conjugated anti-B220/RA3-6B2, anti-HSA/M1/6, anti-CD3, anti-DX5, and anti-BP-1/6C3, PE-conjugated anti-CD43/S7, anti-CD11b/Mac1, anti-CD5/Ly-1, anti-CD4, Cy-Chrome conjugated anti-B220/RA3-6B2 and anti-CD8, and biotinylated anti-HSA/M1/6 and anti-IgM. PE-conjugated anti-IgD was purchased from Southern Biotechnology (Birmingham, AL). Anti-CD23 (B3B4) and anti-CD28/B7.2 (GL1) were purified monoclonal antibodies conjugated to biotin and FITC, respectively, according to standard procedures. Affinity-purified polyclonal rabbit anti-Btk was from Pharmingen. Secondary antibodies used were TriColor- or PE-conjugated streptavidin (Caltag Laboratories, Burlingame, CA), streptavidin-APC (Pharmingen) or FITC-conjugated goat anti-rabbit Ig (Nordic, Capistrano beach, CA).

Ig detection and in vitro immunizations

Levels of Ig subclasses in serum or culture supernatants were measured by sandwich ELISA, using unlabeled and peroxidase-labeled anti-mouse Ig isotype-specific antibodies (Southern Biotechnology). Serially diluted sera were incubated at room temperature for 3 h, and azino-bis-ethylbenzthiazoline sulfonic acid was used as a substrate. Antibody concentrations were calculated by using purified isotype Ig proteins as standards. TD and TI-II immunizations and TNP-specific ELISA were essentially performed as described previously (Maas *et al.*, 1997). Booster doses were given after 5–8 weeks. Serum dilutions were incubated at room temperature for 3 h and the biotinylated TNP-KLH step was overnight at 4°C.

In vitro B cell cultures

Whole spleen cell suspensions from 7- to 10-week-old mice were depleted of erythrocytes by standard NH_4Cl lysis and enriched for B cells by incubation with anti-Thy1.2 (30-H12), anti-CD4 (GK1.5) and anti-CD8 (YTS191) at room temperature for 30 min, and subsequent treatment with rabbit complement (CedarLane Laboratories, Hornby, Ontario, Canada) at 37°C for 30 min. The fractions of $B220^+$ cells remaining after treatment were determined by FACS analyses. The enriched B cells were cultured in round-bottomed microculture plates at a final concentration of 10^5 B cells/well in RPMI 1640 culture medium supplemented with 5% FCS and 5×10^{-5} M 2-mercaptoethanol, in the presence of 50 μg/ml *Escherichia coli* LPS (serotype 026:B6, Difco Laboratories, Detroit, MI), several concentrations of polyclonal goat-anti-mouse IgM (Southern Biotechnology), 10 μg/ml rat anti-mouse CD40 (FGK-45.5, kindly provided by D.Gray), 100 ng/ml rIL-4 (R&D Systems Inc., Minneapolis, MN) or 200 U/ml rINF-γ (R&D Systems Inc.). To measure DNA synthesis, after 2 days of culture cells were pulsed with [^{3}H]thymidine for 16–18 h, harvested and counted using standard methods. Culture supernatants were assayed for Ig production by ELISA on day 7. Expression of CD28/B7.2, CD23 and MHC class II was evaluated by flow cytometry at various time points.

Biochemical analyses

For Western blotting experiments, lysates of 2×10^5 total spleen cells were analyzed as described (Hendriks *et al.*, 1996). Subcellular fractionation, immunoprecipitations and *in vitro* kinase assays were performed essentially as described previously (DeWeers *et al.*, 1994b). A polyclonal rabbit anti-hBtk/GST antiserum (kindly provided by C.Kinnon) was used for Western blotting and for Btk immunoprecipitation. Rabbit anti-hBtk (raised against a peptide containing amino acids 69–88 and used as biotinylated purified Ig; DeWeers *et al.*, 1994b) and anti-pTyr (4G10, Upstate Biotechnology Inc., Lake Placid, NY) were used in immunoblotting.

Immunohistochemistry

Spleens were embedded in OCT compound; frozen 5 μm cryostat sections were fixed in acetone or pararosaniline and single- and double-labeling was performed as previously described (DeJong *et al.*, 1991; Leenen *et al.*, 1998). Monoclonal antibodies anti-B220/RA3-6B2, anti-CD3/KT3, anti-CD11c/N418 (Steinman *et al.*, 1997), ER-TR7 (VanVliet *et al.*, 1986), ER-TR9 and MOMA-1 (Kraal, 1992) were applied as hybridoma culture supernatants; biotinylated anti-IgM was from Pharmingen, anti-IgD was from Southern Biotechnology and biotinylated PNA from Sigma (St Louis, MO). Second-step reagents were peroxidase-labeled goat anti-rat Ig (DAKO, Glostrup, Denmark) or anti-hamster Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-rat Ig alkaline phosphatase (Southern Biotechnology).

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