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Development of nephritis but not sialadenitis in autoimmune-prone BAFF transgenic mice lacking marginal zone B cells

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B cell-activating factor belonging to the TNF family (BAFF) is a B cell survival factor required for B cell maturation. BAFF transgenic (Tg) mice develop autoimmune disorders characterized by autoantibody production, which leads to nephritis and salivary gland destruction (sialadenitis), features reminiscent of systemic lupus erythematosus and Sjögren's syndrome (SS), respectively. Disease in BAFF Tg mice correlates with the expansion of the marginal zone (MZ) B cell compartment and the abnormal presence of MZ-like B cells in the blood, LN and inflamed salivary glands, suggesting a role for these cells in BAFF-induced autoimmunity. Lymphotoxin-β (LTβ)-deficient mice show disrupted splenic architecture, lack MZ B cells and some peripheral LN, and are unable to mount T cell-dependent immune responses. BAFF Tg mice lacking LTβ (LTβ△-BTg) retained these defects, yet still developed nephritis associated with the presence of B-1 B cells in the kidneys. However, in contrast to old BAFF Tg mice, aging LTβ△-BTg mice no longer developed sialadenitis. Thus, autoimmune disorders in BAFF Tg mice are possibly events coordinated by MZ and B-1 B cells at separate anatomical sites.

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Introduction

B cell-activating factor belonging to the TNF family (BAFF, also termed BLyS, TALL-1, zTNF-4, THANK, and TNFSF13b) has emerged as a cytokine critical for peripheral B cell survival and maturation (reviewed in [1–4]). BAFF supports the survival of both splenic immature transitional and mature B cells, and maturation beyond the immature transitional type 1 (T1) stage is impaired in BAFF-deficient mice ($BAFF^{-/-}$) [5–7]. In addition, BAFF supports T-independent isotype switching from IgM to IgA [8, 9]. Mice overexpressing BAFF (BAFF Tg mice) develop autoimmune disorders similar

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Abbreviations: **AP:** alkaline phosphatase · **BAFF:** B cell-activating factor belonging to the TNF family · **Fo:** follicular · **LT:** lymphotoxin · **MZ:** marginal zone · **PerC:** peritoneal cavity · **PLN:** peripheral lymph nodes · **SS:** Sjögren's syndrome · **T1(2):** transitional type 1(2)

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to systemic lupus erythematosus [10] and Sjögren's syndrome (SS) [11], possibly due to improper B cell survival, which predominantly affects splenic immature and marginal zone (MZ) B cell populations [6]. Indeed, when the hen egg lysozyme Tg system was used to assess the effect of excess BAFF production on B cell tolerance, results revealed a BAFF-induced escape of low/intermediate affinity self-reactive B cells, in particular MZ B cells [12]. The number of effector T cells is higher in BAFF Tg mice, yet whether this aspect contributes to disease remains unknown [13, 14].

The splenic MZ B cell compartment contains both non-circulating naïve B cells and some memory B cells, which are particularly responsive to blood-borne Ag in a T cell-independent manner (reviewed in [15, 16]). MZ B cells have been shown to be potentially poly-reactive (dual receptor) and/or self-reactive [17, 18] and their expansion is often associated with autoimmune disorders in mice [19, 20]. In BAFF Tg mice, numbers of splenic MZ B cells are increased, and MZ-like B cells reside outside of the spleen, in the blood, LN and in inflamed salivary glands of BAFF Tg mice [6, 11]. The abnormal presence of MZ-like B cells outside the spleen may be important, as sequestration of MZ B cells in the MZ appears to be essential to preserve immune tolerance [17]. However, a direct connection between MZ B cell activation and kidney damage has never been made. This issue prompted us to study a model in which MZ B cells are absent.

Lymphotoxin-α/β (LTα/β) is a TNF-like ligand essential for peripheral LN (PLN) development, lymphoid organ architecture and T-dependent immune responses (reviewed in [21, 22]). Mice lacking LTα/β lack MZ B cells [23]. LTα/β, expressed on activated lymphocytes, binds to the LTβR, a receptor expressed on non-lymphoid cells, double negative thymocytes and γ/δ T cells [24], and is shared by another TNF-like ligand LIGHT (HVEML) [22]. We generated BAFF Tg mice lacking LTβ (LTβ△-BTg) in which MZ B cells are absent to study the specific role of MZ B cells in the progression of autoimmunity in BAFF Tg mice. We showed that lack of MZ B cells in LTβ$^{-/-}$ mice correlated with impaired survival of the MZ B cell precursors. LTβΔ-BTg mice lacking MZ B cells developed nephritis but were protected from severe sialadenitis.

Results

BAFF overexpression in LTβ$^{-/-}$ mice restores the T2MZ compartment and promotes B-1 B cell expansion

LTβΔ-BTg mice were generated and showed the same lymphoid defects as those described in LTβ$^{-/-}$ mice [25]. As in LTβ$^{-/-}$ mice, LTβ△-BTg mice develop mesenteric LN and cervical LN (data not shown) but not other PLN (Fig. 1A, inguinal PLN shown) or Peyer's patches (data not shown). LTα/β is required for MZ B cell formation, presumably due to its role in maintaining the splenic MZ structure, and expression of adhesion molecules and chemokines essential for MZ B cell differentiation and homing to the MZ [23]. Signaling through the LTβR in splenocytes results in BAFF expression [26], which locally may also be important for MZ B cell differentiation [27]. To investigate the possibility that BAFF overexpression may promote splenic MZ B cell development independently of MZ homing, we analyzed splenic B cell subsets in 6-month-old LTβ△-BTg mice by FACS analysis as previously described [28–30]. FACS analysis confirmed the absence of MZ B cells in both the LTβ$^{-/-}$ and the LTβ△-BTg animals (Fig. 1B and C), indicating that the defect in final MZ B cell development in LTβ$^{-/-}$ mice is independent of LTα/β-mediated BAFF production.

We analyzed two populations of transitional type 2 (T2) B cells, the T2 follicular (Fo) and T2MZ B cells, thought to be precursors of Fo and MZ B cells, respectively [29, 30]. Numbers of T2MZ but not T2Fo B cells were significantly reduced in LTβ$^{-/-}$ mice (Fig. 1C), agreeing with the notion that T2MZ B cells are precursors of MZ B cells [31]. BAFF overexpression in LTβ△-BTg mice normalized T2MZ B cell numbers (Fig. 1C), suggesting that the MZ B cell defect seen in LTβ$^{-/-}$ mice is partially linked to a deficit in T2MZ B cell survival. Similar to BAFF Tg mice, numbers of splenic Fo B cells in LTβ△-BTg mice were increased (Fig. 1C). Numbers of B-1a but not B-1b B cells were reduced in LTβ$^{-/-}$ mice, and overexpression of BAFF in LTβ△-BTg mice normalized B-1a B cell numbers in the spleen (Fig. 1C).

Analysis of B cells in the peritoneal cavity (PerC) of LTβ△-BTg mice revealed significantly greater B cell numbers than in BAFF Tg mice, these were primarily Fo B cells but also B-1a and B-1b B cells (Fig. 1C). Similar to the spleen, this analysis showed that B-1a but not B-1b B cell numbers were reduced in the peritoneum of LTβ$^{-/-}$ mice (Fig. 1C).

As previously described by us, the proportion of effector T cells is greater in BAFF Tg mice compared to control mice [10] and is a B cell-dependent phenomenon [14], perhaps due to increased MZ B numbers, which are particularly efficient as APC to naïve T cells [32]. The proportion of effector T cells was similar in BAFF Tg mice and LTβ△-BTg mice (Fig. 1D), suggesting that in response to excess BAFF production, B cells other than MZ B cells can support the expansion of effector T cells. As LTα/β signaling triggers BAFF expression in the spleen [26], we analyzed BAFF mRNA levels in whole spleen from WT and LTβ$^{-/-}$ mice. As expected, BAFF

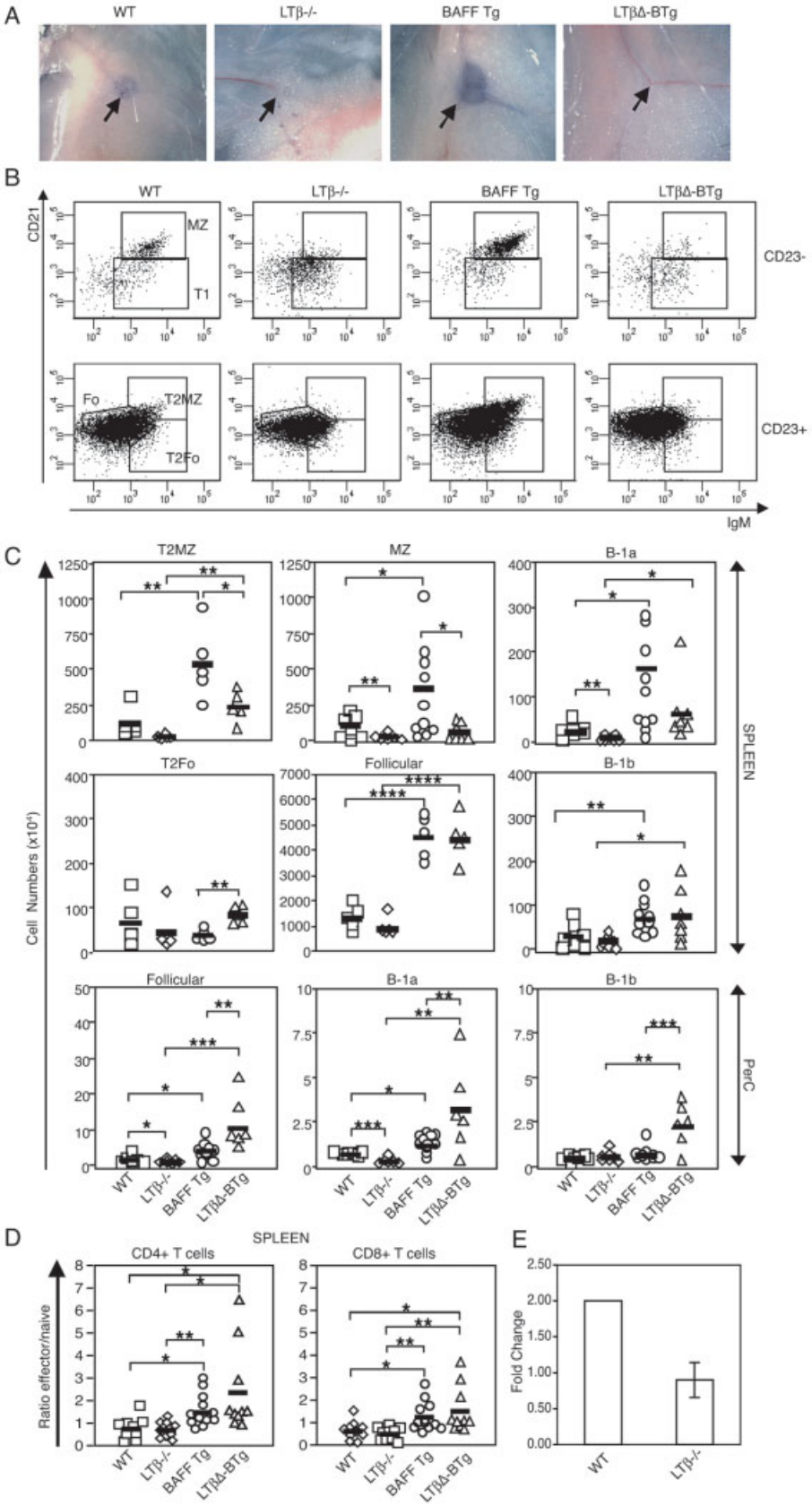

Figure 1. The effect of excess BAFF production in mice lacking LTβ (A) Inguinal LN (indicated by arrows) of WT, LTβ$^{-/-}$, BAFF Tg and LTβ△-BTg mice visualized by footpad injection of ink (magnification 2x). (B) Representative CD21/IgM FACS scatter plots of B220^{+} splenocytes gated on CD23^{+} or CD23^{-} B cell populations as indicated for each mouse group. (C) Absolute numbers of cells in each B lymphocyte subset, as indicated, isolated from the spleen and PerC of WT (□), LTβ$^{-/-}$ (◊), BAFF Tg (○) and LTβ△-BTg (△) mice, stained and gated as described in *Material and methods*. (D) The ratio of splenic effector/memory over naïve T cells for WT (□), LTβ$^{-/-}$ (◊), BAFF Tg (○) and LTβ△-BTg (△) mice, stained and gated as described in *Material and methods*. (E) Relative level of BAFF mRNA expression in splenic tissue from LTβ$^{-/-}$ mice compared to WT expression, shown as fold change (n = 5). In (C) and (D) symbols represent individual mice and lines indicate mean values.

levels were reduced in LTβ$^{-/-}$ spleens when compared to WT (Fig. 1E), although this may also be related to reduced numbers of dendritic cells in LTβ$^{-/-}$ mice [33]. Overexpression of BAFF in LTβ△-BTg mice restored the T2MZ population but not the MZ B cell population presumably due to the absence of splenic MZ structure in LTβ△-BTg mice. The MZ B cell defect in LTβ$^{-/-}$ mice is, therefore, the sum of impaired T2MZ B cell survival and lack of differentiation/homing signals.

BAFF overexpression in LTβ△-BTg mice normalizes serum but not fecal IgA levels

Similar to LTβR$^{-/-}$ mice [34], LTβ$^{-/-}$ mice have reduced serum IgA levels due to impaired migration of B lymphocytes to the lamina propria (Fig. 2A). In contrast, BAFF Tg mice have elevated levels of serum [10, 35] and fecal IgA (Fig. 2A and B). Serum IgA but not fecal IgA levels were normalized in LTβ△-BTg mice compared to LTβ$^{-/-}$ mice (Fig. 2A and B), supporting the notion that blood IgA can originate from a different source than fecal IgA [36, 37]. Like LTβ$^{-/-}$ mice, numbers of mucosal B-1 B cells in the gut of LTβ△-BTg mice remained reduced (data not shown), indicating that B cell homing to the gut is still impaired, which is in line with the reduced fecal IgA levels detected in these animals (Fig. 2B). BAFF promotes isotype switching to IgA [8, 9] and addition of BAFF to cultures of LPS-stimulated B cells led to greater IgA production from MZ B cells compared to Fo [38]. Using an MZ B cell-specific T-independent Ag NP-Ficoll [37, 39], we confirmed that BAFF Tg mice but not control mice secreted NP-specific IgA (Fig. 2C). NP-specific IgA and IgM responses were impaired in LTβ△-BTg mice confirming the absence of a functional MZ and responsive MZ B cells in these mice (Fig. 2C).

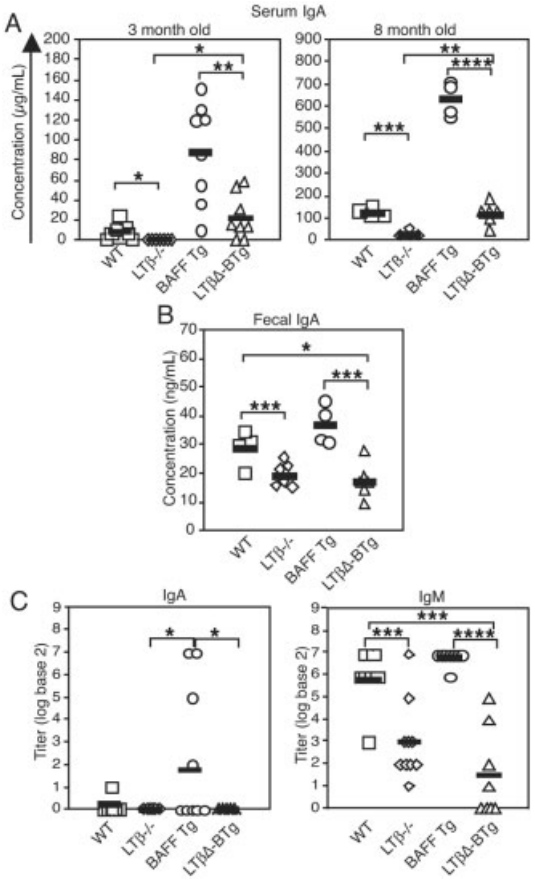

Figure 2. IgA levels are normalized in LTβ△-BTg mice from a source other than the lamina propria, but T-independent immune responses are reduced. (A) Total serum IgA levels, as determined by ELISA, from 3-month-old (left panel) and 8-month-old (right panel) WT (□), LTβ$^{-/-}$ (◊), BAFF Tg (O) and LTβ△-BTg (△) mice. (B) Total fecal IgA levels were determined by ELISA of solubilized fecal samples, from WT (□), LTβ$^{-/-}$ (◊), BAFF Tg(O) and LTβ△-BTg (△) mice. (C) NP-specific IgA and IgM Ab responses from indicated mice 7 days after immunization with the T-independent Ag NP-Ficoll, pre-immune sera from these mice had no detectable NP-specific IgA or IgM (Supplementary Fig. 1). In (A), (B) and (C) symbols represent individual mice and lines indicate mean values.

LTβ△-BTg mice develop nephritis associated with B-1 B cell infiltration in the kidneys

We measured the production of autoantibodies in LTβ△-BTg mice and found that these mice produced high levels of rheumatoid factors, anti-ssDNA, anti-dsDNA and anti-chromatin autoantibodies as seen in BAFF Tg mice (Fig. 3A). These mice also developed splenomegaly similar to BAFF Tg mice (Fig. 3A). Surprisingly, levels of anti-ssDNA autoantibodies and proteinuria were greater in LTβ△-BTg than age-matched BAFF Tg mice (Fig. 3A). Histochemical staining of kidney paraffin sections showed that both LTβ△-BTg and BAFF Tg mice developed nephritis, as shown by abnormally enlarged and segmented glomeruli in the kidneys of these mice (Fig. 3B). Examination of Ig deposition in the kidney of the BAFF Tg animals by immunohistochemistry revealed IgA and IgG2a as the predominant isotypes present (Fig. 3C). In contrast, in the kidney of LTβ△-BTg mice a predominance of IgG1 and IgG2a deposits were observed, but very little IgA (Fig. 3C). We extracted lymphocytes from the kidneys of these animals and analyzed B cells by FACS. We showed that in both BAFF Tg and LTβ△-BTg mice, B-1b cells infiltrate the inflamed kidneys (Fig. 3D). We observed a greater number of B-1a B cells in the kidney of LTβ△-BTg mice compared to BAFF Tg mice (Fig. 3D). Using ELISPOT assays we counted the number of IgG- and IgA-secreting cells in inflamed kidneys and showed increased numbers of IgG- but not IgA-secreting cells in the kidney of LTβ△-BTg mice in contrast to kidneys from BAFF Tg mice in which high numbers of IgA-secreting B cells are present (Fig. 3E). These results indicate that excess BAFF production induces IgA-independent nephritis in

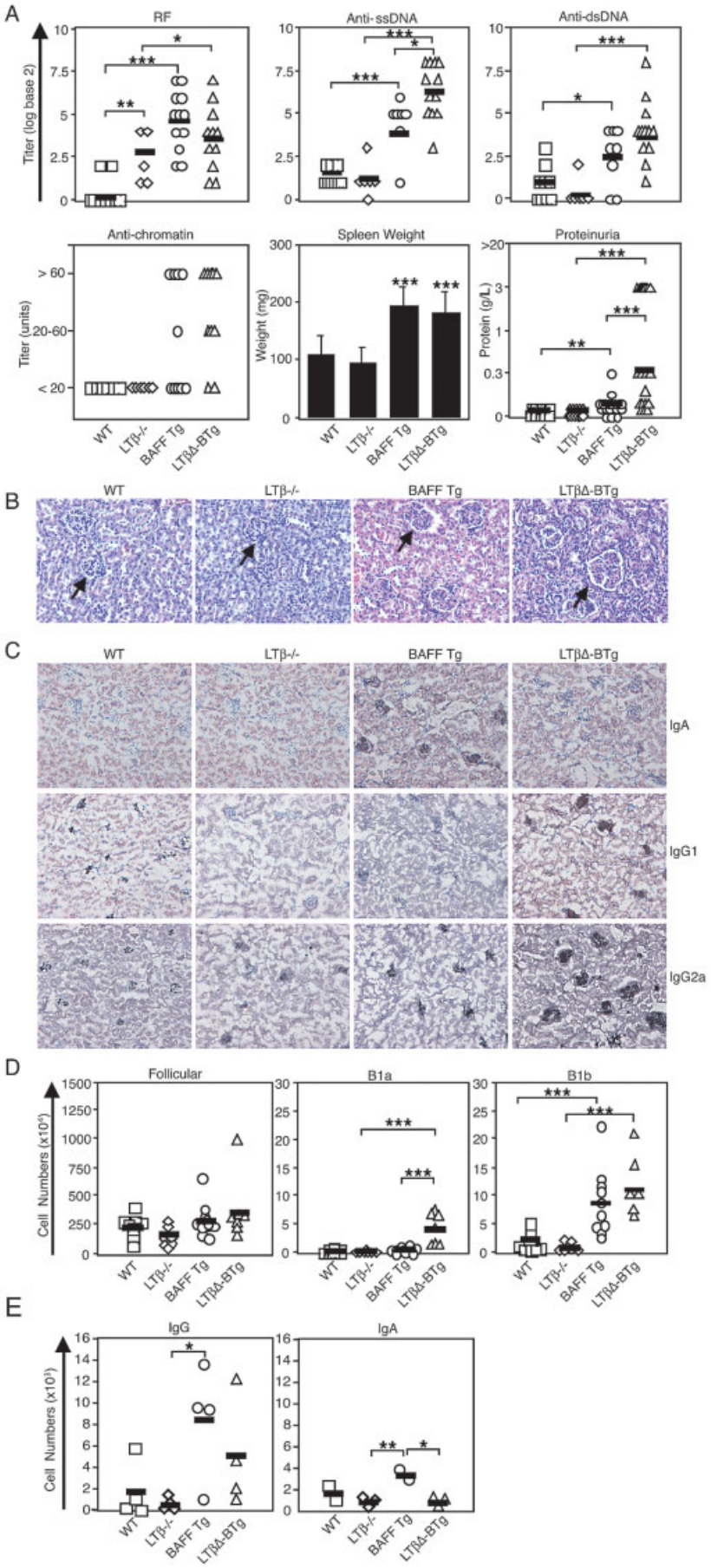

Figure 3. LTβ△-BTg mice develop nephritis and produce high levels of autoantibodies similar to BAFF Tg mice. (A) The 8-month-old WT (□), LTβ$^{-/-}$ (◊), BAFF Tg (O) and LTβ△-BTg (△) mice were assessed for serum autoantibodies for rheumatoid factor (upper left panel), ssDNA (upper middle panel), dsDNA (upper right panel) and chromatin (lower left panel), spleen weights (n = 6) (lower middle panel) and proteinuria (lower right panel). (B) Representative H&E-stained kidney tissue from 8-month-old WT, LTβ$^{-/-}$, BAFF Tg and LTβ△-BTg mice, arrows indicate glomeruli (n = 5) (magnification 10x). (C) Representative immunohistochemical staining for deposition of IgA (top panels), IgG1 (middle panels) and IgG2a (lower panels) in kidney glomeruli of 8-month-old WT, LTβ$^{-/-}$, BAFF Tg and LTβ△-BTg mice (n = 4) (magnification 10x). (D) Enumeration of lymphocytes infiltrating the kidneys of WT (□), LTβ$^{-/-}$ (◊), BAFF Tg (O) and LTβ△-BTg (△) mice, Fo (left panel), B-1a (middle panel) and B-1b (right panel) B cells were stained and gated as described in the *Material and methods*. (E) ELISPOT determination of the absolute number of IgG (left panel) and IgA (right panel) producing lymphocytes isolated from the kidney of WT (□), LTβ$^{-/-}$ (◊), BAFF Tg (O) and LTβ△-BTg (△) mice. In (A), (D) and (E) symbols represent individual mice and lines indicate mean values.

LTβ△-BTg mice even in the absence of most PLN and an organized splenic structure.

Overexpression of LIGHT, another ligand for the LTβR, triggers IgA-nephropathy-like symptoms [13]. We tested the possibility that LIGHT dysregulation may be the cause of nephritis in BAFF Tg mice by treating these animals with LTβR-Fc decoy receptor to neutralize both LTα/β and LIGHT. The treatment reduced MZ B cell numbers and disrupted splenic architecture, but showed no amelioration of glomerulonephritis and proteinuria (data not shown). We also treated LTβ△-BTg mice with LTβR-Fc, however, treatment was unable to protect LTβ△-BTg mice against nephritis (Fig. 4A and B), and did not stop autoantibody production (Fig. 4C) and Ig deposition in the kidneys (Fig. 4D). Thus, nephritis in BAFF Tg mice does not require MZ B cells or LIGHT/LTα/β expression.

LTβ△-BTg mice have reduced sialadenitis and improved saliva production

Aging BAFF Tg mice develop SS-like symptoms characterized by the infiltration of MZ-like B cells in the salivary glands ([11] and Fig. 5A and B)). Histological analysis revealed that salivary ducts are quite normal in 12-month-old LTβ△-BTg mice (Fig. 5A) and minimal cell infiltrates are observed (Fig. 5B and D). Saliva flow was reduced in BAFF Tg mice as previously described ([11] and Fig. 5C) and correlated with tissue destruction and large B cell infiltrates (Fig. 5A, B and D). Saliva flow was also reduced in LTβ$^{-/-}$ mice (Fig. 5C), despite little inflammation and no salivary gland tissue destruction (Fig. 5A and B), and no more B cell (Fig. 5D) and T cell (data not shown) infiltrates than in WT mice. LTβ△-BTg mice exhibited improved saliva flow compared to both LTβ$^{-/-}$ and BAFFTg mice (Fig. 5C). LTβ△-BTg mice also had minimal lymphocytic infiltrates and salivary gland destruction compared to BAFF Tg mice (Fig. 5*A*, B and D). In conclusion, BAFF-induced salivary gland destruction appears to correlate with the presence of large numbers of MZ B cells.

Discussion

Expansion of the MZ B cell compartment is a common feature observed in many mouse models of autoimmune disease [11, 19, 40, 41]. In addition, the MZ B cell compartment contains self-reactive B cells [15]. In the case of BAFF Tg mice, not only are the numbers of splenic MZ B cells augmented, but also MZ-like B cells can be abnormally detected in the blood, LN and in inflamed salivary glands of these mice [11]. Experiments investigating loss of B cell tolerance in BAFF Tg mice using the hen egg lysozyme self-Ag/B cell receptor system

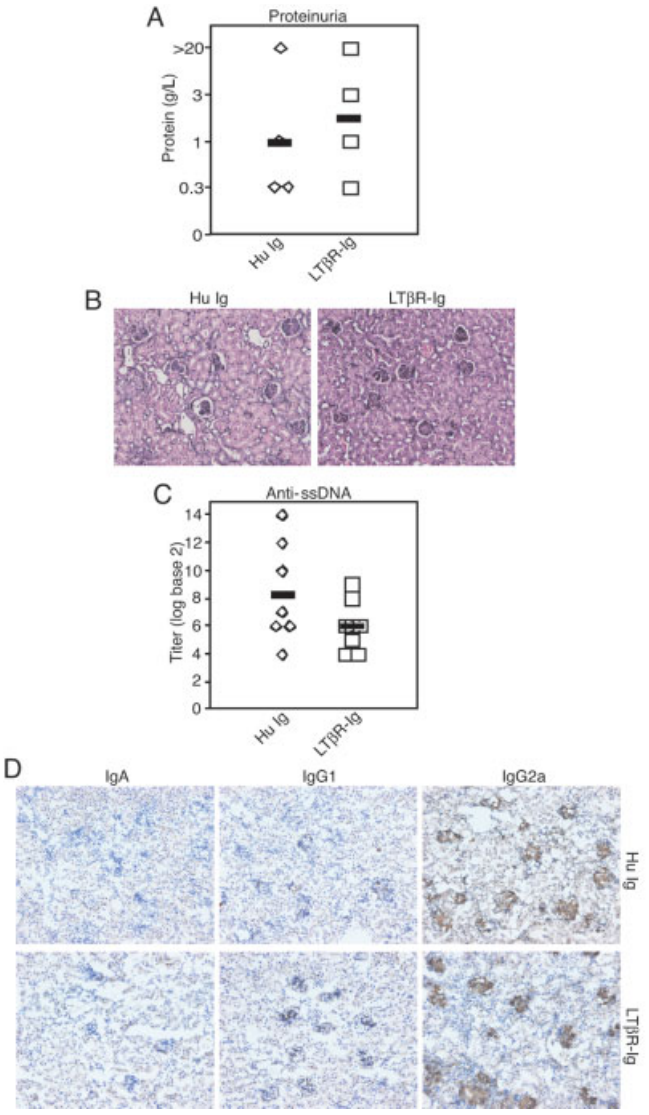

Figure 4. Treatment of LTβ△-BTg mice with LTBR-Fc fusion protein does not ameliorate nephritis or reduce autoantibody levels. (A) Protein levels in the urine of 8-month-old LTβ△-BTg mice treated for five weeks with either Hu Ig (◊) or LTβR-Fc (□) prior to analysis. (B) Representative H&E-stained kidney tissue sections from 8-month-old LTβ△-BTg mice treated with Hu Ig (left panel) or LTβR-Fc (right panel), (n = 4) (magnification 10x). (C) Levels of anti-ssDNA autoantibodies in 8-month-old LTβ△-BTg mice treated with either Hu Ig (◊) or LTβR-Fc (□). (D) Immunohistochemistry of deposition of IgA (left panels), IgG1 (middle panels) and IgG2a (right panels) in the kidney glomeruli of 8-month-old LTβ△-BTg mice treated with either Hu Ig (upper panels) or LTβR-Fc (lower panels) (n = 4) (magnification 10x). In (A) and (C) symbols represent individual mice and lines indicate mean values.

implicate the rescue of low/intermediate affinity self-reactive B cells, mostly MZ B cells, as the source of autoimmune B cells in BAFF Tg mice [12]. However, it is important to note that the size of the MZ B cell compartment is mouse strain-dependent, expansion of the MZ B cell compartment does not always correlate with

disease, and systemic lupus erythematosus symptoms can also develop in mice lacking MZ B cells [42, 43].

The splenic architecture of LTα/β-deficient mice is abnormal and lacks a structured MZ, which prevents MZ B cell development [44]. LTα/β-deficient mice lack some LN and, like $TNF^{-/-}$ mice, cannot mount normal T-dependent immune responses [21, 22], a defect that did not prevent full disease progression in $TNF^{-/-}$ x BAFF Tg mice [45] and was not thought to be an interfering factor in our cross.

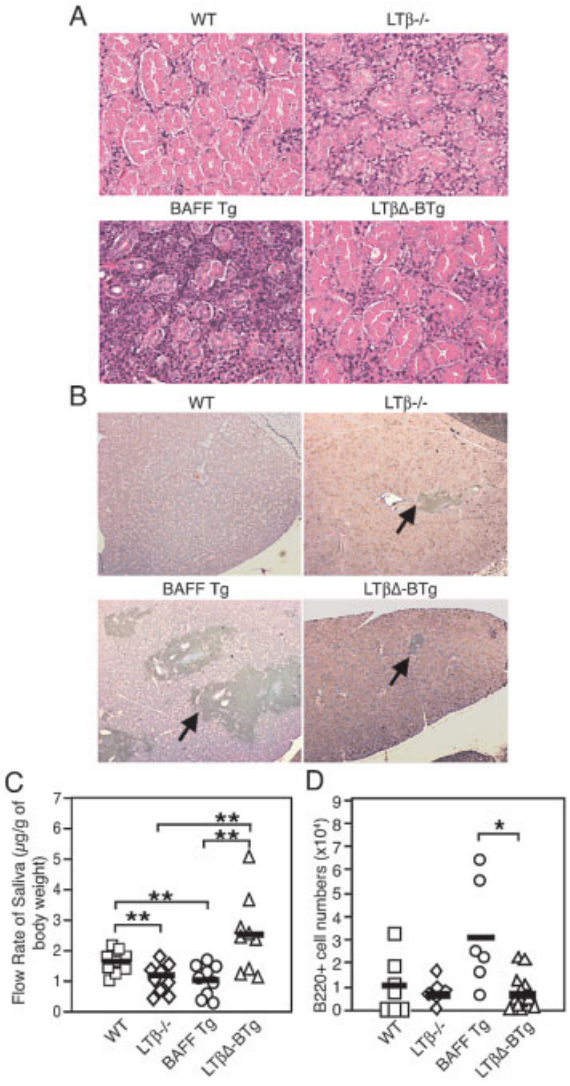

Figure 5. $LT\beta^{-/-}$ mice exhibit minimal salivary gland inflammation, which is not exacerbated by the overexpression of BAFF in LTβ△-BTg mice. (A) Representative H&E staining of sub-maxillary salivary gland tissue sections from 12–14-month-old WT, $LT\beta^{-/-}$, BAFF Tg and LTβ△-BTg mice (n = 5) (magnification 10x). (B) Immunohistochemical detection of $B220^+$ cells (brown) in salivary gland tissue from 12–14-month-old WT, $LT\beta^{-/-}$, BAFF Tg and LTβ△-BTg mice (n = 5) (magnification 10x), B cell infiltrates are indicated with arrows. (C) Assessment of saliva flow rate in 12–14-month-old WT (□), $LT\beta^{-/-}$ (◊), BAFF Tg (O) and LTβ△-BTg (△) mice. (D) Absolute number of $B220^+$ lymphocytes isolated from the submaxillary salivary gland of 12–14-month-old WT (□), $LT\beta^{-/-}$ (◊), BAFF Tg (O) and LTβ△-BTg (△) mice. In (C) and (D) symbols represent individual mice and lines indicate mean values.

LTβ$^{-/-}$ mice display a milder phenotype than LTα$^{-/-}$ or LTβR$^{-/-}$ mice, yet, the reason for this difference remains unclear [46]. The spleen of LTβ$^{-/-}$ mice, while lacking a MZ and MZ B cells, has better T/B cell segregation than other knockout mice in this system and develop cervical and mesenteric LN, a feature we thought could be important for local immune reactions in the salivary glands and kidneys, respectively [25]. A novel aspect of the MZ B cell deficiency in LTβ$^{-/-}$ mice was revealed in LβΔ-BTg mice. LTβ$^{-/-}$ mice have reduced numbers of T2MZ B cells, which are precursors of MZ B cells [30]. Upon BAFF overexpression, the numbers of T2MZ B cells returned to normal in LTβ△-BTg mice. This suggest that lack of MZ B cells in LTβ$^{-/-}$ mice may originate at two levels, impaired survival of T2MZ precursor cells and lack of MZ structure to support final MZ B cell differentiation/homing. T2 B cells express high levels of BAFF-R and are more dependent on BAFF for survival than other B cells [47]. Therefore, it is possible that local signaling through LTβR, which maintains BAFF-producing DC in the spleen, supports T2MZ B cell survival [9, 26], and under sub-optimal BAFF levels (*e.g.* reduced DC numbers), immature T2MZ B cells fail to survive. However, we cannot exclude that LTα/β-driven T2MZ B cell homeostasis is independent of BAFF and that overexpression of BAFF in LTβ△-BTg mice may have served as a surrogate survival signal. In conclusion, signaling through LTβR controls two aspects of MZ B cell development, survival of T2MZ B cell precursors and organization of the splenic MZ, both of which are essential for final MZ B cell differentiation and homing.

LTβ$^{-/-}$ mice have reduced levels of serum IgA, and this has been attributed to a defective migration of IgA precursors to the lamina propria in LTβR$^{-/-}$ mice [34]. LTα/β signaling in the gut tissue is essential to maintain the expression of key adhesion molecules and chemokines, necessary for B cell migration and fecal IgA production [34]. Gut-associated lymphoid tissues appear dispensable in that process [34]. Our study suggests that regulation of IgA production may be a little more complex. A careful analysis of absolute numbers of B-1a B cells, which are thought to be the main source of IgA production [48], revealed that LTβ$^{-/-}$ mice have significantly less of these cells both in the spleen and PerC, and as previously shown, minimal numbers in the gut tissue [34]. As a result, both serum and fecal IgA levels are reduced in these mice. Surprisingly, upon BAFF overexpression in LTβ△-BTg mice, serum but not fecal levels of IgA were restored. This observation correlated with the normalization of B-1a B cell numbers in the spleen of these mice and

augmentation of their numbers in the peritoneal cavity. The increased number of B-1 B cells in the PerC of LTβ△-BTg mice compared to BAFF Tg mice may be explained by impaired B cell migration to the gut in the absence of LTβ, possibly resulting in the accumulation of these cells in the PerC. Thus, serum IgA are not solely produced by B cells in the gut tissue. Previous studies have indeed shown that the specificities of blood IgA differs from that of gut-derived IgA antibodies, suggesting a different site for the activation of B cells responsible for serum IgA levels (reviewed in [36]). Alternatively, BAFF has been shown to promote B cell isotype-switching to IgA [8, 9] and, therefore, B-1a as well as other B cells may have contributed to the production of serum IgA. This work shows that levels of serum IgA are not solely dependent on gut-derived IgA production but can also be influenced by serum BAFF levels.

The proportion of activated T cells is greater in BAFF Tg mice, compared to WT mice [10, 14], an indirect effect due to BAFF-mediated changes to B cell make-up and numbers in BAFF Tg mice [14]. BAFF Tg mice lacking B cells have normal numbers of effector/memory T cells when compared to control mice [14]. MZ B cells have been shown to be efficient APC to naïve T cells [32] and the expansion of this population in BAFF Tg mice has been thought to contribute to the expansion of effector/memory T cells [14]. In LTβ△-BTg mice, which clearly lack functional MZ B cells, the proportion of effector T cells was increased similar to observations in BAFF Tg mice, suggesting that BAFF-affected B cells other than MZ B cells contribute to this phenomenon.

LTβ△-BTg mice revealed that MZ B cells are dispensable for the development of nephritis in BAFF Tg mice. Nephritis in BAFF Tg mice is associated with accumulation of some B-1 B cells in the kidneys and Ig-secreting cells. BAFF is not required for B-1 B cell survival and development [7] but excess production of BAFF has been shown to expand B-1 B cell numbers in one BAFF Tg model [49] and in aging BAFF Tg mice [45]. Nephritis in BAFF Tg mice is associated with the deposition of IgA in the kidneys and shares similarity with IgA nephropathy in humans [50]. However, analysis of Ig deposition in nephritic kidneys of LTβ△-BTg mice revealed that IgG1 and IgG2a but not IgA deposits were predominant features. Overexpression of another ligand of LTβR, LIGHT, has also been shown to trigger kidney disorders similar to IgA nephropathy [13]. However, inhibition of LIGHT in our experiments showed that nephritis in BAFF Tg mice is independent of LIGHT/LTα/β signaling, does not rely on MZ B cells, and is not strictly dependent on IgA deposition.

MZ-like B cells were detected in the salivary glands of BAFF Tg mice and in LN and the blood [11]. A parallel can be made to patients with SS, as MZ B cells disappear from the blood of these patients to accumulate in salivary glands [51]. We, and others, showed that serum BAFF levels in these patients, whether the cause or consequence of the disease, are significantly elevated [11, 52]. Complications with lymphoma are observed occasionally in SS patients and are often described as Marginal Zone Cell Lymphoma (MZCL) [53]. In $TNF^{-/-}$ × BAFF Tg mice MZ B cells are suspected to be the precursors of the B cell lymphomas that develop in these mice [45]. Analysis of cells infiltrating the salivary glands of $LT\beta^{-/-}$ mice showed that B cells numbers were similar to that collected from WT salivary glands. Saliva production in BAFF Tg mice is reduced due to sialadenitis [11]. Despite no apparent tissue destruction or overt inflammation, saliva flow was also reduced in $LT\beta^{-/-}$ mice. In contrast to BAFF Tg mice, excess BAFF production in LTβ△-BTg mice improved salivary gland function and did not promote sialadenitis and tissue destruction. We previously showed that B1 B cell numbers were also increased in the inflamed salivary glands of BAFF Tg mice, however, not to the same extent as MZ B cells, which are the predominant B cell subset, present [11]. As B-1 B cell numbers are also reduced in the salivary glands of LTβ△-BTg mice (Supplementary Fig. 2), we cannot entirely exclude the possibility that B-1 B cells may also participate in the development of sialadenitis in BAFF Tg mice, alongside MZ B cells.

This study clarifies a number of key aspects concerning autoimmune disease development in BAFF Tg mice. Overproduction of BAFF alone has profound effects, triggering autoimmunity in the absence of a full set of LN and Peyer's patches, with immune organs lacking normal T/B organization and despite defects in the splenic architecture. B cells involved in T-independent responses, such as MZ B cells and B-1 B cells, seem to play a central role in the progression of autoimmune disorders in BAFF Tg mice. However, we cannot exclude the possibility that a greater proportion of effector T cells may indirectly influence the function of both MZ and B-1 B cells in BAFF Tg mice. MZ B cells associated with SS-like symptoms, while B-1 B cells associated with nephritis. Our results indicate that autoimmunity in BAFF Tg mice is linked to the function of innate B cell subsets acting at separate anatomical sites.

Materials and methods

Mice

Animals were housed under conventional barrier protection and handled in accordance with guidelines obtained from the Garvan Institute of Medical Research and St Vincent's Hospital Animal Experimentation Ethics Committee, which complies with the Australian code of practice for the care and use of animals for scientific purposes. Lymphotoxin-β knockout

($LT\beta^{-/-}$) mice and BAFF Tg mice were kindly supplied by Biogen-Idec (Cambridge, MA, USA) and have been described previously [10, 25]. BAFF Tg mice were crossed to $LT\beta^{-/-}$ mice, to obtain mice transgenic for BAFF and lacking LTβ (LTβ△-BTg mice). Littermates from this cross (WT, BAFF Tg and $LT\beta^{-/-}$) were used as matched controls in all experiments. LTβ△-BTg and control mice were genotyped by Southern blot analysis and PCR as described previously [25, 45]. Development of nephritis in these mice was monitored by measuring urine protein using Multistix 10 SG reagent strips (Bayer, Elkhart, USA).

Lymph node visualization

LN were visualized by an injection of 20 μL of india ink into the hind footpads, mice were sacrificed 4 h after injection and lymph node images were recorded using a stereoscopic microscope (Leica instruments, Wetzlar, Germany).

Immunizations and ELISA

T-independent type 2 Ab response was tested using the Ag NP-Ficoll (Biosearch Technology, Novarto, USA). Mice were immunized i.p with 30 μg NP-Ficoll in 100 μL of PBS and 100 μL of blood was collected 1 day before and 7 days after immunization to measure NP-specific Ab production by ELISA. NP-specific antibodies were determined by ELISA as described previously [45]. Briefly, ELISA plates were coated with 2 μg/mL NP_3-BSA (Biosearch Technology), serum was added at a starting dilution of 1 in 50 and detected with anti-mouse IgM- and IgA-alkaline phosphatase (AP)-labeled antibodies (Southern Biotechnology, Birmingham, USA), then revealed by p-nitrophenyl phosphate substrate (Sigma-Aldrich, St Louis, MO, USA).

Fecal samples for ELISA were solubilized as described previously [54]. Serum and fecal IgM and IgA levels were measured as described previously [45]. Briefly, plates were coated with 2 μg/mL goat anti-mouse Ig (Southern Biotechnology), samples were added at a dilution of 1 in 100 for serum and 1 in 20 for fecal, and Ig was detected using anti-mouse IgM- and IgA-AP antibodies (Southern Biotechnology), then revealed as described above. ELISA for the detection of anti-dsDNA, anti-ssDNA and rheumatoid factor were performed as described previously on serum from 6–8-month-old mice [10]. Goat anti-mouse IgG-HRP (Jackson Immunoresearch) was used for detection and was revealed by the addition of diaminobenzidine (Sigma-Aldrich). Detection of anti-chromatin antibodies was performed using the Quanta Lite Chromatin ELISA kit (Inova Diagnostics, San Diego, CA, USA), followed by goat anti-mouse IgG-HRP (Jackson Immunoresearch) for detection, which was revealed by the addition of diaminobenzidine (Sigma-Aldrich). Starting serum dilutions were 1 in 100 for rheumatoid factor and 1 in 50 for anti-ds/ssDNA and chromatin autoantibodies. Titer (log base 2) is defined as the serum dilution giving an OD reading four times higher than background.

Flow cytometric analysis

Lymphocyte suspensions were obtained from spleen and mesenteric LN by mechanical disruption. Erythrocytes were removed using an osmotic cell lysis solution (8.34 mg/mL ammonium chloride, 0.84 mg/mL sodium bicarbonate and 1 mM EDTA, pH 8.0). Lymphocytes were resuspended in FACS buffer (1% BSA and 0.02% sodium azide in PBS) at a concentration of 2×10^6 cells/mL for staining. FITC-, PE-, allophycocyanin-, PerCP-, PE Cy7- or biotin-conjugated rat anti-mouse Ab against CD23, CD21/CD35, IgM, B220, CD5, CD11b, IgD, CD4, CD8, CD62L, CD44 (BD PharMingen, San Diego, CA, USA) and AA4.1 (eBioscience, CA, USA) were used for FACS analysis. Biotinylated Ab were detected with either PE Cy7- or PerCP-coupled streptavidin (BD PharMingen). Cells were analyzed using either the BD FACSCalibur flow cytometer with the Cell Quest Pro software or using the LSRII flow cytometer with the FACSDiva software (BD Biosciences, Franklin Lakes, USA). B lymphocytes were gated as follows: T2MZ ($B220^+$, $CD21^{hi}$, $CD23^+$, IgM^{hi}, $AA4^{int}$, IgD^+), T2Fo ($B220^+$, $CD21^{dull}$, $CD23^+$, IgM^{hi}, $AA4^{hi}$, IgD^+), MZ ($B220^+$, $CD21^{hi}$, $CD23^-$, IgM^{hi}, IgD^+), Fo ($B220^+$, $CD21^{int}$, $CD23^+$, IgM^{dull}, IgD^+), B-1a ($B220^{int}$, $CD5^+$, IgM^{hi}) and B-1b ($B220^{int}$, $CD5^-$, $CD11b^+$, IgM^{hi}). T cells were gated according to their CD4 or CD8 cell surface expression, then subgated as naïve ($CD44^-$, $CD62L^+$) and effector ($CD44^+$, $CD62L^-$).

RT-PCR analysis

TRIzol reagent® (Invitrogen life technologies, Carlsbad, CA, USA) was used to prepare RNA from whole thymus and spleen tissues, according to the manufacturers instructions. Of purified RNA, 100 ng was required for cDNA production using the Abgene reverse-IT RTase Blend kit (Abgene, Advanced Diagnostics, UK) according to the manufacturer's instructions. The following intron spanning primers were used to amplify *Baff* cDNA at an annealing temperature of 66°C: *Baff* sense primer, 5′-TACCGAGGTTCAGCAACACC-3′; *Baff* anti-sense primer, 5′-TGCAATCAGC-TGCAGACAGT-3′. For normalization, primers for *Gapdh* were used: sense primer 5′-TTCACCACCATGGAGAAGGC-3′; anti-sense primer, 5′-GGCATGGACTGTGGTCATGA-3′. The Roche LightCycler system (Roche Diagnostics, Germany) was used to perform real-time PCR. The PCR reaction mixes were prepared using the LightCycler Faststart DNA Master SYBR Green 1 reaction kit (Roche) according to the manufacturer's instructions and run on a real-time Roche LightCycler machine.

Histology

Spleen, kidney, salivary glands and LN were either snap-frozen in Optimal Cutting Temperature compound (Tissue-Tek, Sakura, Tokyo, Japan) or fixed in 4% phosphate buffered formaldehyde. Frozen tissue was sectioned at 6 μm, air-dried and fixed in acetone, then stained for 1 h with anti-IgA-, IgG1-, IgG2a- and B220-biotin labeled Abs (BD PharMingen). Streptavidin-HRP (DAKO A/S, Glostrup, Denmark) was used to detect Ab, prior to revealing the HRP activity with diaminobenzidine substrate (Sigma-Aldrich). Sections were then fixed in methanol and counterstained with Wrights

Giemsa solution (Sigma-Aldrich). Kidney sections were also blocked with a cocktail of 5 μg/ml purified rat anti-mouse CD16/CD32 Fc block Ab (BD PharMingen) and 5 μg/mL polyclonal human IgG (Biogen-Idec) in PBS for 10 min prior to staining with biotinylated specific anti-mouse Ig isotype Ab (Southern Biotech).

Formaldehyde fixed sections were embedded in paraffin, and 6-μm tissue sections were H&E stained as described previously [11].

ELISPOT assays

The 96-well MultiScreen-HA filter bottom plates (Millipore, Bedford, USA) were coated with 10 μg/mL goat-anti-mouse Ig (Southern Biotechnology). Plates were washed once with PBS and blocked with PBS/5% BSA for 30 min at 37°C, then once with PBS/0.1% Tween and finally once with PBS. Kidney cell suspensions were obtained by treatment of the kidney with 1 mg/mL collagenase/dispase (Roche) for 1 h at 37°C followed by mechanical disruption. Erythrocytes were removed using an osmotic cell lysis solution. Cells were added at 1×10^6 cells per well in RPMI media (Invitrogen) supplemented with 10% FCS (HyClone, South Logan, USA) and cultured overnight at 37°C. Plates were washed once with PBS/0.1% Tween, then once with H_2O to lyse the cells and twice with PBS. Plates were then incubated with either anti-mouse IgG-biotin (BD PharMingen) or anti-mouse IgA AP (Southern Biotech) for 1 h at 37°C. Plates were washed twice with PBS/0.1% Tween, then once with PBS. Streptavidin-AP (1 in 500) (BD PharMingen) was used for the detection of biotin labeled IgG. Plates were washed once with PBS/0.1% Tween and twice with PBS. Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich) was used to reveal the AP activity on each spot. After color development, plates were washed once with water and air-dried. The number of spots per well was counted using a Carl Zeiss Vision ELISPOT reader (Carl Zeiss, Thornwood, USA).

Treatment of mice with LTβR-Fc

The murine LTβR fused to human Ig Fc domain (LTβR-Fc) (Biogen-Idec) produced from CHO cells and purified by conventional Protein A based affinity chromatography was used in this study, polyclonal human IgG (Hu Ig) was used as a control (Biogen-Idec) [55]. Nephritis development in BAFF Tg and LTβ△-BTg mice was monitored weekly by measurement of proteinuria and mice were enrolled in studies once proteinuria reached 0.3 g/L. Mice were then treated weekly with either 150 μg of LTβR-Fc i.p or 150 μg of Hu Ig i.p for 5 weeks.

Measurement of salivary flow

Saliva flow in mice was measured as described previously [11]. Briefly, Mice were injected i.p with 30 μg of sterile pilocarpine (Sigma-Aldrich) in PBS per 100 g body weight. Five minutes after injection, saliva was collected for 10 min on a cotton swab. The weight of the cotton swab was measured before and after saliva collection, and the difference was used as a value for saliva production.

Statistical analysis

Statistical significance was determined using a Student's *t*-test. Significance is indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

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