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Local increase in thymic stromal lymphopoietin induces systemic alterations in B cell development

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The cytokine thymic stromal lymphopoietin (TSLP) drives immature B cell development *in vitro* and may regulate T helper type 2 responses. Here we analyzed the involvement of TSLP in B cell development *in vivo* with a doxycycline-inducible, keratin 5–driven transgene encoding TSLP (K5-TSLP). K5-TSLP-transgenic mice given doxycycline showed an influx of immature B cells into the periphery, with population expansion of follicular mature B cells, near-complete loss of marginal zone and marginal zone precursor B cells, and 'preferential' population expansion of peritoneal B-1b B cells. These changes promoted cryoglobulin production and immune complex–mediated renal disease. Identical events occurred in mice without T cells, in alternative TSLP-transgenic models and in K5-TSLP-transgenic mice with undetectable systemic TSLP. These observations suggest that signals mediating localized TSLP expression may modulate systemic B cell development and promote humoral autoimmunity.

The generation of a mature B cell compartment results from complex interactions among intrinsic developmental programs and microenvironmental niche constraints. Lymphoid precursors in the bone marrow interact with stromal cells expressing interleukin 7 (IL-7) to become immunoglobulin M–positive (IgM^+) immature B cells, which subsequently emigrate to the periphery[1,2]. Additional developmental steps occur in the spleen, where the recent bone marrow immigrants, called 'transitional B cells', are kept alive by the tumor necrosis factor family member BAFF (also called BLyS). In concert with antigen receptor–mediated signals, cytokines such as IL-7 and BAFF are crucial in promoting B cell maturation[3–5].

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine expressed mainly epithelial cells, including keratinocytes[6]. TSLP binding and signaling occur by means of a heterodimer composed of the IL-7 receptor α-chain (IL-7Rα) and TSLP receptor (TSLPR). TSLPR is expressed mainly on hematopoietic cells, including monocytes, dendritic cells and B cells[7,8]. Originally described as a B cell differentiation factor, when added to bone marrow B cell progenitors, TSLP leads to an increase in both the percentage and absolute number of B lineage cells positive for surface (membrane) IgM ($mIgM^+$)[9–11].

Increased concentrations of TSLP have been associated with the potent development of T helper type 2 cells in several inflammatory diseases[12,13]. For example, lesional skin isolated from patients with atopic dermatitis and bronchoalveolar lavage samples from asthmatic patients have higher TSLP expression than normal controls have[12,13]. Moreover, mice expressing TSLP under the control of skin- or lung-specific promoters develop symptoms consistent with atopic dermatitis and asthma, respectively[14,15]. Such findings have led to a model in which TSLP expression in inflammatory diseases drives the activation of dendritic cells and consequent T helper type 2 cytokine responses and the pathogenesis of allergic disease[16].

Those observations also raise the issue of whether local inflammation-mediated increases in TSLP might lead to alterations in B lymphopoiesis. Several TSLP-transgenic mouse models have been developed, but the physiological effects of systemic TSLP expression remain poorly understood. The first TSLP transgenic line reported, '*Lck*-TSLP' mice, express TSLP under the control of the T cell–specific *Lck*-proximal promoter and develop systemic inflammatory disease[17]. A mouse model with ubiquitous actin-driven expression of a TSLP transgene has also been developed; these mice have many fewer B lymphoid progenitors in the bone marrow and fewer cells of all thymic T cell subsets[18]. These data have been interpreted as suggesting that TSLP actively inhibits lymphopoiesis *in vivo*[18].

Interpretation of the preceding studies has been complicated by the use of nonphysiological promoters to drive TSLP expression. To better understand the effect of altered TSLP expression on B cell development, here we have analyzed B cell populations in a published mouse strain that expresses TSLP under the control of a tetracycline-regulated, skin-specific promoter[15]. We found that local expression of TSLP in this physiologically relevant site caused a substantial increase in bone marrow B cell lymphopoiesis, resulting in premature exodus of immature cells to the periphery. Moreover, TSLP directly

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Table 1 Serum TSLP before and after doxycycline treatment

	Time on 1 mg/ml of doxycycline (weeks)			
	0	1	2	3
NLC	ND	ND	ND	ND
K5-TSLP	ND	ND	359	1,313
K5-TSLP	ND	ND	761	1,197

Serum TSLP is presented as pg/ml. ND, not determined.

stimulated pre–B cell proliferation and specifically induced the expression of genes that promote cell cycle progression. This process, in turn, led to an enlarged pool of immature and naive B cells in the periphery, coupled with complete disappearance of splenic marginal zone B cells and 'preferential' enlargement of the peritoneal B-1b B cell compartment. These combined events led to an increase in antibody-secreting cells, cryoglobulin production and immune-complex renal disease. Our findings indicate that even very low expression of TSLP potently promotes altered B cell development and suggest that such signals may thereby contribute to a loss of B cell tolerance in some inflammatory settings.

RESULTS

Increased TSLP leads to expansion of the immature B cells

We evaluated B cell development in mice expressing TSLP under the control of a keratin 5–driven, tetracycline-regulated promoter ('K5-TSLP mice')[15]. In this model, double-transgenic K5-TSLP mice receiving doxycycline in their drinking water produce TSLP mainly in the skin[15,19]. Analysis of peripheral TSLP concentrations showed no detectable serum TSLP before doxycycline treatment and a progressive increase in serum TSLP concentration after the initial administration of doxycycline (**Table 1**). Systemic TSLP reached steady-state concentrations of about 13 ng/ml within 2–3 weeks of treatment with 1 mg/ml of doxycycline. Systemic TSLP expression was dependent on the doxycycline dosage. Only one of five mice treated with 0.1 mg/ml of doxycycline produced a measurable concentration of serum TSLP, and TSLP was not detectable in mice treated with 0.01 mg/ml of doxycycline (none of six mice; concentration below 25 pg/ml; data not shown). We consistently found altered B cell development, even in mice treated with as little as 0.01 mg/ml of doxycycline (data not shown).

Blood obtained weekly from doxycycline-treated K5-TSLP mice demonstrated direct correlation between increased serum TSLP and an increased proportion of $B220^+AA4.1^+$ B cells (**Fig. 1**). The complement receptor AA4.1 (also called C1aRp) is 'preferentially' expressed in immature B cells[20], and the $AA4.1^+$ B cells in doxycycline-treated mice were mainly negative for surface immunoglobulin or were IgM^+IgD^-, suggesting that TSLP was acting at an early stage in B cell development (data not shown).

TSLP stimulates the population expansion of late pro–B cells

We used a variation of published staining criteria using CD43, BP-1 and heat-stable antigen in addition to surface immunoglobulin[21] to evaluate the development of bone marrow B cells in doxycycline-treated mice. Although their total number of bone marrow cells did not differ from that of normal littermate control mice, doxycycline-treated K5-TSLP mice had a significantly higher percentage and absolute number of bone marrow subsets containing cells expressing the pre–B cell receptor (pre-BCR; $B220^+IgM^-$ population; $P < 0.01$). These subsets included both $CD43^+$ pro–B cells and $CD43^-$ pre–B cells (**Fig. 2a,b**). Additionally, K5-TSLP mice had significantly more IgM^+IgD^- immature B cells ($P < 0.05$). In contrast, there was no significant change in the relative number of IgD^+ recirculating B cells (**Fig. 2a,b**). These data collectively suggested that TSLP was acting on an early B cell precursor.

We used polychromatic flow cytometry to determine the bone marrow B2 developmental subset(s) that composed the primary responder cell type in K5-TSLP mice. Studies using targeted gene disruption have indicated that late pro–B cells are the main TSLP-responsive bone marrow subset[22,23]. Consistent with that finding, the $B220^+IgM^-$ pro–B cell and pre–B cell populations were much greater in K5-TSLP mice (**Fig. 2a**). Additional phenotyping with the BP-1 and CD24 markers showed that mice of both the K5-TSLP and *Lck*-TSLP models had specifically more $B220^+CD43^+CD24^{hi}BP\text{-}1^+$ late pro–B cells (**Fig. 2c** and data not shown).

We reasoned that the TSLP-driven increase in late pro–B cells might result from direct amplification of this subset or, alternatively, from stimulation of the growth or differentiation of its immediate precursor. To distinguish between those two possibilities, we directly analyzed the TSLP-mediated proliferative capacity of highly purified B cell subsets from wild-type mice. We noted robust proliferation (an increase of about fivefold) of isolated late pro–B cells stimulated with TSLP (**Fig. 2d**). In some experiments we also noted a limited response by early pro–B cells, but this finding was not consistently reproducible. In contrast, no other purified bone marrow $B220^+$ B cell fraction proliferated in response to TSLP. Thus, pre-BCR^+ late pro–B cells represent the main post-fetal, TSLP-responsive bone marrow B cell population.

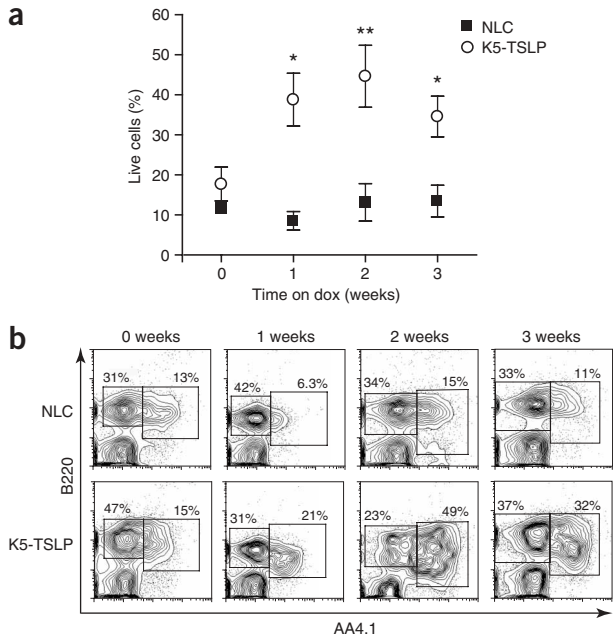

Figure 1 Doxycyline treatment correlates with influx of immature B cells into the periphery. (**a**,**b**) B220 and AA4.1 staining of peripheral blood lymphocytes isolated from normal littermate control mice (NLC) or K5-TSLP mice treated with dietary doxycycline; immature ($B220^+AA4.1^+$) and naive ($B220^+AA4.1^-$) B cells were identified by weekly staining. (**a**) Percent $B220^+AA4.1^+$ (immature) B cells in the blood at each time point. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test). Error bars, s.d. of at least three mice. (**b**) Numbers above boxed areas indicate percent of live cells. Data are one representative of three independent kinetic experiments.

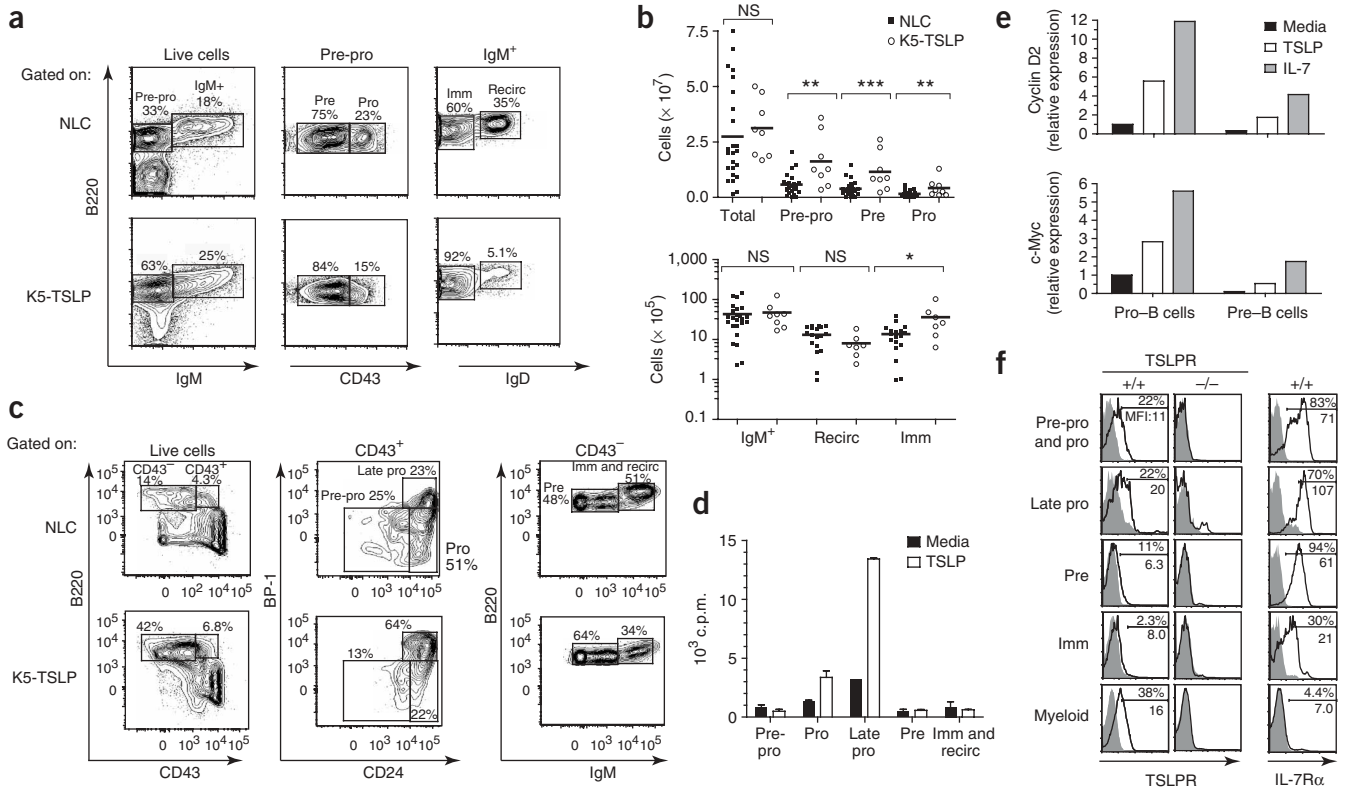

Figure 2 TSLP directly stimulates the population expansion of early B cell precursors in the bone marrow. (**a**) Staining of bone marrow B cell subsets from normal littermate control or K5-TSLP mice treated for 3 weeks with doxycycline. Cells were gated on live lymphocytes, as determined by scatter, and B cell subsets were identified by staining for B220, IgM, CD43 and IgD. Numbers above boxed areas indicate percent of the gated subset. (**b**) Absolute numbers of each bone marrow B cell subset. Small horizontal bars, mean; bottom graph, log scale. (**c**) Polychromatic flow cytometry of bone marrow cells from control or K5-TSLP mice, stained for B220, CD24, CD43, IgM and BP-1. Numbers above boxed areas indicate relative percent of the gated population. (**d**) [^{3}H]thymidine uptake analysis of the proliferation of various developmental subsets sorted from wild-type bone marrow by staining, then stimulated with TSLP or media. Error bars, s.d. of at least three replicates. (**e**) Semiquantitative real-time PCR of the expression of cyclin D2 and c-Myc in isolated B cell subsets stimulated with media, TSLP or IL-7. Expression was normalized to β_2-microglobulin mRNA expression, and the expression ratio was determined by comparison to expression in media-treated pro-B cells (set as 1). (**f**) Flow cytometry of developmental subsets from bone marrow of control mice (+/+) and/or TSLPR-deficient mice (–/–), stained for TSLPR and IL-7Rα. Numbers above bracketed lines (gates) indicate percent TSLPR$^+$ cells (left and middle) or IL-7Rα$^+$ cells (right); numbers below bracketed lines indicate mean fluorescence intensity (MFI). Myeloid, B220$^-$CD43$^+$ bone marrow cells. NS, not significant; Imm, IgM$^+$ immature B cells; Recirc, recirculating. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (Student's *t*-test). Data are representative of at least three (**a**–**e**) or two (**f**) independent experiments.

The signaling cascades 'downstream' of TSLP remain poorly understood. Because TSLP supported late pro–B cell proliferation, we analyzed the expression of candidate cell cycle regulators after TSLP stimulation. In agreement with the proliferation data, we noted upregulation of both c-Myc and cyclin D2 after stimulation with either TSLP or IL-7 (**Fig. 2e**). In contrast, we noted no substantial increase in Bcl-x_L after 15 h of stimulation of bone marrow B cell subsets with TSLP (data not shown).

Given that TSLP can signal through a TSLPR–IL-7Rα heterodimer, we also evaluated which B cell subsets coexpressed these signaling effectors. Initial characterization of TSLPR expression was misleading because of nonspecific staining produced by a commercially available antibody to TSLPR[23]. This reagent produced equivalent positive staining of B cells derived from either normal littermate control or TSLPR-knockout mice (data not shown). In experiments using monoclonal antibody to TSLPR[24], we found that only pre-pro–B cells and pro–B cells expressed both TSLPR and IL-7Rα (**Fig. 2f**). Along with the proliferation data reported above, these observations establish late pro–B cells as the key TSLP-responsive subset driving the B2 B cell developmental abnormalities in K5-TSLP mice.

K5-TSLP mice show peripheral influx of B cell precursors

K5-TSLP mice are reported to develop splenomegaly and lymphadenopathy[15]; however, here the relative proportion of splenic B220$^+$ B cells remained the same as that of normal littermate control mice (**Fig. 3a** and **Supplementary Fig. 1** online). The transgenic B cell pool was 'polarized' to an immature phenotype, with higher absolute numbers and relative percentages of B220$^+$AA4.1$^+$ immature B cells coupled with corresponding relatively lower B220$^+$AA4.1$^-$ mature B cell numbers (**Fig. 3b–d**). The AA4.1$^+$ B cell population was composed mainly of immunoglobulin-negative and IgM$^+$IgD$^-$ immature B cells. In contrast, these populations represented a relatively small percentage of splenic B cells in control mice (**Fig. 3c,d**).

An alternative approach for B cell phenotyping uses CD21, CD24 and CD23 to distinguish immature transitional B cells from mature splenic B cells. 'Transitional 1' B cells are CD24^{hi}CD21$^-$CD23$^{pos–neg}$ and represent the most recent bone marrow emigrants[3,25]. Consistent with the greater B220$^+$AA4.1$^+$ immature population, populations of 'transitional 1' cells were significantly expanded in K5-TSLP mice (**Figs. 3e** and **4a**; $P < 0.001$). These findings collectively suggested that higher systemic TSLP concentrations promote the premature exodus

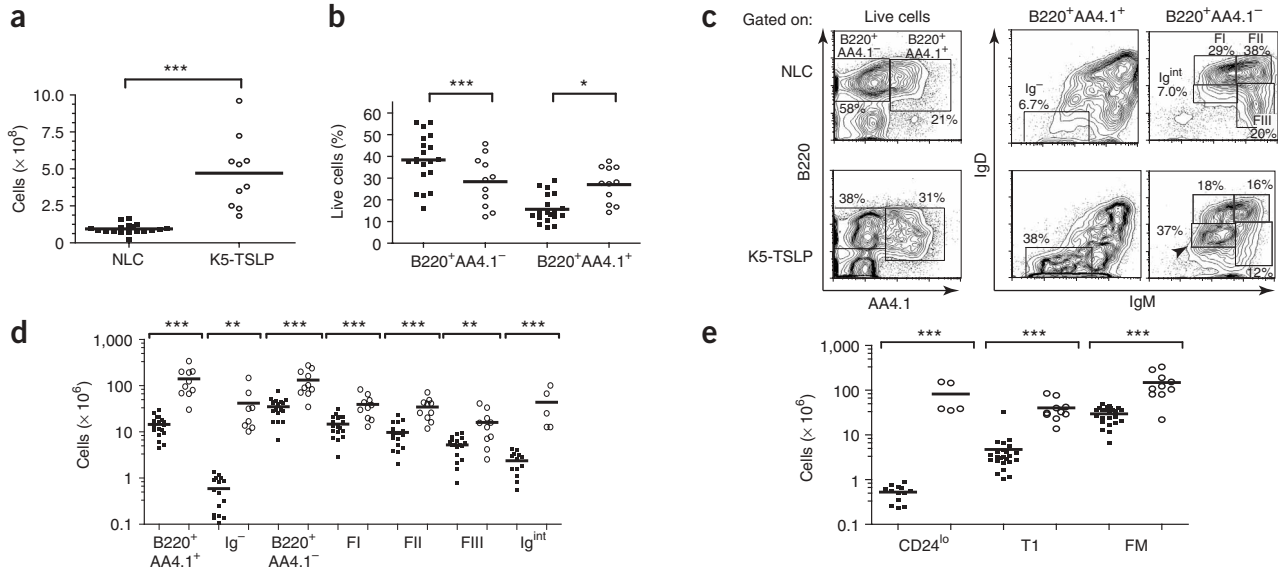

Figure 3 Population expansion of both splenic transitional and follicular mature B cells in K5-TSLP mice. Analysis of splenocytes isolated from normal littermate control mice (filled sqaures) or K5-TSLP mice (open circles) treated for 3 weeks with 1 mg/ml of doxycycline. (**a**) Total splenic cell numbers. Dead cells were excluded by trypan blue staining. (**b**) Flow cytometry to determine the proportion of immature B220$^+$AA4.1$^+$ cells. (**c**) B220, AA4.1, IgM and IgD staining to identify B cell subsets of the splenic B cell compartment. Arrowhead (bottom right) indicates a unique B220$^+$AA4.1$^-$ population with intermediate expression of both mIgD and mIgM. Numbers above boxed areas indicate percent cells in the gated subset. (**d**,**e**) Absolute cell numbers, obtained by multiplication of the total cell number by the percent subsets identified on the basis of B220, AA4.1, IgM and IgD (**d**) or B220, CD24, CD21 and CD23 (**e**). Vertical axes, log scale. At least six mice were analyzed for each stain. Ig$^-$, negative for immunoglobulin expression; Ig^{int}, intermediate immunoglobulin expression; FI–FIII, fractions I–III; T1, transitional 1; FM, follicular mature. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). Data are representative of at least five unique experiments.

of B cell precursors from the bone marrow to the periphery, leading to the accumulation of early B cell progenitors in the bloodstream and spleen.

K5-TSLP mice show expansion of the mature B cell pool

Naive, resting follicular mature B cells are typically AA4.1$^-$mIgD^{hi}CD21^{int}CD24^{int}CD23^{hi}. Analysis of K5-TSLP mice showed population expansion of all mature B cell compartments, except marginal zone and marginal zone precursor B cells (**Figs. 3c** and **4**). The expanded follicular mature population, however, did not show overt upregulation of activation markers, including CD80, CD86 and CD69, suggesting a lack of spontaneous activation (data not shown). In addition, K5-TSLP mice had an unusual CD24^{int}CD21^{lo} population that was IgM^{lo}IgD^{hi}AA4.1$^-$. These cells resembled normal follicular mature B cells but had much lower expression of CD21 (**Figs. 3e** and **4a** and data not shown). Furthermore, we consistently found a unique B220$^+$AA4.1$^-$ population in doxycycline-treated K5-TSLP mice with intermediate expression of both mIgD and mIgM (**Fig. 3c**, arrowhead). Proliferation assays using sorted cells with intermediate expression of immunoglobulin indicated that this population was unlikely to be composed of anergic B cells (data not shown).

K5-TSLP mice lack marginal zone B cells

Marginal zone B cells are located in the splenic marginal zone and respond to a variety of blood-borne, thymus-independent type 2 antigens or pathogens[26]. They are derived from marginal zone precursors (also called 'CD21^{hi}CD24^{hi} transitional 2' B cells)[25,27]. We used CD23 expression to differentiate marginal zone precursors (CD23$^+$) from marginal zone B cells (CD23$^-$). Despite the greater cellularity of transgenic mice, systemic expression of TSLP led to a substantial numerical and proportional reduction of both marginal zone precursors and marginal zone B cells relative to that of control mice (**Fig. 4a**,**b**).

Marginal zone B cells are often identified by CD21 expression, which was consistently downregulated in the B cell pool from transgenic mice (**Fig. 4a**). Thus, we used CD1d as an alternative marker to evaluate marginal zone B cell numbers. Whereas a CD1d$^+$ marginal zone population was readily identified in control mice, this subset was nearly absent from K5-TSLP mice (**Supplementary Fig. 1**). In addition, immunohistochemical analysis of normal littermate control mice delineated IgM^{hi} marginal zones and IgD^{hi} follicular zones, separated by a band of MOMA-1$^+$ metallophilic macrophages (**Fig. 4c**). In contrast, K5-TSLP mice had nearly complete disruption of both marginal zone and follicular organization (**Fig. 4c**).

To further test the connection between immature B cell influx and marginal zone disappearance, we analyzed K5-TSLP mice receiving shorter treatment with doxycycline (1.5 weeks). These mice were phenotypically similar to mice treated for 3 weeks, with splenomegaly and population expansion of immature B cells (data not shown). However, although the mice treated for 1.5 weeks had a proportional loss of marginal zone precursor and marginal zone B cells relative to that of control mice (**Fig. 4d**), the absolute numbers were similar to those of control mice (**Fig. 4e**). Consistent with those results, immunohistochemical analysis showed partial disruption of the MOMA-1$^+$ border and a smaller marginal zone B cell area (**Fig. 4c**). Furthermore, the abundance of mRNA encoding the notch-dependent downstream effector Deltex-1 was significantly lower in B220$^+$ B cells from K5-TSLP mice than in those from control mice, after treatment with doxycycline for 1.5 weeks (**Fig. 4f**). In contrast, the transcript abundance for the Notch ligands Delta, Jagged-1 and Jagged-2 remained unchanged in total splenocytes (**Fig. 4g**). Because Notch

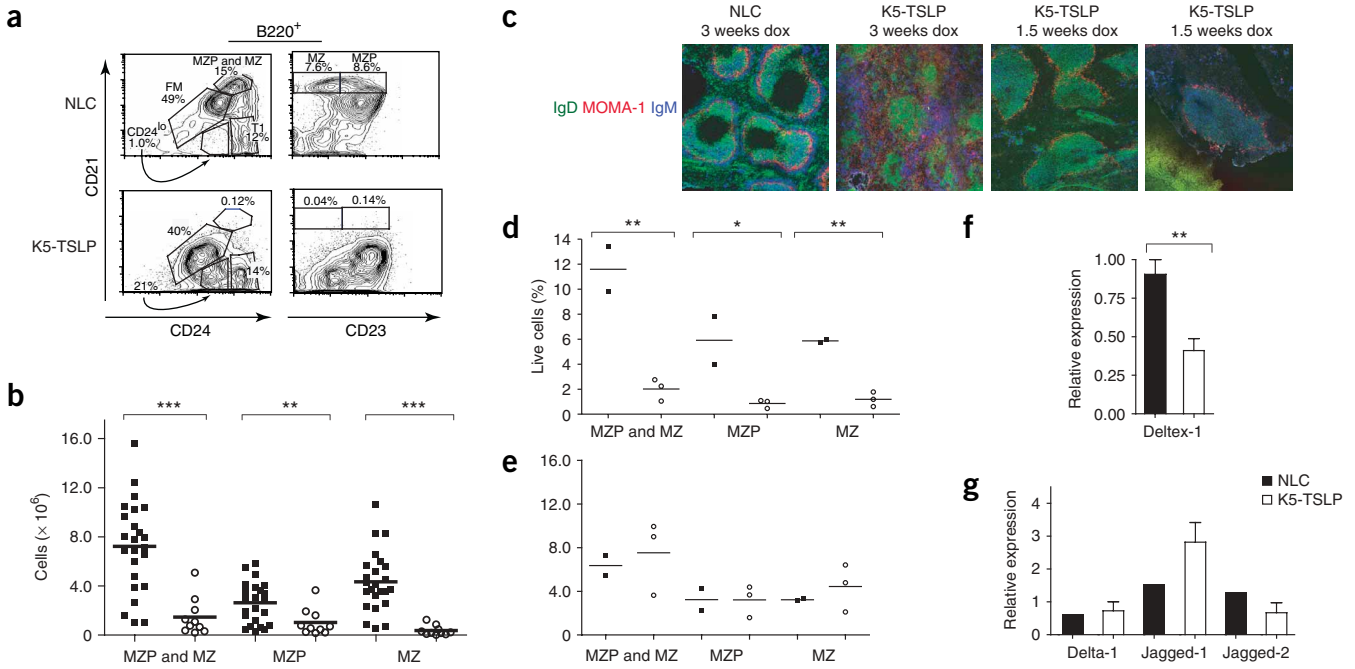

Figure 4 K5-TSLP mice lack marginal zone and marginal zone precursor B cells. Analysis of splenocytes and splenic sections from normal littermate control mice (filled squares) or K5-TSLP mice (open circles) treated with doxycycline. (**a**) B220, CD24, CD21 and CD23 staining of splenocytes from mice treated with doxycycline for 3 weeks. B220$^+$ cells were analyzed; numbers adjacent to outlined areas indicate relative percent B220$^+$ cells in each. (**b**) Absolute numbers of marginal zone precursor (MZP) and marginal zone (MZ) B cells in mice treated with doxycycline for 3 weeks (calculated as described in **Fig. 3d,e**). (**c**) Immunohistochemical analysis of splenic sections at 1.5 or 3 weeks of doxycycline treatment (dox), stained for IgD, IgM and MOMA-1. Images are of sections from one of at least three mice; sections from two different K5-TSLP mice are presented for the 1.5-week time point. Contrast and brightness were adjusted with the 'auto-levels' feature of Adobe Photoshop. Original magnification, ×100. (**d**,**e**) Percent (**d**) and absolute numbers (**e**) of marginal zone and marginal zone precursor cells from mice treated with doxycycline for 1.5 weeks. (**f**,**g**) Semiquantitative real-time PCR analysis of the expression of Deltex-1 (**f**) and Notch ligands (**g**) in B220$^+$ B cells (**f**) or total splenocytes (**g**) obtained from mice treated with doxycycline for 1.5 weeks. Expression was normalized to β_2-microglobulin mRNA expression, and the expression ratio was determined by comparison to expression (set as 1) in B220$^+$ cells (**e**) or total splenocytes (**f**). Small horizontal bars (**b**,**d**,**e**), mean; error bars (**f**,**g**), s.d. of at least three mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). Data are from at least four (**a**,**b**) or two (**c**–**g**) independent experiments.

signaling is crucial for marginal zone B cell development[28], these findings suggested that the disappearance of marginal zone B cells may reflect disruption of Notch–Notch ligand interactions. Alternatively, immature cells may also alter marginal zone structure by competing for cytokines and growth factors required for marginal zone maintenance. In either case, our observations suggest that the TSLP-driven influx of B cell precursors into the spleen probably serves as the catalyst for the progressive loss of marginal zone B cells.

TSLP induces population expansion of B-1b and B-1 progenitors

B-1 B cells are CD11b^{hi}IgM^{hi} and represent the main B cell subset of the peritoneal cavity[29]. In adult mice, this compartment is typically composed of equal numbers of CD5$^+$ B-1a and CD5$^-$ B-1b cells[29]. K5-TSLP mice had a much smaller relative percentage of B-1a B cells coupled with a correspondingly larger B-1b compartment (**Fig. 5a,b**). This result correlated with a consistently greater absolute number of B-1b cells; however, variability in the relative volume of recovered peritoneal lavage fluid prevented full statistical analysis (**Fig. 5c**).

The absence of IL-7Rα on B-1 cells (**Supplementary Fig. 2** online), coupled with a report describing a TSLP-responsive B-1 bone marrow progenitor[30], suggested that the B-1b population expansion in the K5-TSLP mice might be secondary to expansion of a bone marrow B-1 precursor population. CD19$^+$B220$^-$ B-1 progenitors are skewed toward producing B-1a cells rather than B-1b cells during fetal versus neonatal development, respectively[30]. We tested the ability of TSLP to modulate these populations by treating mice with TSLP during neonatal and fetal development. We used 1-week-old K5-TSLP pups that received doxycycline through lactation and collected peritoneal lavage fluid after 2 weeks of doxycycline treatment (3 weeks of age). Although the B-1 compartment in control pups was composed mainly of B-1a B cells, the K5-TSLP neonates had numerically and proportionally more B-1b but not B-1a B cells (**Fig. 5d,e**). Furthermore, K5-TSLP neonates had a 15-fold greater number of CD19$^+$B220$^-$ B-1 bone marrow progenitors, suggesting that TSLP induced the expansion of this subset (**Fig. 5f,g**). We also analyzed *Lck*-TSLP mice, which express TSLP under the control of the proximal *Lck* promoter, which is active during fetal development[17,31]. Analysis of 2-week-old *Lck*-TSLP pups showed expansion of both the B-1a and B-1b compartments, suggesting that an earlier increase in TSLP expression (during both fetal and neonatal stages) promoted both developmental subsets (**Supplementary Fig. 2**). Finally, although 4-week-old *Lck*-TSLP mice showed a progressive increase in the B-1b compartment, we could no longer detect a numerical increase in the B-1a subset (**Supplementary Fig. 2**). This result might reflect CD5 downregulation or a differential self-renewal ability of B-1a versus B-1b cells. These findings collectively suggested that TSLP stimulates bone marrow B-1-committed progenitors, promoting developmentally restricted production of both B-1a and B-1b cells.

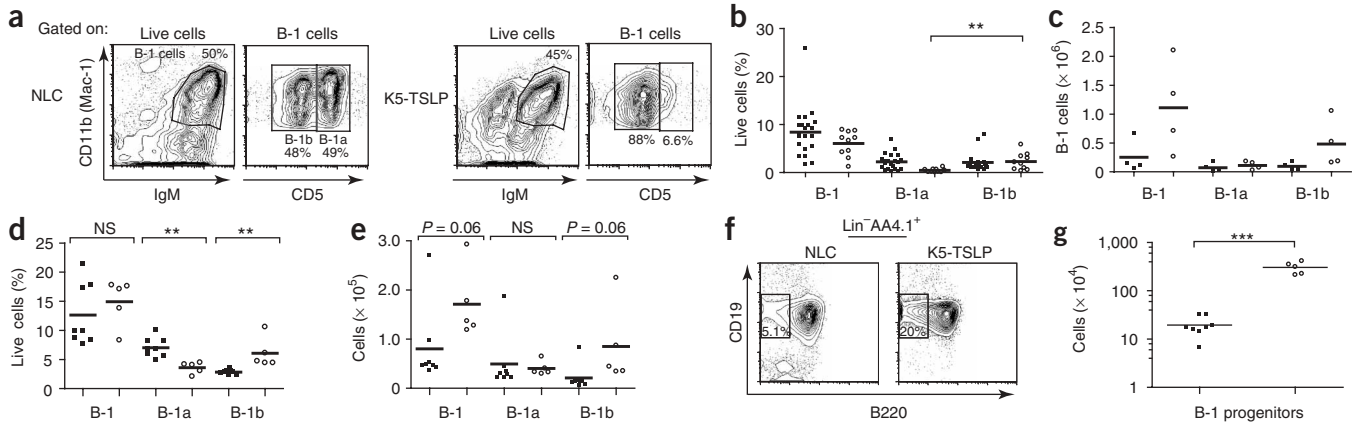

Figure 5 TSLP overexpression promotes the population expansion of peritoneal B-1b B cells and B-1 progenitors in the bone marrow. (**a**–**e**) Analysis of peritoneal exudate cells from normal littermate control mice (filled squares) or K5-TSLP mice (open circles) treated with doxycycline, stained for CD11b (Mac-1), IgM and CD5. (**a**) Flow cytometry of the peritoneal B-1 compartment; numbers adjacent to outlined areas indicate percent of the gated population. (**b**,**c**) Percent (**b**) and absolute numbers (**c**) of B-1 B cells from adult mice treated with doxycycline for 3 weeks. (**d**,**e**) Percent (**d**) and absolute numbers (**e**) of cells obtained from 3-week-old mice treated with doxycycline for 1.5 weeks. (**f**,**g**) Flow cytometry (**f**) and absolute counts (**g**) of lineage-negative (Lin^-) $AA4.1^+B220^-CD19^+$ B-1 progenitors in bone marrow from 3-week-old neonatal mice treated with doxycycline for 1.5 weeks. Vertical axis (**g**), log scale. Small horizontal bars (**b**,**d**,**e**,**g**), mean. **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). Data are from at least three independent experiments.

B cell expansion in K5-TSLP mice is T cell independent

K5-TSLP mice mount a potent T helper type 2 response, with robust $CD4^+$ T cell activation and cytokine production[15]. To exclude the possibility of T cell activation as a modulator of the B cell phenotype, we analyzed the B cell composition of K5-TSLP mice bred onto a T cell–deficient background ('K5-TSLP *Tcrb*$^{-/-}$ mice'). The B cell phenotype of K5-TSLP *Tcrb*$^{-/-}$ mice was indistinguishable from of T cell–sufficient K5-TSLP mice. The K5-TSLP *Tcrb*$^{-/-}$ mice showed expansion of both the pro–B cell and pre–B cell subsets in the bone marrow, influx of $AA4.1^+$ B cells into the periphery, splenomegaly, and expansion of splenic B cell subsets (**Supplementary Fig. 3** online). K5-TSLP *Tcrb*$^{-/-}$ mice also showed a complete loss of

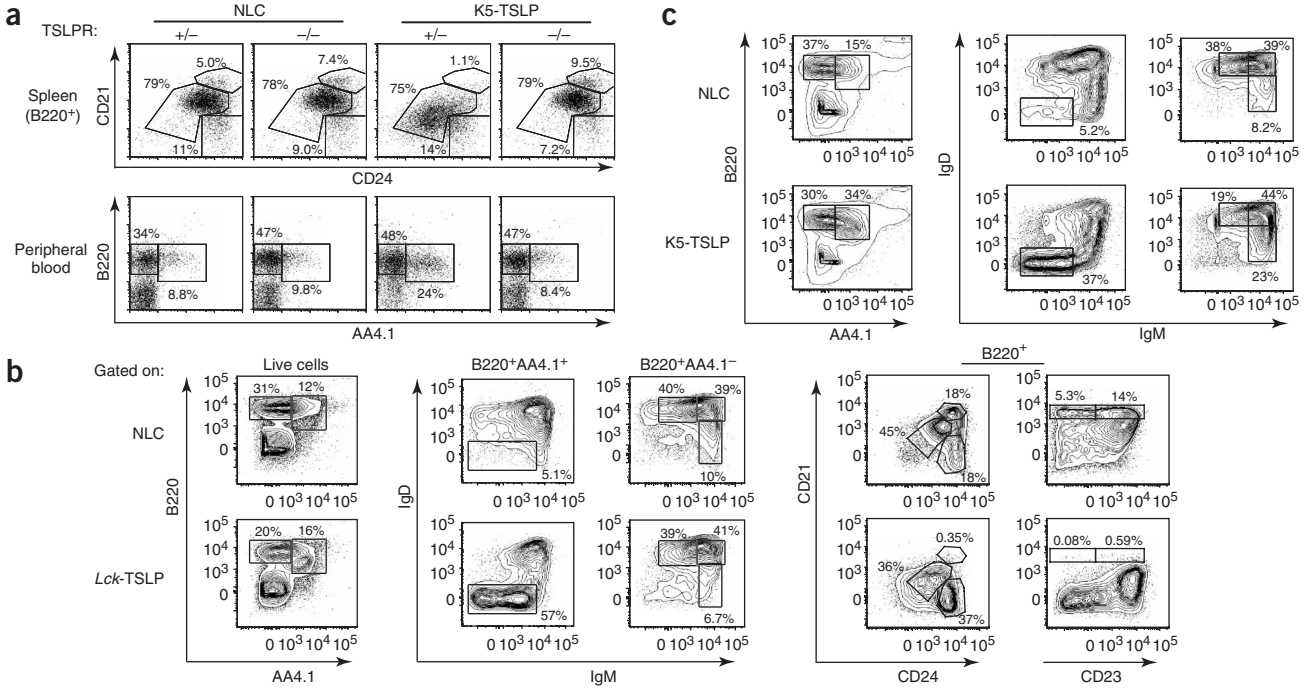

Figure 6 The B cell phenotype of K5-TSLP mice requires TSLPR expression and occurs in other models of TSLP overexpression. (**a**) CD21, CD24 and B220 staining of splenocytes (top) or B220 and AA4.1 staining of peripheral blood lymphocytes (bottom) normal littermate control and K5-TSLP mice bred onto a background of heterozygous (+/–) or homozygous (–/–) TSLPR deficiency. (**b**) B cell composition of splenocyte samples from normal littermate control and *Lck*-TSLP mice; cells stained with various markers were analyzed on the basis of IgM, IgD and AA4.1 (left) or CD24, CD21 and CD23 (right). (**c**) B cell composition of peripheral blood from normal littermate control and K5-TSLP mice treated for 2 weeks with low-dose (0.1 mg/ml) doxycycline, analyzed on the basis of IgM, IgD, B220 and AA4.1 staining. Numbers adjacent to outlined areas indicate percent of gated cells. At least three mice were analyzed for each strain.

Table 2 Cryoglobulin formation in mice treated with doxycycline

Mouse	Sex	Dox	Cryoglobulins
NLC	M	1.0	–
K5-TSLP	M	1.0	+
K5-TSLP	M	1.0	+
NLC	M	0.1	–
NLC	M	0.1	–
NLC	M	0.1	–
NLC	F	0.1	–
NLC	F	0.1	–
NLC	F	0.1	–
K5-TSLP	M	0.1	+
K5-TSLP	M	0.1	+
K5-TSLP	F	0.1	+
K5-TSLP	F	0.1	+
K5-TSLP	F	0.1	+

Dox, doxycycline dose (in mg/ml); M, male; F, female; +, present; –, absent.

marginal zone precursors and marginal zone B cells and expansion of the B-1b subset (**Supplementary Fig. 3** and data not shown). These findings suggested that these aspects of the B cell phenotype in K5-TSLP mice are T cell independent and is the direct consequence of increased systemic TSLP concentrations.

TSLP-dependent B cell expansion occurs in other TSLP models

We next tested the direct involvement of TSLPR in the observed phenotype by crossing K5-TSLP mice with TSLPR-deficient mice[32]. Analysis of the B cell compartment from the resultant K5-TSLP TSLPR-knockout mice showed a normal wild-type B cell developmental profile (**Fig. 6a**). Specifically, the mice did not have greater relative numbers of peripheral immature B cells and their spleens had normal follicular mature and marginal zone B cell compartments (**Fig. 6a**). Therefore, TSLPR-dependent signals are needed to generate the altered B cell phenotype of K5-TSLP mice.

We noted similar patterns of B lineage expansion in an alternative model of TSLP expression and, notably, in conditions leading to very low or undetectable circulating TSLP. *Lck*-TSLP mice had an influx of immature AA4.1$^+$ B cells into the periphery, as well as splenomegaly, with expansion of the immature and mature B cell compartments and loss of marginal zone and marginal zone precursor B cells, as noted for K5-TSLP mice (**Fig. 6b**). We also found an influx of immature B cells in K5-TSLP mice treated with much lower doses of doxycycline (0.1 or 0.01 mg/ml; **Fig. 6c**). In these conditions, we could not detect measurable TSLP in the serum (**Table 1** and data not shown). These observations collectively demonstrated that even very low systemic concentrations of TSLP can specifically modulate B lymphopoiesis.

K5-TSLP mice develop early signs of autoimmunity

Lck-TSLP mice develop cryoglobulinemic glomerulonephritis and systemic inflammatory disease[17]. Cryoglobulinemia is characterized by presence of cryoglobulins, which are antibody complexes that precipitate from sera at low temperatures. As mixed cryoglobulins are often associated with autoimmune disease[33], we sought to determine whether the alterations in B cell development in K5-TSLP mice could lead to similar features. K5-TSLP mice treated with low doses of doxycycline developed cryoglobulins within about 4 weeks of treatment (**Supplementary Fig. 4** online). We found cryoglobulin formation in all mice treated with either high-dose (1 mg/ml) or low-dose (0.1 mg/ml) doxycycline (**Table 2**). Furthermore, using periodic acid Schiff staining, we detected cryoglobulin deposits in subendothelial locations in glomerular capillary walls and in mesangial regions in the

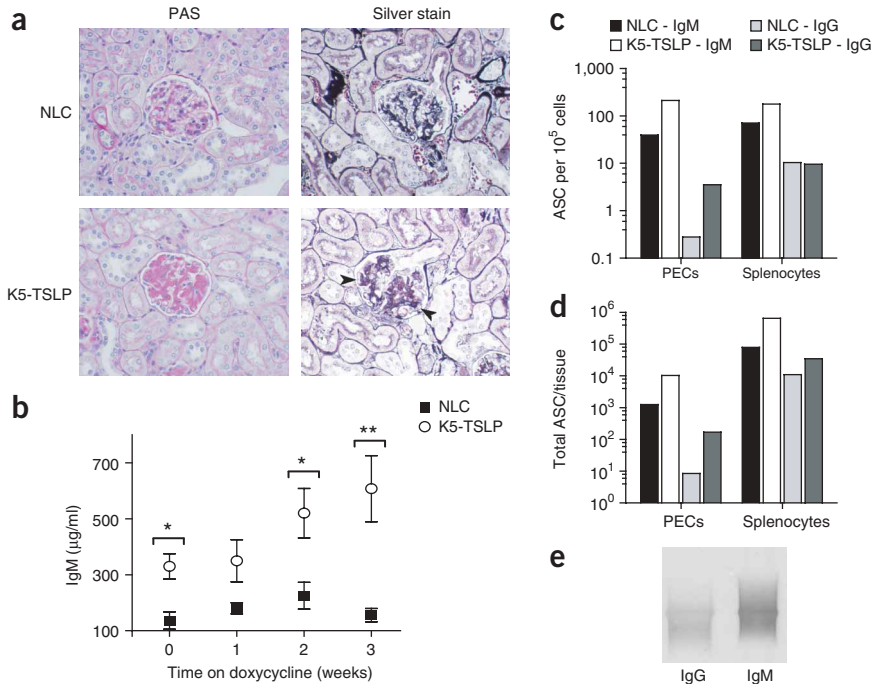

Figure 7 B cell population expansion in K5-TSLP mice leads to cryoglobulinemia and higher antibody production. (**a**) Histological sections of kidneys from normal littermate control or K5-TSLP mice treated with doxycycline (0.1 mg/ml) for 3 weeks, stained with periodic acid Schiff (PAS) or periodic acid–methenamine–silver (Silver stain) to demonstrate cryoglobulin deposits or mesangial matrix expansion, respectively. The periodic acid Schiff staining (left) shows many globules of acellular material typical of cryoglobulin in mesangial regions and in subendothelial and intraluminal locations in glomerular capillaries. Arrowheads (bottom right) indicate increased mesangial matrix. Original magnification, ×400. (**b**) ELISA of sera IgM titers in blood obtained 'sequentially' from mice treated with 1 mg/ml doxycycline. *, $P < 0.05$, and **, $P < 0.01$ (Student's *t*-test). Data are from one of at least two independent experiments; error bars, s.d. of data from four mice. (**c**,**d**) ELISPOT analysis of the proportion (**c**) and total number (**d**) of antibody-secreting cells (ASC) in the spleen (Splenocytes) or peritoneum (PECs). Key includes isotype of antibody secreted (IgG or IgM); vertical axes are on a log scale. Results are averages of data from at least two mice. (**e**) Immunofixation analysis of the isotype composition of cryoglobulins isolated from the serum of mice treated with 1 mg/ml of doxycyline. Representative of data from at least five mice.

kidneys of all K5-TSLP mice (**Fig. 7a**). That finding was coupled with increased mesangial matrix (**Fig. 7a**, silver stain, arrowheads) and irregular thickening and duplication of glomerular capillary basement membranes. Such findings are characteristic of cryoglobulinemic glomerulonephritis and early-stage autoimmune disease.

We analyzed antibody production by K5-TSLP mice to determine the cellular sources mediating the cryoglobulinemia. Compared with normal control mice, K5-TSLP mice had a significant increase in circulating IgM after doxycycline administration ($P < 0.01$; **Fig. 7b**), which correlated with eightfold more IgM-secreting cells in both the spleen and the peritoneum, as determined by enzyme-linked immunospot (ELISPOT) assay (**Fig. 7c**,**d**). Furthermore, the number of IgG-secreting cells was also 20-fold higher in the peritoneum and 3-fold higher in the spleen (**Fig. 7c**,**d**). Consistent with those findings, the cryoglobulins generated by K5-TSLP mice were composed of both polyclonal IgM and IgG (**Fig. 7e**). *Lck*-TSLP mice also had a similar increase in IgG- and IgM-secreting cells (data not shown). These data collectively suggested that both B1- and B2-derived B cell subsets probably contribute to the generation of mixed cryoglobulins in K5-TSLP mice, potentially leading to a breakdown in tolerance and autoimmunity.

DISCUSSION

Despite emerging knowledge about the involvement of TSLP in the development of allergic disease, little is known about how local increases in TSLP may affect B cell homeostasis. We have shown here that physiologically relevant TSLP expression enhanced B cell lymphopoiesis, causing increases in pre-BCR$^+$ B cell subsets and expansion of all immature and mature B cell populations in the periphery, with the notable exception of marginal zone B cells. These TSLP-induced alterations in B cell homeostasis required TSLPR expression and occurred independently of T cell function.

A published report used various genetic models to identify late pro–B cells (B220$^+$CD43$^+$BP-1$^+$CD24$^+$) as the principal TSLP-responsive target[22]. Furthermore, another study has shown that pre-BCR expression is required for TSLP responsiveness[23]. Our data further support those findings by showing that TSLP targets mainly late pro–B cells in adult bone marrow, in part by inducing the transcription of cell cycle–related genes. Based on these findings, we propose that TSLP stimulation promotes cell cycle progression, whereas pre-BCR signaling may act in synergy with these signals in part through upregulation of genes encoding survival molecules such as Bcl-x_L or Bcl-2.

Both TSLP and IL-7 act on early stages in B cell maturation and signal through receptors containing IL-7Rα[7,34]. However, IL-7 acts mainly on early lymphoid stem cells and pre-pro–B cell subsets, whereas TSLP stimulates either fetal or pre-BCR-expressing B cell subsets[22]. Despite such differences, both IL-7- and TSLP-transgenic mice develop splenomegaly and lymphadenopathy with expansion of follicular B cell populations, concurrent with the persistence of immature B cells in the periphery[35–38]. IL-7-transgenic mice, as well as several other models of enhanced bone marrow B cell survival, also have defects in marginal zone development[38–40]. We have shown here that partial disruption of splenic microarchitecture correlated with this decrease in the marginal zone B cell compartment and with less Notch signaling in splenic B cells, despite stable expression of Notch ligands in total splenoctyes. Our data suggest that the enlarged immature B cell compartment may alter the microenvironmental cues, including Notch–Notch ligand interaction, thereby limiting the development and/or maintenance of marginal zone B cells. However, it remains possible that TSLP-induced cytokines such as IL-4 potentiate the survival of immature B cell in the periphery, enhancing microarchitectural disruption and loss of marginal zones. Experiments with cytokine-deficient mice are needed to examine this possibility.

Notably, whereas the B220$^+$CD5$^-$ B-1b B cell subset was 'preferentially' greater in K5-TSLP mice, IL-7-transgenic mice had B-1 cell numbers and a B-1a cell/B-1b cell ratio equivalent to those of wild-type mice[38]. A TSLP-responsive fetal or bone marrow–derived B-1 progenitor population has been identified[30], supporting a model in which the B-1a–versus–B-1b lineage 'choice' is temporally restricted[41]. Our data further support the idea of such a dichotomy, as increasing TSLP concentrations in neonates and adult transgenic mice led to expansion of the B-1b B cell population, whereas TSLP expression during both fetal and neonatal development led to enlargement of both the B-1a and B-1b subsets. B-1 expansion correlated with expansion of the CD19$^+$B220$^-$ B-1 progenitor population, further supporting the idea of a developmental link between these subsets.

Of note, our findings contradict a published report in which multilineage, ubiquitous TSLP overexpression driven by the promoter of the gene encoding β-actin resulted in a decreased rate of bone marrow B cell lymphopoiesis[18]. TSLP can potently stimulate and expand bone marrow myeloid populations[6], and 'actin-TSLP–transgenic mice' have greater myeloid populations in the central and peripheral immune organs[18]. Notably, we have found a similarly extensive population expansion of bone marrow F4/80$^+$Gr-1$^+$ myeloid progenitors in mice expressing TSLP under control of the myeloid-specific *Fes* promoter (S.F.Z., unpublished observations). Those observations suggest that very high systemic and/or local (bone marrow) concentrations of TSLP can lead to less B cell lymphopoiesis due to enhanced myelopoiesis. In contrast, our data indicate that even very low or undetectable serum concentrations of TSLP are sufficient to promote abnormal B cell development, indicating that previous findings regarding a negative function for TSLP in B cell development were probably misinterpreted.

Notably, our findings suggest that B lineage progenitors are responsive to alterations in systemic TSLP concentrations that are undetectable by the present ELISA-based assays (concentration, 25 pg/ml or less). Treatment of K5-TSLP mice with low-dose doxycycline (0.1 or 0.01 mg/ml) was sufficient to accelerate B cell lymphopoiesis, as demonstrated by expansion of immature B cell populations in peripheral blood in the absence of detectable systemic concentrations of TSLP. Although we did not detect serum TSLP or TSLP mRNA in tissues other than the epidermis (data not shown), we did not exclude the possibility that the keratin 5 promoter might induce TSLP expression in the bone marrow. However, additional studies using localized intradermal TSLP injection challenge the idea of such a possibility. In such conditions, mice treated with TSLP intradermally also replicate the B cell phenotype of the K5-TSLP mice. This result also occurs in the absence of detectable serum TSLP (M.R. Comeau and S.F.Z.; unpublished observations). The sensitized response of B cell progenitors to TSLP suggests that this cytokine may have evolved as a potent modulator of humoral immune responses triggered by localized antigenic challenge in the skin or other epithelial sites and that mice deficient in TSLP or TSLPR may be more susceptible to localized infectious challenges.

Although small amounts of TSLP expressed during an infectious challenge may potentiate humoral immunity, increased concentrations of TSLP in allergic inflammatory disease may promote a break in B cell tolerance. Consistent with that idea, small increases (undetectable at present) in circulating TSLP were sufficient to promote B lineage–dependent autoimmunity, as demonstrated by cryoglobulin production and tissue deposition in all mice evaluated. Cryoglobulin

formation is not unique to the K5-TSLP model, as cryoglobulin deposition and kidney disease has also been reported for *Lck*-TSLP mice[17]. The cryoglobulin deposition was associated with more cells secreting IgM and IgG in the peritoneum and the spleen, suggesting a contribution from both B1- and B2-derived subsets. Furthermore, whereas other cytokines may help promote cryoglobulin formation in the periphery, TSLP is required for the initial expansion of both B1 and B2 bone marrow B cell progenitor populations essential for these events.

Although our findings have shown that TSLP can directly stimulate the proliferation of adult B cell precursors, another report has suggested that TSLP can stimulate mouse but not human B cells[6]. That conclusion is in direct contrast, however, to our experiments showing that TSLP promoted the differentiation of $CD19^+VpreB^+$ pre–B cells derived from $CD34^+$ human cord blood progenitors (D.J.R., unpublished data). Based on those observations, we anticipate that alterations in systemic TSLP concentrations probably stimulate early B cell precursors in both humans and mice. Our findings collectively suggest a link between inflammatory triggers and normal B cell homeostasis. Even small increases in systemic TSLP may be sufficient to mediate enhanced B cell lymphopoiesis and expansion of specific B cell subsets, and chronic stimulation may override at least some of the controls orchestrating B cell tolerance. Additional studies with the models described here will allow further testing of these ideas.

METHODS

Mice. The generation of K5-TSLP, K5-TSLP *Tcrb*$^{-/-}$, TSLPR-knockout and *Lck*-TSLP mice has been described[15,17,32]. K5-TSLP mice had a mixed SV29-BALB/c background, so nontransgenic and tetracycline-responsive single-transgenic littermates were used as controls. Unless otherwise stated, mice began receiving 1 mg/ml of doxycycline between 4 and 8 weeks of age and were analyzed after 3 weeks of treatment. All strains were housed in specific pathogen–free conditions and studies were done according to guidelines of the University of Washington, Benaroya Research Institute and Children's Hospital Animal Care and Use Committee.

Antibodies and flow cytometry. Single-cell suspensions from bone marrow, spleen, peripheral blood and the peritoneal cavity were incubated for 15 min at 4 °C with fluorescence-labeled antibodies in staining buffer (0.5% (wt/vol) BSA or 2.5% (vol/vol) FCS in PBS). Data were collected on a FACSCalibur or LSR II flow cytometer (BD Biosciences) and were analyzed with FlowJo software (TreeStar). For experiments with the LSR II, data were analyzed with the biexponential transformation function for complete data visualization[42]. The following antibodies were used for staining: fluorescein isothiocyanate–conjugated antibody to CD24 (anti-CD24; M1/69), phycoerythrin-conjugated anti-CD21 (7G6), biotin-conjugated anti-CD5 (53-7.3), phycoerythrin-conjugated anti-CD11b (anti-Mac1; M1/70), biotin-conjugated anti-CD43 (S7), peridinine chlorophyll protein– and cyanine 5.5–conjugated IgM (II/41) and allophycocyanin-strepatividin (all from BD Biosciences); phycoerythrin-conjugated IgD (11-26; Southern Biotechnology); and indodicarbocyanine-conjugated IgM (Jackson Labs). The following monoclonal antibodies were from eBioscience: phycoerythrin-conjugated anti-IL-7Rα (A7R34), phycoerythrin- and indotricarbocyanine-conjugated anti-B220 (RA3-6B2), biotin-conjugated anti-AA4.1 (AA4.1) and phycoerythrin-conjugated anti-BP1 (FG35.4). Phycoerythrin-indotricarbocyanine-streptavidin conjugates were also from eBioscience. Allophycocyanin-conjugated anti-CD23C (B3B4) and Alexa Fluor 700–conjugated anti-CD19 (6D5) were from Caltag laboratories. Anti-CD11b, anti-Gr-1, anti-CD3 and anti-IgM, all conjugated to fluorescein isothiocyanate, were used for staining for lineage markers. Anti-TSLPR (22H9)[24] was conjugated to Alexa Fluor 647 according to the manufacturer's instructions (Molecular Probes). For sorting, cells were labeled with specific antibodies in staining buffer and were filtered through a 35-μm mesh before sorting. Cells were sorted with a FACSAria and FACSDiVa software (BD Biosciences).

Immunocytochemistry. Mouse spleens were collected, were embedded in optimum cutting temperature compound and were frozen in liquid nitrogen–cooled isopentane. Cryostat sections 7 μm in thickness were air-dried for 30 min and were then fixed for 30 min with cold acetone and were stored at –70 °C until staining. For staining, slides were allowed to reach 25 °C and were washed with PBS. Sections were blocked with a solution of 1% (wt/vol) BSA and 1% (vol/vol) goat serum and were stained with indodicarbocyanine-conjugated IgM (155-177-020; Jackson Labs), fluorescein isothiocyanate-conjugated IgD (clone 11-26c.2a; BD Bioscience) or biotin-conjugated anti-MOMA-1 (clone MOMA-1; BMA Biomedicals). Biotin was detected with allophycocyanin-streptavidin. Slides were mounted in aqueous Gel/Mount (Biomeda) and were viewed on a Leica fluorescent microscope. Pictures were obtained with 10× and 20× lenses and Advanced SPOT software (Diagnostic Instruments). Image contrast and brightness were adjusted with the 'auto-levels' feature in Adobe Photoshop.

Real-time RT-PCR. Splenic B cells were sorted with anti-B220 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). Alternatively, flow cytometry–sorted B cell subsets were cultured for 15 h with TSLP or IL-7 and were 'snap frozen' at –80 °C. RNA was extracted with the RNeasy mini kit (Qiagen) and single-stranded cDNA was synthesized with SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). Primers were designed with Vector NTI software (Invitrogen) and were manufactured by MWG Biotech. Primer sequences were as follows: cyclin D2 forward, 5′-GCCAAGATCACCCACACT-3′, and reverse, 5′-GCTGCTCTTGACGGAACT-3′; c-Myc forward, 5′-TCTCCACTCACCAGCACAACTACG-3′, and reverse, 5′-ATCTGCTTCAGGACCCT-3′; β_2-microglobulin forward, 5′-CTTCAGTCGTCAGCATGGCTCG-3′, and reverse, 5′-CTTCAGTCGTCAGCATGGCTCG-3′. Primers for Deltex and Notch ligands have been published[43]. The iCycler real-time PCR detection system with iQ SYBR Green Supermix were used for PCR, and primer efficiency and change in threshold values were determined with iQ optical analysis software (BioRad). Products were amplified with a standard PCR protocol (35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) and primer specificity was confirmed by melting curve and ethidium bromide gel analysis. Gene expression was determined with the Pfaffl mathematical model for relative quantification of RT-PCR[44], with the 'housekeeping' gene encoding β_2-microglobulin serving as the control.

[^{3}H]thymidine uptake proliferation assay. For the B cell proliferation assay, 5×10^4 cells were cultured in complete medium (RPMI medium with 10% (vol/vol) FCS, glutamine, HEPES, pH 7.2, 2-mercaptoethanol, penicillin and streptomycin) plus 5 ng/ml of TSLP (R&D Systems) or IL-7. Cells were cultured for 48 h in duplicate or triplicate, with 1 μCi [^{3}H]thymidine added for the last 12 h of culture. Proliferation was determined with a scintillation counter.

Renal histology and cryoglobulin detection. Kidneys were collected and fixed as described[17]. Kidneys were fixed in 10% (vol/vol) formalin, were cut into sections 2 μm in thickness and were stained with periodic acid Schiff or with periodic acid–methenamine–silver. Histopathology, including the presence of overt cryoglobulin deposits, mesangial expansion, cellularity, leukocyte infiltration and glomerular capillary wall changes, was evaluated by two independent pathologists 'blinded' to sample identity. For cryoglobulin isolation, blood was allowed to clot and serum was centrifuged and was stored for several days at 4 °C. Cryoprecipitates were identified visually and were redissolved after warming. 'Cryotyping' was done as described[17]. Cryoprecipitates were washed two to three times with PBS, were resuspended in PBS and were separated by 0.8% agarose gel electrophoresis. Gels were incubated with anti-IgM (m8644) and anti-IgG (m1397; Sigma) and were stained with brilliant blue.

ELISA and ELISPOT assay. ELISA for IgM or TSLP was done as described[14]. Nunc Maxisorp ELISA plates (96-well; Fisher) were coated overnight with anti-mouse IgM (1020-01; Southern Biotech), were washed three times with 0.05% (vol/vol) Tween 20 in PBS, were blocked with 2% (wt/vol) BSA in PBS and were incubated for 1 h with serial dilutions of serum, followed by horseradish peroxidase-conjugated anti-mouse IgM (1020-05; Southern Biotech). Plates were washed with 0.05% (vol/vol) Tween 20 in PBS containing Mg^{2+} and were incubated with TMB substrate (3,3′,5,5′-tetramethylbenzidine;

BD Biosciences), and absorbance was measured at 405 nm on a Bio-Rad microplate reader. Immunoglobulin concentrations were quantified by comparison with titrated immunoglobulin standards.

ELISPOT detection of antibody-secreting cells has been described[45]. MultiScreen filter plates were coated overnight with anti-IgM (1020-01) or anti-IgG (1030-01) in PBS (5 μg/ml; Southern Biotech). Plates were washed with PBS and were blocked with 10% (vol/vol) FCS in complete media. Approximately 0.5×10^6 cells were plated in triplicate and were diluted 1:2 in six rows. Cells were cultured overnight and plates were washed six times with PBS before being incubated with horseradish peroxidase-conjugated anti-IgM (1020-05) or anti-IgG (1030-05; 1:2,000 dilution; Southern Biotech). Plates were developed with the AEC developer kit (3-amino-9-ethyl carbazole; Vector Labs) and spots were counted with an ImmunoSpot counter and software (Cellular Technology).

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

D.J.R. and S.F.Z. designed experiments; A.A, M.O., T.N. S.B.-H., M.I. and T.A. designed and did experiments; K.H. and C.E.A. contributed reagents and did kidney immunochemistry; J.D. and A.F generated transgenic mice and contributed reagents; and A.A., S.F.Z. and D.J.R. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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Erratum: Local increase in thymic stomal lymphopoietin induces systemic alterations in B cell development

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In the version of this article initially published, the second subheading on page 523 is incorrect. The correct subheading should be TSLP stimulates the population expansion of late pro–B cells. The error has been corrected in the HTML and PDF versions of the article.