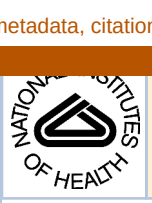

Published in final edited form as:

Mol Immunol. 2007 April ; 44(10): 2719–2728. doi:10.1016/j.molimm.2006.11.023.

Btk Regulates Multiple Stages in the Development and Survival of B-1 Cells

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Abstract

B-1 cells are important players in the first line of defense against pathogens. According to current models for the origin of B-1 cells, they either represent a separate lineage from conventional B-2 cells or differentiate from conventional B-2 cells via an intermediate, B-1^{int}, in response to positive selection by antigen. Here we show that Btk, a Tec family kinase that mediates B cell antigen receptor (BCR) signaling, is required at multiple stages of B-1 cell development. VH12 anti-phosphatidylcholine (PtC) IgH transgenic mice provide a model for the induced differentiation of B-1 cells. This transgene selects for PtC-reactive cells and induces them to adopt a B-1 phenotype. Both processes have been shown to depend on Btk. To determine whether this is secondary to a requirement for Btk in the development of mature B-2 cells, we crossed VH12 transgenic mice to mice expressing low levels of Btk. B-2 cell development occurs normally in Btk^{lo} mice despite reduced responsiveness to BCR crosslinking. Analysis of VH12.Btk^{lo} mice reveals that Btk regulates the B-1^{int} to B-1 transition and/or the survival of splenic B-1 cells, in part via a mechanism independent of its role in BCR signaling. We also show that Btk mediates the survival of, and expression of IL-10 by, those B-1 cells that do develop and migrate to the peritoneum. Multiple roles for Btk in B-1 cell development and maintenance may explain the particular sensitivity of this population to mutations in components of Btk signaling pathways.

Keywords

B cell development; BCR signaling; Lyn; IL-10; phosphatidylcholine

Introduction

There are two major subsets of B lymphocytes in mice, conventional B-2 cells and B-1 cells. B-1 cells are less abundant than B-2 cells and have a restricted repertoire that is skewed towards bacterial and self antigens. They are located predominantly in the peritoneal cavity and display unique cell surface phenotypes and differential regulation of signaling pathways relative to

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B-2 cells (Martin and Kearney, 2001, Hardy and Hayakawa, 2001, Wortis and Berland, 2001, Rothstein, 2002). B-1 cells are important in the first line of defense against pathogens. They produce the majority of natural IgM, which is critical in the early phase of the response to bacterial, viral, and parasitic infections (Paciorkowski *et al*., 2000, Martin *et al*., 2001, Baumgarth *et al*., 2000). They also give rise to mucosal IgA secreting plasma cells (Kroese *et al*., 1989), which are involved in neutralizing pathogens as they enter the organism. B-1 cells have been implicated as a source of autoantibodies in some models of autoimmune disease (Murakami *et al*., 1995), although this is not always the case (Reap *et al*., 1993).

The origin of B-1 cells is controversial. The lineage hypothesis states that B-1 and B-2 cells represent separate lineages derived independently from hematopoietic stem cells. Evidence for this model comes predominantly from bone marrow transplant studies. Adult bone marrow gives rise to B-2, but not B-1, cells when transferred to irradiated hosts. In contrast, fetal or neonatal liver is rich in transplantable progenitors for B-1 cells (Hayakawa *et al*., 1985). Consistent with this observation, fetal liver B cell precursors express reduced levels of TdT relative to adult bone marrow, and antibodies produced by B-1 cells tend to have fewer N regions (Li *et al*., 1993). Indeed, a progenitor which gives rise to B-1, but not B-2, cells, was recently discovered in both fetal and adult bone marrow (Montecino-Rodriguez *et al*., 2006).

Several lines of evidence indicate that B-1 cells can also be induced to differentiate in response to signals from the B cell antigen receptor (BCR). Immunoglobulin (Ig) transgenes derived from B-1 cells force the majority of B cells expressing them to adopt a B-1 phenotype (Arnold *et al*., 1994, Lam and Rajewsky, 1999, Chumley *et al*., 2000, Hayakawa *et al*., 1999, Watanabe *et al*., 1999). When only the Ig heavy chain is provided, strong antigen-dependent positive selection for cells expressing light chains conferring reactivity to the original antigen is observed (Arnold *et al*., 1994, Lam and Rajewsky, 1999, Chumley *et al*., 2000, Hayakawa *et al*., 1999, Watanabe *et al*., 1999). Adoptive transfer experiments in the VH12/Vk4 anti-phosphatidylcholine (PtC) model provide evidence that B-2 cells can differentiate into B-1 cells via an intermediate population, B-1^{int} (Arnold *et al*., 2000). BCR specificity is thus an important regulator of B-1 cell development, suggesting a reason for the restricted repertoire in this population.

Second, there is a strong correlation between BCR signal strength and B-1 cell numbers. Increased expression levels of B-1 derived Ig transgenes leads to increased numbers of B-1 cells (Watanabe *et al*., 1999). In contrast, B-1 favorable Ig transgenes do not force a B-1 phenotype when signals from this BCR are “diluted” by simultaneous expression of a B-2 favorable BCR (1 Watanabe *et al*., 1999). Mice carrying mutations that reduce BCR signaling thresholds (Lyn-/-, me, CD22-/-, PD-1-/-, CD72-/-, CD19 transgene) have increased numbers of B-1 cells (Hardy and Hayakawa, 2001). Conversely, both the transmission of BCR signals and the development of B-1 cells are impaired by loss of function of CD19, BLNK, CD21, vav, p85α, PLCγ2, cyclin D2, oct-2, or Btk (Hardy and Hayakawa, 2001).

Btk is a Tec family kinase that mediates many aspects of B cell development and function (Desiderio, 1997, Satterthwaite and Witte, 2000, Khan, 2001), including transmitting BCR signals. Btk-/- mice and xid mice, which have a point mutation in Btk, have a block at the T2 to mature transition in the B-2 population (Loder *et al*., 1999). B-1 cells are also significantly reduced in these mice (Satterthwaite *et al*., 1997). Previous studies (Clarke and Arnold, 1998) employed VH12 anti-PtC IgH transgenic mice (subsequently referred to as 6-1), a model of induced differentiation of B-1 cells, to define which stages of B-1 cell development require Btk. Clonal expansion and differentiation of anti-PtC B cells beyond B-2 were impaired in 6-1.xid mice (Clarke and Arnold, 1998). Since xid B cells fail to mature efficiently beyond the transitional stage, it is possible that the impaired B-1 cell differentation stems from reduced numbers of mature B-2 cells rather than an inability of mature B-2 cells to adopt a B-1

phenotype. We have previously shown that a transgene expressing 25% of endogenous Btk levels (Btk^{lo}) restores mature B-2 numbers to normal in Btk-/- and xid mice (Satterthwaite *et al*., 1997). However, these cells have reduced, but not completely impaired, responses to BCR crosslinking.

Therefore, we crossed 6-1 transgenic mice to Btk^{lo} mice to determine how Btk regulates stages of B-1 cell development subsequent to the generation of mature B-2 cells. We now demonstrate previously unappreciated roles for Btk in the transition from $B\text{-}1^{int}$ to B-1 cells and/or the survival of differentiated B-1 cells. We also show that efficient differentiation or survival of cells beyond the $B\text{-}1^{int}$ stage requires Btk-mediated, BCR-independent signals.

Methods

Mice

6-1 mice (IgMa) (Arnold *et al.*, 1994) were maintained on a CB17 (IgMb) background and crossed either with Btk^{lo} (Btk-/- + Btk tg) mice (Satterthwaite *et al*., 1997) maintained on a defined C57BL/6 (IgMb) background or Btk^{lo} or Lyn-/-Btk^{lo} (Satterthwaite *et al.*, 1998) mice maintained on a mixed C57BL/6 (IgMb) × 129 (IgMa) background. Mixed background mice used for breeding were screened by flow cytometry of peripheral blood for expression of IgMb but not IgMa since 129 mice carry the IgMa allele. This ensured that the presence of IgMa in the progeny was due to expression of the 6-1 transgene. To minimize potential variability due to mixed genetic backgrounds, littermate controls were used whenever possible. Multiple independent litters were analyzed with similar results. Mice were genotyped by PCR as described (Arnold *et al.*, 1994, Satterthwaite *et al*., 1998).

Flow cytometry

Single cell suspensions of spleen, bone marrow, or peritoneal wash were depleted of red blood cells and stained with combinations of the following reagents: FITC-encapsulated liposomes (Arnold *et al.*, 1994), anti-IgMa FITC, anti-IgMb FITC, anti-IgM PE, anti-CD5 PE, anti-CD43 PE, anti-CD80 PE, anti-B220 PerCP, anti-CD5 PerCP, anti CD23-biotin + streptavidin-APC (Caltag, Burlingame, CA). Unless otherwise noted, antibodies are from BD Pharmingen (San Diego, CA). Samples were run on a Becton-Dickinson (San Jose, CA) FACSCalibur and analyzed with Cellquest (Becton-Dickinson, San Jose, CA) software. Live cells were gated based on forward and side scatter.

Survival Assay

Peritoneal cells from pools of several mice per genotype were depleted of red blood cells and B cells purified by one of two methods: 1) B220+ cells were purified with anti-B220 magnetic beads using the MidiMACS system (Miltenyi Biotec, Auburn, CA). 2) Cells were stained with anti-B220 TriColor (Caltag) and anti-CD5 PE (BD Pharmingen). B220+CD5+ cells were isolated with a MoFlo high-speed sorter (DakoCytomation, Fort Collins, CO). Cells purified by either method were resuspended in complete RPMI media at 10^6 per ml and plated in 96 well plates. The number of viable cells remaining per well (3 wells per time point) was determined by trypan blue exclusion at days 2, 4, and 7.

IL-10 ELISA

Peritoneal cells were depleted of red blood cells, resuspended in complete RPMI media at 2×10^6 per ml and plated in 96 well plates. After 3 days, culture supernatants were harvested and subjected to an IL-10 ELISA (BD Pharmingen).

Real time PCR

Peritoneal cells from pools of several mice per genotype were depleted of red blood cells and B220+ cells were purified from with anti-B220 magnetic beads using the MidiMACS system (Miltenyi Biotec). Total RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA). cDNA was generated with a cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI 7300 Real Time PCR System (Applied Biosystems) using TaqMan reagents (Applied Biosystems) specific for mouse IL-10 and the internal control GAPDH. Data were normalized to GAPDH using the delta Ct method.

Light chain repertoire determination

Splenocytes were depleted of red blood cells and stained with FITC-encapsulated liposomes (Arnold *et al.*, 1994) and anti-CD23 PE (BD Pharmingen). CD23+ liposome- cells were isolated with a MoFlo high-speed sorter (Cytomation). RNA was prepared using Trizol reagent (Life Technologies, Carlsbad, CA). 5'RACE (rapid amplification of cDNA ends; Version 2.0; Life Technologies) was used to amplify Vk light chains as described in Tatu et al (27) with the following modifications. Primer sequences were identical, except those used for PCR did not contain CAU repeats. Amplification products were cloned into the pGEM-T Easy vector (Promega, Madison, WI). Randomly picked colonies were subjected to cycle sequencing with BigDye Terminator chemistry (Applied Biosystems) and an ABI Prism 377 automated sequencer (Applied Biosystems). The GSP2 primer (used for PCR) (Tatu *et al*., 1999) was also used for sequencing. Sequences were analyzed using Ig BLAST (GenBank).

Statistical Analysis

Two-tailed Student's t test was employed.

Results

Btk^{lo} Transgene Partially Restores anti-PtC B Cells in 6-1.Btk-/- Mice

To determine how changes in Btk dosage affect the clonal expansion of PtC-specific B cells, the frequency of cells binding PtC-containing liposomes was assessed in the spleens of 6-1, 6-1.Btk^{lo}, and 6-1.Btk-/- mice (Figure 1a). On average, 17% of total splenocytes bound liposomes in 6-1 mice. In contrast, 6-1.Btk-/- mice had very few detectable anti-PtC cells (1% of total splenocytes, $p = .0003$ vs. 6-1). This is consistent with previous reports describing 6-1.xid mice (Clarke and Arnold, 1998). The Btk^{lo} transgene partially restored this defect. Liposome binding B cells were present at an average of 9% of total splenocytes in 6-1.Btk^{lo} mice ($p = .0007$ vs. 6-1.Btk-/-). However, this was still significantly lower than in 6-1 mice ($p = .02$). The incomplete restoration of PtC reactive B cells was not due to a nonspecific inhibitory effect of the Btk transgene on clonal expansion, because 6-1 mice expressing both the Btk transgene and the wild type endogenous Btk gene had normal numbers of anti-PtC B cells (data not shown).

Btk regulates the $B\text{-}1^{int}$ to B-1 transition

Previous studies have suggested a model in which anti-PtC B cells differentiate in the spleen as follows: B-2 ($CD23^+CD5^-CD43^-$) to $B\text{-}1^{int}$ ($CD23^+CD5^+CD43^+$) to B-1 ($CD23^-CD5^+CD43^+$) (Arnold *et al*., 1994, Arnold *et al*., 2000, Clarke and Arnold, 1998). Cyclosporin, an inhibitor of the Ca++ dependent phosphatase calcineurin, prevents the latter step. Since Btk regulates BCR-induced Ca++ flux (Rigley *et al*., 1989, Fluckiger *et al.*, 1998), we tested whether the $B\text{-}1^{int}$ to B-1 transition was affected by reduced Btk dosage. We used both CD5 and CD43 as independent markers of differentiation as not all B-1 cells express CD5. anti-PtC B cells were skewed towards earlier steps in differentiation in 6-1.Btk^{lo} mice, with increased frequencies of B-2 cells ($p < .003$) and fewer B-1 cells ($p < .01$) as measured

by either CD5 or CD43 expression (Figure 1b). Taking into account the reduced frequency of liposome binding cells, the total number of anti-PtC B-2 and B-1^{int} cells were normal in 6-1.Btk^{lo} mice while anti-PtC B-1 cells were significantly decreased ($p < .05$) (Figure 1c). Thus, we demonstrate here that in addition to its previously appreciated roles in the clonal expansion of anti-PtC B cells and their differentiation beyond B2 (Clarke and Arnold, 1998), Btk also controls the B-1^{int} to B-1 transition and/or survival of fully differentiated B-1 cells. This latter stage is significantly more sensitive than earlier events to subtle changes in Btk signal strength.

Efficient BCR Signaling is Not Sufficient for Normal B-1 Cell Development

To determine the degree to which PtC-reactive B cells from 6-1.Btk^{lo} mice have been stimulated with antigen in vivo, we measured CD80 expression on B cells from the spleens of 6-1 and 6-1.Btk^{lo} mice. anti-PtC cells in other Ig transgenic models express high levels of CD80, indicative of previous antigen exposure (Chumley *et al*., 2002). Similarly, almost all (87 +/- 3%, n=5) liposome-binding cells in 6-1 spleens were CD80+ (Figure 2a). 62 +/- 9% (n=5) of anti-PtC B cells in 6-1.Btk^{lo} spleens expressed CD80. These cells had a B-1^{int} (CD23 +CD5+) or B-1 (CD23-CD5+) phenotype, while the CD80- anti-PtC cells were CD23+CD5- B-2 cells (Figure 2b). Thus, antigen encounter, as measured by CD80 upregulation, is associated with differentiation beyond B-2 but is not sufficient to efficiently mediate the B-1^{int} to B-1 transition.

To further explore the hypothesis that the B-1^{int} to B-1 transition is regulated in part by Btk-dependent, BCR-independent signals, we compared B-1 cell development in the spleens of 6-1.Lyn-/- and 6-1.Lyn-/-Btk^{lo} mice. Lyn deficiency restores the ability of Btk^{lo} B cells to signal through the BCR such that Lyn-/- and Lyn-/-Btk^{lo} B cells are equally sensitive to BCR stimulation as measured by proliferation, Ca++ flux, and ERK and Akt activation (Satterthwaite *et al*., 1998, Whyburn *et al*., 2003). However, 6-1.Lyn-/-Btk^{lo} mice had a reduced frequency of liposome-binding B cells (Figure 3a) and a significant skewing of anti-PtC B cells towards earlier stages of differentiation (B-2, $p < .003$; B-1^{int}, $p < .007$; B-1, $p < .005$) as measured by both CD5 and CD43 expression (Figure 3b) compared to 6-1.Lyn-/- mice. This resulted in a selective reduction in the absolute number of PtC-reactive B-1 cells (Figure 3c). Although we cannot rule out the possibility that there are unidentified differences in BCR responsiveness between Lyn-/- and Lyn-/-Btk^{lo} B cells, these results strongly suggest that differentiation of B-1 cells beyond B-1^{int} and/or the survival of differentiated B-1 cells is mediated in part by Btk via BCR-independent signals.

Btk Regulates the Survival of Peritoneal B-1 Cells

We examined the consequences of the reduction in splenic B-1 cells for the peritoneal B cell compartment of 6-1.Btk^{lo} mice. The total number of peritoneal cells (4.5×10^6 +/- 1.9×10^6, n = 5) was reduced compared to 6-1 mice (12.4×10^6 +/- 4.8×10^6, n = 5) ($p = 0.03$). However, there was a relatively normal frequency of anti-PtC B cells (Figure 4a), the majority of which had a B-1 phenotype (Figure 4b). In contrast, 6-1.Btk-/- mice had few liposome-binding peritoneal B cells (Figure 4a).

B-1 cells normally have a distinct survival advantage over B-2 cells (Chumley *et al*., 2000, Wong *et al*., 2002). The presence of B-1 cells in the peritoneum of 6-1.Btk^{lo} mice allowed us to ask whether Btk regulates the survival of these cells. Since the vast majority of peritoneal B220+ cells have a B-1 phenotype in both 6-1 and 6-1.Btk^{lo} mice (Figure 4b), we isolated peritoneal B-1 cells with anti-B220 magnetic beads. Results were then confirmed by sorting B220+CD5+ cells. These approaches were used to avoid stimulating the cells through the BCR by isolating them via their ability to bind liposomes. With either method, 6-1 Btk^{lo} cells

survived poorly relative to 6-1 cells during one week of culture in media alone (Figure 4c) ($p = .008$ at day 4, $p = .02$ at day 7).

Autocrine production of IL-10 has been shown to mediate the survival of peritoneal B-1 cells (O'Garra *et al*., 1992, Ishida *et al*., 1992, Gary-Gouy *et al*., 2002). We therefore examined expression of IL-10 using real time PCR. A significant reduction ($p = .01$) in IL-10 mRNA expression was observed in peritoneal B-1 cells isolated from 6-1.Btk^{lo} mice compared to 6-1 mice (Figure 4d). Consistent with this observation, peritoneal cells from 6-1.Btk^{lo} mice secreted less IL-10 than their Btk sufficient counterparts (Figure 4e).

Increased Number of non-PtC Reactive CD23+ B Cells in 6-1.Btk^{lo} Mice

A dramatic increase in the frequency (Figure 5a) and total number ($4.3 +/- 1.5 \times 10^6$ vs. $1.4 +/- 0.6 \times 10^6$, $n = 13$, $p = .000016$) of non-PtC reactive (liposome-) CD23+ B cells was observed in the spleens of 6-1.Btk^{lo} mice relative to 6-1 mice. This was not due to an overall increase in the number of B220+ cells but rather a skewing away from reactivity to PtC (Figure 1). These CD23+ liposome- cells still expressed the VH12 transgene as indicated by IgM^a staining (Figure 5a). The CD23+ population of B cells is composed of T2 and mature cells. Of these, only mature B cells enter the long-lived pool (Loder *et al.*, 1999). Thus, an increased frequency of mature cells within the CD23+liposome- population might account for the increased numbers in 6-1.Btk^{lo} mice. This was not the case however as both mice had similar frequencies of T2 and mature cells (Figure 5b).

The relatively limited repertoire of light chains that pair with the VH12 heavy chain results in a reduced number of immature B cells in the bone marrow of 6-1 mice (Tatu *et al.*, 1999). The skewing towards non-PtC reactive B cells in 6-1 Btk^{lo} spleens suggested that this checkpoint may be relaxed upon reduced Btk signaling. However, there was no significant difference in bone marrow B cell populations between 6-1 and 6-1.Btk^{lo} mice, and both strains had fewer IgM+ B cells than wild type mice (Figure 5c). This indicates that restriction of the light chain repertoire in 6-1 mice is independent of Btk signal strength.

To directly address this hypothesis, we compared the light chains expressed by CD23+ liposome- cells in the spleens of 6-1 and 6-1.Btk^{lo} mice (Figure 5d). We did not observe a dramatic overall shift in the light chain repertoire in the context of reduced Btk dosage. If anything,Vk4/5, which can confer PtC reactivity to VH12 (Tatu *et al*., 1999), was present at a slightly increased frequency in CD23+liposome- cells from 6-1.Btk^{lo} mice. Thus, the increased in non-PtC-reactive cells in 6-1.Btk^{lo} mice is not due to either an overall shift in the light chain repertoire or selection against light chains that have the potential to contribute to an anti-PtC antibody.

Discussion

Btk is critical for the development of B-1 cells. The 6-1 transgene has been used to show that clonal expansion of PtC-specific B cells and their differentiation beyond B-2 is impaired in the spleens of xid (Clarke and Arnold, 1998) and Btk-/- mice (Figure 1). When a low level of Btk is provided to B cells in 6-1.Btk-/- mice, normal numbers of PtC-reactive B-2 and B-1^{int} cells are restored. The frequency and total number of anti-PtC B-1 cells remains dramatically reduced, however, revealing an additional role for Btk in either the transition from B-1^{int} to B-1 cells or the survival of differentiated B-1 cells. These observations provide genetic evidence to support the previously proposed model, based on adoptive transfer studies, that splenic B-1^{int} cells can give rise to splenic B-1 cells (Arnold *et al*., 2000). This developmental transition is significantly more sensitive to changes in Btk signal strength than is differentiation from B-2 to B-1^{int}, which requires Btk (Clarke and Arnold, 1998) but occurs normally in 6-1.Btk^{lo} mice.

Despite the relatively severe reduction in the number of anti-PtC B-1 cells in the spleen, peritoneal anti-PtC B cells from 6-1.Btk^{lo} mice have a B-1 cell surface phenotype. Splenic B-1 cells, but not B-2 cells, home to the peritoneum in response to CXCL13 (Chumley *et al*., 2002). Thus, it is possible that the limited number of B-1 cells produced in the spleens of 6-1.Btk^{lo} mice localize normally to the peritoneum. Alternatively, the peritoneal B-1 cells in 6-1.Btk^{lo} mice may derive from B-1 specific progenitors in the bone marrow (Montecino-Rodriguez *et al*., 2006) rather than from splenic B-1^{int} cells. Regardless of their origin, these cells survive poorly compared to their Btk-sufficient counterparts and express reduced levels of the survival factor IL-10 (Figure 4). Since Btk has also been implicated in the response of B cells to IL-10 (Go *et al*., 1990), low levels of this cytokine that may still be produced would be poorly effective. Additional pro-survival mechanisms may also be impaired in the context of reduced Btk dosage. The combination of reduced input from the spleen and/or bone marrow and poor survival of those cells that do reach the peritoneum would lead to the lower absolute number of peritoneal B-1 cells in 6-1.Btk^{lo} mice. Without the 6-1 transgene to focus the repertoire towards "B-1 favorable" BCR specificities, these two defects would render the B-1 population virtually undetectable in Btk^{lo} mice, as we have observed previously (Satterthwaite *et al.*, 1997, Whyburn *et al*., 2003).

The correlation between response to BCR stimulation and B-1 cell numbers (Hardy and Hayakawa, 2001) suggests that BCR signaling is a necessary component of the mechanism by which Btk exerts its effect on B-1 cells. However, this function of Btk is not sufficient for normal development and maintenance of the B-1 population. In the absence of Lyn, Btk^{lo} B cells are equally sensitive to BCR crosslinking as B cells expressing normal levels of Btk (Satterthwaite *et al.*, 1998, Whyburn *et al*., 2003). Despite this, reduced Btk dosage still leads to decreased numbers of B-1 cells in Lyn-/- mice (Figure 3). Thus, Btk-mediated, BCR-independent events are also required. A potential role for IL-10 in this process is described above. In addition, IL-5 also regulates B-1 cell development and survival (Kopf *et al*., 1996, Yoshida *et al*., 1996) and requires Btk to signal (Koike *et al*., 1995). Alternatively, Btk may mediate the contribution of Notch-2 to B-1 cell development. Notch-2 haploinsufficiency reduces B-1 cell numbers (Witt *et al.*, 2003a), and constitutive Notch-2 signaling results in the exclusive development of B-1 cells at the expense of B-2 cells (Witt *et al.*, 2003b). Finally, increased signaling through the small GTPase Rap1 results in an increased frequency of B-1 cells (Ishida *et al*., 2006.). Both Rap1 and Btk have been shown to regulate integrin activity in B cells (McLeod *et al*., 2004, Spaargaren *et al.*, 2003), suggesting another possible connection between Btk and B-1 cell development. The requirement for multiple Btk-mediated signals during the late stages of B-1 cell development and/or maintenance may explain the higher threshold level of Btk required for these processes compared to the earlier transition from B-2 to B-1^{int} cells.

Several studies describing B-1 cell expansion in Lyn-/- mice (Nishizumi *et al*., 1995, Chan *et al*., 1997, Takeshita *et al*., 1998) have recently been contradicted by reports in which no change (Whyburn *et al*., 2003, Harder *et al*., 2001) or a reduction (Hasegawa *et al*., 2001) of B-1 cells was observed. The reason for this discrepancy is unclear. We demonstrate here that in the absence of Lyn, 6-1 mice can generate normal numbers of PtC-reactive B-1 cells (Figure 3). If anything, there is a trend towards an increased frequency of B-1 cells within the anti-PtC population in 6-1.Lyn-/- mice, although this is not statistically significant. Thus, if there is a defect in Lyn-/- mice in the maintenance of B-1 cells, it is not reflected in the processes measurable in the anti-PtC VH12 transgene model employed in the current study.

In addition to a decreased level of anti-PtC B-1 cells, 6-1.Btk^{lo} mice had an increased number of CD23+ B-2 cells that did not bind liposomes (Figure 5). This could not be attributed to differences in the maturity of cells within the CD23+ population, impaired expression of the VH12 transgene, relaxation of a bone marrow checkpoint restricting light chain usage (Tatu

et al, 1999), or reduced usage of light chains that have the potential to confer PtC reactivity (i.e. Vk4/5). The increased representation of mature, non-PtC reactive cells in 6-1.Btk^{lo} mice may result from intermediate BCR signal strength that is sufficient to allow development of mature B-2 cells but too weak to drive strong positive selection of PtC-specific B cells.

Together with previous studies (Arnold *et al*., 2000, Clarke and Arnold, 1998), the data presented here demonstrate that Btk is required for several stages in the skewing of VH12-expressing B cells toward anti-PtC specificity and a B-1 phenotype (Figure 6). These include differentiation from B-2 to B-1^{int}, the transition from B-1^{int} to B-1 and/or the survival of B-1 cells in the spleen, and the survival of differentiated B-1 cells in the peritoneum. The latter two processes have a particularly stringent requirement for Btk, perhaps due to the fact that Btk exerts these effects by transmitting both BCR-mediated and BCR-independent signals. The use of an immunoglobulin transgenic model in this study has facilitated the identification of numerous roles for Btk during the induced differentiation of B-1 cells. However, the recent identification of a progenitor population in fetal and adult bone marrow that gives rise to B-1, but not B-2, cells (Montecino-Rodriguez *et al*., 2006) demonstrates that induced differentiation from B-2 is not the only mechanism by which B-1 cells can be generated. It will be interesting to determine whether Btk-mediated signals are also critical for the activity of this progenitor population.

Acknowledgements

We thank Dr. Clifford Lowell for the Lyn-/- mice, Angela Mobley for flow cytometry, Kelvin Hsu for sequencing, and Heather Wright, Colin Court, and Sandirai Musuka for mouse genotyping. This work was supported by grants from the NIH (R01 AI049248 to A.S., R01 AI29576 and R01 AI43587 to S.C., and T32 AI 005284-28 to R.H.) and the Texas Higher Education Coordinating Board ARP/ATP program (010019-0074-2001 to A.S). C.C. was supported in part by the UT Southwestern SURF Program. A.S. is a Southwestern Medical Foundation Scholar in Biomedical Research.

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Abbreviations

BCR
: B cell antigen receptor

Btk
: Bruton's tyrosine kinase

Btk^{lo}
: transgene expressing low dose (25% of endogenous) of wild type murine Btk under the control of the IgH promoter and enhancer

PtC
: Phosphatidylcholine

Xid
: x-linked immunodeficiency

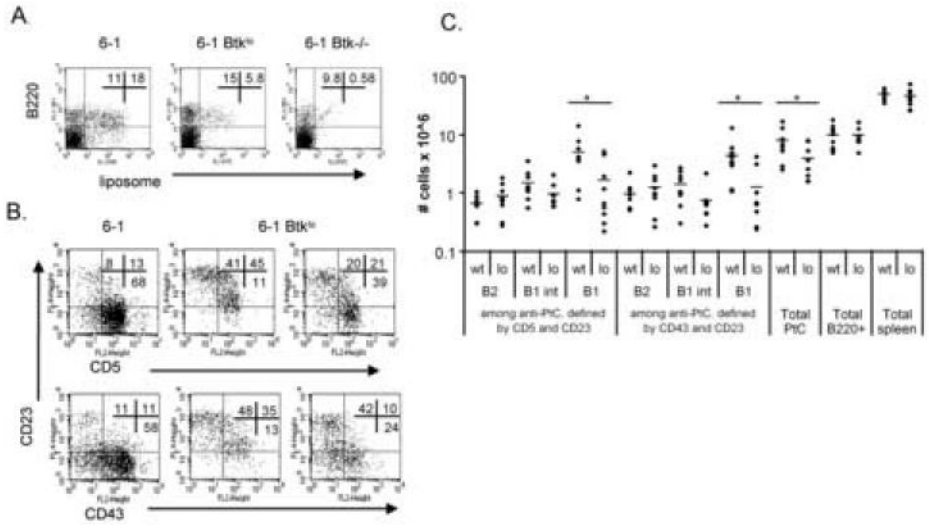

Figure 1. Btk regulates anti-PtC B cell expansion and differentiation from B-1^{int}to B-1

Splenocytes from 6-1, 6-1.Btk^{lo}, and 6-1.Btk-/- mice were stained with FITC-encapsulated liposomes, anti-CD5 PE or anti-CD43 PE, anti-B220 PerCP, and anti-CD23 biotin + streptavidin APC. A) The percentage of lymphocytes in each quadrant is indicated. B) Liposome-binding cells were gated and analyzed for expression of CD5, CD43, and CD23. B-2 cells (CD23+CD5- or CD23+CD43-) are in the upper left quadrant, B-1^{int} cells (CD23+CD5+ or CD23+CD43+) are in the upper right quadrant, and B-1 (CD23-CD5+ or CD23-CD43+) cells are in the lower right quadrant. Quadrants were defined using mice in which clear distinctions between populations were observed. The percentage of gated cells in each quadrant is indicated. Two individual 6-1.Btk^{lo} mice are shown in B) to reflect the range of variability seen in the differentiation state of anti-PtC B cells in these mice. Data in A) and B) are representative of 10 6-1, 10 6-1.Btk^{lo} and 5 6-1.Btk-/- mice. C) The number of splenocytes, B220+ cells, anti-PtC cells, and B-2, B-1^{int}, or B-1 within the anti-PtC population as defined by CD23 vs. CD5 or CD23 vs. CD43 are indicated. Each symbol (diamonds) represents an individual mouse. Bars represent the mean (n = 8-10). * = $p < 0.05$.

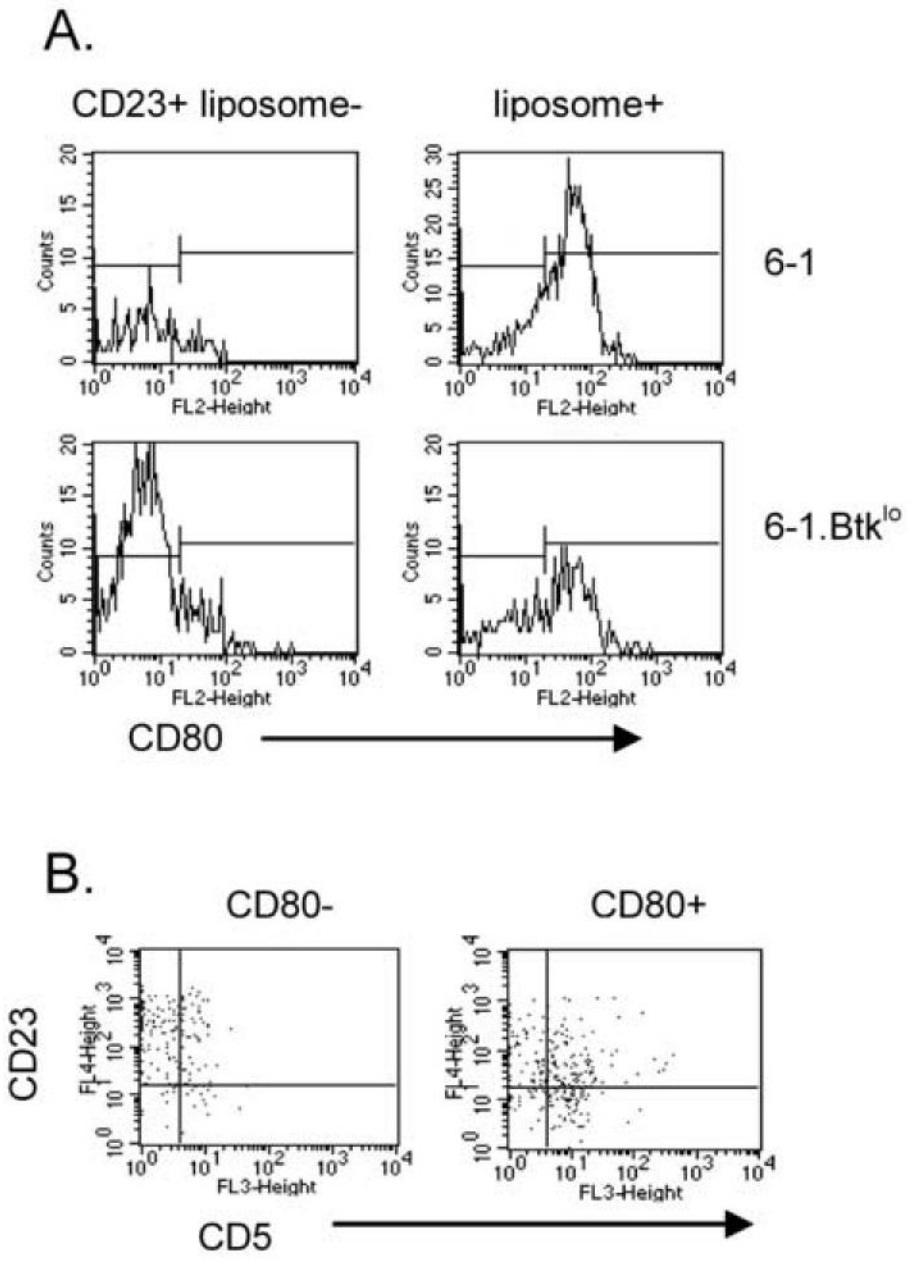

Figure 2. CD80 is upregulated in B-1^{int}and B-1 cells of 6-1.Btk^{lo}mice

Splenocytes were stained with FITC-encapsulated liposomes, anti-CD80 PE, anti-CD5 PerCP, and anti-CD23 biotin + streptavidin APC. A. CD23+liposome- (left) and liposome+ (regardless of CD23 expression) (right) populations were gated and examined for CD80 expression. B. CD80- and CD80+ populations within the liposome-binding gate of 6-1 Btk^{lo} mice (lower right histogram in A) were examined for expression of CD23 and CD5. Results are representative of 5 mice per group.

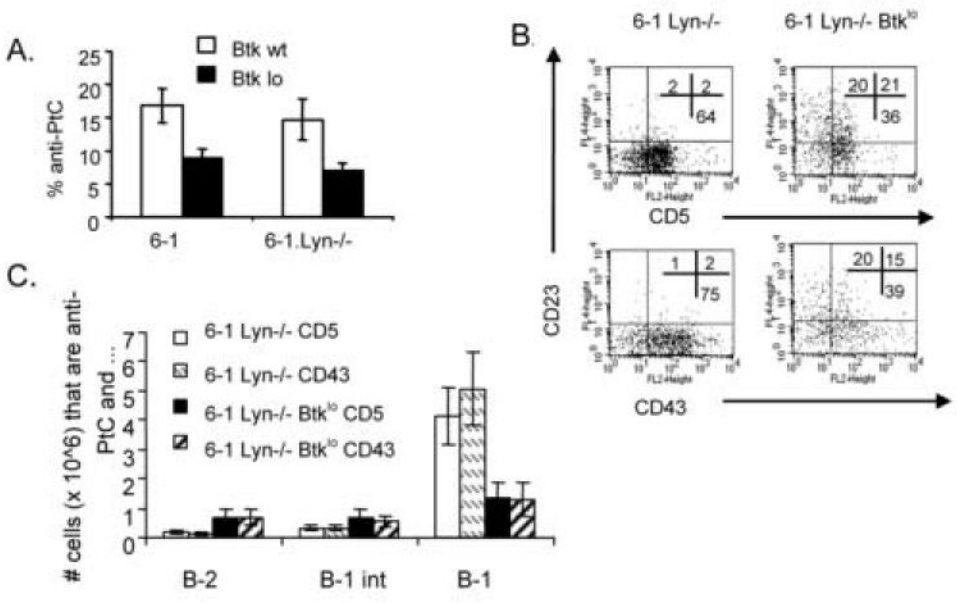

Figure 3. Reduced B-1 cell numbers in 6-1.Lyn-/-Btk^{lo}mice

Splenocytes from 6-1.Lyn-/- and 6-1.Lyn-/-Btk^{lo} mice were analyzed as in Figure 1. A) The frequency of liposome-binding cells is presented as mean +/- SEM. n = 10 for 6-1 and 6-1.Btk^{lo} mice, 6 for 6-1.Lyn-/- and 6-1.Lyn-/-Btk^{lo} mice. B) Data are representative of 6 mice per group. C) The total number of cells that are anti-PtC and B-2, B-1^{int}, or B-1 as defined by CD23 vs. CD5 or CD23 vs. CD43 is indicated. Data are presented as mean +/- SEM, n = 4 for 6-1.Lyn-/- and 5 for 6-1.Lyn-/-Btk^{lo}.

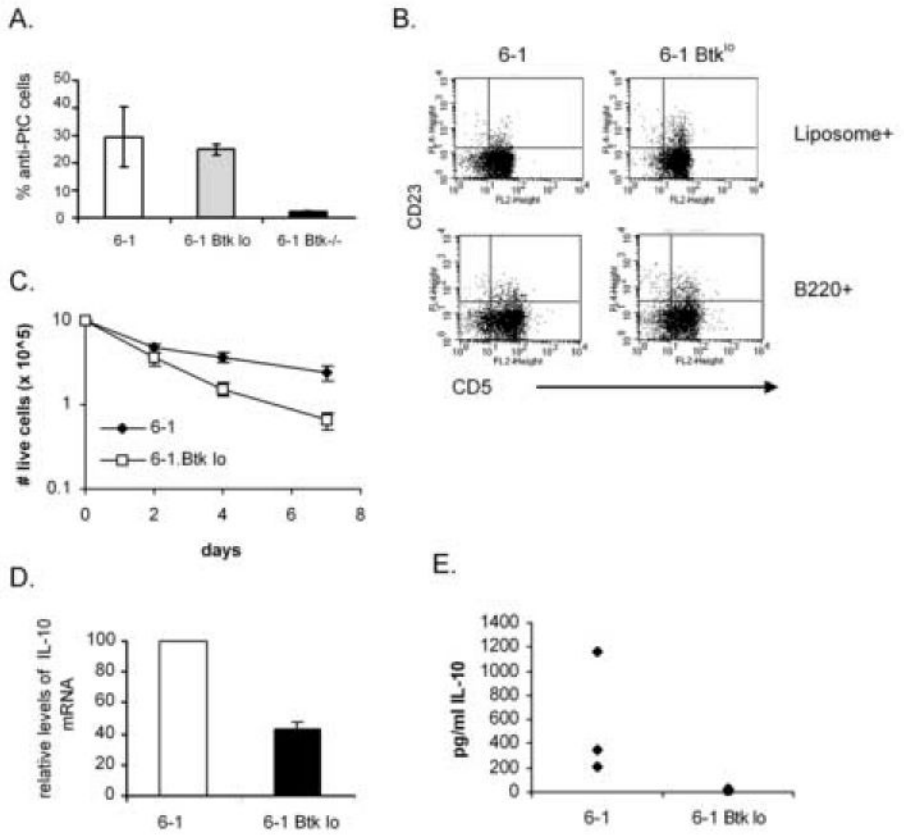

Figure 4. Btk regulates peritoneal B-1 cell survival

A) The frequency of liposome-binding cells among all peritoneal cells is indicated. Data are mean +/- SD, n = 3 for 6-1, 4 for 6-1.Btk^{lo}, and 2 for 6-1.Btk-/-. B) Peritoneal cells were stained with FITC-encapsulated liposomes, anti-CD5 PE, anti-B220 PerCP and anti-CD23 biotin + streptavidin APC. Liposome-binding (top) or B220+ (bottom) cells within the lymphoid gate were examined for CD23 and CD5 expression. Data are representative of 3 mice for 6-1 and 4 for 6-1.Btk^{lo}. C) B220+ cells were purified from the peritoneum of pools of 2-3 mice per group using magnetic beads or flow cytometry. Cells were plated in 96 well plates at 10^6 per well. The number of live cells (as measured by trypan blue exclusion) remaining at the indicated time is indicated. Data represent the mean +/- SEM, n = 6 for 6-1 and 5 for 6-1.Btk^{lo}. D) B220 + cells were purified from the peritoneum of pools of 2-3 mice per group using magnetic beads. IL-10 mRNA levels were measured by quantitative real-time PCR. GAPDH was used as a loading control. The relative expression of IL-10 in 6-1.Btk^{lo} cells compared to 6-1 wt cells (100%) is shown as mean +/- SD, n=3. E) Peritoneal cells were cultured at 2×10^6 per ml for 3 days. IL-10 levels in the culture supernatants were measured by ELISA. Each symbol represents the average of duplicate samples from an individual mouse (n = 3).

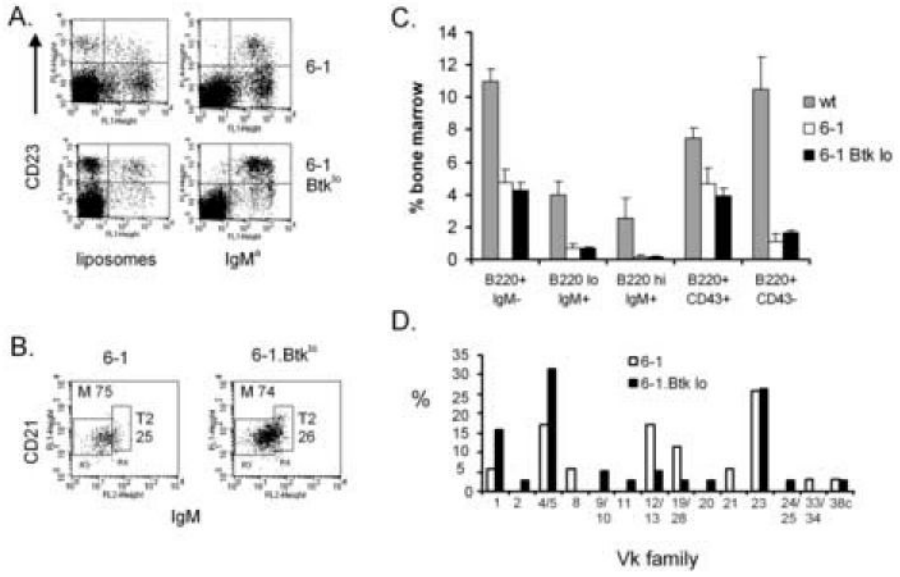

Figure 5. Increased number of CD23+ liposome- B cells in 6-1.Btk^{lo}mice

A) Splenocytes were stained with FITC-encapsulated liposomes or anti-IgMa FITC and anti-CD23 biotin + streptavidin APC. Results are representative of 10 mice per group. B) Splenocytes were stained with anti-CD21-FITC, anti-IgM PE, anti-CD5 PerCP and anti-CD23-biotin + streptavidin APC. CD23+CD5- cells were examined for expression of CD21 and IgM. Regions used to define T2 and mature (M) cells and the percentage of gated cells in each region are indicated. Results are representative of 3 mice per group. C) The frequency of B220+IgM- (pro and pre B cells), B220^{lo} IgM+ (immature B cells), B220^{hi} IgM+ (recirculating B cells), B220+CD43+ (pro B cells) and B220+CD43- (pre, immature, and recirculating B cells) among bone marrow cells is indicated as mean+/- SD, n=3-5. There was no significant difference in the total number of bone marrow cells recovered. D) CD23+ liposome- cells as defined by the upper left quadrant in A) were purified by flow cytometry from pools of 4 6-1 and 2 6-1.Btk^{lo} spleens. RNA was prepared and Ig light chains amplified by 5' RACE. 34 individual clones from 6-1 mice and 38 from 6-1.Btk^{lo} mice were sequenced. The percentage of clones representing each light chain family is shown.

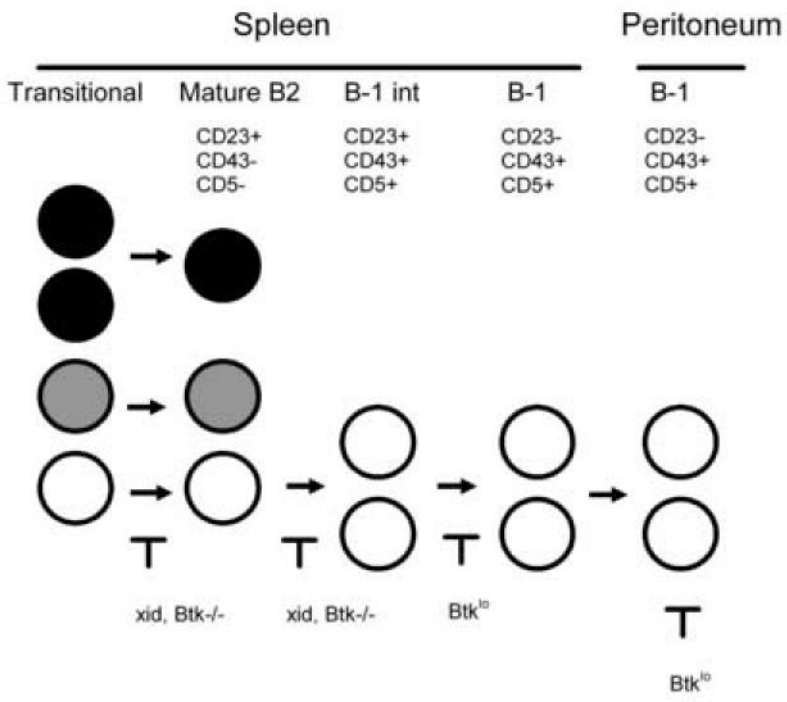

Figure 6. Model for the role of Btk in the focusing of VH12-expressing B cells toward anti-PtC B-1 cells

Circles represent VH12-expressing cells. Black circles express light chains other than Vk4/5, stippled circles represent Vk4/5 expressing cells that do not bind PtC, and empty circles depict anti-PtC cells expressing Vk4/5. In normal mice, the light chain repertoire is focused towards a limited number of light chains including Vk4/5 due to failure of many light chains to pair with VH12 and the inability of some light chains that do pair with VH12 to support the development of mature B cells (Tatu *et al*., 1999). Development of B cells beyond the T2 stage is considerably impaired in the complete absence of Btk signaling (xid and Btk-/-) (Desiderio, 1997, Satterthwaite and Witte, 2000, Khan, 2001, Loder *et al*., 1999) regardless of their specificity. PtC-specific cells that do develop in xid and Btk-/- mice fail to differentiate beyond B2 (Clarke and Arnold, 1998). Btk^{lo} mice can support differentiation of anti-PtC cells through the $B\text{-}1^{int}$ stage, but only limited numbers of B-1 cells are present in the spleen. Some B-1 cells do develop and localize to the peritoneum, but they survive poorly and produce reduced levels of the survival factor IL-10. The current studies do not rule out an additional role for Btk in the newly described B-1 progenitor population present in fetal and adult bone marrow (Montecino-Rodriguez *et al*., 2006).