Studies on the function of *Bruton's tyrosine kinase* in B cell development

Studies on the function of *Bruton's tyrosine kinase* in B cell development

(Onderzoek naar de functie van *Bruton's tyrosine kinase* in de B cel ontwikkeling)

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam op gezag van de
Rector Magnificus
Prof. Dr. P.W.C. Akkermans, M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 16 december 1998 om 11.45 uur

door

Alexander Maas
geboren te Maarheeze

Promotiecommissie

Promotor: Prof. Dr. F. Grosveld

Overige leden: Prof. Dr. W. van Ewijk
Prof. Dr. H.C. Clevers
Dr. E. Dzierzak

Co-promotor: Dr. R.W. Hendriks

Voor mijn ouders

Contents

Chapter 1

General introduction

Introduction

Each individual organism has to protect itself against a large variety of infectious microbial agents, such as bacteria, fungi and parasites to prevent pathological damage and death. In vertebrates, defense mechanisms against foreign substances, antigens, have evolved in the immune system, which has two functional divisions: the 'innate' immune system and the 'adaptive' immune system.

The 'innate' immune system is aspecific and acts as a first line of defense, mediated by cells from the myeloid lineage and soluble factors like complement and lysozyme. The main function of the 'innate' immune system is to avoid entering of microorganisms into the body and to clear it of killed pathogens. In contrast to the 'adaptive' immune system, repeated infection does not improve the resistance of the 'innate' immune system.

If the first line of defense is defeated, the second line of defense, the 'adaptive' immune system, which is very specific and can develop memory to earlier accounted pathogens, is activated. The specific immune response is mediated by lymphocytes belonging to the B and/or T lineages (B and T cells). Both B and T cells express receptor molecules on their cell membrane, which specifically can bind antigens. The T cell receptor (TCR) can only bind antigens if these are processed into small peptides, and presented by major histocompatibility complex (MHC) class I molecules on the surface of host cells and MHC class II molecules on the surface of antigen presenting cells. Intracellular antigens are processed into small peptides and presented on the surface by the MHC class I complex. Recognition of the MHC-class I-peptide complex by the TCR of cytotoxic T cells results in killing of the presenting host cells. MHC class II molecules present processed peptides, which are derived from external endocytosed antigens. Recognition of MHC class II-peptide complexes by the TCR of T helper (T_H) cells results in the production of cytokines and stimulation of cells of the immune system ('cellular' immune response). The B cell receptor (BCR) binds to unprocessed antigens. Stimulation of the BCR results in a 'humoral' immune response, i.e. the secretion of soluble immunoglobulins (Ig), with the same binding specificity as the BCR to the triggering antigen.

Ig molecules are composed of the unique combination of two identical heavy (H) and two identical light (L) chains. Both the H and L chains have a constant amino acid sequence at their carboxyl terminal region (C region), which mediates effector functions, such as membrane expression, complement binding and binding to other cells of the immune system. The amino terminal end contains regions that vary in their amino acid sequence (V region). The amino acid variability of V regions is not distributed evenly throughout the length of these regions. In both H and L chain V regions three hypervariable regions, also referred to as complementary determining regions (CDR), are separated by intervening framework regions (FR). V region CDRs are the antigen binding segments in the Ig. In Fig. 1, a schematic representation of an Ig molecule is given.
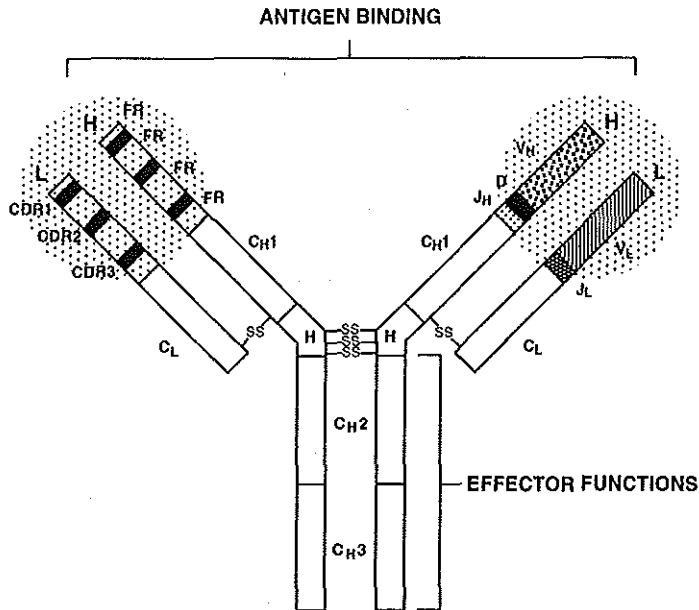
ANTIGEN BINDING

Figure 1. *Diagram of an Ig molecule. Two identical heavy-chain (H) and two identical light-chain (L) polypeptides pair to form two identical antigen-binding sites. The V_H, D, C_H, V_L, J_L and C_L domains are shown; the hinge region is represented by H, disulfide-bridges by -ss-. The approximate locations of the CDR and FR relative to V_H, D, J_H, V_L and J_L are shown by different shadings on the left and right respectively, of the molecule.*

B cell development in the mouse

B cell development is a highly regulated process of ordered events. During this process, a pool of B cells is generated, in which each B cell expresses one specific non-autoreactive Ig with a maximal affinity to a particular antigen. Furthermore, to protect the individual against a large variety of pathogens, the B cell repertoire has to be as diverse as possible. B cell development in mammals can be divided into two phases. The first, antigen-independent, phase is found in the fetal liver and bone marrow and involves differentiation of haematopoietic stem cells to B cells expressing a unique Ig on their membrane. After successful Ig assembly, B cells migrate out of the bone marrow into the peripheral lymphoid tissues. During the second, antigen-dependent, phase recognition of a specific foreign antigen may result in selective expansion and differentiation of the resting B cells into Ig-secreting plasma cells or into memory B cells.

B cell development in the bone marrow

B cell differentiation in the bone marrow was originally characterized by sequential V(D)J recombination of the Ig loci and expression of the Ig H and L chains. In the mouse, B cell differentiation models have been developed using cell surface markers, such as CD45R/B220, CD43, HSA, BP-1, c-kit or interleukin-(IL)-2 receptor and cell size (Osmond, 1990, Hardy *et al.*, 1991, Melchers and Rolink, 1995). Despite much progress in elucidating the cellular stages of B cell development, diverse phenotypic criteria and terminologies are being used, which hampers the comparison of experimental data, even when describing the same process. Here, we use the B cell differentiation model of Osmond *et al.* (1990) depicted in Fig. 2, whereas the corresponding B cell stages according to Hardy *et al.* (1991) and Melchers and Rolink (1995) are given in parentheses, respectively. B cell development in human shows remarkable parallels, as was recently reviewed by Ghia *et al.* (1998).
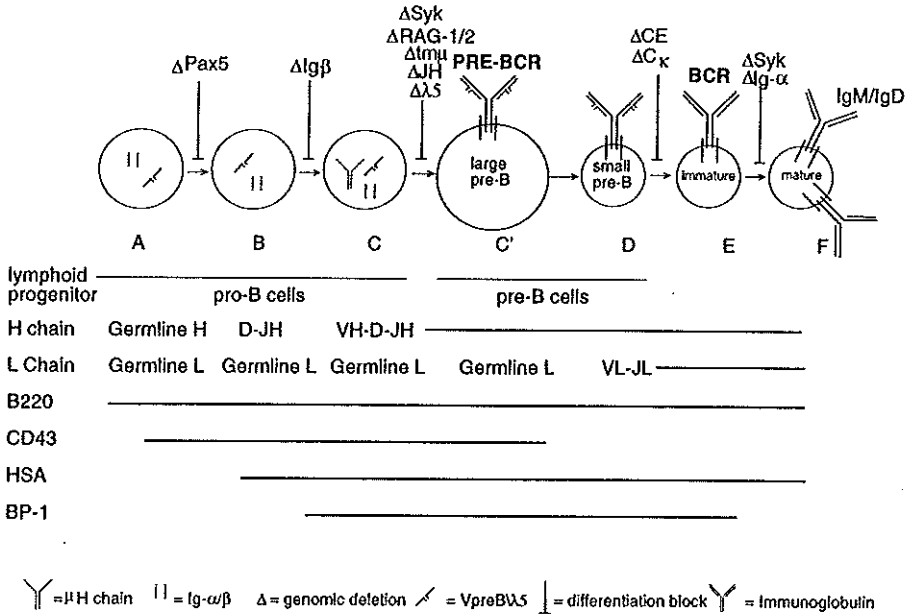
Figure 2. Linear scheme of the B cell differentiation pathway, giving an overview of the temporal expression of the (pre-)BCR component molecules, surface markers molecules and the rearrangement status of the IgH and L chain components. Differentiation blocks result from (partial) deletion of murine genes encoding essential transcription factors (Pax-5, Urbanek et al., 1994, Nutt et al., 1997), the λ5 gene (Kitamura et al., 1992), proteins and DNA regions, necessary for receptor gene recombination (RAG-1, Mombaerts et al., 1992, RAG-2, Shinkai et al., 1992, CE, Hiramatsu et al., 1995, C, Chen et al., 1993, Zou et al., 1993, J$_H$, Chen et al., 1993, Ehlich et al., 1993), signaling elements (Syk, Igα, Torres et al., 1996, Turner et al., 1995, Ig, Gong and Nussenzweig, 1996) and membrane exon of the H chain (tmμ Kitamura et al., 1991).

The earliest cells which are committed to the B cell lineage are the early pro-B cells (fraction A, pro-B/pre-B-I), which constitute 1% to 1.5% of the total BM B-cell population and have all Ig loci in germ-line configuration ($\mu^- L^-$). However, not all cells within the early pro-B cell fraction are committed to the B cell lineage. About 20% to 40% of the cells in this fraction co-express in addition to the B cell markers B220 and CD43, also the NK1.1 surface molecule, but lack VpreB, Ig-β and CD19 expression (Rolink et al., 1996). These cells develop into natural killer (NK) cells. Li et al. (1996) recently reported that cells with B lineage potential form a subpopulation within the early pro-B cell fraction, which is characterized by the expression of the surface marker AA4.1.

Ig H chain V(D)J recombination is initiated at the $B220^+ CD43^+ \mu^- L^-$ intermediate pro-B cell stage (fraction A/B, pro-B/pre-B-I), characterized by the upregulation of HSA expression. In this stage 15% of the cells are in S or G2/M phases (Lin and Desiderio, 1995). Ig H chain loci (Fig. 3) become accessible for recombination of a D (diversity) to a J(joining)$_H$ segment followed by the fusion of a V(variable)$_H$ gene segment to the pre-existing DJ$_H$ segment at the late pro-B cell stage (fraction C, pro-B/pre-B-I). In this V(D)J recombination process any one form of an array of V$_H$ gene segments can be joined to one of many D and J$_H$ gene segments to generate CDR encoding antigen-binding domain of the Ig, which is then located close to the constant region encoding genes of the H chain (C$_H$). Murine B cells lacking Ig-β expression are blocked at the intermediate pro-B cell stage, characterized by the presence of D to J$_H$ but absence of V$_H$ to DJ$_H$ rearrangements (Gong and Nussenzweig, 1996).

Productive Ig μ rearrangement is monitored by the expression on the surface of a μ-pseudo-L chain complex (pre-BCR)(Karasuyama et al., 1990) and coincides with the transition to a large rapidly cycling pre-B cell stage (fraction C', large pre-B-II). This cell stage is characterized by downregulation of CD43 and recombination activating gene (RAG) expression and upregulation of HSA expression. The pseudo L chain consists of $\lambda5$ and Vpre-B gene encoded proteins, homologous to Jλ-Cλ and Vλ respectively (Kudo et al., 1987, Bauer et al., 1988, Sakaguchi et al., 1986, Pillai et al., 1987, Hollis et al., 1989) and is thought to have a chaperone function in expressing the Ig μ chain on the cell membrane.

Expression of the pre-BCR plays a critical role in the transition from the pro-B to the large pre-B cell stage. Disruption of RAG-1 or RAG-2 expression (Mombaerts et al., 1992, Shinkai et al., 1992) prevents the formation and expression of a pre-BCR and results in an arrest at the transition from pro-B to pre-B cells. A developmental blockade was observed at the same stage in mice with a mutation in the membrane exon of the μ chain gene (Kitamura et al., 1991), or a disruption of the Ig J$_H$ gene segments (Chen et al., 1993, Ehlich et al., 1993) or $\lambda5$ gene (Kitamura et al., 1992), preventing membrane deposition of the pre-BCR. However in the latter case, the block appeared to be leaky, leading to the development of a small number of functional B cells. Signaling through the pre-BCR results in a feedback inhibition of H chain V(D)J recombination to ensure the expression of the functionally rearranged Ig H chain locus, whereas the other allele, often still in D-J$_H$ or germline configuration (Alt et al., 1984) is not expressed, a phenomenon called 'allelic exclusion' (Storb et al., 1994).

After an interleukin (IL)-7 dependent proliferative expansion, in which 70% of the cells are in S and G2/M phase (Karasuyama *et al.*, 1994, Rolink *et al.*, 1994), the $\mu^+L^-CD43^{+/-}$ large pre-B cells leave the cell cycle to become $\mu^+L^-CD43^-$ resting small pre-B cells (fraction D and small pre-B-II). Within this population less than 5% of the cells are in S and G2/M phases. At this stage RAG expression is upregulated (Grawunder *et al.*, 1995), followed by the initiation of Ig L chain rearrangements. In mammals, only two isotypes of L chain are produced, Igκ and Igλ. In mice the Igκ locus consists of several hundred V_K gene segments, five J_K gene segments and one C_K gene segment. In the Igλ system there are four $C\lambda$ genes, each with its own $J\lambda$ gene and two $V\lambda$ genes (Fig. 3). Ig L chain gene assembly at the κ locus normally precedes rearrangements of the λ locus (Hieter *et al.*, 1981, Zou *et al.*, 1993). In the majority of B cells and B cell lines κ loci are rearranged, while the λ loci are still in their germ-line configuration leading to a 20-fold overrepresentation of κ+ B cells in mice (McIntrie *et al.*, 1970, Zou *et al.*, 1993). However, Igλ rearrangement does not require rearrangement of the Igκ locus. A small fraction of progenitor B cells rearrange Igλ before Igκ (Zou et al., 1993) and in mutant mice, unable to rearrange their Igκ locus, the Igλ locus is rearranged with an increased efficiency (Takeda *et al.*, 1993). Downstream of C_K there is a κ-deleting element (Kde) known to be involved in deleting the κ genes in those B cells which rearranged their Igλ locus (Siminovitch *et al.*, 1985).

Expression of a complete Ig, rearranged H and L chain at the IgM^+ immature B cell stage (fraction E) is accompanied by a decrease in the expression of Vpre-B, λ5, RAG-1 and RAG-2 (Li *et al.*, 1993) and ensures allelic exclusion, which, in the case of Igκ and Igλ chains, is called isotype exclusion (Rajewsky, 1996). With the acquisition of antigen specificity, maturing B cells pass through a tolerance-susceptible stage (Goodnow *et al.*, 1995) in which $IgM^{low}IgD^-$ cells undergo nested L chain rearrangements (receptor editing) and the more developed $IgM^{high}IgD^-$ cells acquire sensitivity to apoptosis upon auto-antigen binding (Tiegs *et al.*, 1993, Melamed *et al.*, 1988). B cells that do not bind auto-antigen acquire differentiation markers on their cell surface, such as IgD, CD21 and CD23, and migrate out of the bone marrow to colonize peripheral lymphoid organs (fraction F).

A crucial aspect of V(D)J recombination regulation is its target specificity. H chain rearrangements precede L chain rearrangements and both occur only in B lymphocytes. It is now thought that enhancers have a clear function in positioning DNA binding proteins (Serwe and Sablitzky, 1993, Takeda *et al.*, 1993, Xu *et al.*, 1996) and in DNA methylation, thereby affecting DNA accessibility (Lichtenstein *et al.*, 1994). Immediately upstream of each germline V_H and V_L segment, a transcriptional promoter is located. High-level expression from the promoters of rearranged V_H and V_K genes depends on the activity of the intronic IgH enhancer (Eμ) and the Igκ intronic enhancer (Eκ) elements, respectively. These enhancers are located within the J-C intron regions (Blackwell *et al.*, 1989). Powerful enhancers have also been identified downstream of the Cα, Cκ and Cλ genes: the E_H3' (Pettersson *et al.*, 1990), the Igκ3' (Meyer and Neuberger, 1989) end the λ enhancer (Blomberg *et al.*, 1991) respectively. Hiramatsu and co-workers (1995) showed, using transgenic substrates, that the Igκ3' enhancer contained a suppressive element that controls stage and cell type specific Igκ gene rearrangements. Possibly, these enhancer elements are able to

locally open the chromatin structure giving access to the recombination machinery (Stanhope-Baker *et al.*, 1996). It has been reported that low level transcription of unrearranged Ig gene segments (germline transcription) usually precedes V(D)J recombination (Alt *et al.*, 1987, Yancopoulos *et al.*, 1986, Schlissel and Baltimore, 1989). Whether this is a consequence of the open chromatin structure or whether it may also enhance DNA accessibility is not clear yet. In comparison, germline transcription plays an important role in targeting the IgH class switch recombination. Class switching is directed to distinct classes by cytokines, which induce transcription of the targeted DNA sequences. These transcripts are processed, resulting in spliced 'switch' transcripts. Switch recombination can be directed to IgG1 by the heterologous human metallothionein IIA promoter in mutant mice. Induction of the structurally conserved, spliced switch transcripts is sufficient to target switch recombination to IgG1, wheras transcription alone is not (Jung *et al.*, 1993).

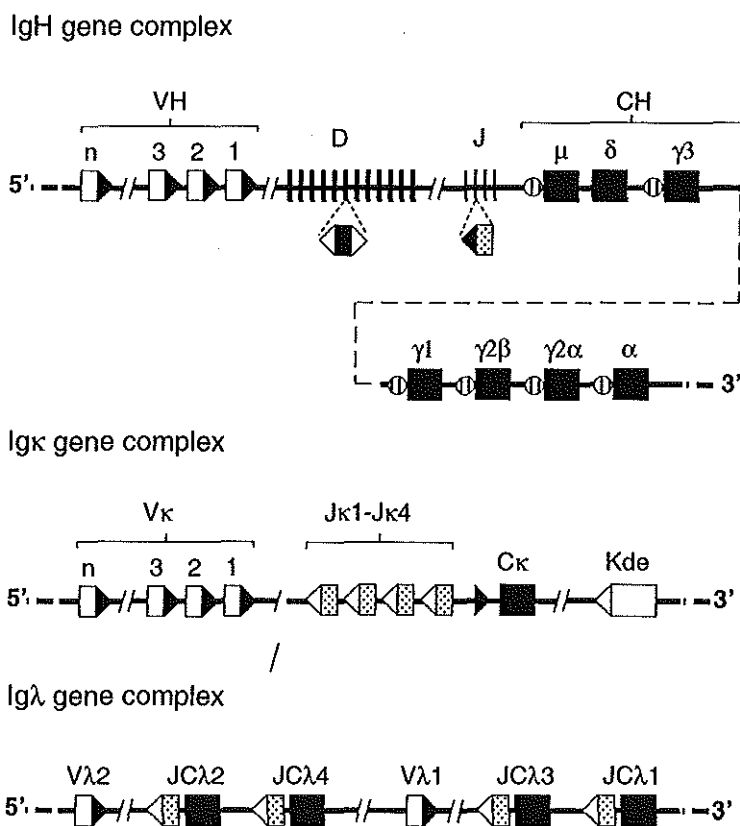## IgH gene complex

Igκ gene complex

Igλ gene complex

Figure 3. *Schematic diagram of murine IgH and IgL genes. Recombinase-recognition sites are depicted by triangles, class switch sequences are represented as hatched circles.*

Enzymes involved in regulation of V(D)J recombination

V(D)J recombination is a process in which both B and T cells make double strand breaks in their somatic DNA. The generation of double strand breaks has to be a tightly regulated process and many enzymes are involved. The *cis* acting elements in the recombination process are the recombination signal sequences (RSS), which consist of a heptamer and a nonamer separated by a spacer of either 12 base pairs (on the 3' end) or 23 base pair (on the 5' end). These RSS form hairpin structures, in which a RSS with a 12 base pair spacer can anneal to a complementary RSS with a 23 base pair spacer, looping out the intervening sequences. Van Gent *et al.* (1995) were the first to show in a cell-free system, that lymphoid specific proteins, encoded by the RAG-1 and -2 genes, were sufficient to catalyze DNA cleavage at RSS in a two step mechanism. In the hairpin structure the 3'OH end, generated by the nicking of DNA at the heptamer sequence of the RSS, attacks the phosphodiester bond of the antiparallel strand, thereby releasing the hairpin signal ends from the hairpin coding ends (Van Gent *et al.*, 1996). Others (Hoim *et al.*, 1997, Grawunder *et al.*, 1997a), have shown that nicking and double strand cleavage at RSS occurs in a stable complex of DNA with the RAG proteins, which disassembles after completion of the reaction. Although RAG-1 and -2 by themselves are able to achieve the DNA cleavage, the efficiency of the reaction is enhanced by other nuclear components, like DNA bending high mobility group proteins (HMG)-1 and 2 (Sawchuk *et al.*, 1997, Van Gent *et al.*, 1997).

After the cleavage step, the hairpinned coding end must be opened to allow further processing and DNA double strand break repair. Enzymes known to be involved in hairpin opening and modification are DNA-dependent protein kinase (DNA-PK), a heterodimeric complex of Ku70 and Ku86, and possibly the RAG-1 and -2 proteins themselves. DNA-PK is probably involved in phosphorylating endonucleases, which might be the RAG proteins, to activate them to open the hairpin. In the natural occurring mutant mouse strain with severe combined immunodeficiency (SCID) (for review see Bosma and Carroll, 1991), due to a defect in the gene coding for DNA-PK, it was demonstrated that coding end structures could not be opened (Roth *et al.*, 1992). The generation of Ku70- and Ku86-deficient mice demonstrated that the heterodimeric complex of Ku70 and Ku86 is involved in the formation of coding and signal joints (Zhu *et al.*, 1996, Nussenzweig *et al.*, 1996). It has been proposed by Gottlieb *et al.* (1993) that the Ku70/86 complex served as a binding site for DNA-PK. However, in Ku70-deficient mice, low levels of normal coding-joint formation occurs, in contrast to mice with defects in DNA-PK, which makes this model less attractive. In addition to the DNA-PK stimulating role (Gottlieb *et al.*, 1993) an additional role for the Ku 70/86 complex in replacing the RAG proteins from the signal ends and thereby allowing their ligation was proposed (Zhu *et al.*, 1996).

After the hairpins are opened, the four DNA ends are modified by other enzymes. To increase the genetic variability of the Ig, terminal deoxy transferase (TdT), which is lymphoid specific, can add nucleotides to coding and signal ends independent of the template. The coding ends, but not the signal ends, could also be subject to nucleotide loss due to the action of yet unidentified nucleases. Most likely, the signal ends are protected from nuclease but not TdT activities (Agrawal *et al.*, 1997)

in a complex where all four ends are close to each other. This would also explain the occurrence of the so-called hybrid joints, in which a signal end is coupled to a coding end (Lewis *et al.*, 1988). The coding ends are then subject to alignment over a very short region of homology. A possible role for the flap endonuclease (FEN)-1, which removes 5' excess DNA, and polymerase β, which fills in DNA gaps, have been proposed in this reaction (Grawunder *et al.*, 1997b), although this is as yet not clear. The blunt signal and the aligned coding ends are then ligated together by a complex formed by XRCC4 and DNA ligase IV (Grawunder *et al.*, 1997b), although others believe it is DNA ligase I, which forms a complex with ERCC4, is responsible for the ligation (Ramsden, *et al.*, 1997).

B cell differentiation in the periphery

Most newly produced IgM$^+$IgD$^-$ B cells from the bone marrow migrate via the blood stream into the B cell areas of the spleen and lymph nodes. The spleen is an important lymphoid organ, involved in immune responses against all types of antigen that appear in the circulation. Its complex anatomical organization, with distinct compartments containing specialized cell types, provides a micro-environment which allows different cell-cell interactions and determines the direction of developing immune responses (Van den Eertwegh *et al.*, 1992). Many of the new B cell emigrants fail to receive a positive selection signal in the spleen and die within a few days (Allman *et al.*, 1993). The small subset of B cells that is positively selected, enters the primary B cell follicles and become recirculating B cells, characterized by the upregulation of the anti-apoptotic protein bcl-2 levels and membrane IgD expression and downregulation of heat stable antigen (HSA) expression (Allman *et al.*, 1993, Hardy *et al.*, 1995, Best *et al.*, 1995). In addition to B cells, the B cell follicles contain a variety of stromal cells, like follicular dendritic cells (FDC), which may produce factors needed for the chemoattraction (Föster *et al.*, 1996) of B cells into the follicle and for upregulating proteins such as bcl-2. As these survival factors are limited, migration into these follicles of newly produced B cells is inhibited by the presence of follicular B cells. Recirculating B cells migrate between the follicles of secondary lymphoid organs and have an average life-span of 6 weeks (Fulcher and Basten, 1997)

    B cell activation of all types of antigen, i.e. T cell independent (TI) type I and II and T cell dependent (TD), leads to accumulation of B cells in the outer T cell zone (Van den Eertwegh *et al.*, 1992, Claassen *et al.*, 1986, Liu *et al.*, 1988, 1991, Jacob *et al.*, 1991). In response to TI-1 (lipopolysaccharide) and TI-II antigens, proliferating B cells undergo plasma cell differentiation within the outer T cell zone or red pulp, but follicular B cell proliferation is moderate. In the primary response to a TD antigen, B cells take up, process and present antigen to T_H cells in the outer T cell zone. Co-stimulation of T_H and B cells is mediated by CD40-CD40L and CD86-CD28 interactions (Fachetti *et al.*, 1995, Van Essen *et al.*, 1995, Grewal *et al.* 1995, Ferguson *et al.*, 1996). Cognate interaction with antigen specific T_H cells results in either the formation of foci of antibody-producing plasma cells in the outer T cell zone or the migration to a network of FDC in B cell follicles where they form germinal centers (GC). Failure of T_H-B cell interaction leads to B cell apoptosis within a few days (Cyster *et al.*, 1995). In the dark zone of the GC, the activated B cell

immigrants differentiate into centroblasts, characterized by high proliferation rates. During this proliferation phase, affinity maturation of the Ig occurs due to random somatic mutations in the Ig H and L chain V regions (Jacob *et al.*, 1991). Centroblasts then migrate into the GC light zone and differentiate into centrocytes. In the GC light zone FDC have sequestered antigens in the form of immune complexes via their complement receptors. Only those centrocytes with a BCR that has a higher affinity than the original antibody can bind to the antigen bound by the FDC. The mechanism of rescuing the B cell by the FDC is not clear yet, but probably involves interaction though adhesion molecules (Lindhout *et al.*, 1993). Competition for antigen and subsequent rescue from apoptosis provides a mechanism for selection of B cells with high affinity BCR, whereas B cells bearing lower affinity BCR due to the somatic mutations are deprived from FDC mediated rescue and go into apoptosis. Rescued B cells retrieve antigen from FDC and present them on the MHC class II molecules to antigen-specific GC T cells. Recent experiments have shown that the syk tyrosine kinase, an effector of the BCR signal transduction pathway, is also involved in the presentation of peptide-MHC-II complexes. (Lankar *et al.*, 1998). If the T cell recognizes the processed antigen-MHC-II complex it will express CD40L and cytokines, including IL-4 and IL-10 (Butch *et al.*, 1993, Lederman *et al.*, 1992, Casamayor-Palleja *et al.*, 1995) which initiate Ig isotype switching (Malisan *et al.*, 1993). In mice the C_H locus contains Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα genes which determine the Ig isotype class; IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE and IgA respectively. An activated B cell can join the V(D)J-rearranged region of the H chain to different constant region genes, allowing the generation of different effector functions. In addition CD40L binds to CD40, expressed on the B cell surface and mediates downregulation on the Fas/CD95 apoptosis pathway (Rathmell *et al.*, 1996). The positively selected centrocytes then differentiate into plasma cells or into memory cells, which will join the recirculating lymphocyte pool (Liu *et al.*, 1991)

B cell populations

Based on the cell surface phenotype, anatomical localization and developmental characteristics, murine B cells can be divided into three subpopulations: B-1a , B-1b and B-2 (conventional) B cells.

B-1 cells are derived from progenitor cells in the omentum and fetal liver, and are characterized by a self-renewal mechanism from mature B-1 cells, whereas *de novo* generation is terminated in post natal life (Lalor *et al.*, 1989). The B-1 subpopulation can be subdivided into B-1a and B-1b populations based on two key differences. Firstly, in contrast to B-1b, B-1a cells express the glycoprotein CD5 on their cell surface. Secondly, upon bone marrow transplantation from adult mice to irradiated recipients, B-1b cell levels are reconstituted to half the normal size, whereas B-1a cells remain absent (Kantor *et al.*, 1992). B-1 cells are predominantly found in the adult peritoneal and pleural cavities (Hayakawa *et al.*, 1985, Kroese *et al.*, 1992) and generate a large percentage of the IgA-producing plasma cells in the gut (Kroese *et al.*, 1989). A detailed description of the origin of murine B-1 cell lineages is given in Herzenberg and Kantor (1993).

B-2, or conventional B cells derive from the fetal liver and bone marrow in adult life and are characterized by a constant *de novo* synthesis from progenitor cells in the bone marrow during post

natal life. Conventional mature B cells are predominantly located in the secondary lymphoid organs, like spleen, lymph node and Peyers patches.

X-linked agammaglobulinemia

Studies on natural occurring immunodeficiency diseases have given us more insight into lymphocyte development. Most of the 50 genetic immunodeficiencies recognized sofar, show an autosomal recessive mode of inheritance (Rosen et al., 1997, Stiehm., 1993), affecting only one generation within a pedigree. In about 70-80% of the cases, patients are males (Conley et al., 1994), reflecting the fact that a substantial fraction of the best characterized immunodeficiencies are inherited on the X chromosome. Due to the X-linked mode of inheritance, affected males are not confined to one generation within a pedigree, making the X-linked immunodeficiency diseases to the most prevalent ones. Immunodeficiency diseases which have been mapped to the X-chromosome are: X-linked severe combined immunodeficiency (XSCID), X-linked hyper-IgM syndrome, Wiskott-Aldrich Syndrome (WAS), X-linked lymphoproliferative syndrome (XLP), Properdin deficiency (PD), X-linked chronic granulomatous disease (X-CGD) and X-linked agammaglobulinemia (XLA). The genes responsible for these X-linked immunodeficiency diseases have been identified using various approaches, including positional cloning (Table 1).

Table 1. X-linked immunodeficiencies

Disorder	Locus	Cells affected	Gene designation	References
X-CGD	Xp21	Granulocytes, Monocytes	Gp91-phox	1-3
PD	Xp11	Macrophages/Complement	Properdin	4,5
WAS	Xp11	Platelets, T/B lymphocytes Granulocytes, Monocytes	WASP	6,7
XLA	Xq22	B lymphocytes	Btk	8,9
X-linked hyper IgM	Xq26	T-B lymphocyte interaction	CD40 ligand	10-14
XLP	Xp25	T/B lymphocytes	SAP/SH2D1A	15-17
XSCID	Xq13	T/B lymphocytes	IL-γ_c receptor	18,19

References: 1: Dinauer et al., 1987, 2: Smith et al., 1991, 3: Teahan et al., 1987, 4: Groundis et al., 1988, 5: Nolan et al., 1991, 6: Derry et al., 1994, 7: Nonoyama and Ochs, 1998, 8: Tsukada et al., 1993, 9: Vetrie et al., 1993, 10: Allen et al., 1993, 11: Korthauer et al., 1993, 12: DiSanto et al., 1993, 13: Aruffo et al., 1993, 14: Ramesh et al., 1993, 15: Skare et al., 1987, 16: Sayos et al., 1998, 17: Coffey et al., 1998, 18: Noguchi et al., 1993, 19: Sugamura et al., 1996.

Three out of 5 X-linked immunodeficiency genes that are involved in specific immunity, encode intracellular signaling molecules (XLA, XLP and WAS).
Clinical aspects of XLA

XLA has an incidence of ~5/10^6 (Sideras et al., 1995) and was the first humoral immunity disorder to be described. Bruton (1952) reported an 8-year-old boy, who had suffered from recurrent

bacterial infections since the age of $4^{1}/_{2}$ years and lacked detectable levels of serum immunoglobulins due to a general inability to synthesize these antibodies. He further noted that the condition of the patient improved dramatically upon administration of gammaglobulins, showing a relationship between control of infection and gammaglobulins.

Maternal antibodies levels, obtained by transplacental passage are protecting the patient against infections during the first months of life, but in the majority the onset of symptoms is within the first year of life (reviewed in Sideras *et al.*, 1995). Patients suffer from serious recurrent pyogenic infections, in most cases confined to upper and lower airways, but meningitis, osteomyelitis, arhtritis and gastro-intestinal manifestations have also been reported (Ochs *et al.*, 1980). Without treatment, patients die from these infections between 2 months and 5 years of age (reviewed in Sideras and Smith, 1995, Rosen *et al.*, 1997, Timmers *et al.*, 1991). Due to the therapy with antibiotics and gammaglobulins, the frequency of bacterial infection decreases, but there is only a weak prophylactic effect (Mease *et al.*, 1981, McKinney *et al.*, 1987, Hermaszewski *et al.*, 1993). With the current treatment, patients can reach adulthood. However, substituted immunoglobulins do not reach the mucous membranes and most patients still suffer from chronic infectious diseases, leading in many patients to pulmonary failure in their third decade.

Cellular aspects of XLA

XLA is generally regarded as a block in B cell development at the level of the pre-B cell. Serum Ig levels of all classes are very low or absent in XLA patients due to the lack of plasma cells in the secondary lymphoid organs. B cells are present in the periphery, although their numbers are severely reduced and they exhibit an immature IgM^{high} phenotype (Conley *et al.*, 1985). In the bone marrow, pre-B cells expressing cytoplasmic Cμ but no surface Ig are present at normal or slightly decreased numbers (Pearl *et al.*, 1978). Since hardly any surface Ig-positive B cells can be detected in XLA, the transition from the pre-B cell stage to later stages in B cell development is affected. However the developmental blockade at the pre-B cell stage is not absolute and there is phenotypic heterogeneity among patients (Wedgwood *et al.*, 1980, Krantman *et al.*, 1981, Landreth *et al.*, 1985, Leikley *et al.*, 1986, Campana *et al.*, 1990). To characterize the heterogeneity among patients and the relation to XLA severity, further analyses on BM samples of a larger group of patients with currently available tools for genetic analysis and recently found B cell markers is needed (Conley and Cooper, 1998).

The XLA defect is intrinsic to B cells as was demonstrated by the unilateral X chromosome inactivation in XLA affected B cells of carrier females (Conley *et al.*, 1986, 1988, Fearon *et al.*, 1987). In females, one of the two X chromosomes of somatic cells is randomly inactivated during early embryogenesis. The inactive state of the X chromosome is maintained in all progeny cells, leading to the inactivation of one X chromosome in one half of the cells while the other X chromosome is active in the other half of the cells. In female XLA carriers, the precursor B cells having the defective XLA gene on their active X chromosome, will be arrested in differentiation, as is found in XLA affected males. No developmental arrest occurs in those B cells, that have the intact gene on the active X-chromosome. As a result, XLA carriers manifest a B cell population with an

unilateral X chromosome inactivation pattern. Unilateral X chromosome inactivation was only demonstrated in the B cell population and not in other haematopoietic lineages such as T cells, monocytes and granulocytes. These findings showed that the XLA defect is intrinsic to and specific for the B cell lineage (Conley *et al.*, 1986, Fearon *et al.*, 1987). The X-chromosome inactivation patterns were used for genetic counseling to analyze XLA carrier status in females at risk (Fearon *et al.*, 1987, Conley *et al.*, 1988, Hendriks and Schuurman, 1991).

Genetics of XLA

The gene affected in XLA patients was suggested to be localized on the X chromosome by the predominant occurrence of the disease in males (Janeway *et al.*, 1953) and this narrowed the search for the gene to one chromosome. Using a series of DNA probes recognizing restriction fragment length polymorphisms (RFLPs) close genetic linkage was shown between the XLA gene and the X chromosome markers DXS17 (S21) and DXS3 (p119-2) at Xq22 (Kwan *et al.*, 1986, Mensink *et al.*, 1986, Malcolm *et al.*, 1987). The availability of flanking RFLP markers provided reliable methods for prenatal diagnosis and carrier detection in XLA pedigrees. No recombination of the XLA gene could be detected in over 100 meioses with DXS178 (p212) in the Xq21.3-Xq22 region, setting the order of loci in this region: centromer-DXS3-(XLA, DXS178)-DXS94-DXS17-telomer (Guioli *et al.*, 1989, Kwan *et al.*, 1990). The 7cM (~7Mb) of DNA, encompassing the region in which the XLA gene was located, was further narrowed by linkage analysis with two other polymorphic markers, DXS442 and DXS101 (Lovering *et al.*, 1993, Parolini *et al.*, 1993, de Weers *et al.*, 1992), reducing the XLA candidate region to 2 Mb of DNA.

Isolation of the gene affected in XLA

The gene defective in XLA was isolated independently by two groups, using different approaches (Vetrie *et al.*, 1993b, Tsukada *et al.*, 1993).

Vetrie and co-workers (1993b) used positional cloning to identify the gene. From a 5.2 Mb Yeast Artificial Chromosome (YAC) contig, a clone positive for DXS178 was chosen and used to enrich region-specific cDNA clones from libraries constructed from B cell lines. In parallel, cosmid clones that hybridized to the YAC were also isolated and used to construct a physical map of the YAC. One of the isolated cDNA clones detected DNA point mutations in two unrelated XLA patients, indicating that the gene from which they were derived was the gene affected in XLA (Vetrie *et al.*, 1993b). The cDNA clone was sequenced and was found to encode a cytoplasmic tyrosine kinase.

The same gene was isolated by Tsukada and co-workers (1993), who were primarily interested in isolating novel tyrosine kinases involved in B cell differentiation. Using the kinase domain of a previously isolated tyrosine kinase, Ltk, as a probe for a mouse B cell progenitor cDNA library, they isolated a gene of which the corresponding human cDNA mapped to the Xq22 region, by fluorescent *in situ* hybridization (FISH). These findings, combined with expression studies in cell lines derived from patients, suggested that this gene was very likely to be the XLA gene.

The X-linked immunodeficiency phenotype is caused by a mutation in the Btk gene

The CBA/N mouse strain, carrying an X-linked immunodeficiency (*xid*), is characterized by a wide array of immune defects (reviewed in Scher *et al.*, 1982). These mice have low levels of serum IgM and IgG3, whereas the levels of other isotype classes are normal (Perlmutter *et al.*, 1979, Slack *et al.*, 1980). Peripheral B cell numbers are moderately reduced and carry an immature phenotype (IgM^{high}/IgD^{low}, Hardy *et al.*, 1983), whereas the B-1 (CD5$^+$) B cells are absent. *Xid* mice do not respond *in* vivo to T cell independent class II (TI-II) antigens, typified by haptenated-Ficoll and other polysaccharide antigens, whereas T cell dependent (TD) responses are normal. Moreover, *xid* B lymphocytes responses to *in vitro* stimuli, such as Ig receptor crosslinking, IL-5, IL-10, CD38 and LPS are absent or impaired (Mond *et al.*, 1983, Go *et al.*, 1990, Hitoshi *et al.*, 1993, Santos-Argumedo *et al.*, 1995, Koike *et al.*, 1995). Like XLA, also *xid* results from an intrinsic B cell defect as was demonstrated by X-inactivation studies in female mice heterozygous for the *xid* mutation (Nahm *et al.*, 1983, Forrester *et al.*, 1987). The gene defective in *xid* was mapped to the mouse X chromosome, between the loci for phosphoglycerate kinase (*Pgk-1*) and the murine GLA gene (*Ags*) (Brown *et al.*, 1993). Comparative mapping studies of the mouse and human X chromosome have shown that the mouse X chromosome containing the *xid* locus is highly conserved and shares homology to the human Xq21.3-Xq22 region, containing the XLA locus. The finding that *xid* and XLA map to the same conserved region of the mouse and human X chromosome, respectively, and the fact that both disorders are only affecting B cells, suggested that also *xid* phenotype could be caused by a mutation in the *Btk* gene. First, it was demonstrated that the Btk gene mapped to the *xid* locus (Thomas *et al.*, 1993, Hendriks *et al.*, 1994) and direct sequencing analysis demonstrated that the *Btk* gene in xid mice carried a mutation converting the arginine residue at position 28 into a cysteine (R28C) (Thomas *et al.*, 1993, Rawlings *et al.*, 1993). Initially, the mild phenotype of the *xid* mice, compared to the human XLA phenotype, was explained by the fact that the R28C mutation was located in a non-catalytic domain of Btk that mediates protein-protein interaction and therefore had only moderate effects on Btk function. Predictions were made that the same mutations in the human *Btk* gene would also result in a mild phenotype (Vorechovsky *et al.*, 1993). However, the identification of a mutation in the human *Btk*, converting Arg$_{28}$ to a histidine and thereby affecting the same codon as is altered in *xid* did not result in a mild phenotype of the patients in one XLA pedigree (De Weers *et al.*, 1994a). This finding suggested that mice do not develop a phenotype as severe as the human XLA, and this was confirmed by gene targeting experiments, resulting in a disrupted Btk kinase domain (Kahn *et al.*, 1995, Chapter 2). In table 2 the features of XLA and *xid* are compared.

Table 2. Comparison of the phenotypes of human XLA and murine *xid*

Studied phenotype	XLA	*xid*
Early pre-B cells in bone marrow	Normal	Normal
Peripheral B lymphocytes	Absent or low (residual cells have IgM^{high} phenotype)	Reduced and show 'immature' IgM^{high} phenotype CD5 positive subset absent
Rearrangement of Ig genes	Normal	Normal
Immunoglobulin levels	All isotypes absent or low	IgM and IgG3 levels reduced No TI-II response
Phenotype of heterozygous female carriers	Normal	Normal
X-inactivation pattern in female carriers	Non-random inactivation in B cells	Non-random inactivation in B cells
Other haematopoietic lineages	Unaffected, random X-inactivation pattern	Unaffected, random X-inactivation pattern

Genomic organization and expression of mouse and human Btk gene

The genomic organization of both murine and human *Btk* were determined using mouse 129SV genomic lambda, P1 phage clones and genomic cosmid clones (Sideras *et al.*, 1994, Hagmann *et al.*, 1993, Ohta *et al.*, 1994, Rawlings *et al.*, 1993). In addition, 93 kb of the human region and 89 kb of the murine region, containing *Btk* and adjacent genes coding for several other proteins, such as α-galactosidase (GLA), the ribosomal protein L44L, the RNA-binding protein FTP-3 and the deafness/dystonia peptide (DDP) have been completely sequenced (Oeltjen *et al.* 1997, GenBank accession no. U78027 and U58105, respectively). A physical map of the murine *Btk* loci is depicted in Fig. 4. The coding regions were divided into 19 exons, spanning approximately 43.5 kb in the mouse and 37.5 kb in humans. Whereas the 5'-UTR is encoded by the first exon and part of the second exon, exon 19 encodes 23 carboxyl terminal amino acids as well as the 3'-untranslated region (3'-UTR). Immediately upstream of the non-coding exon 1, multiple putative transcription start sites have been mapped. Sequencing of the human and murine *Btk* promoter region 5' of exon 1 revealed that there were no obvious TATA or CAAT like motifs present, consistent with the presence of multiple transcription start sites, which is seen more often in TATA-less promoters (Geng *et al.*, 1993, Travis *et al.*, 1991, Matsuzawa *et al.*, 1991).

Btk expression was investigated in human, using cultured cell lines, leukemias and peripheral blood samples (de Weers *et al.,* 1993, Genevier *et al.,* 1994, Smith *et al.,* 1994a, Futatanani *et al.,* 1998). In the mouse, *Btk* expression was analyzed in cultured cell lines, as well as *in vivo*, by flow

cytometric analysis of LacZ activity in a mouse with a targeted, in frame, insertion of a *LacZ* cassette in the *Btk* gene (Chapter 2) and intracellular flow cytometry (Chapter 4). *Btk* is expressed throughout B cell development, from the earliest pro-B cell stage up to mature B cells, except in plasma cells and not in T cells. Btk is also expressed in cells of the myeloid lineage, but is not required for myeloid differentiation, since cells from the myeloid lineage are not affected in XLA nor in *xid* (Sideras and Smith, 1995).

Not much is known about the DNA elements that regulate the *Btk* expression. The tissue-specific and differentiation stage-specific expression may, at least in part, be accomplished by ~450bp of the *Btk* promoter region, which contains several binding sites for the transcription factors Sp1, Sp3 and PU.1 (Himmelman *et al.*, 1996, Müller *et al.*, 1996). Although transient transfection experiments implicate PU.1 as a major regulator, *Btk* expression is not abolished in fetal liver of PU.1-deficient mice (Müller *et al.*, 1996). This implies that other elements may also be required in the absence of PU.1. Such elements, needed for appropriate *Btk* expression might be located further upstream or downstream of the promoter region. To date, the reported transfection studies have only focused on the ~400 bp promoter region and do not address the question of the importance of the *in vivo* chromatin structure for *Btk* gene expression. Recent large-scale sequencing studies which compared 93 kb of the human sequence around *Btk* to 89 kb of the murine sequence in the same region (Oeltjen *et al.*, 1997) revealed highly conserved intronic regions. The importance of the conserved regions was highlighted by an XLA patient with a mutation in a conserved region in intron one, containing an Sp1 binding site (Rohrer and Conley, 1998).
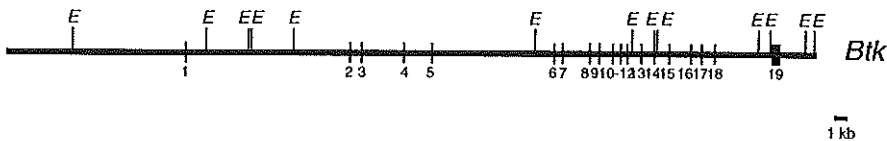
Figure 4. *Scheme of the genomic organization of the murine Btk gene. Exons are represented by filled boxes and numbered 1 to 19, EcoRI restriction sites are indicated as E.*

Btk protein

Both murine and human Btk consist of 659 amino acid, showing 99.3% homology, and have a molecular weight of 77 kDa. Btk is a cytoplasmic Src-like protein tyrosine kinase (PTK) belonging to the Tec family of related tyrosine kinases, which is characterized by the absence of a N-terminal myristylation site and a C-terminal negative regulatory tyrosine residue and the presence of pleckstrin homology (PH), Tec homology (TH), src-homology (SH)3, SH2 and SH1 domains (Rawlings *et al.*, 1994, Smith *et al.*, 1994b). In addition to Btk and Tec (Mano *et al.*, 1990, 1993) other members of the Tec family are the T cell homologue Itk (Siliciano *et al.*, 1992) and Bmx

(Tamagnone *et al.*, 1994). The linear structure of Btk is depicted in Fig. 5 and shows the different domains.

The kinase domain (SH1), located at the C terminal region, consists of about 250 amino acids and shares homology to the Src family of protein kinases. The SH1 region contains 9 invariant and 15 highly conserved residues, involved in ATP binding and catalysis (Hanks *et al.*, 1988, Vihinen *et al.*, 1994). To date, the only substrate associated with Btk *in vivo*, is the DNA-binding protein BAP-135 or TFII-I (Yang *et al.*, 1997, Roy *et al.*, 1997, Grueneberg *et al.*, 1997). PLC-γ is likely to be another substrate of Btk. BCR-induced tyrosine phosphorylation of PLC-γ is compromised in Btk-deficient DT40 cells (Takata *et al.*, 1996), and in *xid* mice, the generation of inositol 1,4,5-triphosphate by PLC-γ is decreased upon BCR crosslinking (Rigley *et al.*, 1989), indicating a role for Btk in the pathway emanating from the BCR and proceeding downstream through PLC-γ. Furthermore, *in vitro* overexpression experiments show that participation in PLC-γ activation is a general property of Tec kinases (Fluckinger *et al.*, 1998, Scharenberg *et al.*, 1998).

Another region that shares homology to the Src-family is the SH2 domain, located at the N-terminus of the SH1 domain. In Btk the SH2 domain includes about 100 amino acids. This domain is found in a large number of signaling molecules and is involved in the specific interaction with phosphorylated tyrosine residues of other signaling molecules (Songyang *et al.*, 1993). SH2 domains have been shown to play a role in modulating enzymatic activity and in the recruitment by tyrosine phosphorylated signaling molecules (Cantley *et al.*, 1991, Walksman *et al.*, 1992).

Next to the SH2 domain, the SH3 domain is located. It consists of about 60 amino acids. SH3 domains were shown to bind to proline and hydrophobic stretches in other proteins (Ren *et al.*, 1993, Pleiman *et al.*, 1994a) and is like SH2 domains involved in locating signaling molecules to certain areas in the cell. Association of the Btk-SH3 domain with signaling molecules, such as the Src-like kinase Lyn (Cheng *et al.*, 1994), the proto-oncogene p120[cbl] (Cory *et al.*, 1995) and WASP (Yang *et al.*, 1995), the protein defective in the Wiskot-Aldrich syndrome and involved in cytoskeletal organization, have been demonstrated *in vitro*, although the physiological significance of these interactions in unknown.

The N-terminal region contains a 100 amino acid long PH domain. Originally, the motif was identified in the protein kinase C substrate Pleckstrin. PH domains recruit signaling molecules to the cell surface through specific interactions with phospholipids and proteins important for the activation of these signaling molecules (reviewed in Lemmon *et al.*, 1996). Direct interactions between the PH domain of Btk and the βγ subunits of G proteins and the α-subunit of the G_q class of G proteins have been demonstrated (Touhara *et al.*, 1994, Tsukada *et al.*, 1994, Bence *et al.*, 1997), but the functional relevance in the Btk signaling pathway still has to be proven. Physical association of the PH domain of Btk with the calcium dependent protein kinase C (PKC) isoforms: α, βI and βII, as well as with the calcium independent isoforms ε and ξ, have also been shown *in vitro*, whereas PKC-βI showed also *in vivo* association with Btk (Yao *et al.*, 1994). Recently, membrane association of Btk by binding of the PH domain to inositol phosphates was shown (Salim *et al.*, 1996, Rameh *et al.*, 1997). Moreover, Btk membrane association depends directly on $PI(3,4,5)P_3$ levels (Bolland *et al.*, 1998, Scharenberg *et al.*, 1998, see below). The R28C mutation in *xid* mice

was the first genetic evidence for the functional importance of this domain (Thomas *et al.*, 1993, Rawlings *et al.*, 1993). The importance of the PH domain was also demonstrated by the E41K Btk gain-of-function mutant, isolated in a random mutagenesis scheme (Li *et al.*, 1995), which showed an increased membrane localization and tyrosine phosphorylation when transfected into 3T3 fibroblasts. The E41K Btk mutant induced growth of 3T3 fibroblast in soft agar cultures and relieved the IL-5 dependence of pre-B cell line Y16.

Between the PH domain and the SH3 domain a stretch of about 70 amino acids is present and is called the TH domain as the domain was first identified in the Tec protein (Smith *et al.*, 1994b). So-far, this TH motif has only been identified in proteins belonging to the Tec family. The TH domain is divided into two subdomains, the Btk motif and a proline stretch. The Btk motif consist of a stretch of 27 conserved amino acids of which 7 are invariant. The function of this region is not yet clear. Next to the Btk motif a prolin stretch is present, which may serve as a binding site for SH3 containing tyrosine kinases, like Fyn, Lyn and Hck (Cheng *et al.*, 1994, Yang *et al.*, 1995). Interestingly, the SH3 domain of Btk itself may bind to this prolin-rich region and in this way have a regulatory function (Smith *et al.*, 1994b).

Finally, adjacent to the PH domain the presence of a Zn-finger motif has been detected (Hyvonen *et al.*, 1997). The function of this motif in Btk is unknown. However, its importance for Btk function was demonstrated by a mutation in this motif resulting in XLA.

The putative binding proteins to the individual Btk domains are summarized in Table 3.

Table 3. putative binding partners for Btk and its domains

Btk domain	Binding proteins	References
PH	PKC-α,βI,βII,ε,ζ isoforms	Yao *et al.*, 1994
	G protein βγ-subunits	Touhara *et al.*, 1994, Tsukada *et al.*, 1994
	G_q protein α-subunit	Bence *et al.*, 1997
	nositol phosphates	Salim *et al.*, 1996, Rameh *et al.*, 1997
SH1	BAP-135/TFII-I	Yang *et al.*, 1997, Roy *et al.*, 1997, Grueneberg *et al.*, 1997
SH2	pTyr proteins	Cantley *et al.*, 1991, Walksman *et al.*, 1992
SH3	Lyn	Cheng *et al.*, 1994
	p120[cbl]	Cory *et al.*, 1995
	WASP	Yang *et al.*, 1995
TH	Fyn, Lyn, Hck	Cheng *et al.*, 1994, Yang *et al.*, 1995

To date over four hundred mutations, including deletions, insertions, missense and non-sense, have been characterized in XLA patients distributed throughout the *Btk* coding region (Vihinen *et al.*, 1998, http://www.helsinki.fi/science/signal/btkbase.html). These studies showed that approximately one third of the human *Btk* mutations cause single amino-acid substitutions (Vihinen *et al.*, 1997, 1998, Saha *et al.*, 1997, Vorechovski *et al.*, 1997, Haire *et al.*, 1997, Brooijmans *et al.*, 1997, de Weers *et al.*, 1997, Gaspar *et al.*, 1998, Futatani *et al.*, 1998, Holinski-Feder *et al.*, 1998). The amino-acid substitutions tend to cluster in the kinase domain, although they have been seen in

all other domains, except the SH3 domain. Remarkably, amino-acid substitutions often lead to the absence of Btk (Futatani *et al.*, 1998, Gaspar *et al.*, 1998). There is no clear correlation between *Btk* genotype and the severity of the phenotype of XLA patients.
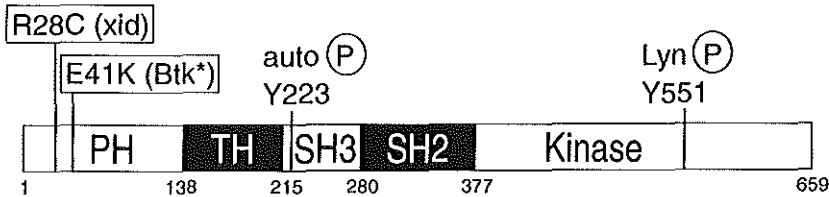

Figure 5. *Linear structure of the Btk protein. Shown are the different domains. The positions of the E41K mutation and the R28C xid mutation are indicated. Numbers represent the amino acid positions. The positions of tyrosine residues Y223 and Y551, involved in Btk activation are shown.*

Regulation of Btk activation

Btk activation is associated with an increased tyrosine phosphorylation and an increase in catalytic activity. Stimulation of the BCR induces tyrosine phosphorylation of Btk *in vivo*, both in human B cell lines and tonsilar lymphocytes (De Weers *et al.*, 1994b, Hinshelwood *et al.*, 1995) as well as in murine B cell lines (Saouf *et al.*, 1994, Aoki *et al.*, 1994) and is accompanied by an increase in its kinase activity *in vitro*. Btk has also been shown to be phosphorylated and activated upon stimulation of receptors for IL-5 (Sato *et al.*, 1994), IL-6 (Matsuda *et al.*, 1995) in B cells and FcεRI in mast cells (Kawakami *et al.*, 1994). Kinetic analyses of protein kinase activation in a murine cell line revealed that Src-like tyrosine kinases were activated immediately after BCR engagement, followed by the activation of Btk within minutes (Saouaf *et al.*, 1994). Two tyrosine residues are important for activation of Btk. One tyrosine residue is located at amino acid position 551 (Y551) in the catalytic domain and the second one, an autophosphorylation site, in the SH3 domain at amino acid position 223 (Y223) (Fig. 5.). The role of the two tyrosine residues in Btk phosphorylation following stimulation of BCR, IL-5-receptor and FcεRI were investigated using antibodies, specific for phosphorylated Y551 and Y223 (Wahl *et al.*, 1997). Following receptor stimulation, Y551 was rapidly phosphorylated, accompanied by autophosphorylation of Y223 and a subsequent dephosphorylation of Y551 followed by dephosphorylation of Y223. Phosphorylation of the Btk SH3 domain may play a role in the negative regulation of Btk activation, as was indicated by the finding that additional Y223 to phelylalanine mutation or deletion of the SH3 domain further activated the transforming activity of the E41K Btk mutant (Park *et al.*, 1996). Src-like tyrosine kinases play important roles in phosphorylating Y551 of Btk as was shown in Btk-Lyn/Fyn co-transfection experiments (Rawlings *et al.*, 1996, Afar *et al.*, 1996, Wahl *et al.*, 1997). Btk

phosphorylation was augmented by increased dosage of Lyn or Fyn, whereas co-expression of Btk with a kinase-inactive Lyn (K275G) did not result in an increase in Btk tyrosine phophorylation, placing Btk downstream of Lyn and Fyn activation. In contrast, co-expression of Btk with Syk failed to increase Btk phosphorylation and activation (Scharenberg *et al.*, 1995).

Co-expression of Btk and PI3-K, which generates the membrane binding site PI(3,4,5)IP$_3$, also result in enhanced Btk phosphorylation (Li *et al.*, 1997), suggesting that membrane targeting of Btk mediated by the PH domain, places Btk in proximity to Lyn, which like other Src-family members can bind via their SH3 domain to the Btk proline stretch (Cheng *et al.*, 1994, Yang *et al.*, 1995), and thereby promoting Lyn-dependent phosphorylation. The importance of this interaction was demonstrated by the impairment of tyrosine phosphorylation of a kinase-inactive Btk with a point mutation in the TH domain that abolished binding of SH3 containing proteins (Yang *et al.*, 1995). Recruitment to the membrane seems to be important for Btk activation as is demonstrated by R28C (*xid*) and E41K alterations of the PH domain leading to loss of function and activation of Btk, respectively (Salim *et al.*, 1996, Ferguson *et al.*, 1995). As the Btk PH domain can also bind inositol phosphates, βγ subunits of G proteins and PKC isoforms, these interactions and those directed by other Btk domains may allow Btk to integrate membrane-targeting signals with those generated by Src-kinases-linked receptor activation. Membrane association and phophorylation of Y551 by Lyn appears to activate Btk by unblocking its catalytic site. Autophosphorylation of Y223 in the SH3 domain may be an early target of the catalytic domain and may lead via further conformational changes of Btk to the creation of binding sites for other signaling proteins known to interact with Btk. Similarly, phosphatases and phosphokinases, influencing Btk phosphorylation and activation may regulate interactions of Btk with other proteins. The phosphatase SHIP has been shown to reduce the levels of P(3,4,5)IP$_3$ and thereby decrease the membrane association of Btk (Bolland *et al.*, 1998, Scharenberg *et al.*, 1998), which is therefore the first pathway identified that, together with PI-3K, can specifically regulate Btk activity (Fig. 6).

Signaling

The B cell receptor complex (BCR)

The BCR belongs to a class of receptors which includes the TCR as well as receptors for the Fc regions. Characteristic for these receptors is that they are multicomponent and that ligand binding and signal transduction are compartmentalized into distinct receptor units (Kurosaki *et al.*, 1997). In the BCR, the ligand binding unit is the membrane Ig. All Ig classes can be expressed on the membrane or be secreted, depending on differential mRNA splicing (Wall *et al.*, 1981). Membrane Ig has only a short cytoplasmic part, and upon Ig crosslinking, intracellular signals are transduced by non-covalently associated, disulfide liked heterodimers Ig-α and Ig-β (CD79α and CD79β respectively, Hombach *et al.*, 1988). Ig-α (34 kDa) is encoded by the *mb-1* gene and Ig-β (38 kDa) by the *B29* gene (Hombach *et al.*, 1990, Campbell *et al.*, 1991). The *B29* gene is expressed throughout the B cell development whereas the *mb-1* gene expression is turned off in plasma cells

(Fig. 3, Sakaguchi *et al.*, 1988, Hermanson *et al.*, 1988). Both Ig-α and Ig-β have one Ig-like extracellular domain, a 22 amino acid transmembrane part and a cytoplasmic domain of respectively 61 and 48 amino acids. The cytoplasmic domain contains a sequence motif of approximately 26 amino acids, which is named immunoreceptor tyrosine based activation motif (ITAM) is characterized by six conserved amino acids in the sequence D/Ex$_7$D/ExxYxxL/Ix$_7$YxxL/I (in single-letter amino acid code, where x represents any amino acid, Reth, 1989, Cambier, 1995). When a μ mutant, unable to associate with Ig-α and Ig-β was introduced into RAG$^{-/-}$ mice, failures in signaling for allelic exclusion and a developmental blockade at the transition from pro- to pre-B cells was observed. The phenotype could be rescued by introducing chimeric molecules, consisting a fusion of the mutant μ to the cytoplasmic domain of Ig-β and Ig-α (Papavasiliou *et al.*, 1995a, 1995b). However, in mice deficient for Ig-β, while still expressing Ig-α, a complete impairment in B cell development was observed before the V$_H$ to DJ$_H$ occurred (Fig. 3, Gong and Nussenzweig 1996). In Ig-α truncated mice, lacking the ITAM, pre-B cells were present, but diminished by a factor of 2 to 4, whereas the number of peripheral B cells was decreased 10-fold (Fig. 3, Torres *et al.*, 1996). These data suggest that, at least parts of, both Ig-α and Ig-β are required for the formation of an Ig-α/Ig-β heterodimeric complex, needed for signaling and/or membrane expression of the (pre-) BCR. It would therefore be interesting to see, at which stage B cell development is blocked in mice that completely lack Ig-α.

The pre-BCR and BCR have critical roles in B cell development. Expression of a pre-BCR in early B cells are needed for maturational progression as has been demonstrated in mice models unable to express a pre-BCR (see Fig. 3) As this progress is thought to occur in the absence of foreign antigen, it is still not clear whether it is an (endogenous) ligand or expression of the pre-BCR by itself that stimulates progression to the more mature phases of B cell development. Like pre-BCR expression is needed for early B cell development, Lam and co-workers (1997) have demonstrated that expression of a BCR is critical for later stages of B cell development. Using Cre-LoxP mediated interferon inducible V$_H$ gene disruption, they created a mouse model in which the effect of the BCR could be investigated without disturbing pre-BCR mediated B cell development. Loss of the V$_H$ gene leads to a decrease of BCR expression and apoptotic death. This presumably means that the B cell must somehow continuously receive signals through the BCR in order to stay alive. Since not all living B cells are activated, the BCR must generate multiple signals or vary its signal strength in order to make a distinction between B cell maintenance and activation.

BCR triggered signal transduction pathways

Signals from the (pre-)BCR and co-receptors such as CD19, CD22, CD45 and FcγIIB (see below) are integrated inside the cell allowing the B cell to react appropriately to a variety of stimuli. The biochemical events triggered by BCR and co-receptor engagement include the recruitment and phosphorylation of proteins and the subsequent activation of downstream signaling cascades. The most rapid event upon BCR crosslinking is the increased tyrosine phosphorylation and activation of Src-family tyrosine kinases (Src-PTKs), including Lyn, Blk, Fyn, Lck and Fgr (Burkhardt *et al.*,

1991, Yamanashi *et al.*, 1992, Wechsler *et al.*, 1995). *In vitro* binding studies have shown that Lyn and Fyn are already associated with the amino-terminal residues of the Ig-α chain from the non-stimulated BCR (Pleiman *et al.*, 1994). The activity of Lyn is regulated by the tyrosine kinase Csk and the transmembrane phosphatase CD45, which can phosphorylate and dephosphorylate the carboxyl-terminal tyrosine residue of Lyn, respectively (Yanagi *et al.*, 1996, Hata *et al.*, 1994). The SH2 domain of Lyn is thought to bind to its own phosphorylated tyrosine, Y527, leading to repression of kinase activity (Cooper *et al.*, 1993). The mechanism for initial Src-PTK activation is still not clear, but it is very likely that intermolecular phosphorylation of the autophosphorylation site in the catalytic domain is involved. Src-PTKs members are able to phosphorylate the two tyrosine residues in the ITAM domains of Ig-α and Ig-β. The phosphorylated ITAMs provide a SH2 binding site for Syk, but also for the Src-PTKs themselves. After binding the activity of Syk is greatly enhanced by phosphorylation. In addition Btk is phosphorylated by Lyn (Rawlings *et al.*, 1996). Src-PTKs, Syk and Btk are likely to contribute to the activation a variety of downstream signaling pathways.

The three most studied pathways stimulated by PTK activation in BCR signaling are the phospho-inositide hydrolysis, the Ras and the PI3-K pathway (Fig. 6).

The phospho-inositide hydrolysis pathway (Fig. 6: A) was the first signaling cascade shown to be activated after BCR cross-linking. Syk, and probably Btk, are required for the activation phopholipase (PL)-C-γ (Takata *et al.*, 1994, 1996), which is involved the hydrolyses of membrane phosphatidylinositol 4,5-biphosphate (PI4,5P$_2$) to form the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$) (Bijsterbosch *et al.*, 1985). DAG is known to activate most of the protein kinase C (PKC) family. Substrates for PKC are the cAMP response element binding protein (CREB) transcription factor (Xie *et al.*, 1995, 1996) and the G protein Ras, thereby activating the mitogen activated protein (MAP) kinase pathway (Gold *et al.*, 1992). An isoform of PKC, PKCμ, is shown to be able to phosphorylate Syk *in vitro* and thereby decreasing its activation, suggesting a negative feedback system (Siderenko *et al.*, 1996). IP$_3$ plays a role in the release of calcium from intracellular stores, which on its turn lead to the activation of enzymes such as calcium-dependent protein kinase II and calcineurin, a protein serine/threonine phosphatase.

A second intracellular signaling pathway activated upon BCR ligation involves Ras (Fig 6: B, Lazarus *et al.* 1993, Harwood *et al.*, 1993), which can also be activated in the absence of PKC activation (Richards *et al.*, 1996). It is currently thought that stimulation of the BCR leads to Syk mediated phosphorylation of Shc (Nagai *et al.*, 1995) and assembly at the membrane of a complex containing one Shc, two Grb2 and one mSOS molecule (Ravichadran *et al.*, 1995, Harmer *et al.*, 1997). The recruitment of the nucleotide exchange factor mSOS to the membrane is sufficient to activate Ras as was shown in experiments using an membrane localized mSOS (Holsinger *et al.*, 1995). The activated form of the G-protein Ras, Ras-GTP, on its turn can activate the classical MAP kinase pathway. The pathway appears to be important for activating transcription via enhancer serum response elements (SRE) (McMahon *et al.*, 1995) found in the genes *c-fos* and *egr-1*, both coding for transcription factors.

BCR stimulation induces a third signaling pathway, the recruitment and activation of phosphatidylinositol 3 kinase (PI3-K, Fig 6: C),). With a proline-rich region PI3-K can bind to the SH3 domains of Lyn and Fyn. (Pleiman *et al.*, 1994a). Little is known about the function of PI3-K. One of the products, PI3,4,5P$_3$, generated by the phosphorylation of PI4,5P$_2$, is a membrane binding site for Btk (Salim *et al.*, 1996, Bolland *et al.*, 1998) and downstream targets for PI3-K products may be Ser/Thr kinases, such as Akt and PKC-ξ (Franke *et al.*, 1995).

Recent experiments in the chicken DT40 lymphoma cell line showed that Btk transduces signals in the pathways leading to activation of ERK and JNK1, but not in the pathway leading to p38 MAP kinase activation (Fig. 6, Jiang *et al.*, 1998).
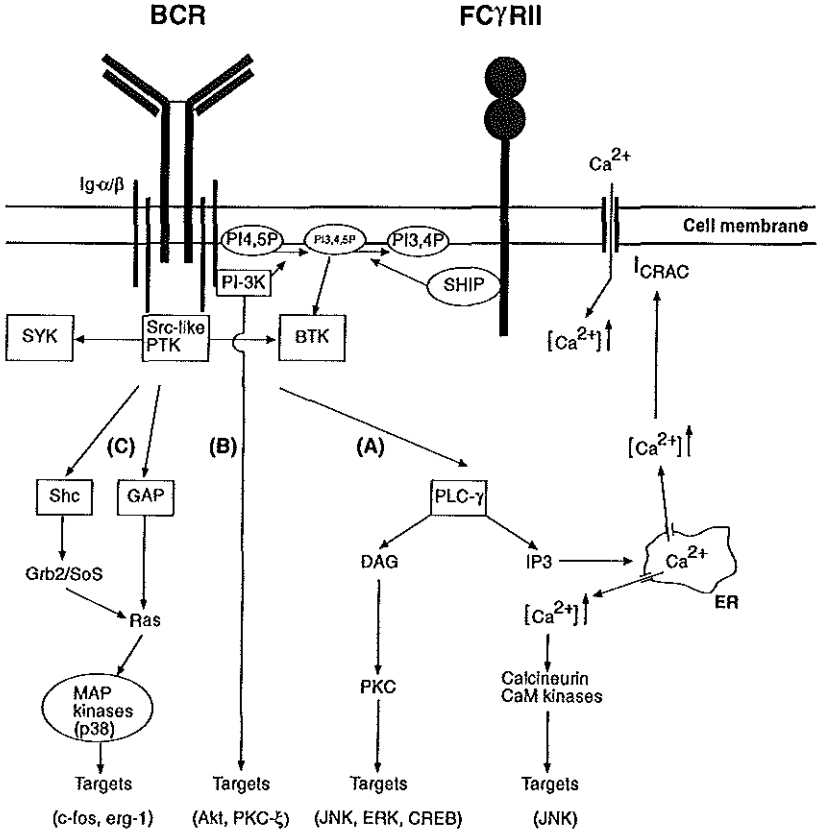
Figure 6. *Signaling pathways activated by the BCR. Boxes indicate the major signaling components that become tyrosine phosphorylated upon BCR engagement. Arrows represent either the phosphorylation of the signaling components by BCR-activated kinases (Src-like PTKs, Syk or Btk) or the connection between these signaling components and downstream signaling events by protein-protein interactions, phosphorylation or the action of second messengers.*

Co-receptors involved in BCR signaling

   The activation of downstream signaling pathways depends on the co-crosslinking of the BCR with other transmembrane proteins. The BCR response may be enhanced or inhibited through the recruitment of SH2-containing signaling proteins by co-receptors, such as CD19, CD22 and FcγIIB, upon activation by a variety of ligands.

   CD19 is a B cell specific 95 kDa glycoprotein with two extracellular Ig-like domains, a transmembrane segment and a large cytoplasmic tail, containing nine conserved tyrosine residues (Zhou *et al.* 1991, Tedder *et al.*, 1989, 1997). Expression of CD19 is regulated by the *pax-5* gene encoded B-cell-specific transcription factor (BSAP, Kozmik *et al.*, 1992) and is found from early pre-B cells until plasma cell differentiation (Zhou *et al.* 1991, Tedder *et al.*, 1989). CD19 forms a complex together with CD21 (receptor for complement factors iC3b, C3d,g and C3d) and other membrane proteins as CD81 and Leu-13. Signaling through the CD19 complex reduces the BCR signaling threshold as was demonstrated in CD19-deficient and CD19-overexpressing mice (Zhou *et al.*, 1994, Engel *et al.*, 1995, Sato *et al.*, 1997). CD19-deficient mice show an unperturbed conventional B cell development, but lack GC formation causing impaired antibody responses, in particularly to T cell dependent antigens (Engel *et al.*, 1995, Rickert *et al.*, 1995). In contrast, numbers of the self-renewing CD5$^+$ B-1 B cells in the peritoneum are severely decreased, suggesting that CD19 plays an important role in signaling needed for the optimal proliferation and maintenance of this population of B cells. Overexpressing of CD19 in transgenic mice lead to a dose dependent blockade at the immature B cell stage in the bone marrow. BCR stimulation results in tyrosine phosphorylation of a small number of BCR-associated CD19 molecules leading to the recruitment of PI-3K by its SH2 domain, and subsequent activation. PI3-K activity is also stimulated by the binding via its SH3 domain to the BCR associated Src-PTKs (Pleiman *et al.*, 1994a). When complement factor C3d, a ligand for CD22, is bound to the BCR antigen and thereby stimulating the co-ligation of the BCR with the CD19 complex, CD19 phosphorylation is enhanced, leading to elevated recruitment and activation of PI-3K (Fearon *et al.*, 1995). So, probably, BCR-CD19 co-ligation results in enhanced recruitment of PI3-K by CD19 and the simultaneously binding of PI3-K to Src-PTK and CD19 will greatly increase its activity.

   FcγIIB receptor stimulation is involved in inhibiting BCR signaling. In the cytoplasmic tail of the FcγIIB receptor a 13 amino acid tyrosine phosphorylated tyrosine immunoreceptor tyrosine inhibitory motif (ITIM) is present, which is probably involved in binding the SH2 domain of the phosphotyrosine phosphatase SHP-1 and subsequent activation. Co-crosslinking of the FcγIIB receptor to the BCR does not have any affect on the overall profile and time course of tyrosine phosphorylation induced by BCR stimulation, except the phosphorylation PLC-γ2, which appears to be only transient upon BCR-FcγIIB co-ligation (Sarkar *et al.*, 1996). The significance of the FcγIIB stimulated inhibitory pathway was demonstrated in moth-eaten mice, which due to a defective SHP-1 (D'Ambrosio *et al.*, 1995), showed an impaired B cell proliferation inhibition. FcγIIB-deficient mice (Takai *et al.*, 1996), displayed a slightly elevated Ig response to both thymus-dependent (TD) and thymus-independent (TI) antigens. In addition, Bolland and co-workers (1998), showed the

inhibitory effect of another SH2 containing inositol polyphosphate phosphatase, named SHIP, that is recruited and activated upon FCγIIB-BCR co-ligation (Fig. 6.). SHIP is able to dephosphorylate the membrane phospholipid PI(3,4,5)P$_3$, creating PI(3,4)P$_2$ and the soluble I(1,3,4,5)P$_4$ to I(1,3,4)P$_3$ (Damen et al., 1996, Lioubin et al., 1996). In their experiments, using wildtype chicken DT40 cells, they showed that reducing PI(3,4,5)P$_3$ levels by SHIP resulted in a decrease of extracellular calcium influx. In SHIP-deficient cells, however, a hyperresponsiveness to BCR stimulation was observed due to increased levels of PI(3,4,5)P$_3$ suggesting an (indirect) role of PI(3,4,5)P$_3$ or associated proteins in opening the calcium channels in the membrane.

A negative role in BCR signaling has been reported for CD22 upon co-cross-linking (O'keefe et al., 1996, Sato et al., 1996, Otipody et al., 1996). CD22 belongs to the Ig superfamily and expression in the mouse is found on all subsets of mature B cells (Erickson et al., 1996). Murine CD22 recognizes α2,6-sialylated carbohydrate chains, which is highly expressed on cells in secondary lymphoid organs (Van der Merwe et al., 1996). CD22 associates with the BCR and tyrosine residues in its cytoplasmic domain are rapidly phosphorylated upon BCR or CD22 cross-linking (Schulte et al., 1992, Peaker et al., 1993, Leprince et al., 1993). Phosphorylation of tyrosine residues in three ITIM motifs are involved in recruiting and activating SHP1 (Doody et al., 1995, Campbell et al., 1995).The cytoplasmic domain also contains two ITAM motifs, by which it can recruit the signaling protein Lyn, Syk, PI3-K and PLC-γ1 (Tuscano et al., 1996a, 1996b, Law et al., 1996), which suggest also a positive role for CD22 in BCR signaling.. It has been shown that ligation of CD22 alone can induce B cell proliferation (Tuscano et al., 1996a). Whether CD22 is a positive or negative regulator might be determined by its association with the BCR. In non-lymphoid microenvironment the level of α2,6-sialylated carbohydrate is low, favoring CD22-BCR association through α2,6-sialylated sugars expressed on IgM and thereby inhibiting BCR signaling. In secondary lymphoid tissues however, high levels of α2,6-sialylated sugars are expressed, leading to dissociation of the BCR associated CD22 and permitting enhanced signaling.

Downstream targets for Btk

BCR engagement leads to the activation of Src-like protein kinases and the subsequent phosphorylation of Syk and Btk followed by the activation of Ras- and PLC-dependent phospholipid hydrolysis signaling pathways. In Btk deficient chicken DT40 cells, following BCR engagement, PLC-γ2 phosphorylation was reduced, accompanied by a decrease in I(3,4,5)P$_3$ generation and subsequent Ca^{2+} release (Takata et al., 1996). In contrast, the Ras pathway, which can be activated by DAG, a product of PLC-γ activation, was unperturbed in Btk deficient DT40 cells (Takata et al., 1996). Syk is also essential for the PLC and Ras activated signaling pathways (Nagai et al., 1995, Takata et al., 1994). However, Syk activation was not impaired in Btk deficient DT40 cells, but disruption of Syk expression also lead to loss of calcium signaling (Takata et al., 1994), suggesting that Syk and Btk have distinct non-redundant functions in Ca^{2+} signaling. Moreover, overexpression of wildtype or E41K mutated Btk in Ramos B cells lead to an enhanced sustained extracellular calcium influx, whereas it had only a limited effect on the initial calcium release. In contrast,

overexpression of wildtype Syk had no effect on the sustained calcium influx, while overexpression of dominant-negative Syk, dramatically inhibited the initiation of calcium signaling (Fluckinger *et al.*, 1998). As Btk activation is preceded by Syk activation upon BCR engagement, the increase in $I(3,4,5)P_3$ levels by Btk above those generated by Syk, may lead to sustained increases in $[Ca^{2+}]$ through the opening of biochemically differently operating calcium channels in the cell membrane and the inhibition of calcium re-uptake by the intracellular stores (Fluckinger *et al.*, 1998). This is consistent with the finding that opening of calcium release-activated calcium (CRAC) channel occurs only upon generation of high levels of $I(3,4,5)P_3$ (Parekh *et al.*, 1997).

Another downstream target for Btk is transcription factor BAP-135/TFII-I, which has recently been found to associate *in vivo* with Btk and is transiently phosphorylated in response to BCR crosslinking (Yang and Desiderio, 1997). However, the localization in the cell of these interactions remain unclear, since there are no studies yet, reporting a nuclear localization for Btk, although the presence of a nuclear localization signal in Btk has been reported (Vetrie *et al.*, 1993b).

Aim of the thesis

X-linked agammaglobulinemia (XLA) is one of the most frequent inherited immunodeficiency diseases in man and characterized by an almost complete arrest of B cell differentiation at the pre-B cell stage. The gene defective in XLA has been identified to code for a cytoplasmic protein tyrosine kinase, Bruton's tyrosine kinase (Btk). The natural occurring R28C mutation in the *Btk* gene of CBA/N mice results in a relative mild B cell disorder. The *Btk* gene is expressed throughout B cell differentiation, from the earliest stage identified, the pro-B cells up to mature B cells, except in plasma cells. To date, numerous interactions of the individual domains of Btk with various protein or lipid molecules have been reported. To study the impact of these interactions on B cell differentiation *in vivo*, we have developed mouse models, in which wild-type or mutated *Btk* can be appropriately expressed as transgenes. For these studies we used transgene constructs containing the MHC-II Locus control region or the CD19 promoter, which were shown to provide position independent, copy number-dependent expression and could therefore be used to target genes to B lineage cells. To be able to answer the central question why B cell development is arrested in XLA patients, the aim of this thesis is to investigate the role of Btk at the individual steps of B cell development. In addition, we wanted to study the regulatory elements that are responsible for *Btk* gene expression, to be able to manipulate *Btk* expression *in vivo*.

In **chapter 2**, the generation of a Btk-deficient mouse, by an in-frame insertion of a *LacZ* gene is described. Using the *LacZ* activity, *Btk* expression could be studied *in vivo*. The phenomenon of X-chromosome inactivation in *Btk* [+/-] heterozygous female mice enabled us to evaluate the *in vivo* competition between B cell progenitors expressing wildtype *Btk* and those expressing the *Btk* [-]/*LacZ* allele in each successive step of development. This model enabled the detection of subtle defects of Btk-deficient cells, characterized by only minor selective disadvantages, which were not recognized in the phenotype of affected male. In addition, this model has been used for back-crosses with other transgenic *Btk* mouse models.

In **chapters 3 and 4** the generation of two yeast artificial chromosome (YAC)-transgenic mouse strains is described, in which high-level expression of human *Btk* is provided by endogenous regulatory *cis*-acting elements. These mouse models were generated to address the question, whether human Btk can compensate the absence of Btk in the mouse. We could also investigate whether regulatory sequences are conserved between humans and mice. These experiments are the first step to analyze the regulation of the *Btk* gene expression *in vivo* (and to subsequently manipulate *Btk in vivo*). We choose to start with transgenic expression of a 340 kb YAC clone, as essential regulatory elements may be located far upstream or downstream of the *Btk* gene itself.

In **chapter 3** we describe the generation of YAC transgenic mouse strains, using a 340-kb transgene, Yc340-h*Btk*, which contained the *Btk* gene and 100 kb of 5' flanking DNA and 200 kb 3' flanking DNA. The expression pattern of the transgenic human *Btk* was found to parallel that of the endogenous murine gene. Furthermore, transgenic human *Btk* expression on a murine Btk-deficient background fully corrected the *xid* phenotype.

In chapter 4 the expression pattern of *Btk* is studied in various haematopoietic lineages, using intracellular flow cytometry. The expression pattern of murine *Btk* was compared to the expression pattern of the human *Btk* from a Yc240-h*Btk* transgene, which in addition to the entire *Btk* gene, contained 5 kb 5' flanking DNA and 200 kb 3' flanking DNA. As was for Yc340-h*Btk* transgene, *Btk* expression from the Yc240-h*Btk* transgene on a Btk-deficient background fully corrected the *xid* phenotype. In parallel, (putative) regulatory elements within the Btk gene itself were characterized. The role of Sp1/3 and PU.1, binding to the Btk promoter was studied, using *in vitro* transfection assays as well as targeted disruption of the genes coding for Sp1 and Sp3 *in vivo*. Finally, possible regulatory sequences within the human *Btk* gene were identified using DNAseI hypersensitivity site mapping.

To identify signaling pathways that are activated by Btk late in B cell development *in vivo* (chapter 5), we generated MHC-II-h*Btk* transgenic mice, which either overexpress wild-type h*Btk*, or various levels of the E41K gain-of-function *Btk* mutant, which represent a constitutive activated form of Btk. Whereas wildtype *Btk* overexpression had no adverse effects on B cell function, and essentially corrected the xid phenotype, E41K-mutant *Btk* expression resulted in a block in the development of follicular recirculating B cells.

We also wanted to analyze the functions of Btk activity early in B cell differentiation in the bone marrow. In chapter 6, the *in vivo* effect of the E41K-*Btk* was investigated using the early active, B cell specific CD19 promoter. While wildtype CD19-driven *Btk* expression resulted in the correction of the xid phenotype, E41K-*Btk* expression, driven by the CD19 promoter lead to a developmental block at the transition of IgM^{low} to IgM^{high} immature B cells in the bone marrow. Furthermore a role for Btk in the regulation of Ig L chain assembly was observed.

References

Afar, D.E.H., Park, H., Howell, B.W., Rawlings, D.J., Cooper, J. and Witte, O.N. (1996) Regulation of Btk by Src family tyrosine kinases. *Mol. Cell. Biol.* 16:3465-3471.

Agrawal, A. and Schatz, D.G. (1997) RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. *Cell* 89:43-53.

Allen, R.C., Armitage, R.J., Conley, M.E., Rosenblatt, H., Jenkins, N.A., Copeland, N.G., Bedell, M.A., Edelhoff, S., Disteche, C.M. and Simoneaus, D.K. (1993) CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 259:990-993.

Allman, D.M., Ferguson, S.E., Lentz., V.M. and Cancro, M.P. (1993) Perpheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol.* 151:4431-4444.

Alt, F.W., Blackwell, T.K. and Yancopoulos, G.D. (1987) Development of the primary antibody repertoire. *Science* 238:1079-1087.

Alt, F.W., Yancopoulos, G., Blackwell, Y.K., Wood, C., Thomas, E., Boss, M., Coffman, R., Rosenberg, N., Tonegawa, S. and baltimore, D. (1984) Ordered rearrangment of immunoglobulin heavy chain variable region gene segments. *EMBO J.* 3:1209-1219.

Aoki, Y., Isselbacher, K.J. and Pillai, S. (1994) Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligand B cells. *Proc. Natl. Acad. Sci. USA* 91:10606-10609.

Aruffo, A., Farrington, M., Hollenbaugh, D., Li, X., Milatovich, A., Nonoyama, S., Bajorath, J., Grosmaire, L.S., Stenkamp, R. and Neubauer, M. (1993) The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper IgM syndrome. *Cell* 72:291-300.

Bauer, S.R., Kudo, A. and Melchers, F. (1988) Structure and pre-B lymphocyte restricted expression of the VpreB gene in humans and conservation of its structure in other mammalian species. *EMBO J.* 7:111-116.

Bence, K., Ma, W., Kozasa, T. and Huang, X.Y. (1997) Direct stimulation of Bruton's tyrosine kinase by G_q-protein α-subunit. *Nature* 389:296-299.

Best, C.G., Kemp, J.D. and Waldtschmidt, T.J. (1995) Murine B cell subsets defined by CD23. *Methods: A companion Methods Enzymol.* 8:3-10.

Bijsterbosch, M.K. and Klaus, G.G.B. (1985) Crosslinking of surface immunoglobulin and Fc receptors on B lymphocytes inhibits stimulation of inositol phosphate breakdown via the antigen receptor . *J. Exp. Med.* 162:1825-1827.

Blackwell, T.K. and Alt, F.W. (1989) Mechanism and developmental program of immunoglobulin gene rearrangment in mammals. *Annu. Rev. Genet.* 23:605-636.

Blomberg, B.B., Rudin, C.M. and Storb, U. (1991) Identification and localization of an enhancer for the human Lambda L chain Ig gene complex. *J. Immunol.* 147:2354-2358.

Bolland, S., Pearse, R.N., Kurosaki, T. and Ravetch, J.V. (1998) SHIP modulates immune receptor responses by regulating membrane associations of Btk. *Immunity* 8:509-516.

Bosma, M.J. and Carrol, A.M. (1991) The SCID mouse mutant: definition, characterization, and potential uses. *Annu. Rev. Immunol.* 9:323-350.

Brown, S.D.M., Avner, P., Boyd, Y., Chapman, V., Rastan, S., Sefton, L., Thomas, J.D. and Herman, G.E. (1993) Encyclopedia of the mouse genome III. October 1993. Mouse X-chromosome. *Mamm. Genome* 4:269-281.

Brooijmans, R.A., van den Berg, A.J.A.M., Rijkers, G.T., Sanders, L.A.M., van Amstel, J.K.P., Tilanus, M.G.J., Grubben, M.J.A.L. and Zegers, B.J.M. (1997) Identification of novel Bruton's tyrosine kinase mutations in 10 unrelated subjects with X-linked agammaglobulinemia. *J. Med. Genet.* 34:484-488.

Bruton, O.C. (1952) Agammaglobulinemia. *Pediatrics* 9:722-728.

Burkhardt, A.L., Brunswick, M., Bolen, J.B. and Mond, J.J. (1991) Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 88:7410-7414.

Butch, A.W., Chung, G.H., Hoffmann, J.W. and Nahm, N.H. (1993) Cytokine expression by germinal center cells. *J. Immunol.* 150:39-47.

Cambier, J.C. (1995) Antigen and Fc receptor signalling. *J. Immunol.* 152:3281-3285.

Campana, D., Farrant, J., Inamdar, N., Webster, A.D. and Janossy, G. (1990) Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. *J. Immunol.* 145:1675-1680.

Campbell, K.S., Hager, E.J., Friedrich, R.J. and Cambier, J. (1991) IgM antigen receptor complex contains phosphoprotein products of B29 and mb-1 genes. *Proc. Natl. Acad. Sci. USA* 88:3982-3986.

Campbell, M.A. and Klinman, N.R. (1995) Phosphotyrosine-dependent association between CD22 and the protein tyrosine phosphatase 1C. *Eur. J. Immunol.* 25:1573-1579.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kappeller, R. and Soltoff, S. (1991) Oncogenes and signal transduction. *Cell* 64:281-302.

Casamayor-Palleja, M., Khan, M. and MacLennan, I.C.M. (1995) A subset of $CD4^+$ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor. *J. Exp. Med.* 181:1293-1301.

Chen, J., Young, F., Bottaro, A., Stewart, V., Smith, R.K. and Alt, F.W. (1993). Mutations of the IgH enhancer and its flanking sequences differentially affect accessibility of the J_H locus. *EMBO J.* 12:4635-4645.

Cheng, G., Ye, Z-S. and Baltimore, D. (1994) Binding of Bruton's tyrosine kinase to Fyn, Lyn, or Hck through a Src homology 3 domain-mediated interaction. *Proc. Natl. Acad. Sci. USA* 91:8152-8155.

Claassen, E., Kors, N., Dijkstra, C.D. and Van Rooijen N. (1986) Marginal zone of the spleen and the development and localization of specific antibody forming cells against thymus-dependent and thymus-independent antigens. *Immunology* 57:399-403.

Coffey, A.J., Brooksbank, R.A., Brandau, O., Oohashi, T., Howell, G.R., Bye, J.M., Cahn, A.P., Durham, J., Heath, P., Wray, P., Pavitt, R., Wilkinson, J., Leversha, M., Huckle, E., Shaw-Smith, C.J., Dunham, A., Rhodes, A., Schuster, V., Porta, G., Yin, L., Serafini, P., Sylla, B., Zollo, M., Franco, B., Bentley, D.R. et al. (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet.* 20:129-135.

Conley, M.E. (1985) B cells in patients with X-linked agammaglobulinemia. *J. Immunol.* 134:3070-3074.

Conley, M.E. (1994) X-linked immunodeficiencies. *Curr. Op. Gen. Dev.* 4:401-406.

Conley, M.E., Brown, P., Pickard, A.R., Buckley, R.H., Miller, D.S., Raskind, W.H., Singer, J.W. and Fialkow, P.J. (1986) Expression of the gene defect in X-linked agammaglobulinemia. *N. Engl. J. Med.* 315:564-570.

Conley, M.E. and Cooper, M.D. (1998) Genetic basis of abnormal B cell development. *Curr. Op. Immunol.* 10:399-406.

Conley, M.E. and Puck (1988) Carrier detection in typical and atypical X-linked agammaglobulinemia. *J. Pediatr.* 112:688-694.

Cooper, J.A. and Howell, B. (1993) The when and how of *Src* regulation. *Cell* 73:1051-1054.

Cory, G.O.C., Lovering, R.C., Hinshelwood, S., MacCarthy-Morrogh, L., Levinsky, R.J. and Kinnon, C. (1995) The protein product of the c-cbl protooncogene is phosphorylated after B cell receptor stimulation and binds the SH3 domain of Bruton's tyrosine kinase. *J. Exp. Med.* 182:611-615.

Cyster, J.G. and Goodnow, C.C. (1995) Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity* 3:691-701.

D'Ambrosio, D., Hippen, K.L., Minskoff, S.A., Mellman, I., Pani, G., Siminovitch, K.A. and Cambier, J.C. (1995) Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by FcγRIIB1. *Science* 268:293-297.

Damen, J.E., Liu, L., Rosten, P., Humphries, R.K., Jefferson, A.B., Majerus, P.W. and Krystal, G. (1996) The 145-kDa protein induced to associate with Shc multiple cytokines is an inositol tetrapjosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. USA* 93:1689-1693.

Derry, J.M.J., Ochs, H.D. and Franke, U. (1994) Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell* 4:1127-1135.

De Weers, M., Brouns, G.S., Hishelwood, S., Kinnon, C., Schuurman, R.K.B., Hendriks, R.W. and Borst, J. (1994b) B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. *J. Biol. Chem.* 269:23857-23860.

De Weers, M., Dingjan, G.M., Brouns, G.S., Kraakman, M.E.M., Mensink, G.G.J., Lovering, R.C., Schuurman, R.K.B., Borst, J. and Hendriks, R.W. (1997) Expression of Bruton's tyrosine kinase in B lymphoblastoid cell lines from X-linked agammaglobulinemia patients. *Clin. Exp. Immunol.* 107:235-240.

De Weers, M., Mensink, R.G.J., Kenter, M. and Schuurman, R.K.B. (1992) Three dinucleotide rerpeat polymorphisms at the DXS178 locus. *Hum. Mole. Genet.* 1:653.

De Weers, M., Mensink, R.G.J., Kraakman, M.E.M., Schuurman, R.K.B. and Hendriks, R.W. (1994a) Mutation analysis of the Bruton's tyrosine kinase gene in X-linked agammaglobulinemia: identification of a mutation which affects the same codon as is altered in immunodefecient *xid* mice. *Hum. Molec. Genet.* 3:161-166

De Weers., Verschuren, M.C.M., Kraakman, M.E.M., Mensink, R.G.J., Schuurman, R.K.B., Van Dongen, J.J.M. and Hendriks, R.W. (1993) The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur. J. Immunol.* 23:3109-3114.

Dinauer, M.C., Orkin, S.H., Brown, R., Jesaitis, A. and Parkos, C.A. (1987) The glycoprotein encoded by the C-linked chronic granulmatous disease locus is a component of the neutrophil cytochrome b complex. *Nature* 327:717-720.

DiSanto, J.P., Bonnefoy, J.Y., Gauchat, J.F., Fischer, A. and de Saint Basile, G. (1993) CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature* 361:541-543.

Doody, G.M., Justement, L.B., Delibrias, C.C., Matthews, R.J., Lin, J., Thomas, M.L. and Fearon, D.T. (1995) A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science* 269:242-244.

Ehlich, A., Schaal, S., Gu, H., Kitamura, D., Müller, W. and Rajewsky, K. (1993) Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. *Cell* 72:695-704.

Engel, P., Zhou, L-J., Ord, D.C., Sato, S., Koller, B. and Tedder, T.F. (1995) Abnormal B lymphocyte development, activation and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity* 3:39-50.

Erickson, L.D., Tygrett, L.T., Bhatia, S.K., Grabstein, K.H. and Waldschmitt, T.J. (1996) Differential expression of CD22 (Lyb8) on murine B cells. *Int. Immunol.* 8:1121-1129.

Fachetti, F., Appiani, C., Salvi, L., Levy, L. and Notarangelo, L.D. (1995) Immunohistologic analysis of ineffective CD40-CD40 ligand interaction in lymphoid tissues from patients with X-linked immunodeficiency with hyper-IgM. Abortive germinal center cell reaction and severe depletion of follicular dendritic cells. *J. Immunol.* 156:4576-4581.

Fearon, D.T. and Carter, R.H. (1995) The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acuired immunity. *Annu. Rev. Immunol.* 13:127-149.

Fearon, E.R., Winkelstein, J.A., Civin, C.I., Pardoll, D.M. and Vogelstein, B. (1987) Carrier detection in X-linked agammaglobulinemia by analysis of X-chromosome inactivation. *N. Engl. J. Med.* 316:427-431.

Ferguson, K.M., Lemmon, M.A., Sclessinger, J. and Sigler, P.B. (1995) Structure of the high affinity complex of inositol triphosphate with a phospholipase C pleckstrin homology domain. *Cell* 83:1037-1046.

Ferguson, S.E., Han, S.H., Kelsoe, G. and Thompson, C.B. (1996) CD28 is required for germinal center formation. *J. Immunol.* 156:4576-4581.

Fluckinger, A.C., Li, Z., Kato, R.M., Wahl, M.I., Ochs, H.D., Longnecker, R., Linet, J.P., Witte, O.N., Scharenberg, A.M. and Rawlings, D.J. (1998) Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation. *EMBO J.* 17:1973-1985.

Forrester, L.M., Ansell, J.D. and Micklem, H.S. (1987) Development of B lymphocytes in mice heterozygous for the X-linked immunodefeiciency (xid) mutation: xid inhibits development of all splenic and lymph node B cells at a stage subsequent to their initial formation in bone marrow. *J. Exp. Med.* 165:949-958.

Förster, R., Mattis, A.E., Kremmer, E., Wolf, E., Brem, G. and Lipp, M. (1996) A putative chemokine receptor BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 87:1037-1047.

Franke, T.F., Yang, S-I., Chan, T.O., Datta, K., Kaziauskas, A., Morrison, D.K., Kaplan, D.R. and Tsichlis, P.N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81:727-736.

Fulcher, D.A. and Basten, A. (1997) Influences on the lifespan of B cell subpopulations defined by different phenotypes. *Eur. J. Immunol.* 27:1188-1199.

Futatani, T., Miyawaki, T., Tsukada, S., Hashimoto, S., Kunikata, T., Arai, S., Kurimoto, M., Niida, Y., Matsuoka, H., Sakiyama Y., Iwata, T., Tsuchiya, S., Tatsuzawa, O., Yoshizaki, K. and Kishimoto, T. (1998) Deficient expression of Bruton's tyrosine kinase in monocytes from X-linked agammaglobulinemia as evaluated by flow cytometric analysis and its clinical application to carrier detection. *Blood* 91:595-602.

Gaspar, H.B., Lester, T., Levinsky, R.J. and Kinnon, C. (1998) Bruton's tyrosine kinase expression and activity in X-linked agammaglobulinemia (XLA): the use of protein analysis as a diagnostic indicator of XLA. *Clin. Exp. Immunol.* 111:334-338.

Genevier, H.C., Hinshelwood, S., Caspar, H.B., Rigley, K.P., Brown, D., Sealand, S., Rousset, F., Levinsky, R.J., Callard, R.E., Kinnon, C. and Lovering, R.C. (1994) Expression of Bruton's tyrosine kinase protein within the B cell lineage. *Eur. J. Immunol.* 24:3100-3105.

Geng, Y. and Johnson, F. (1993) Lack of an initiator element is responsible for multiple transcriptional initiation sites of the TATA less mouse thymidine synthase promotor. *Mol Cell. Biol.* 13:4894-4903.

Ghia, P., Ten Boekel, E., Rolink, A.G. and Melchers, F. (1998) B-cell development: a comparison bewteen mouse and man. *Immunol. Today* 19:480-485.

Gottlieb, T. and Jackson, S.P. (1993) The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell* 72:131-142.

Go, N.F., Castle, B.E., Barrett, R., Kastelein, R., Dang, W., Mosmann, T.R., Moore, K.W. and Howard, M. (1990) Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X-Chromosome-linked immunodefeciency B cells. *J. Exp. Med.* 172:1625-1631.

Gold, M.R., Sanghera, J.S., Stewart, J. and Plelech, S.L. (1992) Selective activation of p42 MAP kinase in murine B lymphoma cell lines by membrane immunoglobulin crosslinking. Evidence for protein kinase C-independent and -dependent mechanisms of activation. *Biochem. J.* 286:269-276.

Gong, S. and Nussenzweig, M.C. (1996) Regulation of an early developmental checkpoint in the B cell pathway by Igβ. *Science* 272:411-414.

Grawunder, U. and Lieber, M.R. (1997a) A complex of RAG-1 and RAG-2 persists on the DNA after single-strand cleavage at V(D)J recombination signal sequences. *Nucleic Acid Res.* 25:1375-1382.

Grawunder, U., Leu, T.M.J., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F. and Winkler, T.H. (1995). Down-regulation of *RAG1* and *RAG2* gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. Immunity 3, 601-608.

Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T.E., Mann, M. and Lieber, M.R. (1997b) Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature* 388:492-495.

Grewal, I.S., Xu, J.C. and Flavell, R.A. (1995) Impairement of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature* 378:617-620.

Groundis, D. and Reid, K.B.M. (1988) Properdin, the terminal complement components, thrombospondin and circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature* 335:82-85.

Grueneberg, D.A., Henry, R.W., Brauer, A., Novina, C.D., Chriyath, V., Roy, A.L. and Gilman, M. (1997) A multifunctional DNA-binding protein that promotes the formation of serum response factor/homeodomain complexes. identity to TFII-I. *Genes Dev.* 11:2482-2493.

Guioli, S., Arveiler, B., Bardoni, B., Notarangelo, L.D., Panina, P., Duse, M., Ugazio, A., Oberlé,I., de Saint-Basile, G., Mandel, J.L. and Camerino, G. (1989) Close linkage of prope p212 (DXS178) to X-linked agammaglobulinemia. *Hum. Genet.* 84:19-21.

Hagemann, T.L., Chen, Y., Rosen, F.S. and Kwan, S-P. (1994). The genomic structure of the Btk gene and its use in direct DNA sequence analysis for mutations in patients with X-linked agammaglobulinemia. *Hum. Mol. Genet.* 3:1743-1749.

Haire, R.N., Ohta, Y., Strong, S.C., Litman, R.T., Liu, Y., Prchal, J.T., Cooper, M.D. and Litman, G.W. (1997) Unusual patterns of exon skipping in Bruton's tyrosine kinase are associated with mutations involving the intron 17 3' splice site. *Amer. J. Hum. Genet.* 60:798-807.

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) The protein kinase family: Conserved features and deduced phylogeny of catalytic domains. *Science* 241:42-52.

Hardy, R.R. and Hayakama, K. (1995) B-lineage differentiation stages resolved by multiparameter flow cytometry. *Ann. N. Y. Acad. Sci.* 764:19-24.

Hardy, R.R., Carmack, C.E., Shinton, S.A., Kemp, J.D. and Hayakawa, K. (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213-1225.

Hardy, R.R., Hayakawa, K., Parks, D.R. and Herzenberg, L.A. (1983) Demonstration of B cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence. *Nature* 306:270-272.

Harmer, S. and DeFranco, A.L. (1997) Shc posesses two Grb2 binding sites needed for the efficient formation of complexes with SOS in B lymphocytes. *Mol. Cell. Biol.* 17:4087-4095.

Harwood, A.E. and Cambier, J.C. (1993) B cell antigen receptor cross-linking triggers rapid protein kinase C independent activation of p21^{ras}. *J. Immunol.* 151:4513-4522.

Hata, A., Sabe, H., Kurosaki, T., Takata, M. and Hanafusa, H. (1994) Functional analysis of Csk in signal transduction through the B-cell antigen receptor. *Mol. Cell.Biol.* 14:7306-7313.

Hayakawa, K., Hardy, R.R., Herzenberg, L.A. and Herzenberg, L.A. (1985) Progenitors for Ly-1 B cells are distict from progenitors for other B cells. *J. Exp. Med.* 161:1554-1568.

Hieter, P.A., Korsmeyer, S.L., Waldmann, T.A. and Leder, P. (1981) Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambda-producing B cells. *Nature* 290:368-372.

Hitoshi, Y., Sonoda, E., Kikuchi, Y., Yonehara, S., Nakauchi, H. and Takatsu, K. (1993) IL-5 receptor positive B cells, but not eosinophils, are functionally and numerically influenced in mice carrying the X-linked immune defect. *Int. Immunol.* 5:1183-1190.

Hendriks, R.W., Reed, V. and Boyd, Y. (1994) Mapping of the agammaglobulinemia tyrosine kinase gene on the mouse X chromosome with interspecific crosses. *Mamm. Genome* 5:60-61.

Hendriks, R.W. and Schuurman, R.K.B. (1991) Genetics of X-linked immunodeficiencies. *Clin. Exp. Immunol.* 85:182-192.

Hermanson, G.G., Eisenberg, D., Kincade, P.W. and Wall, R. (1988) B29: A member of the immunoglobulin gene superfamily exclusively expressed on B-lineage cells. *Proc. Natl. Acad. Sci. USA* 85:6890-6894.

Hermaszewski, R.A. and Webster, A.D.B. (1993). Primary hypogammaglobulinemia: A survey of clinical manifestations and complications. *Q. J. Med.* 86:31-42.

Herzenberg, L.A. and Kantor, A.B. (1993) B-cell lineages exist in the mouse. *Immunol. Today* 14:79-83.

Himmelmann, A., Thevenin, C., Harrison, K. and Kehrl, J.H. (1996) Analysis of the Bruton's tyrosine kinase gene promotor reveals critical PU.1 and Sp1 sites. *Blood* 87:1036-1044.

Hishelwood, S., Lovering, R.C., Genevier, H.C., Levinsky, R.J. and Kinnon, C. (1995) The protein defective in X-linked agammaglobulinemia, Bruton's tyrosine kinase, shows increased autophosphorylation *in vitro* when isolated from cells in which the B cell receptor has been cross-linked. *Eur. J. Immunol.* **25**:1113-1116.

Hiramatsu, R., Akagi, K., Matsuoka, M., Sakumi, K., Nakamura, H., Kingsbury, L., David, C., Hardy, R.R., Yamamura, K-I. and Sakano, H. (1995) The 3' enhancer region determines the B/T specificity and pro-B/pre-B specificity of immunoglobulin V_K-J_K joining. *Cell* **83**:1113-1123.

Hoim, K. and Gellert, M. (1997) A stable RAG1-RAG2-DNA complex that is active in V(D)J cleavage. *Cell* **88**:65-72.

Hollis, G.F., Evans, R.J., Stafford-Hollis, J.M., Korsmeyer, S.J. and McKearn, J.P. (1989) Immunoglobulin light chain related genes 14.1 and 16.1 are expressed in pre-B cells and may encode the human immunoglobulin ω light-chain protein. *Proc. Natl. Acad. Sci. USA.* **86**:5552-5556.

Holsinger, L.J., Spencer, D.M., Austin, D.J., Schreiber, S.L. and Crabtree, G.R. (1995) Signal transduction in T lymphocytes using a conditional allele of SoS. *Proc. Natl. Acad. Sci. USA* **92**:9810-9814.

Holsinski-Feder, E., Weiss, M., Brandau, O., Jedele, K.B., Nore, B., Bäckesjö, C.M., Vihinen, M., Hubbard, S.R., Belohradsky, B.H., Smith, C.I.E. and Meindl, A. (1998) Mutation screening of the Btk gene in 56 families with X-linked agammaglobulinemia (XLA): 47 unique mutations without correlation to clinical course. *Pediatrics* **101**:276-284.

Hombach, J., Leclerq, L., Radbruch, A., Rajewski, L. and Reth, M. (1998) A novel 34 kD protein co-isolated with the IgM molecule in surface IgM-expressing cells. *EMBO J.* **7**:3451-3456.

Hombach, J., Lottspeich, F. and Reth, M. (1990) Identification of the genes encoding the IgMα and IgMβ components of the IgM antigen receptor complex by amino-terminal sequencing. *Eur. J. Immunol.* **20**:2795-2799.

Hyvonen, M. and Saraste, M. (1997) Structure of the PH domain and Btk motif from Bruton's tyrosine kinase: molecular explanations for X-linked agammaglobulinemia. *EMBO J.* **16**:3396-3404.

Jacob, J., Kassir, R. and Kelsoe, G. (1991) In situ studies of the primary immune sesponse to (4-hydroxy-3-nitrophenyl) acetyl.I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* **173**:1165-1175.

Janeway, C.A., Apt, L. and Gitlin, D. (1953) Agammaglobulinemia. *Trans. Assoc. Am. Physicians* **66**:200-202.

Jiang, A., Craxton, A., Kurosaki, T. and Clark, E.A. (1998) Different protein tyrosine kinases are required for B cell antigen receptor-mediated activation of extracellular signal-related kinase, c-Jun NH_2-terminal kinase 1, and p38 mitogen-activated protein kinase. *J. Exp. Med.* **188**:1297-1306.

Jung, S., Rajewski, K. and Radbruch, A. (1993) Shut down of class switch recombination by deletion of switch region control element. *Science* **259**:984-987.

Kantor, A.B., Stall, A.M., Adams, S. and Herzenberg, L.A. (1992) Adoptive transfer of murine B cell lineages. *Ann. N.Y. Acad. Sci.* **651**:168-169.

Karasuyama, H., Kudo, A. and Melchers, F. (1990) The proteins encoded by the V_{pre-B} and λ5 pre-B cell-specific genes can associate with each other and with μ heavy chains. *J. Exp. Med.* **172**:969-972.

Karasuyama, H., Rolink, A., Shinkai, Y., Young, F., Alt, F.W. and Melchers, F. (1994) The expression of VpreB/lambda 5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. *Cell* **77**:133-143.

Kawakami, Y., Yao, L., Miura, T., Tsukada, S., Witte, O.N. and Kawakami, T. (1994) Tyrosine phosphorylation and activation of Bruton's tyrosine kinase upon Fc epsilon RI cross-linking. *Mol. Cell. Biol.* **14**:5108-5113.

Khan, W.N., Gerstein, F.W., Malynn, B.A., Larsson, I., Rathburn, G., Davidson, L., Müller, S., Kantor, A.B. and Herzenberg, L.A. (1995) Defective B cell development and function in Btk-deficient mice. *Immunity* **3**:283-299.

Kitamura, D. and Rajewski, K. (1991) Targeted disruption of μ chain membrane exon causes loss of heavy chain allelic exclusion. *Nature* **356**:154-156.

Kitamura, D., Kudo, A., Schaal, S., Müller, W., Melchers, F. and Rawjewski, K. (1992) A critical role of λ5 in B cell development. *Cell* **69**:823-831.

Koike, M., Kikuchi, Y., Tominaga, A., Takai, S., Agaki, K., Miyazaki, J-I., Yamamura, K.I. and Takatsu, K. (1995) Defective IL-5 mediated signalling in B cells of X-linked immunodefecient mice. *Int. Immunol.* **7**:21-30.

Korthauer, U., Graf, D., Mages, H.W., Briere, F., Padayachee, M., Malcolm, S., Ugazio, A.G., Notarangelo, L.D., Levisky, R.J. and Kroczek, R.A. (1993) Expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* **361**:539-541.

Kozmik, Z., Wang, S., Dörfler, P., Adams, B. and Busslinger, M. (1992) The promotor of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol. Cell. Biol.* **12**:2662-2672.

Krantman, H.J., Saxon, A., Stevens, R.H. and Stieham, E.R. (1981) Phenotypic heterogeneity in X-linked infantile agammaglobulinemia with in vitr monocyte suppression of immunoglobulin synthesis. *Clin. Immunol. Immunopathol.* **20**:170-178

Kroese, F.G.M., Butcher, E.C., Stall, A.M., Lalor, P.A. Adams, S. and Herzenberg, L.A. (1989) Many of the IgA producing plasma cells in the murine gut are derived from self-replenishing pre-cursors in the peritoneal cavity. *Int. Immuno.* 1:75-84.

Kroese, F.G.M., Ammerlaan, W.A.M. and Deenan, G.J. (1992) Location and dynamics of B-cell lineages. *Ann. N.Y. Acad. Sci.* 651:44-48.

Kudo, A. and Melchers, F. (1987) A second gene, VpreB in the λ5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. *EMBO J.* 6:2267-2272.

Kurosaki, T. (1997) Molecular mechanisms in B cell antigen receptor signaling. *Curr. Opin. Immunol.* 9:309-318.

Kwan, S-P., Kunkel, L., Bruns, G., Wedgwood, R.J., Latt, R.J. and Rosen, F.S. (1986) Mapping of the X-linked agammaglobulinemia locus by use of restriction fragment polymorphism. *J. Clin. Invest.* 77:649-652.

Kwan, S-P., Terwilliger, J., Parmley, R., Raghu, G., Sandkuyl, L.A., Ott, J., Ochs, H., Wedgwood, R. and Rosen, F. (1990) Identification of closely linked DNA marker, DXS178 to further refine the X-linked agammaglobulinemia locus. *Genomics* 6:238-242.

Lalor, P.A., Herzenberg, L.A., Adams, S. and Stall, A.M. (1989) Feedback regulation of murine Ly-1 B cell development. *Eur. J. Immunol.* 19:507-513.

Lam, K-P., Kühn, R. and Rajewsky, K. (1997) In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90:1073-1083.

Landreth, K.S., Engelhard, D., Anasetti, C., Kapoor, N., Kincade, P.W. and Good, R.A. (1985). Pre-B cells in agammaglobulinemia: evidence for disease heterogeneity among affected boys. *J. Clin. Immunol.* 5:84-89.

Lankar, D., Briken, V., Adler, K., Weiser, P., Cassard, S., Blank, U., Viguier, M. and Bonnerot, C. (1998) Syk tyrosine kinase and B cell antigen receptor (BCR) immunoglobulin-α subunit determine BCR-mediated major histocompatibility complex class II-restricted antigen presentation. *J. Exp. Med.* 188:819-831.

Law, C-L., Sidorenko, S.P., Chandran, K.A., Zhao, Z., Shen, S-H., Fischer, E.H. and Clark, E.A. (1996) CD22 associates with protein tyrosine phosphatase 1C, syk and phospholipase C-γ1 upon B cell activation. *J. Exp. Med.* 183:547-560.

Lazarus, A.H., Kawauchi, K., Rapoport, M.J. and Delovitch, T.L. (1993) Antigen-induced B lymphocyte activation involves the p21^{ras} and RasGAP signalling pathway. *J. Exp. Med.* 178:1765-1769.

Lederman, S., Yellin, M.J., Inghirami, G., Lee, J.J., Knowles, D.M. and Chess, L. (1992) Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles: role of T-cell activating molecule (5c8 antigen) and CD40 in contact-dependent help. *J. Immunol.* 149:3817-3826.

Leickley, F.E. and Buckley, R. (1986). Variability in B cell maturation and differentiation in CX-linked agammaglobulinemia. *Clin. Exp. Immunol.* 65:90-99.

Lemmon, M.A., Ferguson, K.M. and Schlessinger, J. (1996) PH domains: diverse sequences with a common fold recruit signalling molecules to the cell surface. *Cell* 85:621-624.

Leprince, C., Draves, K.E., Geahlen, R.L., Ledbetter, J.A. and Clark, E.A. (1993) CD22 associates with the human surface IgM-B cell antigen receptor complex. *J. Immunol.* 90:32336-32340.

Lewis, S., Hesse, J.E., Mizuuchi, K. and Gellert, M. (1988) Novel strand exchanges in V(D)J recombination. *Cell* 55:1099-1107.

Lichtenstein, M., Keini, G., Cedar, H. and Bergman, Y. (1994) B cell-specific demethylation: a novel role for the intronic κ chain enhancer sequence. *Cell* 76:913-923.

Lindhout, E., Mevissen, M.L.C.M., Kwekkeboom, J., Tager, J. and de Groot, C. (1993) Direct evidence that human follicular dendritic cells (FDC) rescue germinal centre B cells from death by apoptosis. *Clin. Exp. Immunol.* 91:330-336.

Lin, W-C. and Desiderio, S. (1995) V(D)J recombination and the cell cycle. *Immunol. Today* 16:279-289.

Li, T., Tsukada, S., Satterthwaite, A., Havlik, M.H., Park, H., Takatsu, K. and Witte, O.N. (1995) Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. *Immunity* 2:451-460.

Li, Y.S., Hayakawa, K. and Hardy, R.R. (1993) The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. (1993) *J. Exp. Med.* 178:951-960.

Li, Y.S., Wasserman, R., Hayakawa, K. and Hardy, R.R. (1996) Identification of the earliest B lineage stage in the mouse bone marrow. *Immunity* 5:527-535.

Li, Z.M., Wahl, M.I., Eguinoa, A., Stephens, L.R., Hawkins, P.T. and Witte, O.N. (1997) Phosphatidylinositol 3-kinase-gamma activates Bruton's tyrosine kinase in concert with Src family kinases. *Proc. Natl. Acad. Sci. USA* 94:13820-13825.

Lioubin, m.N., Algate, P.A., Tsai, S., Carlberg, K., Aebersold, R. and Rohrschneider, L.R. (1996) p150^{ship}, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev.* 10:1085-1095.

Liu, Y-J., Oldfiled, S. and MacLennan, I.C.M. (1988) Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones. *Eur. J. Immunol.* 18:355-362.

Liu, Y-J., Zhang, J., Lane, P.J.L., Chan, E.Y-T. and MacLennan, I.C.M. (1991) Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol.* 21:2951-2962.

Lovering, R.C., Middleton-Price, H.R., O'Reilly, M.A., Genet, S.A., Parkar, M., Sweatman, A.K., Bradley, L.D., Alterman, L.A., Malcolm, S. and Morgan, G. (1993) genetic linkage analysis idintifies new proximal and distal flanking markers for the X-linked agammaglobulinemia gene locus, refining its localization in Xq22. *Hum. Mol. Genet.* 2:139-141.

Malcolm, S., de Saint-Basile, G., Arveiler, B., Lau, Y., Szabo, P., Fisher, A., Griscelli, C., Debre, M., Mandel, J.L., Callard, R.F., Robertson, M.E., Goodship, J.A., Pembrey, M.E. and Levinski, R.J. (1987) Close linkage of random DNA fragments from Xq21.3-22 to X-linked agammaglobulinemia (XLA). *Hum. Genet.* 77:172-174.

Malisan, F., Briere, F., Bridon, J.M., Harindranath, N., Mills, F.C., Max, E.E., Banchereau, J. and Martinez-Valdez, H. (1996) Interleukin-10 induces immunoglobulin G isotype switch recombination in human CD40-activated naive B lymphocytes. *J. Exp. Med.* 183:937-947.

Mano, H., Ishikawa, F., Nishida, J., Harai, H. and Takaku, F. (1990) A novel protein-tyrosine kinase, tec, is preferentially expressed in the liver. *Oncogene* 5:1781-1786.

Mano, H., Mano, K., Tang, B., Koehler, M., Yi, T., Gilber, D.J. Jenkins, N.A., Copeland, N.G. and Ihle, J.N. (1993) Expression of a novel form of Tec kinase in hematopoietic cells and mapping of the gene to chromosome 5 near Kit. *Oncogene* 8:417-424.

Matsuda, T. *et al.* (1995) Association and activation of Btk and Tec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines. *Blood* 85:627-633.

Matsuzawa, Y., Semba, K., Kamamura-Tsuzuki, J., Sudo, T., Ishii, S., Toyoshima, K. and Yamamoyo, T. (1991) Characterisation of the promotor region of the c-yes proto-oncogene; the importance of the GC boxes on its promotor activity. *Oncogene* 6:1561-1567.

McIntrie, K.R. and Rouse, A.M. (1970) Mouse Ig light chains alteration of κ/λ ratio. *Fed. Proc.* 29:704-708.

McMahon, S.B. and Monroe, J.G. (1995) A ternary complex factor-dependent mechanism mediates induction of erg-1 through selective serum response elements following antigen receptor crosslinking in B lymphocytes. *Mol. Cell. Biol.* 15:1086-1093.

McKinney, R.E., Katz, S.L. and Wilfert, C.M. (1987) Chonic enteroviral meningo-encephalitis in agammaglobulinemic patients. *Rev. Infect. Dis.* 9:334-356.

Mease, P.J., Ochs, H.D. and Wedgwood, R.J. (1981) Successful treatment of echovirusmenigoencephalitis and myositis-fasciitis with intravenous immune globulin therapy in apatient with X-linked agammaglobulinemia. *N. Engl. J. Med.* 304:1278-1281

Melamed, D., Benschop, R.J., Cambier, J.C., and Nemazee, D. (1998). Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor editing. *Cell* 92:173-182.

Melchers, F., Rolink, A. and Grawunder, U., Winkler, T.H., Karasuyama, H., Ghia, P., and Anderson (1995) Positive and negative selection events during B lymphopoiesis. *Curr. op .immunol.* 7:214-227.

Meyer, K.B. and neuberger, M.S. (1989) The immunoglobulin kappa locus contains a second, stronger B-cell specific enhancer which is located downstream of the constant region. *EMBO J.* 8:1959-1964.

Mombaerts, P., Lacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S. and Papaionnou, V.E. (1992) GAG-1 deficient mice have no mature B and T lymphocytes. *Cell* 68:869-877.

Mond, J.J., Schaefer, M.E., Smith, J. and Finkelman, F.D. (1983) Lyb-5 B cells of CBA/N mice can be induced to synthesize DNA bu culture with insolubilized but no soluble anti-Ig. *J. Immunol.* 31:2107.

Müller, S., Sideras, P., Smith, C.I.E. and Xanthopoulos, K.G. (1996) Cell specific expression of human Bruton's agammaglobulinemia tyrosine kinase gen (Btk) is regulated by Sp-1 and Spo-1/PU.1-family members. *Oncogene* 13:1955-1964.

Nagai, K., Takata, M., Yamamura, H. and Kurosaki, T. (1995) Tyrosine phosphorylation of Shc is mediated through Lyn and Syl in B cell receptor signalling. *J. Biol. Chem.* 270:6824-6829.

Nahm, M.H., Paslay, J.W. and Davie, J.M. (1983) Unbalanced X chromosome mosaicism in B cells of mice with X-linked immunodeficiency. *J. Exp. Med.* 158:920-931.

Noguchi, M., Yi, H., Rosenblatt, H.M., Filipovitch, A.H., Asdelstein, S., Modi, W.S., McBride, O.W. and Leonard, W.J. (1993) Interleukin-2 receptor gamma chain mutation result in X-linked severe combined immunodeficiency in humans. *Cell* 73:147-157.

Nolan, K.F., Schweable, W., Kaluz, S., Dierich, M.P. and Reid, K.B.M. (1991) Molecular cloning of the cDNA coding for properdin, a positive regulator of the alternative pathway of human complement. *Eur. J. Immunol.* 21:771-776.

Nonoyama, S. and Ochs, H.D. (1998) Characterization of the Wiskott-Aldrich syndrome protein and its role in the disease. *Curr. Opin. Immunol.* 10:407-412.

Nussenzweig, A., Chen, C., da Costa Soares, V., Sanchez, M., Sokol, K., Nussenzweig, M.C. and Li, G.C. (1996) Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature* 382:551-555.

Nutt, S.L., Urbanek, P., Rolink, A., Busslinger, M., (1997) Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V to DJ recombination at the IgH locus. *Genes Dev.* 11:476-491.

Ochs, H.D. and Wedgewood, R.J. (1980) In 'Immunological disorders in infants and children' (E.R. Stiehm and V.A. Fulginiti, Eds), p.239, Saunders, Philidelphia, PA.

Oeltjen, J.C., Malley, T.M., Muzny, M., Miller, W., Gibbs, R.A. and Belmont, J.W. (1997) Large-scale comparative sequence analysis of the human and murine Bruton's tyrosine kinase loci reveals conserved regulatory domains. *Genome Res.* 7:315-329.

Ohta, Y., Haire, R.N., Litman, R.T., Fu, S.M., Nelson, R.P., Kratz, J., Kornfeld, S.J., de la Morena, M., Good, R.A. and Litman, G.W. (1994). Genomic organization and structure of Bruton agammaglobulinemia tyrosine kinase: localization of mutations associated with varied clinical presentations and course in X-linked agammaglobulinemia. *Proc. Natl. Acad. Sci. USA* 91:9062-9066.

O'Keefe, T.L., Williams, G.T., Davies, S.L. and Neuberger, M.S. (1996) Hyperresponsive B cells in CD22-defecient mice. *Science* 274:798-801.

Osmond, D.G. (1990) B cell dvelopment in the bone marrow. *Semin. Immunol* 2:173-180.

Otipody, K.L., Andersson, K.B., Draves, K.E., Klaus, S.J., Farr, A.G., Kerner, J.D., Perlmutter, R.M., Law, C-L. and Clark, E.A. (1996) CD22 regulates thymus-independent responses and lifespan of B cells. *Nature* 5:551-562.

Papavasiliou, F., Jankovic, M., Suh, H. and Nussenzweig, M.C. (1995a) The cytoplasmic domains of immunoglobulin (Ig)α and (Ig)β can independently induce the precursor B cell transition and allelic exclusion. *J. Exp. Med.* 182:1389-1394.

Papavasiliou, F., Misulovin, Z., Suh, H. and Nussenzweig, M.C. (1995b) The role of Igβ in precursor B cell transition and allelic exclusion. *Science* 268:408-411.

Parekh, A.B., Fleig, A. and Penner, R. (1997) The store operated calcium current I_{CRAC}: nonlinear activation and dissociation from calcium release. *Cell* 89:973-980.

Park, H., Wahl, M.I., Afar, D.E.H., Turck, C.W. Rawlings, D.J., Tam, C., Scharenberg, A.M., Kinet, J-P. and Witte, O.N. (1996) Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity* 4:515-525.

Parolini, O., Hejtmancik, J.F., Allen, R.C., Belmont, J.W., Lassiter, G.L.,Henry, M.J., Barker, D.F. and Conley, M.E. (1993) Linkage analysis and physical mapping near the gene for X-linked agammaglobulinemia at Xq22. *Genomics* 15:342-349.

Pillai, S. and Baltimore, D. (1987) Formation of disulphide-linked $\mu_2\omega_2$ tetramers in pre-B cells by the 18K ω-immunoglobulin light chain. *Nature* 329:172-176.

Peaker, C.J.G. and Neuberger, M.S. (1993) Association of CD22 with the B-cell antigen receptor. *Eur. J. Immunol.* 23:1359-1362.

Pearl, E.R., Vogler, L.B., Okos, A.J., Christ, W.M., Lawton, III A.R. and Coopmer M.D. (1978) B lymphocyte precursors in human bone marrow: an analysis of normal individuals and patients with antibody-deficiency states. *J. Immunol.* 120:1169-1175.

Perlmutter, R.M., Nahm, M., Stein, K.E., Slack, J., Zitron, I., Paul, W.E. and Davie, J.M. (1979) Immunoglobulin subclass-specific immunodeficiency in mice with an X-linked B-lymphocyte defect. *J.Exp. Med.* 149:993-998.

Pleiman, C.M., Abrams, C., Timson Gauen, L.T., Bedzyk, W., Jongstra, J., Shaw, A.S. and Cambier, J. (1994b) Distinct p53/56^{lyn} and p59^{fyn} domains associate with nonphosphorylated and phosphorylated Ig-α. *Proc. Natl. Acad. Sci. USA* 91:4268-4277.

Pleiman, C.M., Hertz, W.M. and Cambier, J.C. (1994a) Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. *Science* 263:1609-1612.

Rameh, L.E., Tolias, K.F., Duckworth, B.C. and Cantley, L.C. (1997) A new pathway for the synthesis of phosphatidylinositol-4,5-biphosphate. *Nature* 390:192-196.

Rajewski, K. (1996) Clonal selection and learning in the antibody system. *Nature* 381:751-758.

Ramesh, N., Fuleihan, R., Ramesh, V., Lederman, S., Yellin, M.J., Sharma, S., Chess, L.,Rosen, F.S. and Geha, R.S. (1993) Deletions in the ligand for CD40 in X-linked immunoglobulin defeciency with normal of elevated IgM (HIGMX-1). *Int. Immunol.* 5:769-773.

Ramsden, D., Paull, T. and Gellert, M. (1997) Cell-free V(D)J recombination. *Nature* 388:488-491.

Rathmell, J.C., Townsend, S.E., Xu, J.C., Flavell, R.A. and Goodnow, C.C. (1996) Expansion or elimination of B cells in vivo: dual roles for Cd40 and Fas (CD95)-ligands modulated by the B cell antigen receptor. *Cell* 87:319-329.

Ravichandran, K.S., Lorenz, U., Shoelson, S.E. and Burakoff, S.J. (1995) interaction of Shc with Grb2 regulates association with mSOS. *Mol. Cell. Biol.* 15:593-600.

Rawlings, D.J., Saffran, D.C., Tsukada, S., Largaespada, D.A., Grimaldi, J.C., Cohen, L., Mohr, R.N., Bazan, J.F., Howard, M., Copeland, M.G., Jenkins, N.A. and Witte, O.N. (1993) Mutation of the unique region of Bruton's tyrosine kinase in immunodeficient mice. *Science* 261:358-361.

Rawlings, D.J. Scharenberg, A.M., Park, H., Wahl, M.I., Lin, S., Kato, R.M., Fluckinger, A-C., Witte, O.N. and Kinet, J-P. (1996) Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* 271:822-825.

Rawlings, D.J. and Witte, O.N. (1994) Bruton's tyrosine kinase is a key regulator in B cell development. *Immunol. Rev.* 138:105-119.

Ren, R., Mayer, B.J., Thiel, G. and Baltimore, D. (1993) Identification of a ten-aminoacid proline-rich-SH3 binding site. *Science* 259:1157-1161.

Reth. M (1989) Antigen receptor tail clue. *Nature* 338:383-384.

Richards, J.D., Gold, M.R., Hourihane, S.L., DeFranco, A.L.and Matsuuchi, L. (Reconstitution of B cell antigen receptor-induced signaling events in a nonlymphoid cell line by expressing the Syk protein-tyrosine kinase. *J. Biol. Chem.* 271:6458-6466.

Rickert, R.C., Rajewsky, K. and Roes, J. (1995) Impairment of T-cell-dependent B-cell responses and B-1 development in CD19-deficient mice. *Nature* 376:352-355.

Rigley, K.P., Harnett, M.M., Philips, R.J. and Klaus, G.G.B. (1989) Analysis of signalling via surface immunoglobulin receptors on B cells from CBA/N mice. *Eur. J. Immunol.* 19:2081-2086.

Rohrer, J. and Conley, M.E. (1998) Transcriptional regulatory elements within the first intron of Bruton's tyrosine kinase *Blood* 91:214-221.

Rolink, A., Grawunder, U., Winkler, T.H., Karasuyama, H. and Melchers, F. (1994) IL-2 receptor a chain (CD25, TAC) expression defines a crucial stage in pre-B cell development. *Int. Immunol.* 6:1257-1264.

Rolink, A., Ten Boekel, E., Melchers, F., Fearon, D.T., Krop, I. and Anderson, J. (1996) A subpopulation of B220^{+} cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. *J. Exp. Med.* 183:187-194.

Rosen, F. *et al.* (1997) Primary immunodeficiency diseases: Report of a WHO scientific group. *Clin. Exp. Immunol.* 109:1-28.

Roth, D.B., Menetski, J.P., Nakajiaama, P., Bosma, M.J. and Gellert, M. (1992) V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in SCID mouse thymocytes. *Cell* 70:983-991.

Roy, A.L., Du, H., Gregor, P.D., Novina, C.D., Martinez, E. and Roeder, R.G. (1997) Cloning of an Inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. *EMBO J.* 16:7091-7104.

Saha, B.K., Curtis, S.K., Vogler, L.B. and Vihinen, M. (1997) Molecular and structural characterization of five novel mutations in the Bruton's tyrosine kinase gene from patients with X-linked agammaglobulinemia. *Mol. Med.* 3:477-485.

Sakaguchi, N. and Melchers, F. (1986) λ5, new light-chain-related locus selectively expressed in pre-B lymphocytes. *Nature* 324:579-582.

Sakaguchi, N., Kashiwamura, S-I, Kinoto, M., Thalmann, P. and Melchers, F. (1988) B lymphocyte lineage restricted expression of mb-1, a gene with CD3-like structural properties. *EMBO J.* 7:3457-3464.

Sarkar, S., Schlottmann, K., Cooney, D. and Coggeshall, K.M. (1996) Negative signaling via FcγRII in B cells blocks phospholipase Cγ2 tyrosine phosphorylation but not Syk or Lyn activation. *J. Biol. Chem.* 271:20182-20186.

Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, E.C.I. and Discoll, P.C. (1996) Distict specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.* 15:6241-6250.

Santos-Argumedo, L., Lund, F.E., Heath, A.W., Solvason, N., Wu, W.W., Grimaldi, J.C., Parkhouse, R.M. and Howard, M (1995) CD38 unressponsineness of xid cells implicates Bruton's tyrosine kinase (btk) as a regulator of CD38 induced signal transduction. *Int. Immunol.* 7:163-170.

Saouf, S.J., Mahajan, s., Rowley, R.B., Kut, S.A., Fargnoli, J., Burkhardt, A.L., Tsukada, S., Witte, O.N. and Bolen, J.B. (1994) Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc. Natl. Acad. Sci. USA* 91:9524-9528.

Sato, S., Miller, A.S., Inaiki, M., Bock, C.B., Jansen, P.J., Tang, M.L.K. and Tedder, T.F. (1996) CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signalling in CD22-defecient mice. *Immunity* 5:551-562.

Sato, S., Steeber, D.A., Jansen, P.J. and Tedder, T.F. (1997) CD19 expression levels regulate B lymphocyte develpopment: human CD19 restores normal function in mice lacking endogenous CD19. *J. Immunol.* 158:4662-4669.

Sato, S., Katagiri, T., Takaki, S., Kikuchi, Y., Hitoshi, Y., Yonehara, S., Tsukada, S., Katamura, D., Watanabe, T., Witte, O.N. and Takatsu, K. (1994) IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus kinases. *J. Exp. Med.* 180:2101-2111.

Sawchuk, D., Weis-Garcia, F., Malik, S., Besmer, E., Bustin, M., Nussenzweig, M. and Cortes, P. (1997) V(D)J recombination: modulation of RAG1 and RAG2 cleavage activity on 12/23 substrates by whole cell extracts and DNA-bending proteins. *J.Exp. Med.* 185:2025-2032.

Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., Van Scaik, S., Notarangelo, L., Geha, R., Rincarolos, M.G., Oetttgen, H., De Vries, J.E., Aversal, G. and Terhorst, C. (1998) The X-linked lymphoproliferative disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* 395:462-469.

Scharenberg, A.M., Lin, S., Cuenod, B., Yamamura, H. and Kinet, J.P. (1995) Reconstitution of interactions between tyrosine kinases and the high affinity IgE receptor which are controlled by receptor clustering. *EMBO J.* 14:3385-3394.

Scharenberg, A.M., EL-Hillal, O., Fruman, D.A., Beitz, L.O., Li, Z., Lin, S., Gout, I., Cantley, L.C., Rawlings, D.J. and Kinet, J-P. (1998) Phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P_3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J.* 17:1961-1972.

Scher, I. (1982). The CBA/N mouse strain: An experimental model illustrating the influence of the X-chromosome on immunity. *Adv. Immunol.* 33:1-71.

Schlissel, M.S. and Baltimore, D. (1989) Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. *Cell* 58:1001-1007.

Schulte, R.J., Cambell, M-A, Fisher, W.H. and Sefton, B.M. (1992) Tyrosine phosphorylation of CD22 during B cell activation. *Science* 258:1001-1004.

Serwe, M. and Sablitzky, F. (1993) V(D)J recombination in B cells is impaired but not blocked by targeted deletion of the immunoglobuloin heavy chain intron enhancer. *EMBO J.* 12:2321-2327.

Shinkai, Y., Rathbun, G., Lam, K-P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M. and Alt, F.W. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855-867.

Sideras, P., Müller, S., Shiels, H., Jin, H., Khan, W.N., Nilsson, L., Parkinson, E., Thomas, J.D., Brandén, L., Larsson, I., Paul, W.E., Rosen, F.S., Alt, F.W., Vetrie, D., Smith, C.I.E. and Xanthopoulos, K.G. (1994) Genomic organisation of mouse and human Bruton's agammaglobulinemia tyrosine kinase (Btk) loci. *J. Immunol.* 153:5607-5617.

Sideras, P. and Smith, C.I.E. (1995) Molecular and Cellular aspects of X-linked agammaglobulinemia. *Adv. In Immunol.* 59:135-223.

Siderenko, S.P., Law, C-L., Kalus, S.J., Chandran, K.A., Takata, M., Kurosaki, T. and Clark, E.A. (1996) Protein kinase Cμ (PKCμ) associates with the B cell antigen receptor complex and regulates lymphocyte signalling. *Immunity* 5:353-363.

Siliciano, L.D., Morrow, T.A. and Desiderio, S.V. (1992) Itk, a T cell specific tyrosine kinase gene inducible by interleukin 2. *Proc. Natl. Acad. Sci. USA* 89:11194-11198.

Siminovitch, K.A, Bakshi, A., Goldmoan, P. and Korsmeyer, S.J. (1985) A uniform deleting element mediates the loss of kappa genes in human B cells. *Nature* 316:260-262.

Skare, J.C., Milunsky, A., Byron, R.S. and Sullivan, J.L. (1987) Mapping the X-linked lymphoproliferative syndrome. *Proc. Natl. Acad. Sci. USA* 84:2015-2018.

Slack, J., Der-Balian, G.P., Nahm, M. and Davie, J.M. (1980). Subclass restriction of murine antibodies. II. The IgG plaque forming cell response to thymus independent type 1 and type 2 antigens in normal and mice expressing a X-linked immunodeficiency. *J. Exp. Med.* 151:853-862.

Smith, C.I.E., Baskin, B., Humire-Greiff, P., Zhou, J.N., Olsson, P.G., Maniar, H.S., Kjellen, P., Lambris, J.D., Christensson, B., Hammarström, L., Bentley, D., Vetrie, D., Islam, K.B., Vorechovsky, I. and Sideras, P. (1994a) Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J. Immunol.* 152:557-565.

Smith, C.I.E., Islam, K.B., Vorechovsky, I., Olerup, O., Wallin, E., Rabbani, H., Baskin, B. and Hammarström, L. (1994b). X-linked agammaglobulinemaia and other immunoglobulin defeciencies. *Immunol. Rev.* 138:159-183.

Smith, R.M. and Curnette, J.T. (1991) Molecular basis of chronic granulomatous disease. *Blood* 77:673-686.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G.,Pawson, T., Hase, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., Nells, B.G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hanafusa, H., Schaffhausen, B. and Cantley, L.C. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell* 64:693-702.

Stanhope-Baker, P., Hudson, K.M., Shaffer, A.L., Constantinescu, A. and Schlissel, M.S. (1996) Cell type-specific chomatin structure determines the targeting of V(D)J recombinase acivity in vitro. *Cell* 85:887-897.

Stiehm, E.R. (1993) New and old immunodeficiencies. *Pediatr. Res.* 33:S2-S7.

Storb, U., Roth, P. and Kutz, B.K. (1994) γ2b transgenic mice as a model for the rule of immunoglobulins in B cell development. *Immunol. Res.* 13:291-298.

Sugamura, K., Asao, H., Kondo, M., Tanaka, N., Ishii, N, Ohbo, K., Nakamura, M. and Takeshita, T. (1996) The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu. Rev. Immunol.* 14:179-205.

Takai, T., Ono, M., Hikida, K.L., Ohmori, H. and Ravetch, J.V. (1996) Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature* 379:346-349.

Takata, M. and Kurosaki, T. (1996) A role for Bruton's tyrosine kinase in B cell antigen receptor -mediated activation of phospholipase C-γ2. *J. Exp. Med.* 184:31-40.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H. and Kurosaki, T. (1994) Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. *EMBO J.* 13:1341-1349.

Takeda, S., Zou, Y-R., Bluethmann, H., Kitamura, D., Müller, U. and Rajewski, K. (1993) Deletion of the immunoglobulin κ chain intron enhancer abolishes κ chain gene rearrangemnt in *cis* but not λ chain gene rearrangement in *trans*. *EMBO J.* 12:2329-2336.

Tamagnone, L., Lahtinen, I., Mustonen, T., Virtaneva, K., Muscatelli, F., Francis, F., Alitalo, R., Smith, C.I.E., Larsson, C. and Alitalo, K. (1994) BMX/TXK, a novel cytoplasmic tyrosine kinase gene of the BTK/ITK/TEC family located in chromosome Xp22.2. *Oncogene* 9:3683-3688.

Teahan, C., Rowe, P., Parkar, P., Totty, N. and Segal, A.W. (1987) The X-linked chronic granulomatous disease gene codes for the β-chain of cytochrome b-245. *Nature* 327:720-721.

Tedder, T.F., Inaoki, M. and Sato, S. (1997) The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity* 6:107-118.

Tedder, T.F. and Isaacs, C.M. (1989) Isolation of cDNAs encoding the CD19 antigen of human and mouse B lymphocytes: a new member of the immunoglobulin superfamily. *J. Immunol.* 143:712-717.

Thomas, J.D., Sideras, P., Smith, C.I.E., Vorechovsky, I., Chapman, V. and Paul, W.E. (1993) Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiecy genes. *Science* 261:355-358.

Tiegs, S.L., Russell, D., aand Nemazee, D. (1993). Receptor editing in self-reactive bone marrow B cells. J. Exp. Med. *177*, 1009-1020.

Timmers, E., de Weers, M., Alt, F.W., Hendriks, R.W. and Schuurman, R.K.B. (1991) X-linked agammaglobulinemia. *Clin. Immunol. Immunopathol.* 61:83-93.

Torres, R.M., Flaswinkel, H., Reth, M. and Rajewski, K. (1996) Aberant B cell development and immune responses in mice with a compromised BCR complex. *Science* 272:1804-1808.

Touhara, K., Inglese, J., Pitcher, J.A., Shaw, G. and Lefkowitz, R.J. (1994) Binding of G protein βγ-subunits to pleckstrin homology domains. *J. Biol. Chem.* 269:10217-10220.

Travis, A., Hagman, J. and Grosschedel, R. (1991) Heterogeneity initiated transcription from the pre-B and B cell specific mb-1 promotor: analysis of the requirement for upstream factor binding sites and initiation site sequences. *Mol. Cell. Biol.* 11:5756-5766.

Tsukada, S., Saffran, D.C., Rawlings, D.J., Parolini, O., Allen, R.C., Klisak, I., Sparkes, R.S., Kubagawa, H., Thuluvancheri, M., Quan, S., Belmont, J.W., Cooper, M.D., Conley, M.E. and Witte, O.N. (1993). Defecient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 72:279-290.

Tsukada, S., Simon, M.I., Witte, O.N. and Katz, A. (1994) Binding of βγ subunits of heterotrimeric G-proteins to the PH domain of Bruton's tyrosine kinase. *Proc. Natl. Acad. Sci. USA* 91:11256-11260.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L. and Tybulewicz, V.L. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 378:298-302.

Tuscano, J., Engel, P., Tedder, T.F., Agarwal, A. and Kehrl, J.H. (1996b) Involvement of p72syk kinase, p53/56lyn kinase and phosphatidyl inositol-3 kinase in signal transduction via human B lymphocyte antigen CD22. *Eur. J. Immunol.* 26:1246-1252.

Tuscano, J., Engel, P., Tedder, T.F. and Kehrl, J.H. (1996a) Engagement of the adhesion receptor CD22 triggers a potent stimulatory signal for B cells and blocking CD22/CD22L interactions impairs T-cell proliferation. *Blood* 87:4723-4730.

Urbanek, P., Wang, Z-Q., Fetka, I., Wagner, E.F. and Busslinger, M. (1994) Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* 79:901-912.

Van den Eertwegh, A.J.M., Boersma, W.J.A. and Classen, E. (1992) Immunological functions and *in vivo* cell-cell interactions of T cells in the spleen. *Clin. Rev. Immunol.* 11:337-380.

Van der Merwe, P.A., Crocker, P.R., Vinson, M., Barclay, A.N., Schauer, R. and Kelm, S. (1996) Localization of the putative sialic acid-binding site on the immunoglobulin superfamily cell-surface molecule CD22. *J. Biol. Chem.* 271:9273-9280.

Van Essen, D., Kikutani, H. and gray, D. (1995) CD40 ligand-transduced co-stimulation of t cells in the development of helper function. *Nature* 378:620-623.

Van Gent, D.C., Hoim, K., Paull, T. and Gellert, M. (1997) Stimulation of V(D)J cleavage by high mobility group proteins. *EMBO J.* 16:2665-2670.

Van Gent, D.C., McBlane, J.F., Ramsden, D.D., Romeo, C., Cuomo, C.A., Gellert, M. and Oettinger, M.A. (1995) Initiation of V(D)J recombination in a cell-free system. *Cell* 81:387-395.

Van Gent, D.C., Ramsden, D.A. and Gellert, M. (1996) The RAG1 and RAG2 proteins establish the 12/23 rule in V(D)J recombination. *Cell* 85:107-113.

Vetrie, D., Bobrow, M. and Harris, A. (1993a) Construction of a 5.2-megabase physical map of the human X chromosome at Xq22 using pulsed-field gel electrophoresis and yeast artificial chromosomes. *Genomics* 15:631-642.

Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammerström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C.I.E. and Bentley, D.R. (1993b) The gene involved in X-linked agammaglobulinemia is a member of the *src* family of protein tyrosine kinases. *Nature* 361:226-233.

Vihinen, M., Brandau, O., Brandén, O., Kwan, S-P., Lappalainen, I., Noordzij, J.G., Ochs, H.D., Ollila, J., Pienaar, S.M., Rukonen, P., Saha, B.K. and Smith, C.I.E. (1998) Btkbase, mutation database for X-linked agammaglobulinemia. *Nucl. Acid Res.* 1:244-250.

Vihinen, M., Nore, B.F., Mattsson, P.T., Bäckesjö, C.M., Nars, M., Koutaniemi, S., Watanabe, C., Lester, T., Jones, A., Ochs, H.D. and Smith, C.I.E. (1997) Missense mutations affecting a conserved cysteine pair in the TH domain of Btk. *FEBS Lett.* 413:205-210.

Vihinen, M., Vetrie, D., Maniar, H.S., Ochs, H.D., Zhu, Q., Vorechovsky, I., Webster, A.D.B., Notarangelo, L.D., Nilsson, L., Sowadski, L. and Smith, C.I.E. (1994) Structural basis for chromosome X-linked agammaglobulinemia. A tyrosine kinase disease. *Proc. Natl. Acad. Sci. USA* 91:12803-12807.

Vorechovski, I., Luo, L., Hertz, J.M., Frøland, S.S., Klemola, T., Fiorini, M., Quinti, I., Paganelli, R., Ozsahin, H., Hammarströmm, L., *et al.*(1997) Mutation pattern in the Bruton's tyrosine gene in 26 unrelated patients with X-linked agammaglobulinemia. *Hum. Mutat.* 9:418-425.

Vorechovski, I., Zhou, J-N., Hammerström, L., Smith, C.I.E., Thomas, J.D., Paul, W.E., Notarangelo, L.D. and Bernatowska-Matuszkiewicz, E. (1993) Absence of xid mutation in X-linked agamma-globulinemia. *Lancet* 342:552.

Wahl, M.I., Fluckinger, A-C., Kato, R.M., Park, H., Witte, O.N. and Rawlings, D.J. (1997) Phosphorylation of two regulatory tyrosine residues in the activation of Bruton's tyrosine kinase via alternative receptors. *Proc. Natl. Acad. Sci. USA* 94:11526-11533.

Walksman, G., Kominos, D., Robertson, S.C., Pant, N., Baltimore, D., Birge, R.B., Cowburn, D., Hananfusa, M., Mayer, B.J., Overduin, M., Resh, M.D., Rios, C.B., Silverman, L. and Kuriyan, J. (1992) Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. *Nature* 358:646-653.

Wall, R., Choi, E. and Carter, C. (1981) RNA processing in immunoglobulin gene expressing. *Cold Spring Harb. Symp. Quant. Biol.* 2:879-885.

Wechsler, R.J. and Monroe, J.G. (1995) Src-family tyrosine kinase p55^{fgr} is expressed in murine splkenic B cells and is activated in response to antigen receptor cross-linking. *J. Immunol.* 154:3234-3244.

Wedgwood, R.J. and Ochs, H.D. (1980) Variability in the expression of X-linked agammaglobulinemia: The co-existence of classic XLA (Bruton type) and 'common variable immunodeficiency' in the same families. *In* 'Primary Immunodefeciencies' (M. Seligmann and W.H. Hitzid, Eds.), p.69-78. Elsevier/ North-Holland Biomedical Press, Amsterdam.

Xie, H. and Rothstein, T.L. (1995) Protein kinase C mediates activation of nuclear cAMP response element-binding protein (CREB) in B lymphocytes. *J. Immunol.* 154:1717-1723.

Xie, H., Wang, Z. and Rothstein, T. (1996) Signalling pathways for antigen receptor-mediated induction of transcription factor CREB in B lymphocytes. *Cell Immunol.* 169:264-270.

Xu, Y., Davidson, L., Alt, F.W. and Baltimore, D. (1996) Deletion of the Ig Kappa light chain intronic enhancer/matrix attachment impairs but does not abolish V kappa J Kappa rearrangement. *Immunity* 4:377-385.

Yamanashi, Y., Kukui, Y., Wongsasant, W., Kinoshita, Y., Ichimori, Y., Toyoshima, K. and Yamamori, T. (1992) Activation of Src-like protein-kinase Lyn and its association with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signalling. *Proc. Natl. Acad. Sci. USA* 89:1118-1122.

Yamashita, Y., Miyake, K., Kikuchi, Y., Takatsu, K., Noda, S. and Kosugi, A. (1995) A monoclonal antibody against a murine CD38 homologue delivers a signal to B cells for prologation of survival and protection against apoptosis *in vitro*. *Immunol.* 85:248-255.

Yancopoulos, G.D. and Alt, F.W. (1986) Regulation of the assembly and expression of variable-region genes. *Ann. Rev. Immunol.* 4:339-368.

Yanagi, S., Sugawara, H., Kurosaki, M., Sabe, H., Yamamura, H. and Kurosaki, T. (1994) CD45 modulates phosphorylation of both autophosphorylation and negative regulatory tyrosines of Lyn in B cells. *J. Biol. Chem.* 271:30487-30492.

Yang, W-Y. and Desiderio, S. (1997) BAP-135, a target for Bruton's tyrosine kinase in response to B cell receptor engagement. *Proc. Natl. Acad. Sci. USA* 94:604-609.

Yang, W-Y., Malek, S.N. and Desiderio, S. (1995) A sequence motif conserved in Btk and related tyrosine kinases mediates SH3-dependent interactions *in vitro* and *in vivo*. *J. Biol. Chem.* 270:20832-20840.

Yao, L., Kawakami, Y. and Kawakami. T. (1994) The pleckstrin homology domain of Bruton's tyrosine kinase interactes with protein kinase C. *Proc. Natl. Acad. Sci. USA* 91:9175-9170.

Zhou, L-J., Ord, D.C., Hughes, A.L. and Tedder, T.F. (1991) Structure and domain organization of the CD19 antigen of human, mouse and guinea pig B lymphocytes: conservation of the extensive cytoplasmic domain. *J. Immunol.* 147:1424-1432.

Zhou, L-J., Smith, H.M., Waldschmidt, T.J., Schwarting, R. and Tedder, T.F. (1994) Tissue-specific expression of the human CD19 gene in transgenic mice inhibits antigen-independent B-lymphocyte developmen. *Mol. Cell. Biol.* 14:3884-3894.

Zhu, c., Bogue, M.A., Lim, D.A., Hasty, P. and Roth, D.B. (1996) Ku86-deficient mice exhibit severe combined immunodeficiecy and defective processing of V(D)J recombination intermediates. *Cell* 86:379-389.

Zou, R., Takeda, S. and rawjewski, K. (1993) Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangemnts in Ig kappa. *EMBO J.* 12:811-820.

Chapter 2

Inactivation of *Btk* by insertion of *LacZ* reveals defects in B cell development only past the pre-B cell stage

Rudolf W. Hendriks, Marella F.T.R. de Bruijn, Alex Maas, Gemma M. Dingjan, Alar Karis and Frank Grosveld

Inactivation of *Btk* by insertion of *lacZ* reveals defects in B cell development only past the pre-B cell stage

Rudolf W.Hendriks[1,2,3],
Marella F.T.R.de Bruijn[4], Alex Maas[1,2],
Gemma M.Dingjan[1], Alar Karis[1] and
Frank Grosveld[1]

Departments of [1]Cell Biology and Genetics and [4]Immunology, Faculty of Medicine, Dr Molewaterplein 50, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam and [2]Department of Immunohaematology, University Hospital Leiden, Rijnsburgerweg 10, Leiden, The Netherlands

[3]Corresponding author

Bruton's tyrosine kinase (Btk) is a cytoplasmic protein kinase that is defective in X-linked agammaglobulin-aemia in man and in X-linked immunodeficiency in the mouse. There is controversy regarding the stages of B cell development that are dependent on Btk function. To determine the point in B cell differentiation at which defects in Btk become apparent, we generated a mouse model by inactivating the *Btk* gene through an in-frame insertion of a *lacZ* reporter by homologous recombination in embryonic stem cells. The phenomenon of X-chromosome inactivation in *Btk*^{+/-} heterozygous female mice enabled us to evaluate the competition between B cell progenitors expressing wild-type *Btk* and those expressing the *Btk*[-]/*lacZ* allele in each successive step of development. Although *Btk* was already expressed in pro-B cells, the first selective disadvantage only became apparent at the transition from small pre-B cells to immature B cells in the bone marrow. A second differentiation arrest was found during the maturation from IgD[low]IgM[high] to IgD[high]IgM[low] stages in the periphery. Our results show that *Btk* expression is essential at two distinct differentiation steps, both past the pre-B cell stage.
Keywords: B cell development/Btk/immunodeficiency/XLA/*xid*

Introduction

B lymphocyte differentiation proceeds through successive steps involving two distinct phases (Figure 1). The first antigen-independent phase occurs in the bone marrow and includes the ordered rearrangement of immunoglobulin (Ig) heavy and light chain variable region genes, leading to the generation of surface IgM-bearing B cells (Alt *et al.*, 1987; Rolink and Melchers, 1991). In the mouse, the IgM[-] precursor B cells can be divided into four groups based on their differential expression of the cell surface markers CD43, heat stable antigen (HSA) and BP-1 (Hardy *et al.*, 1991). In the second phase, the immature IgM^{+} B cells leave the bone marrow and undergo antigen-driven proliferation and selection in secondary lymphoid tissues, where they gain an IgD[high]IgM[low] surface phenotype and

differentiate into either plasma cells or memory B cells (Hardy *et al.*, 1983; Nossal, 1993).

Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that is essential for B cell differentiation; mutations in the *Btk* gene lead to X-linked agamma-globulinaemia (XLA) in man and to X-linked immuno-deficiency (*xid*) in the mouse (reviewed in Conley *et al.*, 1994; Rawlings and Witte, 1994; Sideras and Smith, 1995). *Btk* encodes a 659 amino acid protein that, like the *src* family of tyrosine kinases, contains an SH2 and an SH3 domain and a single catalytic domain (Tsukada *et al.*, 1993; Vetrie *et al.*, 1993). *Btk*, together with the homologous *Tec*, *Itk*, *Bmx* and *Txk* genes, comprise a subfamily with a unique N-terminal region that contains a pleckstrin homology (PH) domain (Musacchio *et al.*, 1993) and a proline-rich *Tec* homology domain (Vihinen *et al.*, 1994) but lacks a myristylation site for membrane anchoring.

XLA patients display a large variety of genomic mutations, including deletions, insertions and point mutations in all domains, but missense mutations were found to be confined to the PH, SH2 and kinase domains (Vihinen *et al.*, 1996). XLA patients suffer from protracted and recurrent bacterial infections caused by very low serum concentrations of all Ig classes (Bruton, 1952). In the peripheral blood, surface Ig[+] B cell numbers are severely reduced, and in lymphoid tissues, plasma cells are virtually lacking; the small numbers of B cells that remain have an aberrant IgM[high] surface phenotype (Conley, 1985). In the bone marrow, the number of pre-B cells is either normal or decreased, and those pre-B cells present have a decreased proliferative activity (Pearl *et al.*, 1978; Campana *et al.*, 1990). Therefore, XLA reflects an almost complete failure of B cell precursors to differentiate into mature B cells. This defect is intrinsic to the B cell, because female XLA carriers manifest a unilateral X-chromosome inactivation in the peripheral blood B lymphocyte population (Conley *et al.*, 1986; Fearon *et al.*, 1987) because of a selective disadvantage of cells that have the defective *Btk* gene on the active X chromosome.

A less severe B cell deficiency has been observed in the CBA/N mouse strain carrying the *xid* mutation, which alters a highly conserved arginine (Arg28) in the PH domain into cysteine (Rawlings *et al.*, 1993; Thomas *et al.*, 1993). *xid* mice have ~50% fewer B cells in the spleen than normal, and the residual cells manifest an IgM[high]IgD[low] profile and cannot make antibodies to type-2 thymus-independent antigens (reviewed in Scher, 1982). In the serum of these mice, IgM and IgG3 concentrations are low, but other Ig classes are normal.

The milder phenotype of murine *xid* compared with human XLA cannot be explained by the nature of the mutations involved. Mutation of the same Arg28 amino
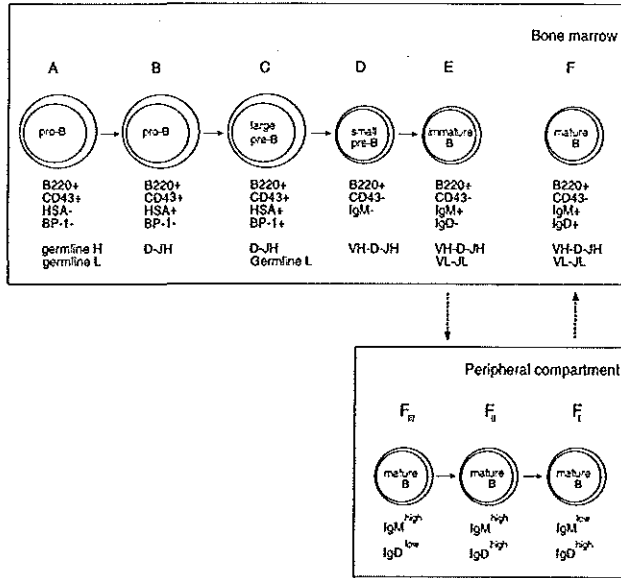
Fig. 1. B cell differentiation in the mouse. The classification of B lineage cells into bone marrow fractions A–F is according to the expression of cell surface molecules (Hardy et al., 1991) and the status of the Ig loci (Ehlich et al., 1993). The three mature B cell populations are distinguished on the basis of their differential expression of IgM and IgD (Hardy et al., 1982). The dotted arrows indicate that fraction F in the bone marrow reflects recirculating cells that have been selected in the periphery.

acid into histidine has been observed in patients with classic severe XLA phenotypes (de Weers et al., 1994a; Ohta et al., 1994). Furthermore, the extensive phenotypic analysis of mice deficient for Btk in their germline (Btk-kin−), which were generated recently by gene targeting, showed that the general absence of Btk protein also results in a xid phenotype (Kahn et al., 1995).

Btk is expressed throughout B cell differentiation, except in plasma cells (de Weers et al., 1993; Smith et al., 1994), and is implicated in several receptor-coupled signal transduction pathways, such as interleukin (IL)-5R, IL-6R, IL-10R, CD38 and CD40 (Go et al., 1990; Hasbold and Klaus, 1994; Sato et al., 1994; Matsuda et al., 1995; Santos-Argumedo et al., 1995). Stimulation of the BCR induced tyrosine phosphorylation of Btk in vivo and its kinase activity in vitro (Aoki et al., 1994; de Weers et al., 1994b; Saouaf et al., 1994).

At present it is not known which of the signalling functions of Btk are responsible for the immunodeficient phenotypes of XLA and xid. Moreover, the exact stage in B cell differentiation at which defects in Btk become apparent is also unclear. In the XLA patients analysed, the numbers of pre-B cells in the bone marrow varied: they were either not detectable or present in reduced or normal numbers (Pearl et al., 1978; Campana et al., 1990). Incidentally, pre-B cells exhibited a rare TdT+ phenotype. In the mouse, the xid or Btk-kin− mutations do not appear to affect pre-B cell numbers or proliferative capacities (Reid and Osmond, 1985; Kahn et al., 1995). The only obvious effect of the absence of Btk on the bone marrow B lineage fractions was a deficiency of recirculating

mature IgM+IgD+ B cells (Figure 1, fraction F), which would imply that B cell differentiation up to the immature IgM+IgD− B cell is normal (Kahn et al., 1995). However, the analyses of X-chromosome inactivation patterns in bone marrow from mice heterozygous for the xid mutation (Forrester et al., 1987) produced evidence for a slightly earlier arrest in differentiation: from the pre-B cell (fraction D) to the immature B cell (fraction E). In contrast with these findings, in vivo competition experiments of normal C57BL/6- and Btk− ES-derived B cell precursors indicated a deficit in the ability to expand between the pro-B/large pre-B (fractions A–C) and the pre-B cell (fraction D) (Kerner et al., 1995). Thus, the differentiation stage of B cell precursors in which Btk expression appears to be critical in mice and humans remains puzzling.

To gain further insight into the role of Btk in the expansion and differentiation of B cell precursors, we have also generated a mouse model in which the Btk gene is inactivated but have labelled the cells at the same time by inserting a lacZ reporter by homologous recombination in ES cells. Because of the process of random X-chromosome inactivation in Btk+/− heterozygous female mice, each X chromosome is active in about half of the cells within the bone marrow and the peripheral compartment. This phenomenon enabled us to evaluate the competition between cells expressing wild-type Btk and those expressing the Btk−/lacZ allele in each successive step of B cell development. This approach allows the novel detection of subtle defects that only lead to minor selective disadvantages, which may not be recognized in the phenotype of affected males.

Results

Generation of Btk⁻/lacZ-expressing mice

To inactivate the *Btk* gene, we constructed a vector that contained an in-frame fusion of 1.5 kb 5′ genomic sequences of *Btk* (at the *MunI* site in exon 8) to the nuclear localization signal (nls) of the SV40 large T antigen and the *Escherichia coli lacZ* gene (Figure 2A). The targeting vector included the herpes simplex virus (HSV) thymidine kinase gene for negative selection, the neomycin resistance gene for positive selection and 8 kb of 3′ *Btk* homology.

The linearized plasmid was transfected into E14 ES cells and, after double selection, surviving clones were screened by Southern blotting of *Eco*RI digests (Figure 2B). Four homologous recombination events were obtained out of 356 clones. These were karyotyped and subjected to further analysis to ensure that they carried intact *Btk* exons 1–7 and only a single *lacZ* copy (data not shown). Two of the *Btk*⁻ clones with a 70–90% normal karyotype were injected into mouse blastocysts to generate chimeric mice that transmitted the *Btk*⁻ allele through the germline. Male chimeras were bred to FVB or C57BL/6 mice to obtain heterozygous (*Btk*⁺/⁻) females or hemizygous (*Btk*⁻/Y) males (Figure 2B).

Western blotting experiments were performed on cell lysates from 5 week-old mice, using a polyclonal rabbit antiserum specific for Btk amino acids 163–218 to show the absence of Btk protein. The normal *Btk* gene encodes a 77 kDa protein that was detected in the spleen of *Btk*⁺ male and *Btk*⁺/⁻ female mice, but not in the spleen of *Btk*⁻ males nor in any thymus samples (Figure 2C). Transcription of the targeted allele would result in a hybrid mRNA, containing *Btk* sequences fused to nls*lacZ*/poly(A). By utilizing the regular first ATG in the *Btk* gene in exon 2, Btk (amino acids 1–234)–nls*lacZ* fusion proteins of 140–150 kDa would be generated in splenic B cells of *Btk*⁻ male mice. No such proteins could be detected in Western blotting analyses using the available Btk-specific antiserum (Figure 2C). However, evidence for transcriptional and translational activity of the targeted locus was provided by the detection of β-galactosidase activity in *Btk*⁻ male and *Btk*⁺/⁻ female mice (see below).

The Btk⁻/Y mice manifest an xid-like immunological phenotype

The effect of the *Btk*⁻/*lacZ* mutation on B cell maturation was investigated by flow cytometric analyses of cell samples from the spleen, mesenteric lymph node (MLN), thymus and peritoneal cavity (Table I). No significant differences could be detected between normal male or female mice and the *Btk*⁺/⁻ females. The phenotype of the *Btk*⁻/Y mice was characterized by decreased numbers of B220⁺ B cells in the periphery and a severe deficiency of CD5⁺ B cells in the peritoneum. Thymocyte and T cell numbers were not affected. In the myeloid compartment, no differences between the *Btk*⁻/Y and their normal littermates were observed (data not shown).

In 5 week-old *Btk*⁻/Y mice the serum concentrations of IgM and IgG3 were reduced significantly (50 and 20% of normal, respectively). The *in vitro* antibody responses to lipopolysaccharide of spleen cells demonstrated a severe deficiency in the production of IgG3 (0.11 ± 0.04 μg/ml

in *Btk*⁻/Y mice and 6.5 ± 1.2 μg/ml in normal littermates), whereas the production of IgM, IgG1 and IgG2a was equivalent in the two groups. Together with the IgM^high IgD^low surface profile of peripheral B cells (see below), these findings indicate that the immunological defects of the *Btk*⁻/Y mice are similar to those observed in *xid* or *Btk-kin*⁻ mice (Kahn *et al.*, 1995).

Expression of the Btk⁻/lacZ allele in the haematopoietic compartment

The introduction of the *lacZ* gene allowed us (i) to determine the *Btk* expression pattern in the affected male mice and (ii) to evaluate the competition between cells expressing wild-type *Btk* and those expressing the *Btk*⁻/*lacZ* allele in heterozygous females by using fluorescein-di-β-D-galactopyranoside (FDG) as a fluorogenic substrate in conjunction with flow cytometry.

A majority of the B220⁺ B cell precursors in the bone marrow of *Btk*⁻ males, and virtually all mature B cells in the periphery, expressed *lacZ* (Figure 3A). Because of the process of random X-chromosome inactivation, ~40–50% of the B220⁺ bone marrow cells were *lacZ*⁺ in *Btk*⁺/⁻ heterozygous female mice, with fluorescence intensities comparable with those in males. In contrast, only a small fraction of peripheral B cells in these mice expressed *lacZ* (Figure 3A). These findings illustrated a profound selection against those B cells that lack Btk, which has also been observed in *xid* (Nahm *et al.*, 1983; Forrester *et al.*, 1987) and XLA (Conley *et al.*, 1986; Fearon *et al.*, 1987).

No *lacZ* activity was detected in the peripheral CD4⁺ or CD8⁺ T cell populations. In the thymus of *Btk*⁻ males, *lacZ* expression was restricted to a small fraction (8.3 ± 0.3%) of the CD4⁻CD8⁻ cells. These cells, which could not be detected in *Btk*⁺/⁻ females, were found to be surface IgM⁺ and B220⁺, and most probably represent B cells present at very low numbers in the thymus (Myama-Inaba *et al.*, 1988). The *Btk*⁻/Y mice manifested *lacZ* activity in nearly all the ER-MP20^high cells (de Bruijn *et al.*, 1994) of the monocyte lineage in the bone marrow and the Mac-1⁺ peritoneal macrophages (Figure 3B). The ER-MP20^medium fraction of granulocyte precursors in the bone marrow manifested heterogeneous levels of *lacZ* expression. The mature granulocyte population in the spleen (Mac-1⁺/Gr-1⁺) contained 52 ± 6% of cells with a significant *lacZ* activity. There was no evidence for a critical function of *Btk* in the expansion or proliferation of cells in the myeloid lineage: in the heterozygous females the numbers of *lacZ*⁺ myeloid cells were approximately half those found in the *Btk*⁻ males. The variation in the fractions of *lacZ*⁺ cells between different female mice (e.g. 28–63% in peritoneal macrophages) most probably reflected individual differences in the X-chromosome inactivation ratios of the haematopoietic compartment.

Btk⁻ B cells show a defect in the maturation to IgM^low IgD^high stages in the periphery

Cell samples from spleen, MLN, peripheral blood and peritoneal cavity of 5 week-old mice were evaluated for *lacZ* activity in conjunction with their surface expression of IgM and IgD (Figure 4 and Table II). The *Btk*⁻/Y mice exhibited a selective deficiency of the mature IgM^low IgD^high population in the peripheral compartment, as found previously for the *xid* or *Btk-kin*⁻ mice (Hardy
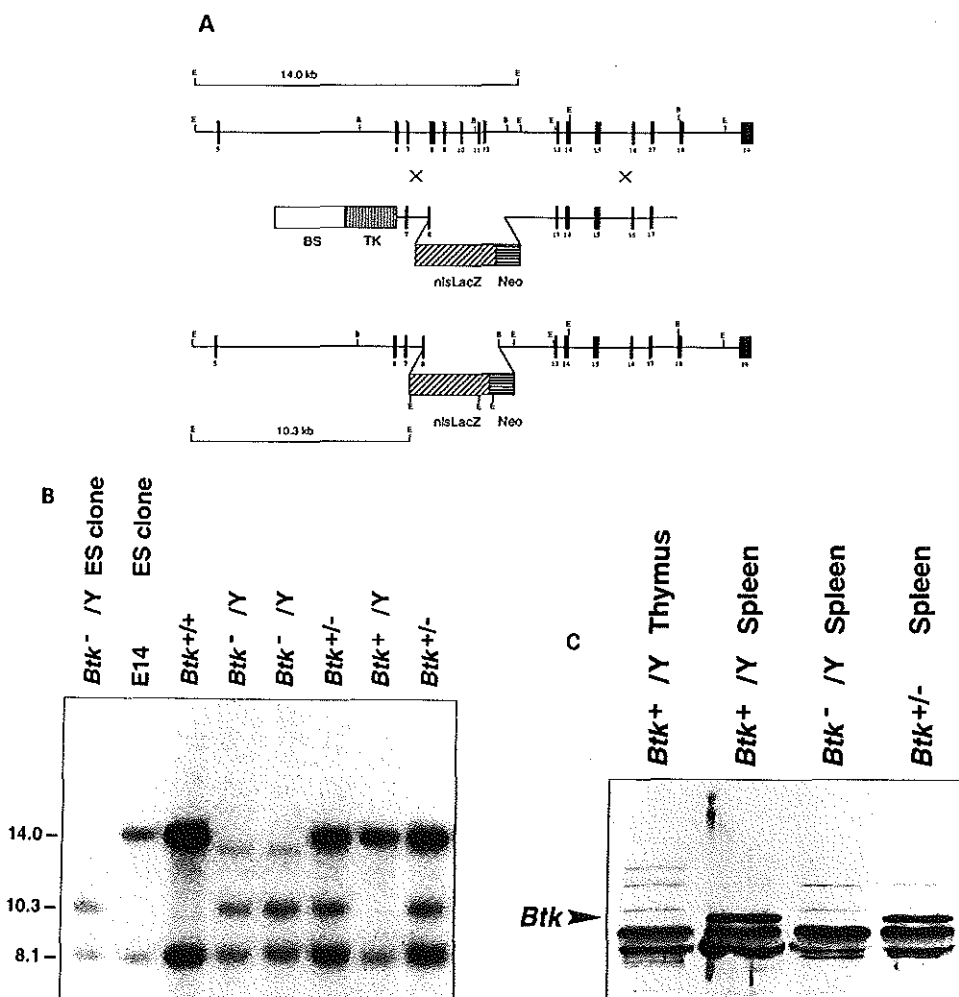
Fig. 2. Insertion of a *lacZ* reporter to disrupt the murine *Btk* gene. (A) A partial genomic map of the wild-type (top) and the targeted (bottom) murine *Btk* gene (Sideras *et al.*, 1994), encompassing exons 5–19, which are highlighted as closed boxes. In between the gene-targeting construct is shown, containing BS, the herpes simplex TK gene, the nuclear localization signal (nls) of simian virus large T fused to *E.coli lacZ* and the neomycin resistance gene (neo). The expected *EcoRI* fragments and their sizes before and after homologous recombination are indicated. B, *Bam*HI; E, *EcoRI*. (B) Southern blot analysis of DNA from a targeted (*Btk⁻/Y*) and the wild-type E14 ES cell clone, and of DNA from mouse tails, demonstrating germline transmission of the mutation. DNA samples were digested with *EcoRI* and probed with a human *Btk* cDNA fragment encompassing exons 2–11 (de Weers *et al.*, 1993), which shows an unaltered 8.1 kb band corresponding to exons 2 and 3. The 14 kb fragment is from the wild-type allele and the 10.3 kb fragment is from the targeted allele. The weak ~13 kb fragment (in the two *Btk⁻/Y* lanes) probably represents incomplete digestion of the *EcoRI* site in the nls because the fragment also hybridizes to a *lacZ* probe. The genotypes are indicated at the top [wild-type male (*Btk⁺/Y*) or female (*Btk⁺/⁺*), mutated male (*Btk⁻/Y*) and heterozygous female (*Btk⁺/⁻*)]. (C) Western blot analysis of Btk protein expression in total cell lysates from spleen cells or thymocytes. Btk was detected by a polyclonal rabbit antiserum raised against a fusion protein of glutathione S-transferase and amino acids 163–218 of the human *Btk* gene. The genotypes are given at the top; the position of the wild-type 77 kDa Btk band is indicated.

et al., 1982, 1983; Kahn *et al.*, 1995). Because a small fraction of peripheral B cells in *Btk⁺/⁻* heterozygous females express *lacZ*, it was possible to specifically analyse the surface phenotype of those cells that have the *Btk⁻/lacZ* allele on the active X chromosome. The gated *lacZ⁻* mature B cells exhibited an IgM/IgD surface profile similar to that of B cells in normal mice, whereas the gated *lacZ*-expressing cells manifested an IgMʰⁱᵍʰ phenotype reminiscent of the B cell population found in *Btk⁻/Y* mice (Figure 4). This phenomenon, which was

Table I. Flow cytometric analysis of the peripheral lymphocyte compartment

	Btk⁻/Yᵃ		Btk⁺/Y	
Spleen				
Total nucleated cells (×10⁶)	91	± 21	178 ± 58	
B220⁺ cells (%)	28	± 10	59	± 5.3
CD4⁺ cells (%)	12	± 1	8.6 ± 1.4	
CD8⁺ cells (%)	6.3 ± 0.2		5.9 ± 1.2	
MLN				
B220⁺ cells (%)	24	± 8.8	42	± 2.5
CD4⁺ cells (%)	47	± 6.6	36	± 0.9
CD8⁺ cells (%)	26	± 0.9	19	± 1.4
Peritoneum				
CD5⁺IgM⁺ B cells (%)	0.3 ± 0.1		8.5 ± 4.2	
CD5⁻IgM⁺ B cells (%)	2.7 ± 0.7		6.4 ± 1.8	
CD5⁺IgM⁻ T cells (%)	2.2 ± 1.0		2.3 ± 0.6	

ᵃMice were 6–8 weeks old. Numbers of mice analysed are two to four for spleen and MLN and four to eight for peritoneal cells.

Table II. Expression of *lacZ* in the peripheral B cell compartment in female *Btk*⁺/⁻ mice

Tissue	Age (weeks)	Percentage of *lacZ*⁺ cells		
		IgMʰⁱᵍʰIgDˡᵒʷ	IgMʰⁱᵍʰIgDʰⁱᵍʰ	IgMˡᵒʷIgDʰⁱᵍʰ
Spleen (*n* = 3)	5	35.7 ± 5.5	17.1 ± 5.3	2.8 ± 1.0
Spleen (*n* = 4)	20	21.6 ± 6.9	7.3 ± 2.8	1.2 ± 0.4
MLN (*n* = 2)	5	31.1 ± 6.9	8.6 ± 2.0	1.6 ± 0.2
Blood (*n* = 4)	5	30.0 ± 7.0	16.2 ± 6.5	2.7 ± 1.5
Peritoneum (*n* = 3)	5	1.8 ± 1.7¹	3.5 ± 3.2	1.7 ± 0.6

The percentages (mean ± standard deviations) of *lacZ*⁺ cells are given within the gated population. These values are corrected for individual X-chromosome inactivation ratios using the *lacZ* expression values from both peritoneal macrophages and ER-MP20ʰⁱᵍʰ monocytes in the bone marrow. In the *Btk*⁻/Y mice, *lacZ* activity was detected in 90–95% of cells; the background values in wild-type male or female mice were 1–4% for peritoneal cells and <2% for the other lymphoid tissues.
*The IgMʰⁱᵍʰIgDˡᵒʷ B cell fraction in the peritoneum consists mainly of CD5⁺ cells.
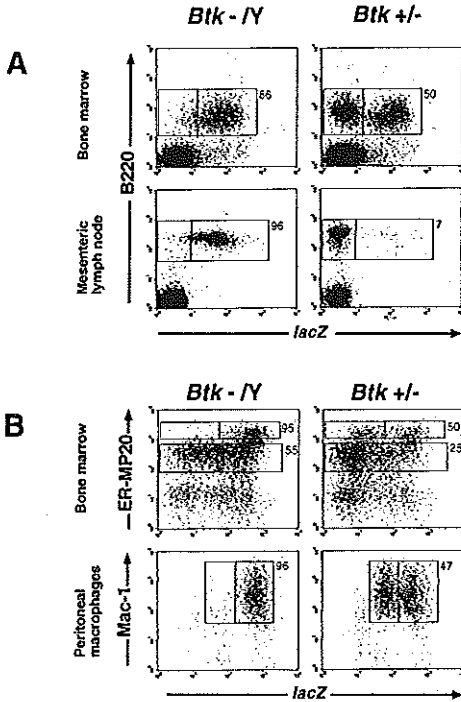

Fig. 3. Selection against *Btk*⁻*lacZ*⁺ cells in the B lineage but not in the myeloid lineage. Two-colour flow cytometric analysis of *lacZ* expression using FDG in 5 week-old *Btk*⁻/Y males and heterozygous *Btk*⁺/⁻ females. (A) The B cell compartment was analysed by staining with anti-B220-APC for bone marrow and anti-B220-PE for MLN cells. (B) Two distinct bone marrow myeloid populations are revealed by staining with ER-MP20: monocytes are ER-MP20ʰⁱᵍʰ and granulocytes are ER-MP20ᵐᵉᵈⁱᵘᵐ. The peritoneal macrophage population was gated by forward and side scatter, and revealed by staining with anti-Mac-1-PE. Data are shown as dot plots representative of the mice examined. The percentages of *lacZ*⁺ cells within the indicated gates are shown.

found in the spleen, MLN and peripheral blood, also implied that the fraction of *lacZ*-expressing cells in the heterozygous females decreased during maturation, from ~30% in IgMʰⁱᵍʰIgDˡᵒʷ to almost undetectable levels in IgMˡᵒʷIgDʰⁱᵍʰ mature B cells (Table II). The finding that *lacZ* expression in the IgMʰⁱᵍʰIgDˡᵒʷ fraction in *Btk*⁺/⁻ females was less than half that in males implied a selective disadvantage of *Btk*⁻*lacZ*-expressing cells at the stage in which the B cells from the bone marrow emerge into the periphery.

To investigate whether selection against *Btk*-deficient cells becomes more pronounced with increasing age, we analysed spleens from mice that were aged >4 months. The fraction of *lacZ*-expressing cells was found to be reduced when compared with 5 week-old mice, but nevertheless >20% of cells within the IgMʰⁱᵍʰIgDˡᵒʷ population still expressed the mutated allele.

In the peritoneum of *Btk*⁺/⁻ female mice, the development of both the conventional and the CD5⁺ B cells that express *lacZ* appeared to be compromised. The fraction of *lacZ*⁺ cells within the CD5⁺ (2.5 ± 0.8%, *n* = 5) or in the conventional B cell population (4.2 ± 1.5%, *n* = 5) did not exceed background values. Similar results were obtained when peritoneal B cells were classified on the basis of the Ig expression pattern (Table II).

Btk⁻ B cell progenitors show impaired differentiation from the pre-B cell stage onwards

A flow cytometric analysis of bone marrow cell suspensions of 5 week-old *Btk*⁻/Y mice and normal littermates revealed no significant alterations in the fractions of the myeloid or lymphoid/erythroid lineages or in the total size of the B220⁺ B cell precursor compartment (Table III). *Btk*⁻/Y mice had fewer IgM⁺IgD⁻ immature B cells (fraction E; ~70% of normal) and showed a significant decrease in the IgMˡᵒʷIgDʰⁱᵍʰ mature B cells (fraction F₁; ~10% of normal; Table III). Similar to the observations made on peripheral B lymphocytes, the numbers of IgMˡᵒʷIgDʰⁱᵍʰ mature B cells (fraction F₁) were reduced severely in both the affected males and the *lacZ*⁺ population in heterozygous females (Figure 5A). The analyses of gated *lacZ*⁻ and *lacZ*⁺ populations of B220⁺ bone
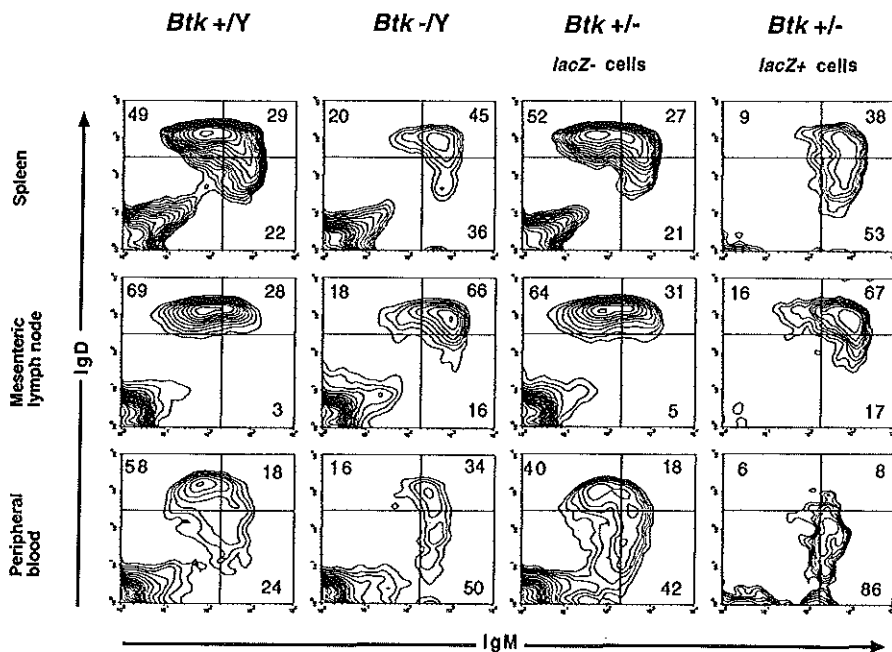
Fig. 4. The effect of the *Btk⁻* mutation on the surface IgM/IgD profile of peripheral B cells. Tri-colour flow cytometric analysis of spleen, MLN and peripheral blood from 5 week-old *Btk⁺/Y* normal males, *Btk⁻/Y* affected males and gated *lacZ⁻* and *lacZ⁺* cells from *Btk⁺/⁻* heterozygous females. The distribution of cells over the three compartments (I, IgM^{low}IgD^{high}; II, IgM^{high}IgD^{high}; III, IgM^{high}IgD^{low}; Hardy *et al.*, 1982) in percentages is indicated. Cells were loaded with FDG and stained with anti-IgD-PE, biotinylated anti-IgM and streptavidin-TriColor. Data are shown as 5% (or 10% for the gated *lacZ⁺* cells from *Btk⁺/⁻* females) probability contour plots. Dead cells were gated out, based on forward and side scatter characteristics.

Table III. Flow cytometric analysis of bone marrow cell populations

Cell fraction	Surface markers	Btk⁻/Y (n = 4)	Wild-type (n = 8)
Monocytes	ER-MP20^{high}	10 ± 2.7	10 ± 0.4[a]
Granulocytes	ER-MP20^{med}	40 ± 3.0	32 ± 7.5
Lymphoid/erythroid	ER-MP20⁻	48 ± 8.8	55 ± 10
Total B lineage	B220⁺	23 ± 12	24 ± 7
Pro-B (A, B and C)	B220⁺CD43⁺	5.3 ± 2.7	4.6 ± 1.6
Pre-B (D)	B220⁺CD43⁻IgM⁻	11 ± 4	12 ± 5
Immature B (E)	B220⁺IgM⁺IgD⁻	4.1 ± 0.7	6.1 ± 1.7[b]
Mature B (F)			
F$_{III}$	IgM^{high}IgD^{low}	0.62 ± 0.08	0.66 ± 0.16
F$_{II}$	IgM^{high}IgD^{high}	0.40 ± 0.12	0.63 ± 0.37
F$_{I}$	IgM^{low}IgD^{high}	0.08 ± 0.02	0.84 ± 0.62[c]

Total cell numbers (one femur and one tibia) were 53.0×10⁶ ± 7.1×10⁶ for the *Btk⁻/Y* mice and 46.0×10⁶ ± 6.6×10⁶ for wild-type mice.

The percentages (mean ± standard deviation) from total nucleated cells with the indicated phenotype are given.

[a]For non-parametric consideration of the hypothesis that the *Btk⁻/Y* mice manifested significant differences in cell numbers compared with wild-type littermates, the Mann–Whitney *U*-test was applied. Except for B lineage populations E and F$_{I}$, the values were not significantly different.

[b]The decrease in *Btk⁻/Y* mice was just significant, with a one-tailed *P* value of 0.036.

[c]The decrease in *Btk⁻/Y* mice was significant, with a one-tailed *P* value of 0.002.

marrow cells from *Btk⁺/⁻* female mice further revealed abundant *lacZ* expression in IgM⁻IgD⁻ cells (fractions A–D) and IgM⁺IgD⁻ cells (fraction E).

To identify the precise stage at which B-cell differentiation is impaired in the *Btk⁻* precursors, we performed four-colour flow cytometry using markers specific for the successive stages of B cell development. In the earliest stage (B220⁺CD43⁺HSA⁻, fraction A), ~59% of cells in *Btk⁻/Y* males were found to express *lacZ* (Figure 5B and C). In all following differentiation stages, *lacZ* activity was detected in the majority of cells. In the *Btk⁺/⁻* heterozygous female mice, ~30% of fraction A cells were *lacZ⁺*, i.e. about half the number found in the *Btk⁻* males. Therefore, the expression of *Btk* did not appear to be essential for the survival of B cell progenitors at this very early stage. Selection against the *Btk⁻/lacZ⁺* cells was also not observed in the pro-B cells of fraction B or in the pre-B cells (fractions C and D) (Figure 5C). These results indicate that development up to the large pre-B cell (fraction D) is not affected by the *Btk⁻* mutation. The first selective disadvantage of *Btk*-deficient cells became apparent at the transition from pre-B cells to immature B cells (fraction E). The percentages of *lacZ*-expressing cells in heterozygous females decreased from 43% in fraction D to 29% in fraction E (Figure 5C). When the immature B cells develop into the IgM^{high}IgD^{low} B cells of fraction F, the percentage of *lacZ*-expressing cells remains at ~30%, a value which was also found for the IgM^{high}IgD^{low}
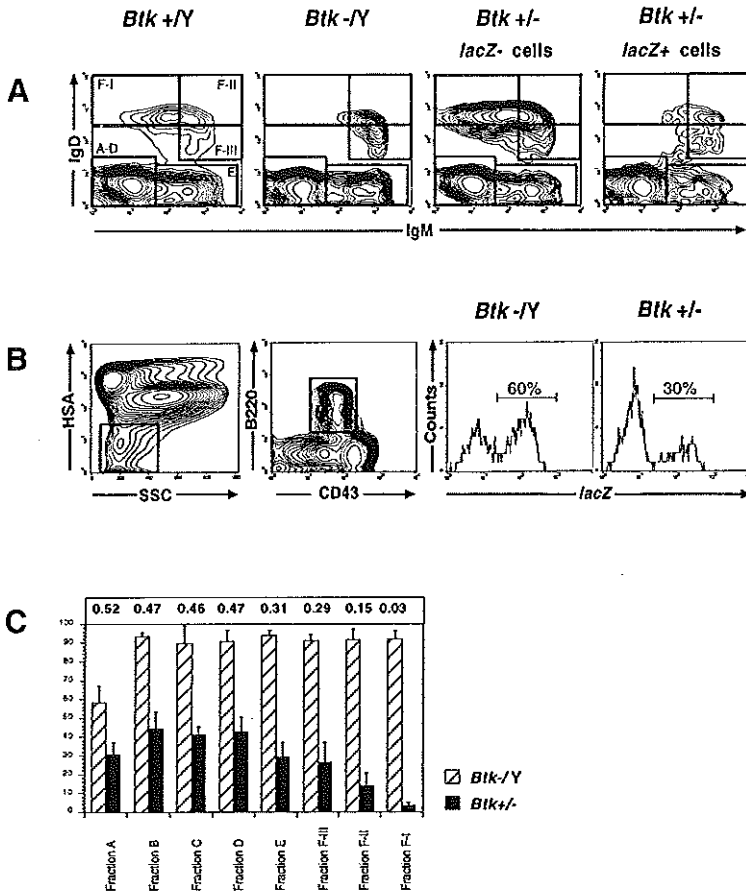
Fig. 5. The *Btk⁻* mutation affects the development of B cell precursors from the small pre-B to the mature B cell stage. (A) Bone marrow cell suspensions from 5 week-old *Btk⁺/Y* normal males, *Btk⁻/Y* affected males and *Btk⁺/⁻* heterozygous females were stained with FDG for *lacZ*, anti-B220-APC, anti-IgD-PE and anti-IgM (biotinylated)/streptavidin–Red 613. Gated B220⁺ (male samples), B220⁺/*lacZ⁻* and B220⁺/*lacZ⁺* cells (from a heterozygous female) are displayed. Data are shown as 5% probability contour plots. The gates for fractions A–D and E and the individual F fractions are shown. Dead cells were gated out, based on forward and side scatter characteristics. (B) Bone marrow cell suspensions from 5 week-old mice were loaded with FDG and stained with anti-B220-APC, anti-CD43-PE and anti-HSA (biotinylated)/streptavidin–Red 613. Fraction A was identified by analysing the low side scatter and the HSA⁻ fraction (gate in left contour plot) for the expression of CD43 and B220 (right contour plot), shown for a *Btk⁻/Y* mouse. Fraction A (B220⁺CD43⁺HSA⁻) was analysed for *lacZ* expression, and the result is displayed as a histogram. The percentage of *lacZ⁺* cells within fraction A is indicated for an affected male and a heterozygous female mouse. (C) Quantitation of the percentage of *lacZ⁺* cells in the individual B lineage fractions in the bone marrow of *Btk⁻/Y* male and *Btk⁺/⁻* female mice. Bone marrow cell suspensions were analysed for *lacZ* expression using FDG and stained with anti-B220-APC. The B220⁺ fractions were classified on the basis of the expression of two additional markers labelled with PE (anti-IgD, anti-CD43 or anti-HSA) or streptavidin–Red 613 (biotinylated anti-IgM or anti-BP-1) as follows: HSA⁻CD43⁺ (fraction A), HSA^{d-II}BP-1⁻ (fraction B), BP-1⁺CD43⁺ (fraction C), CD43⁺IgM⁻ (fraction D), IgM⁺IgD⁻ (fraction E), IgM^{high}IgD^{low} (fraction F$_{III}$), IgM^{high}IgD^{high} (fraction F$_{II}$) and IgM^{low}IgD^{high} (fraction F$_I$). Data shown are the mean values ± standard deviations obtained from the analyses in four *Btk⁻* male mice and five (fractions A–D) or seven (fractions E and F) heterozygous female mice. The values for the female mice are corrected for individual X-chromosome inactivation ratios (see legend to Table II). The numbers above the bars represent the mean value of the ratio between *lacZ⁺* cell percentages in heterozygous females and in the *Btk⁻/Y* males for each B lineage cell fraction. Using the non-parametric two-tailed Wilcoxon signed-rank test, these values were significantly different from 0.50 for fractions E, F$_{III}$, F$_{II}$ and F$_I$ ($P = 0.016$ in all four fractions).

B cells in the peripheral lymphoid compartment (Table II). During further maturation within the F fraction, selection against *Btk*-deficient cells was more profound. Finally, in the IgM^{low}IgD^{high} fraction, *lacZ* expression was virtually absent.

Discussion

To determine the stage in B cell development at which defects in Btk become apparent, we generated a mouse model in which the *Btk* gene is inactivated through an in-

frame insertion of a *lacZ* reporter. The *Btk*-/Y mice had moderately reduced B cell numbers in the periphery, a specific deficiency of IgM^{low}IgD^{high} mature B cells, an absence of peritoneal CD5$^+$ B cells, reduced IgM and IgG3 serum concentrations and their splenic B cells exhibited defective IgG3 production upon lipopolysaccharide stimulation *in vitro*. These findings confirm those of Kahn *et al.* (1995), that mice deficient for *Btk* in their germline exhibit an *xid* phenotype and that the elimination of *Btk* function does not lead to an almost complete block in B cell development, which is typical for XLA in man.

The presence of the *lacZ* reporter enabled us to determine the *Btk* expression profile *in vivo*. Moreover, because of the competition between cells expressing wild-type *Btk* and those expressing the *Btk*-/*lacZ* allele in heterozygous females, our strategy allowed the detection of the proliferative disabilities of *Btk*-deficient cells in each successive step of B cell development. The results clearly show that defects in Btk do not generally affect proliferation or survival throughout B cell differentiation, as has been proposed (Conley, 1985), but rather that Btk is essential at two distinct steps, i.e. at the transition from small pre-B cells to immature B cells in the bone marrow, and during the antigen-driven maturation of selected B cells in the periphery.

The expression pattern of Btk

The *Btk*-/*lacZ* mutation was designed to visualize the *Btk*-deficient cells in affected males and heterozygous females on the basis of *lacZ* activity using FDG as a substrate and resulting in fluorescence values that were ~20–30 times background levels. The absence of any detectable *Btk*–*lacZ* fusion proteins of the expected size in Western blotting probably indicates that our anti-Btk antiserum does not recognize such fusion proteins. The alternative explanation, of a translation start further downstream, is not very likely because only the 77 kDa protein starting at the first ATG is normally present *in vivo* (Tsukada *et al.*, 1993) and the ATG at the introduced *lacZ* cassette does not fulfil the sequence context criteria for an initiation codon (Kozak, 1987).

The *Btk* expression profile was determined using the *lacZ* gene activity under the control of the endogenous *Btk* promoter and regulatory elements. Consistent with previous findings in cell lines (de Weers *et al.*, 1993; Tsukada *et al.*, 1993; Genevier *et al.*, 1994; Smith *et al.*, 1994), we found expression of the *Btk* gene to be restricted to the myeloid and B cell compartments. Within the monocyte–macrophage lineage, *Btk* expression appeared to be abundant in all cells. In the granulocyte population in bone marrow and spleen, *Btk* was expressed in a major fraction of the cells but at variable levels. Peripheral T cells did not contain detectable amounts of *Btk* and, moreover, we did not find any evidence for *Btk* expression in the earlier stages of T cell development in the thymus.

The analyses of B220$^+$ cells in the bone marrow and the peripheral compartment showed that the *Btk* gene is expressed in the vast majority of cells throughout B cell differentiation. The finding of *Btk*-expressing cells in the very early fraction of HSA$^-$ pro-B cells in the bone marrow, fraction A, implies that *Btk* expression precedes the expression of many other genes associated with early

B cell development, such as TdT, Mb-1, V$_{pre-B}$, λ5, RAG-1 and RAG-2 (Li *et al.*, 1993). The fact that not all cells in fraction A uniformly express Btk may partly be explained by its heterogeneous nature because it appears that not all the cells are completely committed to the B lineage (Hardy *et al.*, 1991; Rolink *et al.*, 1996).

Btk is not required for the generation of mature myeloid cells

Btk was found to be expressed in the bone marrow myeloid cells, in splenic granulocytes and in peritoneal macrophages. The absence of any selective disadvantage of *Btk*-deficient cells in heterozygous females demonstrates that *Btk* expression as such is not required for the expansion or differentiation of the various mature myeloid cell populations. This conclusion is further supported by the apparently normal macrophage function observed in *xid* mice (Scher, 1982) and the random X-chromosome inactivation patterns found in peripheral blood granulocytes of XLA carriers (Conley *et al.*, 1986; Fearon *et al.*, 1987). The neutropenia that is frequently observed in XLA patients has been assumed to be secondary to ongoing infection because it resolves after Ig substitution therapy (Kozlowski and Evans, 1991). The absence of any detectable effects of *Btk* deficiency on myeloid cells may by explained by redundancies within tyrosine kinase pathways or may indicate that *Btk* is involved in a signalling pathway that is not essential for myeloid cell survival.

Btk is critical only past the pre-B cell stage

The analyses of *lacZ* expression in the bone marrow of *Btk*$^{+/-}$ heterozygous female mice showed that *Btk*-deficient B cell progenitors develop to the small pre-B cell stage at normal frequencies. Furthermore, because none of the pro-B or pre-B fractions contained >50% *lacZ*$^+$ cells, there was also no accumulation of B cell precursors that are blocked in differentiation. The earliest detectable selective disadvantage of *Btk*-deficient cells was recognized at the transition from small pre-B to immature B cells. In the heterozygous females only 29% (reduced from 50%) of the immature B cells expressed *lacZ*, which is in good agreement with the reduction in size of this population in affected males (70% of normal). These findings agree with previous findings that *xid* pro-B or pre-B cells are unaffected by this mutation, whether in a hemizygous male (Reid and Osmond, 1985) or in competition with normal cells in a heterozygous female (Forrester *et al.*, 1987). In contrast, Kahn *et al.* (1995) noticed an increase in the pro-B cell population in both CBA/N *xid* and *Btk-kin*$^-$ mice, indicating some role for *Btk* in the transition from pro-B to pre-B cells. However, the significance of this finding is unclear because *xid* mice with a C57BL/6 background manifested a slightly reduced pro-B cell fraction size. Our results are in apparent conflict with those obtained by Kerner *et al.* (1995), who observed a severe failure of Btk$^-$ cells to expand to the small pre-B cell stage in *in vivo* competition assays, in which *Btk*$^-$ ES cells were injected into wild-type blastocysts. We cannot formally exclude the possibility that there are functional differences in the respective mutations, but both targeted disruptions almost certainly represent true null alleles. Alternatively, it is conceivable that the selection against

Btk⁻ pre-B cells may result from genetic background differences between 129 ES cell-derived H-2^{b}/Ly9.1^{+} cells and C57BL/6 blastocyst-derived H-2^{b}/Ly9.2^{+} cells. In this context, the same *Btk⁻* ES cells only partially rescued peripheral B cell numbers in RAG-2-deficient hosts (1–15% of normal), whereas blastocyst complementation by *Btk-kin⁻* ES cells (Kahn et al., 1995) resulted in chimeras with B cell numbers comparable with those in *xid* mice (60–70% of normal). Because in heterozygous mice *Btk*-expressing and *Btk*-deficient cells arise through the process of X-chromosome inactivation, there are no additional genetic differences between the two cell types in our *Btk-/lacZ* mouse model.

A dramatic selection against *Btk⁻* cells only occurs late in B cell differentiation, during antigen-driven proliferation and maturation of the B cells. In the heterozygous females, the more immature IgM^{high}IgD^{low} cell population still contains a substantial proportion of *Btk*-deficient cells, whereas in the IgM^{low}IgD^{high} mature B cell population these cells are virtually absent. With increasing age, selection against the *Btk*-deficient B cells becomes more pronounced. In this respect, there are no differences between our *Btk*-deficient mice and the *xid* mice (Forrester et al., 1987). The major differentiation arrest in the periphery is consistent with a crucial function of *Btk* in B cell receptor signalling. This hypothesis is also supported by the observed lower responses of *xid* B cells to anti-Ig antibodies (Rigley et al., 1989), and is substantiated further by the findings of tyrosine phosphorylation of Btk *in vivo* accompanied by an increase in its kinase activity *in vitro* after B cell receptor stimulation (Aoki et al., 1994; de Weers et al., 1994b; Saouaf et al., 1994).

Because affected males lack CD5^{+} B cells in the peritoneum, it was not surprising to see that *Btk*-deficient CD5^{+} B cells were also absent in heterozygous females. However, the finding that *lacZ*-expressing cells were also not detected within the conventional B cell population in the peritoneum would argue for a more critical role for *Btk* in the development of conventional B cells in the peritoneum compared with other peripheral lymphoid compartments.

Functional roles of Btk in early B cell development

Our findings imply that in the mouse Btk is not involved in signalling pathways essential for the proliferation of pro-B and early pre-B cells, which is stroma cell-dependent and driven by ILs such as IL-4, IL-5 and in particular IL-7 (Rolink and Melchers, 1991). In addition, the fraction of *lacZ*$^{+}$ cells in *Btk*$^{+/-}$ female mice remains the same (~30%) between the immature B cells in the bone marrow (fraction E) and the IgM^{high}IgD^{low} B cells in the periphery (Figure 5C and Table II). Together with earlier findings, that the homing properties and recirculating abilities of *xid* B lymphocytes are normal (Sprent et al., 1985), our results indicate that *Btk* expression is not relevant for the migration process of immature B cells from the bone marrow into the periphery, or the initiation of surface IgD expression.

A role for *Btk* in signalling at the stage between pre-B cells and B cells still remains puzzling. Moreover, the outcome of defective *Btk* function appears to be different between mice (which exhibit an impaired transition to immature B cells) and man (where an almost complete

block is observed at this stage). These defects may well involve the same stage of B cell development, but with quantitative differences between mice and man. In addition, a large variation in the numbers of pre-B cells present in the bone marrow of XLA patients (Pearl et al., 1978; Fu et al., 1980; Campana et al., 1990) would point towards a role for *Btk* in the survival of pre-B cells in humans. In this context, it is interesting to note that mice and humans show differences in their expression patterns of Ig surrogate light (SL) chains at the transition from pre-B to immature B cells (Lassoued et al., 1993; Karasuyama et al., 1994). Although both murine and human pre-B cell lines express SL chains on the cell surface in association with µH chain, the surface expression of this µH–SL complex on normal bone marrow cells can only be detected in humans (on late stage pre-B cells; Lassoued et al., 1993). This offers the possibility of an interaction of the µH–SL complex with an environmental ligand to promote the transition from pre-B to immature B cells in humans. In the mouse, the surface expression of SL ceases once µH is produced; the small pre-B cells express µH intracellularly but do not express SL in either the surface or the cytoplasm. An attractive model for *Btk* function in the pre-B cell would be its involvement in signal transduction from the µH–SL complex, which in humans would be essential for the enhanced survival of pre-B cells and further differentiation into the immature B cell. In mice, the role for *Btk* appears to be less critical because immature B cells are generated at only moderately reduced numbers.

In summary, our *Btk-/lacZ* mouse model allows the novel detection in heterozygous females of subtle defects that may only lead to minor selective disadvantages for *Btk*-deficient cells. In future studies, such as transgenic rescue experiments or crosses with other knock-out strains, these mice will again offer the same possibility of the identification of defects which may not be recognized from the phenotype of only affected males.

Materials and methods

Construction of the targeting vector

Btk-containing cosmid clones were isolated from a 129 mouse genomic DNA library (Nuez et al., 1995) by screening with a human *Btk* cDNA probe (de Weers et al., 1993). A 2.0 kb SalI–SacI end fragment encompassing exons 7 and 8, and a 7.4 kb BamHI fragment encompassing exons 13–17, were cloned into Bluescript (BS). A 4.6 kb BspHI fragment, with the nls*lacZ* gene and the *neo*-selectable marker (Nuez et al., 1995), was blunted and cloned into a partially digested and blunted MunI fragment at exon 8. The in-frame insertion of the *lacZ* gene at *Btk* Leu235 was verified by a DNA sequence analysis. A KpnI–SpeI fragment containing the PyEn-HSV thymidine kinase (TK) gene was subsequently introduced in the BS polylinker upstream of exon 7. The same 4.6 kb BspHI nls*lacZ*–*neo* fragment was also cloned into the SmaI site of the BS polylinker at the 5' end of the 7.4 kb BamHI fragment. The targeting vector was constructed by combining the two plasmids, one containing the TK gene, 5' *Btk* homology and nls*lacZneo*, and one containing nls*lacZ*–*neo* and 3' *Btk* homology, using a unique ClaI site in the *lacZ* gene and KpnI sites in the BS polylinkers.

Generation of mutant mice

E14 ES cells were cultured in BRL-conditioned medium supplemented with 1000 U/ml leukaemia inhibitory factor. The targeting vector was linearized with NotI and electroporated into ES cells (8 µg DNA per 10^{7} cells in 500 µl), as described previously (Zhou et al., 1995). Selection with 0.2 mg/ml G418 and 0.2 µM FIAU was started 24 h after electroporation. After 8–10 days, double-resistant clones were selected and expanded, partly frozen in 10% dimethylsulfoxide and partly used

to isolate genomic DNA. Screening for homologous recombinants was performed by Southern blotting of EcoRI-digested DNA and hybridization to a human Btk cDNA fragment clone (133–1153 bp) hybridizing to exons 2–11 (de Weers et al., 1993). Four mutant E14 clones were identified and verified by Southern blot analyses of EcoRI, BamHI and HindIII digests, using genomic probes specific for Btk exon 1 (1.8 kb mouse genomic EcoRI fragment), exon 2 (0.9 kb mouse genomic PstI fragment), exons 12–19 (1.0 kb human 3' fragment cDNA probe; de Weers et al., 1993) and a lacZ probe. Two ES clones carrying the Btk-lacZ mutation were injected into blastocysts of C57BL/6 mice (Robertson, 1987) and transplanted into BL6/CBA foster mothers. The resulting male chimeras that had >80% agouti were mated to C57BL/6 females. Germline transmission of the targeted Btk allele to female offspring was verified by a Southern blot analysis. These females were further bred to C57BL/6 males; therefore the mice analysed had a mixed 129/C57BL/6 background.

Western blotting
For the Western blotting analysis, lysates of 5×10^5 cells were separated on 10% SDS–PAGE gels under reducing conditions and transferred to nitrocellulose. Blots were blocked with 5% non-fat dry milk in 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20 and incubated with first- and second-step reagents in the same buffer with 1% non-fat dry milk. The polyclonal Btk antiserum was raised against fusion proteins of glutathione S-transferase and amino acids 163–218 of the human Btk protein (Katz et al., 1994). The antibody, which also detects murine Btk because amino acids 163–206 are completely conserved, was used in a 1:1000 dilution with horseradish peroxidase-conjugated swine anti-rabbit Ig as a second step and developed by enhanced chemiluminescence, as described previously (de Weers et al., 1994b).

Flow cytometric analysis
Single-cell suspensions were prepared from lymphoid organs in PBS, essentially as described previously (Slieker et al., 1993). Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). β-Galactosidase activity was analysed by loading 2×10^5 cells in 50 μl Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS) with an equal volume of 2 mM FDG (Molecular Probes Europe, Leiden, The Netherlands) at 37°C for 60 s (Nolan et al., 1988). To stop the reaction, 1 ml ice-cold DMEM/FBS was added. FDG-loaded cells were allowed to react with the FDG substrate for 30 min (on ice), and subsequently stained with monoclonal antibodies. When kept on ice, FDG staining was found to be stable up to 7 h after loading.

Approximately $1-2\times10^6$ cells were incubated with antibodies in PBS/0.5% bovine serum albumin (BSA)/20 mM NaN₃ in 96-well U-bottomed plates for 30 min at 4°C. Cells were washed twice in PBS/0.5% BSA/NaN₃. For each sample, 3×10^4 cells were scored using a FACScan analyser (Becton Dickinson, Sunnyvale, CA) equipped with appropriate filters for a three-colour analysis. For four-colour immunofluorescence of the bone marrow samples, $1-3\times10^5$ events were scored using a FACS Vantage dual laser (488 and 595 nm excitation) facility at Becton Dickinson (Erembodegem, Belgium). FACS data were analysed using CellQuest version 1.0 computer software; statistical evaluations were performed with Instat version 2.0 (Graphpad Software, San Diego, CA).

The following monoclonal antibodies were purchased from PharMingen (San Diego, CA): PE-conjugated anti-CD5/Ly-1, anti-CD11b/Mac-1, anti-HSA/M1/69 and anti-CD43/S7; biotinylated anti-HSA/M1/6, anti-BP-1/6C3 and anti-IgM; and Cy-Chrome- and APC-conjugated anti-B220/RA3-6B2. Fluorescein isothiocyanate-conjugated anti-B220/RA3-6B2 was obtained from Sigma (St Louis, MO), and PE-conjugated anti-IgD was purchased from Southern Biotechnology (Birmingham, AL). PE-conjugated anti-CD4 was purchased from Becton Dickinson. Anti-CD8/53-6.7, anti-Gr-1/RB6-8C5 and ER-MP20 were purified monoclonal antibodies conjugated to biotin (de Bruijn et al., 1994). Secondary antibodies used were TriColor- or PE-conjugated streptavidin (Caltag Laboratories, CA) or streptavidin–Red 613 (Becton Dickinson).

In vitro cultures and Ig ELISA
Whole-spleen cell suspensions from 5 week-old mice were prepared and cultured with 50 μg/ml E.coli lipopolysaccharide as described previously (Savelkoul et al., 1988). The culture supernatants were assayed for the production of IgM, IgG1, IgG2a and IgG3 on day 7 in an isotype-specific ELISA using antisera purchased from Southern Biotechnology.

References

Alt,F.W., Blackwell,T.K. and Yancopoulos,G.D. (1987) Development of the primary antibody repertoire. Science, 238, 1079–1087.

Aoki,Y., Isselbacher,K.J. and Pillai,S. (1994) Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells. Proc. Natl Acad. Sci. USA, 91, 10606–10609.

Bruton,O.C. (1952) Agammaglobulinemia. Pediatrics, 9, 722–728.

Campana,D., Farrant,J., Inamidar,N., Webster,D.B. and Janossy,G. (1990) Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. J. Immunol., 145, 1675–1680.

Conley,M.E. (1985) B cells in patients with X-linked agammaglobulinemia. J. Immunol., 134, 3070–3074.

Conley,M.E., Brown,P., Pickard,A.R., Buckley,R.H., Miller,D.S., Raskind,W.H., Singer,J.W. and Fialkow,P.J. (1986) Expression of the gene defect in X-linked agammaglobulinemia. N. Engl. J. Med., 315, 564–570.

Conley,M.E., Parolini,O., Rohrer,J. and Campana,D. (1994) X-linked agammaglobulinemia: new approaches to old questions based on the identification of the defective gene. Immunol. Rev., 138, 5–21.

de Bruijn,M.F.T.R., Slieker,W.A.T., Van der Loo,J.C.M., Voerman,J.S.A., Van Ewijk,W. and Leenen,P.J.M. (1994) Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. Eur. J. Immunol., 24, 2279–2284.

de Weers,M., Verschuren,M.C.M., Kraakman,M.E.M., Mensink,R.G.J., Schuurman,R.K.B., Van Dongen,J.J.M. and Hendriks,R.W. (1993) The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. Eur. J. Immunol., 23, 3109–3114.

de Weers,M., Mensink,R.G.J., Kraakman,M.E.M., Schuurman,R.K.B. and Hendriks,R.W. (1994a) Mutation analysis of the Bruton's tyrosine kinase gene in X-linked agammaglobulinemia: identification of a mutation which affects the same codon as is altered in immunodeficient xid mice. Hum. Mol. Genet., 3, 161–166.

de Weers,M., Brouns,G.S., Hinshelwood,S., Kinnon,C., Schuurman, R.K.B., Hendriks,R.W. and Borst,J. (1994b) B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. J. Biol. Chem., 269, 23857–23860.

Ehlich,A., Schaal,S., Gu,H., Kitamura,D., Müller,W. and Rajewski,K. (1993) Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. Cell, 72, 695–704.

Fearon,E.R., Winkelstein,J.A., Civin,C.I., Pardoll,D.M. and Vogelstein,B. (1987) Carrier detection in X-linked agammaglobulinemia by analysis of X chromosome inactivation. N. Engl. J. Med., 316, 427–431.

Forrester,I.M., Ansell,J.D. and Micklem,H.S. (1987) Development of B lymphocytes in mice heterozygous for the X-linked immunodeficiency (xid) mutation. J. Exp. Med., 165, 949–958.

Fu,S.M., Hurley,J.N., McCune,J.M., Kunkel,H.G. and Good,R.A. (1980) Pre-B cells and other possible precursor lymphoid cell lines derived from patients with X-linked agammaglobulinemia. J. Exp. Med., 152, 1519–1526.

Genevier,H.C. et al. (1994) Expression of Bruton's tyrosine kinase protein within the B cell lineage. Eur. J. Immunol., 24, 3100–3105.

Go,N.F., Castle,B.E., Barret,R., Kastelein,R., Dang,W., Mosmann,T.R., Moore,K.W. and Howard,M. (1990) Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X-chromosome-linked immunodeficiency B cells. J. Exp. Med., 172, 1625–1631.

Hardy,R.R., Hayakawa,K., Haaijman,J. and Herzenberg,L.A. (1982) B-cell subpopulations identified by two-colour fluorescence analysis. Nature, 297, 589–591.

Hardy,R.R., Hayakawa,K., Parks,D.R. and Herzenberg,L.A. (1983) Demonstration of B cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence. Nature, 306, 270–272.

Hardy,R.R., Carmack,C.E., Shinton,S.A., Kemp,J.D. and Hayakawa,K. (1991) Resolution and characterization of pro-B and pre-B cell stages in normal mouse bone marrow. *J. Exp. Med.*, 173, 1213–1225.

Hasbold,J. and Klaus,G.G.B. (1994) B cell from CBA/N mice do not proliferate following ligation of CD40. *Eur. J. Immunol.*, 24, 152–157.

Kahn,W.F. *et al.* (1995) Defective B cell development and function in *Btk*-deficient mice. *Immunity*, 3, 283–299.

Karasuyama,H., Rolink,A., Shinkai,Y., Young,F., Alt,F.W. and Melchers,F. (1994) The expression of V$_{pre-B}$/λ5 surrogate light chain in early bone marrow precursor B cells or normal and B cell-deficient mutant mice. *Cell*, 77, 133–143.

Katz,F.E., Lovering,R.C., Bradley,L.A.D., Rigley,K.P., Brown,D., Cotter,F., Chessels,J.M., Levinsky,R. and Kinnon,C. (1994) Expression of the X-linked agammaglobulinemia gene, *btk*, in B-cell acute lymphoblastic leukemias. *Leukaemia*, 8, 574–577.

Kerner,J.D., Appleby,M.W., Mohr,R.N., Chien,S., Rawlings,D.J., Maliszewski,C.R., Witte,O.N. and Perlmutter,R. (1995) Impaired expansion of mouse B cell progenitors lacking *Btk*. *Immunity*, 3, 301–312.

Kozak,M. (1987) An analysis of 5′ noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.*, 15, 8125–8148.

Kozlowski,C. and Evans,D.I.K. (1991) Neutropenia associated with X-linked agammaglobulinemia. *J. Clin. Pathol.*, 44, 388–390.

Lassoued,K., Nuñez,C.A., Billips,L., Kubagawa,H., Nonteiro,R.C., LeBien,T.W. and Cooper,M.D. (1993) Expression of surrogate light chain receptors is restricted to a late stage in pre-B cell differentiation. *Cell*, 73, 73–86.

Li,Y.-S., Hayakawa,K. and Hardy,R.R. (1993) The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. *J. Exp. Med.*, 178, 951–960.

Matsuda,T. *et al.* (1995) Association and activation of Btk and Tec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines. *Blood*, 85, 627–633.

Musacchio,A., Gibson,T., Rice,P., Thompson,J. and Saraste,M. (1993) PH domains: a common piece in the structural patchwork of signalling proteins. *Trends Biochem. Sci.*, 18, 343–348.

Miyama-Inaba,M., Kuma,S., Inaba,K., Ogata,H., Iwai,H., Yasumizu,R., Muramatsu,S., Steinman,R.M. and Ikehara,S. (1988) Unusual phenotype of B cells in the thymus of normal mice. *J. Exp. Med.*, 168, 811–816.

Nahm,M.H., Paslay,W.J. and Davie,J.M. (1983) Unbalanced X-chromosome mosaicism in mice with X-linked immunodeficiency. *J. Exp. Med.*, 158, 920–931.

Nolan,G.P., Fiering,S., Nicolas,J.-F. and Herzenberg,L.A. (1988) Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*. *Proc. Natl Acad. Sci. USA*, 85, 2603–2607.

Nossal,G.J.V. (1992) The molecular and cellular basis of affinity maturation in the antibody response. *Cell*, 68, 1–2.

Nuez,B., Michalovich,D., Bygrave,A., Ploemacher,R. and Grosveld,F. (1995) Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature*, 375, 316–318.

Ohta,Y. *et al.* (1994) Genomic organization and structure of Bruton agammaglobulinemia tyrosine kinase: localization of mutations associated with varied clinical presentations and course in X chromosome-linked agammaglobulinemia. *Proc. Natl Acad. Sci. USA*, 91, 9062–9066.

Pearl,E.R., Vogler,L.B., Okos,A.J., Christ,W.M., Lawton,A.R.,III and Cooper,M.D. (1978) B lymphocyte precursors in human bone marrow: an analysis of normal individuals and patients with antibody-deficiency states. *J. Immunol.*, 120, 1169–1175.

Rawlings,D.J. and Witte,O.N. (1994) Bruton's tyrosine kinase is a key regulator in B cell development. *Immunol. Rev.*, 138, 105–119.

Rawlings,D.J. *et al.* (1993) Mutation of the unique region of Bruton's tyrosine kinase in immunodeficient mice. *Science*, 261, 358–361.

Reid,G.K. and Osmond,D.G. (1985) B lymphocyte production in the bone marrow of mice with X-linked immunodeficiency (*xid*). *J. Immunol.*, 135, 2299–2302.

Rigley,K.P., Harnett,M.M., Phillips,R.J. and Klaus,G.G.B. (1989) Analysis of signaling via surface immunoglobulin receptors on B cells from CBA/N mice. *Eur. J. Immunol.*, 19, 2081–2085.

Robertson,E.J. (1987) *Teratocarcinomas and Embryonic Stem Cells*. IRL Press at Oxford University Press, Oxford, UK.

Rolink,A. and Melchers,F. (1991) Molecular and cellular origins of B lymphocyte diversity. *Cell*, 66, 1081–1094.

Rolink,A., ten Boekel,E., Melchers,F., Fearon,D.T., Krop,I. and Andersson,J. (1996) A subpopulation of B220+ cells in murine bone

marrow does not express CD19 and contains natural killer cell progenitors. *J. Exp. Med.*, 183, 187–194.

Santos-Argumedo,L., Lund,F.E., Heath,A.W., Solvason,N., Wu,W.W., Grimaldi,C., Parkhouse,R.M.E. and Howard,M. (1995) CD38 unresponsiveness of *xid* B cells implicates Bruton's tyrosine kinase (*btk*) as a regulator of CD38 induced signal transduction. *Int. Immunol.*, 7, 163–170.

Saouaf,S.J., Mahajan,S., Rowley,R.B., Kut,S.A., Fargnoli,J., Burkhardt,A.L., Tsukada,S., Witte,O.N. and Bolen,J.B. (1994) Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc. Natl Acad. Sci. USA*, 91, 9524–9528.

Sato,S. *et al.* (1994) IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. *J. Exp. Med.*, 180, 2101–2111.

Savelkoul,H.F.J., Lebman,D.A., Benner,R. and Coffman,R.L. (1988) Increase of precursor frequency and clonal size of murine IgE-secreting cells by IL-4. *J. Immunol.*, 141, 749–755.

Scher,I. (1982) The CBA/N mouse strain: an experimental model illustrating the influence of the X-chromosome on immunity. *Adv. Immunol.*, 33, 1–71.

Sideras,P. and Smith,C.I.E. (1995) Molecular and cellular aspects of X-linked agammaglobulinemia. *Adv. Immunol.*, 59, 135–223.

Sideras,P. *et al.* (1994) Genomic organization of mouse and human Bruton's agammaglobulinemia tyrosine kinase (*Btk*) loci. *J. Immunol.*, 153, 5607–5617.

Slieker,W.A.T., de Rijk-de Bruijn,M.F.T.R., Leenen,P.J.M. and van Ewijk,W. (1993) ER-MP12 antigen, a new surface marker on mouse bone marrow cells with thymus-repopulating ability. I. Intrathymic repopulating ability of ER-MP12-positive bone marrow cells. *Int. Immunol.*, 5, 1093–1098.

Smith,C.I.E. *et al.* (1994) Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J. Immunol.*, 152, 557–565.

Sprent,J., Bruce,J., Ron,Y. and Webb,S.R. (1985) Physiology of B cells in mice with X-linked immunodeficiency. I. Size, migratory properties, and turnover of the B cell pool. *J. Immunol.*, 134, 1442–1448.

Thomas,J.D., Sideras,P., Smith,C.I.E., Vorechovsky,I., Chapman,V. and Paul,W.E. (1993) Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science*, 261, 355–358.

Tsukada,S. *et al.* (1993) Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*, 72, 279–290.

Vetrie,D. *et al.* (1993) The gene involved in X-linked agammaglobulinemia is a member of the *src* family of protein tyrosine kinases. *Nature*, 361, 226–233.

Vihinen,M., Nilsson,L. and Smith,C.I.E. (1994) Tec homology (TH) adjacent to the PH domain. *FEBS Lett.*, 350, 263–265.

Vihinen,M., Iwata,T., Kinnon,C., Kwan,S.-P., Ochs,H.D., Vorechovsky,I. and Smith,C.I.E. (1996) BTKbase, mutation database for X-linked agammaglobulinemia (XLA). *Nucleic Acids Res.*, 24, 160–165.

Zhou,X.Y. *et al.* (1995) Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes Dev.*, 9, 2623–2634.

Chapter 3

The X-linked immunodeficiency defect in the mouse is corredted by expression of human *Bruton's tyrosine kinase* from a yeast artificial chromosome transgene.

Alex Maas, Gemma M. Dingjan, Huub F.J. Savelkoul, Christine Kinnon , Frank Grosveld and Rudolf W. Hendriks

Alex Maas[1,2],
Gemma M. Dingjan[1],
Huub F. J. Savelkoul[3],
Christine Kinnon[4],
Frank Grosveld[1] and
Rudolf W. Hendriks[1,2]

[1] Department of Cell Biology and
Genetics, Faculty of Medicine,
Erasmus University Rotterdam,
Rotterdam, The Netherlands
[2] Department of
Immunohematology, University
Hospital Leiden, Leiden, The
Netherlands
[3] Department of Immunology,
Faculty of Medicine, Erasmus
University Rotterdam, Rotterdam,
The Netherlands
[4] Molecular Immunology Unit,
Institute of Child Health, London,
GB

The X-linked immunodeficiency defect in the mouse is corrected by expression of human *Bruton's tyrosine kinase* from a yeast artificial chromosome transgene

Mutations in the gene for Bruton's tyrosine kinase result in the B cell differentiation defects X-linked agammaglobulinemia in man and X-linked immunodeficiency in mice. Here we describ the generation of two yeast artificial chromosome (YAC)-transgenic mouse strains in which high-level expression of human *Btk* is provided by endogenous regulatory cis-acting elements that are present on a 340-kb transgene, Yc340-h*Btk*. The expression pattern of the transgenic human *Btk* was found to parallel that of the endogenous murine gene. When the Yc340-h*Btk*-transgenic mice were mated onto a *Btk*-deficient background, the *xid* B cell defects were fully corrected: conventional and CD5+ B-1 B cells were present in normal numbers, serum IgM and IgG3 levels as well as responses to T cell-independent type II antigens were in the normal ranges. *In vivo* competition experiments in *Btk*^{+/-} female mice demonstrated that in the conventional B cell population the Yc340-h*Btk* transgene could fully compensate the absence of expression of endogenous murine *Btk*. We conclude that in the YAC-transgenic mice *Btk* is appropriately expressed in the context of native regulatory sequences.

1 Introduction

Bruton's tyrosine kinase (Btk) is a non-receptor protein tyrosine kinase that is mutated in X-linked agammaglobulinemia (XLA) in man and X-linked immunodeficiency in the mouse [1–4]. XLA is characterized by severe and recurrent bacterial infections. Affected males have very low serum levels of all Ig classes. In the periphery, surface Ig+ B cell numbers are severely decreased and plasma cells are virtually missing. Because in BM of XLA patients pre-B cells are present, the disease is manifested as an arrest in differentiation of pre-B cells to later B cell stages (for review see [5]). Although *Btk*-deficient mice exhibit a less severe B cell deficiency, the first selective disadvantage of *Btk*-deficient cells was also found at the transition from small pre-B to immature B cells in the BM [6]. Both CBA/N mice carrying an Arg[28] pleckstrin homology (PH) domain mutation, and mice with targeted disruptions of *Btk* in their germ line display the x-linked immunodeficiency (*xid*) phenotype. The disorder is characterized by a decrease of peripheral B cell numbers, specifically of mature surface IgM[low]IgD^{high} cells, an absence of perito-

[I 17084]

neal CD5+ B-1 B cells, low levels of serum IgM and IgG3 and severely impaired responses to T cell-independent type II (TI-II) antigens [6–8].

The *Btk* gene encodes a 659-amino acid protein that contains a single kinase domain, the *src* homology domains SH2 and SH3, and an N-terminal region with a PH domain and a unique proline-rich Tec homology (TH) domain [1–5]. Several molecules, including *Src* family kinases, protein kinase C, βγ subunits of heterotrimeric G proteins, and the 120-kDa protein encoded by the c-*cbl* proto-oncogene have been shown to interact with the individual domains of *Btk*, mainly by studies *in vitro* (reviewed in [9]). *Btk* has been implicated in signaling events induced by cross-linking of the surface Ig receptor, IL-5, IL-6, CD38 and CD40 in B cells and FcεRI in mast cells and basophils [5, 9].

The expression pattern of the *Btk* gene was investigated in mice and man using cultured cell lines [10, 11], as well as by analysis of β-galactosidase activity *in vivo* in mice with a targeted in-frame insertion of a *lacZ* reporter in the *Btk* gene [6]. *Btk* is expressed throughout B cell development, from the earliest identifiable pro-B cell stage (B220+CD43+HSA-Ig-; 12) up to mature B cell stages. At the transition from mature B cells to plasma cells, expression is down-regulated. *Btk* is not expressed in the T cell lineage. Although *Btk* is also expressed in myeloid cells, it is not required for myeloid differentiation, since myeloid cells are not affected in XLA or in *xid* [5].

The tissue-specific and differentiation stage-specific *Btk* expression may -- at least in part -- be accomplished by the *Btk* promoter region, which contains several binding sites for the transcription factors Sp1 and PU.1 [13, 14]. Although transient transfection experiments implicate PU.1 as a major regulator for *Btk* expression in B cells and myeloid cells, other elements may well be required, especially because *Btk* expression is not abolished in fetal liver of PU.1-deficient mice [14].

To develop methods to investigate the effects of *Btk* mutations *in vivo* and to analyze the developmental control of *Btk* gene expression, we generated *Btk*-transgenic mice. Because far upstream or downstream cis-acting regulatory elements are essential for correct expression of genes [15-17], large DNA transgenes are needed to exclude any influence of the chromatin environment at the position of integration in the mouse genome [17, 18]. We have used a 340-kb yeast artificial chromosome (YAC) clone containing the human *Btk* gene to generate transgenic mice in which the expression of *Btk* is under the control of native regulatory sequences. By mating these *Btk* transgenic mice onto a *Btk*-deficient background, we show that the *xid* phenotype in the mouse is completely corrected by expression of human *Btk* from the YAC transgene.

2 Materials and methods

2.1 Southern and Western blotting

High molecular weight DNA was extracted and processed by Southern blotting analyses using standard methods [19]. The 340-kb YAC clone Yc340-h*Btk* was from the ICI human DNA YAC library [20] and was selected by hybridization to probes specific for the DXS178 locus. The DXS101, DXS178, DXS265, DXS366, DXS442, 5D8/DDP and GLA probes and cDNA, for which the Yc340-h*Btk* YAC was screened, have been described [21, 22]. In addition, the following probes were applied: *Btk.1* (human *Btk* cDNA bp 133-1153 [23]; *Btk.2* (human *Btk* cDNA bp 1049-2159), *FTP-3*, a 1.8-kb PCR fragment from position 9373 to 10749 [24], *YcL* (YAC left arm probe; a 2.3-kb *PvuII-EcoRI* fragment of pBR322 [25]) and *YcR* (YAC right arm probe; a 1.4 *PvuII-SalI* fragment of pBR322 [25]).

Western blotting was performed as described [6].

2.2 Isolation of YAC DNA and generation of transgenic mice

Isolation of the YAC was performed essentially as described [18], except that the agarose blocks of yeast cells were made in 1 % low-melting point agarose in SCE (1 M sorbitol, 100 mM sodium citrate pH 5.8, 10 mM EDTA) and a final zymolyase concentration of 4 mg/ml. The agarose blocks were incubated in SCE containing 10 mM DTT at 37 °C for 2 h, followed by incubation in 400 mM EDTA pH 7.5, 1 % sarkosyl and 2 mg/ml proteinase K at 50 °C overnight. Size-fractionation of yeast chromosomes, isolation and preparation of YAC DNA were as described [18]. Purified YAC DNA was micro-injected into pronuclei of FVB fertilized oocytes [26] at various concentrations (2-10 ng/ml).

2.3 Reverse transcription polymerase chain reaction

Isolation of RNA and cDNA synthesis have been described [27]. PCR reactions were performed in 50 μl PCR buffer (Life Technologies, Paisley, GB), 1 pmol/μl of each primer and 0.3 μCi of [α-32P] dATP. Amplification was for 25 cycles with denaturation at 94 °C for 30 s, fol-lowed by annealing at 58 °C for 30 s and extension at 72 °C for 45 s. PCR products were visualized by electrophoresis on a 6 % polyacrylamide 1X TBE gel followed by autoradiography. Quantifications were performed using IMAGEQUANT (Molecular Dynamics). Primers specific for human *Btk* (5'-GACTGCTGAACACATTGCCC-3' and 5'-AGAAGTAGAACCAAGAAGCTTAT-3') and murine *Btk* (5'-GACAGCAGAACACATTGCTCA-3' and 5'-CTCAGGAAACATGGAATTAGGT-3') were in exons 18 and 19. Murine hypoxanthine phosphoribosyl-transferase (*HPRT*) primers have been described [27].

2.4 Flow cytometric analyses

Preparation of single-cell suspensions, loading of cells with fluorescein-di-β-D-galactopyranoside to measure β-galactosidase activity and three-color flow cytometry have been described [6]. Cells (3×10^4-5×10^4) were scored using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). mAb obtained from Pharmingen (San Diego, CA) included PE-conjugated anti-CD4, anti-CD5, anti-CD11b/Mac-1 and anti-CD43; biotinylated anti-IgM and anti-CD8; FITC-conjugated anti-B220/RA3-6B2 and anti-CD3. PE-conjugated anti-IgD was purchased from Southern Biotechnology Associates (SBA, Birmingham, AL). TriColor-conjugated streptavidin (Caltag Laboratories, CA) was used as a secondary antibody.

2.5 Serum Ig detection and immunizations *in vitro*

Serum levels of Ig subclasses were determined by isotype-specific sandwich ELISA [28]. To measure TI-II responses, mice were injected i.p. with 50 μg DNP-Ficoll in PBS and DNP-specific Ig subclasses were analyzed at day 7 by ELISA. To measure thymus-dependent (TD) responses, mice were immunized with 100 μg TNP-KLH precipitated with alum; after 3 months a booster dose of 100 μg TNP-KLH was given and TNP-specific Ig subclasses were analyzed at day 7 after the booster. For DNP/TNP-specific sandwich ELISA assays, plates were coated with isotype-specific antibodies and serum dilutions were incubated over night. Subsequent steps were biotinylated TNP-KLH, streptavidin-coupled peroxidase and azino-bis-ethylbenzthiazoline-sulfonic acid (ABTS) as a substrate. Absorbance (A) was read at 419 nm.

3 Results

3.1 The Yc340-h*Btk*-transgenic mice contain the human *Btk* gene

The YAC clone Yc340-h*Btk* was isolated from the ICI YAC library [20] on the basis of hybridization with the DXS178 probe, which is 80-150 kb distal to *Btk* [1, 21]. Yc340-h*Btk* was found to contain the 40 kb human *Btk* gene, approximately 100 kb of 5' flanking DNA and 200 kb of 3' flanking DNA (Fig. 1A). The YAC also included several other genes, such as α-galactosidase A, the ribosomal protein L44L, the RNA-binding protein FTP-3 and the deafness/dystonia peptide (DDP) [22, 24], as well as the polymorphic markers DXS178 and DXS265. The YAC was negative for more distant markers, including DXS366,
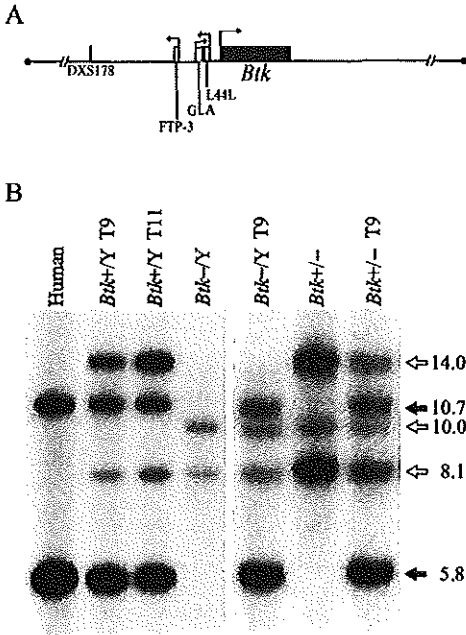
A

B

Figure 1. Generation of Yc340-h*Btk*-transgenic mice. (A) Map of the 340-kb YAC transgene construct, showing the location of the *Btk* gene (filled box), the *L44L*, α-galactosidase A (GLA) and *FTP-3* genes (open boxes) and the polymorphic marker DXS178. Arrows indicate the direction of transcription. (B) Southern blot analysis of DNA from the two different Yc340-h*Btk*-transgenic mouse lines T9 and T11 on various backgrounds, wild-type (*Btk+*), knockout (*Btk-*), or heterozygous females (*Btk+/-*), as well as from human and mouse controls. DNA samples were digested with *Eco*RI and probed with a human *Btk* cDNA fragment (*Btk.1*). The closed arrows indicate the 10.7-kb and 5.8-kb Yc340-h*Btk*-transgenic bands. The open arrows indicate the endogenous murine bands, which are 8.1 kb and 14 kb (wild-type allele) or 8.1 and 10.0 (knockout allele). The genotypes are indicated at the top.

DXS442 and DXS101. In a comparison of Yc340-h*Btk* with other DXS178-positive YAC and genomic DNA, by pulse-field gel electrophoresis, Southern blotting and Alu fingerprinting, we did not find evidence that the Yc340-h*Btk* is rearranged, deleted or a hybrid clone (data not shown).

The Yc340-h*Btk* YAC was isolated by pulse-field electrophoresis, purified and microinjected. Out of 50 offspring born, two h*Btk*-transgenic mouse lines, T9 and T11, were obtained (Fig. 1B). In Southern blotting analyses using *Bam*HI, *Eco*RI, *Hin*dIII, *Xba*I and *Pvu*II and probes specific of the entire *Btk* gene, FTP-3 and DXS178, all restriction fragments present in the Yc340-h*Btk* YAC clone were also found in the transgenic lines; both lines contained left and right YAC arm sequences. Densitometric quantifications indicated that T9 and T11 were low-copy transgenic lines, containing two to three copies of the h*Btk* gene (data

not shown). Fluorescence *in situ* hybridization of metaphase chromosomes to human *Btk* sequences demonstrated single integration spots at chromosome 14 for T9 and chromosome 3 for T11. In the transgenic lines, no developmental defects or any increased susceptibility to infectious diseases or malignancies were observed on various genetic backgrounds (FVB, 129 or C57BL/6) for over 10 months of age.

3.2 The expression pattern of transgenic human Btk

To investigate whether the expression of the transgenic human *Btk* gene is comparable to that of the endogenous murine *Btk* gene, RT-PCR experiments were performed on various tissues from Yc340-h*Btk*-transgenic mice (Fig. 2A). Both the human and the murine *Btk* gene were found to be highly expressed in spleen and BM, whereas

A

B
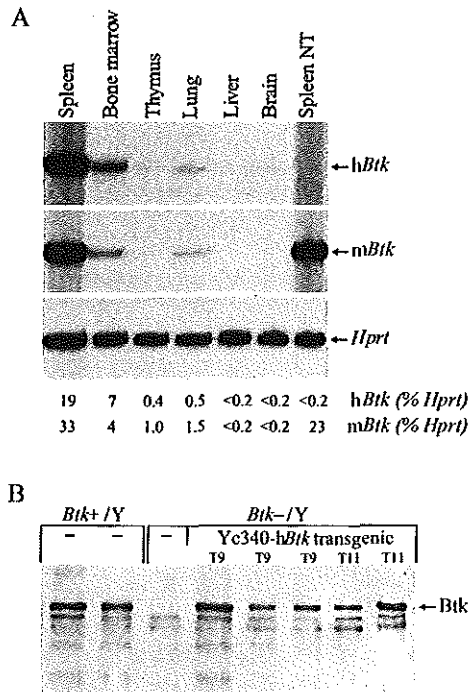
Figure 2. Expression of the Yc340-h*Btk* transgene. (A) RT-PCR analysis of cDNA samples from the indicated tissues from a Yc340-h*Btk*-transgenic *Btk+/+* mouse. Spleen from a non-transgenic mouse was included as a control (NT). The human and the murine *Btk*-specific amplified products were ~200 and ~230 bp, respectively. Yields were normalized using *HPRT* RT-PCR products (~250 bp). At the bottom, the relative densities of the human and the murine *Btk* RT-PCR products are given as percentages of the values of the *HPRT* RT-PCR products. (B) Western blot analysis of *Btk* expression in total cell lysates (2 × 10⁵ cells/lane) from spleen from 2-month-old wild-type (*Btk+/Y*), knockout (*Btk-/Y*) and Yc340-h*Btk*-transgenic *Btk-/Y* mice. The position of the 77-kDa *Btk* band is indicated.

Table 1. Flow cytometric analysis of the lymphocyte compartments in Yc340-h*Btk*-transgenic mice[a]

Tissue	Cell population	Non-transgenic		Yc340-h*Btk*-transgenic	
		Btk⁺/Y	*Btk*⁻/Y	*Btk*⁺/Y	*Btk*⁻/Y
Bone marrow	B220⁺ cells, %	16 ± 3	15 ± 2	19 ± 4	19 ± 1
	Pro-B (CD43⁺), %	3 ± 1	3 ± 1	3 ± 1	3 ± 1
	Pre-B (CD43⁻IgM⁻), %	5 ± 2	5 ± 2	7 ± 1	6 ± 1
	Immature B (IgM⁺IgD⁻), %	4 ± 2	3 ± 1	4 ± 1	3 ± 1
	Mature B (IgM⁺IgD⁺), %	4 ± 1	1.0 ± 0.4	4 ± 1	5 ± 2
Spleen	Nucleated cells, × 10⁶	192 ± 33	82 ± 15	173 ± 28	155 ± 27
	B220⁺ cells, %	51 ± 10	29 ± 12	55 ± 3	55 ± 7
	IgM^{low}IgD^{high} B cels, %	31 ± 5	6 ± 3	30 ± 3	31 ± 4
	CD3⁺CD4⁺ T cells, %	20 ± 3	19 ± 5	19 ± 2	18 ± 1
	CD3⁺CD8⁺ T cells, %	11 ± 4	10 ± 3	10 ± 3	11 ± 3
MLN	B220⁺ cells, %	24 ± 4	12 ± 2	24 ± 4	23 ± 6
	IgM^{low}IgD^{high} B cells, %	21 ± 2	3 ± 1	17 ± 4	18 ± 4
	CD3⁺CD4⁺ T cells, %	48 ± 7	52 ± 9	45 ± 3	38 ± 8
	CD3⁺CD8⁺ T cells, %	23 ± 3	27 ± 6	23 ± 3	23 ± 4
Peritoneum	CD5⁺ B cells, %	4 ± 2	0.3 ± 0.3	3 ± 2	9 ± 5
	CD5⁻ B cells, %	10 ± 6	2 ± 1	10 ± 4	15 ± 9
	CD5⁺ T cells, %	4 ± 2	4 ± 2	3 ± 3	4 ± 2

a) Mice were 6–10 weeks old. Numbers of mice anlyzed are: 7 for Yc340-h*Btk*-transgenic *Btk*⁺/Y mice and 4–7 for the other groups.

low but detectable signals were present in thymus and lung. Quantification of the densities of the amplified products indicated that the expression levels of the human and the murine *Btk* genes were similar. Murine or human *Btk* RT-PCR products were not detected in liver, brain, kidney, intestine, ovary or heart (Fig. 2A, and data not shown), indicating the absence of additional, integration site-dependent, expression of the Yc340-h*Btk* transgene.

The Yc340-h*Btk*-transgenic mice were mated onto a *Btk*⁻ background, *i.e.* mice with a targeted inactivation of the *Btk* gene by an in-frame insertion of a *LacZ* cassette [6]. Btk protein expression was evaluated by Western blotting of total spleen cell lysates from Yc340-h*Btk*-transgenic *Btk*⁻/Y male mice (Fig. 2B). Using a polyclonal rabbit antiserum which recognizes both human and murine Btk, a 77-kDa band was observed in wild-type *Btk*⁺/Y mice and absent in knockout *Btk*⁻/Y mice. In the two Yc340-h*Btk*-transgenic lines T9 and T11 on the *Btk*⁻/Y background, Btk protein was detected at levels comparable to those in wild-type mice (Fig. 2B). In all further analyses described below, the two Yc340-h*Btk* lines did not exhibit any differences and, therefore, data from the two transgenic lines were combined.

3.3 Transgenic Yc340-h*Btk* expression restores B cell numbers

To investigate whether expression of the Yc340-h*Btk* transgene could fully compensate the absence of the endogenous murine *Btk* gene, we examined the lymphoid populations in four groups of 6–10-week-old male mice: wild-type and *Btk*⁻/Y mice, either non-transgenic or Yc340-h*Btk*-transgenic (Table 1; Fig. 3). Flow cytometric analyses of BM, spleen, mesenteric lymph nodes (MLN), thymus and peritoneum displayed the *xid* phenotype in the *Btk*⁻/Y mice [6–8]. The numbers of B lymphocytes, in particular recirculating IgM⁺IgD⁺ B cells in the BM and mature IgM^{low}IgD^{high} B cells in the periphery, were significantly reduced and the population of CD5⁺ B-1 B lymphocytes in

the peritoneal cavity was missing. In contrast, the *Btk*⁻/Y mice that expressed the Yc340-h*Btk* transgene had normal numbers of mature B cells in BM, spleen and MLN, and of CD5⁺ B-1 B cells in the peritoneum (Table 1; Fig. 3). The additional expression of the Yc340-h*Btk* transgene on the wild-type background did not have any effect on the B cell numbers. The four groups of mice analyzed did not show significant differences in the numbers of myeloid or T lineage cells.

3.4 Transgenic Yc340-h*Btk* expression restores serum Ig levels and responses to TI-II antigens

Although Yc340-h*Btk*-transgenic *Btk*⁻/Y mice had normal B cell numbers, a possibility remained that B cell function was not fully restored.

Serum levels of all Ig isotypes were determined by ELISA in 2-month-old mice from the four genotype groups described in Sect. 3.3. The *Btk*⁻/Y knockout mice had decresed concentrations of IgM and IgG3 in the serum (Fig. 4A), whereas the levels of IgG1, IgG2a, IgG2b, IgA or IgE were in the normal range (data not shown). The Yc340-h*Btk*-transgenic mice, both on the wild-type background and on the *Btk*⁻/Y background, manifested serum Ig levels that were in the normal range for all isotypes, including IgM and IgG3 (Fig. 4A).

To analyze the responsiveness to TI-II antigens *in vivo*, 2-month-old mice were injected i.p. with DNP-Ficoll, and after 7 days DNP-specific antibodies were measured by ELISA (Fig. 4B). In *Btk*-deficient mice, DNP-specific IgM or IgG3 was completely absent: absorbance measured did not differ from the values of unimmunized animals. In contrast, the TI-II responses of Yc340-h*Btk*-transgenic mice, both on a wild-type and on a *Btk*⁻/Y background, were comparable to those of wild type mice. As a control, secondary responses were measured after immunization *in vivo* with the T cell-dependent antigen TNP-KLH. The TNP-specific IgM, IgG1 and IgG2a responses were not sig-
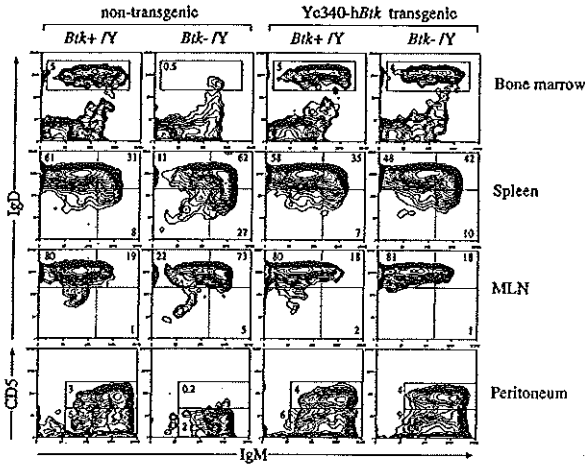
Figure 3. The effect of the Yc340-h*Btk* transgene on peripheral B lymphocyte populations. Three-color flow cytometric analysis of BM, spleen, MLN, and peritoneal cavity from 6–8-week-old non-transgenic and Yc340-h*Btk*-transgenic *Btk*+/Y or *Btk*-/Y mice. Spleen, MLN and BM cell suspensions were stained with biotinylated anti-IgM and streptavidin-TriColor, PE-conjugated anti-IgD, and FITC-conjugated anti-B220, and B220+ cells are displayed. Percentages of total BM cells that are B220+IgM+IgD+ are displayed in the boxes (Fraction F; [12, 29]). For spleen and MLN the proportions of cells that are IgM^{low}IgD^{high} (Fraction I; [29]), IgM^{high}IgD^{high} (Fraction II), and IgM^{high}IgD^{low} (Fraction III) are shown. Peritoneal cells were stained with anti-B220, anti-IgM anti-CD5-PE and B220+ cells are displayed. The percentages of total cells (including peritoneal macrophages) that are B220+CD5+ B cells or conventional B220+CD5- B cells are indicated. Data shown are representative of the mice examined and shown as 5% probability contour plots.

nificantly different between the four groups of mice analyzed (Fig. 4C).

In summary, these results show that transgenic Yc340-h*Btk* expression did not affect serum Ig concentrations of TI-II or TD responses *in vivo* in the presence of endogenous murine *Btk*. More importantly, the selective IgM and IgG3 deficiency in the serum and the unresponsiveness *in vivo* to TI-II antigens of *Btk*-/Y knockout mice was restored by transgenic *Yc340*-h*Btk* expression.

3.5 *In vivo* competition between B cells expressing only Yc340-h*Btk* and B cells that also express endogenous murine *Btk*

In the *Btk*-/lacZ mice, expression of the disrupted *Btk* allele can be monitored by *lacZ* activity: in *Btk*- males, virtually all B220+ cells, except for HSA-CD43+ pro-B cells in the BM, express *lacZ*. Because of the process of random X-chromosome inactivaton, about half of the B-lineage cells in female *Btk*+/- heterozygous mice will have the disrupted *Btk* allele on the active X chromosome. When these cells reach a developmental stage at which *Btk* is required, they are arrested in differentiation. We previously identified selective disadvantages of *Btk*- cells within B cell subpopulations by a reduction of the proportion of *lacZ*+ cells below the value of 50% [6]. In the BM of *Btk*+/- heterozygous mice such selective disadvantages were absent up to the B220+CD43- pre-B cell stage, but were first exposed at the transition to the immature IgM+IgD-B cell and, more markedly, in mature IgM+IgD+ B cells [6].

In the Yc340-h*Btk*-transgenic mice, we used *lacZ* expression anlysis as an *in vivo* competition assay to compare the capacities of B cells that only express transgenic human *Btk* and B cells that express the transgenic human *Btk* in addition to the endogenous murine *Btk*. Cells from BM, spleen, MLN and peritoneum from *Btk*+/- females, either non-transgenic of Yc340-h*Btk*-transgenic, were analyzed for *lacZ* expression by flow cytometry. Fluorescein di-β-D-

galactopyranoside was used as a substrate, in conjunction with surface markers that define various B cell populations. The proportions of *lacZ*-expressing cells in the non-transgenic females decreased during B cell maturation
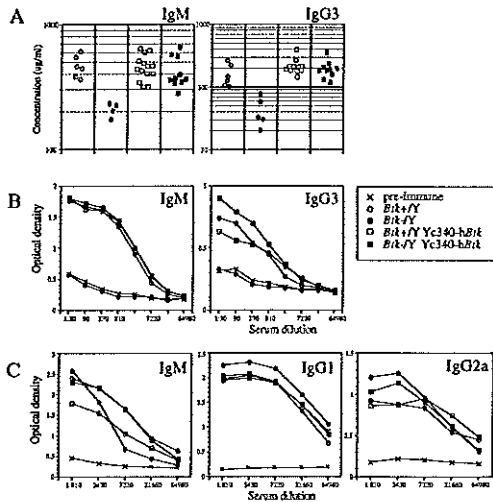
Figure 4. Expression of the Yc340-h*Btk* transgene corrects serum Ig levels and responses *in vivo* to TI-II antigens. (A) Serum concentrations of IgM and IgG3 in unimmunized 2-month-old mice from the four different groups indicated. (B) For TI-II responses, serum concentrations of DNP/TNP-specific IgM and IgG3 antibodies were determined 7 days after i.p. injection of DNP-Ficoll. For each serum dilution, the A (OD) are shown as mean values from 6 to 12 mice in each group. The values for pre-immune sera (*n* = 8, 2 from each group) were not significantly different between the four groups. (C) To determine secondary TD responses, serum concentrations of DNP/TNP-specific IgM, IgG1 and IgG2a were measured 7 days after an i.p. booster with TNP-KLH.
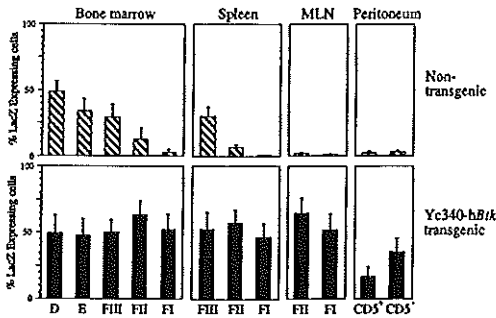
Figure 5. The effect of the Yc340-h*Btk* transgene in female *Btk*^{+/-} mice. Cell suspensions of non-transgenic and Yc340-h*Btk*-transgenic *Btk*^{+/-} mice were loaded with FDG to asses *LacZ* activity, and subsequently stained with anti-B220-FITC, biotinylated anti-IgM/streptavidin-TriColor, and either PE-conjugated anti-IgD, PE-conjugated anti-CD5 or PE-conjugated anti-CD43. For the indicated developmental B cell fractions in the BM and the peripheral organs [12, 29], the proportions of cells that express *LacZ* are shown. Data are given as mean values and standard deviations from three to nine non-transgenic mice and five to nine Yc340-h*Btk*-transgenic mice that were 7–10 weeks of age. D = B220^{+}CD43^{-} pre-B cells, E = IgM^{+}IgD^{-} immature B cells, F_{III} = IgM^{high}IgD^{low}, F_{II} = IgM^{high}IgD^{high}, F_I = IgM^{low}IgD^{high} mature B cells.

from ~ 50 % in CD43^{-} pre-B cells (Fig. 5, fraction D), to ~ 30 % in immature IgM^{+} B cells (fraction E) in BM, and to almost undetectable levels in mature IgM^{low}IgD^{high} B cells (fraction F_I) in BM, spleen and MLN, as previously reported [6]. In contrast, in the Yc340-h*Btk*-trangenic *Btk*^{+/-} heterozygous females, *lacZ*-expressing cells represented 50 % of the B cell population at every developmental stage in the BM (Fig. 5). Likewise, no selective disadvantages of *lacZ*-expressing cells were observed in the various peripheral mature B cell fractions F_{III}(IgM[high]IgD[low]), F_{II}(IgM[high]IgD[high]), and F_I(IgM[low]IgD[high]) in spleen and MLN. These findings indicate that the Yc340-h*Btk* transgene could fully compensate the absence of the endogenous murine *Btk* gene.

In the CD5^{+} B-1 B cells or the conventional CD5^{-} B cell populations in the peritoneum from non-transgenic *Btk*^{+/-} mice, *lacZ*^{+} cells were hardly detectable (Fig. 5 and [6]). In contrast, the Yc340-h*Btk*-transgenic mice manifested *lacZ* activity in significant proportions of their CD5^{+} B-1 B cells (16% ±7%) and conventional CD5^{-} B cells (35% ± 11%) (Fig. 5). These values did not appear to result from a down-regulation of *Btk* expression in a significant proportion of peritoneal B cells, because *lacZ* expression was found in 97% ± 0.4% of the CD5^{+} B-1 and in 98% ± 0.3% of the conventional peritoneal B cells of Yc340-h*Btk*-transgenic *Btk*^{-}/Y mice.

4 Discussion

Although *Btk* has been implicated in several receptor-mediated signal transduction pathways, it is currently unknown which of these signaling functions account for the disease phenotypes of XLA and *xid*. Moreover, the

function of *Btk* in early pre-B cell stages or the molecular mechanisms that lead to an arrest of B cell development at the pre-B cell level in XLA are unknown. The reported interactions of *Btk* with various proteins have mainly been studied *in vitro* ([9] and references therein). The significance of these findings still has to be established *in vivo*, *e.g.* by studies in transgenic mice using mutated *Btk* transgenes. For most genes, expression in transgenic mice is influenced by surrounding chromatin at the site of integration, leading to low expression levels of the transgene, or to expression in cell lineages that normally do not express the transgene. Therefore, transgenes should contain the essential regulatory sequences, such as transcriptional enhancers or suppressors that are located either in introns or at the 5' or 3' flanking regions of genes, to obtain position-independent copy number-related expression.

We have generated two transgenic mouse strains in which expression of human *Btk* is provided by the endogenous regulatory cis-acting elements present on the 340-kb YAC clone. Our results indicate that all relevant regulatory sequences that are required for high-level expression of *Btk* during B cell development are contained within the 340 kb of genomic DNA.

The RT-PCR and Western blot experiments showed that the expression of the Yc340-h*Btk* transgene is similar to the expression of the endogenous murine *Btk* gene, providing evidence for the absence of position effects.

When the Yc340-h*Btk*-transgenic mice were mated onto a *Btk*-deficient background, the Yc340-h*Btk* transgene could fully compensate the absence of the endogenous murine *Btk* gene. In BM and peripheral tissues, the numbers of B cells and their surface IgM/IgD profile were normal. In the peritoneum normal amounts of CD5^{+} B-1 B lymphocytes were present. Furthermore, the serum concentrations of IgM and IgG3, as well as the responses *in vivo* to TI-II antigens were restored.

The *in vivo* competition experiments in *Btk*^{+/-} female mice demonstrated that expression of human *Btk* from the Yc340-h*Btk* transgene completely eliminates the selective disadvantages of *Btk*-deficient cells at the transition from small pre-B cells to immature B cells in the bone marrow, as well as the severe arrest during maturation of conventional B cells from IgD[low]IgM[high] to IgD[high]IgM[low] stages in the peripheral tissues. These findings indicate the absence of any dominant negative effects of the expression product of the targeted *Btk-lacZ* allele, and also show that the human *Btk* can functionally substitute the murine protein. In this respect, the Yc340-h*Btk* transgenic rescue differs from the correction of the *xid* phenotype by transgenic expression of human *Btk* driven by the MHC class II Ea locus control region, which we previously reported [27]. In those mice, the fractions of *lacZ*-expressing cells in the MHCII-h*Btk* transgenic *Btk*^{+/-} females did not reach 50 %, but only ~ 10 % in the most mature IgM[low]IgD^{high} B cells in the periphery. Although the *xid* defects were completely corrected, apparently there were some minor insufficiencies in the expression of the MHCII-h*Btk* transgene that were only detected by competition *in vivo* in heterozygous females. The native regulatory sequences present on the Yc340-h*Btk* transgene, however, appear to provide appropriate expression of *Btk* in conventional B cells.

In contrast, within the peritoneal CD5$^+$ B-1 B cell population of Yc340-h*Btk*-transgenic *Btk*$^{+/-}$ mice, the fractions of *lacZ*-expressing cells still only reached 10–20 %. This finding implies that the human Btk protein has a slight intrinsic inability to substitute for murine Btk in peritoneal B cells. Twelve out of 659 amino acid residues are different between murine and human Btk, three of which are clustered at positions 207–214 in the TH domain thought to be involved in the interaction of Btk with SH3 domains of other tyrosine kinases [1, 2, 27, 30]. The alternative explanation of a selective down-regulation of *Btk* expression in a major fraction of CD5$^+$ B-1 B cells seems unlikely, because in the Yc340-h*Btk*-transgenic *Btk*$^{-/Y}$ mice, *lacZ* was found to be expressed in nearly all CD5$^+$ B cells.

The finding that in the Yc340-h*Btk*-transgenic mice *Btk* is appropriately expressed in the context of native regulatory sequences, is a starting point for the identification of the elements involved in the regulation of *Btk* expression in the hematopoietic system. Using the inherent homologous recombination system of yeast, various deletions within the *Btk* locus or its flanking regions can be readily introduced in the Yc340-h*Btk* YAC. After transfer of these modified YAC into transgenic mice, functional analyses should allow the identification of essential regulatory elements. Using similar methods, precise site-specific mutations can be introduced into YAC [31, 32], enabling the analysis of the effects *in vivo* of *Btk* gain-of-function mutations [34], or *Btk* variants that lack the function of specific domains. In these mouse models, *Btk* transgenes should be expressed at natural levels, with a stage and tissue specificity that parallels the endogenous *Btk* locus.

Knowledge of the regulatory elements involved in *Btk* gene expression would not only help to explain XLA cases without mutations in *Btk* coding region [34], but would also be essential for the development of gene therapy strategies for XLA: the constructs that will eventually be used to express an intact *Btk* gene in pre-B cells of XLA patients should preferably contain regulatory sequences from the *Btk* gene itself. For restoration *in vivo* of B cell function in XLA, a *Btk* mini-locus could be designed that contains genomic *Btk* sequences and the major regulatory elements. The development of gene therapy vectors with less strict size limitations, *e.g.* based on receptor-mediated delivery [35], would make such a *Btk* mini-locus DNA construct applicable for use in gene therapy to achieve physiological levels of *Btk* expression during B cell development.

5 References

1 Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammerström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C. I.E. and Bentley, D. R., *Nature* 1993. *361:* 226.

2 Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klisak, I., Sparkes, R. S., Kubagawa, H., Thulu-

vancheri, M., Quan, S., Belmont, J. W., Cooper, M. D., Conley, M. E. and Witte, O. N., *Cell* 1993. *72:* 279.

3 Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, M. G., Jenkins, N. A. and Witte, O. N., *Science* 1993. *261:* 358.

4 Thomas, J. D., Sideras, P., Smith, C. I. E., Vorechovsky, I., Chapman, V. and Paul, W. E., *Science* 1993. *261:* 355.

5 Sideras, P. and Smith, C. I. E., *Adv. Immunol.* 1995. *59:* 135.

6 Hendriks, R. W., de Bruijn, M. F. T. R., Maas, A., Dingjan, G. M., Karis, A. and Grosveld, F., *EMBO J.* 1996. *15:* 4862.

7 Scher, I., *Adv. Immunol.* 1982. *33:* 1.

8 Kahn, W. F., Alt, F. W., Gerstein, R. M., Malynn, B. A., Larsson, I., Rathbun, G., Davidson, L., Müller, S., Kantor, A. B., Herzenberg, L. A., Rosen, F. S. and Sideras, P., *Immunity* 1995. *3:* 283.

9 Mattson, P. T., Vihinen, M. and Smith, C. I. E., *Bioessays* 1996. *18:* 825.

10 Smith, C. I. E., Baskin, B., Humire-Greiff, p., Zhou, J. N., Olsson, P. G., Maniar, H. S., Kjellen, P., Lambris, J. D., Christensson, B., Hammarström, L., Bentley, D., Vetrie, D., Islam, K. B., Vorechovsky, I. and Sideras, P., *J. Immunol.* 1994. *152:* 557.

11 De Weers, M., Mensink, R. G. J., Kraakman, M. E. M., Schuurman, R. K. B. and Hendriks, R. W., *Hum. Mol. Genet.* 1994. *3:* 161.

12 Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D. and Hayakawa, K., *J. Exp. Med.* 1991. *173:* 1213.

13 Himmelmann, A., Thevenin, C., Harrison, K., and Kehrl, J. H., *Blood* 1996. *87:* 1036.

14 Müller, S., Sideras, P., Smith, E. and Xanthopoulos, K. G., *Oncogene* 1996. *13:* 1955.

15 Carson, S. and Wiles, M. V., *Nucleic Acids Res.* 1993. *21:* 2065.

16 Grosveld, F., van Assendelft, G. B., Graeves, D. and Kollias, D., *Cell* 1987. *51:* 975.

17 Graeves, D. R., Wilson, F. D., Lang, G. and Kiousis, D., *Cell* 1989. *56:* 979.

18 Yannoutsos, N., IJzermans, J. N. M., Harkes, C., Bonthuis, F., Zhou, C., White, D., Marquet, R. L. M. and Grosveld, F., *Genes to Cells* 1996. *1:* 409.

19 Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular cloning – A Laboratory Manual*, Cold Spring Harbor Pres, Cold Spring Harbor, New York 1989.

20 Anand, R., Riley, J. H., Butler, H., Smith, J. C. and Markham, A. F., *Nucleic Acids Res.* 1990. *18:* 1951.

21 Sweatman, A. K., Bradley, L. A. D., Lovering, R. C, O'Reilly, M.-A. J., Levinsky, R. and Kinnon, C., *Hum. Genet.* 1994. *94:* 624.

22 Jin, H., May, M., Tranebjaerg, L., Kendall, E., Fontán, G., Jackson, S., Subramony, S. H., Arena, F., Lubs, H., Smith, S., Stevenson, R., Schwartz, C. and Vetrie, D., *Nat. Genet.* 1996. *14:* 177.

23 De Weers, M., Brouns, G. S., Hinshelwood, S., Kinnon, C., Schuurman, R. K. B., Hendriks, R. W. and Borst, J., *J. Biol. Chem.* 1994. *269:* 23857.

24 Oeltjen, J. C., Liu, X., Lu, J., Allen, R. C., Muzny, D., Belmont, J. W. and Gibbs, R. A., *Mamm. Genome* 1995. *6:* 334.

25 6th Wellcome Summer School. London, *DNA related methods in human genetics. Yac cloning in genome analysis*, 5th July–13th July 1991.

26 Hogan, B., Beddington, R., Costanini, F. and Lacy, E., *Manipulating the mouse embryo, a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York 1994.

27 Drabek, D., Raguz, S., de Wit, T. P. M., Dingjan, G. M., Savelkoul, H. F. J., Grosveld, F. and Hendriks, R. W., *Proc. Natl. Acad. Sci. USA* 1997. *94:* 610.

28 Essers, J., Hendriks, R. W., Swagemakers, S. M. A., Troelstra, C., de Wit, J., Hoeijmakers and Kanaar, R., *Cell* 1997. *89:* 195.

29 Hardy, R. R., Hayakawa, K., Haaijman, J. and Herzenberg, L. A., *Nature* 1982. *297:* 589.

30 Chen, G., Ye, Z.-S. and Baltimore, D., *Proc. Natl. Acad. Sci. USA* 1994. *91:* 8152.

31 Barton, M. C., Hoekstra, M. F. and Emerson, B. M., *Nucleic Acids. Res.* 1990. *18:* 7349.

32 Duff, K., McGuigan, A., Huxley, C., Schulz, F. and Hardy, J., *Gene Ther.* 1994. *1:* 70–75.

33 Li, T., Tsukada, S., Satterthwaite, A., Havlik, M. H., Park, H., Takatsu, K. and Witte, O. N., *Immunity* 1995. *2:* 451.

34 Hashimoto, S., Tsukada, S., Matsushita, M., Miyawaki, T., Niida, Y., Yachie, A., Kobayashi, S., Iwata, T, Hayakawa, H., Matsuoka, H., Tsuge, I., Yamadori, T., Kunikata, T., Arai, S., Yoshizaki, K., Taniguchi, N. and Kishimoto, T., *Blood* 1996. *88:* 561.

35 Cotten, M., Wagner, E., Zatloukai, K., Philips, S., Curiel, D. T. and Birnstiel, M. L., *Proc. Natl. Acad. Sci. USA* 1992. *89:* 6094.

Chapter 4

Studies on the regulation of *Btk* expression

Alex Maas, Gemma M. Dingjan, Susanne Müller, Sjaak Philipsen, C.I.Edvard Smith, Frank Grosveld and Rudolf W. Hendriks

Parts of these studies are submitted

Studies on the regulation of *Btk* expression

Alex Maas[1], Gemma M. Dingjan[1,2], Susanne Müller[3], Sjaak Philipsen[1], C.I.Edvard Smith[3,4], Frank Grosveld[1] and Rudolf W. Hendriks[1]

Departments of [1]Cell Biology and Genetics and [2]Immunology, Faculty of Medicine, Dr. Molewaterplein 50, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, [3]Center for BioTechnology, Department of Biosciences, Karolinska Institute, NOVUM, S-14157, Huddinge, Sweden and [4]Division of Clinical Immunology, Department of Immunology, Microbiology, Pathology and Infectious Diseases, Huddinge Hospital, S-14186, Sweden.

Abstract

Defects in the gene for Bruton's tyrosine kinase (Btk) result in X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice. *Btk* is expressed throughout B cell development, but not in plasma cells. *Btk* is also expressed in other lineages of the haematopoietic system, except in T cells. In this chapter we addressed the question how this unique expression pattern is accomplished. Using intracellular flow cytometry we show that *Btk* is already expressed at significant levels early in B cell development. We describe the generation of yeast artificial chromosome (YAC) transgenic mouse strains in which the human *Btk* (h*Btk*) expression is provided by endogenous regulatory *cis*-acting elements that are present on a 240 kb YAC transgene, Yc240-h*Btk*, which contained the *Btk* gene and ~6 kb 5' flanking DNA and ~200 kb 3' flanking DNA. When the Yc240-*hBtk* transgenic mice were mated onto a Btk-deficient background, B cell numbers were fully restored. The expression pattern and levels of the transgenic h*Btk* were found to parallel the endogenous murine *B*tk gene. The expression level of Btk did not change significantly during the subsequent stages of B cell development. To identify *cis*-acting regulatory elements, within the *Btk* locus itself, DNAseI hypersensitive site (HSS) mapping was performed. Nine HSS were identified, one of which was found to be B cell specific and conserved between mouse and men. Furthermore we show that although Sp1, Sp3 and PU.1 are able to transactivate the *Btk* promoter synergistically in *in vitro* transfection assays. However, targeted deletion of Sp1 or Sp3 does not result in decreased *Btk* expression *in vivo*. We conclude that not only the Btk protein itself is conserved between mice and men, but also the regulatory elements that drive *Btk* expression. This study provides a foundation for the development of a *Btk* mini-locus to develop strategies to manipulate *Btk* expression *in vivo*.

Introduction

Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that plays a crucial role in B lymphocyte development and function. Mutations affecting the *Btk* gene lead to X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice (Tsukada *et al.*, 1993, Vetrie *et al.*, 1993, Rawlings *et al.*, 1993, Thomas *et al.*, 1993). Affected patients display a

large variety of mutations in the *Btk* gene (Vihinen *et al.*, 1998). XLA is characterized by an almost complete block in B cell development at the pre-B cell stage. Both CBA/N mice with a R28C mutation in the PH domain as well as mice with a targeted disruption of the germline *Btk* locus display the *xid* phenotype (Khan *et al.*, 1995, Hendriks *et al.*, 1996), which is a less severe B cell disorder than XLA.

The *Btk* gene encodes a 659-amino acid protein that contains a single kinase domain, the *src* homology domains SH2 and SH3, and a N terminal region with a PH domain and a unique proline- and cysteine-rich Tec homology (TH) domain (see for review: Sideras and Smith, 1995, Desiderio, 1997).

Btk expression has been investigated in human using cultured cell lines, leukemias and peripheral blood samples (de Weers *et al.*, 1993, Genevier *et al.*, 1994, Smith *et al.*, 1994, Futatani, *et al.*, 1998). In the mouse, Btk expression has been analyzed in cultured cell lines as well as *in vivo*, by flow cytometric analysis of LacZ activity in a mouse with a targeted insertion in frame of a *LacZ* cassette in the *Btk* gene (Tsukada *et al.*, 1993, Hendriks *et al.*, 1996). Btk is expressed throughout B cell development, from the earliest pro-B cell stage up to mature B cells, except in plasma cells. Btk is also expressed in cells of the myeloid lineage, but is not required for myeloid differentiation, since cells from the myeloid lineage are not affected in XLA nor in *xid* (Sideras and Smith, 1995). Btk expression is not found in T-lineage cells.

It is not clear to date, how the tissue-specific expression pattern of *Btk* is accomplished. Knowledge of the regulatory elements involved in *Btk* gene expression would not only help to explain XLA cases without mutations in *Btk* coding region, but would also be essential for the development of gene therapy strategies for XLA: the constructs that will eventually be used to express an intact *Btk* gene in pre-B cells of XLA patients should preferably contain regulatory sequences from the *Btk* gene itself.

Sequence analyses of the ~280 bp *Btk* promoter region revealed no obvious TATA box, but several E boxes and PU.1, Sp1 and Sp3 binding sites were found (Sideras *et al.*, 1994, Himmelmann *et al.*, 1996). Sp1, Sp3, Spi-B and PU.1 were shown to bind *in vitro* to this part of the promoter region (Müller *et al.*, 1996, Himmelman *et al.*, 1996). A construct, containing the first 450 bp of the *Btk* promoter showed high activity in *Btk* expression in B cell lines and the erythroleukemia line, K562, but not in Btk negative Hela cells or various T cell lines. Moreover, PU.1 and Spi-B can transactivate the *Btk* promoter in the T-cell line Jurkat which does not contain these factors endogenously. However, in fetal liver of PU.1 homozygous mutant mice, Btk was still expressed, albeit at a slightly reduced level (Müller *et al.*, 1996). The transcription factors Sp1 and Sp3 are closely related members of a gene family of zinc finger proteins with similar structural features. They share a highly conserved DNA binding domain and recognize a GGGCGGGG- or GGGTGGGG-box with identical binding affinities (Hagen *et al.*, 1992). Like Sp1, Sp3 is ubiquitously expressed (Hagen *et al.*, 1992, Supp *et al.*, 1996). The transcription factor PU.1 belongs to the Ets family of sequence specific DNA binding proteins which specifically bind to purine-rich sequences characterized by an invariant GGA core sequence (Klemsz *et al.*, 1990, Moreau-Gachelin 1994 and references therein). PU.1 is expressed in haematopoietic cells, with the highest expression

in B-cells, myeloid cells and erythroid cells but PU.1 is absent in T-cells (Su *et al.*, 1996). Numerous target genes for PU.1 have so far been identified in the B cell and monocyte lineages, such as mb-1, Ig J chain and Ig μ, κ and λ chains (Eisenberg *et al.*, 1995, Pongubala *et al.*, 1997, Mittrucker *et al.*, 1997).

Besides the promoter region, additional far upstream, downstream or intragenic *cis*-acting regulatory elements, such as enhancers and locus control regions are often essential for correct expression of genes (Carson *et al.*, 1993, Grosveld *et al.*, 1989, Graeves *et al.*, 1989, Yannoutsos *et al.*, 1996). Multiple regions in *Btk* introns showed extensive conservation, to over 95% identity, between mice and men (Oeltjen *et al.*, 1997), which suggests that these regions may contain *cis*-acting regulatory sequences. As a first step in the characterization of the regulation of *Btk* gene expression, we have previously described the generation of yeast artificial chromosome (YAC) transgenic mice strains, Yc340-h*Btk*, in which human *Btk* (h*Btk*) expression is provided by endogenous regulatory *cis*-acting elements present on a 340 kb YAC transgene (Maas *et al.*, 1997). When the Yc340-h*Btk* mice were mated onto a Btk ⁻ background, all features of the *xid* defect were fully corrected. We have now shortened the 340 kb YAC by homologous recombination in yeast and reduced the ~100 kb flanking the 5' end of the *Btk* gene to ~6 kb. The shortened 240 kb YAC was used to generate transgenic mice. By mating these *Btk*-transgenic mice onto a Btk-deficient background, we show that the *xid* phenotype in the mouse is corrected, and that the *in vivo* expression patterns and levels of the transgenic h*Btk* are similar to that of the endogenous murine *Btk* .

The chromatin structure of promoter and enhancer regions of actively transcribed genes is often hypersensitive to DNAseI endonucleases, which reflects the accessibility of the DNA *in vivo* for interaction with proteins, like transcription factors and nucleases (Gross, 1988). We performed DNAseI hypersensitive site analyses to identify any *cis*-acting regulatory sequences within the *Btk* region.

To elucidate the role of the transcription factors binding to the Btk promoter we have performed transfection experiments with expression vectors for Sp1, Sp3 and PU.1. In addition, the effect on *Btk* expression by targeted deletion of Sp1 and Sp3 was studied.

Results

1. *In vivo* expression of murine *Btk*

We have previously described the generation of a Btk-deficient mouse model by inactivating the *Btk* gene through an in-frame insertion of a *LacZ* reporter gene (Hendriks *et al.*, 1996). Expression of LacZ activity, driven by the endogenous *Btk* promoter and regulatory elements, allowed us to determine the *Btk* expression pattern in affected male mice. Expression of the *Btk* gene was found to be restricted to cells of the myeloid and B cell lineages. In the bone marrow, the ER-MP20^{high} precursor cells of the monocyte lineage (De Bruijn *et al.*, 1994) showed lacZ activity, whereas the ER-MP20^{med} fraction of granulocyte precursors manifested heterogeneous levels of *LacZ* expression (Hendriks *et al.*, 1996, and Fig. 1). In the erythoid lineage (Ter-119^{+}), *LacZ* activity was only detected in the immature population of large erythroid precursors, representing the (pro)erythroblast stages.

To study the *in vivo* murine Btk protein expression, we applied intracellular flow cytometry, using a rabbit polyclonal antibody to a recombinant Btk-fusion protein, containing aa 25-173. Cells from Btk-deficient mice served as negative controls (Fig. 2A and C). As shown in the upper panel of Fig. 2A, in the bone marrow, Btk is expressed throughout B cell development from the pro-B cell stage up to the mature IgM^{+} IgD^{+} B cell stage. From the intensities of the fluorescence signals, it was clear that *Btk* was already expressed at significant levels in early B cell development. During B cell differentiation, only minor changes in the *Btk* expression levels were observed. In the spleen and mesenteric lymph node Btk expression is found from the immature IgM^{high}IgD^{low} up to the mature IgM^{low}IgD^{high} cell stages as well as in activated B lymphoblasts, as identified by large FSC B220^{+} cells (shown for spleen in Fig. 2A, middle panel). In the peritoneum Btk was found to be expressed in conventional CD5^{-}IgM^{+} B cells and in CD5^{+}IgM^{+} B-1 B cells. For the B-1 B cell population, the fluorescence signals in wild-type could not be compared with knock-out cells, since these cells are absent in Btk-deficient mice (Hendriks *et al.*, 1996).

In the BM, very low levels of Btk expression were observed in the ER-MP20^{high} monocyte and the ER-MP20^{med} granulocyte precursors (Fig. 2B, upper panel). Btk could not be detected in the Ter-119^{+} erythroid lineage of the bone marrow. In the periphery (Fig. 2B, lower panel), Btk was found to be expressed both in the Gr-1^{-} Mac-1^{+}monocytes in the spleen as well as in the peritoneal Mac-1^{+} macrophages. In the Gr-1^{+} Mac-1^{+} granulocyte populations, the expression level was found to vary between different mice (shown for two wild-type mice in Fig. 2B, lower panel).

Btk could not be detected in any of the thymocyte subpopulations, nor in T cells or NK cells in the spleen (Fig. 2C).
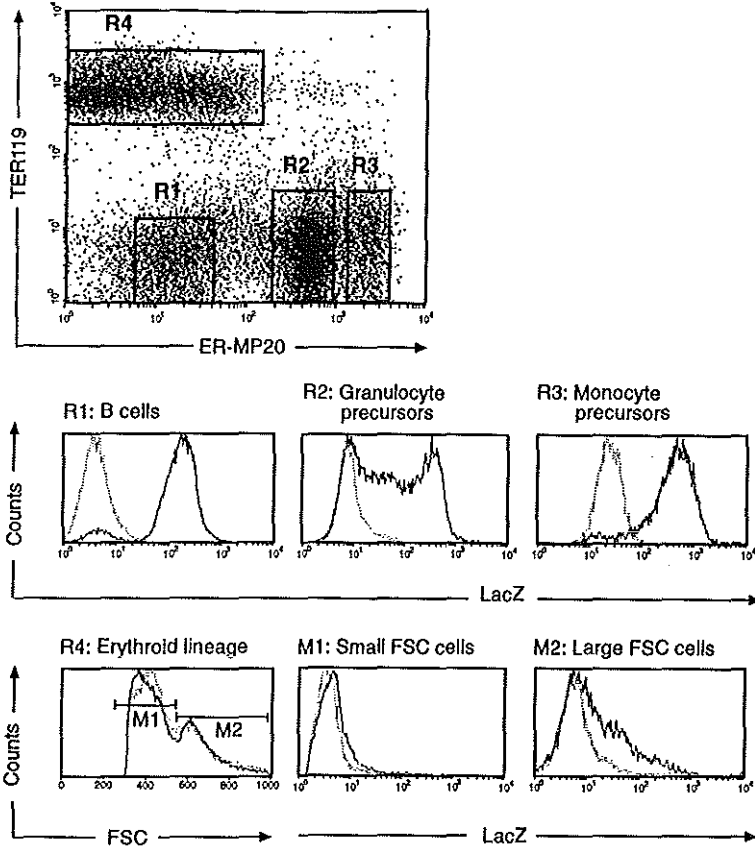
Figure 1. *Surface profile of the ER-MP20 and Ter-119 markers of cells from the bone marrow (top). The gated (R) subpopulations were analyzed for LacZ expression and the results are displayed as histograms. NK cells were Mac-1^{low}. The Ter-119$^+$-ER-MP20$^-$ erythroid cells (R4) were subdivided, using forward side scatter profiles (FSC) into small (M1) and large (M2) erythroid cells. Subsequently, LacZ activity was analyzed in these subpopulations and displayed as histograms. Solid lines represent cell populations of Btk$^-$/LacZ -mice, dashed lines represent the background staining as determined in wild-type mice.*

A   B-lymphoid

B   Non-lymphoid

C   T-lymphoid and NK cells
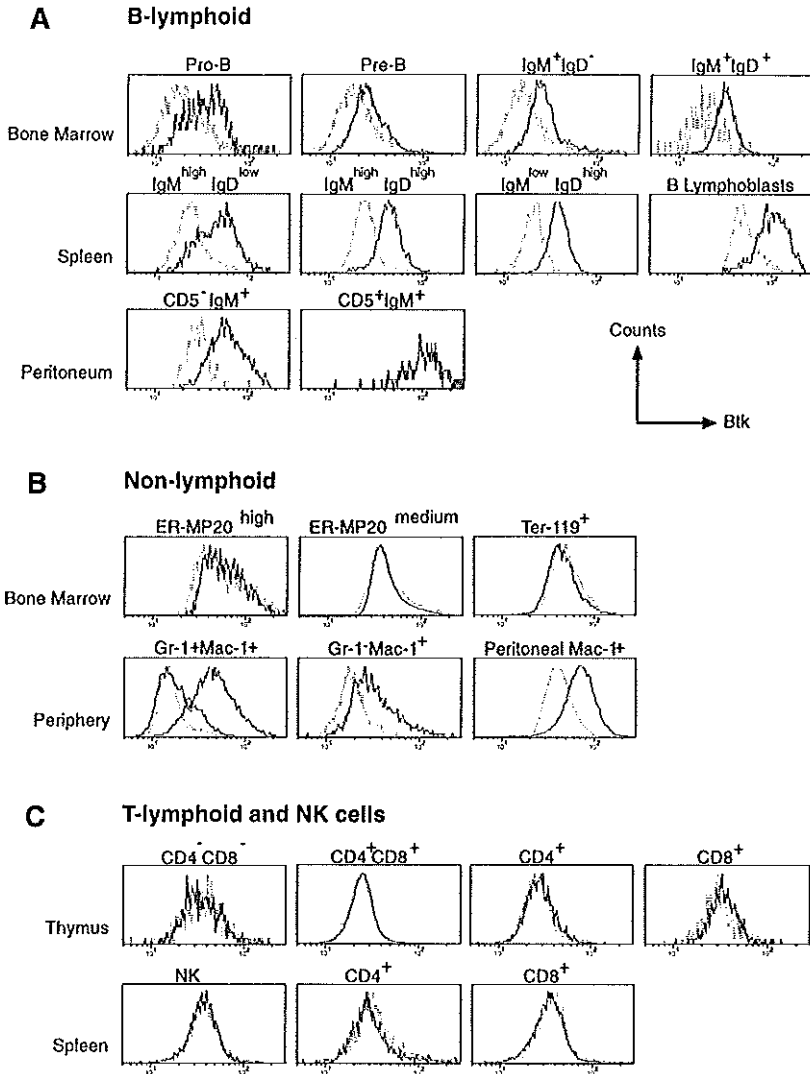
Figure 2. *Intracellular expression of the endogenous murine Btk and transgenic human Btk in 8-10 week-old wild-type or Btk⁻ mice and Yc240-hBtk transgenic mice on the Btk⁻ background. The indicated populations were gated and analyzed for Btk expression. The results are displayed as histograms, cell populations are given at the top, originating tissues at the left. Pro-B cells were defined as B220⁺CD43⁺, pre-B cells as B220⁺CD43⁻. ER-MP20^high cells are monocytes (precursors) and ER-MP20^medium cells are granulocyte (precursors). The peripheral Gr-1⁺-*

D **B-lymphoid**

| | Pro-B | Pre-B | IgM$^+$IgD$^-$ | IgM$^+$IgD$^+$ |

Bone Marrow

IgM^{high} IgD^{low} | IgM^{high} IgD^{high} | IgM^{low} IgD^{high} | B Lymphoblasts

Spleen

CD5$^-$IgM$^+$ | CD5$^+$IgM$^+$

Peritoneum

Counts

Btk

E **Non-lymphoid**

ER-MP20^{high} | ER-MP20^{medium} | Ter-119$^+$

Bone Marrow

Gr-1$^+$Mac-1$^+$ | Gr-1$^-$Mac-1$^+$ | Peritoneal Mac-1$^+$

Periphery

F **T-lymphoid and NK cells**

CD4$^-$CD8$^-$ | CD4$^+$CD8$^+$ | CD4$^+$ | CD8$^+$

Thymus

NK | CD4$^+$ | CD8$^+$
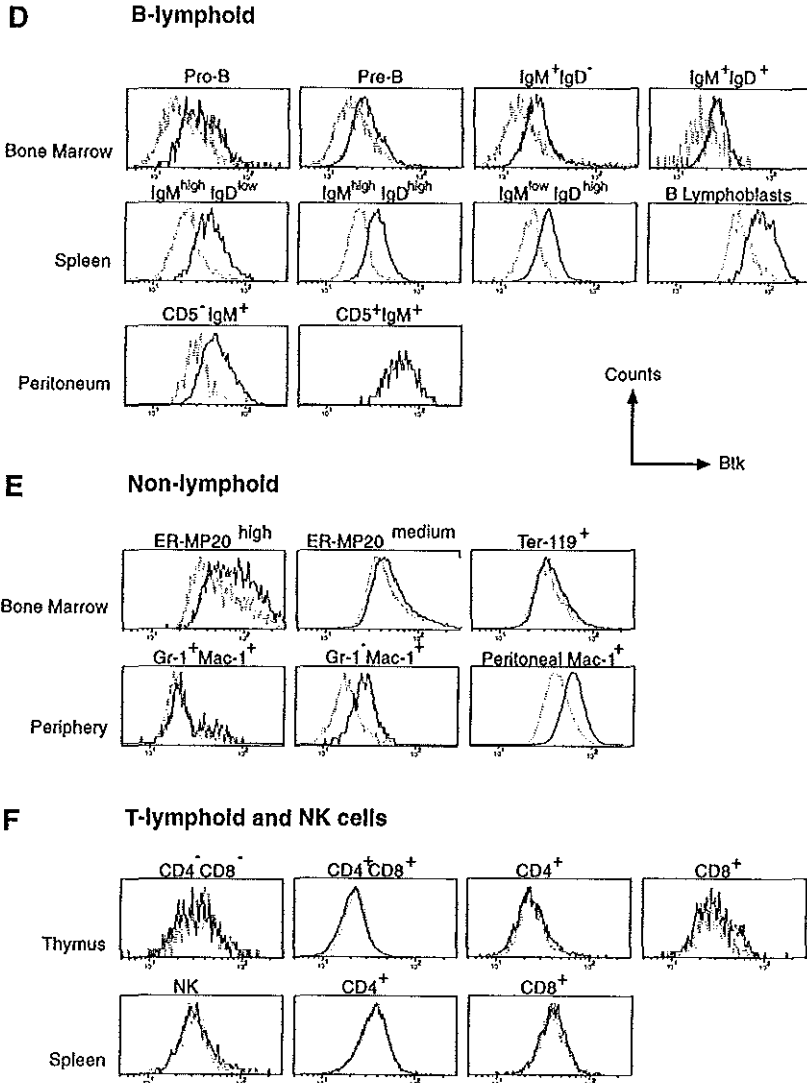
Spleen

Mac-1$^+$ population represents the granulocyte fraction and the Gr-1$^-$Mac-1$^+$ population the macrophage fraction of the spleen. Data are obtained from 10^5-10^6 total events. (A-C) Btk expression in wild-type (solid lines) and background Btk staining as determined in Btk$^-$ mice (dashed lines). (D-F) Btk expression from the Yc240-hBtk transgene (solid lines) and background staining (dashed lines).

2. *In vivo* expression of human *Btk* from a 240 kb Yc240-h*Btk* transgene

2.1 Generation of Yc240-hBtk YAC transgenic mice

The 340 kb YAC clone 9DA6, containing the human *Btk* gene and ~100 kb 5' flanking and ~200 kb 3' flanking DNA (Maas *et al.*, 1997) was shortened by homologous recombination, using a 7.5 kb *Hind*III genomic DNA fragment encompassing the first exon of the *Btk* gene (Fig. 3A) The obtained 240 kb YAC, Yc240-h*Btk* was isolated by PFGE, purified and micro-injected. Out of 50 offspring born, three independent h*Btk*-transgenic mouse lines, Yc240-h*Btk* S2, S3 and S30 were generated. In Southern blotting analyses, using *Hind*III, *Pvu*II, *Xba*I and *Bam*HI and probes specific for the entire *Btk* gene and Yc240-h*Btk* itself, all restriction fragments present in the Yc240-h*Btk* YAC clone were also found in the Yc240-h*Btk* transgenic lines. Densitometric estimations indicated that S2, S3 and S30 contained 1-3 copies of the transgene (data not shown). Fluorescence *in situ* hybridization of metaphase chromosomes to human *Btk* sequences demonstrated single integration spots at chromosome 2 for S2, chromosome X for S3 and chromosome 8 for S30. As the transgene was integrated on the X chromosome in line S3, we did not use this line for further analyses.

The two other lines manifested expression of the *Btk* transgene as was evaluated in cells from spleen and peritoneal cavity by RT-PCR using a range of cDNA concentration dilutions (shown for spleen of line S2 in Fig. 3B). In the transgenic lines S2 and S30, neither developmental defects nor any increased susceptibility to infectious diseases or malignancies were observed on various genetic backgrounds (FVB, 129 or C57BL/6) for over 12 months of age.
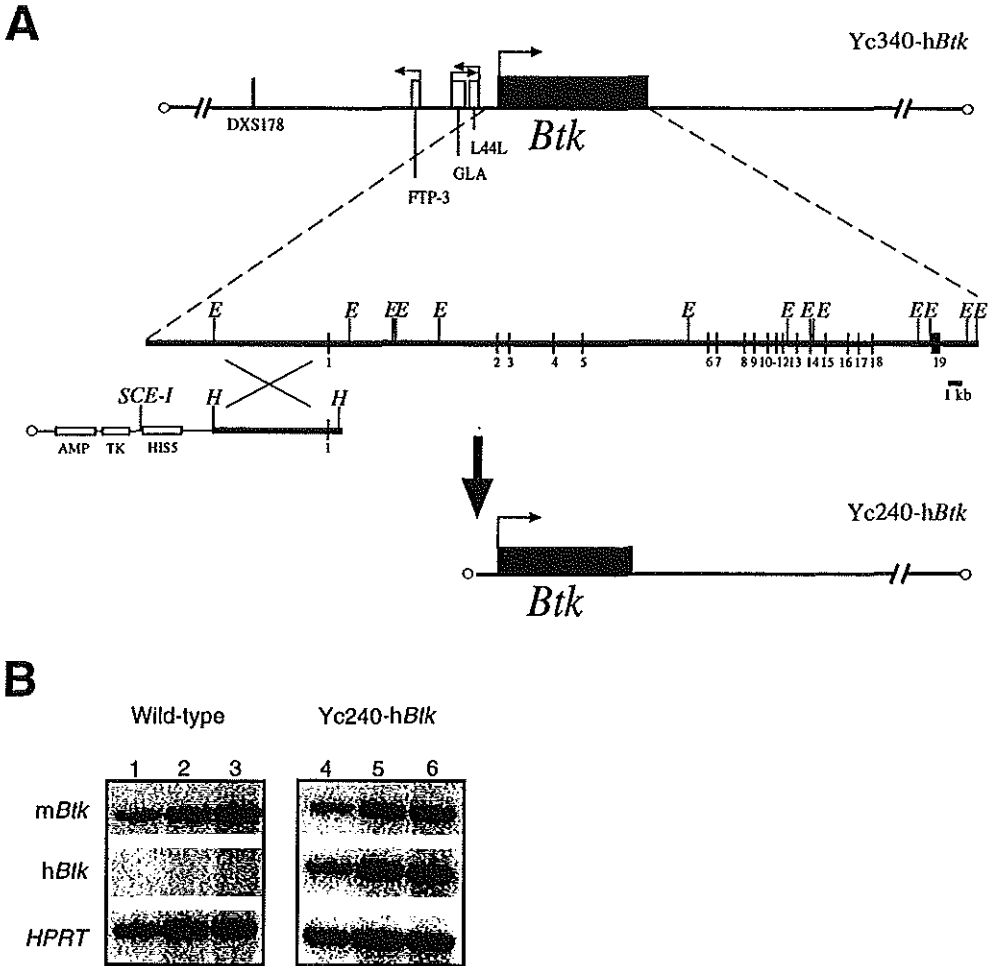
Figure 3. *Generation of Yc240-hBtk-transgenic mice (A) Map of the 340kb Yc340-hBtk YAC (top), showing the location of the Btk gene (filled box) the L44L, -galactosidase (GLA) and FTP-3 genes (open boxes) and the polymorphic marker DXS178 (Maas et al., 1997). In the middle section, the genomic organization of the murine Btk gene, including the position of the 19 exons (Sideras et al., 1994) and the retrofitting vector are shown. At the bottom, the targeted construct Yc240-hBtk is shown, indicating the location of the Btk gene. E, EcoRI; H, HindIII. (B) RT-PCR analysis of cDNA samples from spleen of Yc240-hBtk transgenic line S2 and wild-type. Shown is a PCR concentration range using 0.3-1-3 l cDNA, for wild-type (lanes 1-3) and Yc240-hBtk transgenic mice (lanes 4-6).*

2.2 Transgenic Yc240-hBtk expression restored B cell numbers and paralleled endogenous Btk expression

To investigate whether expression of the Yc240-h*Btk* could fully compensate the absence of the endogenous murine *Btk* gene, transgenic mice were bred onto a Btk-deficient background (Hendriks *et al.*, 1996). As shown in Table 1, flow cytometric analyses of spleen, mesenteric lymph nodes, peritoneum and bone marrow of Btk-deficient mice displayed the *xid* phenotype (Hendriks *et al.*, 1996, Scher, 1982, Khan *et al.*, 1995), characterized by a reduction of $IgM^{low}IgD^{high}$ peripheral B cells and a lack of $CD5^+$ B cells in the peritoneum. Expression of Yc240-h*Btk* on the Btk-deficient background resulted in correction of the B cell numbers to levels comparable to those of wild-type (Btk^+/Y) mice. No differences between the two lines were observed and, therefore, data of the two transgenic lines were combined.

Table 1. Frequencies of lymphocyte populations

Compartment Cell population	Non-transgenic		Yc240-h*Btk*
	Btk^+/Y	Btk^-/Y	Btk^-/Y
Spleen			
Nucleated cells, x 10^6	200±37	128±11	160±29
$B220^+$ cells, %	51±5	33±5	57±4
Of which, $IgM^{high}IgD^{low}$ B cells, %	8±3	32±7	10±3
Of which, $IgM^{high}IgD^{high}$ B cells, %	25±4	39±5	26±5
Of which, $IgM^{low}IgD^{high}$ B cells, %	67±6	30±3	64±7
$CD3^+$ T cells, %	35±5	48±0.3	35±1
Lymph Node			
$B220^+$ cells, %	28±4	7±1	21±4
Of which, $IgM^{high}IgD^{low}$ B cells, %	7±1	15±1	5±1
Of which, $IgM^{high}IgD^{high}$ B cells, %	45±3	67±1	51±2
Of which, $IgM^{low}IgD^{high}$ B cells, %	47±2	19±2	44±1
Peritoneum			
$CD5^+IgM^+$ B cells, %	5±1	<1	12±4
$CD5^-IgM^+$ B cells, %	35±16	28±3	49±7
$CD5^+IgM^-$ T cells, %	33±11	44±8	20±8
Bone Marrow			
$B220^+$ cells, %	35±15	34±4	40±4
Of which, IgM^- pre-B cells, %	18±10	22±2	24±5
Of which, IgM^+IgD^- B cells, %	7±3	8±1	8±1
Of which, IgM^+IgD^+ B cells, %	9±1	2±1	7±2

The phenotype of lymphocyte population was determined by flow cytometry; dead cells and high side scatter cells were excluded by gating. Data are mean values ± standard deviations from 2-6 mice analyzed per group. Mice were 8-10 weeks of age.

Using intracellular flow cytometry, the expression of human Btk from the Yc240-h*Btk* transgene was evaluated in cells of the bone marrow, spleen, thymus and peritoneum. (Fig. 2E-F). The transgenic human *Btk* expression pattern was similar to the murine endogenous *Btk* expression pattern and, moreover, expression levels were in the same range. As was found for the endogenous murine Btk (Fig. 2A), the levels of transgenic human Btk were found to be similar in the individual stages of B cell development.

3. The role of transcription factors Sp1, Sp3 and PU.1 in activation of the human Btk promoter

3.1 Sp1, Sp3 and PU.1 activate the Btk promoter in SL2 cells

To assess the individual role of transcription factors binding to a 280 bp fragment containing the Btk promoter (-280-Btk-Luc, Fig. 4A) we performed cotransfection experiments in *Drosophila* Schneider (SL2) cell, which are devoid of endogenous Sp factors or PU.1-like activities (Courey and Tjian, 1988). Nuclear extracts prepared form SL2 cells transfected with either Sp1, Sp3 or PU.1 expression plasmids contained complexes that were able to bind to sites in the Btk promoter, specific for these transcription factors, as was verified in electromobility shift assay (EMSA, data not shown). This binding was specific since it could be competed with specific oligonucleotides but not with unspecific oligonucleotides.

To determine the range in which expression of Sp1, Sp3 and PU.1 were in a linear range as referred to the amount of transfected plasmid, 2 ng to 2 µg of the expression vectors were transfected into SL2 cells, and luciferase activity was determined (Fig. 4B). Transactivation was clearly detected with 200 ng of each of the expression vectors. For Sp1 and Sp3 a plateau was reached with about 1 µg expression plasmid.

3.2 PU.1, Sp1 and Sp3 activate the Btk promoter synergistically.

We performed cotransfection experiments using the Sp1, Sp3 and PU.1 expression plasmids either alone or in combination. Although relative luciferase activity were found to vary between the individual experiments, consistent effects of cotransfection of individual plasmids were observed. Using different amounts of the Sp3 and Sp1 vectors, it was shown that the two transcription factors had additive stimulatory effects (Fig. 4C). Transfection of 200 ng of the PU.1 expression plasmid resulted in about five-fold activation of the Btk promoter, whereas the same amount of expression plasmid for Sp1 and Sp3 was able to transactivate the Btk promoter six-fold and 2.5-fold, respectively (Fig. 4D). Cotransfection of PU.1 with either the Sp1 or Sp3 vectors, activated the Btk promoter 62 fold and 32 fold, respectively. Transfection of all three expression plasmids together activated the Btk promoter 124-fold (Fig. 4D). These data show that PU.1 is able to act synergistically with Sp1 and Sp3.

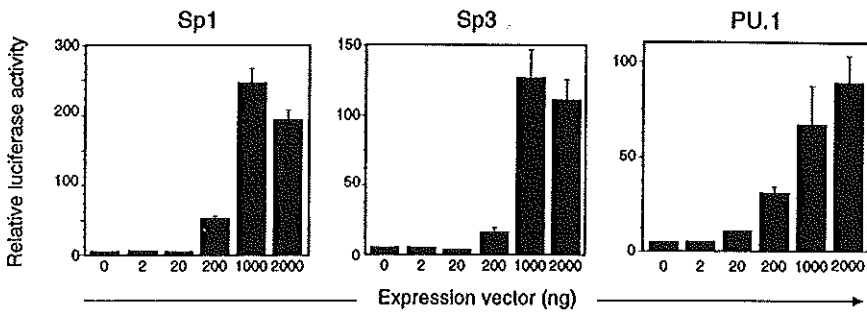The contribution of each of the two individual Sp-binding sites (GC-, and GT-box, Fig. 4A) to the transcriptional activation of *Btk*, was investigated by mutating them separately. When 200 ng of the respective expression plasmid was transfected, mutation of either Sp-binding site reduced the inducibility of -280-Btk-Luc by Sp1 or Sp3, indicating that both Sp binding sites are functional in SL2 cells (Fig. 4D). The effect was more prominent when 400 ng of the expression plasmids were used (data not shown). To address the question if the two Sp-binding sites could contribute equally to the synergistic effect with PU.1, the Sp1-, Sp3- and PU.1-expression plasmids were cotransfected with either wild-type or mutant reporter constructs. Upon coexpressing PU.1 with either Sp1 or Sp3, the mutation of the GT-box had a more pronounced effect than the mutation of the GC-box. Since the GT-box and the PU-box are in close proximity in the Btk promoter (see Fig. 4D), these findings suggest a functional interaction between PU.1 and Sp factors binding to the GT-box.

Figure 4. *Btk promoter studies. (A) Schematic representation of the human Btk promoter. Shown is the promoter area from -250 to +30 and cis-acting regulatory elements that have been identified in this region. The major transcription initiation site is determined as +1, GC and GT represent the two Sp1/Sp3 binding sites, PU the binding site for PU.1 and E represents an E-box. Black bars indicate the position of the binding sites for Sp1/3 and PU.1. (B) PU.1, Sp1 and Sp3 activate the Btk promoter. SL2 cells were transiently transfected with 8 g of the 280-Btk-Luc construct and variable amounts of the Sp1, Sp3 or PU.1 expression plasmids, as indicated. (C) Sp1 and Sp3 have additive stimulatory effects. 8 g of the 280-Btk-Luc construct was transfected together with variable amounts of Sp1 and Sp3 vectors, as indicated. (D) Cotransfection of Sp1, Sp3 and PU.1 in SL2 cells. 8 g of a reporter construct was transfected together with 200 ng of the Sp1, Sp3 and PU.1 expression plasmids, respectively, as indicated. Filled bars represent data obtained by transfecting the reporter construct 280-Btk-Luc, open bars by transfecting 280-Btk-GC-M-Luc, and shaded bars by transfecting 280-Btk-GT-M-Luc. (B-C) The relative activity measured after transfection of the empty expression vector was set 1. Data represent the mean and the standard deviation of a representative experiment.*

A

GC

PU GT

-200

HMG   E   E

+30

AATGAAGGGCGGGGACAGTTG   AAAAAAAGGGAACTGAGTGGCTGCGAAAGGGTGGGGTTTG

B

Sp1

Sp3

PU.1

Relative luciferase activity

0 2 20 200 1000 2000

0 2 20 200 1000 2000

0 2 20 200 1000 2000

Expression vector (ng)

C

Relative luciferase activity

Sp1 (ng) 0 200 0 200 400 0 400
Sp3 (ng) 0 0 200 200 0 400 400

D

Relative Luciferase activity

■ -280-Btk-Luc
□ -280-Btk-GC-M-Luc
▨ -280-Btk-GT-M-Luc

62±10

124±1

—   Sp1   Sp3   PU.1   Sp1
PU.1

Sp3
PU.1

Sp1
Sp3
PU.1

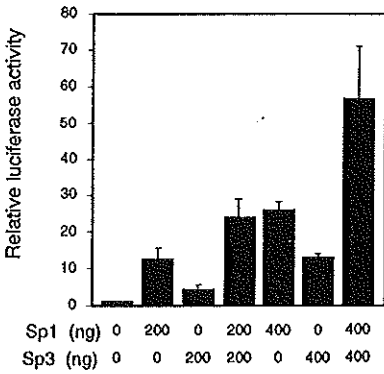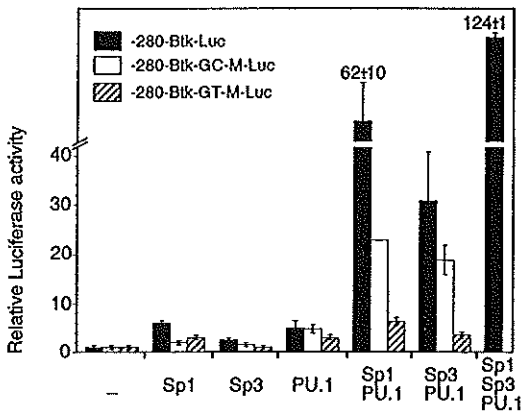*3.3 Btk expression in the absence of Sp1 or Sp3 in vivo.*

To study the functional importance of Sp1 for *in vivo* Btk expression, Sp1$^{+/+}$ and Sp1$^{-/-}$ embryonic stem (ES) cells were examined using RT-PCR (Fig. 5A). In these experiments, densitometric values were normalized to the HPRT RNA content, since the expression levels of this gene were shown to be indistinghuisable between wild-type, Sp1$^{+/-}$ and Sp1$^{-/-}$ mice (Marin *et al.,* 1997). As shown in Fig. 5A, the expression of Btk was not affected in Sp1$^{-/-}$ ES cells. Due to lethality of Sp1$^{-/-}$ mice at embryonic day 9-10 (Marin *et al.,* 1997) no lymphoid tissues of Sp1$^{-/-}$ mice can be analyzed. The low expression of the Btk gene in day 8.5 embryos, which was at the borderline of detection, precluded a quantitative comparison of Btk levels in Sp1$^{+/+}$, Sp1$^{+/-}$ and Sp1$^{-/-}$ total embryo's. However, in at least one Sp1$^{-/-}$ day 8.5 embryo we detected significant levels of Btk (data not shown). Subsequently, we analyzed day 11.5 embryos at which time point, due to the lethality of Sp1-deficient embryos, no Sp1$^{-/-}$ mice can be found. Densitometric analyses demonstrated that expression levels of Btk were similar in wild-type and Sp1$^{+/-}$ heterozygous embryos (Fig. 5B). We also did not detect differences in Btk expression between Sp1$^{+/+}$ and Sp1$^{+/-}$ day 14.5 fetal liver samples (data not shown). These results demonstrated that Sp1 is not absolutely essential for Btk expression *in vivo* and can be compensated for by other factors.

One of these compensating factors might be transcription factor Sp3. As Sp3$^{-/-}$ mice die perinatally (Peter Bouman, unpublished data), we analyzed fetal livers of day 19 dpc Sp3$^{+/+}$ and Sp3$^{-/-}$ embryo's to study the functional importance of Sp3 for *in vivo* Btk expression. As shown in Fig. 5C, Btk was expressed at comparable levels in fetal liver cells of both Sp3$^{+/+}$ and Sp3$^{-/-}$ mice, as detected by Western blotting experiments.
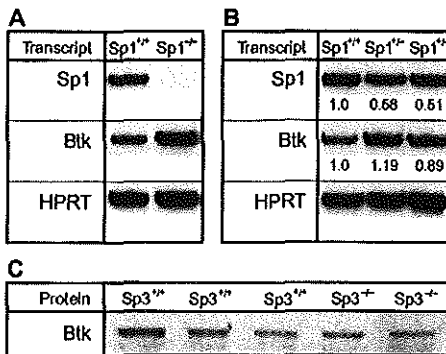
Figure 5. *Effect of Sp1 and Sp3 on Btk expression. (A) RT-PCR analysis of the indicated transcripts in Sp1$^{+/+}$ and Sp1$^{-/-}$ ES cell clones. (B) RT-PCR analysis of the indicated transcripts in day 11.5 embryos. Genotypes are given at the top. Under the transcripts, the relative densities of the yield are given. Using HPRT, the relative densitometric values of Sp1 and Btk transcripts of the Sp1$^{+/+}$ embryo were set to 1.0. (C) Western blot analysis of Btk expression in total cell lysates (2 x 10^5 cells/lane) from spleens of day 19.5 embryos. Genotypes are indicated at the top.*

4. DNAseI hypersensitive site mapping of the human *Btk* gene

To identify regions within the human *Btk* gene that are possibly involved in regulation of *Btk* expression, we performed DNAseI hypersensitivity experiments on human tonsil cell suspensions. Nuclei of these cells were incubated with DNAseI and, DNA was subsequently extracted, and digested with restriction enzymes, and fragments were separated on agarose gels. Digests of *Eco*RI, *Bgl*II, *Bam*HI, *Pst*I and *Hind*III were hybridized to a large series of single-copy probes from the *Btk* region. Fig. 6A shows the genomic organization of the human *Btk* locus and indicates the 9 DNAseI hypersensitive sites (HSS) that were detected. The localization of the identified HSS was compared to the regions identified by Oeltjen *et al.* (1997), in which there is strong conservation of intronic DNA sequence between mice and man (Fig. 6A). Five of the HSS were strong, and representative blots, showing hypersensitive fragments, hybridizing to the indicated probes p1, p2 and p3 are shown in Fig. 6B-F. Using probe p1, two HSS could be detected in a 7.5 kb *Hind*III fragment encompassing exon 1 (I and II in Fig. 6A and B). With probe p3 a HSS site 3' of exon 19 in a 3.0 kb *Bgl*II fragment could be detected (IX in Fig. 6A and C). Two additional HSS could be detected in a 19 kb *Bam*HI fragment with probe p2 (V and VI in Fig. 6A and D). The localization of these HSS was confirmed in genomic digests of multiple restriction enzymes, using probes located both at the 5' end and at the 3' end of the restriction fragments (data not shown). To test B cell specificity of the strongest HSS, we isolated B and T cell fractions from human tonsils, using standard E-rozetting techniques. HSS V and VI appeared to be B cell specific, as DNA isolated from tonsilar B cells was very sensitive to DNAseI, whereas DNA isolated from tonsilar T cells was much more resistant to DNAseI treatment (Fig. 6E and F). When we analyzed the B cell specificity of the other HSS, only minor differences between the B and T cell fractions were detected (data not shown). HSS VI was found to co-localize to a 229 bp intronic region that showed strong conservation between men and mice (Oeltjen *et al.*, 1997) (Fig. 6A). The region contained binding sites for PU.1 and transcription factor C/EBP (Lee *et al.*, 1997) that were conserved between men and mice.

Figure 6. *DNAse I hypersensitive site assays on human tonsilar B and T cells. (A) Map showing the genomic organization of the Btk gene, including the position of the 19 exons (Sideras et al., 1994) and intronic regions of more than 80% conservation between human and mouse (open boxes). Restriction fragments and the position of the probes (p) are indicated at the bottom. Roman numbers represent the HSS detected. E, EcoRI; H, HindIII; B, BamHI; Bg, BglII. (B-F) Southern blot analysis of the DNAseI treated DNA from tonsilar lymphocytes (B-D), B cells (E) and T cells, (F). Black triangles above the blots indicate the increase in DNAseI-concentration (0-6 units/ml). The restriction enzymes and probes used are indicated at the bottom. The size of the uncleaved restriction fragment and the HSS (roman numbers) are given on the right.*

A

B HindIII / probe : p1

C BglII / probe : p3

D BamHI / probe : p2

E B-cells

F T-cells
BamHI / probe : p2
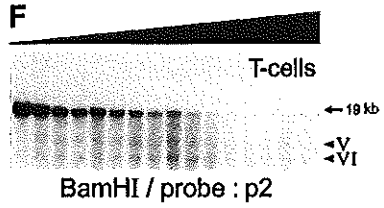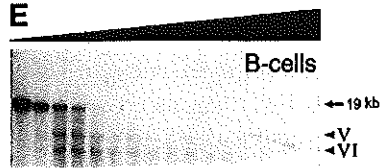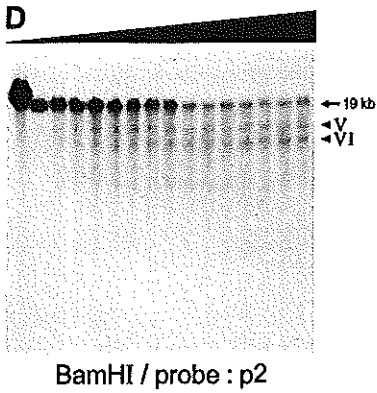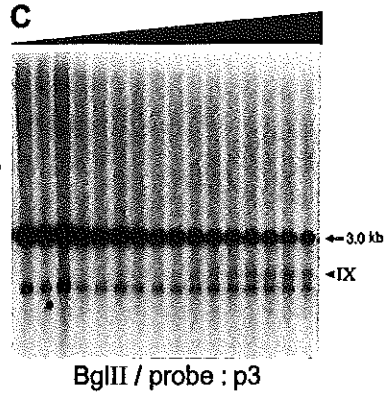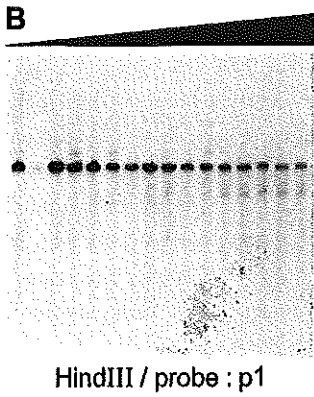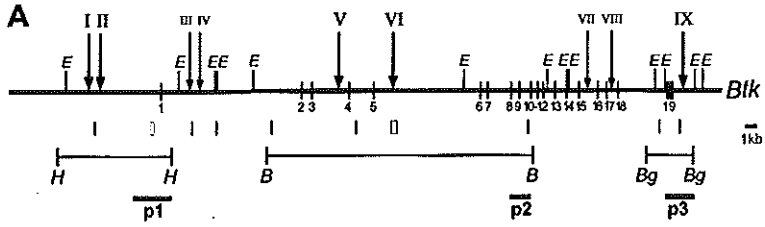
Discussion

Btk expression patterns in vivo

Although *Btk* has been demonstrated to be expressed throughout the B cell development, with the exception of plasma cells, and in other cells, such as monocytes, granulocytes and megakaryocytes (De Weers *et al.*, 1993, Smith *et al.*, 1994, Genevier *et al.*, 1994, Tsukada *et al.*, 1993) these studies were mainly done in cultured cell lines or leukemia samples. Therefore, data on the expression pattern *in vivo* or the levels of Btk in the individual stages of b cell development were not available. To study murine *Btk* expression *in vivo* we used the *Btk⁻/ LacZ* mutation (Hendriks *et al.*, 1996), intracellular flow cytometry and RT-PCR. Consistent with published data in the mouse and in human, we found that *Btk* was expressed throughout the B cell differentiation, from the early pro-B cell stage up to the activated B lymphoblasts. In contrast to the enormous differences in expression levels of the BCR on the cell surface (*e.g.* between IgM^{low} and IgM^{high} immature B cells) the expression of Btk appeared to be constant during B cell development. In the myeloid cell lineage, *Btk* was found to be expressed in the different stages of monocyte/macrophage development, whereas Btk expression in granulocytes and their precursors in the bone marrow was low or variable. In contrast to the *LacZ* activity observed in the Ter-119^{+} (pro-)erythroblast population in the bone marrow of *Btk⁻/Y* mice (Fig. 1), indicating the expression of *Btk*, we could not detect *Btk* expression using flow cytometry in this cell population. Although it can not be excluded that there are erythroid-specific negative regulator sequences present in the region encompassing exon 8 to 13, which is deleted in *Btk⁻/Y* mice (Hendriks *et al.*, 1996), the discrepancy is very likely to reflect differences in sensitivity between the two methods.

Regulation of Btk expression

Surrounding chromatin at the site of integration influences the expression of most transgenes, leading either to low expression, or to expression in cell populations that normally do not express the transgene. Therefore, to obtain position-independent and copy-number related expression, transgenes should contain the essential regulatory sequences, such as transcriptional enhancers or suppressers that are located either in introns or at the 5' or 3' flanking regions of genes (Carson *et al.*, 1993, Grosveld *et al.*, 1989, Graeves *et al.*, 1989, Yannoutsos *et al.*, 1996). We have previously reported that the human *Btk* gene was appropriately expressed in transgenic mice from a 340 kb YAC transgene, Yc340-h*Btk* (Maas *et al.*, 1997). We have now generated a YAC clone, Yc240-h*Btk*, by homologous recombination in yeast, in which ~100 kb upstream of the human *Btk* is deleted. With this ~240 kb Yc240-h*Btk* clone we have generated two transgenic mouse strains. When the Yc240-h*Btk* transgenic mice were mated onto a Btk-deficient background, the Yc240-h*Btk* could fully compensate the absence of the endogenous murine *Btk* gene. B cell numbers in the bone marrow and peripheral tissues were in the same range as compared to the wild-type littermates. The intracellular flow cytometric experiments showed that the expression pattern and expression levels

of the Yc240-h*Btk* transgene were similar to the endogenous murine *Btk*. Our results indicate that native regulatory sequences present on the 240 kb YAC are sufficient for appropriate expression of the human *Btk* gene and that *cis*-acting elements, which determine the cell lineage specificity of Btk as well as the expression level of Btk during the different stages of B cell development are highly conserved between mice and humans.

We furthermore studied the effect of Sp1, Sp3 and PU.1 binding on *Btk* expression. Although each individual transcription factor was able to transactivate the reporter construct, we have furthermore shown that transcription factor PU.1 is able to act synergistically with either Sp1 or Sp3. This synergistic effect is mediated mainly through the PU-box and the adjacent GT-box since mutation of the GT-box, resulted in a much greater reduction of activation than mutation of the GC-box, which is located about 100 bp further upstream. It has been shown that an exchange of cytosine in a typical GC-box (GGGCGG) for a thymidine, creating a GT-box (GGGTGG) weakens the binding of Sp1 (Letovsky *et al.*, 1989). It seems therefore that the position of the Sp-site is more important than the affinity. It is interesting to note in this context that the PU-box and the adjacent GT-box are highly conserved between the human and the mouse promoter whereas the more distant GC-box in the human promoter is replaced by a GT-box in the mouse promoter (Sideras *et al.*, 1994). The functional importance of the PU-box was recently confirmed by the finding that a point mutation (agggaac→aggggac) in this site leads to XLA (Holinski-Feder *et al.*, 1998). The synergistic effect can be mediated by several different mechanisms. One possibility is that Sp1 and Sp3 interact directly with PU.1, as was shown for Sp1 with several transcription factors (Perkins *et al.*, 1994, Li *et al.*, 1991, Lee *et al.*, 1997, Sif *et al.*, 1996). This is possible through the different functional domains of Sp1, such as the DNA binding domain, as described for the interaction with E2F and p65 (Karlseder *et al.*, 1996, Perkins *et al.*, 1994) or the activation domains, as shown for the interaction with C/EBPβ (Lee *et al.*, 1997). Also for PU.1 interactions with several other transcription factors have been described (Nagullapalli *et al.*, 1995, Pongubala *et al.*, 1992, Hagemeier *et al.*, 1993). Even though a direct interaction between Sp1 and Ets-1, another member of the Ets family of proteins, has not yet been demonstrated, it has been shown that Sp1 can act synergistically with Ets-1. Both proteins are able to form a ternary complex in the presence of DNA and bind cooperatively to their cognate sites (Gégonne *et al.*, 1993, Dittmer *et al.*, 1994). Further analysis will be necessary to reveal the nature of the synergism in the Btk promoter.

Although Sp1 and Sp3 are clearly able to transactivate the promoter construct in transfection assays, *Btk* expression is not abolished in mice with targeted deletions of the genes coding for Sp1 (Marin *et al.*, 1997) or Sp3, suggesting they can compensate for each other. It will therefore be interesting to analyze Btk expression in Sp1$^{-/-}$Sp3$^{-/-}$ double knock-out mice.

Large scale comparative sequence analysis of the human and murine *Btk* loci revealed highly conserved clusters within intronic regions (Oeltjen *et. al.*, 1997). It is very likely that these conserved regions contain transcription control elements, as has been shown for introns of several other lymphoid genes, including the immunoglobulin (Staudt *et al.*, 1991), T-cell receptor (Leiden *et al.*, 1992), IL-4 (Henkel *et al.*, 1992) and CD4 genes (Sawada *et al.*, 1994). Recently, a negative regulator for *Btk* expression in pre-B cells and two Sp1 binding sites were identified within the first

500 bp of intron 1 of *Btk* that may account for the fine tuning of *Btk* expression (Rohrer and Conley, 1998). Furthermore, intron 10 contains two E-boxes, a silencer element and methylated site in pre-B cells that becomes specifically demethylated in B cells (Parolini *et al.*, 1995). However, a regulatory role in Btk expression in B cells is not supported by our HSS analysis, since we did not find any HSS in this intronic region.

Using the homologous recombination system of Yeast, further regions can be deleted from the 240 kb YAC and precise site specific mutations within the *Btk* locus or its flanking regions can be introduced (Kaiser *et al.*, 1994). After transfer of these modified YAC into transgenic mice, functional analyses should allow the determination of the *in vivo* importance of these mutated sites. Knowledge of the complex regulation of the expression of *Btk* would be essential for the construction of a *Btk* mini locus, containing endogenous regulatory sequences, to develop further strategies to restore *in vivo* the B cell function in XLA patients.

Materials and Methods

Construction of targeting vector and shortening of YAC by homologous recombination

A 7.5 kb *Hind*III fragment encompassing the first exon of the *Btk* gene was isolated from a Btk[+] cosmid (De Weers *et al.*, 1997) and cloned into the *Hind*III site of pBluescript (BS). Subsequently, this 7.5 kb fragment was excised from pBluescript, using a *Eco*RV/*Sal*I digestion and cloned into the *Cla*I (blunted) and *Sal*I site of pCGS990HIS5-I-SceI retrofitting vector. The construct was linearized by a *Sal*I digestion and transformed into Yeast strain AB1380 containing the 9DA6 YAC (Trp and Ura selectable) (Maas *et al.*, 1997) using the lithium acetate method (Kaiser *et al.*, 1994). Colonies were plated out on Ura⁻, His⁻ selective plates and after five days colonies were picked. DNA was isolated from these colonies and checked for the presence of a shortened YAC using pulse field gel electrophoresis (PFGE). Out of 20 colonies picked, one colony contained a ~240 kb YAC, Yc240-h*Btk*, positive for the *Btk* gene and was chosen for further analyses. No abnormalities in restriction enzyme pattern of Yc240-h*Btk* were observed with *Pst*I, *Bgl*I, *Hind*III, *Eco*RI, *Bam*HI and *Pvu*I, compared to YAC clone 9DA6 and human genomic DNA using *Btk*-cDNA and the YAC arms (Maas *et al.*, 1997) as probes. The following probes were applied: *Btk*-cDNA (human *Btk* cDNA), *Btk.1* (human *Btk* cDNA bp 133-1153) and *Btk.2* (human *Btk* cDNA bp 1094-2159) (De Weers *et al.*, 1997, Maas *et al.*, 1997).

Isolation of YAC DNA and generation of transgenic mice

Size-fractionation of yeast chromosomes and preparation of YAC DNA was essentially performed as described previously (Maas *et al.*, 1997). Yeast DNA, embedded in agarose blocks were digested with meganuclaese I-Sce-I according the manufactures protocol (Boehringer Mannheim, Mannhein Germany) before size-fractionation of the yeast chromosomes. Purified YAC DNA was micro-injected into pronuclei of FVB fertilized oocytes (Hogan *et al.*, 1994) at ~ 8 ng/ml. Oocytes were implanted into pseudopregnant CBA/N x C57/BL6 female mice. Tail DNA was analyzed by Southern blotting and transgenic offspring was crossed with *Btk* ⁻/*LacZ* mice of mixed 129/Sv x C57BL/6 background (Hendriks *et al.*, 1996).

Flow cytometric analyses

Preparation of single-cell suspensions, loading of cells with fluorescein-di-β-D-galactopyranoside to measure β-galactosidase activity, intracellular flow cytometric detection of cytoplasmic Btk protein, and three- or four-color flow cytometry have been described (Hendriks *et al.*, 1996, Dingjan *et al.*, 1998).

Plasmid construction

Luciferase constructs -280-Btk-Luc, -280-Btk-GC-M-Luc, -280-Btk-GT-M-Luc and -280-PU-M-Luc have been described previously (Müller *et al.*, 1996). The plasmid pPACSp1, PACUSp3, pPAC and the β-galactosidase expression plasmid p97b have been described (Hagen *et al.*, 1994, Dennig *et al.*, 1996). For the expression in *Drosophila* Schneider cells we generated the PU.1 expression plasmid pPACPU.1.

The PU.1 cDNA from ΔEB Spi-1 (a kind gift from Dr. Dominique Ray-Gallet) was cloned into pBluescript II KS. A 1.4 kb *Bam*HI/*Xho*I fragment was subsequently subcloned into pPACSp1 plasmid from which the Sp1 cDNA was removed by *Bam*HI/*Xho*I cleavage. To achieve an in frame protein a PCR product was generated between the following primer pair: 5'-GCGGGATCCATGTTACAGGCGTGC-3' and 5'-CTCTGGAGCTCCGTGAAG T-3' and cloned between the *Bam*HI and *Sst*I sites (translational fusion). The structure of the construct was verified by nucleotide sequencing (Sanger, 1977).

Cell culture and transfections

The human B-cell line DG75 (Epstein-Barr virus-negative Burkitt's lymphoma) was cultured under conditions previously described (Sideras *et al.*, 1994). *Drosphila* Schneider (SL2) cells (Schneider 1972) were maintained in Schneider medium supplemented with 10 % fetal bovine serum and 2 mM glutamine at 25°C. One day prior to transfection cells were plated at a density of 3.6×10^6 cells in 6 cm dishes. Cells were transfected by the calcium phosphate method. (DiNocera *et al.*, 1983). Each plate received 14 μg of plasmid with 4 μg of the β-galactosidase reporter plasmid p97b as internal control. DNA amount was kept constant by adding the empty expression vector pPAC. After 14 h the medium was changed and cells were harvested 48 h post transfection.

Luciferase and -galactosidase assays

Cells were harvested after 2 washes in phosphate buffered saline in 200 μl lysis buffer (25 mM Tris-Acetate pH 7.8, 2 mM DTT, 1 mM EDTA, 10 % Glycerol, 1 % Triton-X-100). 100 μl lysate was mixed with 400 μl Luciferase reagent (20 mM Tris-Acetate pH 7.8, 33 mM DTT, 1 mM EDTA, 3.74 mM MgSO4, 0.27 mM Coenzyme A, 0.47 mM luciferin, 0.53 mM ATP) and assayed using standards procedures (Maniatis *et al.*, 1989). Transfections were carried out in duplicates which were repeated three times and normalized to the β-Galactosidase activity. β-Galactosidase activity as determined according to Hall et al. (1983).

Reverse transcription (RT)-PCR and Western blotting

Sp1$^{-/-}$ ES cells and embryos have been described (Marin *et al.*, 1997). Isolation of RNA, cDNA synthesis and PCR reactions were essentially performed as described previously (Maas *et al.*, 1997).

Amplification was for 25, 33 or 35 cycles with denaturation at 94°C for 1 min, followed by annealing at 56°C for 1 min and extension at 72°C for 45 s. The PCR products were visualized by electrophoresis on a 6 % polyacrylamide 1 x TBE gel followed by autoradiography. IMAGEQUANT (Molecular Dynamics) was used for quantifications. Specific primers for murine Btk, human Btk, HPRT and Sp1 have been described (Maas *et al.*, 1997, Marin *et al.*, 1997).

Western blotting was performed as described (Hendriks *et al.*, 1996) using 2×10^4 spleen cells per lane.

Mapping of DNAseI hypersensitive sites

Human tonsilar lymphomononuclear cells (MNC) were isolated by standard Ficoll-isoplaque gradient centrifugation. T-cells were separated from non-T cells by incubating 2×10^7 cells/ml on ice for 40 minutes with 2-aminoethylisothiouroniumbromide (AET)-treated sheep erythrocytes and erythrocyte free fetal calf serum (FCS). The T-cell-AET- rosettes were separated from the non-T cells by centrifugation on a Ficoll-isoplaque gradient (Schuurman *et al.*, 1980). B-cells were isolated from the interphase and washed twice with PBS. T-cells were isolated by resuspending the cell-pellet in lysisbuffer (0.155M NH4CL, 0.01M KHCO3, 0.1 mM Na-EDTA pH 7.4) on ice to lyse the erythocytes, followed by washing with RPMI medium supplemented with 10% FCS.

Nuclei of T and B cells were isolated by incubating 5×10^8 cells in HS-buffer (15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 5% glycerol, 1 mM DTT, 0.15 mM Spermine, 0.1 mM Spermidine, 1 mM PMSF) on ice. Nonidet NP40 was added to a final concentration of 0.1 % and the cell suspensions were transferred to an all glass Dounce homogenizer. The lysis of the cells was monitored by visually examination of samples. Nuclei were collected by centrifugation at 1500 rpm at 2°C and resuspended in ice-cold HS-buffer at a concentration of 3×10^7 nuclei/ml.

DNAseI treatment was performed in a total volume of 2 ml of HS-buffer, containing 3×10^7 nuclei, supplemented with 20 mM MgCl2, 1mM CaCl2 and various amounts (0-6 units/ml) of DNAseI (Boehringer Mannheim) and incubated on ice for 30 minutes. Reactions were stopped by adding equal volumes of 2x proteinase-K buffer (0.2 M Tris-HCl pH 8, 0.4 M NaCl, 1% SDS) and 200 µg/ml proteinase K, followed by an incubation overnight at 45°C. DNA was isolated and digested with restriction endonucleases followed by agarose gel electrophoresis and southern blotting. Southern blots were hybridized with various probes covering the *Btk*. Probe p1 (Fig. 1) is a 2.4 kb *PstI-Hind*III genomic fragment containing exon 1, probe p2 is a 1.6 kb genomic PCR product from exon 8 to exon 10 and probe p3 is a genomic 2.1 kb *Eco*RI fragment including exon 19.

Acknowledgments

References

De Bruijn, M.F.T.R., Slieker, W.A.T., Van der Loo, J.C.M., Voerman, J.S.A., Van Ewijk, W. and Leenen, P.J.M. (1994) *Eur. J. Immunol.* 24:2279-2284.

Carson, S. and Willis, M.V. (1993) *Nucl. Acid Res.* 21:2065-2072.

Courey, J.A. and Tjian, R. (1988) *Cell* 55:5659-5667.

Denning, J., Beato, M. and Suske, G. (1996) *EMBO J.* 15:5659-5667.

Desiderio, S. (1997) *Curr. Op. Immunol.* 9:534-540.

De Weers,M., Dingjan, G.M., Brouns, G.S., Kraakman, M.E.M., Mensink, R.G.J., Lovering, R.C., Schuurman, R.K.B., Borst, J. and Hendriks, R.W. (1997) *Clin. Exp. Immunol.* 107:235-240.

De Weers, M., Verschuren, M.C., Kraakman, M.E., Mensink, R.G., Schuurman, R.K.B., van Dongen, J.J. and Hendriks, R.W. (1993) *Eur. J. Immunol.* 23:3109-3114.

Dingjan, G.M., Maas, A., Nawijn, M.C., Smit, L., Voerman, J.S.A., Grosveld, F. and Hendriks, R.W. (1998) *EMBO J.* 18:5309-5320.

DiNocera, P.P. and Dawid, I.B. (1983) *Proc. Natl. Acad. Sci. USA* 80:7095-7098.

Dittmer, J., Gégonne, A., Gitlin, S.D., Ghysdael, J. and Brady, J.N. (1994) *J. Biol. Chem.* 269:21428-21434.

Eisenberg, C.F., Singh, H. and Storb, U. (1995) *Genes Dev.* 9:1377-1387.

Futatani, T., Miyawaki, T., Tsukada, S., Hashimoto, S., Kunikata, T., Arai, S., Kurimoto, M., Niida, Y., Matsuoka, H., Sakiyama, Y., Iwata, T., Tsuchiya, S., Tatsuzawa, O., Yoshizaki, K. and Kishimoto, T. (1998) *Blood* 91:595-602.

Gégonne, A., Bosselut, R., Bailly, R.A. and Ghysdael, J. (1993) *EMBO J.* 12:1169-1178.

Genevier, H.C., Hishelwood, S., Caspar, H.B., Rigley, K.P., Brown, D., Saeland, S., Rousset, F., Levinsky, R.J., Callard, R.E., Kinnon, C. and Lovering, C. (1994) *Eur. J. Immunol.* 24:3100-3105.

Greaves, D.R., Wilson, F.D., Lang, G. and Kiousis, D. (1989) *Cell* 56:979-986.

Gross, D.S. and Garrard, W.T., (1988) *Ann. Rev. Biochem.* 57:159-197.

Grosveld, F., van Assendelft, G.B., Greaves, D. and Kiousis, D. (1989) *Cell* 51:975-985.

Hagemeier, C., Bannister, A.J., Cook, A. and Kouzarides, T. (1993) *Proc. Natl. Acad. Sci. USA* 90:1580-1584.

Hagen, G., Müller, S., Beato, M. and Suske, G. (1994) *EMBO J.* 13:3843-3851.

Hagen, G., Müller, S., Beato, M. and Suske, G. (1992) *Nucleis Acids Res.* 20:5519-5525.

Hall, C.V., Jacob, P.E., Ringold, G.M. and Lee, F (1983) *J. Mol. Appl. Genet.* 2:101-109.

Hendriks, R.W., de Bruijn, M.F.T.R., Maas, A., Dingjan, G.M., Karis, A. and Grosveld, F. (1996) *EMBO J.* 15:4862-4872.

Henkel, G., Weiss, D.L., McCoy, R., Deloughery, T., Tara, D. and Brown, M.A. (1992) *J. Immunol.* 149:3239-3246.

Himmelmann, A., Thevenin, C., Harrison, K. and Kehrl, J.H. (1996) *Blood* 87:1036-1044.

Holinski-Feder, E., Weiss, M., Brandau, O., Jedele, K.B., Nore, B., Bäckesjö, M., Vihinen, M., Hubbard, S.R., Belohradsky, B.H., Smith, C.I.E. and Meindl, A. (1998) *Pediatrics* 1:276-284.

Kaiser, C., Michaelis, S. and Mitchell, A. (1994) Methods in Yeast genetics, A cold Spring Harbor laboratory course manual, 1994 ed. Cold Spring Harbor, NY.

Karlseder, J., Rotheneder, H. and Wintersberger, E. (1996) *Mol. Cell. Biol.* 16:1659-1667.

Khan, W.F., Alt, , F.W., Gerstein, R.M., Malynn, B.A., Larsson, I., Rathbun, G., Davidson, L., Müller, S., Kantor, A.B., Herzenberg, L.A., Rosen, F.S. and Sideras, P. (1995) *Immunity* 3:283-299.

Kerner, J.D., Appleby, M.W., Mohr, R.N., Chein, S., Rawlings, D.J., Maliszeweski, C.R., Witte, O.N. and Perlmutter, R. (1995) *Immunity* 3:301-312.

Klemsz, M.J., McKercher, S.R., Celada, A., van Beveren, C. and Maki, R.A. (1990) *Cell* 61:113-124.

Lee, Y.H., Williams, S.C., Baer, M., Sterneck, E., Gonzalez, F.J. and Johnson, P.F. (1997) *Mol. Cell. Biol.* 17:2038-2047.

Leiden, J.M. (1992) *Immunol. Today* 13:22-30.

Letovsky, J. and Dynan, W.S. (1989) *Nucleic Acids Res.* 17:2639-2653.

Li, R., Knight, J.D., Jackson, S.P., Tjian, R. and Botchan, M.R. (1991) *Cell* 65:493-505.

Maas, A., Dingjan, G.M., Savelkoul, H.F.J., Kinnon, C., Grosveld, F. and Hendriks, R.W. (1997) *Eur. J. Immunol.* 27:2180-2187.

Maniatis, Y., Fritsch, E.F. and Sambrook, J. (1989) *Molecular cloning: A laboratory manual* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marin, M., Karis, A., Visser, P., Grosveld, F. and Philipsen, S. (1997) *Cell* 89:619-628.

Mittrucker, H-W., Matsuyama, T., Grossman, A., Kundig, T.M., Potter, J., Shahinian, A., Wakeham, A., Patterson, B., Ohashi, P.S. and Mak, T.W. (1997) *Science* 275:540-543.

Moreau-Gachelin, F. (1994) *Biochim. Biophys. Acta* 1198:149-163.

Müller, S., Sideras, P., Smith, C.I.E. and Xanthopoulos, K.G. (1996) *Oncogene* 13:1955-1964.

Nagulapali, S., Pongubala, J.M. and Atchison, M.L. (1995) *J. Immunol.* 155:4330-4338.

Oeltjen, J.C., Malley, T.M., Muzny, D.M., Miller, W., Gibbs, R.A. and Belmont, J.W. (1997) *Genome Res.* 7:315-329.

Parolini, O., Rohrer, J., Shapiro, L.H. and Conley, M.E. (1995) *Immunogenetics* 42:129-135.

Perkins, N.D., Agranoff, A.B., Pascal, E. and Nabel, G.J. (1994) *Mol. Cell. Biol.* 14:6570-6583.

Pongubala, J.M.R. and Atchison, M.L. (1997) *Biochemistry* 94:127-132.

Pongubala, J.M.R., Nagulapalli, R.S., Klemsz, M.J., McKercher, S.R., Maki, R.A. and Atchison, M.L. (1992) *Mol. Cell. Biol.* 12:368-372.

Rawlings, D.J., Saffran, D.C., Tsukada, S., Largaespada, D.A., Grimaldi, J.C., Cohen, L., Mohr, R.N., Bazan, J.F., Howard, M., Copeland, M.G., Jenkins, N.A. and Witte, O.N. (1993) *Science* 261:358-361.

Rohrer, J. and Conley, M.E. (1998) *Blood* 91:214-221.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74:5463-5467.

Sawada, S., Scarborough, J.D., Killeen, N. and Littmann, D.R. (1994) *Cell* 77:917-....

Scher, I.. and Wicker, (1982) *Adv. Immunol.* 33:1-71.

Schneider, I. (1972) *J. Embryol. Exp. Morphol.* 27:353-365.

Schuurman, R.K.B., Gelfand, E.W., Matheson, D., Zimmerman, B. and Dosch, H-M. (1980) *J. Immunol.* 124:1924-1928.

Sideras, P., Müller, S., Shiels, H., Jin, H., Khan, W.N., Nilsson, L., Parkinson, E., Thomas, J.D., Brandén, L., Larsson, I., Paul, W.E., Rosen, F., Alt, F.W., Vetrie, D., Smith, C.I.E. and Xanthopoulos, K.G. (1994) *J. Immunol.* 153:5607-5617.

Sideras, P. and Smith, C.I.E. (1995) *Adv. Immunology* 59:135-223.

Sif, S. and Gilmore, T.D. (1994) *J. Virol.* 68:7131-7138.

Smith, C.I.E., Baskin, B., Humire-Greiff, P., Zhou, J.N., Olsson, P.G. Maniar, H.S., Kjellén, P., Lambris, J.D., Christensson, B., Hammarström, L., Bentley, D., Vetrie, D., Islam, K.B., Vorechovsky, I. and Sideras, P. (1994) *J. Immunol.* 152:557-565.

Staudt, L.M. and Lenardo, M.J. (1991) *Ann. Rev. Immunol.* 9:373-398.

Su, G.H., Ip, H.S., Cobb, B.S., Lu, M.M., Chen, H.M. and Simos, M.C. (1996) *J. Exp. Med.* 184:203-214.

Supp, D.M., Witte, D.P., Branforrd, W.W., Smith, E.P. and Potter, S.S. (1996) *Dev. Biology* 176:284-299.

Thomas, J.D., Sideras, P., Smith, C.I.E., Vorechovsky, I., Chapman, V. and Paul, W.E. (1993) *Science* 261:355-358.

Tsukada, S., Saffran, D.C., Raelings, D.J., Parolini, O., Allen, R.C., Klisak, I., Sparkes, R.S., Kubagawa, H., Thuluvancheri, M., Quan, S., Belmont, J.W., Cooper, M.D., Conley, M.E. and Witte, O.N. (1993) *Cell* 72:279-290.

Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammerström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C.I.E. and Bentley, D.R. (1993) *Nature* 361:226-233.

Vihinen, M., Brandau, O., Brandén, O., Kwan, S-P., Lappalainen, I., Noordzij, J.G., Ochs, H.D., Ollila, J., Pienaar, S.M., Rukonen, P., Saha, B.K. and Smith, C.I.E. (1998) *Nucl. Acid Res.* 1:244-250.

Yannoutsos, N., Ijzermans, J.N.M., Harkes, C., Bonthuis, F., Zhou, C., White, D., Marquet, R.L.M. and Grosveld, F. (1996) *Genes to Cells* 1:409-419.

Chapter 5

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

Gemma M. Dingjan, Alex Maas, Martijn C. Nawijn, Linda Smit, Jane S.A. Voerman, Frank Grosveld and Rudolf W. Hendriks

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@chl.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 *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
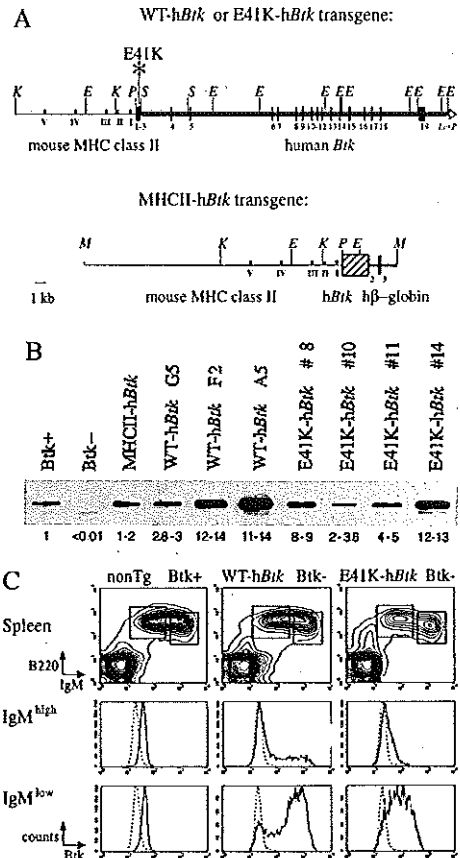
Fig. 1. Structure and protein expression of the h*Btk* transgenes.
(A) Map of the transgene constructs, showing the locations of the five DNase I hypersensitivity sites present in the 10.6 *Kpn*I–*Pvu*I mouse MHC class II upstream Ea fragment. The WT-h*Btk* and E41K-h*Btk* transgenes contain a 27.4 kb 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 (2×10⁵ 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 nontransgenic 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 contrast, 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-hBtk and E41K-hBtk transgenic mice

Compartment	Cell population	Non-transgenic		WT-hBtk		E41K-hBtk	
		[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).

A Lymph Node
B220 / IgM
Btk+ 17, Btk- 8
WT-hBtk Btk- 9, E41K-hBtk Btk- 2

B Peritoneal Cavity
CD5 / IgM
Btk+ 27, 20, Btk- 1, 16
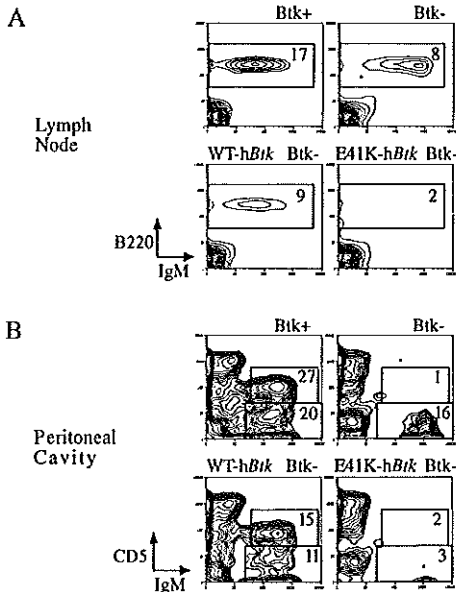WT-hBtk Btk- 15, 11, E41K-hBtk Btk- 2, 3

Fig. 2. Depletion of peripheral B cells in E41K-hBtk 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-hBtk or E41K-hBtk 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-hBtk 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-hBtk transgenic mice and a 3- to 4-fold reduction in E41K-hBtk 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-hBtk and WT-hBtk 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-hBtk 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-hBtk transgene expression (Drabek et al., 1997). In the WT-hBtk transgenic mice on the Btk$^+$ background, serum Ig subclass levels were in the same ranges. In the E41K-
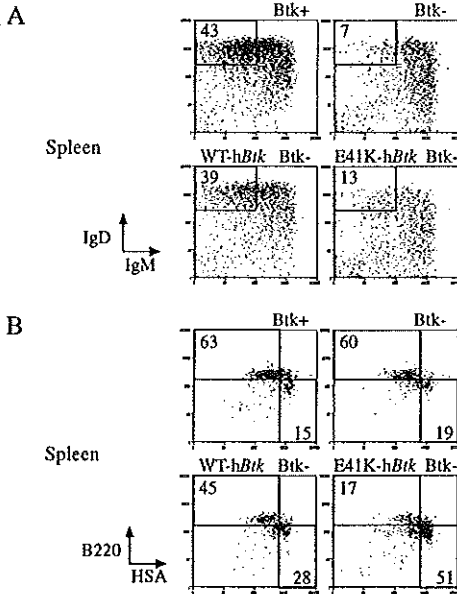
Fig. 3. Expression of E41K Btk induces a dominant maturational defect in peripheral B cells. (A) Surface IgM-IgD profiles of splenic B cells. Percentages of B220+ cells that are IgM^low IgD^high are given. These cells are mature B cells, whereas IgM^high IgD^low cells are more immature (Hardy *et al.*, 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-hBtk 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-hBtk and E41K-hBtk 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-hBtk (*n* = 7) and E41K-hBtk (*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.

hBtk 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-hBtk mice decreased significantly with age. In 6-month-old E41K-hBtk 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-hBtk 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-hBtk 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-hBtk and E41K-hBtk 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 absorbance measured did not differ from the values of unimmunized animals. On the Btk+ background, the TI-II response of WT-hBtk mice was comparable with the response of normal mice, while on the Btk− background, expression of the WT-hBtk transgene could only partially restore the

A

TI-II
response

IgM   IgG3

B

TD
primary
response

IgM   Optical density

Serum dilution

```
□ Btk+
■ Btk-
○ WT-hBtk   Btk+
● WT-hBtk   Btk-
△ E41K-hBtk Btk+
▲ E41K-hBtk Btk-
✕ pre-immune
```

C

TD
secondary
response

IgM   IgG1
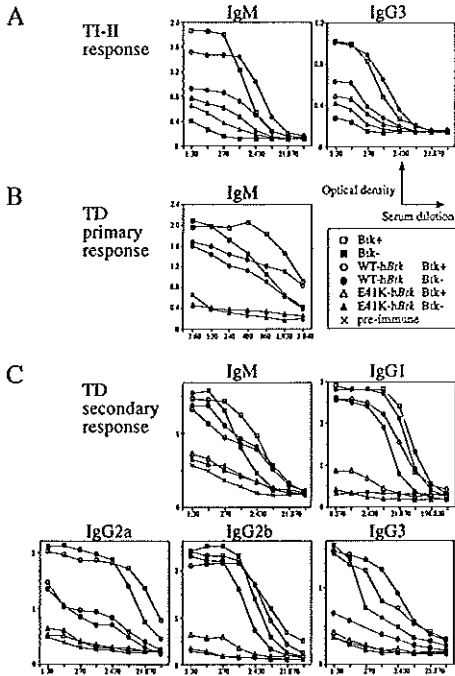
IgG2a   IgG2b   IgG3

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 litter-mates, 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 [³H]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-h*Btk* 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
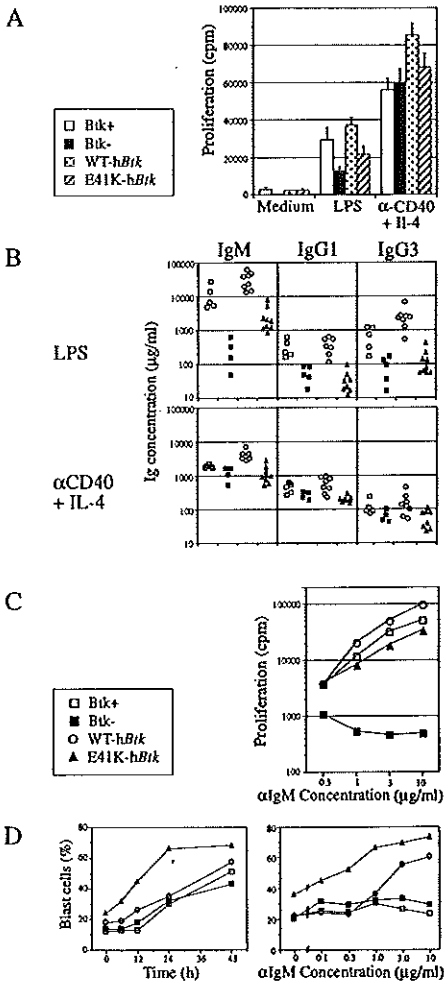
Fig. 6. Transgenic E41K-h*Btk* B cells show aberrant responses to mitogenic signals *in vitro*. (A) Proliferation, determined by [³H]thymidine incorporation, of cells cultured in medium alone, in the presence of LPS, or anti-CD40 antibodies and IL-4. (B) Ig levels of supernatants of 7-day cultures in the presence of LPS or anti-CD40 and IL-4. (C) Proliferation, determined by [³H]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
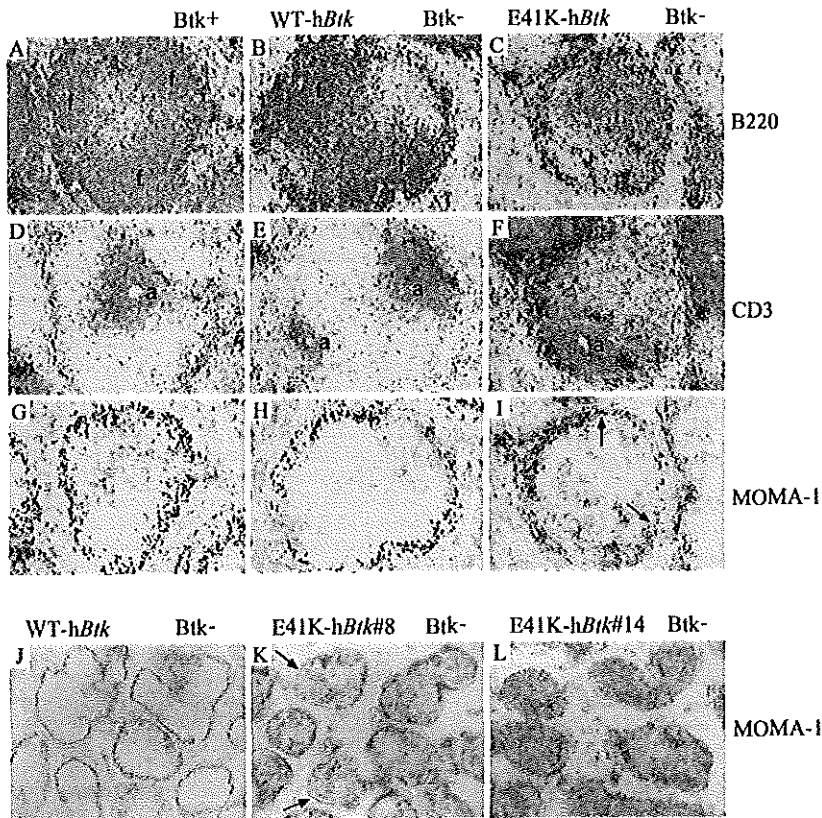
Fig. 7. Disrupted splenic architecture in E41K-h*Btk* transgenic mice. Immunohistochemical analysis of 5 μm splenic frozen sections from Btk+ mice, WT-h*Btk* Btk‾ mice and E41K-h*Btk* transgenic mice [(C), (F), (I) and (K), line #8, and (L), line #14] on the Btk‾ background. Sections were stained with 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 immuno-histochemical 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 hBtk cDNA clone phBtk2.55 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 hBtk 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 hBtk 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 hBtk 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 hBtk 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 hBtk 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).

References

Allman,D.M., Ferguson,S.E. and Cancro,M.P. (1992) Peripheral B cell maturation I. Immature peripheral B cells in adults are heat-stable antigen (hi) and exhibit unique signalling characteristics. *J. Immunol.*, 149, 2533–2540.

Allman,D.M., Ferguson,S.E., Lentz,V.M. and Cancro,M.P. (1993) Peripheral B cell maturation II. Heat-stable antigen (hi) splenic B cells are an immune developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol.*, 151, 4431–4444.

Anderson,J.S., Teutsch,M., Dong.Z. and Wortis,H.H. (1996) An essential role for Bruton's tyrosine kinase in the regulation of B-cell apoptosis. *Proc. Natl Acad. Sci. USA*, 93, 10966–10971.

Aoki,Y., Isselbacher,K.J. and Pillai,S. (1994) Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B and antigen receptor-ligated B cells. *Proc. Natl Acad. Sci. USA*, 91, 10606–10609.

Bence,K., Ma,W., Kozasa,T. and Huang,X.Y. (1997) Direct stimulation of Bruton's tyrosine kinase by G$_q$-protein α-subunit. *Nature*, 389, 296–299.

Caamaño,J.H., Rizoo,C.A., Durham,S.K., Barton,D.S., Raventos-Suarez,C., Snapper,C.M. and Bravo,R. (1998) Nuclear Factor (NF)-κB2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J. Exp. Med.*, 187, 185–196.

Carson,S. and Wiles,M.V. (1993) Far upstream regions of class I MHC Ea are necessary for position-independent, copy-dependent expression of Ea transgene. *Nucleic Acids Res.*, 9, 2065–2072.

Cook,M.C., Basten,A. and Fazekas de St Groth,B. (1997) Outer periarteriolar lymphoid sheath arrest and subsequent differentiation of both naive and tolerant immunoglobulin transgenic B cells is determined by B cell receptor occupancy. *J. Exp. Med.*, 186, 631–643.

DeJong,J.P., Voerman,J.S.A., Leenen,P.J., Van der Sluijs-Gelling,A.J. and Ploemacher,R.E. (1991) Improved fixation of frozen lympho-haemopoietic tissue sections with hexazotized pararosaniline. *Histochem. J.*, 23, 392–401.

Desiderio,S., (1997) Role of *Btk* in B cell development and signalling. *Curr. Opin. Immunol.*, 9, 534–540.

DeWeers,M., Verschuren,M.C.M., Kraakman,M.E.M., Mensink,R.G.J., Schuurman,R.K.B., Van Dongen,J.J.M. and Hendriks,R.W. (1993) The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur. J. Immunol.*, 23, 3109–3114.

DeWeers,M., Mensink,R.G.J., Kraakman,M.E.M., Schuurman,R.K.B. and Hendriks,R.W. (1994a) Mutation analysis of the Bruton's tyrosine kinase gene in X-linked agammaglobulinemia: identification of a mutation which affects the same codon as is altered in immunodeficient *xid* mice. *Hum. Mol. Genet.*, 3, 161–166.

DeWeers,M., Brouns,G.S., Hinshelwood,S., Kinnon,C., Schuurman, R.K.B., Hendriks,R.W. and Borst,J. (1994b) B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. *J. Biol. Chem.*, 269, 23857–23860.

DeWeers,M., Dingjan,G.M., Brouns,G.S., Kraakman,M.E.M., Mensink, R.G.J., Lovering,R.C., Schuurman,R.K.B., Borst,J. and Hendriks,R.W. (1997) Expression of Bruton's tyrosine kinase in B lymphoblastoid cell lines from X-linked agammaglobulinemia patients. *Clin. Exp. Immunol.*, 107, 235–240.

Drabek,D., Raguz,S., De Wit,T.P.M., Dingjan,G.M., Savelkoul,H.F.J., Grosveld,F. and Hendriks,R.W. (1997) Correction of the Xid phenotype by transgenic expression of human *Btk* under the control of the class II MHC Ea locus control region. *Proc. Natl Acad. Sci. USA*, 94, 610–615.

Franzoso,G. *et al.* (1997) Critical roles for the Bcl-3 oncoprotein in T cell-mediated immunity, splenic microarchitecture and germinal center reactions. *Immunity*, 6, 479–490.

Franzoso,G. *et al.* (1998) Mice deficient in nuclear factor (NF)-κB/p52 present with defects in humoral responses, germinal center reactions and splenic microarchitecture. *J. Exp. Med.*, **187**, 147–159.

Fukuda,M., Kojima,T., Kabayama,H. and Mikoshiba,K. (1996) Mutation of the pleckstrin homology domain of Bruton's tyrosine kinase in immunodeficiency impaired inositol 1,3,4,5-tetrakisphosphate binding capacity. *J. Biol. Chem.*, **48**, 30303–30306.

Fulcher,D.A. and Basten,A. (1994) Reduced life span of tolerant self-reactive B cells in a double transgenic model. *J. Exp. Med.*, **179**, 125–134.

Fulcher,D.A., Lyons,A.B., Korn,S.L., Cook,M.C., Koleda,C., Parish,C., Fazekas de St Groth,B. and Basten,A. (1996) The fate of self-reactive B cells depends primarily on the degree of antigen receptor engagement and availability of T cell help. *J. Exp. Med.*, **183**, 2313–2328.

Fulcher,D.A. and Basten,A. (1997) Influences on the lifespan of B cell subpopulations defined by different phenotypes. *Eur. J. Immunol.*, **27**, 1188–1199.

Goodnow,C.C. *et al.* (1995) Self-tolerance checkpoint in B lymphocyte development. *Adv. Immunol.*, **59**, 279–368.

Gu,H.D., Tarlinton,D., Muller,W., Rajewsky,K. and Förster,I. (1991) Most peripheral B cells are ligand selected. *J. Exp. Med.*, **173**, 1357–1371.

Hardy,R.R., Hayakawa,K., Haaijman,J. and Herzenberg,L.A. (1982) B-cell subpopulations identified by two-color fluoresence analysis using a dual-laser FACS. *Nature*, **297**, 589–591.

Hardy,R.R., Carmack,C.E., Shinton,S.A., Kemp,J.D. and Hayakawa,K. (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.*, **173**, 1213–1225.

Hendriks,R.W., de Bruijn,M.F.T.R., Maas,A., Dingjan,G.M., Karis,A. and Grosveld,F. (1996) Inactivation of *Btk* by insertion of *lacZ* reveals defects in B cell development only past the pre-B cell stage. *EMBO J.*, **15**, 4862–4872.

Hyvönen,M. and Saraste,M. (1997) Structure of the PH domain and Btk motif from Bruton's tyrosine kinase: molecular explanations for X-linked agammaglobulinemia. *EMBO J.*, **16**, 3396–3404.

Kahn,W.F. *et al.* (1995) Defective B cell development and function in *Btk*-deficient mice. *Immunity*, **3**, 283–299.

Kerner,J.D., Appleby,M.W., Mohr,R.N., Chien,S., Rawlings,D.J., Maliszeweski,C.R., Witte,O.N. and Perlmutter,R. (1995) Impaired expansion of mouse B cell progenitors lacking Btk. *Immunity*, **3**, 301–312.

Kraal,G. (1992) Cells in the marginal zone of the spleen. *Int. Rev. Cytol.*, **132**, 31–74.

Kumararatne,D.S. and MacLennan,I.C.M. (1981) Cells of the marginal zone of the spleen are derived from recirculating precursors. *Eur. J. Immunol.*, **11**, 865–869.

Leenen,P.J.M., Radosevic,K., Voerman,J.S.A., Salomon,B., Van Rooijen,N., Klatzmann,D. and Van Ewijk,W. (1998) Heterogeneity of mouse spleen dendritic cells: *in vivo* phagocytic activity, expression of macrophage markers and subpopulation turnover. *J. Immunol.*, **160**, 2166–2173.

Lemmon,M.A., Ferguson,K.M. and Schlessinger,J. (1996) PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell*, **85**, 621–624.

Li,T., Tsukada,S., Satterthwaite,A., Havlik,M.H., Park,H., Takatsu,K. and Witte,O.N. (1995) Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its Pleckstrin Homology (PH) domain. *Immunity*, **2**, 451–460.

Li,Z., Wahl,M., Eguinoa,A., Stephens,L.R., Hawkins,P.T. and Witte,O.N. (1997) Phosphatidylinositol 3-kinase-γ activates Bruton's tyrosine kinase in concert with Src family kinases. *Proc. Natl Acad. Sci. USA*, **94**, 13820–13825.

Lund,T., Grosveld,F. and Flavell,R.A. (1982) Isolation of transforming DNA by cosmid rescue. *Proc. Natl Acad. Sci. USA*, **79**, 520–524.

Maas,A., Dingjan,G.M., Savelkoul,H.F.J., Kinnon,C., Grosveld,F. and Hendriks,R.W. (1997) The Xid defect in the mouse is corrected by expression of human *Btk* from a YAC transgene. *Eur. J. Immunol.*, **27**, 2180–2187.

Mason,D.Y., Jones,M. and Goodnow,C.C. (1992) Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. *Int. Immunol.*, **4**, 163–175.

Matsumoto,M., Fu,Y.-X., Molina,H. and Chaplin,D.D. (1997) Lymphotoxin-α-deficient and TNF receptor-I-deficient mice define developmental and functional characteristics of germinal centers. *Immunol. Rev.*, **156**, 137–144.

Mattsson,P.T., Vihinen,M. and Smith,C.I.E. (1996) X-linked agammaglobulinemia (XLA): a genetic tyrosine kinase (Btk) disease. *Bioassays*, **18**, 825–834.

Melamed,D., Benschop,R.J., Cambier,J.C. and Nemazee,D. (1998) Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. *Cell*, **92**, 173–182.

Oeltjen,J.C., Malley,T.M., Muzny,D.M., Miller,W., Gibbs,R.A. and Belmont,J.W. (1997) Large scale comparative sequence analysis of the human and murine Bruton's tyrosine kinase loci reveals conserved regulatory domains. *Genome Res.*, **7**, 315–329.

Park,H., Wahl,M.I., Afar,D.E.H., Turck,C.W., Rawlings,D.J., Tam,C., Scharenberg,A.M., Kinet,J.-P. and Witte,O.N. (1996) Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity*, **4**, 515–525.

Rathmell,J.C., Townsend,S.E., Xu,J.C., Flavell,R. and Goodnow,C.C. (1996) Expansion or elimination of B cells *in vivo*: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. *Cell*, **87**, 319–329.

Rawlings,D.J. *et al.* (1993) Mutation of the unique region of Bruton's tyrosine kinase in immunodeficient mice. *Science*, **261**, 358–361.

Rawlings,D.J., Scharenberg,A.M., Park,H., Wahl,M.I., Lin,S., Sato,R.M., Fluckiger,A.-C., Witte,O.N. and Kinet,J.-P. (1996) Activation of Btk by a phosphorylation mechanism initiated by SRC family kinases. *Science*, **271**, 822–825.

Ridderstad,A., Nossal,G.J.V. and Tarlinton,D.M. (1996) The *xid* mutation dimishes memory B cell generation but does not affect somatic hypermutation and selection. *J. Immunol.*, **157**, 3357–3365.

Salim,K. *et al.* (1996) Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.*, **15**, 6241–6250.

Saouaf,S.J., Mahajan,S., Rowley,R.B., Kut,S.A., Fargnoli,J., Burkhardt,A.L., Tsukada,S., Witte,O.N. and Bolen,J.B. (1994) Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc. Natl Acad. Sci. USA*, **91**, 9524–9528.

Sideras,P. and Smith,C.I.E. (1995) Molecular and cellular aspects of X-linked agammaglobulinemia. *Adv. Immunol.*, **59**, 135–223.

Steinman,R.M., Pack,M. and Inaba,K. (1997) Dendritic cells in the T-cell areas of lymphoid organs. *Immunol. Rev.*, **156**, 25–37.

Tedder,T.F., Inaoki,M. and Sato,S. (1997) The CD19–CD21 complex regulated signal transduction thresholds governing humoral immunity and autoimmunity. *Cell*, **6**, 107–118.

Thomas,J.D., Sideras,P., Smith,C.I.E., Vorechovsky,I., Chapman,V. and Paul,W.E. (1993) Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science*, **261**, 355–358.

Torres,R.M., Flaswinkel,H., Reth,M. and Rajewsky (1996) Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science*, **272**, 1804–1808.

Tsukada,S. *et al.* (1993) Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*, **72**, 279–290.

Tsukada,S., Simon,M.I., Witte,O.N. and Katz,A. (1994) Binding of βγ subunits of heterotrimeric G proteins to the PH domain of Bruton tyrosine kinase. *Proc. Natl Acad. Sci. USA*, **91**, 11256–11260.

Turner,M., Gulbranson-Judge,A., Quinn,M.E., Walters,A.E., MacLennan,I.C.M. and Tybulewicz,V.L.J. (1997) Syk tyrosine kinase is required for the positive selection of immature B cells into the recirculating B cell pool. *J. Exp. Med.*, **186**, 2031–2021.

VanVliet,E., Melis,M., Foidart,J.M. and Van Ewijk,W. (1986) Reticular fibroblast in peripheral lymphoid organs identified by a monoclonal antibody. *J. Histochem. Cytochem.*, **34**, 883–890.

Vetrie,D. *et al.* (1993) The gene involved in X-linked agamma-globulinemia is a member of the *src* family of protein tyrosine kinases. *Nature*, **361**, 226–233.

Vihinen,M. *et al.* (1998) Btkbase, mutation database for X-linked agamma-globulinemia (XLA). *Nucleic Acids Res.*, **26**, 242–247.

Wahl,M.I., Fluckiger,A.-C., Kato,R.M., Park,H., Witte,O.N. and Rawlings,D.J. (1997) Phosphorylation of two regulatory residues in the activation of Bruton's tyrosine kinase via alternative receptors. *Proc. Natl Acad. Sci. USA*, **94**, 11256–11533.

Wicker,L.S. and Scher,I. (1986) X-linked immune deficiency (*xid*) of CBA/N mice. *Curr. Opin. Microbiol. Immunol.*, **124**, 87–101.

Yao,L., Kawakami,Y. and Kawakami,T. (1994) The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc. Natl Acad. Sci. USA*, **91**, 9175–9179.

Chapter 6

A role for Btk in the development of immunoglobulin light chain assembly at the pre-B to immature B cell transition

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

A role for Btk in the development of immunoglobulin light chain assembly at the pre-B to immature B cell transition

Alex Maas[1], Gemma M. Dingjan[1,2], Frank Grosveld[1] and Rudolf W. Hendriks[1]

[1]Department of Cell Biology and Genetics and [2]Department of Immunology, Faculty of Medicine, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam.

Abstract

We generated transgenic mice that express a constitutively activated form of the cytoplasmic tyrosine kinase Btk, the E41K gain-of-function mutation, under the control of the CD19 promoter. In these mice, B cell development was arrested at the transition of IgM^{low} to $IgM^{high}IgD^-$ immature B cells in the bone marrow, representing the first immune tolerance checkpoint where auto-reactive B cells become susceptible to apoptosis. A correlation was observed between Btk activity and Ig κ/λ L chain usage: the proportion of Ig λ L chain expressing B cells was increased in E41K-Btk transgenic mice and decreased in mice with a targeted deletion of the Btk gene. We also observed that Btk^- cells accumulated within a subpopulation of small resting pre-B cells. These findings in the mouse define a role for Btk in Ig L chain assembly at the transition of pre-B to immature B cells, coinciding with the developmental arrest in X-linked agamma-globulinemia in man.

Introduction

B lymphocyte development in the bone marrow is characterized by assembly of immunoglobulin (Ig) heavy (H) chain and light (L) chain variable region genes from component V, (D) and J gene segments (reviewed in Tonegawa, 1983; Rajewski, 1996). V(D)J recombination is a highly ordered process, initiated by DNA rearrangements at the H chain loci in pro-B cells. The expression of a functional μ H chain is monitored through the formation of a pre-B cell receptor (pre-BCR) complex, together with $\lambda 5$ and V_{preB} proteins (Melchers et al., 1995). Signaling through the pre-BCR results in feedback inhibition of H chain V(D)J recombination to ensure allelic exclusion and IL-7 dependent proliferative expansion. The rapidly proliferating pre-B cells then exit cell cycle and perform Ig L chain gene rearrangements (reviewed in Lin & Desiderio, 1995). Productive L chain expression leads to the formation of complete Ig molecules and the transition of pre-B cells into surface IgM^+IgD^- immature B cells. With the acquisition of antigen specificity, maturing B cells pass through a tolerance-susceptible stage (Goodnow, 1996). Auto-antigen binding in newly generated $IgM^{low}IgD^-$ B cells results in continued Ig L chain rearrangement, i.e. receptor editing. In constrast, B cells that have advanced to the IgM^{high} stage lose this ability and concomitantly acquire sensitivity to antigen-mediated apoptosis (Tiegs et al., 1993; Melamed et al., 1998). IgM^{high} B cells

that do not bind to auto-antigen acquire various differentiation markers on their cell surface, such as IgD, CD21 and CD23, and migrate out of the bone marrow to colonize peripheral lymphoid organs.

One of the molecules that is involved in BCR signaling and directs B cell development is the Tec family non-receptor tyrosine kinase Btk (Bruton's tyrosine kinase), which when mutated results in X-linked agammaglobulinemia in humans and X-linked immunodeficiency (*xid*) in mice (reviewed in Sideras and Smith, 1995; Desiderio, 1997). Patients with XLA have very low B cell numbers in the peripheral blood, and those few B cells present exhibit an immature IgM^{high} surface phenotype (Conley, 1985). As the numbers of pre-B cells in the bone marrow are not significantly reduced, XLA reflects impaired developmental progression or increased cell death at the pre-B to B cell transition. XLA patients manifest protracted recurrent bacterial infections due to a profound reduction of serum Ig of all classes.

The *xid* phenotype, present both in CBA/N mice carrying an Arg_{28} mutation and in mice with targeted disruptions of *Btk* in their germ line, is distinct from XLA (Scher, 1982; Kahn *et al.*, 1995; Hendriks *et al.*, 1996). Compared to normal mice, Btk-deficient mice have ~50% fewer B cells in the periphery, with an over-representation of immature $IgM^{high}IgD^{low}$ cells. The $CD5^+$ B-1 B cell population is absent, and the levels of IgM and IgG3 in the serum are reduced. Although Btk-deficient mice do not make antibodies to a subset of T cell independent (TI) type II antigens, they are able to respond to most T cell dependent (TD) antigens. Btk-deficient B cells do not enter S phase when triggered through the B cell receptor (Scher, 1982; Kahn, 1995). Whereas *xid* is characterized by a defect in peripheral B cell maturation and function, there is no substantial block in early B cell development, as in XLA. Nevertheless, by analysis of competition *in vivo* between wild-type and Btk-deficient cells, it was shown that the first selective disadvantage of Btk-deficient cells in the mouse is also at the transition from pre-B to immature B cell (Hendriks *et al.*, 1996).

Btk is a 659 amino acid protein that contains a single C-terminal catalytic domain, the *Src* homology domains SH2 and SH3 and a unique pleckstrin homology (PH) domain at the amino terminus, with an adjacent proline- and cysteine-rich Tec homology (TH) domain (see for review: Sideras and Smith, 1995; Desiderio, 1997). Btk is expressed throughout B cell development, but not in plasma cells (de Weers *et al.*, 1993; Sideras and Smith, 1995; Hendriks *et al.*, 1996). It has been shown that Btk tyrosine phosphorylation and *in vitro* kinase activity increase upon BCR stimulation (Saouaf *et al.*, 1994; de Weers *et al.*, 1994; Aoki *et al.*, 1994). BCR engagement leads to activation of phosphatidylinositol 3-kinase (PI3-K), which generates the second messenger phosphatidylinositol-[3,4,5]triphosphate (PtdIns-3,4,5,-P_3). In concert with *Src* family kinases (Rawlings *et al.*, 1996; Wahl *et al.*, 1997), PtdIns-3,4,5,-P_3 initiates Btk activation by targeting the kinase to the plasma membrane through interactions with the Btk PH domain (Salim *et al.*, 1996; Scharenberg *et al.*, 1998; Bolland *et al.*, 1998). These interactions are critical to the activity of Btk and result in PLC-γ tyrosine phosphorylation, inositol triphosphate (IP_3) production and calcium mobilization. This pathway is inhibited by engagement of the Fc receptor γIIB through the activity of SH2-containing inositol polyphosphate (SHIP) which regulates the association of Btk with the membrane by reducing the level of PIP_3 (Bolland *et al.*, 1998; Scharenberg *et al.*, 1998).

Btk-deficient B cells exhibit a reduced IP_3 peak level and Ca^{2+} flux following BCR cross-linking (Fluckiger *et al.*, 1998). Conversely, a consitutively active form of Btk, E41K, a Glu-to-Lys mutant at position 41 in the PH domain close to the predicted inositol phosphate binding site (Hyvönen and Saraste, 1997) is associated with increased membrane localization and tyrosine phosphorylation of Btk (Li *et al.*, 1995). Expression of the E41K Btk mutant was shown to stimulate growth of NIH 3T3 fibroblasts in soft agar (Li *et al.*, 1995), to enhance the sustained increase in $[Ca^{2+}]$ following BCR cross-linking in Ramos B cells (Fluckiger *et al.*, 1998), and to enhance blast formation of splenic B cells in response to BCR cross-linking (Dingjan *et al.*, 1998). Transgenic expression of the E41K Btk gain-of-function mutant driven by the MHC class II Ea gene locus control region resulted in an immunodeficient phenotype that is more severe than *xid*: recirculating B cells were lacking, *in vivo* B cell responses to TD or TI-II antigens were essentially absent and splenic architecture was disrupted (Dingjan *et al.*, 1998). The arrest in development in the spleen coincided with a marked increase in expression of the transgene during maturation from IgM^{high} to IgM^{low} B cells.

To be able to study the effect of constitutive activation of Btk in early B cell development, we generated transgenic mice in which expression of wild-type or E41K human Btk ($hBtk^{WT}$ or $hBtk^{E41K}$) is driven by the human CD19 promoter, which contains a critical high-affinity binding site for the B-cell-specific transcription factor BSAP/pax-5 (Kozmik *et al.*, 1992). Expression of a human CD19 transgene was reported to be completely restricted to the B cell lineage and to appear early in development (Zhou *et al.*, 1994). Here, we show that transgenic expression of the E41K Btk mutant driven by the CD19 promoter resulted in an arrest at the transition of IgM^{low} to IgM^{high} immature B cells in the bone marrow, and in an increase of the proportion of Ig λ L chain expressing cells. Conversely, mice with a targeted deletion of the Btk gene (Hendriks *et al.*, 1996) had decreased proportions of λ expressing B cells. A role for Btk in Ig L chain assembly was further supported by the observed accumulation of Btk-deficient cells within a subpopulation of small resting pre-B cells.

Results

Transgenic expression of $hBtk^{WT}$ and $hBtk^{E41K}$ under the control of the CD19 promoter

A 6.3 kb genomic fragment with the CD19 promoter, containing the critical BSAP/pax-5 site (Kozmik *et al.*, 1992), and 5' flanking DNA was used to express the $hBtk^{WT}$ and $hBtk^{E41K}$ transgenes (Figure 1A). To construct the transgenes, a 27.4 kb $hBtk$ cDNA-genomic DNA fusion segment containing either the wild-type or the E41K mutant human Btk was ligated at an *MspI* site in exon 1 of the human CD19 gene, located 18 bp 5' of the ATG translation start. After micro-injection into fertilized oocytes, two CD19-$hBtk^{WT}$ and four CD19-$hBtk^{E41K}$ founder mice were obtained. These founders were mated onto a Btk-deficient background, in which the *Btk* gene is inactivated by a targeted in-frame insertion of a *lacZ* reporter (Hendriks *et al.*, 1996). The offspring

of these founders did not exhibit developmental defects or any increased susceptibility to malignancies for over 9 months of age.

In the transgenic lines, expression of Btk was detected by Western blotting analyses (data not shown) and intracellular flow cytometry using a polyclonal rabbit antiserum specific for Btk (Fig. 1B). Expression of transgenic Btk was restricted to B220^{+} cells and increased while bone marrow B cell progenitors matured to surface Ig[+] peripheral B cells. As compared to the endogenous levels, both CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenic B cells manifested a significant overexpression of Btk protein, in particular in the peripheral lymphoid tissues. T cells, granulocytes, monocytes and macrophages did not express detectable amounts of transgenic Btk (data not shown).
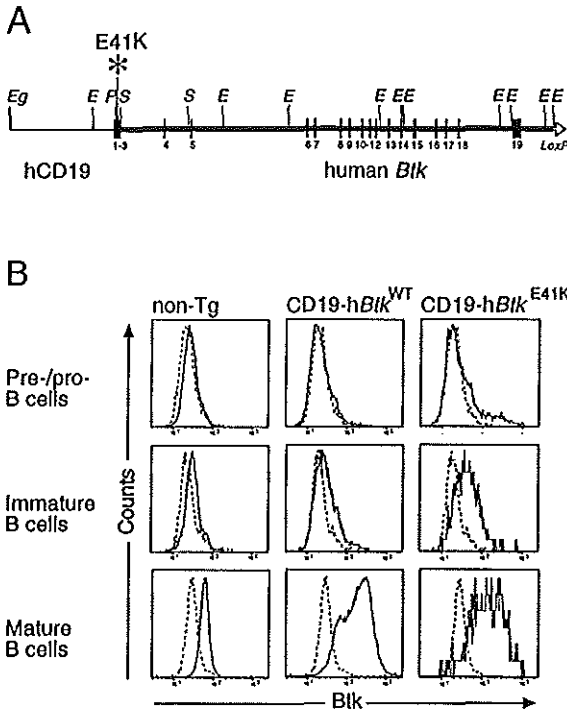
Figure 1. *Structure and expression of the CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenes.* (A) *Map of the transgene constructs, showing the 6.3 kb human CD19 promoter region and the 27.4 kb hBtk cDNA-genomic DNA fusion segment with exons 1 to 19, as well as a loxP sequence. The position of the E41K mutation in exon 3 is indicated. E=EcoRI, Eg= EagI; P=PvuI; S=SwaI.* (B) *Intracellular Btk expression in B cells from 2-month-old non-transgenic Btk$^+$ or Btk$^-$ mice, and CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenic mice on the Btk$^-$ background. Cell suspensions were stained for surface B220 and IgM and subsequently for intracellular Btk. The indicated populations of pro-/pre-B cells (B220^{low}IgM$^-$) and immature B cells (B220^{low}IgM$^+$) from bone marrow, as well as mature B220$^+$IgM$^+$ B cells from spleen were gated and analyzed for Btk expression. The results are displayed as histograms of the indicated mice (solid lines), together with the background staining as determined in Btk$^-$ mice (dashed lines). Data are from 10^5 total events (B cell numbers were significantly lower in the CD19-hBtk^{E41K} transgenic mice, see Table 1).*

Table 1.
Frequencies of lymphocyte populations in CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenic mice

Compartment	Cell population	Non-transgenic		CD19-hBtk^{WT}		CD19-hBtk^{E41K}	
		Btk$^+$ [a]	Btk$^-$	Btk$^+$	Btk$^-$	Btk$^+$	Btk$^-$
Spleen [b]	Nucleated cells, x10^{-6}	114±29	58±13	103±17	88±21	47±14	40±11
	B220$^+$ cells, %	42±5	22±3	38±7	42±5	0.2±0.1	0.5±0.4
	CD3$^+$CD4$^+$ T cells, %	27±3	29±2	23±1	26±4	41±5	42±2
	CD3$^+$CD8$^+$ T cells, %	14±3	19±1	14±1	14±1	30±5	30±7
Lymph Node	B220$^+$ cells, %	18±2	6±0.4	20±0.3	19±1	<0.1	<0.1
	CD3$^+$CD4$^+$ T cells, %	50±4	56±1	47±2	47±2	65±4	63±4
	CD3$^+$CD8$^+$ T cells, %	25±3	32±2	26±4	26±1	34±3	36±4
Blood	B220$^+$ cells, %	52±2	27±0.2	ND [c]	ND	<0.1	<0.1
Peritoneum	CD5$^+$ IgM$^+$ B cells, %	24±8	0.5±0.2	22±14	19±5	0.9±0.2	1±0.4
	CD5$^-$ IgM$^+$ B cells, %	34±7	19±4	24±1	28±6	0.6±0.5	0.7±0.7
	CD5$^+$ IgM$^-$ T cells, %	30±6	48±1	28±1	30±5	64±15	62±9
Bone Marrow	B220$^+$ cells, %	48±5	35±8	46±8	47±3	27±4	25±5
	Ig$^-$ pro/pre B cells, %	29±4	23±5	32±6	30±2	22±4	21±4
	IgM$^+$IgD$^-$ B cells, %	10±2	9±2	9±2	10±5	4±1	3±1
	IgM$^+$IgD$^+$ B cells, %	8±3	3±1	5±2	7±2	<0.1	0.2±0.2

The phenotype of lymphocyte populations were determined by flow cytometry; dead cells and high side scatter cells were excluded by gating. [a] Btk$^+$ mice were Btk$^+$/Y males or Btk$^{+/-}$ females, Btk$^-$ mice were Btk$^-$/Y males (Hendriks et al., 1996). [b] mice were 6-9 weeks old. Data are mean values standard deviations from 5-10 mice analyzed per group, except for blood, where values are from 2-4 mice per group. [c] ND = not determined.

Transgenic CD19-hBtk^{E41K} expression blocks B cell development at the immature B cell stage

To examine the effect of CD19-hBtk^{WT} and CD19-hBtk^{E41K} expression on B cell development, total cell suspensions from various lymphoid tissues from transgenic and non-transgenic mice, either on a Btk$^+$ or a Btk$^-$ background were analyzed by flow cytometry (Table 1; Figure 2). As previously described (Hendriks et al., 1996), the Btk$^-$ mice had fewer mature B cells (~30-50% of normal) in spleen, lymph node, peripheral blood, and bone marrow (B220^{high}IgM$^+$ fraction; Figure 2A). In addition, these mice exhibited a specific deficiency of mature IgM^{low}IgD^{high} B cells in the spleen (Figure 2B), and CD5$^+$ B-1 B cells in the peritoneal cavity (Figure 2C). In mice that expressed the CD19-hBtk^{WT} transgene on the Btk$^-$ background, a complete correction of the B cell numbers was observed in all lymphoid tissues analyzed. On the Btk$^+$ background, expression of CD19-hBtk^{WT} did not appear to have any effect on the sizes of B cell subpopulations (Table 1; Figure 2).
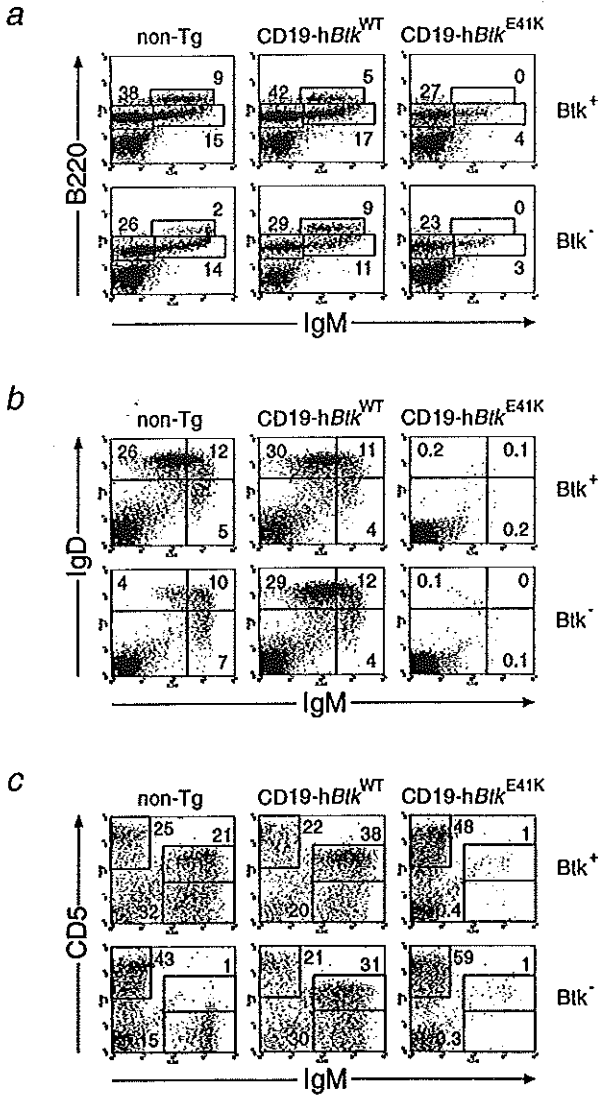
Figure 2. *The effects of transgenic CD19-hBtk^{WT} and CD19-hBtk^{E41K} expression on B cell development. Flow cytometric analyses of (A) bone marrow, (B) spleen, and (C) peritoneal cavity of the indicated mice on the Btk^{+} or Btk^{-} background. Single cell suspensions were stained with biotinylated anti-IgM and streptavidin-TriColor, and either FITC-conjugated anti-B220, PE-conjugated IgD or PE-conjugated anti-CD5. Data are displayed as dot plots of total lymphocytes, which were gated on the basis of their forward and side scatter characteristics. Percentages of total lymphocytes within the indicated gates are given. Data shown are representative of the mice examined (Table 1).*

In contrast, transgenic CD19-hBtk^{E41K} expression resulted in a block of B cell development at the immature B cell (B220^{low}IgM$^+$IgD$^-$) stage in the bone marrow, irrespective of the Btk$^+$ or Btk$^-$ background. Compared with wild-type or Btk$^-$ mice, the size of the immature B cell population was reduced by a factor of two to three, and lacked IgM^{high} cells (Table 1; Figure 2). The almost complete absence of the B220^{high}IgM$^+$ population of mature recirculating B cells in bone marrow and peripheral lymphoid compartments indicated a reduction of the mature B cell pool to <1% of the normal size (Table 1; Figure 2). In addition to the defect in conventional B cells, the CD19-hBtk^{E41K} transgenic mice also lacked both CD5$^+$ and CD5$^-$ B-1 B cells in the peritoneal cavity. The six groups of mice analyzed did not manifest significant differences in pro-B or pre-B cell fractions in the bone marrow, as assayed by expression of the surface markers B220, CD43, heat stable antigen and BP-1

(Hardy *et al.*, 1991); they also did not manifest differences in the thymocyte subpopulations (data not shown). In the CD19-hBtk^{E41K} transgenic mice a relative increase of the proportions of T cells was observed in spleen, lymph node and peritoneal cavity (Table 1).

IgM is present, but antigen responses are defective in CD19-hBtk^{E41K} transgenic mice

To evaluate the capacities of the residual B cells present in the CD19-hBtk^{E41K} transgenic mice, we analyzed serum Ig levels and *in vivo* immune responses.

Serum Ig levels were determined in 2-month-old CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenic mice, with non-transgenic Btk$^+$ and Btk$^-$ mice as controls (Figure 3A). The levels of IgM and IgG3 in the sera of Btk$^-$ mice were decreased as compared with those in control Btk$^+$ littermates (Scher, 1982; Kahn *et al.* 1995; Drabek *et al.*, 1997). This defect was corrected by transgenic CD19-hBtk^{WT} expression: IgM levels were somewhat elevated and IgG3 levels were in the normal range. By contrast, in CD19-hBtk^{E41K} transgenic mice the serum levels of IgM were low, with values between those for Btk-deficient and control mice. Notably, the serum levels of all other subclasses were strongly reduced, whether on the Btk$^+$ or Btk$^-$ background (Figure 3A). In the CD19-hBtk^{E41K} transgenic mice the serum IgM levels increased with age: when the mice were 6-7 months old, the IgM concentration in the serum was elevated, as compared to normal or CD19-hBtk^{WT} transgenic mice (Figure 3B). At this age, IgG1 and IgG2a remained undetectable, IgG2b and IgG3 were in the range of Btk$^-$ mice and IgA was variable.

The finding of substantial IgM levels in CD19-hBtk^{E41K} transgenic mice implied that - in spite of the reduction of the mature B cell pool to <1% of the normal size - significant numbers of IgM secreting plasma cells were present. To investigate whether functional antibodies could be produced, we analyzed the immune responses to a TI type II antigen, dinitrophenol(DNP)-Ficoll, and a TD antigen, trinitrophenol-keyhole limpet haemocyanin (TNP-KLH).

Consistent with the reported findings (Scher, 1982, Kahn *et al.*, 1995, Drabek *et al.*, 1997), the Btk$^-$ mice were unresponsive to DNP-Ficoll: the detected NP-specific IgM or IgG3 levels at day 7 after i.p. injection did not differ from the values of unimmunized animals. Whereas expression of the CD19-hBtk^{WT} transgene restored the TI type II response, expression of the CD19-hBtk^{E41K} transgene abolished this response, already on the Btk$^+$ background (Figure 3C).

The primary IgM response at day 7 or the secondary IgG1, IgG2a or IgG2b responses, at day 7 after booster injection with TNP-KLH, were not significantly different between Btk[+], Btk[-] or CD19-hBtk[WT] transgenic mice. However, the CD19-hBtk[E41K] transgenic mice were unable to mount detectable primary or secondary humoral immune responses against the TD antigen TNP-KLH (Figure 3D).

In summary, we conclude that those few B cells that populated the peripheral immune system and were able to develop into plasma cells did not respond productively to TD or TI type II antigens.

Follicular and marginal zone B cells are absent but plasma cells are present in CD19-hBtk[E41K] mice

Seven days after booster injection with the TD antigen TNP-KLH, the spleens from 3-month-old non-transgenic, CD19-hBtk[WT] and CD19-hBtk[E41K] transgenic mice, each on the Btk[+] or Btk[-] background, were examined by immunohistology. Double labelings of serial spleen sections with monoclonal antibodies specific for B cells (anti-IgM, anti-IgD and anti-B220), T cells (anti-CD3), metallophilic macrophages (MOMA-1) or interdigitating dendritic cells (anti-CD11c/N418), as well as peanut agglutinin (PNA) are shown in Figure 4. The spleens of non-transgenic or CD19-hBtk[WT] mice, either on the Btk[+] or Btk[-] background, demonstrated a characteristic histological organization, in terms of separate T and B cell areas in the white pulp, with T cells surrounding central arterioles (Figure 4, a-d) and B cells in follicles (a-c,e-g,i-k) with PNA[+] germinal centers (e-g). In these mice, the marginal zones at the outer boundaries of the white pulp contained IgD^{low} B cells, as well as MOMA-1^{+} macrophages interrupted with nests of strongly N418-expressing dendritic cells that form bridging channels into the red pulp (Steinman et al., 1997).

By contrast, in the CD19-hBtk[E41K] transgenic mice only few B cells were detected. They were present partly as isolated cells in the T cell areas, and partly in small clusters, close to the N418^{high} dendritic cells at the periphery of the white pulp nodule (Figure 4, d,h,j). There was no evidence for the presence of a distinct marginal zone, as the outer boundary of the white pulp did not contain B220^{+} cells and MOMA-1^{+} metallophilic macrophages were largely absent, except for the incidental presence of some MOMA-1^{+} cells close to the small B cell clusters (Figure 4p). In the red pulp, IgM^{+} plasma cells were present in apparently normal numbers (Figure 4d). Consistent with the absence of TD immune responses in vivo, PNA[+] germinal center B cells were not detectable (Figure 4h).

In summary, these findings show that in CD19-hBtk[E41K] transgenic mice, those few B cells that emerged from the bone marrow did not develop into follicular or marginal zone B cells, but were present in T cell areas and were subsequently driven into plasma cell differentiation.

The splenic B cells in CD19-hBtk[E41K] transgenic mice have an aberrant surface phenotype

The residual splenic B cells of CD19-hBtk[E41K] transgenic mice were further characterized by three- and four-color flow cytometry. As shown in Figure 5A, those B220^{+} B cells present manifested close to normal IgM expression on the membrane, but the level of cell surface IgD was decreased, as compared to CD19-hBtk[WT] transgenic B cells. The B cells manifested a normal

forward scatter profile and did not appear to belong to the B-1 lineage, as CD5 or Mac-1 were not present on the cell surface (data not shown). The B cells had a B220^{low}HSA^{high} phenotype, reminiscent of recent emigrants from the bone marrow that have not yet differentiated into mature B220^{high}HSA^{low} cells of the long-lived B cell pool (Allman *et al.*, 1993). The lack of B220^{high}HSA^{low} cells (Fig. 5A) indicated that in CD19-hBtk^{E41K} transgenic mice those few immature B cells that have left the bone marrow failed to mature in the spleen into long-lived recirculating cells.

Figure 3. *Serum Ig levels and humoral immune responses in CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenic mice.* **(A, B)** *Serum concentrations of the indicated Ig subclasses in non-transgenic Btk^{+} or Btk^{-} mice, CD19-hBtk^{WT} transgenic mice on the Btk^{+} or Btk^{-} background, as well as CD19-hBtk^{E41K} transgenic mice on the Btk^{+} or Btk^{-} background. Mice were 2 months (A) or 6-7 months (B) of age.* **(C)** *IgM and IgG3 responses against the TI type II antigen DNP-Ficoll at day 7 after i.p. injection.* **(D)** *Primary IgM responses against the TD antigen TNP-KLH (determined 7 days after i.p. injection) and secondary TD responses of the indicated subclasses (determined 7 days after an i.p. booster injection approximately 4 weeks after the primary immunization). Pre-immune levels were <10 U/ml for IgM, <2 U/ml for IgG3, <5 g/ml for IgG2a and <10 g/ml for IgG1 and IgG2b. Mice were 2 months old, total or NP-specific Ig levels were determined by ELISA and each symbol indicates an individual animal. (Next page).*

A

B

C

	Blk+	Blk-
non-Tg	□	▨
CD19-h*Blk*WT	○	●
CD19-h*Blk*E41K	△	▲

D
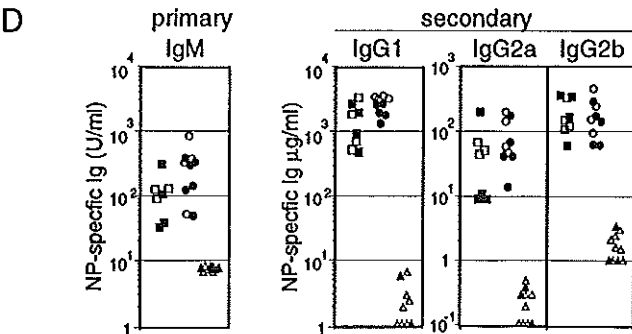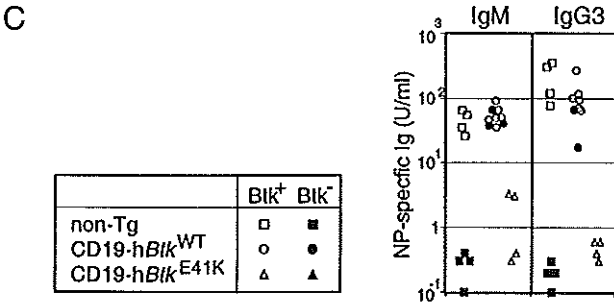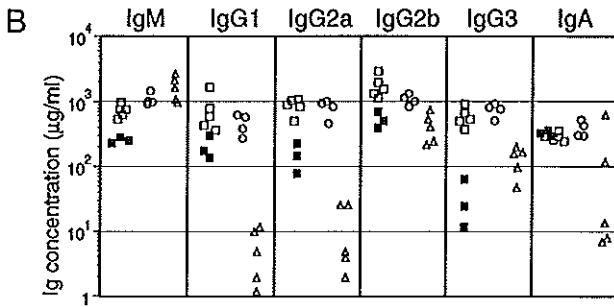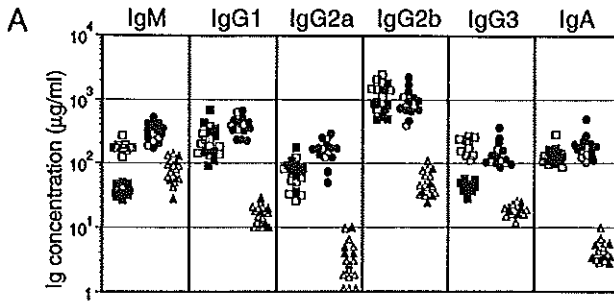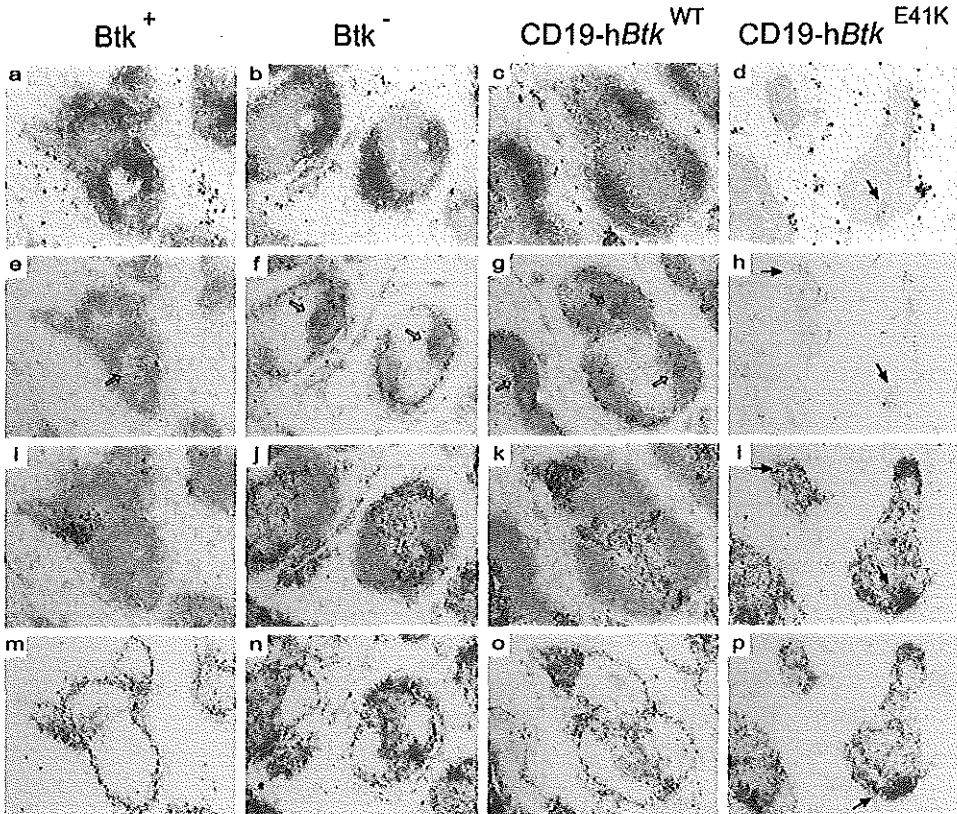
Figure 4. *Histology of the spleen from CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenic mice. Immunohistochemical analysis of 5 m splenic frozen sections from Btk^+ mice, Btk^- mice, as well as CD19-hBtk^{WT} and CD19-hBtk^{E41K} transgenic mice on the Btk^- background. Sections were stained with anti-CD3 (blue, a-d) for T cells, anti-IgM (brown, a-d), anti-IgD (blue, e-h) or anti-B220 (blue, i-l) for B cells, PNA (brown, e-h) for germinal center B cells, anti-CD11c/N418 (brown, i-p) for dendritic cells and MOMA-1 (blue, m-p) for metallophilic marginal zone macrophages. Open arrows indicate the presence of plasma cells in a-d or PNA^+ germinal centers in e-g. Closed arrows indicate the location of small clusters of B cells (d,h,l) or MOMA-1^+ cells (p) in CD19-hBtk^{E41K} transgenic mice. Original magnifications are 16x.*

A

CD19-h*Btk*^{WT} CD19-h*Btk*^{E41K}

B
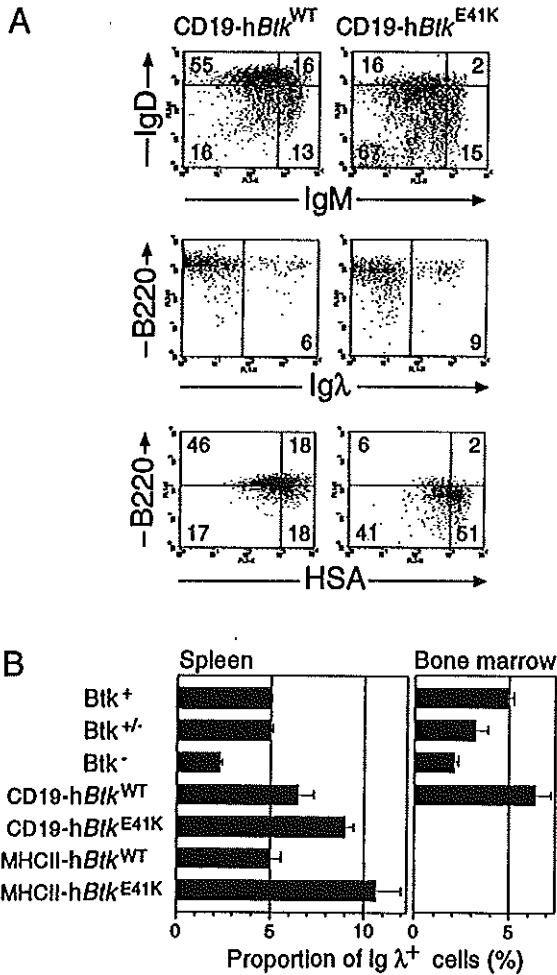
Figure 5. *Aberrant surface phenotype of CD19-hBtk*^{E41K} *transgenic mice.* **(A)** *Surface profiles of splenic B cells. B220*⁺ *cells were gated and analyzed for IgM and IgD expression (top). CD19*⁺ *cells were gated and analyzed for B220 and HSA expression (middle) or B220 and Ig λ L chain (bottom). Data are displayed as dot plots, and the percentages of gated cells within the indicated quadrants are shown.* **(B)** *The proportions of Ig λ L chain expressing cells of the total B220*⁺*CD19*⁺ *fraction in the spleen (left) and of the Ig*⁺*B220*^{low}*CD19*⁺ *immature B cell fraction in the bone marrow (right) of the indicated mice as determined by flow cytometry. Data are shown as mean ± standard error from 5-10 mice per group, except for the MHCII-hBtk*^{E41K} *mice where 3 mice were analyzed.*

Btk and Ig λ L chain expression

In normal mice ~95% of B cells express Ig κ L chains, while only ~5% express λ L chain. In CD19-hBtk^{E41K} transgenic mice the proportion of Ig λ L chain expressing cells in the spleen was found to be increased to ~9.0%, compared to CD19-hBtk^{WT} transgenic mice (~6.5%) or wild-type mice (~4.9%) (Figure 5AB). Conversely, in Btk⁻ mice we noticed a significant reduction of the proportion of Ig λ⁺ cells to ~2.3%. When we analyzed the transgenic mice in which the expression of the E41K Btk mutant is driven by the MHC class II LCR (Dingjan *et al.*, 1998), also a significant increase in the proportion of Ig λ L chain expressing cells in the spleen was observed (Figure 5B).

To further investigate whether the expression of λ L chain is more dependent on Btk function than κ L chain, we investigated the competition *in vivo* between Btk⁺ and Btk⁻ λ⁺ B cells in female Btk$^{+/-}$ heterozygous mice. In these mice, expression of the disrupted Btk allele could be monitored by *lacZ* expression (Hendriks *et al.*, 1996). Because of the process of random X-chromosome inactivation, ~50% of the B-lineage cells will have the disrupted Btk allele on the active X chromosome. When these cells reach a differentiation stage at which Btk is required, their further development is arrested and the proportions of *lacZ*⁺ cells decrease below this value of 50% (Hendriks *et al.*, 1996). In 4-month-old female Btk$^{+/-}$ heterozygous mice, we found that the fraction of *lacZ*⁺ cells in the most immature population of IgD^{low}B220⁺ B cells in the spleen was 22% ± 5 (n=5). However, within the subpopulation of surface Ig λ L chain positive IgD^{low}B220⁺ B cells, the fraction of *lacZ*⁺ cells was only 11% ± 4 (n=5). Consistent with our findings in wild-type and Btk⁻ mice (Figure 5B), we found that within the immature IgD^{low}B220⁺ splenic B cell population of heterozygous Btk$^{+/-}$ females, the proportion of Ig λ L chain expressing cells was 4.4% ± 0.7 for *lacZ*⁻ Btk⁺ cells, and 1.9% ± 0.5 for *lacZ*⁺ Btk⁻ cells.

The reduction of Ig λ L chain expression in Btk⁻ mice was confirmed in an Ig λ-specific ELISA: the percentage of λ L chain containing IgM in the serum was found to be <0.4% in Btk⁻ mice, 4.4%±0.4 in wild-type littermates and 6.9%±0.8 in CD19-hBtk^{WT} transgenic mice. In the CD19-hBtk^{E41K} transgenics this fraction was rather variable, ranging between 1.1% and 8.7%.

The observed differences in k/λ ratios could partly result from differences in peripheral B cell selection mechanisms between the mouse groups. Therefore, λ L chain expression was also analyzed in the fraction of B220^{low}Ig⁺ immature B cells in the bone marrow, and was found to be ~2.0% in Btk⁻ mice, ~3.2% in heterozygous Btk$^{+/-}$ females, ~4.9% in wild-type mice and ~6.6% in CD19-hBtk^{WT} transgenic mice (Figure 5B). The low levels of Ig expression on the surface of immature B cells in CD19-hBtk^{E41K} transgenic mice (Figure 2A) precluded a reliable estimation of κ/λ L chain distribution. These data demonstrated that the correlation between Btk activity and Ig κ/λ L chain usage was already present at the level of the immature B cell in the bone marrow.

Accumulation of Btk⁻ cells within the pre-B cell population

We previously reported a selective disadvantage of Btk⁻ cells to contribute to the population of immature B cells in the bone marrow, as in heterozygous *Btk*$^{+/-}$ female mice the percentage of lacZ expressing cells decreased from ~50% in pro-B and pre-B cells to ~30% in immature IgM⁺ B cells. (Hendriks *et al.*, 1996). This finding could reflect a selective disability of Btk⁻ cells in Ig L chain

assembly at the small pre-B cell stage, or alternatively a decreased survival of Btk⁻ cells within the immature B cell population. To discriminate between these two possibilities, we tried to identify subpopulations of cells within the pre-B cell or immature B cell stage that would manifest an accumulation of cells that are blocked or delayed in development. In such subpopulations, the proportion of lacZ+ cells was expected to be >50%.

A four-color flow cytometric analysis of bone marrow suspensions of 5 week-old Btk^+ and Btk^- males, as well as heterozygous $Btk^{+/-}$ females was performed, using the markers B220, CD43/S7, IgM and *lacZ*. The earliest stages of B cell development, pro-B cells and large cycling pre-B cells express CD43 on their cell surface but the expression is down-regulated at the transition to small resting pre-B cells which are IgM⁻CD43$^{dull/-}$ (Hardy *et al.*, 1991). Within this IgM⁻CD43$^{dull/-}$ population of small resting B cells, we identified a subpopulation of IgM⁻CD43⁻ cells, in which Btk⁻ cells appeared to accumulate: the size of this subpopulation was 1.5% ± 0.4 of all B220$^+$ in wild-type mice (n=3), 7.1% ± 2.2 in Btk⁻ mice (n=4) and 4.4% ± 1.8 in $Btk^{+/-}$ heterozygous females (n=5) (Figure 6). Most importantly, when Btk⁻ cells were in competition with wild-type cells *in vivo* in the $Btk^{+/-}$ heterozygous female mice (n=5), a substantial accumulation of *lacZ*$^+$ Btk⁻ cells was observed within this IgM⁻CD43⁻ subpopulation, which contained 72% ± 9 *lacZ*$^+$ cells (Figure 6).

Within the fraction of immature B cells (IgM$^+$IgD⁻), we could not detect any accumulation of Btk⁻ cells: the fraction of *lacZ*$^+$ cells was ~30%, irrespective of the surface IgM expression level (data not shown). In summary, these findings in male Btk⁻ and female Btk$^{+/-}$ mice indicate an accumulation of Btk⁻ cells within the pre-B cell population, as a result of impaired progression into the stage of immature sIgM expressing B cells.
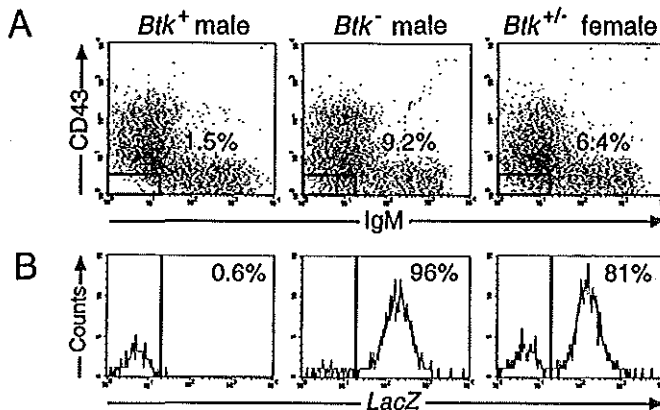
Figure 6. *Btk⁻ cells are arrested at the pre-B cell stage.* **(A)** *Surface CD43-IgM profile of B220⁺ cells from bone marrow of the indicated mice. For each sample the percentage of total B220⁺ cells which is within the indicated gate of CD43⁻IgM⁻ pre-B cells is indicated.* **(B)** *The gated subpopulation of CD43⁻IgM⁻ pre-B cells were analyzed for lacZ expression and the results are displayed as histograms. The percentages of lacZ⁺ cells within fraction A are indicated. The data shown are representative of 3-5 mice examined within each group.*

Discussion

We have generated transgenic mice in which expression of wild-type human Btk or the E41K Btk mutant is under the control of the promoter and 5' flanking region of the CD19 gene. High-level Btk expression was obtained, specifically in B cells, but since the transgene constructs used contained ~27 kb of genomic DNA from the Btk gene itself, it is very well possible that Btk endogenous regulatory elements contributed to the expression patterns of the transgene. When the CD19-h*Btk*^{WT} transgenic mice were mated onto a Btk⁻ background, correction of all *xid* features was observed, comparable to our earlier findings in transgenic mice in which Btk expression was driven by either the MHC class II Ea gene locus control region or endogenous regulatory regions in a 340kb yeast artificial chromosome (Drabek et al., 1997; Maas et al., 1997; Dingjan et al., 1998).

Constitutive activation of Btk leads to a developmental block at the immature B cell stage

The present data demonstrate that expression of a constitutively activated form of Btk, the E41K mutant, causes an almost complete arrest of B cell development within the IgM⁺IgD⁻ immature B cell population at the progression from IgM^{low} to IgM^{high} IgD⁻ B cells. Since the IgM^{high}IgD⁻ immature B cell stage reflects the first immune tolerance checkpoint where auto-reactive B cells become susceptible to apoptosis, we conclude that constitutive activation of Btk mimicked B cell occupancy by self-antigens. An alternative explanation, that expression of the CD19-h*Btk*^{E41K} transgene would impede basal BCR signaling, which is thought to direct developmental progression of B cells, seems less likely, because in mice which lack essential signaling components of the BCR complex, such as the CD79*a* (Igα) cytoplasmic tail or the *syk* tyrosine kinase, B cell development is arrested at a slightly later stage, *i.e.* the stage of recruitment of IgM^{high}IgD⁻ immature B cells into the circulating B cell pool (Torres et al., 1996; Turner et al., 1997).

The phenotype differed markedly from our previously reported MHCII-h*Btk*^{E41K} transgenic mice, which did not show detectable defects in developing B cells in the bone marrow, but manifested a deficiency of recirculating B cells (Dingjan *et al.*, 1998). The differences between the two Btk^{E41K} expressing mouse strains most likely resulted from differences in the expression level of the transgene in the bone marrow, which was found to be higher in the CD19-h*Btk*^{E41K} transgenic mice.

Constitutive activation of Btk drives peripheral B cells into plasma cell differentiation

In the CD19-hBtk^{E41K} transgenic mice the block at the IgM^{high} immature B cell stage was leaky, allowing very small numbers of B cells to populate the peripheral immune system. Such cells were almost exclusively found in the spleen as B220^{low}HSA^{high} cells, which normally represent immature B cells that have just left the bone marrow. In parallel to our findings in the of MHCII-hBtk^{E41K} transgenic mice (Dingjan et al., 1998), the expression of Btk^{E41K} further impeded follicular entry of these cells, as B220^{high}HSA^{low} cells were absent in the spleen and recirculating B cells in the bone marrow or lymph nodes were completely lacking.

In spite of the reduction of the mature B cell pool to <1% of the normal size, significant numbers of IgM secreting plasma cells were present in the splenic red pulp. Serum IgM levels were substantial, and increased with age to levels that were elevated, as compared to those in normal mice. However, specific TD or TI antibody responses were lacking, indicating that B cells failed to interact productively with T cells or antigen presenting cells. Therefore, we conclude that constitutive activation of Btk drives the residual B cells that have been able to emerge from the bone marrow into plasma cell differentiation, apparently without functional selection.

Btk and Ig L chain assembly

Our findings in the Btk-deficient mice and the CD19-hBtk^{E41K} transgenic mice indicate a role for Btk during the L chain rearrangement process at the transition of pre-B cells into immature B cells.

Btk-deficient cells accumulated within a subpopulation of small resting B cells, suggesting a delay in the onset of L chain rearrangement. In this respect, Btk⁻ deficient mice show resemblance with mice carrying a targeted deletion in the Ig κ locus, which manifest an increase in the percentage of small resting CD43⁻ pre-B cells (Zou et al., 1993; Chen et al. 1993). It is possible that Btk is an essential transducer of signals that initiate rearrangement of the Ig L chain locus, e.g. by regulating the re-expression of the RAG gene products, which are absent in the large cycling pre-B cells and re-activated in the small resting pre-B cells (Grawunder et al., 1995). This would be supported by the finding that expression of the RAG genes is dependent on BCR signaling in immature IgM^{low} B cells (Melamed et al., 1998). Btk could also transduce signals that result in changes in chromatin that allow the recombinase access to the Ig L chain gene segments. This would be analogous to findings in the T cell system, in which anti-CD3e treatment of CD4⁻CD8⁻ cells of RAG-deficient mice induces the opening of the TCR a locus, as monitored by germ-line J_a transcription and methylation status (Villey et al., 1997). Concomitantly, large numbers of CD4$^+$CD8$^+$ thymocytes are generated, a process that was also seen when an lck transgene encoding a constitutively active kinase was introduced into RAG-deficient mice (Mombaerts et al., 1994).

The proportion of λ L chain expressing cells was decreased in Btk⁻ mice (~2%), and significantly increased in CD19-hBtk^{E41K} transgenic mice (~9%), as compared to normal mice (~5%). Low-level expression of the E41K mutant in bone-marrow of MHCII-hBtk^{E41K} transgenic mice already increased the proportion of λ$^+$ B cells to ~10.6% (Figure 5B). Also overexpression of wild-type Btk appeared to increase λ L chain usage, as we found 6-7% λ$^+$ cells in CD19-hBtk^{WT}

transgenic mice. Collectively, these findings indicate a critical role for Btk activity at the pre-B to immature B cell transition. Also in human the absence of Btk results in a deviation from the normal κ^+ to λ^+ ratio of 50:50, as was shown by the finding of only ~25% λ^+ cells in the peripheral blood B cell population or in B lymphoblastoid cells lines from XLA patients (Conley, 1985; Timmers *et al.*, 1993).

The observed ratio of κ^+ to λ^+ B cells of 95:5 in normal mice is thought to reflect the earlier initiation of rearrangements at the κ locus and the ~3-fold higher probability of productive κ^+ versus λ^+ rearrangements per chromosome, within the limited life span of the small pre-B cell (Hieter *et al.* 1981; Ramsden and Wu, 1991; Arakawa *et al.*, 1995). It was estimated that only those small pre-B cells that failed to produce κ L chain after up to three sequential κ rearrangement attempts per allele, would proceed with λ rearrangements (Arakawa *et al.*, 1995). As L chain rearrangement occurs without cell division or substantial cell loss (Osmond, 1991), the κ to λ ratio of the immature B cell population directly reflects the probability of productive κ^+ and λ^+ rearrangements (Arakawa *et al.*, 1995). Therefore, our finding of reduced λ L chain expression in Btk$^-$ mice, indicates that the absence of Btk results in a delay in the initiation of L chain rearrangements, a reduced survival of pre-B cells that are in the process of L chain rearrangement, and/or impaired receptor editing of immature IgM^{low} B cells.

In this context, the CD19-hBtk^{E41K} transgenic mice parallel transgenic mice with enforced expression of the Bcl-2 apoptotic inhibitor in B cells, which also showed a significant increase in the percentage of λ^+ B cells, either *in vivo* in the presence or absence of auto-reactive Ig transgenes (Tiegs et al., 1993; Lang et al. 1997) or in bone marrow cultures *in vitro* (Rolink et al., 1993). These results were explained by assuming that B cells normally have too little time to take full advantage of the potential λ L chain repertoire that could rescue non-functional or auto-reactive B cells (Lang *et al.*, 1997). It is attractive to hypothesize that, like *Bcl-2* transgene expression, also CD19-hBtk^{E41K} expression results in an extended life-span of pre-B cells (or immature IgM^{low} B cells) providing an extended time window per cell for L chain rearrangement or editing. Conversely, the absence of Btk would limit the survival of pre-B cells that are in the process of L chain rearrangement and therefore result in low λ/κ ratios. A role for Btk in (pre-)B cell survival would be supported by the findings that Bcl-2 expression is reduced and sIg-mediated bcl-x_L induction is absent in *xid* B cells (Anderson *et al.*, 1996; Choi *et al.*, 1996; Woodland *et al.*, 1996). In addition, the large variation found in the numbers of pre-B cells present in the bone marrow of XLA patients (Sideras and Smith, 1995) could be explained by a crucial role for Btk in pre-B cell survival. Furthermore, this would be consistent with experiments that indicate that a certain level of basal BCR signaling is required for developmental progression or survival of (pre-)B cells (Gu *et al.*, 1991, Lam *et al.*, 1997).

Btk transduces signals at multiple checkpoints in B cell development

The phenotypes of the xid and the MHCII-hBtk^{E41K} transgenic mice already indicated that Btk is essential in signaling pathways that govern the maturation of peripheral B cells (Scher, 1982;

Khan *et al.*, 1995, Dingjan *et al.*, 1998). Now, we report that constitutive activation of Btk can drive peripheral B cells into terminal plasma cell differentiation.

Our findings in the mouse show that Btk also transduces signals at several check-points in B cell development in the bone marrow. The lack of progression of Btk^{E41K} B cells into the IgM^{high}IgD$^-$ cell stage, would place Btk in the BCR signal pathway that eliminates auto-reactive B cells in the bone marrow. Moreover, the present data provide the first evidence for a role for Btk in Ig L chain assembly at the transition of pre-B cells to immature B cells, whereby defective Btk function results in an almost complete developmental arrest in XLA and only a minor selective disadvantage of Btk-cells in *xid*. In the absence of Btk, signaling through the pre-BCR complex still mediates its normal checkpoint function (Rajewski, 1996) by effecting Ig H chain allelic exclusion, Il-7 driven proliferative expansion and progression to the resting small pre-B cell stage. However, the subsequent assembly of Ig L chain, resulting in progression into the IgM$^+$ immature B cell stage, is impaired when Btk is defective, *e.g.* because of defective re-expression of the RAG genes or insufficient accessibility of Ig L chain loci, or because of reduced survival of pre-B cells that are in the process of L chain rearrangement. Further experiments are needed to directly demonstrate the involvement of Btk signaling in any of these events.

Experimental procedures

Generation of CD19-h*Btk*^{WT} and CD19-h*Btk*^{E41K} transgenic mice

The transgene constructs CD19-h*Btk*^{WT} and CD19- h*Btk*^{E41K}, in which the E41K mutation was introduced by site-directed mutagenesis (Dingjan *et al.*, 1998), are shown in Figure 1A. The cosmid clone containing the human CD19 promoter region (kindly provided by M. Busslinger, Research Institute of Molecular Pathology, University of Vienna, Austria) was isolated from a human genomic cosmid library by screening with a homologous murine CD19 cDNA probe (Kozmik *et al.*, 1992). A ~16 kb *Xba*I fragment containing 5' flanking DNA, and a 317 bp *Xba*I-*Msp*I(blunted) fragment (positions 1072-1389 of the human CD19 gene; Kozmik *et al.*, 1992) were cloned into cosmid vector pTL5 (Lund *et al.*, 1982), using a *Bgl*II-*Mlu*I-*Xba*I-*Sma*I-*Pvu*I-*Not*I-*Bgl*II polylinker. A unique *Eag*I site in the 16 kb *Xba*I fragment was used to isolate a 6.3 kb *Eag*I(blunted)-*Pvu*I fragment and to ligate it to the 27.1 kb *Pvu*I-*Not*I fragment, containing 400 bp (*Btk* exon 1-3) of the wild-type or E41K mutated h*Btk* cDNA, a ~27 kb genomic DNA fragment (*Btk* exon 3-19), and a 109 bp LoxP fragment (Dingjan *et al.*, 1998).

The ~34 kb *Mlu*I-*Not*I inserts of the CD19-h*Btk*^{WT} and CD19-h*Btk*^{E41K} constructs were excised from the vector and gel-purified. DNA (~2 ng/µl) was injected into the pronulcei of FVBxFVB fertilized oocytes, which were subsequently implanted into pseudopregnant foster mice. Tail DNA was analyzed by Southern blotting of *Bam*HI digests and hybridization to a partial h*Btk* cDNA probe (h*Btk* pos. 133-1153), to determine the genotype of the founder mice and next generations generated by crosses with *Btk-/LacZ* mice of mixed 129/Sv x C57BL/6 background (Hendriks *et al.*, 1996).

Southern and Western blotting techniques have previously been detailed (Hendriks *et al.*, 1996).

Flow cytometric analyses

Preparation of single-cell suspensions, and three- or four-color flow cytometry have previously been described (Hendriks *et al.*, 1996; Dingjan *et al.*, 1998). The following monoclonal antibodies were obtained from Pharmingen (San Diego, CA): FITC-conjugated anti-B220/RA3-6B2, anti-Igκ /R5-240, anti-HSA/M1/6, anti-CD3 and anti-BP-1/6C3, PE-conjugated anti-CD43/S7, anti-CD5/Ly-1, anti-CD4, anti-CD19, anti-CD11b/Mac-1, CyChrome-conjugated anti-B220/RA3-6B2 and anti-CD8, and biotinylated anti-HSA/M1/6, anti-Igλ/R26-46 and anti-IgM. PE-conjugated anti-IgD was purchased from SBA (Birmingham, AL). Affinity-purified polyclonal rabbit-anti-Btk (Pharmingen) was used for intracellular flow cytometric detection of cytoplasmic Btk protein, as previously described (Dingjan *et al.*, 1998). 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).

Serum Ig detection, *in vitro* immunizations and immunohistochemistry

Total or NP-specific levels of serum Ig were measured by subclass-specific sandwich ELISA as described previously (Dingjan *et al.*, 1998). In these assays TNP-specific standards were used for IgG1, IgG2a and IgG2b, whereas for IgM and IgG3 values were calculated as arbitrary units, using a reference serum sample. Immunizations with TD and TI type II antigens were essentially performed as described (Maas *et al.*, 1997). Booster doses were given after 4 weeks.

For immunohistochemical analyses, tissue samples were embedded in OCT compound and frozen 5 µm cryostat sections were acetone-fixed and single and double labelings were performed using standard procedures (Leenen *et al.*, 1998). Monoclonal antibodies anti-B220/RA3-6B2, anti-CD3/KT3, anti-CD11c/N418 (Steinman *et al.*, 1997) and MOMA-1 (Kraal, 1992) were applied as hybridoma culture supernatants; biotinylated anti-IgM was from Pharmingen, biotinylated PNA from Sigma (St. Louis, MI) and anti-IgD from SBA. The second-reagents used have been previously described (Dingjan *et al.*, 1998).

References

Allman, D.M., Ferguson, S.E., Lentz., V.M. and Cancro, M.P. (1993). Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. J. Immunol. *151*, 4431-4444.

Anderson, J.S., Teutsch, M., Dong, Z. and Wortis, H.H. (1996). An essential role for Bruton's tyrosine kinase in the regulation of B-cell apoptosis. Proc. Natl. Acad. Sci. USA *93*, 10966-10971.

Aoki, Y., Isselbacher, K.J. and Pillai, S. (1994). Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligand B cells. Proc. Natl. Acad. Sci. USA *91*, 10606-10609.

Arakawa, H., Shimizu, T. and Takeda, S. (1995). Re-evaluation of the probabilities for productive rearrangements on the k and l loci. Int. Immunol. *8*, 91-99.

Bolland, S., Pearse, R.N., Kurosaki, T. and Ravetch, J.V. (1998). SHIP modulates immune receptor responses by regulating membrane associations of Btk. Immunity *8*, 509-516.

Chen, J., Troustine, M., Kurahara, C., Young, F., Kuo, C.C., Xu, Y., Loring, J.F. Alt, F.W. and Huszar, D. (1993). B cell development in mice that lack one ore both immunoglobulin light chain genes. EMBO J. *12*, 821-830.

Choi, M.S.K., Holman, M., Atkins, C.J. and Klaus, G.G.B. (1996). Expression of bcl-x during mouse B cell differentiation and following activation by various stimuli. Eur. J. Immunol. *26*, 676-682.

Conley, M.E. (1985). B cells in patients with X-linked agammaglobulinemia. J. Immunol. *134*, 3070-3074.

Desiderio, S. (1997). Role of *Btk* in B cell development and signalling. Curr. Op. Immunol. *9*, 534-540.

Goodnow, C.C., Cyster, J.G., Hartley, S.B., Bell, B.E., Cooke, M.P., Healy, J.I., Akkaraju, S., Rathmell, J.C., Pogue, S.L. and Shokat, K.P. (1995). Self-tolerance checkpoint in B lymphocyte development. Adv. Immunol. *59*, 279-368.

De Weers, M., Brouns, G.S., Hishelwood, S., Kinnon, C., Schuurman, R.K.B., Hendriks, R.W. and Borst, J. (1994). B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. J. Biol. Chem. *269*, 23857-23860.

De Weers, M., Verschuren, M.C.M., Kraakman, M.E.M., Mensink, R.G.J., Schuurman, R.K.B., Van Dongen, J.J.M. and Hendriks, R.W. (1993). The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. Eur. J. Immunol. *23*, 3109-3114.

Dingjan, G.M., Maas, A., Nawijn, M.C., Smit, L., Voerman, J.S.A., Grosveld, F. and Hendriks, R.W. (1998). Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. EMBO J.*17*, 5309-5320.

Drabek, D., Raguez, S., De Wit, T.P.M., Dingjan, G.M., Savelkoul, H.F.J., Grosveld, F. and Hendriks, R.W. (1997). Correction of the Xid phenotype by transgenic expression of human *Btk* under the control of class II MHC Ea locus control region. Proc. Natl. Acad. Sci. USA *94*, 610-615.

Fluckinger, A.C., Li, Z., Kato, R.M., Wahl, M.I., Ochs, H.D., Longnecker, R., Linet, J.P., Witte, O.N., Scharenberg, A.M. and Rawlings, D.J. (1998). Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation. EMBO J. *17*, 1973-1985.

Grawunder, U., Leu, T.M.J., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F. and Winkler, T.H. (1995). Down-regulation of *RAG1* and *RAG2* gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. Immunity *3*, 601-608.

Gu, H., Tarlinton, D., Müller, W., Rajewski, K. and Förster, I. (1991). Most peripheral B cells in mice are ligand-selected. J. Exp. Med. *173*, 1357-1371.

Hardy, R.R., Carmack, C.E., Shinton, S.A., Kemp, J.D. and Hayakawa, K. (1991). Resolution and characterisation of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J. Exp. Med. *173*, 1213-1225.

Hendriks, R.W., De Bruijn, M.F.T.R., Maas, A., Dingjan, G.M., Karis, A., and Grosveld, F. (1996). Inactivation of *Btk* by insertion of *LacZ* reveals defects in B cell development only past the pre-B cell stage. EMBO J. *15*, 4862-4872.

Hieter, P.A., Korsmeyer, S.L., Waldmann, T.A. and Leder, P. (1981). Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambda-producing B cells. Nature *290*, 368-372.

Hyvonen, M. and Saraste, M. (1997). Structure of the PH domain and Btk motif from Bruton's tyrosine kinase: molecular explanations for X-linked agammaglobulinemia. EMBO J. *16*, 3396-3404.

Khan, W.N., Alt, F.W., Gerstein, F.W., Malynn, B.A., Larsson, I., Rathbun, G., Davidson, L., Müller, S., Kantor, A.B., Herzenberg, L.A., Rosen, F.S. and Sideras, P. (1995). Defective B cell development and function in Btk-deficient mice. Immunity *3*, 283-299.

Kozmik, Z., Wang, S., Dörfler, P., Adams, B. and Busslinger, M. (1992). The promotor of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. Mol. Cell. Biol. *12*, 2662-2672.

Kraal, G. (1992). Cells in the marginal zone of the spleen. Int. Rev. Cytol. *132*, 31-74.

Lam, K.-P., Kühn, R. and Rajewsky, K. (1997). In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell *90*, 1073-1083.

Lang, J., Arnold, B., Hammerling, G., Harris, A.W., Korsmeyer, S., Russell, D., Strasser, A. and Nemazee, D. (1997). Enforced Bcl-2 expression inhibits antigen-mediated clonal elimination of peripheral B cells in an antigen dose-dependent manner and promotes receptor editing in autoreactive, immature B cells. J. Exp. Med. *186*, 1513-1522.

Leenen, P.J.M., Radosevic, K., Voerman, J., Salomon, B., Van Rooijen, N., Klatzman, D. and Van Ewijk, W. (1997). Heterogeneity of mouse spleen dendritic cells: *in vivo* phagocytic activity, expression of macrophage markers and subpopulation turnover. J. Immunol. *160*, 2166-2173.

Li, T., Tsukada, S., Satterthwaite, A., Havlik, M.H., Park, H., Takatsu, K. and Witte, O.N. (1995). Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. Immunity *2*, 451-460.

Lin, W.-C. and Desiderio, S. (1995) V(D)J recombination and the cell cycle. Immunol. Today *16*, 279-289.

Maas, A., Dingjan, G.M., Savelkoul, H.F.J., Kinnon, C., Grosveld, F. and Hendriks, R.W. (1997). The X-linked immunodeficiency defect in the mouse is corrected by expression of human *Bruton's tyrosine kinase* from a yeast artificial chromosome transgene. Eur. J. Immunol. *27*, 2180-2187.

Melamed, D., Benschop, R.J., Cambier, J.C., and Nemazee, D. (1998). Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor editing. Cell *92*, 173-182.

Melchers, F., Rolink, A. and Grawunder, U., Winkler, T.H., Karasuyama, H., Ghia, P. and Anderson (1995). Positive and negative selection events during B lymphopoiesis. Curr. Op. Immunol. *7*, 214-227.

Mombaerts, P., Anderson, S.J., Perlmutter, R.M., Mak, T.W. and Tonegawa, S. (1994) An activated form of lck transgene promotes thymocyte development in RAG-1 deficient mice. Immunity *1*, 261-268.

Osmond, D.G. (1991). Proliferation kinetics and the lifespan of B cells in central and peripheral lymphoid organs. Curr. Opin. Immunol. *3*, 179-185.

Rajewski, K. (1996). Clonal selection and learning in the antibody system. Nature *381*, 751-758.

Ramsden, D.A. and Wu, G.E. (1991). Mouse κ light chain recombination signal sequences mediate recombination more frequently than those of λ light chain. Proc. Natl. Acad. Sci. USA *88*, 10721-10725.

Rawlings, D.J. Scharenberg, A.M., Park, H., Wahl, M.I., Lin, S., Kato, R.M., Fluckinger, A-C., Witte, O.N. and Kinet, J-P. (1996). Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. Science *271*, 822-825.

Rolink, A., Grawunder, U., Haasner, D., Strasser, A. and Melchers, F. (1993). Immature surface Ig$^+$ B cells can continue to rearrange kappa and lambda L chain gene loci. J. Exp. Med. *178*, 1263-1270.

Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, E.C.I. and Discoll, P.C. (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. EMBO J. *15*, 6241-6250.

Saouf, S.J., Mahajan, S., Rowley, R.B., Kut, S.A., Fargnoli, J., Burkhardt, A.L. Tsukada, S., Witte, O.N. and Bolen, J.B. (1994). Temporal differences in the activation of three classes on non-transmembrane protein tyrosine kinases following B-cell antigen surface engagement. Proc. Natl. Acad. Sci. USA *91*, 9524-9528.

Schaffer, A.L. and Schlissel, M.S. (1997). A truncated heavy chain protein relieves the requirement for surrogate light chains in early B cell development. J. Immunol. *159*, 1265-1275.

Scharenberg, A.M., EL-Hillal, O., Fruman, D.A., Beitz, L.O., Li, Z., Lin, S., Gout, I., Cantley, L.C., Rawlings, D.J. and Kinet, J-P. (1998). Phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signalling pathway: a target for SHIP-mediated inhibitory signals. EMBO J. *17*, 1961-1972.

Scher, I. (1982). The CBA/N mouse strain: An experimental model illustrating the influence of the X-chromosome on immunity. Adv. Immunol. *33*, 1-71.

Sideras, P. and Smith, C.I.E. (1995). Molecular and Cellular aspects of X-linked agammaglobulinemia. Adv. in Immunol. *59*, 135-223.

Steinman, R.M., Pack, M. and Inaba, K. (1997). Dendritic cells in the T-cell areas of lymphoid organs. Immunol. Rev. *156*, 25-37.

Tiegs, S.L., Russell, D. and Nemazee, D. (1993). Receptor editing in self-reactive bone marrow B cells. J. Exp. Med. *177*, 1009-1020.

Timmers, E., Hermans, M.M., Kraakman, M.E.M., Hendriks, R.W. and Schuurman, R.K.B. (1993). Diversity of immunoglobulin κ light chain gene segment rearrangement in B lymphoblastoid cell lines from X-linked agammaglobulinemia patients. Eur. J. Immunol. *21*, 2355-2363.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature *302*, 575-581.

Torres, R.M., Flaswinkel, H., Reth, M. and Rajewski, K. (1996). Aberrant B cell development and immune responses in mice with a compromised BCR complex. Science *272*, 1804-1808.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L. and Tybulewicz, V.L. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature 378, 298-302.

Turner, M., Gulbranson-judge, A., Quinn, M.E., Walters, A.E., MacLennan, I.C.M. and Tybulewicz, V.L.J. (1997). Syk tyrosine kinase is required for the positive selection of immature B cells into the recirculating B cell Pool. J. Exp. Med *186*, 2013-2021.

Villey, I., Quartier, P., Selz, F and Villartay, J.P. (1997) Germ-line transcription and methylation status of the TCR-Jʔ locus in its accessible configuration. Eur. J. Immunol. 27:1619-1625.

Wahl, M.I., Fluckinger, A-C., Kato, R.M., Park, H., Witte, O.N. and Rawlings, D.J. (1997). Phosphorylation of two regulatory tyrosine residues in the activation of Bruton's tyrosine kinase via alternative receptors. Proc. Natl. Acad. Sci. USA *94*, 11526-11533.

Woodland, R.T., Schmidt, M.R., Korsmeyer, S.J. and Gravel, K.A. (1996). Regulation of B cell survival in *xid* mice by the proto-oncogene *bcl-2*. J. Immunol. *156*, 2143-2154.

Zhou, L-J., Smith, H.M., Waldschmidt, T.J., Schwarting, R. and Tedder, T.F. (1994). Tissue-specific expression of the human CD19 gene in transgenic mice inhibits antigen-independent B-lymphocyte development. Mol. Cell. Biol. *14*, 3884-3894.

Zou, R., Takeda, S. and Rawjewski, K. (1993). Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangements in Ig kappa. EMBO J. *12*, 811-820.

Chapter 7

General discussion

General discussion

xid as a model for XLA

B lymphopoiesis is regulated by multiple signals derived from stromal cell contact, cytokines, antigens and helper T cells. *In vitro* biochemical and gene targeting *in vivo* experiments have implicated tyrosine kinases as key regulators of many of these signaling pathways (Satterthwaite and Witte, 1996).

Btk is one of several kinases involved in regulating the differentiation of the B cell lineage. Defects in the Btk protein lead to XLA in humans. Since the discovery of the *Btk* gene (Vetrie *et al.*, 1993, Tsukada *et al.* 1993) over four hundred mutations, distributed over the entire *Btk* coding region have been characterized in XLA patients (Vihinen *et al.*, 1998). There is phenotipical heterogeneity among patients, even among patients from single XLA pedigrees (Vihinen *et al.*, 1998, Holsinki-Feder *et al.*, 1998, Bykowsky *et al.*, 1996). Sofar, it has not been possible to correlate severity of the phenotypic presentation with the genotype. Also the precise arrest of B cell development in XLA patients has not been revealed (Campana *et al.*, 1990). Recently developed new techniques, such as single cell PCR, and the characterization of cell surface markers could help to study the *Btk* expression pattern and its role in B cell development in human. However, the large genetic variety, differences in habitat or therapeutic interventions between patients might complicate these studies. Mouse models therefore could serve as an alternative to study the effect of *Btk* mutations and its role in B cell development, since they exhibit an uniformly genetic background and can be kept under comparable conditions. However, mice do not develop a phenotype as severe as the human XLA (Khan *et al.*, 1996, Kerner, *et al.*, 1995, see introduction, Table 2). The molecular bases of these differences between the two species are not well understood. In addition, severity of the *xid* phenotype is dependent on the genetic background of mice (Bona *et al.*, 1980, Khan *et al.*, 1996). Differences in phenotype severity between human and mouse can be caused by the relative contribution of alternative pathways of B cell differentiation in a Btk-independent manner. This hypothesis is supported by the observation that Btk-deficient mice that also lack CD40 have decreased numbers of peripheral B cells and lower serum immunoglobulin levels, comparable to XLA patients (Oka *et al.*, 1996, Kahn *et al.*, 1997). This would imply that the mechanism by which mice can compensate to some degree for Btk-deficiency is T cell dependent. An alternative explanation, are differences in availability or functionality of Btk-relatives that may compensate Btk function. The BCR-induced hydrolysis of phospholipids in Btk-deficient chicken DT40 and XLA cell lines is severely reduced, leading to a strong reduction in Ca^{2+} flux, whereas *xid* B cells demonstrate only a twofold reduction in phosphatidyl-inositide hydrolysis (Takata *et al.*, 1996, Fluckinger *et al.*, 1998). Expression of wild-type Btk as well as other members of the Tec family including human Itk and murine Tec, restored Ca^{2+} flux in these cells (Fluckinger *et al.*, 1997). While Itk expression is restricted to the T and NK cells, Tec is expressed in multiple haematopoietic lineages, including myeloid cells and B cells, as well as in liver, kidney ovary and heart (Siliciano *et al.*, 1992, Heyeck *et al.*, 1993, Mano *et al.*, 1993, Sato *et al.*, 1994). Since murine Tec expression

was able to correct impaired Ca^{2+} flux in cell lines derived from XLA patients, the expression of Tec endogenously in xid B cells may compensate, at least in part, for the absence of Btk. Therefore the differences in phenotype between XLA and *xid* may be caused by differences in expression level or expression patterns of Btk-relatives (Tec). An alternative, but not mutually exclusive, explanation could be a higher degree of homology between murine *Btk* and Tec. It would therefore interesting to see the phenotype of mice lacking both Btk and Tec.

In spite of the obvious differences in severity of the phenotype, Btk appears to be a conserved key factor involved in both murine and human B cell development. Btk has been shown to be involved in the BCR signal transduction pathways in both human (Hinshelwood *et al.*, 1995, De Weers *et al.*, 1994) and murine (Aoki *et al.*, 1994, Saouf *et al.*, 1994) B cells. *In vivo* competition experiments in female *Btk* $^{+/-}$ mice between B cells either expressing the wild-type *Btk* gene or the *LacZ* gene from the disrupted *Btk* allele, demonstrated that the first defects of Btk⁻ cells became apparent at the transition from the pre-B cell stage to the immature B cell stage (chapter 2). Moreover, the accumulation of Btk⁻ cells within a subpopulation of pre-B cells indicated that Btk⁻ cells are defective in the pre-B to B cell transition (chapter 6). This arrest of B cell transition is the hallmark of XLA. Moreover, the genomic organization of the murine *Btk* gene is very homologous to the human *Btk* gene and the Btk proteins share 99.3% homology. As is described in chapters 3-6, transgenic human *Btk* could fully compensate for the absence of murine Btk, indicating that the essential sites for Btk interaction with other signal transduction components are conserved between human and mouse. In addition, large scale comparative sequence analysis of the human and murine *Btk* loci revealed clusters of sequence conservation in non-coding regions throughout the loci (Oeltjen *et al.*, 1997), which may play an essential role in the complex gene regulation. Moreover, when human Btk was expressed under the control of the endogenous *cis*-acting elements, the expression pattern of transgenic Btk paralleled that of the endogenous Btk. We concluded that also *cis*-acting elements that regulate Btk expression have been conserved. Studies on Btk using mouse models, therefore, lead to a better understanding of the regulation of expression and the role of Btk and its domains in human B cell development.

Mouse models for other X-linked immunodeficiency diseases

A comparison between the XLA- and *xid*-phenotypes is given in the general introduction (chapter 1, Table 2.). From this comparison it is clear that *xid* represents a less severe phenotype. A similar B cell differentiation disorder results from defects in the λ5 pseudo-light chain component. The difference in severity of B cell deficiency resulting from the *Btk* gene mutation in mouse and man is reminiscent of that which arises due to mutations in the λ5 gene, resulting in a less severe phenotype in mice, and a more severe phenotype CVID in man (Conley *et al.*, 1994, Scher, 1982). The effects of the human B-lineage developmental defect appear to be more severe than those seen in λ5-defective mice. While normal numbers of CD19⁺ TdT⁺ pro- and pre-B cells were detectable in CVID patients, as in the λ5-defective mice, there were even fewer pre-B-II cells in mouse, and the peripheral B cells were IgM^{low} and CD38^{low}. Since the patient's father was heterozygous for the

$\lambda 5(14.1)$ mutation and had normal numbers of B cells, an additional dominant negative mutation contributing to the severity of the B cell defect appears unlikely. In other X-linked immunodeficiencies, also differences between the phenotypes of human immunodeficiency patients and phenotypes of mouse models for these diseases are observed. A comparison of these differences in phenotypes for the XSCID, X-linked-hyper-IgM-syndrome and Wisckott-Aldrich syndrome (WAS) immunodefciencies are summarized in Table 1. Mouse models tend to have a less severe phenotype as the human patients, although the bases for these differences are not clear yet.

Table 1. Comparison of phenotypes of human immunodeficiencies and mouse models

human	mouse
XSCID (IL-2 R):	
Severe impairment of B and T cell function	Severe impairment of B and T cell function
T cells absent or low	T cells absent or low
Peripheral B cell numbers normal or increased	Peripheral B cell numbers decreased
Ig class switch defects	Ig class switch defects
NK cell numbers normal or reduced	NK cells absent
Thymus lacks cortex/medullar demarcation, Hassall's corpuscles not detectable	Size of thymus diminished, differentiation of cortical and medullary zones, Hassall's corpuscles present
With age, T cell numbers remain low	Development of a peripheral pool of (activated) $CD4^+$ T cells with age
X-linked hyper-IgM (CD40L)	
Low levels of serum IgA and IgE IgM serum levels generally elevated	Low serum levels of IgG1, IgG2b and IgA, lack of IgE, IgG3 levels slightly reduced, IgM serum levels normal
Immune responses are impaired	Immune responses are impaired
No germinal centers	No germinal centers
Normal B and T cell numbers	Normal B and T cell numbers
WAS :	
Severely decreased platelet numbers, reduced platelet size	Modest reduction in platelet number, normal platelet size
Defective T cell activation	Defective T cell activation
Eczema	No eczema
Variable B cell defects	Normal antigen-induced B cell stimulation
Poor responses to carbohydrate and protein antigens	Normal Ab responses to TD and TI-II antigens
Lymphoreticular malignancies	No haematopoietic malignancies
No chronic colitis	Development of chronic colitis

References: XSCID: Fischer *et al.*, 1997, Sugamura *et al.*, 1996, Cao *et al.*, 1995, DiSanto *et al.*, 1995; X-Linked-hyper-IgM: Foy *et al.*, 1996, Renshaw *et al.*, 1994; WAS: Snapper *et al.*, 1998.

XLA, mouse models and gene therapy

For the development of future strategies towards gene therapy as a means of correcting the XLA defect, experiments in which mouse models are used, will be essential. Using mouse models, the efficiency of gene transfer strategies, the final *Btk* expression levels in B cells and the obtained correction of the *xid* phenotype can be investigated.

Despite the fact that most XLA patients respond quite well to current treatments, it is obvious that repetitive administration of antibodies (gammaglobulin) or antibiotics, is inferior to a natural immune response. Many XLA patients manifest chronic pulmonary infections of a disabling character. Gene therapy is the obvious future choice for correction of this hereditary disease, as it would restore an active immune response to infection. Several features of the *Btk* gene make XLA an attractive model to develop methods for somatic gene therapy. Firstly, XLA, which is one of the most frequent inherited immunodeficiencies in man, affects only one single cell lineage, without any defects outside the B cell system. Secondly, the findings that (1) the first selective disadvantage of Btk⁻ B cells occurs at the transition from pre-B to B cells (chapter 2), and (2) *Btk* expression from the pre-B cell stage onwards is sufficient to correct the *xid* phenotype (Drabek *et al.*, 1997), indicates that the *Btk* gene does not need to be introduced in the very small population of haematopoietic stem cells to restore B cell development in XLA patients. Instead, *Btk* gene transfer to any of the precursor B cell stages up to the small pre-B cell stage would correct the XLA defect, thereby increasing the target population to ~10 % of total cells in the bone marrow. In addition, Btk expression only needs to be transient, since Btk is not required in plasma cells (Sideras and Smith, 1995, De Weers *et. al.*, 1993). Furthermore, bone marrow cells are easily accessible for *ex vivo* gene transfer, and there is a natural selective advantage for developing B cells that have been corrected (Drabek *et al.*, 1997, chapter 3). Finally, in contrast to the fact that certain protein tyrosine kinases have been implicated in tumor formation when mutated or overexpressed, we have shown that overexpression of Btk or the E41K mutated form of Btk did not lead to the development of lymphomas or leukemias in mice (chapter 4 and 5). As the construct that eventually will be used to express an intact *Btk* gene in pre-B cells of XLA patients should preferably contain regulatory sequences from the *Btk* gene itself, it is of importance to identify all elements involved in the regulation of *Btk* gene expression.

Multiple roles of Btk in B cell development

The main subjects of the research described in this thesis was the analysis of the regulation of *Btk* gene expression and the function of Btk in the individual subsequent stages of B cell development *in vivo*. Therefore we have generated one Btk-knock-out mouse model and several transgenic mouse models that express either wild-type or E41K mutated Btk under the control of different promoters or regulatory elements (chapters 2- 6). As the data described in each chapter have already been discussed at length at the end of each chapter and represent our current view, this section will overview the involvement of Btk during B cell development (Fig. 1).

Each successive step in B cell differentiation, as well as the mature B cell response to antigen, depend on signals mediated by the (pre-)BCR complex (Kitamura *et al.*, 1991, Lam *et al.*, 1997, Gu *et al.*, 1991). The majority of B-lineage cells that are generated in the bone marrow do not migrate to peripheral tissues to participate in an immune response but, instead, undergo apoptosis and are disposed of by resident macrophages (Osmond *et al.*, 1994). Two reasons for the death of B cell precursors are the non-productive assembly of the heavy or light chain segments and the generation of a BCR that is self-reactive. A third, related reason for pre-mature cell-death may be that cells have exhausted their potential for additional V_L chain gene rearrangements by receptor editing.

Studies on the *in vivo* expression pattern of murine Btk, using the *LacZ*-labeled disrupted *Btk* allele (Chapter 2) or intracellular flow cytometric Btk detection (Chapter 4) demonstrated that Btk is expressed *in vivo* throughout B cell development, from the pro-B cell stage to the most mature IgM^{low}IgD^{high} peripheral B cell and activated B lymphoblasts stage. These expression patterns were consistent with other Btk expression pattern studies in cell lines and leukemias (De Weers *et al.*, 1993, Genevier *et al.*, 1994, Smith *et al.*, 1994, Tsukada *et al.*, 1993). The finding that Btk is expressed throughout B cell development and the involvement of Btk in BCR-signaling pathways (De Weers *et al.*, 1994, Aoki *et al.*, 1994, Saouf *et al.*, 1995, Hinshelwood *et al.*, 1994), support the idea that signaling cascades activated by Btk have a crucial role during the B cell differentiation process, as well as in the activation of mature B cells in the periphery (Fig. 1).

(0) Btk is not required in pro and large pre-B cells

A critical role for Btk in the assembly of the μ H chain and the subsequent transition from the pro-B to the small pre-B cell stage is very unlikely. Firstly, this transition is not affected in human XLA, since pre-B cells are generally present at normal numbers in XLA patients (Sideras and Smith, 1995). Secondly, in addition, in mice, the absence of Btk did not result in a selective disadvantage up to the small pre-B cell stage, as was shown by the *in vivo* competition assays in female *Btk* $^{+/-}$ mice (chapter 2). Thirdly, the *xid* phenotype can be corrected by transgenic expression of Btk from the pre-B cell stage onwards (Drabek *et al.*, 1997). Finally, proliferation of pre-B cells that have successfully rearranged their IgH chain locus and have performed allelic exclusion (both indicated by pre-BCR signaling) are not affected in Btk-deficient mice. We conclude that Btk is not required in pro- and large pre-B cell stages (Fig. 1).

(1) A role for Btk in Ig L chain assembly

The first role for Btk becomes apparent at the transition of the small resting pre-B cell stage to the IgM^{low}IgD$^-$ immature B cell stage (Fig. 1). This transition is affected both in XLA patients (Conley, 1985) and in mice, in which B cells that lack Btk show a selective disadvantage compared to B cells that express Btk (Chapter 2). The observed accumulation of Btk-deficient cells within a subpopulation of small resting pre-B cells (Chapter 6) further indicates that Btk is involved in signaling pathways, leading to the initiation of IgL chain rearrangement and/or expression of a

complete IgM-molecule on the membrane, needed for further B cell maturation (Rajewski, 1996). It might be possible that Btk is an essential transducer of signals, involved in regulating the re-expression of the *RAG* gene products, which are absent in the large cycling pre-B cells and reactivated in small resting pre-B cells (Grawunder *et al.*, 1995) needed for Ig L chain rearrangements. This hypothesis is consistent with the finding that expression of the *RAG* genes is dependent on BCR signaling in immature IgM^{low} B cells that are in the process of receptor editing (Melamed *et al.* 1998). Alternatively, Btk could also transduce signals that result in chromatin structure changes that allow the recombinase access to the IgL chain gene segments.

A role for Btk in Ig L chain rearrangement events is also suggested by the finding that the proportion of λ L chain expressing B cells is decreased in Btk-deficient mice whereas proportion of λ L chain expression B cells is increased in CD19-h*Btk*^{E41K} transgenic mice, compared to wildtype mice (Chapter 6). The observed 95:5 ratio of κ:λ expressing L chain B cells in normal mice is thought to reflect the earlier rearrangement initiation at the κ locus and the higher probability of productive κ$^+$ rearrangements within the limited life span of the small pre-B cell (Hieter *et al.*, 1981, Ramsden and Wu, 1991, Arakawa *et al.*, 1995). As L chain rearrangement occurs without cell division or substantial cell loss (Osmond, 1991), the κ/λ ratio of the immature B cell population directly reflects the probability of productive κ and λ Ig L chain rearrangements. The reduced λ L chain expression found in Btk-deficient mice, therefore, indicates that the absence of Btk results in a delay in the initiation of Ig L chain rearrangements (*e.g.* by defective RAG activity upregulation, or L chain locus accessibility), a reduced survival of pre-B cells that are in the process of L chain rearrangement, and/or impaired receptor editing of immature IgM^{low} B cells.

The increased proportion of λ L chain expressing cells in the E41K transgenic mice may be due to early initiation of the IgL chain rearrangement, caused by the constitutively activated form of Btk, or, alternatively be a result of an extended life-span of pre-B cells (or immature IgM^{low} B cells), each of which would increase the limited time window for Ig L chain rearrangements. In the latter case, the CD19-h*Btk*^{E41K} transgenic mice parallel transgenic mice with enforced expression of the anti-apoptotic protein bcl-2 in B cells, which also showed a significant increase in the percentage of λ L chain expressing B cells, either *in vivo* in the presence or absence of auto-reactive Ig transgenes (Tiegs *et al.*, 1993, Lang *et al.*, 1997) or in bone marrow cultures *in vitro* (Rolink *et al.*, 1993). The high λ\κ ratio in Bcl-2 transgenic B cells were explained by assuming that B cells normally have too little time to take full advantage of the potential λ L chain repertoire that could rescue non-functional or autoreactive B cells (Lang *et al.*, 1997). Conversely, the absence of Btk would limit the survival of pre-B cells that are in the process of L chain rearrangement and therefore result in low λ/κ ratios. A role for Btk in (pre-)B cell survival would be supported by the findings that bcl-2 expression is reduced and sIg mediated bcl-x$_L$ induction is absent in *xid* B cells (Anderson *et al.*, 1996, Choi *et al.*, 1996, Woodland *et al.*, 1996).

(2) A role for Btk in central immune tolerance

With the expression of the IgM and the resulting antigen specificity on the cell surface of immature B cells, they become susceptible to immune tolerance (Goodnow, 1996). Recently, two subpopulations within the immature B cell stage with differences in apoptosis sensitivity were described (Melamed and Nemazee, 1997). Upon BCR stimulation in an *in vitro* IL-7 driven BM culture system, IgM^{high}IgD$^-$ immature B cells, representing an intermediate between early IgM^{low}IgD$^-$ immature and recirculating B cells, were extremely sensitive to apoptosis, whereas stimulation of the BCR of IgM^{low}IgD$^-$ immature B cells promoted nested L chain rearrangements, *i.e.* receptor editing.

In mice, the absence of Btk did not affect the transition from the IgM^{low}IgD$^-$ to IgM^{high}IgD$^-$ immature cell stage (chapter 6), indicating that the absence of Btk expression is not critical for transition to the IgM^{high}IgD$^-$ cell stage. Conversely, expression of the constitutively activated E41K Btk mutant driven by the CD19 promoter resulted in an almost complete absence of IgM^{high}IgD$^-$ immature B cells (Chapter 6). The IgM^{high}IgD$^-$ immature B cell stage reflects the first immune tolerance checkpoint where autoreactive B cells become susceptible to apoptosis. Therefore, the absence of IgM^{high}IgD$^-$ immature B cells in CD19-h*Btk*^{E41K} transgenic mice, suggests that constitutive activation of Btk mimics autoreactive BCR crosslinking. The absence of the IgM^{high}IgD$^-$ B cells would imply that Btk is involved in the BCR signaling pathway that eliminates auto-reactive B cells at the IgM^{high}IgD$^-$ B cell stage. An alternative explanation for the absence of IgM^{high}IgD$^-$ B cells could be that the E41K Btk mutant blocks the transition from the IgM^{low}IgD$^-$ to IgM^{high}IgD$^-$ immature cell stage, because of sustained receptor editing (Melamed and Nemazee, 1997). Finally, cells might die due to exhausted potential for nested L chain rearrangements.

(3) A role for Btk in follicular entry

In contrast to the phenotype of the CD19-h*Btk*^{E41K} transgenic mice, mice in which E41K Btk expression is driven by the MHC class II LCR (MHCII-h*Btk*^{E41K} mice) display normal B cell development in the bone marrow, but manifest a deficiency of recirculating follicular B cells (Chapter 5). Cells that were present in the spleen were IgM^{high}HSA^{high}B220^{low} immature B cells that have recently left the bone marrow. The differences between the two Btk^{E41K} expressing mouse strains can be explained by the escape of negative selection in the bone marrow of the MCHII-h*Btk*^{E41K} expressing cells because the expression level had not reached a critical threshold value. Due to the nature of the MHCII-LCR, transgenic Btk^{E41K} expression is upregulated after cells have arrived in the spleen (chapter 5). As a result only late in B cell development an active state of the B cell is mimicked. In this context, MHC-II-E41K Btk transgenic B cells may resemble transgenic B cells, autoreactive for antigens present in the periphery (Eibel *et al.*, 1994, Goodnow *et al.*, 1995, Russel *et al.*, 1991). In these mice, immature B cell numbers in the bone marrow were present in normal numbers but were eliminated shortly after leaving the bone marrow, bearing an immature phenotype. In comparison, cell transfer studies with antigen-specific transgenic B cells have shown that naive cells could migrate into follicles, but were retained in the outer T cell zones in the

presence of antigen, even without competition of follicular B cells (Cyster and Goodnow, 1995). This is consistent with the observation that antigen-specific B cells accumulate in the outer T cell zone within the first days of an immune response where they are in close contact with T helper cells, specifically recognizing the presented antigen. As the outer T cell zone is a major trafficking region for T cells, it is likely that selective retention of antigen-reactive B cells in this zone functions to promote encounter between antigen-specific T and B cells. Activated B cells that are excluded from follicles can be rescued from cell death and stimulated to proliferate and differentiate, in response to stimuli, provided by the activated T helper cells. Likewise, the activated state of the MHCII-$hBtk^{E41K}$ B cells may result in follicular exclusion. Concomitantly, the immature $IgM^{high}HSA^{high}B220^{low}$ phenotype may inhibit rescue by T helper cells, thereby resembling the apoptosis-sensitive $IgM^{high}IgD^{-}$ immature B cells in the bone marrow, in which apoptosis is induced upon strong BCR signaling.

(4) Follicular maturation and survival

B cells are positively selected to become long-lived $IgM^{low}IgD^{high}$ recirculating follicular B cells by BCR mediated signaling (Gu *et al.*, 1991, Lam *et al.*, 1997). Mutations in BCR signaling components such as Ig-α or Syk, block the entry of B cells into follicles (Torres *et al.*, 1996, Turner *et al.*, 1995). Btk-deficient B cells are not excluded from B cell follicles, but fail to develop from the immature $IgM^{high}IgD^{low}$ stage into the long-lived recirculating follicular $IgM^{low}IgD^{high}$ B cell stage (Wicker and Scher, 1986, Khan *et al.*, 1995, chapter 2). As *xid* B cells express only low levels of bcl-2 and undergo spontaneous apoptosis more rapidly than wild-type B cells *in vitro* (Woodland *et al.*, 1996), Btk must play a critical role in survival and maturation, mediated by low-level BCR signaling (Gu *et al.*, 1991, Lam *et al.*, 1997), to become long-lived recirculating B cells (Fig. 1).

(5) Plasma cell differentiation

Another role for Btk was observed in the CD19-hBtk^{E41K} transgenic mice. As the block at the $IgM^{high}IgD^{-}$ immature B cell stage was leaky, B cells were found in the peripheral organs in very low numbers, almost exclusively bearing a $IgM^{high}HSA^{high}B220^{low}$ immature phenotype. In parallel to the MHCII-hBtk^{E41K} transgenic mice, the expression of Btk^{E41K} impeded follicular entry of these immature B cells and their subsequent maturation. Despite the severe reduction of the mature B cell pool, significant numbers of IgM secreting plasma cells were present in the splenic red pulp. Serum IgM concentrations were substantial and increased with age as compared to those in normal mice. Therefore, we conclude that Btk activation quite efficiently induced the terminal differentiation of the residual B cells into IgM-producing plasma cells. In the CD19-hBtk^{E41K} transgenic mice, serum levels of the IgG and IgA subclasses were severely decreased, confirming that constitutive BCR signaling in the absence of co-stimulation by CD40-CD40L interactions does not induce B cells to perform IgH chain class switch or germinal center formation (Foy *et al.*, 1996).
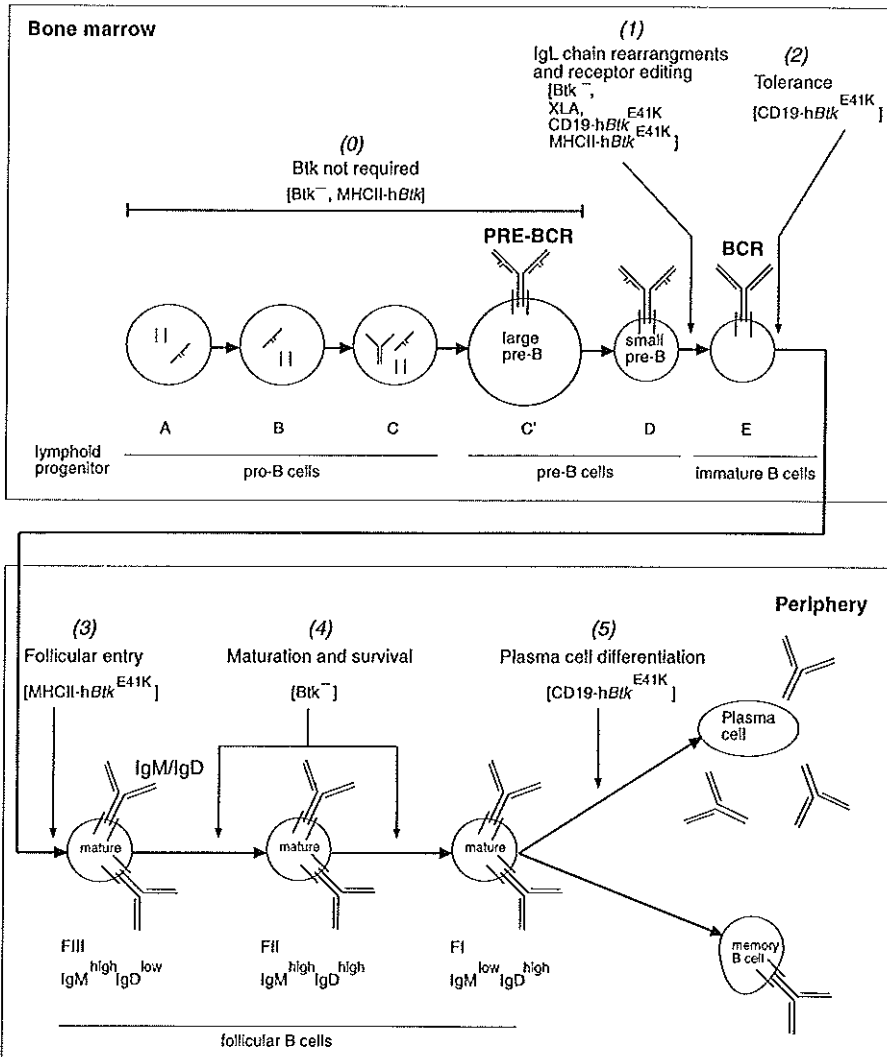
Figure 1. *Scheme of the B cell differentiation. The multiple stages at which Btk is involved are shown, together with the mouse models that provided evidence for a role of Btk (in parenthesis).*

Downstream targets of Btk activation during B cell development

Although our analysis of transgenic and knock-out mice *in vivo* have defined at least five stages of B cell development in which Btk activity is involved, it is unknown by which downstream signaling pathways these effects on B cell development are accomplished. Only two possible targets of Btk have been identified so far (see also introduction). Btk-deficient cell lines display a reduced phosphorylation of PLC-γ upon BCR cross-linking (Takata *et al.*, 1996, Fluckinger *et al.*, 1998). PLC-γ is involved in the hydrolysis of phosphatidyl-inositol 4,5-biphosphate (PI4,5-P2) into diacylglycerol (DAG) and IP_3. (Bijsterbosch *et al.*, 1985). DAG activates most protein kinase C (PKC) isoforms and IP3 generates an increase in Ca^{2+} flux. The involvement of Btk in the PLC-γ signaling pathway is suggested by the phenotype of PKCβ-deficient mice, which is similar to *xid*. Moreover, deletion of PLC-γ2 in DT40 cells, abolished IP3 production and Ca^{2+} mobilization (Takata *et al.*, 1995). As a result, the induction of apoptosis, which normally follows BCR stimulation in these cultured cells, was blocked. Increases in the intracellular $[Ca^{2+}]$ are required for the induction of the mitogen-activated protein kinase (MAPK), c-JunN terminal kinase (c-JNK) and p38 cascades, as well as the calcium-dependent transcription factors NF-AT and NF-κB (Dolmesch *et al.*, 1997, Healy *et al.*, 1997). These are important signals that lead to cell proliferation and differentiation (Fluckinger *et al.*, 1998). In contrast, intermittent or subthreshold oscillation in calcium concentrations result in activation of only a subset of these downstream signals and lead to decreased survival and apoptotic death (Fluckinger *et al.*, 1998). In mast cells, c-JNK plays a role in apoptosis in a Btk dependent way (Kawakami *et al.*, 1997). It would be interesting to investigate whether apoptosis, stimulated by BCR-mediated signaling, in immature $IgM^{high}IgD^-$ B cells is also regulated via c-JNK in a Btk dependent manner.

Another important downstream signaling pathway, activated by Btk may involve proteins leading to the activation of cell survival proteins, such as bcl-2 and bcl-x_L (see discussion section of chapter 6).

Recently, the widely expressed transcription factor BAP-135/TFII-I was shown to be tyrosine phosphorylated by Btk in response to BCR crosslinking *in vivo* (Yang *et al.*, 1997). It is presently unclear, whether Btk is also present in the nucleus. BAP-135/TFII-I, interacts with E-boxes and initiator (Inr) sequences (Grueneberg *et al.*, 1997, Roy *et al.*, 1997). Moreover, the protein is suggested to be involved in the formation of a multiprotein complex at the promoter of the early B cell response gene *c-fos* (Grueneberg *et al.*, 1997). C-fos is involved in the regulation of the Ig κ L chain transcription (Schanke *et al.*, 1994) and it is intriguing to hypothesize that c-fos may cause an increase in the accessibility of the IgL chain loci for recombination. The almost complete block of B cell development at the pre-B cell stage in XLA could then be explained by defective initiation of Ig L chain rearrangement.

Concluding remarks

Our findings in the mouse show that Btk is expressed throughout B cell development, except in plasma cells, and that signaling cascades activated by Btk are critical at several checkpoints throughout B cell differentiation. The transition into IgM$^+$ immature cell stage is impaired when Btk is defective, indicating a role for Btk in re-expression of the RAG genes, insufficient accessibility of Ig L chain loci, or a reduced survival of pre-B cells that are in the process of L chain rearrangement. Expression of the CD19-h*Btk*^{E41K} transgene resulted in a lack of progression of B cells into the IgM^{high}IgD$^-$ immature cell stage, placing Btk in the BCR signaling pathway that eliminates auto-reactive B cells in the bone marrow. A similar role for Btk was observed in the periphery: in transgenic mice expressing MHCII-h*Btk*^{E41K}, the development of recirculating follicular B cells is blocked, showing that constitutive activation of Btk induces the elimination of virgin B cells. Btk-deficient B cells manifest an impaired survival and maturation in the periphery, indicating a role for Btk in basal BCR signaling needed to direct developmental progression of B cells. Finally, the phenotype of CD19-h*Btk*^{E41K} transgenic mice, provide evidence that Btk activation (in the absence of co-stimulatory signals) drives B cells into IgM$^+$ plasma cell differentiation.

Our studies show remarkable similarities between XLA and *Xid*. XLA patients show a block in B cell development at the transition of the small pre-B cell stage to the immature B cells. In comparison, in Btk-deficient mice, the first selective disadvantage for Btk-deficient B cells occurred at the same transition. Moreover, the proportion of λ L chain expressing cells is decreased in Btk-deficient mice comparable to humans, in which the absence of Btk results in a deviation from the normal κ$^+$ to λ$^+$ ratio of 50:50, to only ~25% λ$^+$ cells. We therefore conclude that *xid* is a good animal model for XLA.

The molecular mechanisms by which Btk mediates B cell development, cell activation and cell death need further elucidation and further identification of downstream signaling targets and pathways. Knowledge of the regulatory elements involved in *Btk* gene expression *in vivo* would help the design of a *Btk* mini-locus. Such a mini-locus would not only be essential for the progress of gene therapy strategies as a means to correct the B cell defect in XLA patients, but would also be helpful for the achievement of physiological levels of *Btk* expression in studies of the *in vivo* function of the separate Btk-domains in transgenic mouse models. Different domains can easily be mutated and the effect on interacting proteins and signaling pathways activated by Btk can be studied. The biochemical characterization of proteins that associate with the individual domains of Btk at the subsequent stages of B cell development could be facilitated by the generation of transgenic mice that express a tagged form of Btk. Similar approaches could be used for other signaling molecules implicated in BCR signaling which will eventually lead to a better insight in B cell development and function.

References

Anderson, J.S., Teutsch, M., Dong, Z. and Wortis, H.H. (1996). An essential role for tyrosine kinase in the regulation of Bruton's B-cell apoptosis. *Proc. Natl. Acad. Sci. USA* 93: 10966-10971.

Aoki, Y., Isselbacher, K.J. and Pillai, S. (1994) Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligand B cells. *Proc. Natl. Acad. Sci. USA* 91:10606-10609.

Arakawa, H., Shimizu, T. and Takeda, S. (1995) Re-evaluation of the probabilities for productive rearrangements on the κ and λ loci. *Int. Immunol.* 8:91-99.

Bijsterbosch, M.K. and Klaus, G.G.B. (1985) Crosslinking of surface immunoglobulin and Fc receptors on B lymphocytes inhibits stimulation of inositol phosphate breakdown via the antigen receptor . *J. Exp. Med.* 162:1825-1827.

Bona, C., Mond, J.J. and Paul, W.E. (1980) Synergistic genetic defect in B-lymphocyte funtion. I. Defective responses to B-cell stimulants and their genetic basis. *J. Exp. Med.* 151:224-234.

Bykowsky, M.J., Haire, R.N., Ohta, Y., Tang, H., Sing, S.S., Veksler, E.S., Green, J.M., Fu, S.M., Litman, G.W. and Sullivan, K.E. (1996). *Am. J. Hum. Genet.* 58:477-483.

Campana, D., Farrant, J., Inamidar, N., Webster, D.B. and Janossy, G. (1990) Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. *J. Immunol.* 145:1675-1680.

Cao, X., Shores, E.W., Hu-Li, J., Anver, M.R., Kelsall, B.L., Russell, S.M., Drago, J.,, Noguchi, M., Grinberg, A., Bloom, E.T., Paul, W.E. and Leonard, W.J. (1995) Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity* 2;223-238.

Choi, M.S.K., Holman, M., Atkins, C.J. and Klaus, G.G.B. (1996). Expression of bcl-x during mouse B cell differentiation and following activation by various stimuli. *Eur. J. Immunol.* 26:676-682.

Conley, M.E. (1985) B cells in patients with X-linked agammaglobulinemia. *J. Immunol.* 134:3070-3074.

Conley, M.E., Parolini, O., Roher, J. and Campana, D. (1994) X-linked agammaglobulinemia: new aprroaches to old questions based on the identification of the defective gene. *Immunol. Rev.* 138:5-21.

Cyster, J.G. and Goodnow, C.C. (1995) Antigen-induced exclusion from follicles and anergy are seperate and complemantary processes that influence B cell fate. *Immunity* 3:691-701.

DiSanto, J.P., Müller, W., Guy-Grand, D., Fischer, A. and Rajewski, K. (1995) Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl. Acad. Sci. USA* 92:377-381.

De Weers, M., Brouns, G.S., Hinshelwood, S., Kinnon, C., Schuurman, R.K.B., Hendriks, R.W. and Borst, J. (1994) B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. *J. Biol. Chem.* 269:23857-23860.

De Weers., Verschuren, M.C.M., Kraakman, M.E.M., Mensink, R.G.J., Schuurman, R.K.B., Van Dongen, J.J.M. and Hendriks, R.W. (1993) The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur. J. Immunol.* 23:3109-3114.

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. and Healy, J.I. (1997) Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration. *Nature* 386:855-858.

Drabek, D., Raguz, S., De Wit, T.P.M., Dingjan, G.M., Savelkoul, H.F.J., Grosveld, F. and Hendriks, R.W. (1997) Correction of the X-linked immunodeficiency phenotype by transgenic expression of human Bruton's tyrosine kinase under the control of the class II major histocompatibility complex Ea locus control region. *Proc. Natl. Acad. Sci. USA* 94:610-615.

Eibel, H., Fiedler, P. and Köhler, G. (1994) Transgenic mouse model for peripheral B cell tolerance. *In* 'Transgenesis and targeted mutagenisis in Immunology' (H. Bluethmann and P. Ohashi, Eds.), pp. 251-265. Academic Press, San Diego.

Fischer, A., Cavazzana-Calvo, M., De Saint Basile, G., DeVillartay, J.P., DiSanto, J.P., Hivroz, C., Rieux-Laucat, F. and Le Deist, F. (1997) Naturally occuring primary defeciencies of the immune system. *Annu. Rev. Immunol.* 15:93-124.

Fluckinger, A.C., Li, Z., Kato, R.M., Wahl, M.I., Ochs, H.D., Longnecker, R., Linet, J.P., Witte, O.N., Scharenberg, A.M. and Rawlings, D.J. (1998) Btk/Tec kinases regulate sustained increases in intracellular Ca²⁺ following B-cell receptor activation. *EMBO J.* 17:1973-1985.

Foy, T.M., Aruffo, A., Bajorath, J., Buhlmann, J.E. and Noelle, R.J. (1996) Immune regulation by CD40 and its ligand GP39. *Annu. Rev. Immunol.* 14:591-617.

Genevier, H.C., Hinshelwood, S., Caspar, H.B., Rigley, K.P., Brown, D., Sealand, S., Rousset, F., Levinsky, R.J., Callard, R.E., Kinnon, C. and Lovering, R.C. (1994) Expression of Bruton's tyrosine kinase protein within the B cell lineage. *Eur. J. Immunol.* 24:3100-3105.

Goodnow, C.C. (1996) Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc. Natl. Acad. Sci. USA* 93:2264-2271.

Goodnow, C.C., Cyster, J.G., Hartley, S.B., Bell, S.E., Cooke, M.P., Healy, J.I., Akkarju, S., Ratmell, J.C., Pogue, S.L. and Shokat, K.P. (1995) Self-tolerance checkpoints in B lymphocyte development, *Adv. Immunol.* 59:279-368.

Grawunder, U., Leu, T.M.J., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F. and Winkler, T.H. (1995). Down-regulation of *RAG1* and *RAG2* gene expression in pre-B cells after functional immunoglobulin heavy chain rearrangement. *Immunity* 3:601-608.

Grueneberg, D.A., Henry, R.W., Brauer, A., Novina, C.D., Chriyath, V., Roy, A.L. and Gilman, M. (1997) A multifunctional DNA-binding protein that promotes the formation of serum response factor/homeodomain complexes. identity to TFII-I. *Genes Dev.* 11:2482-2493.

Gu, H., Tarlinton, D., Müller, W., Rajewski, K. and Förster, I. (1991). Most peripheral B cells in mice are ligand-selected. *J. Exp. Med.* 173:1357-1371.

Healy, J.I., Dolmetsch, R.E., Timmerman, L.A., Cyster, J.G., Thomas, M.L., Crabtee, G.R., Lewis, R.S. and Goodnow, C.C. (1997) Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity* 6:419-428.

Heyeck, S.D. and Berg, L.J. (1993) Developmental regulation of a murine T-cell-specific tyrosine kinase gene, *Tsk. Proc. Natl. Acad. Sci. USA* 90:669-673.

Hieter, P.A., Korsmeyer, S.L., Waldmann, T.A. and Leder, P. (1981). Human immunoglobulin kappa light-chain genes are deleted or rearranged in lambda-producing B cells. Nature *290*, 368-372.

Hinshelwood, S., Lovering, R.C., Genevier, H.C., Levinsky, R.J. and Kinnon, C. (1995) The protein defective in X-linked agammaglobulinemia, Bruton's tyrosine kinase, shows increased autophosphorylation *in vitro* when isolated from cells in which the B cell receptor has been cross-linked. *Eur. J. Immunol.* 25:1113-1116.

Holsinki-Feder, E., Weiss, M., Brandau, O., Jedele, K.B., Nore, F.A.A.A.P.B., Bäckesjö, M., Vihinen, M., Hubbard, S.R., Belohradsky, B.H., Smith, C.I.E. and Meindl, A. (1998) Mutation screening of the Btk gene in 56 families with X-linked agammaglobulinemia (XLA): 47 unique mutations without correlation to clinical course. *Pediatrics* 101:276-284.

Kawakami, Y., Miura, T., Bissonette, R., Hata, D., Khan, W.N., Kitamura, T., Maeda-Yamamoto, M., Hartman, S.E., Yao, L. and Alt, F.W. (1997) Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. *Proc. Natl. Acad. Sci. USA* 94:3938-3942.

Kerner, J.D., Appleby, M.W., Mohr, R.N., Chien, S., Rawlings, D.J., Maliszeweski, C.R., Witte, O.N. and Perlmutter, R. (1995) Impaired expansion of mouse B cell progenitors lacking *Btk. Immunity* 3:301-312.

Kitamura, D. and Rajewski, K. (1991) Targeted disruption of μ chain membrane exon causes loss of heavy chain allelic exclusion. *Nature* 356:154-156.

Khan, W.N., Gerstein, F.W., Malynn, B.A., Larsson, I., Rathburn, G., Davidson, L., Müller, S., Kantor, A.B. and Herzenberg, L.A. (1996) Defective B cell development and function in Btk-deficient mice. *Immunity* 3:283-299.

Khan, W.N., Nilsson, A., Mizoguchi, E., Castigli, E., Forsell, J., Bahn, A.K., Geha, R., Sideras, P. and Alt, F.W. (1997) Impaired B cell maturation in mice lacking Bruton's tyrosine kinase (Btk) and CD40. *Int. Immunol.* 9:395-405.

Lam, K-P., Kühn, R. and Rajewsky, K. (1997) In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90:1073-1083.

Lang, J., Arnold, B., Hammerling, G., Harris, A.W., Korsmeyer, S., Russell, D., Strasser, A. and Nemazee, D. (1997). Enforced Bcl-2 expression inhibits antigen-mediated clonal elimination of peripheral B cells in an antigen dose-dependent manner and promotes receptor editing in autoreactive, immature B cells. *J. Exp. Med.* 186:1513-1522.

Mano, H., Mano, K., Tang, B., Koehler, M., Yi, T., Gilbert, D.J., Jenkins, N.A., Copeland, N.G. and Ihle, J.N. (1993) Expression of a novel form of *Tec* kinase in heatopoietic cells and mapping of the gene to chromosome 5 near *Kit. Oncogene* 8:417-424.

Melamed, D., Benschop, R.J., Cambier, J.C., and Nemazee, D. (1998). Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor editing. *Cell* 92:173-182.

Melamed, D. and Nemazee, D. (1997) Self-antigen does not accelerate immature B cell apoptosis, but stimulates receptor editing as a consequence of developmental arrest. *Proc. Natl. Acad. Sci. USA* 94:9267-9272.

Oeltjen, J.C., Malley, T.M., Muzny, D., Miller, W., Gibbs, R.A. and Belmont, J.W. (1997) Large-scale comparative sequence analysis of the human and murine Bruton's tyrosine kinase loci reveals conserved regulatory domains. *Genome Res.* 7:315-329.

Oka, Y., Rolink, A., Andersson, J., Kamanaka, M., Uchida, J., Yasui, T., Kishimoto, T., Kikutani, H. and Melchers, F. (1996) Profound reduction of mature B cell numbers, reactivities and serum Ig levels in mice which simultaneously carry the xid and CD40 deficiency genes. *Int. immunol.* 8:1675-1685.

Osmond, D.G. (1991). Proliferation kinetics and the life span of B cells in central and peripheral lymphoid organs. *Curr. Opin. Immunol.* 3:179-185

Osmond, D.G., Rico-Vargas, S., Valenzona, H., Fauteux, L., Liu, L., Janani, R., Lu. L. and Jacobsen, K. (1994) Apoptosis and macrophage-mediated cell deletion in the regulation of B Lymphopoiesis in mouse bone marrow. *Immunol. Rev.* 142:209-230.

Rajewski, K. (1996) Clonal selection and learning in the antibody system. *Nature* 381:751-758.

Ramsden, D.A. and Wu, G.E. (1991). Mouse κ light chain recombination signal sequences mediate recombination more frequently than those of λ light chain. *Proc. Natl. Acad. Sci. USA* 88:10721-10725.

Renshaw, B.R., Fanslow III, W.C., Armitage, R.J., Campbell, K.A., Liggitt, D., Wright, B., Davidson, B.L. and Maliszewski, C.R. (1994) Humoral immune responses in CD40 ligand-deficient mice. *J. Exp. Med.* 180:1889-1900.

Rolink, A., Grawunder, U., Haasner, D., Strasser, A. and Melchers, F. (1993). Immature surface Ig⁺ B cells can continue to rearrange kappa and lambda L chain gene loci. *J. Exp. Med.* 178:1263-1270.

Roy, A.L., Du, H., Gregor, P.D., Novina, C.D., Martinez, E. and Roeder, R.G. (1997) Cloning of an Inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. *EMBO J.* 16:7091-7104.

Russell, D.M., Dembic, Z., Morahan, G., Miller, J.F.A.P., Bürki, K. and Nemazee, D. (1991) Peripheral deletion of self-reactive B cells. *Nature* 354:308-311.

Saouf, S.J., Mahajan, s., Rowley, R.B., Kut, S.A., Fargnoli, J., Burkhardt, A.L., Tsukada, S., Witte, O.N. and Bolen, J.B. (1994) Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc. Natl. Acad. Sci. USA* 91:9524-9528.

Sato, K., Mano, H., Ariyama, T., Inazawa, J., Yazaki, Y. and Hirai, H. (1994) Molecular cloning and analysis of the human Tec protein-tyrosine kinase. *Leukemia* 8:1663-1672.

Satterthwaite, A. and Witte, O. (1996) Genetic analysis of tyrosine kinase function in B cell development. *Annu. Rev. Immunol.* 14:131-154.

Schanke, J.T., Marcuzzi, A., Podzorski, R.P. and Van Ness, B. (1994) An AP1 binding site upstream of the kappa immunoglobulin intron enhancer binds inducible factors and contributes to expression. *Nucl. Acids Res.* 22:5425-5432.

Scher, I. (1982) The CBA/N mouse strain: An experimental model illustrating the influence of the X-chromosome on immunity. *Adv. Immunol.* 33:1-71.

Sideras, P. and Smith, C.I.E. (1995) Molecular and cellular aspects of X-linked agammaglobulinemia. *Adv. In Immunol.* 59:135-223.

Siliciano, J.D., Morrow, T.A. and Desiderio, S.V. (1992) *Itk*, a T-cell-specific tyrosine kinase inducible by interleukin-2. *Proc. Natl. Acad. Sci. USA.* 89:11194-11198.

Smith, C.I.E., Baskin, B., Humire-Greiff, P., Zhou, J-N., Olsson, P.G., Maniar, H.S., Kjellén, P., Lambris, J.D., Christensson, B., Hammarström, L., Bentley, D., Vetrie, D., Islam, K.B., Vorechovsky, I. and Sideras, P. (1994) Expression of Bruton's agammaglobulinemia tyrosine kinase gene, *Btk*, is selectively down-regulated in T lymphocytes and plasma cells. *J. Immunol.* 152:557-565.

Snapper, S.B., Rosen, F.S., Mizoguchi, E., Cohen, P., Khan, W, Liu, C-H., Hageman, T.L., Kwan, S-P., Ferrini, R., Davidson, L., Bhan, A.K. and Alt, F.W. (1998) Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* 9:81-91.

Sugamura, K., Asao, H., Kondo, M., Tanaka, N., Ishii, N., Ohbo, K., Nakamura, M. and Takeshita, T. (1996) The interleukin-2 receptor γ chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu. Rev. Immunol.* 14:179-205.

Takata, M. and Kurosaki, T. (1996) A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-γ2. *J. Exp. Med.* 184:31-40.

Takata, M., Homma, Y. and Kurosaki, Y. (1995) Requirement of phospholipase C-γ2 activation in surface IgM-induced B cell apoptosis. *J. Exp. Med.* 182:907-914.

Tiegs, S.L., Russell, D., and Nemazee, D. (1993). Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* 177:1009-1020.

Torres, R.M., Flaswinkel, H., Reth, M. and Rajewski, K. (1996). Aberant B cell development and immune responses in mice with a compromised BCR complex. *Science* 272:1804-1808.

Tsukada, S., Saffran, D.C., Rawlings, D.J., Parolini, O., Allen, R.C., Klisak, I., Sparkes, R.S., Kubagawa, H., Thuluvancheri, M., Quan, S., Belmont, J.W., Cooper, M.D., Conley, M.E. and Witte, O.N. (1993). Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 72:279-290.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L. and Tybulewicz, V.L. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 378:298-302.

Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C.I.E. and Bentley, D.R. (1993) The gene involved in X-linked agammaglobulinemia is a member of the *src* family of protein kinases. *Nature* 361:226-223.

Vihinen, M., Brandau, O., Brandén, O., Kwan, S-P., Lappalainen, I., Noordzij, J.G., Ochs, H.D., Ollila, J., Pienaar, S.M., Rukonen, P., Saha, B.K. and Smith, C.I.E. (1998) Btkbase, mutation database for X-linked agammaglobulinemia. *Nucl. Acid Res.* 1:244-250.

Wicker, L.S. and Scher, I. (1986) X-linked immune deficiency (*xid*) of CBA/N mice. *Curr. Top. Microbiol. Immunol.* 124:87-101.

Woodland, R.T., Schmidt, M.R., Koersmeyer, S.J. and Gravel, K.A. (1996). Regulation of B cell survival in *xid* mice by the proto-oncogene *bcl-2*. *J. Immunol.* **156**:2143-2154.

Yang, W-Y. and Desiderio, S. (1997) BAP-135, a target for Bruton's tyrosine kinase in response to B cell receptor engagement. *Proc. Natl. Acad. Sci. USA* **94**:604-609.

Summary

X-linked agammaglobulinemia (XLA) is one of the most frequent inherited immunodeficiency diseases in man and is characterized by severe, recurrent bacterial infections. Affected males manifest a dramatic decrease in mature B cell numbers and immunoglobulin (Ig) levels of all isotypes. Current treatment of XLA patients includes gammaglobulin substitution and antibiotics. XLA reflects an arrest in B cell differentiation at the pre-B cell stage. The gene defective in XLA has been identified to code for a cytoplasmic signaling protein, named *Bruton's tyrosine kinase* (Btk). In the mouse, inactivation of Btk results in a relative mild B cell disorder, *xid*, characterized by a reduction of mature $IgM^{low}IgD^{high}$ B cells in spleen and lymph nodes, lack of $CD5^+$ B-1 B cells and low serum levels of IgM and IgG3. In contrast to XLA patients, Btk-deficient mice generally make functional antibodies to proteins, however, they manifest impaired responses to T cell independent type II antigens. *Btk* is expressed throughout B cell development, except for plasma cells and is implicated in several receptor coupled signal transduction pathways, including the B cell antigen receptor (BCR).

To study the role of Btk in B cell differentiation *in vivo*, we have developed mouse models, in which wild-type or mutated human *Btk* are expressed as transgenes. The aim of the thesis was to investigate the role of Btk at the individual steps of B cell development, to answer the central question why B cell development is arrested at the level of the pre-B cell in XLA patients.

Chapter 1 is the general introduction and presents an overview of murine B cell development. Furthermore, identification and cloning of the *Btk* gene as well as clinical and molecular aspects of XLA and *xid* are described.

Chapter 2 describes the generation of a Btk-deficient mouse model by inactivating the *Btk* gene through an in-frame insertion of a *LacZ* reporter by homologous recombination in embryonic stem cells. The phenomenon of X-chromosome inactivation in $Btk^{+/-}$ heterozygous female mice enabled us to evaluate the competition between B cell progenitors expressing wild-type *Btk* and those expressing the *Btk/LacZ* allele in each successive step of development. Although *Btk* was already expressed in pro-B cells, the first selective disadvantage of Btk-deficient cells only became apparent at the transition from small pre-B cells to immature B cells in the bone marrow. A second differentiation arrest was found during the maturation from $IgM^{high}IgD^{low}$ to $IgM^{low}IgD^{high}$ stages in the periphery.

Chapter 3 describes the generation of two yeast artificial chromosome (YAC)-transgenic mouse strains in which high-level expression of human *Btk* was provided by endogenous regulatory *cis*-acting elements that are present on a 340-kb transgene, Yc340-h*Btk*, which contained the *Btk* gene and ~100 kb 5' flanking DNA and ~200 kb 3' flanking DNA. The expression pattern of the transgenic human *Btk* was found to parallel that of the endogenous murine gene. When the Yc340-h*Btk*-transgenic mice were mated onto a *Btk*-deficient background, all features of *xid* were corrected. We conclude that expression of human Btk can fully compensate for the absence of endogenous Btk in the mouse, and that in the YAC-transgenic mice *Btk* was appropriately expressed in the context of native regulatory sequences.

Chapter 4 describes the generation of yeast artificial chromosome (YAC) transgenic mouse strains in which the human *Btk* is expressed from a 240 kb YAC transgene Yc240-h*Btk*, containing the entire *Btk* gene and ~6 kb of 5' flanking DNA and ~200 kb of 3' flanking DNA. When the Yc240-h*Btk* transgenic mice were mated onto a Btk-deficient background, B cell development was fully restored. Using intracellular flow cytometry, the expression patterns and expression levels of the transgenic human *Btk* were found to parallel the endogenous murine *Btk* gene, indicating the conservation of Btk regulatory sequences between mice and men.

As a first step to identify *cis*-acting elements, involved in the regulation of expression of *Btk*, DNAseI hypersensitive site mapping (HSS) were performed within the *Btk* genomic region. Nine HSS were identified, one of which was found to be B cell specific and strongly conserved between mouse and man. Furthermore, we show that Sp1, Sp3 and PU.1 are able to transactivate synergistically the *Btk* promoter in *in vitro* transfection assays, while targeted deletion of Sp1 or Sp3 does not result in decreased *Btk* expression *in vivo*.

Chapter 5 describes the generation of transgenic mice, in which Btk expression was 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 *xid* phenotype in Btk⁻ mice. By 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*. We demonstrate that expression of constitutively activated Btk blocked the development of follicular recirculating B cells. The levels in the serum of most Ig subclasses decreased with age and *in vivo* B cell responses were essentially absent. Expression of the E41K Btk mutant enhanced blast formation of splenic B cells in response to anti-IgM stimulation *in vitro*.

Chapter 6 describes the generation of transgenic mice that express the E41K Btk mutant under the control of the human CD19 promoter. In these mice B cell development was arrested at the transition of IgM^{low} to IgM^{high}IgD⁻ immature B cells in the bone marrow, and the proportion of λ L chain expressing cells was increased. Conversely, Btk-deficient mice manifested a decrease in the proportion of λ expressing B cells. Together with the observed accumulation of Btk-deficient cells within the small pre-B cell stage, these results indicate a role for Btk in the developmental control of Ig L chain assembly at the transition from pre-B to immature B cells, coinciding with the arrest of B cell development in XLA patients.

Chapter 7 discusses the data presented in this thesis and gives an overview of the role of Btk in B cell development. Our findings in the mouse show that Btk is expressed throughout B cell development and that signaling cascades activated by Btk are critical at multiple checkpoints throughout B cell differentiation. When Btk is defective, the transition from pre-B cell into IgM⁺ immature cell stage is impaired and the proportion of λ⁺ cells is reduced. These findings indicate a role for Btk in re-expression of the recombination activating genes, that accomplish V(D)J recombination, the regulation of accessibility of Ig light (L) chain loci for recombination, or the survival of small pre-B cells that are in process of Ig L chain rearrangement. Expression of the CD19-h*Btk*^{E41K} transgene resulted in a lack of progression of immature B cells into the IgM^{high}IgD⁻ cell stage in the bone marrow, placing Btk in the BCR signaling pathway that eliminates auto-

reactive B cells. A similar role for Btk, but at a late stage in the periphery, was observed in mice expressing transgenic MHCII-hBtk^{E41K}, which resulted in a block in the development of recirculating follicular B cells. In the periphery, Btk is essential for B cell survival and maturation, indicating a role for Btk in basal BCR signaling needed to direct developmental progression of B cells. Finally, the phenotype of CD19-hBtk^{E41K} transgenic mice, provided evidence that Btk activation in peripheral B cells drives them into terminal IgM$^+$ plasma cell differentiation.

Samenvatting

X-gebonden agammaglobulinemia (XLA) is een van de meest voorkomende erfelijke immuundefeciëntie ziekten bij de mens en wordt gekenmerkt door ernstige, vaak terugkomende bacteriële infecties. In XLA patiënten is het aantal rijpe B cellen in perifeer bloed sterk verlaagd. Antistoffen van alle sub-klassen zijn nauwelijks detecteerbaar in het serum. De huidige behandeling van XLA patiënten bestaat vooral uit het toedienen van gammaglobuline en antibiotica. XLA wordt gekenmerkt door een blokkade in de B cel differentiatie op het pre-B cel niveau. Het gen dat defect is in XLA codeert voor een cytoplasmatisch signalering eiwit, *Bruton's tyrosine kinase* (Btk). In de muis, mutaties in het *Btk* gen resulteren in een relatief mild B cel defect, *xid*, gekenmerkt door een afname van rijpe immuunglobuline (Ig)-$M^{laag}IgD^{hoog}$ B cellen in milt en lymfeklieren, de afwezigheid van $CD5^+$ B-1 B cellen en een verlaging van de antistof sub-klassen IgM en IgG3 in het serum. Btk-deficiënte muizen maken, in tegenstelling tot XLA patiënten, normaliter wel functionele antistoffen tegen eiwitten, maar een respons tegen T cel onafhankelijke type 2 antigenen is afwezig. Het *Btk* gen komt gedurende de hele B cel ontwikkeling tot expressie, maar niet in plasma cellen. Het Btk eiwit maakt deel uit van signaaltransductie routes van verschillende receptoren, o.a. de B cel antigeen receptor (BCR).

Om de *in vivo* rol van Btk gedurende de B cel differentiatie te bestuderen hebben we transgene muizen modellen ontwikkeld, waarin wildtype of gemuteerd humaan *Btk* tot expressie wordt gebracht. Het doel van het onderzoek, beschreven in dit proefschrift, was het bestuderen van de rol van Btk gedurende de individuele stappen van de B cel differentiatie, om de centrale vraag, waarom in XLA patiënten de B cel ontwikkeling is geblokkeerd op het niveau van de pre-B cel, te beantwoorden.

Hoofdstuk 1 vormt de algemene inleiding en geeft een overzicht van de B cel ontwikkeling in muizen. Verder is in dit hoofdstuk de identificatie en klonering van Btk beschreven, alsmede de klinische and moleculaire aspecten van XLA en *xid*.

Hoofdstuk 2 beschrijft een Btk-deficiënt muizenmodel, dat werd verkregen door inactivatie van het *Btk* gen door middel van een in-frame insertie van een *LacZ* reporter gen door homologe recombinatie in embryonale stamcellen. Door het fenomeen van X-chromosoom inactivatie in $Btk^{+/-}$ heterozygote vrouwtjes muizen, waren we in staat de competitie tussen vroege B cellen die het wildtype *Btk* allel en B cellen die het *Btk/LacZ* allel tot expressie brachten te onderzoeken, gedurende elke opeenvolgende stap van de B cel ontwikkeling. Ondanks het feit dat *Btk* al in pro-B cellen tot expressie kwam, trad het eerste selectieve nadeel voor Btk-deficiënte B cellen op bij de overgang van het kleine pre-B cel stadium naar het immature B cel stadium in het beenmerg. Een tweede blokkade in de differentiatie werd gevonden tijdens de maturatie van het $IgM^{hoog}IgD^{laag}$ naar $IgM^{laag}IgD^{hoog}$ B cel stadium in de periferie.

Hoofdstuk 3 beschrijft de ontwikkeling van twee transgene muizen stammen, waarin de expressie van het humane *Btk* gen wordt verzorgd door regulerende elementen die aanwezig zijn op een 340-kb transgen. Dit transgen (Yc340-h*Btk*) bestond uit een 'yeast artificial chromosome' (YAC), met het *Btk* gen en ~100 kb DNA aan de 5' zijde en ~200 kb DNA aan de 3' zijde. Het

expressie patroon van het transgene humane *Btk* kwam overeen met dat van het endogene muize *Btk*. Na het inkruisen van de Yc340-h*Btk* transgene muizen op een *Btk*-deficiënte achtergrond, werd het *xid* B cel defect volledig gecorrigeerd. We concluderen dat expressie van humaan *Btk*, de afwezigheid van Btk in de muis kan compenseren en dat in de YAC-transgene muizen de regulerende elementen die aanwezig zijn op de 340-kb YAC zorgen voor een juiste expressie van het humane *Btk*.

Hoofdstuk 4 beschrijft de ontwikkeling van YAC-transgene muizen stammen waarin humaan *Btk* werd geëxpresseerd door een 240 kb YAC transgen, Yc240-h*Btk*, dat het hele *Btk* gen bevat en ~6 kb 5' DNA en ~200 kb 3' DNA. Het inkruisen van de Yc240-h*Btk* transgen op een Btk-deficiënte achtergrond herstelde de B cel ontwikkeling volledig. Met behulp van intracellular flow cytometrie werd gevonden dat de expressie patronen en expressie niveaus van het transgene humane *Btk* overeen kwamen met die van het endogene muizen *Btk* gen.

Als eerste stap in de identificatie van regulerende elementen, betrokken bij de regulatie van *Btk* expressie, werden 'DNAseI hypersensitive sites' (HSS) in kaart gebracht. Er werden negen HSS gevonden, waarvan er een B cel specifiek bleek te zijn en geconserveerd binnen muis en mens. Verder laten we zien in *in vitro* experimenten, dat de transcriptiefactoren Sp1, Sp3 en PU.1 samen in staat waren de *Btk* promoter additief te activeren, maar dat de afwezigheid van Sp1 of Sp3, door specifieke inactivatie van het desbetreffende gen, niet resulteerde in vermindering van *Btk* expressie niveaus *in vivo*.

Hoofdstuk 5 beschrijft de ontwikkeling van transgene muizen, waarin *Btk* expressie wordt gereguleerd door de 'Major Histocompatibility Cluster' (MHC) class II Ea gen locus control region. Overexpressie van Btk had geen significante effecten op de functie van B cellen en corrigeerde in essentie het *xid* fenotype. Expressie van een E41K gemuteerde vorm van Btk, resulterend in constitutieve activatie, leidde tot een B cel defect dat ernstiger was dan *xid*. In deze transgene muizen was het B cel compartiment in de milt, lymfknoop, perifeer bloed en in de buikholte, sterk gereduceerd. De serum concentraties van de meeste antistof sub-klassen gingen omlaag naarmate de muize ouder werden en *in vivo* B cel waren in essentie afwezig. Onze resultaten laten zien dat de ontwikkeling van folliculaire recirculerende B cellen werd geblokkeerd door de expressie van constitutief actief Btk. Expressie van de E41K gemuteerde Btk verhoogde de vorming van B cel blasten cellen van B cellen uit de milt na anti-IgM stimulatie *in vitro*.

Hoofdstuk 6 beschrijft de ontwikkeling van transgene muizen waarin expressie van het E41K gemuteerde Btk wordt gereguleerd door de humane CD19 promoter. In deze muizen trad een blokkade op in de differentiatie van het IgM^{laag} naar het IgM^{hoog}IgD$^-$ immature B cel stadium in het beenmerg. De fractie van Ig λ lichte keten expresserende cellen was verhoogd, in tegenstelling tot Btk-deficiënte muizen, waarin deze fractie juist was verlaagd. Samen met de waargenomen opeenhoping van Btk$^-$ cellen binnen het kleine pre-B cel stadium suggereren deze bevindingen dat Btk is betrokken bij de regulatie van Ig lichte keten assemblage tijdens de differentiatie van het pre-B naar het immature B cel stadium, samenvallend met de blokkade in de B cel ontwikkeling in XLA patiënten.

Hoofdstuk 7 bediscussieert de gepresenteerde resultaten van dit proefschrift en geeft een overzicht van de rol van Btk in de B cel ontwikkeling. Onze resultaten in de muis geven aan dat Btk tot expressie komt gedurende de hele B cel ontwikkeling en dat signalering cascades, geactiveerd door Btk, kritiek zijn op verscheidende controle punten in de B cel differentiatie. Wanneer Btk defect is, is de overgang van het kleine pre-B cel stadium naar de IgM$^+$ immature B cel stadium gestoord en het percentage Ig λ, lichte keten expresserende cellen significant verlaagd. Dit betekent dat Btk betrokken zou kunnen zijn bij her-expressie van de recombinatie activerende genen, die verantwoordelijk zijn voor de recombinatie van Ig gen segmenten, de regulatie in toegangkelijkheid van de Ig lichte keten gen segmenten voor het recombinase complex, of de overleving van kleine pre-B cellen, die bezig zijn met Ig L keten recombinatie. Expressie van het CD19-hBtk^{E41K} transgen resulteerde in een blokkade in de progressie naar het IgM^{boog}IgD$^-$ immature B cel stadium. Dit geeft aan dat Btk een functie heeft in de BCR signalering route die verantwoordelijk is voor de eliminatie van auto-reactieve B cellen. Een vergelijkbare rol voor Btk, maar in een later stadium van de B cel ontwikkeling, werd gevonden in muizen die het MHCII-hBtk^{E41K} transgen tot expressie brachten, hetgeen resulteerde in een blokkade in de ontwikkeling van recirculerende folliculaire B cellen. In de periferie blijkt Btk essentieel voor B cel overleving en maturatie, hetgeen betekent dat Btk betrokken is bij de basale BCR signalering, die essentieel is voor het sturen van de B cel ontwikkeling. Tenslotte geeft het fenotype van de CD19-hBtk^{E41K} transgene muizen aan dat activatie van Btk in perifere B cellen de differentiatie naar IgM$^+$ plasma cellen stimuleert.

Abbreviations

Ab	Antibody
Bp	Basepair
BCR	B cell receptor
BM	Bone marrow
Btk	Bruton's tyrosine kinase
C	Constant
CD	Cluster of designation
CDR	Complementary determining region
cM	Centimorgan
CRAC	Calcium release activated calcium
CREB	cAMP response element binding protein
D	Diversity
DAG	Diacylglycerol
DNP	2,4-dinitrophenyl
ER	Endoplasmic reticulum
FDC	Follicular dentritc cell
FISH	Fluorenscense in situ hybridization
FITC	Fluorescein isothiocyanate
FR	Framework region
GC	Germinal center
H	Heavy
h*Btk*	Human *Btk*
Ig	Immunoglobulin
IL	Interleukin
IP$_3$	Inositol 1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
J	Joining
Kb	Kilobase
KDa	Kilodalton
Kde	κ deleting element
KLH	Keyhole limpet hemocyanin
L	Light
LCR	Locus control region
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
Mb	Megabase
m*Btk*	murine *Btk*

MHC	Major histocompatibility complex
NK	Natural killer
PD	Properdin deficiency
PH	Pleckstrin homology
PI3-K	Phospho-inositol-3 kinase
PI	Phosphatidylinositol
PLC	Phospholipase C
PKC	Protein kinase C
PTK	Protein tyrosine kinase
RAG	Recombination activating gene
RFLP	Restiction frangment lenght polymorphism
RSS	Recombination signal sequence
SH	Src-homology
SHIP	SH2 containing inositol 5'-phosphatase
Sos	Son of Sevenless
SRE	Serum response element
TCR	T cell receptor
TD	Thymus dependent
TH	Tec homology
T$_H$	T helper cell
TI-I	Thymus independent class I
TI-II	Thymus independent class II
TNP	2,4,6-trinitrobenzenesulfonic acid
UTR	Untranslated region
V	Variable
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
X-CGD	X-linked chronic granulomatous disease
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia
XLP	X-linked lymphoproliferative syndrome
XSCID	X-linked severe combined immunodeficiency

Dankwoord

Het onderzoek, beschreven in dit proefschrift, is niet het werk van een persoon, maar reflecteert de gezamenlijke inspanningen van velen. Een aantal mensen zijn hierbij heel belangrijk geweest en die wil ik dan ook bij naam noemen.

Allereerst Rudi, jou wil ik bedanken voor de enthousiaste manier waarop je het onderzoek geleid hebt, het vele dat ik van je geleerd heb en de prettige manier van samenwerken. Frank, jou wil ik bedanken voor de mogelijkheid om in je lab te mogen werken en de vele no-nonsense discussies tijdens de werkbesprekingen, al gingen deze ook vaak niet over het werk. Gemma, allereerts bedankt voor de vele experimenten, die je van me overgenomen hebt (soms ook saaie, waar ik weining zin in had) en voor het paranimf zijn. Dan zijn er nog de (oud)-bewoners van Lab 706; Dubi (het is weer 'terrasjesweer'), Rick (ondanks de 'tegenwerking'), Jacky (thanks for all the solutions, they were of perfect quality!) en Nikos (mr. YAC), bedankt voor gezelligheid, geduld en de vaak goede sfeer in het lab. Verder, Sylvia en DJ bedankt voor de 'koffie-pauzes' en het gezeur dat jullie vaak aan hebben moeten horen. Verder wil ik Lien, Tjeerd en Daniëlle van het "muizenhuis" bedanken voor de prettige samenwerking.

Ook wil ik een aantal mensen bedanken voor de steun en gezelligheid buiten het lab. Ard, Cuno (mijn tweede paranimf) en Severene, dankzij jullie ben ik toch heelhuids het afgelopen jaar doorgekomen (livin' on the edge?).

Als laatste wil ik mijn naaste familie, Manfred en Inge en mijn ouders bedanken voor hun onvoorwaardelijk vertrouwen en steun en voor het vormen van een 'vangnet' wanneer ik dat nodig heb, al doet een simpel dankjewel jullie veel te kort.

Alex

Curriculum Vitae

De schrijver van dit proefschrift werd op 3 maart 1970 geboren te Maarheeze (N-Br). In 1988 behaalde hij het antheneum-b diploma op het Bisschoppelijk College te Weert. Na een jaar chemische technologie aan de Technische Universiteit Eindhoven werd in 1989 aangevangen met de studie Biologie aan de Universteit Utrecht. In 1994 behaalde hij het doctoraal diploma met als hoofdvak: moleculaire microbiologie (prof. Hoekstra) en bijvak immunohaematologie (prof. De Gast). Zijn promotie-onderzoek (1994-1998) heeft hij verricht onder begeleiding van Dr. R.W. Hendriks en prof. F. Grosveld, op het project 'Studies on the function of *Bruton's tyrosine kinase* in B cell development' aan de Erasmus Universiteit Rotterdam bij de vakgoep Celbiologie en Genetica.

Na zijn promotie gaat hij werken als postdoc bij de Human-Genetics Unit van het Molecular Medicine Centre (University of Edinburgh) onder begeleiding van Dr. J. Nasir.

Publications

Pettersson, A., Maas, A. and Tommassen, J. (1994). Identification of the iroA gene product of Neisseria meningitidis as a lactoferrin receptor. *J. Bacteriol.* **176**:1764-1766.

Pettersson, A., Maas, A., van Wassenaar, D., van der Ley, P. and Tommassen, J. (1995). Molecular charactirization of FrpB, the 70-kilodalton iron-regulated outer membrane protein of Neisseria meningitidis. *Infect. Immun.* **63**:4181-4181.

Hendriks, R.W., de Bruijn, M.F.T.R., Maas, A., Dingjan, G.M., Karis, A. and Grosveld, F. (1996). Inactivation of *Btk* by insertion of *LacZ* reveals defects in B cell development only past the pre-B cell stage. *EMBO J.* **15**:4862-4872.

Maas, A., Dingjan, G.M., Savelkoul, H.F.J, Kinnon, C., Grosveld, F. and Hendriks, R.W. (1997). The X-linked immunodeficiency defect in the mouse is corrected by expression of human Bruton's tyrosine kinase from a yeast artificial chromosome transgene. *Eur. J. Immunol.* **27**:2180-2187.

Dingjan, G.M., Maas, A., Nawijn, M.C., Smit, L., Voerman, J.S., Grosveld, F. and Hendriks, R.W. (1998). Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. *EMBO J.* **17**:5309-5320.

Müller, S., Maas, A., Islam, T.C., Sideras, P., Suske, G., Philipsen, S., Xanthopoulos, K.G., Hendriks, R.W. and Smith, C.I.E. (1998). Synergistic activation of the human Btk promoter by transcription factors Sp1/3 and PU.1. *submitted.*

Maas, A., Dingjan, G.M., Grosveld, F. and Hendriks, R.W. (1998). A role for Btk in the developmental control of immunoglobulin light chain assembly at the pre-B to immature B cell transition. *submitted.*