Severe B Cell Deficiency in Mice Lacking the Tec Kinase Family Members Tec and Btk

By Wilfried Ellmeier,*‡ Steffen Jung,* Mary Jean Sunshine,*‡ Farah Hatam,*‡ Yang Xu,§ David Baltimore,‖ Hiroyuki Mano,¶ and Dan R. Littman*‡

*From the *Molecular Pathogenesis Program, Skirball Institute of Biomolecular Medicine, and the ‡Howard Hughes Medical Institute, New York University School of Medicine, New York, New York 10016; the §Department of Biology, University of California at San Diego, San Diego, California 92093; the ‖Office of the President, California Institute of Technology, Pasadena, California 91125; and the ¶Division of Functional Genomics, Jichi Medical School, Tochigi 329-0498, Japan*

Abstract

The cytoplasmic protein tyrosine kinase Tec has been proposed to have important functions in hematopoiesis and lymphocyte signal transduction. Here we show that Tec-deficient mice developed normally and had no major phenotypic alterations of the immune system. To reveal potential compensatory roles of other Tec kinases such as Bruton's tyrosine kinase (Btk), Tec/Btk double-deficient mice were generated. These mice exhibited a block at the $B220^+CD43^+$ stage of B cell development and displayed a severe reduction of peripheral B cell numbers, particularly immunoglobulin (Ig)$M^{lo}IgD^{hi}$ B cells. Although Tec/Btk^{null} mice were able to form germinal centers, the response to T cell–dependent antigens was impaired. Thus, Tec and Btk together have an important role both during B cell development and in the generation and/or function of the peripheral B cell pool. The ability of Tec to compensate for Btk may also explain phenotypic differences in X-linked immunodeficiency (*xid*) mice compared with human X-linked agammaglobulinemia (XLA) patients.

Key words: gene targeting • B cell development • lymphocytes • signaling • X-linked immunodeficiency

Introduction

Nonreceptor protein tyrosine kinases (PTKs)[1] are essential for the development and activation of B and T lymphocytes. It has been shown that the stimulation of the antigen-specific receptors (TCR on T cells, and B cell receptor [BCR] on B lymphocytes) activates several members of both the Src kinase (Lck, Fyn, Lyn) and Syk kinase (ZAP-70 and Syk) family. Their critical involvement in signal transduction pathways during these processes has been demonstrated by a large number of studies and great detail about their molecular functions and enzymatic activities is known (1, 2).

Members of the Tec kinase family (Tec, Bruton's tyrosine kinase [Btk], Itk, Rlk, and Bmx) form another class of PTKs that are activated during lymphocyte development and activation (3–5). Like Src kinases, Tec kinases have a modular structure containing a short so-called Tec homology (TH) domain, followed by src homology (SH) domains SH3, SH2, and a kinase domain (SH1). However, unlike Src kinases, they do not have an NH_2-terminal myristylation signal or a COOH-terminal negative regulatory tyrosine. Instead they contain an NH_2-terminal Pleckstrin homology (PH) domain (except Rlk). PH domains, found in several intracellular signaling molecules, are able to bind phosphatidylinositols and in some cases also mediate protein–protein interactions (6). TH domains, containing proline-rich sequences, have been proposed to be involved in the autoregulation of Tec kinases. It has been shown that the TH and SH3 domains of Itk are able to interact intramolecularly, thus preventing the interaction of these domains with their prospective ligands (7).

The understanding of the physiological roles of the different members of the Tec kinase family is still at an early

Address correspondence to Wilfried Ellmeier at his present address, Institute of Immunology, University of Vienna, Brunner Strasse 59, 1235 Vienna, Austria. Phone: 43-1-4277-64986; Fax: 43-1-4277-64991; E-mail: wilfried.ellmeier@univie.ac.at

[1]*Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; APC, allophycocyanin; BCR, B cell receptor; bio, biotin; BM, bone marrow; Btk, Bruton's tyrosine kinase; ECL, enhanced chemiluminescence; ES, embryonic stem; PH, Pleckstrin homology; PTK, protein tyrosine kinase; SH, src homology; TD, thymus-dependent; TH, Tec homology; TI-II, thymus-independent type II; *xid*, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

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stage. Btk expressed in the B cell lineage and in myeloid cells is the most extensively studied member of the Tec kinase family. Mutations in Btk are the cause of X-linked agammaglobulinemia (XLA) in humans (8). A mutation in Btk has also been found in X-linked immunodeficiency (*xid*) mice (9, 10) and targeted disruption of Btk in mice confirmed that *xid* is caused by loss of Btk function (11, 12). The phenotype of *xid* or Btk knockout mice (*xid*/Btk) resembles partially human XLA. The number of peripheral B cells is about two- to threefold lower and the mature IgM^{lo}IgD^{hi} B cell population is severely reduced. *xid*/Btk mice lack peritoneal CD5$^+$ B cells and have reduced serum IgM and IgG3 levels. B cells isolated from these mice do not proliferate in response to anti-IgM treatment and the proliferation in response to LPS is reduced. In addition, *xid*/Btk mice are not able to mount a thymus-independent type II (TI-II) response to antigens (11, 12). The effect of Btk deficiency on B cell development remains controversial. Some groups noticed either a small increase in the pro-B cell fraction (B220$^+$CD43$^+$) of Btk-deficient and *xid* mice (12) or a severe failure of Btk^{null} cells to expand to the small pre-B cell stage in in vivo competition experiments (11). In contrast, another group observed a developmental disadvantage of Btk^{null} B cells only past the pre-B cell stage (13). It is likely that part of this controversial data may be explained both by the different experimental approaches and by the different genetic background of the Btk-deficient embryonic stem (ES) cells and blastocysts used in complementation or competition experiments.

The generation of Itk-deficient mice revealed that this kinase has an important function during both T cell development and activation (14, 15). Itk has also been suggested to have a role in the CD28 costimulatory pathway, although it remains to be determined whether Itk acts as a positive or negative regulator of this signaling pathway (16–18). Recently, evidence for an additional function for Itk in the polarization of Th cells towards Th1 and Th2 has been provided. Itk^{null} T cells were unable to polarize towards a Th2 phenotype, even if primed under Th2-polarizing conditions (19).

Rlk/Txk is another member of the Tec kinase family that, in contrast to other members, lacks the PH domain. Rlk has been shown to be expressed in the T cell lineage, preferentially in Th1 cells, and in mast cells and testis (20–23). Deletion of Rlk by homologous recombination did not reveal any major developmental alteration or signaling defects within the T cell lineage (24). However, the generation of Itk/Rlk double-deficient mice and the analysis of Itk/Rlk^{null} T cells have shown that Rlk is involved in TCR-mediated signaling events, as indicated by an almost completely absent proliferative response to anti-CD3 stimulation. In addition, a further decrease in IL-2 and IFN-γ production in Itk/Rlk double-deficient T cells compared with Itk^{null} T cells was observed. Furthermore, double-deficient mice showed an increased susceptibility to the intracellular pathogen *Toxoplasma gondii* (24).

Tec, the founding member of the Tec kinase family, is expressed in T and B cells, in myeloid cells, and in liver (25–27). Recent in vitro studies have shown that Tec is activated in response to BCR (27) or TCR/CD28 stimulation (28). It has also been shown that CD28 engagement leads to a recruitment of Tec (via its SH3 domain) to a proline-rich motif within the cytoplasmic tail of CD28, and overexpression of Tec activates both the IL-2 and IL-4 promoters (28, 29). Besides BCR- and TCR/CD28-mediated signals, Tec is also activated by anti-CD19 (27) and anti-CD38 (27) ligation, and by stimulation of the cytokine receptors for IL-3 (30, 31), IL-6 (31), stem cell factor (32), thrombopoietin (33), and GM-CSF (34).

The different studies on Tec function have not investigated its role in primary T or B cells. To extend the analysis of Tec function to an in vivo model, we generated Tec-deficient mice by means of homologous recombination in ES cells. Initial analysis of T and B lymphocytes, and of other hematopoietic lineage cells, indicated no major phenotypic alterations. To reveal potential functions of Tec that may be masked in lymphocytes by the expression of the Tec kinase family member Btk, we intercrossed Tec-deficient mice with Btk-deficient mice. Phenotypic analysis of these mutant mice indicated that Tec and Btk have redundant functions in B cell development and activation.

Materials and Methods

Generation of the Tec Targeting Construct. A genomic clone containing the region around Tec exon 2 was isolated from a 129 genomic library (Stratagene) and subcloned into pBluescript (pBS; Stratagene). The short and the long arms of the targeting construct were isolated as 4-kb BamHI-HindIII or 5.0-kb XhoI-HindIII fragments, respectively. The fragments were cloned into a pBS-based vector containing a polylinker with suitable cloning sites and the thymidine kinase gene driven by the herpes simplex virus enhancer/promoter elements (35). A pgk(G)neo(pA) cassette was inserted between the short and the long arm of the targeting construct. Therefore, proper targeting will result in the deletion of a genomic fragment containing exon 2 (186 bp) and surrounding intronic sequences. All cloning steps were performed according to standard procedures (36). E14.1 ES cells (37) were transfected with 30 μg NotI-linearized targeting vector and cultured on mitomycin C–treated murine embryonic fibroblasts. 2 d after transfection, G418 (Geneticin; GIBCO BRL) was added to a final concentration of 350 μg/ml and after another 2 d, gancyclovir (GANC) was added (2 μM final concentration) for negative selection. There was approximately threefold enrichment between plates with and without GANC. 9 d after transfection, individual ES cell colonies were isolated, expanded, and subsequently Southern blot analysis was performed to detect homologous recombinants. Two targeted ES cell clones were injected into E3.5 C57BL/6 blastocysts and transferred into (B6/D2) F1 pseudopregnant females. Chimeric mice obtained were then backcrossed to C57BL/6 mice and transmission of the targeted allele was confirmed by Southern blot analyses of tail DNA.

Btk-deficient Mice. Btk-deficient mice (12) were purchased from The Jackson Laboratory.

Generation of A-MuLV–transformed Cell Lines. Pre-B cell lines were Abelson murine leukemia virus (A-MuLV)–transformed as described (38). 2×10^6 bone marrow (BM) cells from the various knockout mice were infected with A-MuLV in RPMI (supplemented with 20% FCS, 50 μM β-mercaptoethanol, 2 mM L-glu-

tamine, and 50 U/ml penicillin/streptomycin) in the presence of 10 μg/ml polybrene, and incubated for 10 d. Transformed pre-B cells were then split into new wells and grown in RPMI (supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, and 50 U/ml penicillin/streptomycin).

Immunoprecipitation and Western Blotting. For immunoprecipitation and Western blotting experiments, splenocytes from one spleen or 50×10^6 pre-B cells were lysed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, and 50 mM Tris-HCl) supplemented with protease inhibitors (Complete; Roche). The lysates were cleared from nuclei and debris and the Ab used for immunoprecipitation was added (rabbit anti–C-term Tec [39]). After incubating for 3 h at 4°C, the immune complexes were precipitated by addition of 30 μl protein A (50% slurry, prewashed in lysis buffer) followed by an additional incubation for 1 h at 4°C. After several washes with lysis buffer, the Ab–protein A complexes were loaded on a 9% polyacrylamide-SDS gel. 100 μg of protein was used when Western blotting experiments were performed with total cell lysates. Proteins were transferred onto a polyvinylidene difluoride (PVDF) nylon membrane (Roche), blocked, and incubated with an Ab suitable for Western blotting (rabbit anti–SH3-Tec [30]) in PBS-Tween (supplemented with 5% nonfat dry milk). After the incubation with the secondary Ab (protein A–horseradish peroxidase; Zymed Laboratories), the signals were detected with the enhanced chemoluminescence (ECL) system (Pierce Chemical Co.).

B Cell Proliferation Assays. To obtain enriched B cell populations, splenocytes were stained with a PE-conjugated Ab mix containing anti-CD43 (clone S7), anti–Gr-1 (RB6-8C5), and anti-CD11b (M1/70). The cells were washed and stained with anti-PE magnetic beads and loaded onto a negative enrichment column (Miltenyi Biotec). The purity of the B cell population was ~50–80% for the Tec/Btk double knockout and >85% for the other genotypes. The purified cells (i.e., an equivalent of 5×10^4 $B220^+$ B cells) were then incubated with the appropriate stimuli in 160 μl proliferation medium (RPMI supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, and 50 U/ml penicillin/streptomycin). Anti-IgM ($F[ab']_2$ fragment) and LPS were used at concentrations of 0.3, 1, and 5 μg/ml.

ELISA Assay. Isotype-specific Ab titers were detected according to standard ELISA protocols. In brief, ELISA plates (Dynax) were coated overnight with 2 μg/ml isotype-specific goat anti–mouse Abs (Southern Biotechnology Associates, Inc.). Diluted serum samples (isolated from 7–10-wk-old mice) were added. After overnight incubation, the bound isotype-specific Abs were detected with an alkaline phosphatase–conjugated goat anti–mouse Ig (H plus L) Ab (Southern Biotechnology Associates, Inc.). Alkaline phosphatase activity was determined with *p*-nitrophenyl phosphate (pNPP) tablets as a substrate (Sigma-Aldrich). Abs used as standards were purchased from either BD PharMingen or Southern Biotechnology Associates, Inc.

TI-II Antigen Immunization. 10 μg TNP-Ficoll (in PBS) was intraperitoneally injected. TNP-specific Ab titers were determined 5 d later by ELISA. ELISA plates were coated overnight with 1 μg/ml TNP-BSA. Several dilutions of both preimmunization and postimmunization serum were added. Isotype-specific Abs were detected with an alkaline phosphatase–conjugated goat anti–mouse Ig (H plus L) Ab (Southern Biotechnology Associates, Inc.). Alkaline phosphatase activity was determined with pNPP tablets as a substrate (Sigma-Aldrich).

Thymus-dependent Antigen Immunization. 20 μg TNP-KLH was allowed to adsorb on 2 mg of aluminum hydroxide for 1 h at 4°C. Nonadsorbed TNP-KLH was washed away with PBS, and the TNP-KLH/aluminum hydroxide mixture was resuspended in 200 μl PBS and intraperitoneally injected. KLH-specific serum titers were determined 13 d later by ELISA. For secondary immune responses, mice were injected 8 wk after the primary immunization with TNP-KLH/aluminum hydroxide (20 μg in 200 μl PBS) and KLH-specific serum titers were determined 7 d after the secondary injection by ELISA. ELISA plates were coated overnight with 1 μg/ml KLH-BSA and several dilutions of the preimmunization and postimmunization serum were added. Isotype-specific Abs were detected with an alkaline phosphatase–conjugated goat anti–mouse Ig (H plus L) Ab (Southern Biotechnology Associates, Inc.). Alkaline phosphatase activity was determined with pNPP tablets as a substrate (Sigma-Aldrich).

Immunohistochemistry. Spleens were frozen in Tissue-Tek (Sakura Finetek). Sections (8–12 μm) were stained overnight with the appropriate Abs. Afterwards, the sections were washed and subsequently incubated with the secondary Abs for 3 h at room temperature. For germinal center detection, mice were immunized with TNP-KLH/aluminum hydroxide and spleens were isolated 11 d after immunizations. The reagents used for immunohistochemistry were FITC-conjugated anti-B220 (clone RA3-6B2; Caltag), biotin (bio)-conjugated anti-TCRβ (H57-597; BD PharMingen), rat IgM anti–murine MOMA-1 (40), Cy3-strepatvidin and Cy5-streptavidin (Cedarlane), Cy3-goat anti–rat IgM and Cy3-goat anti–rat IgG (Jackson ImmunoResearch Laboratories), and bio–peanut agglutinin (PNA; Vector Laboratories). Visual data were acquired with an Axioplan 2 fluorescent microscope (ZEISS).

Flow Cytometric Analysis and Abs. Thymus, lymph nodes, and spleen were removed from killed animals (5–12 wk of age) and placed into 60-mm tissue culture dishes containing staining buffer (PBS supplemented with 2% FCS and 0.1% sodium azide). Single cell suspensions were made by passing the tissue through a 70-μm nylon cell strainer. BM was isolated from both femurs and pooled. The cell suspensions were washed once with staining buffer and $1–5 \times 10^5$ cells were incubated on ice with Fc block (BD PharMingen) for 5 min and subsequently with the appropriate Abs for 30 min. Afterwards, the cells were washed once with staining buffer and analyzed or incubated with secondary Abs on ice for 30 min. The following Abs were used for FACS® staining. From Caltag: FITC–, PE–, or bio–anti-B220 (RA3-6B2), PE– or bio–anti-mCD3 (clone 500-A2), PE–anti-mCD8α (CT-CD8α), Tricolor–anti-mCD4 (CT-CD4), and Tricolor-streptavidin; from BD PharMingen: allophycocyanin (APC)–anti-B220 (clone RA3-6B2), PE–anti-CD43 (S7), FITC– or bio–anti-IgM (II/41), FITC–anti-IgD (11-26c.2a), FITC–anti-CD3ε (145-2C11), PE–anti-CD5 (53-7.3), bio–anti-Vα11 (RR8-1), bio–anti-Vβ8.1/8.2 (MR5-2), PE–anti-Gr-1 (RB6-8C5), PE–anti-CD11b (M1/70), PE–anti-CD11c (HL-3), APC–anti-mCD4 (RM4-5), FITC– or bio–anti-HSA (M1/69), bio–anti-CD69 (H1.2F3), bio–anti-mTCR-γ/δ (GL3), and bio–anti–mTCR-α/β (H57-597). Cells were analyzed using a FACScan™ flow cytometer and CELLQuest™ software (Becton Dickinson).

Results

Generation of Tec-deficient Mice by Homologous Recombination

The murine *Tec* gene contains 18 exons spanning >86 kb (41). Two major splice variants have been described: Tec IV, encoding the full-length protein, and Tec III, containing a 66-bp deletion within exon 8. Minor splice

forms, Tec I and II, are generated by usage of alternative exons 18a and 4a instead of exons 18 and 4, respectively (26, 41). Specific roles of the different isoforms are not known, and it remains unclear whether all of the potential protein isoforms indeed exist in vivo. To ensure the inactivation of all the possible splice forms, we deleted exon 2, which harbors the translation initiation codon and 45 additional amino acids of the PH domain. A similar targeting strategy has been used successfully for the generation of Btk-deficient mice (12). Therefore, a HindIII-XhoI fragment containing exon 2 plus 5′ and 3′ intronic sequences was replaced with pgk(G)neo(pA). The deletion of the first 46 amino acids should result in the complete inactivation of Tec protein synthesis. However, as two potential translation initiation codons are located either at the beginning of the TH (exon 5) or SH3 (starting at exon 7) domain, it remained possible that truncated forms of Tec (of a size of 60 and 52 kd, respectively) would be generated (Fig. 1 A). To confirm that the targeting strategy resulted in abrogation of Tec expression (and to determine whether potential truncated forms of Tec were generated), a series of immunoblotting experiments was performed on A-MuLV–transformed pre-B cells generated from mutant and wild-type littermate control mice. Immunoprecipitation of Tec from lysates with a rabbit anti–mouse COOH-terminal Tec Ab followed by Western blotting with a rabbit anti–mouse Tec-SH3 domain Ab confirmed the absence of the full-length 73-kd Tec protein (Fig. 1 D). Direct Western blotting of A-MuLV–transformed pre-B cells and of total splenocyte lysates with the anti–Tec-SH3 Ab detected no truncated forms of Tec, indicating that the deletion of exon 2 resulted in the inactivation of the gene (Fig. 1 E).

Phenotypic Analysis of Tec-deficient Mice

Tec-deficient mice were viable and fertile, and did not show any gross developmental defects. Even though it has

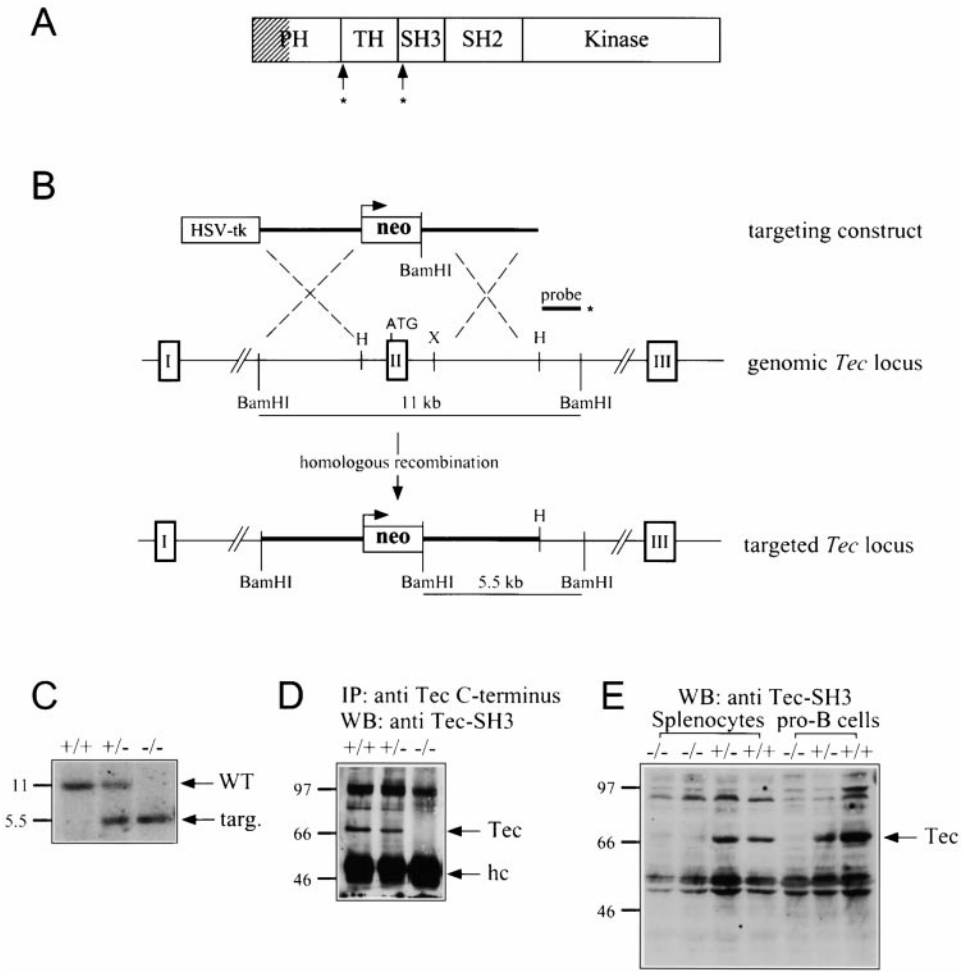

Figure 1. Deletion of the *Tec* gene by homologous recombination in ES cells. (A) Schematic map of Tec kinase indicating the PH domain, the TH domain, and the SH domains SH3, SH2, and SH1 (kinase domain). The vertical arrows with an asterisk indicate potential translation initiation sites downstream of the first ATG within the *Tec* coding region. The hatched area within the PH domain indicates the region of the Tec kinase deleted by the applied targeting strategy. (B) Schematic map of the targeting construct (top), and of the *Tec* locus before and after homologous recombination (middle and bottom, respectively). Only restriction sites important for the targeting strategy are shown. The horizontal thick black line (in the top and bottom) indicates the region of homology between the targeting construct and the endogenous locus. The bar with an asterisk in the middle part represents the probe used for Southern blotting to detect homologous recombination. Thick-lined rectangles in the middle and lower part represent exons. Exon 1 contains the 5′ untranslated region, and exon 2 (186 bp) contains the first 46 amino acids including the initiation codon (reference 41). The length of introns 1 and 2 are >30 kb and ~23 kb, respectively (reference 41). Horizontal bars with numbers (indicating the size in kb) represent the expected genomic fragments after digestion with BamHI. X, XhoI; H, HindIII. (C) Southern blot of BamHI digested tail DNAs showing wild-type (+/+), heterozygous (+/−), and homozygous (−/−) knockout mice. The origin of the detected wild-type (WT) and targeted (targ.) fragments is indicated in B. (D) Immunoprecipitation (IP) and Western blot (WB) analysis of Tec kinase from A-MuLV–transformed pre-B cell lines. Tec kinase was immunoprecipitated from NP-40 lysates with a rabbit Ab against the COOH-terminal region of Tec. Tec kinase was detected with a rabbit Ab against the SH3 domain of Tec followed by ECL. The strong signal around 50 kd is due to the rabbit heavy chain (hc) of the rabbit Ab used for the immunoprecipitation. (E) Western blot (WB) analysis of Tec kinase from NP-40 lysates of splenocytes or A-MuLV–transformed pro-B cell lines. Tec kinase was detected with a rabbit Ab against the SH3 domain of Tec followed by ECL.

been shown that Tec is expressed within the T and B cell lineage and in cells of myeloid origin, no developmental alterations were observed within these cell populations. The number of splenic T and B cells in $Tec^{-/-}$ mice was similar to that in wild-type littermate controls (Fig. 2, A and B). The IgM versus IgD expression pattern on peripheral B cells (Fig. 2 C) and the developmental profile of B cells in the BM (Fig. 3) were indistinguishable between wild-type and Tec-deficient mice. Peritoneal $CD5^+$ B cells were present in the knockout mice (not shown). In addition, T cell development appeared to be normal in Tec-deficient mice and FACS® analysis of BM cells or splenocytes for the expression of the surface molecules TER119, CD61, Gr-1, CD11b, and NK1.1 did not reveal any differences in Tec-null mice compared with wild-type mice (data not shown). Thus, Tec kinase is not essential for the development of cells in the hematopoietic system.

To reveal potential nonredundant functions of Tec during T cell signaling, both in vitro and in vivo experiments were performed. Purified T cells from Tec-deficient mice proliferated like their wild-type counterparts in response to anti-CD3 stimulation and showed similar induction of CD25 and CD69 (data not shown). As Tec has been implicated in the signaling pathways mediated by CD28 costimulation (28), the effect of anti-CD28 Ab treatment upon suboptimal anti-CD3 stimulation was also determined. Tec-deficient T cells responded to CD28 stimulation like wild-type T cells, with respect to both proliferation and IL-2 production (data not shown). In agreement with the in vitro results, the ex vivo responses to KLH of Tec^{null} or

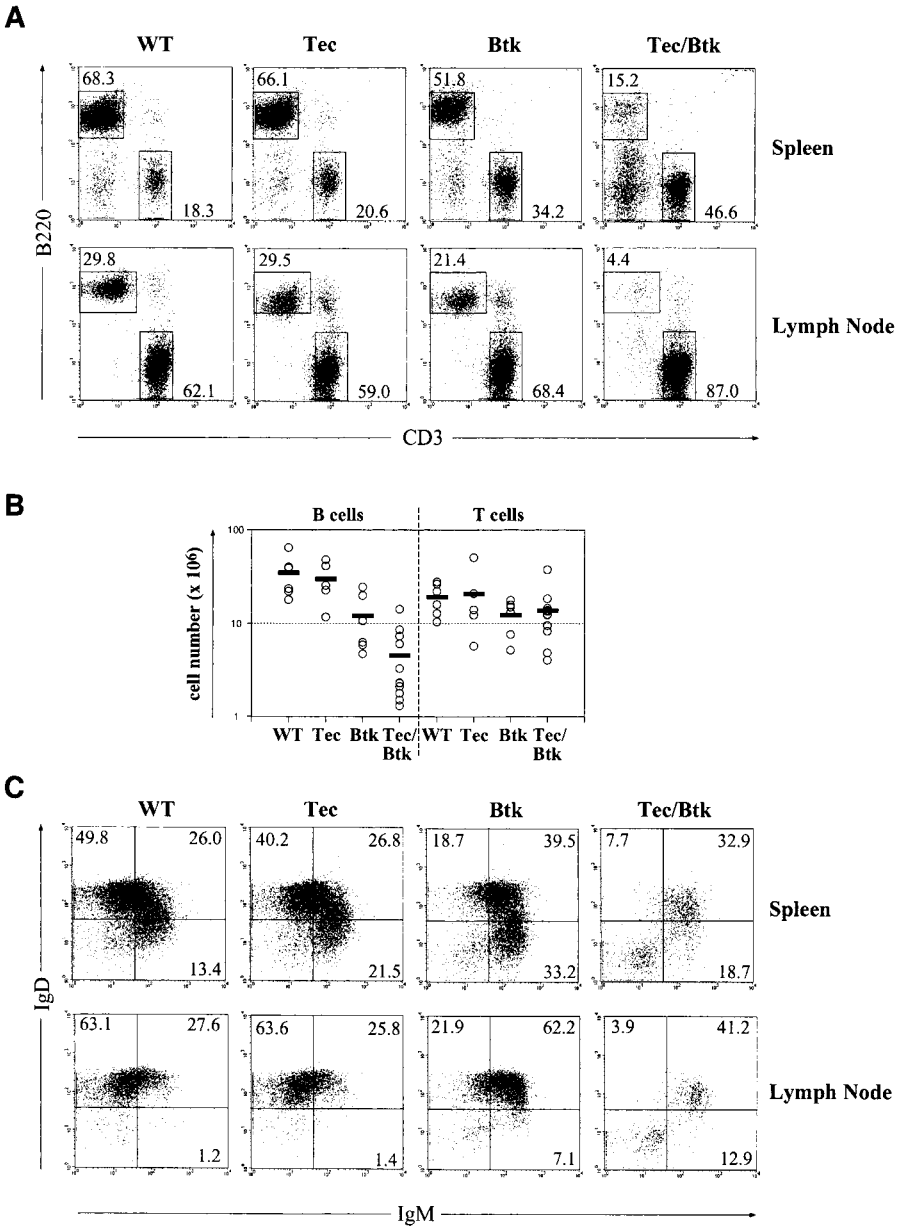

Figure 2. Reduced B cell numbers and altered IgM/IgD profile in the periphery of Tec/Btk double-deficient mice. (A) Flow cytometric analysis of spleen (top) and lymph nodes (bottom) isolated from the mice of the indicated genotype. The lymphocytes were stained with anti-B220 and anti-CD3 Abs. Numbers next to the gates in the dot plots indicate percentage of gated cells. (B) The numbers of $B220^+$ and $CD3^+$ lymphocytes in the spleen. Open circles represent individual mice (5–11 spleens per genotype were counted). Thick solid bars indicate average numbers for B cells (34.4 ± 17.3, 29.5 ± 14.4, 11.9 ± 8.2, and 4.5 ± 4.0 million for wild-type, Tec-deficient, Btk-deficient, and Tec/Btk double knockout mice, respectively). (C) IgM versus IgD FACS® profile of B220-gated lymphocytes from spleen (top) and lymph nodes (bottom) isolated from mice of the indicated genotype. The numbers in the dot plots show the percentage of cells within each quadrant. (A, B, and C) Wild-type (WT), Tec-deficient (Tec), Btk-deficient (Btk), and Tec/Btk double-deficient (Tec/Btk) mice were littermates. (A and C) 50,000 splenocytes were collected for FACS® analysis.

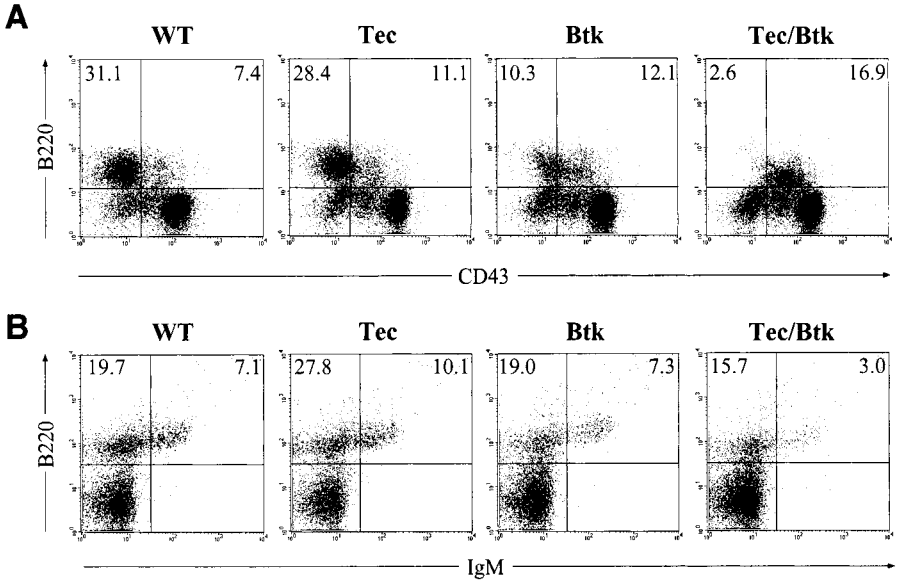

Figure 3. Partial block in B cell development in Tec/Btk double-deficient mice. (A) BM cells of the indicated genotype were analyzed for the expression of B220 and CD43 by FACS® analysis. The numbers in the dot plots show the percentage of cells within each quadrant. (B) B220 versus IgM profile of BM cells isolated from mice of the indicated genotype. The numbers in the dot plots show the percentage of cells within each quadrant. (A and B) Wild-type (WT), Tec-deficient (Tec), Btk-deficient (Btk), and Tec/Btk double-deficient (Tec/Btk) mice were littermates. The numbers in the dot plots show the percentage of cells within each quadrant.

wild-type T cells isolated from the draining lymph nodes of KLH-immunized mice were indistinguishable (data not shown).

To identify a potential role for Tec in signaling pathways induced by BCR stimulation or by polyclonal B cell activators, purified B cells from $Tec^{-/-}$ and littermate control mice were stimulated either with anti-IgM Ab or with LPS. As shown in Fig. 4, B cells from both sets of mice showed similar proliferative responses. In addition, Tec-deficient B lymphocytes responded like wild-type B cells to anti-CD40 or anti-CD40 plus IL-4 stimulation, and anti-IgM cross-linking induced a similar calcium flux in Tec^{null} B cells compared with their wild-type counterparts (data not shown).

Phenotypic Analysis of Mice Deficient in Both Tec and Btk

B Cell Development. Because the initial phenotypic characterization of $Tec^{-/-}$ mice did not reveal any major developmental or signaling defect within B and T cell lineages, we decided to test whether other Tec kinases compensate for Tec in *Tec*-deficient mice. To reveal compensatory pathways for the B cell lineage, Tec knockout mice were crossed to Btk-deficient mice (12).

Btk knockout and *xid* mice display an approximately two- to threefold reduction of splenic B cells and the remaining $B220^+$ cells are reduced in numbers that express the mature $IgM^{lo}IgD^{hi}$ surface phenotype. Peritoneal $CD5^+$ B cells are missing and the mice have low IgM and IgG3 serum levels (11, 12). FACS® analysis of cells from spleen and lymph nodes of Tec/Btk double-deficient mice revealed a dramatic reduction of B cells compared with Btk single knockout mice (Fig. 2, A and B). A similar reduction in $B220^+$ cells was also observed in peripheral blood lymphocytes (data not shown). Analyses of the maturational status of the Tec/Btk double-deficient B cells revealed a further relative decrease of the mature $IgM^{lo}IgD^{hi}$ population (Fig. 2 C).

To investigate whether the deletion of both Tec and Btk also affects B cell development, BM cells from the various mutant mice were isolated and analyzed for the expression of B220, CD43, and IgM. Pro-B cells (or fractions A–C; nomenclature according to references 42 and 43, respectively) express CD43, whereas pre-B cells (fractions D–F) lose its expression. As shown in Fig. 3 A, the majority of wild-type or Tec-deficient $B220^+$ BM cells did not express CD43. A small increase in $B220^+CD43^+$ B lymphocytes was observed in Btk^{null} mice, as described previously (12, 13). In contrast, Tec/Btk double-deficient mice displayed an accumulation of $CD43^+$ B cell precursors, indicating a partial developmental block at the large pre-B cell stage. At this stage, developing B cells become dependent on pre-

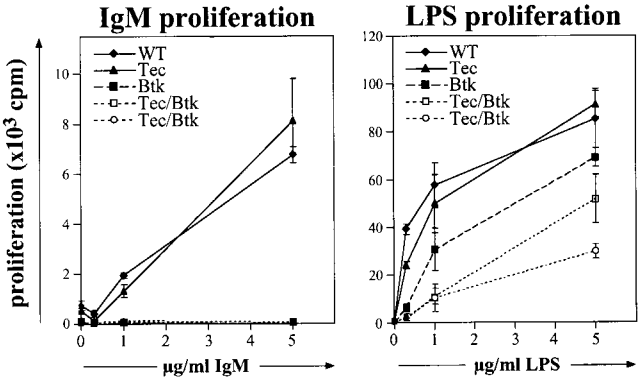

Figure 4. Proliferative response of B cells to anti-IgM and LPS stimulation. Purified splenic B cells isolated from mice of the indicated genotypes were stimulated with the indicated amounts of anti-IgM (left) or LPS (right). Cell proliferation was measured by [^{3}H]thymidine incorporation. Experiments were done in triplicates and standard deviations are indicated with error bars. WT, wild-type; Tec, Tec-deficient; Btk, Btk-deficient; Tec/Btk, Tec/Btk double-deficient.

BCR–mediated signaling for their further maturation into surface IgM-expressing immature B cells (44). However, some surface IgM^+ immature B cells did develop in Tec/Btk double-deficient mice (Fig. 3 B).

B Cell Proliferation. The ability of purified splenic B cells from the different mutant strains to respond to external signals was determined by culturing them in the presence of anti-IgM or LPS. Neither Btk^{null} (as described previously [12]) nor Tec/Btk double-deficient B cells proliferated in response to anti-IgM treatment (Fig. 4, left) and the response to LPS was further reduced in Tec/Btk^{null} compared with Btk^{null} B cells (Fig. 4, right). The response to anti-CD40 treatment was slightly reduced in Tec/Btk double-deficient B cells compared with Btk-deficient cells, although addition of IL-4 to anti-CD40 treated B cells resulted in similar levels of proliferation independent of the B cell genotype (data not shown).

Serum Ig Levels and Antigen-specific Responses

To investigate whether the decrease in peripheral B cell numbers correlates with a reduction of serum Ig levels, ELISAs were performed. As shown in Fig. 5, serum Ig levels in Tec/Btk mice were similar to those in Btk knockout mice, reflected by a decrease in the amount of IgM, IgG3, and IgG2a subtypes. In addition, IgG1 and IgA levels were slightly lower in the double knockout mice compared with the other mice. This indicates that still significant numbers of Ig-producing plasma cells were induced in Tec/Btk double-deficient mice.

To test the ability of Tec/Btk double knockout mice to mount an antigen-specific immune reaction, we determined the response to both TI-II and thymus-dependent (TD) antigens. For TI-II responses, four mice of each genotype were intraperitoneally injected with TNP-Ficoll and 5 d later TNP-specific IgM and IgG3 Abs levels were detected by ELISA. Btk-deficient mice, in agreement with previous findings (11, 12), lacked TI-II responses, as did Tec/Btk knockout mice. Tec-deficient mice responded like wild-type mice to TI-II antigens (Fig. 6 A).

The primary response to TD antigens was measured 13 d after intraperitoneal injection of TNP-KLH adsorbed on aluminum hydroxide. As shown in Fig. 6 B, equal TNP-specific IgM and IgG1 responses were observed in wild-type and Tec-deficient mice. In agreement with previous studies (12), the TNP-specific IgM and IgG1 response was reduced in Btk-deficient mice compared with wild-type mice. Tec/Btk double knockout mice showed a further reduction in the primary TNP-IgG1 response despite a TNP-specific IgM response similar to that of Btk^{null} mice. To examine the secondary immune response, mice were boosted 6 wk after the primary immunizations by injection of TNP-KLH/aluminum hydroxide and the TNP-specific Ig isotype titers were determined 7 d later. As shown in Fig. 6 C, Tec/Btk-deficient mice had a lower secondary IgG1 response than Btk^{null} mice. Strikingly, the IgG2a response was barely detectable in Tec/Btk^{null} mice. This indicates an important role for Tec and Btk in the generation of an antigen-specific immune response.

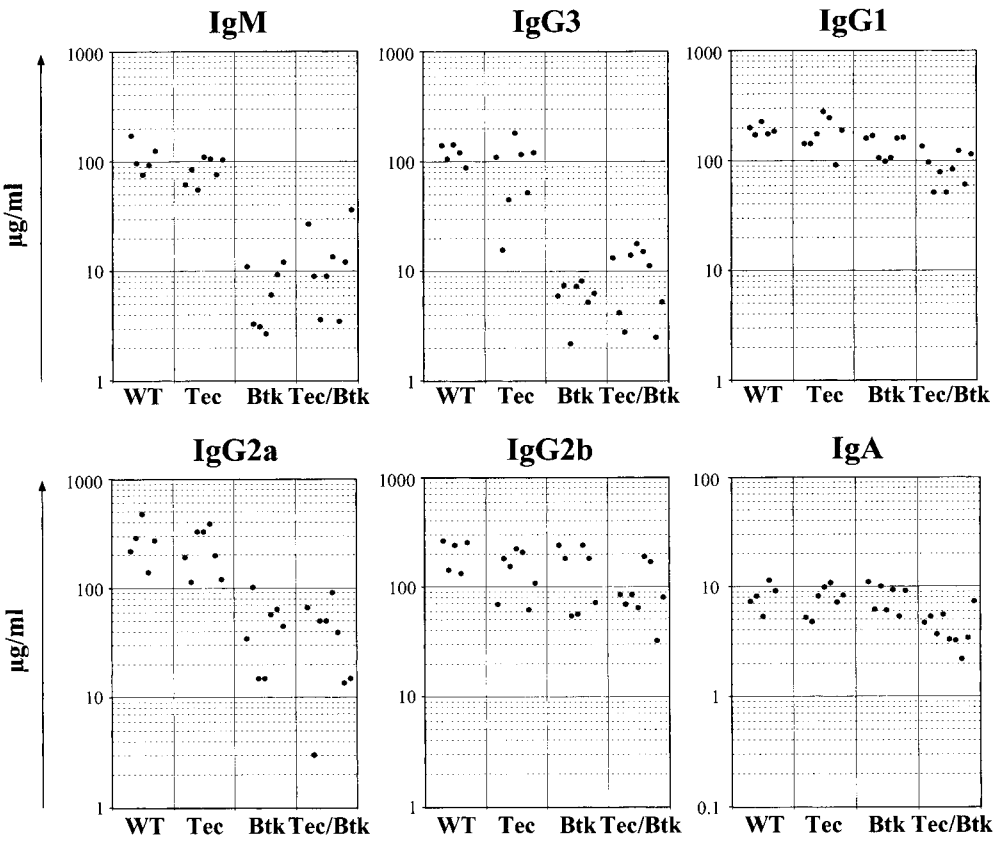

Figure 5. Similar levels of serum Ig in Btk- and Tec/Btk-deficient mice. Serum Ig levels of 7–10-wk-old mice of the indicated genotype were determined by ELISA. WT, wild-type; Tec, Tec-deficient; Btk, Btk-deficient; Tec/Btk, Tec/Btk double-deficient..

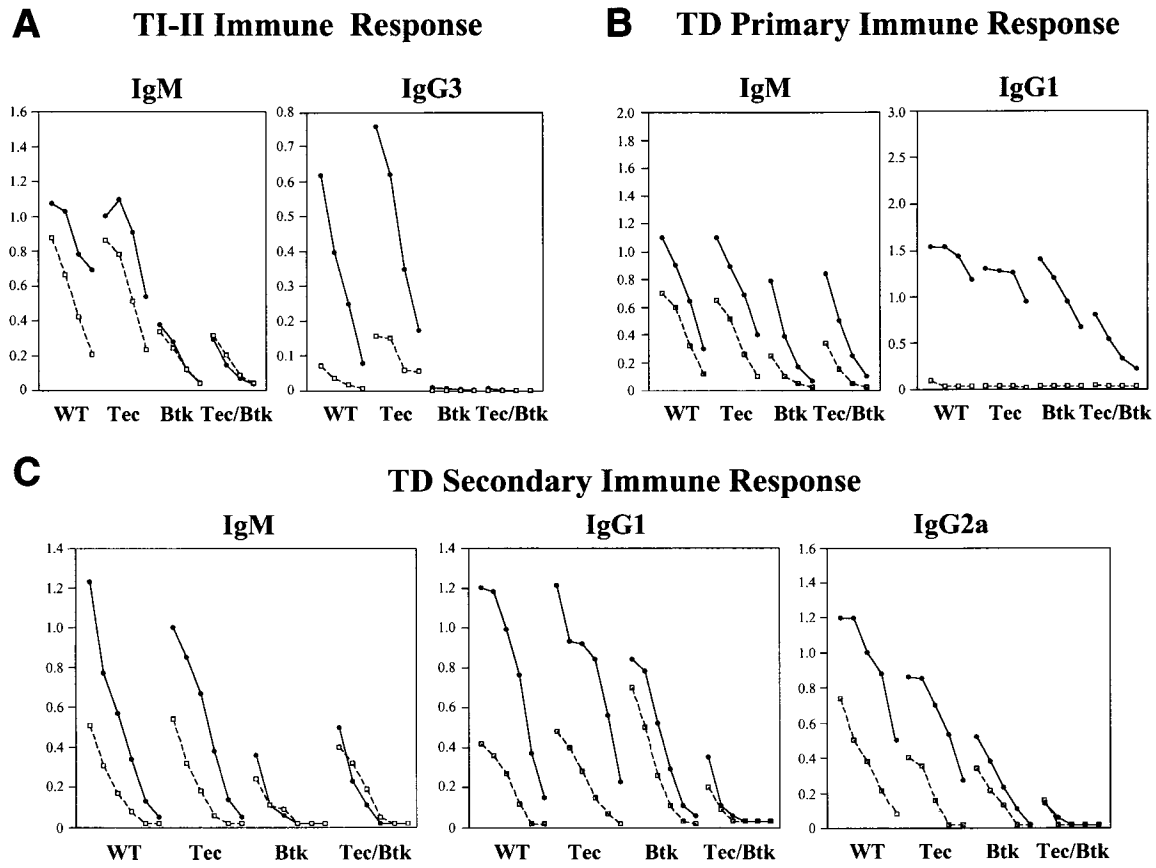

Figure 6. In vivo response to TI-II and TD antigens. (A) Response to TI-II antigen: four mice of the indicated genotype were immunized with TNP-Ficoll and the TNP-specific IgM and IgG3 isotype levels are shown in the left and right panel, respectively. A representative mouse is shown for each genotype. Dotted lines with open squares indicate levels in the preimmune serum. Serum dilution steps were threefold starting from 1:100. (B) Primary immune response to TD antigen: six mice of the indicated genotype were immunized with TNP-KLH and TNP-specific IgM and IgG1 are shown in the left and right panel, respectively. Dotted lines with open squares indicate levels in the preimmune serum. Serum dilution steps were threefold starting from 1:100 for IgM and 1:30 for IgG1. (C) Secondary immune response to TD antigens: 8 wk after the primary immunization, mice were reimmunized with TNP-KLH and TNP-specific IgM, IgG1, and IgG2a are shown in the left, middle, and right panel, respectively. Dotted lines with open squares indicate serum levels before the second TNP-KLH injection. Serum dilution steps were threefold starting from 1:100 for IgM and IgG2a and 1:900 for IgG1. (B and C) The average serum isotype titers of six mice are shown for each dilution. Standard deviations were omitted from the graphs, because of variation in the absolute levels of Ig isotypes among individual mice within each genotype group. However, the shape of the dilution curves is similar for each mouse within each genotype group. (A, B, and C) Wild-type (WT), Tec-deficient (Tec), Btk-deficient (Btk), and Tec/Btk double-deficient (Tec/Btk) mice were age-matched controls.

Disrupted Splenic Architecture in Tec/Btk Double-deficient Mice. To investigate whether the reduced number and the immature phenotype of peripheral B cells in Tec/Btk-deficient mice also affected the general architecture of the spleen, cryosections were stained with anti-B220, anti-TCR, and anti–MOMA-1 Abs to reveal the localization of B cells, T cells, and metallophilic marginal zone macrophages, respectively. The morphology of the white pulp appeared normal in Tec-deficient mice. B and T cell areas surrounded by a well-defined marginal zone could be identified (Fig. 7 B). In *Btk*-deficient mice, large B and T cells areas were also clearly detected, although we observed a discontinuity of the MOMA-1^+ marginal zone macrophage layer (Fig. 7 C). In contrast, the structure of the white pulp in Tec/Btk double-deficient mice was altered (Fig. 7 D). Only few clearly defined but small follicular structures were observed. The majority of the $B220^+$ B cell areas appeared to be loosely associated with T cell areas and no clear and distinct surrounding anti–MOMA-1 staining was observed, indicating an increased discontinuity of the marginal zone compared with Btk^{null} mice. Marginal zone B cells were present, however, as revealed by anti-CD21 and anti-CD23 FACS® staining (data not shown). Germinal centers, identified by PNA staining after primary immunization with TNP-KLH, were clearly observed in wild-type and Tec-deficient mice (Fig. 7, E and F, respectively). In agreement with other studies (45), we also could detect GCs in Btk^{null} mice (Fig. 7 G), although their frequency appeared to be reduced compared with wild-type and Tec-deficient mice. Germinal centers were also detected in Tec/Btk double-deficient mice (Fig. 7 H). Their frequency was further reduced compared with Btk^{null} mice, most likely due to the lower numbers of B cells in Tec/Btk-deficient mice.

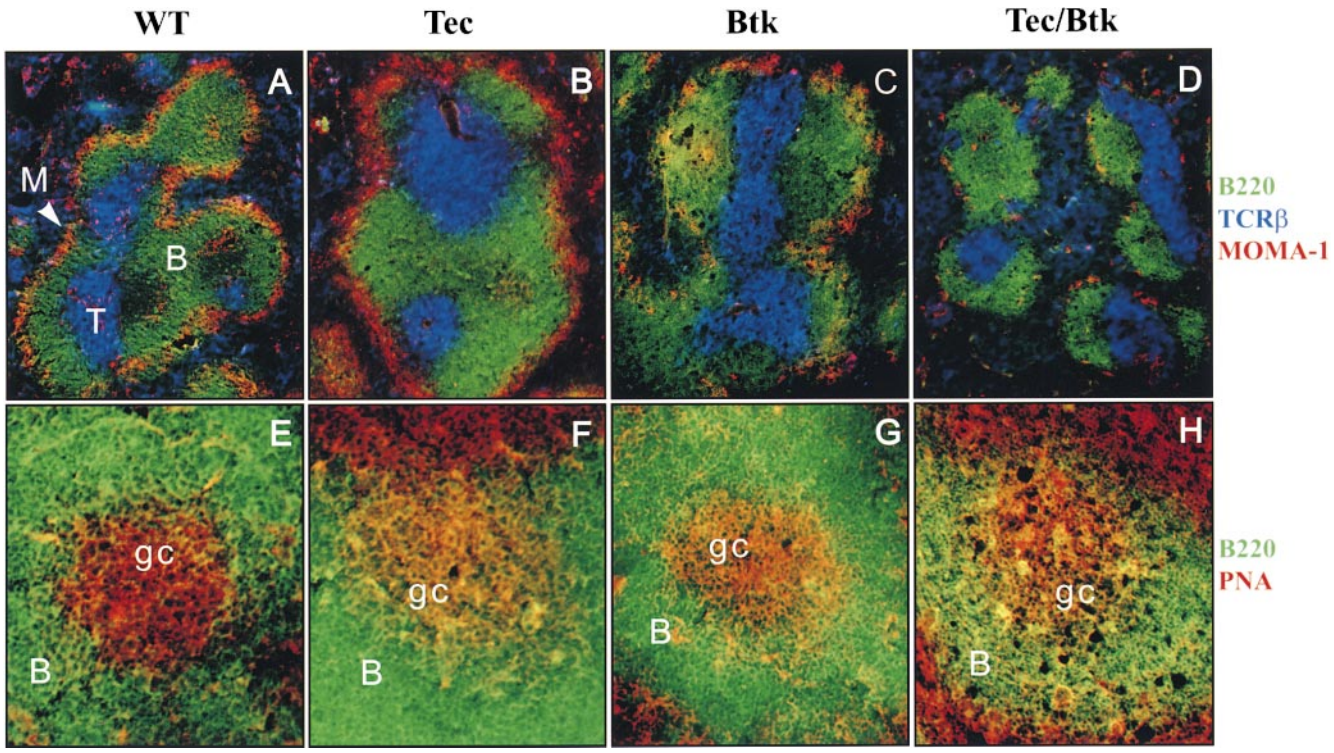

Figure 7. Disrupted splenic architecture in Tec/Btk double-deficient mice. (A–D) Cryosections of spleens of the indicated genotype were stained with anti-B220 (green), anti-TCRβ (blue), and anti–MOMA-1 (red). Original magnifications: ×50. M, Moma-1$^+$ marginal zone macrophage layer; T, T cell area. (E–H) Cryosections of spleens from mice immunized with TNP-KLH. B cell follicles (B) are shown in green (anti-B220) and PNA$^+$ areas (in red) within the B cell follicles indicate germinal centers (gc). Original magnifications: ×200.

Discussion

Members of the Tec kinase family are nonreceptor PTKs that are activated during lymphocyte development and activation (3–5). Tec is expressed in cells of the myeloid lineage, in T and B cells, and in liver (25–27). In this study, we generated Tec-deficient mice to gain insight into the biological role of Tec. Mice homozygous for the disruption of Tec were viable and fertile, and FACS® analysis of *Tec*$^{-/-}$ mice did not reveal any developmental defects within the hematopoietic lineage. In addition, Tec-deficient B and T lymphocytes behaved like wild-type cells in both in vitro proliferation assays and in vivo immunization experiments. To investigate whether Btk compensated for the loss of Tec in B cells, Tec-deficient mice were intercrossed with Btk knockout mice (12). Tec/Btk double-deficient mice displayed a severe reduction of peripheral B cell numbers, most likely caused by a partial block of B cell development at the B220$^+$CD43$^+$ to B220$^+$CD43$^-$ transition. Furthermore, the percentage of mature IgM^{lo}IgD^{hi} B cells within the peripheral B cell pool was further reduced in the double knockouts compared with the Btk single knockout and secondary TD immune responses were almost absent. Our study thus reveals a differential dependency of peripheral and developing murine B cells for the function of members of the Tec family. During B cell development, Tec and Btk share common functions and thus the activity of either Tec or Btk is required for the proper generation of immature B cells. Btk function appears to be more important than that of Tec, as a moderate reduction of pre-B cells was observed in Btk-deficient mice, but not in those lacking Tec. In the periphery, Btk appears to have additional unique functions that are only partially shared by Tec.

Tec Is Not Essential for B and T Cell Development and Function. Studies have shown that Tec is expressed in several human B cell lines that represent various stages of B cell development (27). Cross-linking of the BCR with anti-IgM Abs induces tyrosine phosphorylation of Tec in mature B cell lines and also causes an increase in the tyrosine kinase activity of Tec. Similarly, in immature B cell lines, Tec becomes tyrosine phosphorylated upon stimulation via various cell surface molecules. In T cells it has been demonstrated that Tec becomes activated upon anti-CD3 or anti-CD28 stimulation and that Tec physically interacts with CD28 in an activation-dependent manner (28). Furthermore, overexpression of a wild-type or kinase-dead form of Tec enhanced or suppressed IL-2 expression, respectively (28). Taken together, these studies suggested that Tec might have important functions both during B cell development and in signaling pathways in mature lymphocytes. However, analysis of Tec-deficient mice did not reveal any defect within the B and T cell lineage. All major B cell populations, both in the BM and in the periphery, were present and the total numbers of splenic B and T cells

were similar to wild-type control mice. In addition, $CD5^+$ peritoneal B cells were present in the Tec knockout mice. Tec has also been implicated to function in myeloid cells (30, 46). Although the experiments described in this study did not address whether $Tec^{-/-}$ myeloid cells are still functional, FACS® analysis of Tec-deficient mice did not show any developmental alterations within the myeloid lineages.

To identify potential signaling defects in B or T cells, both in vitro and in vivo experiments were performed. Neither anti-IgM, LPS, or anti-CD40 treatment of B cells nor anti-CD3 or anti-CD3 plus anti-CD28 treatment of T cells revealed any proliferative defect due to the absence of Tec. Anti-IgM cross-linking induced a calcium flux in Tec-deficient B cells that was similar to that in wild-type counterparts. Anti-CD3 plus anti-CD28 stimulation also induced similar levels of IL-2 in $Tec^{-/-}$ T cells compared with wild-type T cells. In vivo, TI-II and TD immune responses were normal as were the ex vivo T cell responses to KLH. Further experiments such as exposing $Tec^{-/-}$ mice to different pathogens are required to finally determine whether Tec-deficient mice indeed have normal immune responses.

Several Tec kinase family members are expressed within the B and/or T cell lineage. For example, B cells express Btk and Tec and low levels of Bmx, and T cells express Itk, Tec, and Rlk. The absence of any developmental and/or signaling defect in Tec-deficient mice could thus be explained by the fact that other Tec family kinases are able to compensate for the loss of Tec. Indeed, in Btk-deficient human XLA B cells or in chicken DT-40 cells, the defect in phospholipase Cγ2 phosphorylation and Ca^{2+} mobilization can be overcome by overexpression of any Tec kinase family member (47, 48). The ability of these kinases to at least partially replace each other has also been shown for murine T cells. Itk/Rlk double-deficient mice have a more severe phenotype than Itk single-deficient mice, indicating that Rlk partially compensates for the loss of Itk (24). In contrast, Itk and perhaps other members of the Tec kinase family are able to fully compensate for the loss of Rlk, as those mice have no major developmental or signaling defect within the T cell lineage (24).

Tec and Btk Are Essential for B Cell Function and Development. To test whether Btk is able to compensate for the loss of Tec in the B cell lineage and therefore masks pathways using Tec kinase, we intercrossed Tec-deficient mice with Btk knockout mice (12). FACS® analysis of PBLs and secondary lymphoid organs of Tec/Btk double-deficient mice showed that both Tec and Btk are required for the proper maturation of peripheral B cells. Not only was the percentage of mature $IgM^{lo}IgD^{hi}$ cells within the peripheral B cell pool further decreased compared with Btk^{null} cells, but Tec/Btk-null mice also had severely reduced numbers of peripheral B cells. The absolute numbers of $B220^+$ splenocytes were three- to fourfold reduced in Tec/Btk-deficient compared with Btk^{null} mice. In addition to the defect in the peripheral B cell compartment, Tec/Btk-deficient mice exhibited a defect during B cell development. There was an accumulation of $B220^+CD43^+$ pro- and pro/pre-B cells, at the stage at which developing B cells become dependent on pre-BCR signals for their further maturation into surface IgM–expressing immature B cells (44). The activity of either Tec or Btk is thus required to allow progression through this crucial developmental stage. In the absence of both Tec and Btk, only a few cells are able to develop into immature B cells. This indicates an essential role for Tec family kinases during pre-BCR signaling in the murine system.

The partial block during B cell development could explain the dramatic drop in peripheral B cell numbers in mice lacking both Tec and Btk. Therefore, one may expect to observe in these mice a slow accumulation of peripheral B cells that could develop in the absence of both Tec and Btk with increasing age. However, as older mice (4–5 mo) still showed low numbers of peripheral B cells (Ellmeier, W., and D.R. Littman, unpublished data), it remains possible that the absence of both Tec and Btk causes additional defects within the B cell lineage. Peripheral B cells could have a decreased life span due to defects in BCR signaling (49). In addition, the selection of immature B cells in the BM and/or the emigration out of the BM could be impaired (50).

Despite the reduced number of peripheral B cells in Tec/Btk double-deficient mice, their serum Ab titers were similar to those in Btk knockout mice at 8–12 wk of age. It has been shown that Btk is essential for an immune response to TI-II antigens. Thus, it was not surprising that Tec/Btk-deficient mice also failed to respond to TNP-Ficoll. Btk^{null} mice, however, are able to respond to TD antigens such as TNP-KLH. Both Btk- and Tec/Btk-deficient mice mounted similar IgM responses, but the primary IgG1 response was dramatically reduced in Tec/Btk-deficient mice. The secondary IgG1 and IgG2a immune responses were low in Btk^{null} mice but were virtually absent in Tec/Btk-deficient mice. Thus, the formation and/or activation of memory B cells are strongly dependent on signals transmitted by either Tec or Btk, and these processes are impaired despite formation of germinal centers (although of small size) in Tec/Btk-deficient mice. The requirement for both Tec and Btk in B cell signaling pathways is also indicated by the reduced proliferative response to LPS stimulation in the double-deficient B cells compared with Btk-deficient B lymphocytes. However, as the B cell areas and the marginal zones of the spleen seem to be altered in Tec/Btk^{null} mice, it remains possible that the failure to respond properly to TD antigens is a consequence of the altered splenic microenvironment rather than to an intrinsic B cell defect.

The reason for the alterations of the splenic B cell areas and marginal zones is not known. Further experiments will be required to determine whether the change in the splenic architecture is caused by the defect in the B cell lineage and/or by additional defects in other lineages (e.g., metallophilic marginal zone macrophages).

The phenotype of the Tec/Btk^{null} mice resembles partially the phenotype of mice expressing the E41K gain of function mutant of Btk (51). Both types of mice showed a

similar reduction of peripheral follicular B cell numbers with a similar immature IgM/IgD profile, no TI-II, and almost absent TD immune responses, and altered B cell areas and marginal zones in the spleen. However, proliferation in response to anti-IgM stimulation was observed in transgenic E41K Btk mice, even on a Btk^{null} background. Thus it remains to be determined whether the similarity in the phenotypes is caused by the same mechanisms. It has been suggested that E41K Btk mimics BCR engagement and therefore transgenic B cells may resemble autoreactive B cells that are eliminated (51). The absence of mature B cells in Tec/Btk^{null} mice is more likely explained by a reduction in the life span and/or a block in the maturation of immature splenic B cells as a consequence of lack of BCR signaling.

Differential Requirement for Tec and Btk in Humans versus Mice: How Is Specificity Regulated among Tec Kinases? A question only indirectly addressed in this study is why mutations in Btk result in a more severe phenotype in humans than in mice. Our results clearly demonstrate that Tec functionally compensates for loss of Btk in Btk^{null} mice during B cell development, thus providing an explanation for why B cell development is little affected in Btk-deficient mice. However, inactivation of human Btk causes a severe block during B cell development. It remains to be determined whether Tec is expressed in primary human pro- or pre-B cells, although it has been shown that Tec is expressed in human pro-B, pre-B, and mature B lymphoid cell lines, and even in EBV-transformed B lymphoblastoid cell lines derived from XLA patients (27). This raises the important question of how specificity is regulated among Tec kinases. In murine B cells, Btk is able to fully compensate for loss of Tec, but Tec is not able to fully compensate for loss of Btk. Similar observations have also been made in T cells for the Tec kinase family members Rlk and Itk: loss of Rlk, unlike Itk, does not affect T cell function, although combined deletion of both kinases results in a stronger phenotype (24). Whether specificity is determined by specific substrates or interacting proteins, or by different affinities to the same binding partners remains to be studied. Another possibility is that the expression levels of the different kinases determine whether one kinase is able to compensate for another. It has been shown that the ability of a transgene to fully rescue the *xid* defect depends on its expression levels. Low level expression of Btk was sufficient to generate normal numbers of peripheral B cells, but rescue of signaling defects of *xid* B cells was inefficient (52). Dosage dependency has also been observed during the analysis of Tec/Btk double-deficient mice. On a Btk-deficient background, $Tec^{+/-}$ mice had an intermediate B to T cell ratio phenotype compared with $Tec^{+/+}$ and $Tec^{-/-}$ mice (Ellmeier, W., and D.R. Littman, unpublished data), again suggesting that dosage of Tec kinases influences B cell numbers.

In summary, the generation of Tec kinase–deficient mice revealed that the absence of Tec kinase causes no major phenotypic alteration of the immune system. By generating Tec/Btk double-deficient mice, we could show that Btk compensates for the loss of Tec in the B cell lineage and that the activity of either Tec or Btk is required for the proper generation of immature B cells. However, as Btk-deficient mice, but not Tec^{null} mice, show a moderate reduction of pre-B cells, Btk function appears to be more important than that of Tec during B cell development. In the periphery, Btk appears to have additional unique functions that are only partially shared by Tec. The availability of mice deficient for the various members of the Tec kinase family will also facilitate further studies that address the regulation of specificity among Tec kinases in hematopoietic cells in more detail.

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