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Development of autoimmune nephritis in genetically asplenic and splenectomized BAFF transgenic mice

Carrie A. Fletcher [a], Joanna R. Groom [a], Blanche Woehl [a], Helen Leung [a], Charles Mackay [c], Fabienne Mackay [a,b,*]

[a] Autoimmunity Research Unit, Immunology and Inflammation Research Program, The Garvan Institute of Medical Research, Sydney, NSW, Australia
[b] Department of Immunology, AMREP, Central Clinical School, Monash University, 89 Commercial Road, Melbourne, Victoria 3181, Australia
[c] Department of Immunology, School of Biomedical Sciences, Monash University, Clayton, Victoria, Australia

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ABSTRACT

B cell activating factor belonging to the TNF family (BAFF or BLyS) is a critical B cell survival factor essential for B cell maturation. BAFF transgenic (Tg) mice develop autoimmunity resembling Systemic Lupus Erythematosus (SLE) in a T cell-independent but toll-like receptor (TLR) signalling-dependent manner, requiring TLR-induced innate B cell-derived pro-inflammatory autoantibody deposition in the kidneys. Importantly, neutralizing BAFF in the clinic shows efficacy in patients with SLE, confirming its critical role in the progression of this disease in both humans and mouse models. The specific B cell types that produce autoantibodies in BAFF Tg mice are TLR-activated innate marginal zone (MZ) B cells and B1 cells, but not follicular B cells. Interestingly, in BAFF Tg mice MZ-like B cells infiltrate salivary glands whereas B1 B cells infiltrate the kidneys. To ascertain the relevance of B1 and MZ-like B cells in the development of nephritis in BAFF Tg mice, we generated genetically asplenic as well as splenectomized BAFF Tg animals. BAFF Tg mice born without a spleen lack MZ B cells, have very reduced B1a B cell numbers but a normal B1b B cell compartment. Loss of these B cell subsets failed to protect BAFF Tg mice against nephritis indicating that B1b B cells are an important subset for the development of autoimmune nephritis in BAFF Tg mice. Thus the spleen is dispensable for the development of autoimmune nephritis in BAFF Tg mice and points toward a pathogenic role for innate B1 B cells. Identifying similar innate B cells in humans may offer the possibility of more targeted B cell therapies.

1. Introduction

B cell activating factor belonging to the TNF family (BAFF, also termed BLyS, TALL-1, zTNF-4, THANK, and TNFSF13b) is a key B cell survival factor (reviewed in [1–5]). This factor is critical for B cell maturation in the periphery, particularly the progression B cells from the immature transitional type I (T1) to the immature transitional type II (T2) maturing stage [6]. In mice lacking BAFF, B cell maturation beyond the T1 stage is impaired [7–9]. In addition, neutralization of BAFF *in vivo* leads to the disappearance of mature B cells [10].

B cell tolerance to self-antigen is achieved by eliminating potentially self-reactive B cells at various immune checkpoints during the development of these cells [11,12]. A careful balance between life and death at these various checkpoints is key to ensure proper elimination of self-reactive B cells. BAFF transgenic (Tg) mice develop autoimmune disorders that resemble systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) in humans [13]. This suggested that excessive BAFF production (hence, excessive B cell survival) led to a breakdown of B cell tolerance. Yet, studies looking at the deletion of self-reactive anti-hen egg lysozyme (HEL) B cells in mice expressing HEL as a self-antigen, revealed that excess BAFF did not prevent negative selection of high affinity self-reactive B cells competing with normal B cells. Rather, this led to the expansion of low affinity self-reactive B cells, particularly marginal zone (MZ) B cells [14]. These low affinity self-reactive B cells alone seemed unlikely to be the main driver of the autoimmune disease in BAFF Tg mice. The role of T cells in lupus is also important (reviewed in [15]), however, BAFF Tg mice lacking all T cells develop autoimmune disorders indistinguishable from that of T cell-sufficient BAFF Tg mice [16]. Moreover, development of disease in BAFF Tg mice depends on the expression of the Toll-like receptor (TLR) adaptor MyD88 in B cells [16]. The

* Corresponding author. Department of Immunology, AMREP, Central Clinical School, Monash University, 89 Commercial Road, Melbourne, Victoria 3181, Australia. Tel.: +61 3 9903 0712; fax: +61 3 9903 0038.
E-mail address: Fabienne.Mackay@monash.edu (F. Mackay).

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current model suggests that T cell-independent but TLR-dependent activation of innate B cells is the underlying pathogenic event that leads to the production of pro-inflammatory autoantibodies able to activate complement and inflammation in the kidneys [16]. While it is clear that B cells drive disease in BAFF Tg mice, it is still not clear which B cell subset contributes to disease pathogenesis. The MZ B cell compartment is enlarged in BAFF Tg mice and MZ B cells which are normally only found in the spleen are abnormally detected in lymph nodes (LN) but also the inflamed salivary glands of BAFF Tg mice [6,17]. Unlike other B cells the B1 B cell compartment does require BAFF for survival but is also enlarged in BAFF Tg mice as they age [18] and these cells but not MZ B cells have been detected in the inflamed kidneys of these mice [19]. Work using BAFF Tg mice lacking lymphotoxin, which prevented MZ B cell formation, were protected against inflammation of the salivary gland but not nephritis [19]. Finally, TLR activation of MZ and B1 but not follicular B cells isolated from BAFF Tg mice led to production of autoantibodies reinforcing the notion that these subsets of B cells are central contributors to disease in BAFF Tg mice [16]. This preliminary work suggested that MZ and B1 B cells played a role in inflammation in BAFF Tg mice via the production of pro-inflammatory autoantibodies but at separate anatomical sites, the salivary glands and the kidney, respectively.

To confirm this idea we studied two mouse models lacking the spleen, Hox-11$^{-/-}$ asplenic mice crossed onto BAFF Tg mice and splenectomized BAFF Tg mice. Both models are characterized by the elimination of splenic MZ B cells but also led to the loss of B1a B cells [20]. However, we showed here that the spleen is dispensable for the development of nephritis in BAFF Tg mice and suggest that B1b cell-derived pro-inflammatory autoantibodies are sufficient to drive kidney destruction.

2. Materials and methods

2.1. 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. BAFF Tg mice were kindly supplied by Biogen-Idec Inc. (Cambridge, USA) and have been described previously [13]. BAFF Tg mice were crossed to Hox11$^{-/-}$ mice (the Jackson Laboratory, Maine, USA), to obtain mice transgenic for BAFF and lacking Hox11 (Hox11Δ-BTg mice). Littermates from this cross (WT, BAFF Tg and Hox11$^{-/-}$) were used as matched controls in all experiments. Hox11Δ-BTg and control mice were genotyped by Southern Blot analysis and PCR as described previously [13,21,22]. Development of nephritis in these mice was monitored by measuring urine protein using Multistix 10 SG reagent strips (Bayer, Elkhart, USA) [13]. Mice were splenectomized at 8 wk of age as previously described [23].

2.2. Mouse B cell activation

Lymph node (LN) cells were prepared and activated for 48 h in RPMI supplemented with 10% FCS and/or BAFF 50 ng/ml (Alexis Biochemicals, San Diego, CA, USA) and 5 μg/ml goat anti-mouse μ antibodies or control goat antibodies (Southern Biotechnology, Birmingham, AL, USA).

2.3. Immunizations and ELISA

T-independent type 2 Ab response was tested using the Ag NP-Ficoll (Biosearch Technology, Novato, 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.

Ab response to the polysaccharide antigens present on the encapsulated bacteria *Streptococcus pneumoniae* was determined by i.p immunization of mice with 10 μl of Pneumovax® 23 in 100 μl of PBS. 100 μl of blood was collected 1 day before and 7 days post-immunization.

ELISA plates were coated with 2 μg/ml NP40-BSA (Biosearch Technology) to determine NP-specific Ab response or 5 μl Pneumovax® 23 per well for determination of anti-Pneumovax Ab response. NP ELISA plates were blocked for 1 h at 37 °C with 100 μl of 4% skim milk powder in PBS and Pneumovax® 23 ELISA plates were blocked with 100 μl of 1% BSA in PBS. Serum was added at a starting dilution of 1 in 50, and detected with anti-mouse IgM- and IgA-alkaline phosphatase (AP) labelled antibodies (Southern Biotechnology), then revealed by p-nitrophenyl phosphate substrate (Sigma–Aldrich, St Louis, USA).

ELISA for the detection of Basal IgM, anti-dsDNA, anti-ssDNA and RF were performed as described previously on serum from 12-mo-old mice [13]. Briefly, ELISA plates were coated with 2 μg/ml of goat anti-mouse Ig (H + L) (Jackson Immunoresearch, West Grove, PA, USA) or normal goat Ig (Jackson) or 10 μg/ml methylated BSA (Invitrogen, Carlsbad, CA, USA) for basal IgM, RF and anti-DNA levels respectively. For the detection of anti-dsDNA and anti-ssDNA Abs, plates were coated with 2 μg/ml dsDNA or ssDNA prepared from grade I calf thymus DNA (Sigma) prior to the addition of serum. Purified mouse IgM was used as a standard for basal IgM levels (Southern Biotechnology). ELISAs were detected with anti-mouse isotype-specific-alkaline phosphatase (AP) labelled antibodies (Southern Biotechnology), and revealed by p-nitrophenyl phosphate substrate (Sigma).

2.4. Flow cytometric analysis

Lymphocyte suspensions were obtained from lymph nodes (LNs), peripheral LN (PLN) and mesenteric LN (MLN) by mechanical disruption and peritoneal lavage cells were obtained by flushing the peritoneal cavity with 5 ml of PBS. Lymphocytes were resuspended in FACS buffer (1% BSA and 0.02% sodium azide in PBS) at a concentration of 2 × 106 cells/ml for staining. FITC-, PE-, allophycocyanin-, PerCP-, PE Cy7-, APC-Cy7, Pacific Blue or biotin-conjugated rat anti-mouse Ab against IgM, B220, CD5, CD11b, IgD (BD PharMingen, San Diego, CA, USA) and CD93, CD23, CD21/CD35 (eBioscience, San Diego, CA, USA) were used for FACS analysis. Biotinylated Abs were detected with either PE Cy7- or PerCP-coupled streptavidin (BD PharMingen). Cells were analysed 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}, CD93^{int}, IgD^{+}), T2Fo (B220^{+}, CD21^{dull}, CD23^{+}, IgM^{hi}, CD93^{hi}, IgD^{+}), MZ (B220^{+}, CD21^{hi}, CD23^{-}, IgM^{hi}, IgD^{-}, CD1d^{+}), Fo (B220^{+}, CD21^{int}, CD23^{+}, IgM^{dull}, IgD^{+}), B1a (B220^{int}, CD5^{+}, IgM^{hi}) and B1b (B220^{int}, CD5^{-}, CD11b^{+}, IgM^{hi}).

2.5. Histology

LN, kidneys and salivary glands were either snap-frozen in OCT (Tissue-Tek, Sakura, Tokyo, Japan) or fixed in 4% phosphate buffered formaldehyde. Frozen tissue was sectioned at 6 μm, air-dried and fixed for 10 min in ice-cold acetone. Kidney sections were blocked for 30 min with a cocktail of 5 μg/ml purified rat anti-mouse CD16/CD32 Ab (BD PharMingen) and 5 μg/ml polyclonal human IgG (Biogen-Idec Inc. Cambridge, MA) in PBS, prior to staining for 1 h with anti-B200, IgA-, IgM-, IgG1-, IgG2b- and IgG2c and IgG3-FITC labelled Abs, anti-CD1d-PE labelled Abs (BD PharMingen),

anti-complement component C3-FITC (Cedarlane, labs, Burlington, Ontario, Canada). Sections were analysed by fluorescent microscopy (Carl Zeiss, Thornwood, NY, USA).

Formaldehyde fixed sections were embedded in paraffin, and 6 μm tissue sections were H&E stained as described previously [17].

2.6. ANA immunofluorescence

Serum obtained by tail bleed from 12-mo old mice was diluted 1:25 in PBS and used for indirect immunofluorescence on fixed Hep-2a slides (Antibodies Inc, Davis, CA, USA) to determine ANA. Mouse antibody binding was detected with Anti-mouse-IgA-, IgM- or IgG-FITC labelled Ab (BD PharMingen). Slides were analysed by fluorescent microscopy (Carl Zeiss).

2.7. Statistical analysis

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

3. Results

3.1. Normal B cell numbers in the peripheral lymph nodes (PLN) of Hox11$^{-/-}$ mice, and normal ex-vivo response to activation

Hox11$^{-/-}$ mice are used as a model of congenital asplenia [21,22]. Prior to combining this model with the BAFF Tg model we wanted to ensure that the status of B cells in lymph nodes (LN) was the same as that of LN B cells from WT mice. Peripheral LN (PLN) cells were prepared from WT and age-matched Hox11$^{-/-}$ mice and stained with antibodies to B220, CD21, IgM, AA4^{+} (CD93) and CD23 as previously described [24]. Fraction I (Fr I: contains T1 and T2 follicular (T2Fo) B cells), Fraction II (Fr II: contains transitional type 3 (T3) and follicular (Fo) B cells) and Fraction III (Fr III: contains T2MZ and MZ B cells). These were gated as previously described [24] and shown in Fig. 1A and B. As previously noted, MZ B cells are solely found in the spleen of WT mice [25] and not LN. This is also the case for B cells from Hox11$^{-/-}$ PLN (Fig. 1B). The staining shows that both WT and Hox11$^{-/-}$ PLN contained mostly Fo B cells with few T2Fo, T3 and T2MZ B cells, but not T1 and no MZ B cells (Fig. 1B and C). Absolute numbers of immature B cells in WT and Hox11$^{-/-}$ PLN were similar (Fig. 1C), suggesting that lack of a spleen in Hox11$^{-/-}$ mice has not displaced B cell maturation and MZ B cell formation to the lymph nodes.

PLN cells from WT and Hox11$^{-/-}$ mice were put in cultures supplemented or not with BAFF + anti-μ to stimulate B cell activation as previously shown [26]. FACS analyses looking at surviving activated cells in the B220^{+} gate showed a similar response of WT and Hox11$^{-/-}$ B cells to activation in the presence of BAFF (Fig. 1D). Finally, WT and Hox11$^{-/-}$ B cells expressed similar BAFF receptor levels (data not shown). In conclusion, B cells in Hox11$^{-/-}$ lymph nodes (LN) are present in normal numbers and respond normally to BAFF + anti-μ activation. Therefore, the Hox11$^{-/-}$ model despite lack of a spleen has normal B cells and is a suitable model to study in combination with excess BAFF production.

3.2. Splenectomy and genetic asplenia have different effects on MZ B cell numbers in the blood and LN of BAFF Tg mice

BAFF Tg mice were crossed onto Hox11$^{-/-}$ mice and LN cells from the resulting Hox11Δ-BTg were stained as in Fig. 1, analysed by flow cytometry for the presence of MZ B cells and compared to littermate controls. As previously shown, numbers of MZ-like

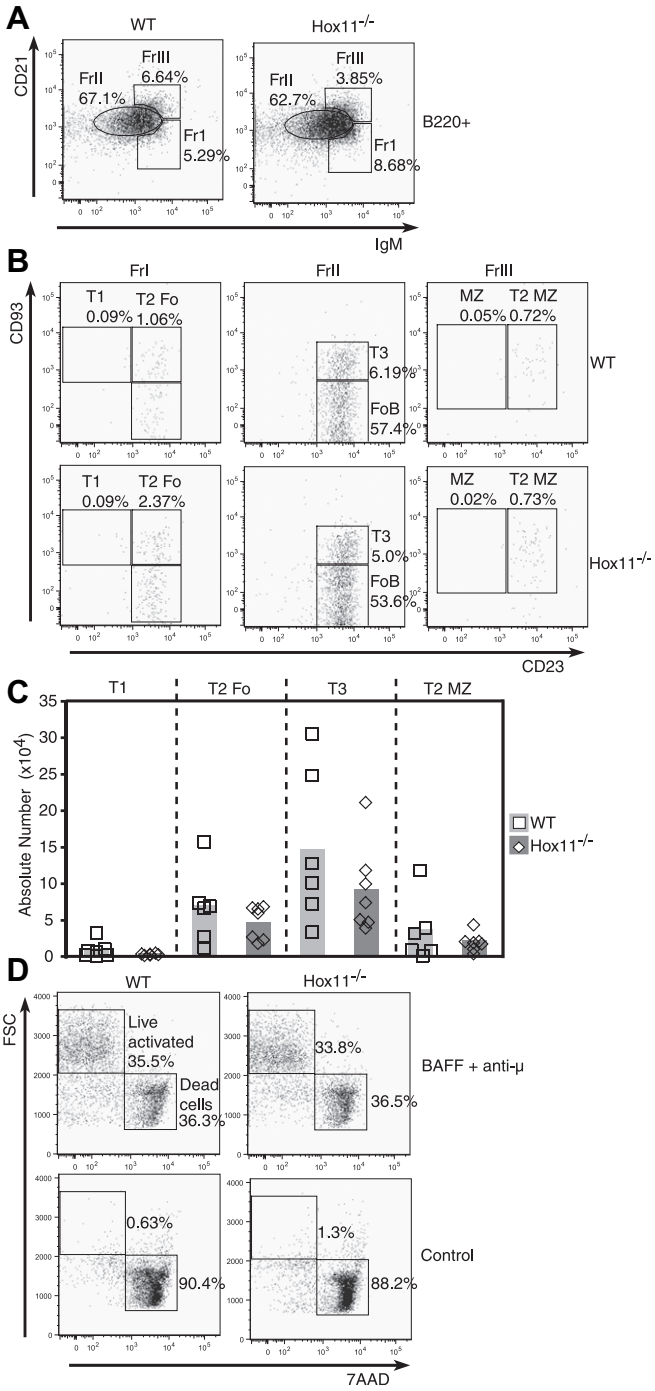

Fig. 1. Hox11$^{-/-}$ lymph node B cells are present in normal numbers and proportion, and respond normally to activation. (A) Immune cells isolated from peripheral lymph nodes were stained with antibodies to B220, CD21, CD23, CD93 and IgM. B220^{+} gated B cells of WT (left) and Hox11$^{-/-}$ (right) mice were further sub-gated into fractions: Fr I, Fr II and FR III as indicated. Percentages for each fraction are shown. (B) B cells in Fr I, Fr II and FR III were further gates as T1, T2Fo, T3, Fo B cells (FoB), T2MZ and MZ based on expression of CD93 and CD23 as indicated. Percentages for each B cell subset are shown. Plots in (A) and (B) are representative of at least 6 animals analysed per group. (C) Absolute numbers of T1, T2Fo, T3 and T2MZ B cells in the lymph nodes of WT (□) and Hox11$^{-/-}$ mice (◇). The mean B cell number is shown with a light grey bar (WT) and a darker grey bar (Hox11$^{-/-}$). 6–7 animals per group are shown. (D) Lymph node cells from WT (left panels) and Hox11$^{-/-}$ (right panels) mice were stimulated for 48 h in culture with BAFF and anti-μ. Cells were stained with 7AAD and analysed by FACS. Live activated and dead cells are gated for control cultures (media only, bottom panels) and cultures supplemented with BAFF and anti-μ (top panels). Percentages for each gate are indicated. These plots are representative of 3 independent experiments.

B cells are significantly elevated in PLN and MLN of BAFF Tg mice [6,17] and Fig. 2A. However, the MZ-like B cells did not develop/expand in Hox11Δ-BTg (Fig. 2A). This result suggests that MZ-like B cells present in the LN of BAFF Tg mice may have originated from the spleen. MZ-like B cells in BAFF Tg mice are in terms of phenotype and function no different than splenic MZ B cells in WT animals [17]. The term "like" only indicates that their homing and positioning pattern in immune organs differs from that of WT MZ B cells.

To see whether loss of the spleen could affect MZ-like B cells that are present in the LN of BAFF Tg mice, 2 mo old BAFF Tg mice were splenectomized and LN B cells analysed 8 mo post-splenectomy (Fig. 2B). Elevated numbers of MZ-like B cells were present in the LN and blood of sham-operated BAFF Tg mice (Fig. 2B). These numbers were reduced in splenectomized BAFF Tg mice but this was only significant in the blood but not in PLN or MLN (Fig. 2B). The MZ-like B cells expressed higher levels of CD1d compared to Fo B cells in the MLN of both sham-operated and splenectomized BAFF Tg mice (Fig. 2C), confirming further the MZ-like nature of these B cells. This suggests that the spleen may supply MZ B cells normally found in the blood of BAFF Tg mice.

To check whether the remaining MZ B cells seen in splenectomized BAFF Tg mice retained the activation characteristics of

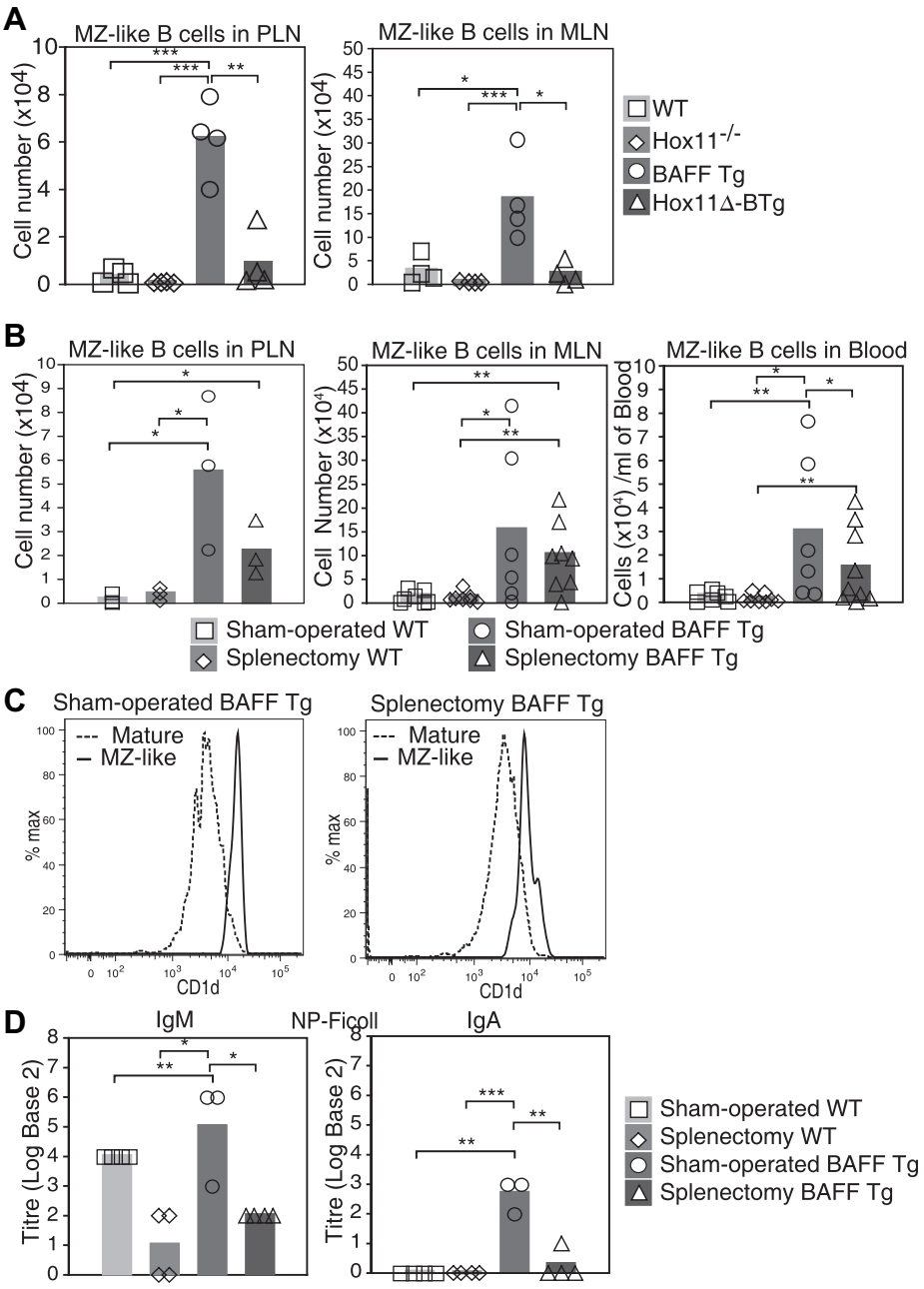

Fig. 2. Reduced numbers of MZ B cells in the LN of asplenic BAFF Tg mice and impaired response to the T-independent antigen NP-Ficoll. (A) Absolute numbers of MZ B cells (gated as in Fig. 1) in the PLN (left) and MLN (right) of WT (□), Hox11$^{-/-}$ (◇), BAFF Tg (○) and Hox11Δ-BTg (△) mice, as indicated. (B) Absolute numbers of MZ B cells in the PLN (left), MLN (middle) and blood (right) of sham-operated WT (□), BAFF Tg (○), splenectomized WT (◇) and splenectomized BAFF Tg (△) mice, as indicated. (C) Histograms showing CD1d expression on gated MLN Fo (dotted line) or MZ (black line) B cells from sham-operated BAFF Tg (left) or splenectomized BAFF Tg (right) mice. (D) NP-specific IgM (left) and IgA (right) titres in the serum of sham-operated WT (□), BAFF Tg (○), splenectomized WT (◇) and splenectomized BAFF Tg (△) mice, as indicated, 7 d post-immunization. In (A), (B) and (C), mean values are shown with graph bars and 4–10 animals per group have been analysed. Statistical significance is indicated.

splenic MZ B cells, sham-operated and splenectomized mice were immunized with the T-independent type 2 (TI-2) antigen NP-Ficoll, known to preferentially activate MZ B cells [19,27]. In addition, BAFF-stimulated MZ B cells but not Fo B cells secrete IgA in response to T cell-independent activation [28]. As previously noted for BAFF Tg mice [19], sham-operated BAFF Tg mice mount greater NP-specific IgM and IgA antibody responses to NP-Ficoll compared to sham-operated WT mice (Fig. 2D). Splenectomy of WT or BAFF Tg mice led to a strong reduction in the T-independent antibody response to NP (Fig. 2D), suggesting that the greater number of MZ-like B cells detected in LN and blood of the splenectomized BAFF Tg mice are unable to sustain an antibody response to NP-Ficoll. This work confirms that the splenic MZ is a unique structure for MZ B cell activation, without which these cells are unable to respond well to TI-2 antigens [29].

In conclusion, genetic asplenia eliminates all MZ-like B cells in Hox11Δ-BTg mice and remaining MZ-like B cells in splenectomized BAFF Tg mice have impaired responses to a T-independent antigen.

3.3. Genetic asplenia but not splenectomy eliminates peritoneal B1a B cells and antibody response to pneumovax in BAFF Tg mice

An examination of basal serum IgM levels in WT and Hox11$^{-/-}$ mice showed no significant difference (Fig. 3A). As previously noted, these levels were elevated in BAFF Tg mice but also in Hox11Δ-BTg mice and to a similar level (Fig. 3A). This result indicated that elevated IgM levels in BAFF Tg mice could be attributed to non-splenic B cells. As previously observed, Hox11$^{-/-}$ mice have markedly reduced numbers of peritoneal B1a B cells [20] and the same observation was made in Hox11Δ-BTg mice (Fig. 3B and C). In contrast, peritoneal B1b cell numbers were greater in Hox11$^{-/-}$ animals, BAFF Tg mice and Hox11Δ-BTg mice compared to WT mice but not different between Hox11$^{-/-}$ and Hox11Δ-BTg mice, suggesting that over-expression of BAFF does not expand B1b cells (Fig. 3C). Yet, elevated levels of B1b cells in Hox11$^{-/-}$ and Hox11Δ-BTg mice did not allow an immune response to Pneumovax, which relied on the presence of B1a cells (Fig. 3D).

Splenectomy had no effect on B1b cell numbers of WT [20] and BAFF Tg mice (data not shown). However, as previously described splenectomy markedly reduced peritoneal B1a cell numbers in WT mice ([20] and Fig. 3E) and this reduction correlated with a reduced response to Pneumovax immunization (Fig. 3F). As previously noted, BAFF Tg mice have elevated B1a cell numbers [18] (Fig. 3E). Splenectomy reduced the number of B1a B cells in BAFF Tg mice but to numbers comparable to that of WT sham-operated mice (Fig. 3E). As a result, response to Pneumovax in splenectomized BAFF Tg mice was identical to that of sham-operated WT and BAFF Tg mice (Fig. 3F). Collectively, these results confirm the role of the spleen as an important immune organ maintaining B1a B cells and potentially supplying these cells. However, our work showed that BAFF over-expression compensated for the loss of B1a cells due to splenectomy but not that due to genetic asplenia, which allowed responses to the T-independent antigen Pneumovax to be maintained at normal levels in splenectomized animals but not genetically asplenic mice.

3.4. Reduced rheumatoid factor levels but elevated anti-DNA autoantibodies in Hox11Δ-BTg mice

We have previously shown that disease in BAFF Tg mice is a T cell-independent process requiring the expression of the TLR adaptor MyD88 in B cells [16]. We have also shown that lack of MZ B cells does not protect BAFF Tg mice against nephritis [19]. However, B1a cells have also been associated with autoimmunity (reviewed in [30]). The Hox11Δ-BTg mice have reduced B1a B cell numbers, which correlated with impaired responses to the T-independent antigen Pneumovax (Fig. 3). Therefore, we tested whether these mice were able to produce autoantibodies. BAFF Tg mice produce elevated levels of rheumatoid factors (RF) (Fig. 4A and [13]), however this was not the case in Hox11Δ-BTg mice (Fig. 4A). In contrast, elevated levels of anti-double stranded (ds) and single stranded (ss) DNA autoantibodies were detected in both BAFF Tg and Hox11Δ-BTg mice (Fig. 4A), suggesting that the T-independent process known to control this aspect [16], did not require spleen-derived B cells, MZ or B1a B cells, and most likely relied on the presence of B1b cells, as previous work showed that BAFF Tg-derived Fo B cells do not produce anti-DNA autoantibodies [16]. A similar observation was made when assessing anti-nuclear autoantibody (ANA) using Hep2 cell-coated slides (Fig. 4B), suggesting that maintaining ANA production in BAFF Tg mice does not rely solely on the spleen, MZ or B1a B cells. In conclusion, Hox11Δ-BTg mice lacking a spleen have no MZ B cells, no B1a B cells but have B1b cells and produce elevated levels of anti-DNA autoantibodies. Since autoantibody production in BAFF Tg mice is a T cell-independent event driven by innate B cells [16], these results implicate innate B1b cells in particular in Hox11Δ-BTg mice.

3.5. Genetic asplenia or splenectomy does not protect BAFF Tg mice against autoimmune nephritis

Examination of 10 mo old animals revealed that proteinuria, an indication of nephritis, is not as severe in Hox11Δ-BTg mice compared to BAFF Tg mice (Fig. 5A). However, examination of older 12 mo old mice did not show any difference in the level of proteinuria detected in BAFF Tg and Hox11Δ-BTg mice (Fig. 5A), suggesting that disease is not reduced but delayed in mice genetically asplenic. In fact, hematoxylin and eosin (H&E) staining of paraffin-embedded section of kidneys from BAFF Tg and Hox11Δ-BTg mice revealed a similar degree of inflammation, and the glomeruli of both mutants were enlarged and segmented (Fig. 5B).

Our previous studies showed that inflammation in the kidney of BAFF Tg mice depended on the deposition of IgG2b and 2c and C3 complement fixation in the kidneys [16]. Deposits of IgG2b and 2c as well as C3 complement fixation were detected in the kidneys of both BAFF Tg and Hox11Δ-BTg mice but not WT mice (Fig. 5C). Interestingly, mild IgG2b deposition was detected in the kidneys of Hox11$^{-/-}$ mice but that did not lead to significant C3 complement fixation (Fig. 5C) and subsequent activation that leads to signs of inflammation (Fig. 5B). In previous studies we showed that B cells infiltrated the kidneys of BAFF Tg mice [19], and this was also the case for Hox11Δ-BTg mice (Fig. 5D).

Examination of proteinuria in 8 and 12 mo old splenectomized BAFF Tg mice (6 and 10 mo post-splenectomy, respectively) showed no difference with sham-operated BAFF Tg mice and both groups developed nephritis at the same rate (Fig. 5E). Similar IgG2b and 2c deposition as well as C3 complement fixation and B cell infiltrates to that of Hox11Δ-BTg mice were seen in splenectomized and sham-operated BAFF Tg mice (Supplementary Fig. 1). Nephritis was confirmed looking at H&E-stained kidney tissue sections, revealing the same degree of kidney damage in sham-operated and splenectomized BAFF Tg mice (Fig. 5F). In conclusion, this work showed that T cell-independent nephritis developing in BAFF Tg mice does not require the spleen, MZ and B1a B cells.

4. Discussion

Using two separate animal models, this work demonstrated conclusively that nephritis in BAFF Tg mice does not require cellular activation or differentiation processes taking place in the spleen. B1b B cells are the only innate B cells left in asplenic BAFF Tg mice

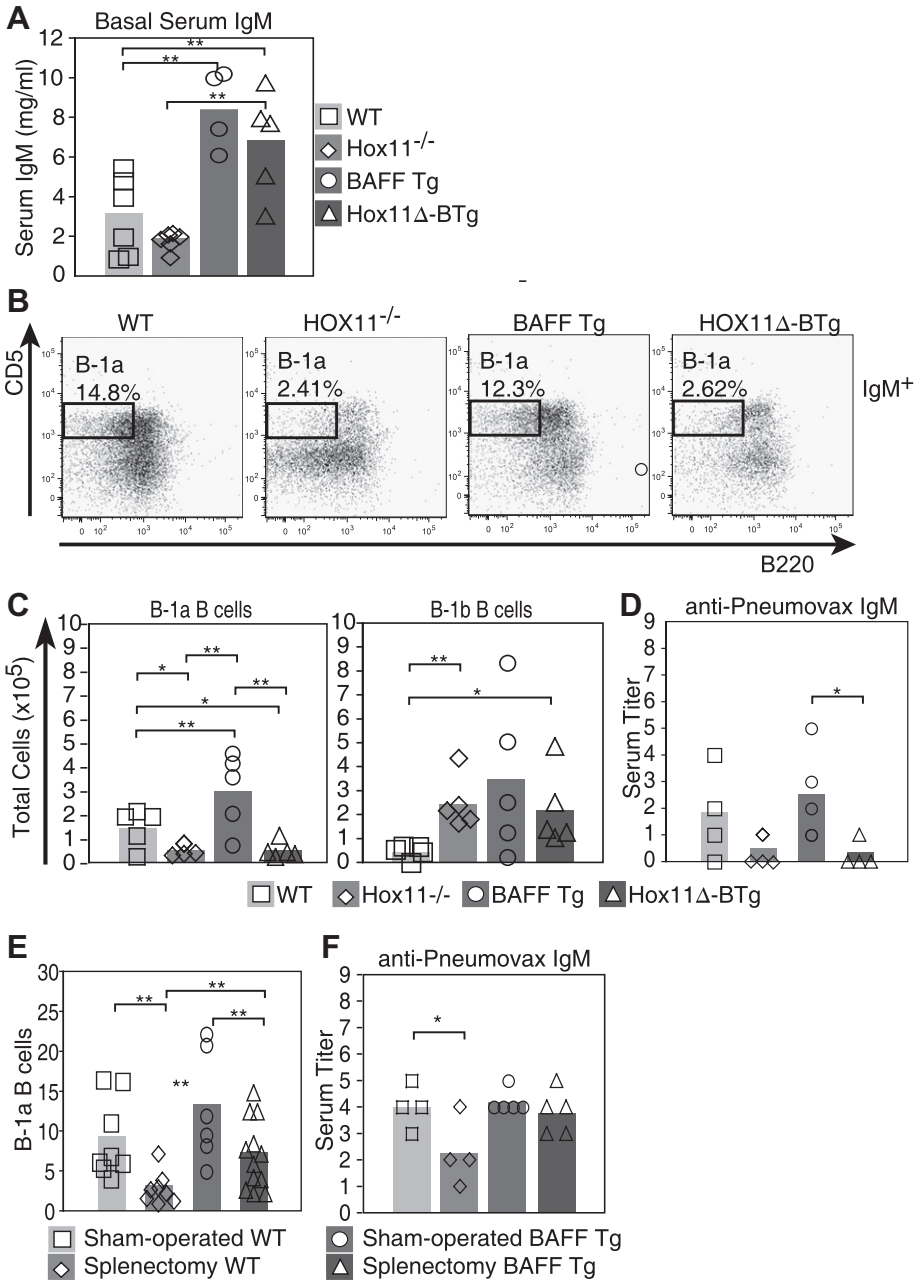

Fig. 3. Loss of B1a B cells in asplenic but not splenectomized BAFF Tg mice, correlating with impaired and normal response to Pneumovax, respectively. (A) Titres of total IgM in the serum of WT (□), Hox11$^{-/-}$ (◇), BAFF Tg (○) and Hox11Δ-BTg (△) mice, as indicated. (B) Dot plots showing gated IgM+/IgDlo peritoneal B cells from WT, Hox11$^{-/-}$, BAFF Tg and Hox11Δ-BTg mice. B1a B cells are gated on the plots (B220^{int} and CD5^{+}) and percentages of these cells are indicated. These plots are representative of 5 mice analysed per group. (C) B1a B cells were gated as in (B) and B1b cells gated as B220^{int}, IgM^{hi}, IgD^{lo} and CD5^{-}. Absolute numbers of peritoneal B1a (left) and B1b (right) per mouse are shown for WT (□), Hox11$^{-/-}$ (◇), BAFF Tg (○) and Hox11Δ-BTg (△) mice, as indicated. (D) Serum Pneumovax-specific IgM titres in WT (□), Hox11$^{-/-}$ (◇), BAFF Tg (○) and Hox11Δ-BTg (△) mice, 7 d post-immunization, as indicated. (E) Absolute numbers of peritoneal B1a B cells in sham-operated WT (□), BAFF Tg (○), splenectomized WT (◇) and splenectomized BAFF Tg (△) mice, as indicated. (F) Serum Pneumovax-specific IgM titres in sham-operated WT (□), BAFF Tg (○), splenectomized WT (◇) and splenectomized BAFF Tg (△) mice, 7 d post-immunization, as indicated. In (A), (C), (D), (E) and (F) mean values are shown with graph bars and 4–10 animals per group have been analysed. Statistical significance is indicated.

that are capable of producing autoantibodies in this model [16] and have been shown to infiltrate kidneys of BAFF Tg mice [19]. As such our experiments enabled us to single out B1b cells as important contributors to nephritis in BAFF Tg mice. MZ B cells are abnormally present in the LN of BAFF Tg mice [6]. Interestingly, this work showed that MZ B cells were not found in the LN of genetically asplenic BAFF Tg mice, suggesting that the supply for these cells probably originated from the spleen in the original BAFF Tg mice. Of note, while some MZ-like B cells can still be detected in the LN of splenectomized BAFF Tg mice (for ethical reasons splenectomy could not be done at an earlier age prior to detection of these cells in LN), significantly less MZ B cells were detected in the blood, again suggesting that once the spleen is gone, supply of MZ B cells into the periphery of BAFF Tg mice is affected. Moreover, MZ-like cells present in the LN of splenectomized mice were unable to respond normally to immunization with T-independent type II antigens. This result fits very nicely with a recent study suggesting that the splenic MZ is a very important site allowing greater activation of

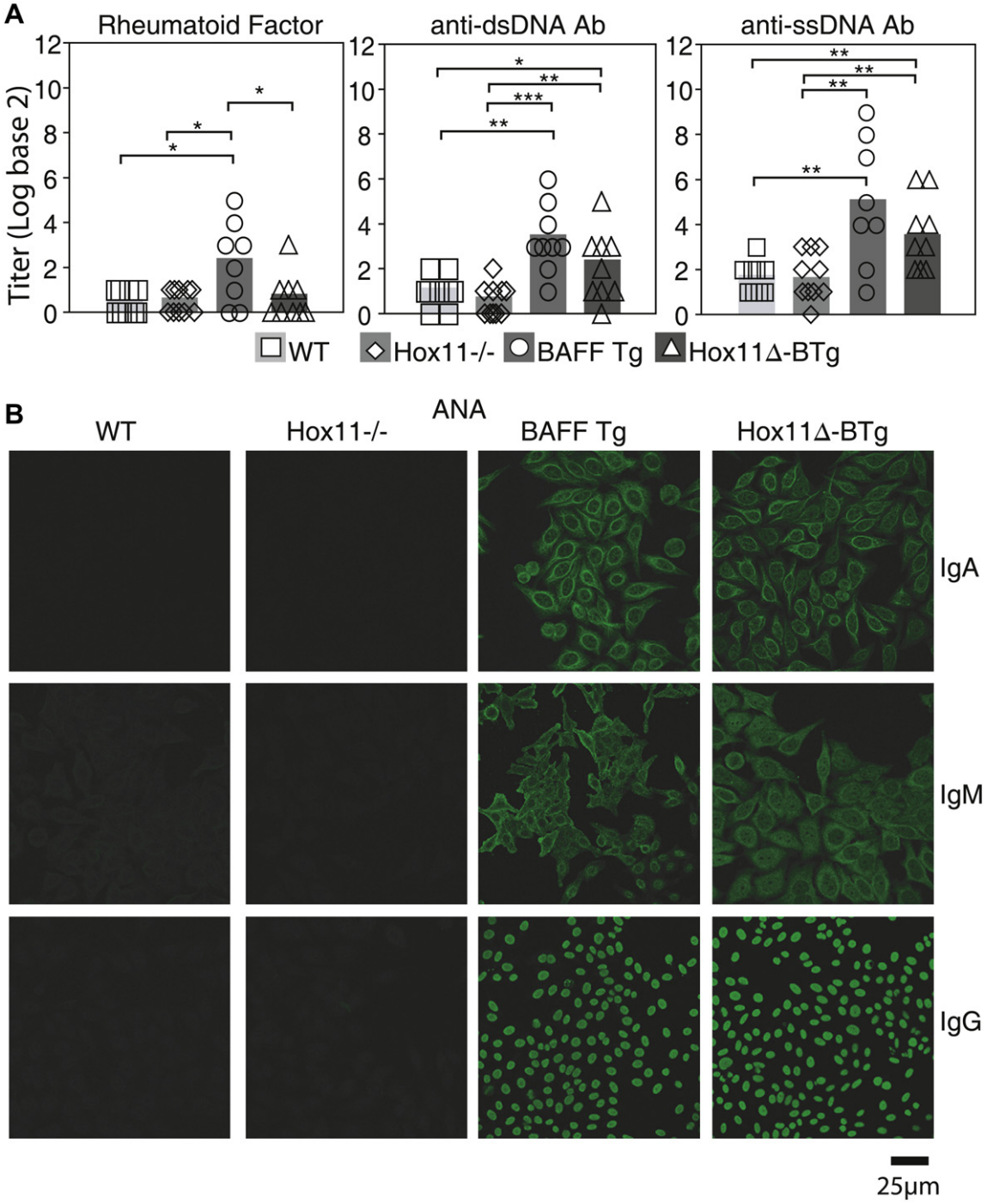

Fig. 4. Reduced production of rheumatoid factors but not anti-DNA autoantibodies in asplenic BAFF Tg mice. (A) Levels of rheumatoid factors (left), anti- double stranded (ds) DNA (middle) and single stranded (ss) DNA autoantibodies in the serum of WT (□), Hox11$^{-/-}$ (◇), BAFF Tg (○) and Hox11Δ-BTg (△) mice, as indicated. Mean values are shown with graph bars and 10 animals per group have been analysed. Statistical significance is indicated. (B) Staining of Hep2 cell-coated slides for the presence of IgA, IgM and IgG anti-nuclear autoantibodies (ANA) in the serum of WT, Hox11$^{-/-}$, BAFF Tg and Hox11Δ-BTg mice as indicated and detected with FITC-labelled anti-IgA, anti-IgM and anti-IgG antibodies, respectively (green staining). Pictures are representative of 10 animals analysed per groups.

B cells [31] and in LN, MZ-like B cells may be unable to be efficiently activated due to the environment not adapted for this type of response.

BAFF is a key B cell survival factor, and excessive BAFF production is associated with autoimmunity in humans and BAFF Tg mice [1]. Belimumab, a therapeutic antibody neutralizing human BAFF has recently met the primary endpoints in a phase III clinical trials with patients suffering from SLE [32]. This is the first successful late stage clinical trial with SLE patients in over 40 years. For many years, targeting T cell function and activation has dominated new therapeutic strategies around SLE [15,33]. Indeed numerous reports have linked defects in T cell regulation with SLE in mouse models and SLE patients [15,34]. Yet, some success in the clinic with the use of Rituximab, a B cell-depleting agent, and now Belimumab,

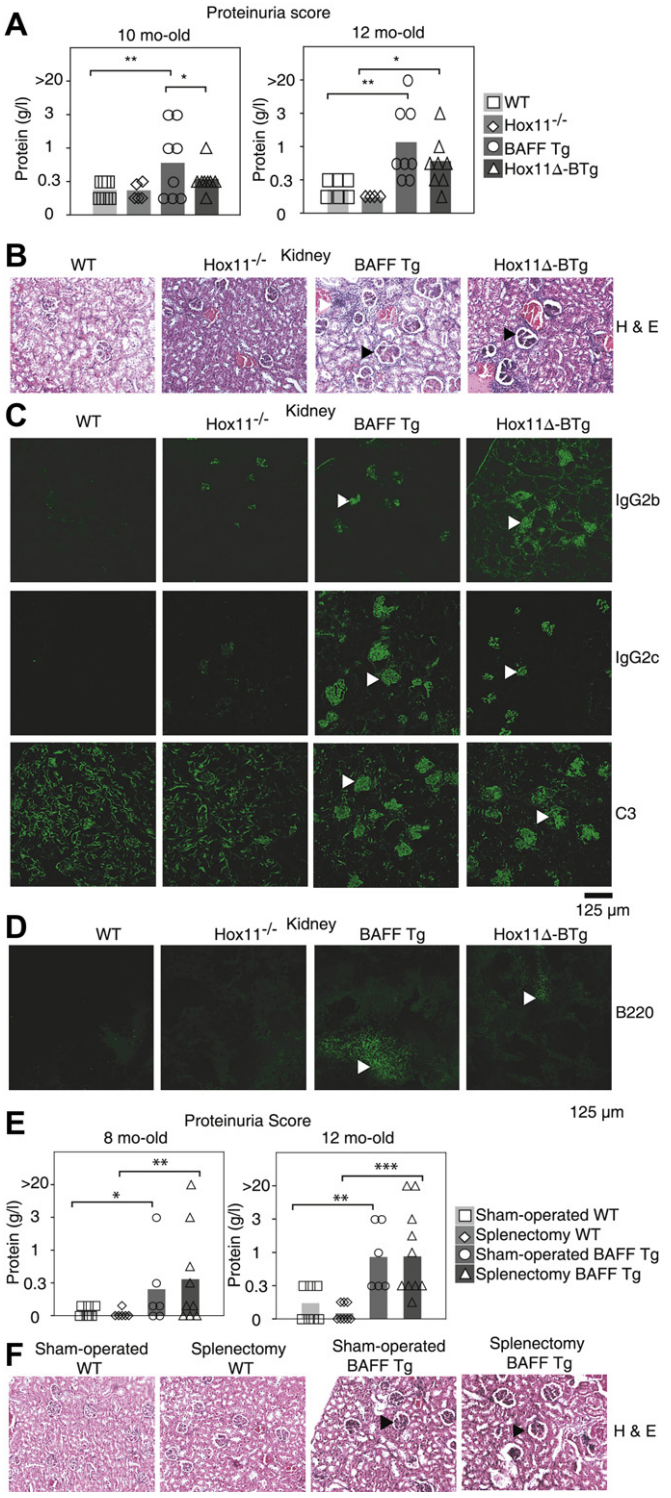

Fig. 5. Delayed development of nephritis in asplenic BAFF Tg mice but severity similar to that seen in BAFF Tg mice. (A) Proteinuria score in the urine of 10 mo (left) and 12 mo (right) WT (□), Hox11$^{-/-}$ (◇), BAFF Tg (○) and Hox11Δ-BTg (△) mice. (B) H&E staining of paraffin tissue sections of kidneys from 12 mo old WT, Hox11$^{-/-}$, BAFF Tg and Hox11Δ-BTg mice as indicated. Abnormal glomeruli are shown with black arrows. (C) IgG2a and IgG2c deposition and C3 complement fixation detected on frozen sections of kidneys from WT, Hox11$^{-/-}$, BAFF Tg and Hox11Δ-BTg mice as indicated. Ig deposition and C3 fixation are shown with white arrows. (D) B220 staining of kidney tissue sections described in (C). Arrows show B cell infiltrates. (E) Proteinuria score in the urine of 8 mo old (left) and 12 mo old sham-operated WT (□), BAFF Tg (○), splenectomized WT (◇) and splenectomized BAFF Tg (△) mice, as indicated. (F) H&E staining of paraffin tissue sections of kidneys from 12 mo old sham-operated WT (□), BAFF Tg (○), splenectomized WT (◇) and splenectomized BAFF Tg (△) mice, as indicated. Abnormal glomeruli are shown with black arrows. In (A) and (E), mean values are shown with graph bars and significant statistical differences are shown. Histology in (B), (C), (D) and (F) is representative of a minimum of 8 animals analysed per group.

highlight the dominant pathogenic role of B cells in SLE [32,35]. B cells may have several pathogenic roles, as producers of pathogenic autoantibodies triggering complement-dependent tissue inflammation and destruction, but also as antigen-presenting cells to T cells, fuelling the inflammatory response [36]. The common view on the mechanism of pathogenicity in SLE and other autoimmune conditions is that B cells and T cells cooperate to drive autoimmunity [36]. However, our work with BAFF Tg mice demonstrated that T cells are dispensable for autoimmunity, at least in this model [1]. Therefore, in view of the exciting positive outcome from BAFF inhibition in phase III SLE clinical trials [32], it is still important to understand the mechanisms whereby BAFF inhibition led to a therapeutic effect in patients.

Initial views have blamed inadequate survival of autoreactive B cells as the main cause of BAFF-mediated autoimmunity. Yet sophisticated animal models that allowed a dissection of B cell tolerance showed that excessive BAFF production does not lead to a catastrophic breakdown of B cell tolerance, in particular when self-reactive B cells compete with normal B cells [14]. Instead, it leads to the expansion of low affinity self-reactive B cells, in particular MZ and B1 B cells [14]. These B cells referred to as 'innate' B cells are particularly good responders to TLR activation and respond with rapid and high levels of Ig production [37]. In addition, TLR-activated MZ and B1 B cells (but not Fo B cells) from BAFF Tg mice produce autoantibodies in response to TLR activation and this effect is enhanced by the presence of BAFF [16]. The innate nature of the mechanism underlying autoimmunity in BAFF Tg mice is also supported by the fact that disease progressed independently of T cells and is dependent on the expression of MyD88 in B cells, a signalling adaptor for TLR [16].

Surprisingly, in the absence of T cells BAFF Tg mice produce large amounts of IgG2b and IgG2c autoantibodies, which deposit in the kidneys and are particularly effective at trapping complement component C3 [16]. C3 complement will ultimately trigger an inflammatory cascade that leads to kidney damage [38]. In summary, TLR-activated innate B cells such as MZ and B1 B cells produce pro-inflammatory autoantibodies, which drive tissue destruction via complement activation. This finding is particularly critical in view of the data from the phase II SLE clinical trial with Belimumab, which missed the primary endpoint [32]. A closer look at the data showed that 28% of patients in the phase II study were seronegative [32]. When the data was recalculated excluding seronegative patients, results showed a significant therapeutic benefit from Belimumab (anti-BAFF/BLyS mAb) [32]. These findings led to a redesigned phase III clinical trial with Belimumab, which met the primary endpoint [32]. This observation in SLE patients supports our results suggesting that disease driven by BAFF in SLE patients is B cell- and autoantibody-dependent [16].

Having established that BAFF-mediated autoimmunity in mice is T cell-independent but innate B cell-dependent, the remaining question is whether or not some innate B cell subsets, such as MZ or B1 B cells play a greater role in driving BAFF-mediated autoimmunity. The role of MZ and B1 cells in autoimmunity has already been suggested [30,37], however, in the case of MZ B cells, expansion of this population does not always correlate with development of nephritis [39] and, conversely, mice defective in MZ B cells can develop nephritis [40]. In fact, our published work showed that while MZ-like B cells were abnormally found in the salivary glands of BAFF Tg mice [17,18], none of these cells were

detected in the kidneys [19]. Moreover, BAFF Tg mice lacking MZ B cells develop nephritis symptoms undistinguishable from that of MZ B cell-sufficient BAFF Tg mice [19], suggesting a connection between MZ B cells and sialadenitis but not with nephritis. An interesting parallel has been observed in patients with Sjögren's syndrome. $CD27^+$ B cells (a marker on human memory but also MZ B cells) are absent in the blood of patients with Sjögren's syndrome but accumulate in their salivary glands as well as transitional B cells [41,42]. Collectively, a picture emerged suggesting that dysregulated MZ B cell function correlated with salivary gland inflammation in BAFF Tg mice, whereas nephritis in these animals involved B1a/b cells.

The work presented here has allowed us to dissect further the role of B1 B cells in BAFF Tg mice. B1a, in particular, have been involved in autoimmune diseases [30], in contrast a lot less is known about B1b cells, yet our work here shows for the first time that B1b cells can play an active role in driving disease on their own. Here, we took advantage of two models: mice lacking either MZ or B1a B cells, these being $Hox11^{-/-}$ mice and splenectomized mice respectively. The $Hox11^{-/-}$ model had the advantage of avoiding a possible role of inflammation occurring following the surgical procedure of splenectomy, and as shown here, the Hox11 mutation does not affect B cell numbers and activation. Combining these two models with the BAFF Tg model gave very similar results and revealed that the spleen, MZ and B1a cells are dispensable for the development of autoimmune nephritis in BAFF Tg mice. B1b cells also infiltrate the kidneys of BAFF Tg mice [19] and in contrast to Fo B cells, these cells can mount long-lasting T cell-independent immune responses [43]. Therefore, B1b cells appear to be the only candidate B cell population capable of producing autoantibodies long-term in a T-independent fashion and driving nephritis in BAFF Tg mice. Development of nephritis in Hox11Δ-BTg is delayed compared to that of BAFF Tg mice, and in BAFF Tg mice, both B1a and B1b cells infiltrated the kidneys [19]. This result suggested that autoantibodies from both B1a and B1b may contribute to drive faster nephritis in BAFF Tg mice [19]. Our work in spleen-deficient BAFF Tg mice showed that B1b but not B1a cells are still present and in time, production of pro-inflammatory autoantibodies from these cells will compensate for the loss of autoantibody production from B1a or MZ B cells.

There is however one point which remains unclear. Work done using $BAFF^{-/-}$ mice has shown that B1 B cells do not require BAFF for survival and BAFF inhibition does not affect survival of these cells [7,8,44]. Assuming that similar cells may exist and contribute to SLE in humans, the question arises how would BAFF inhibition affect their function? Interestingly, B1 B cells express high levels of the BAFF receptor TACI [45]. TACI is critical for T-independent activation of B cells and class switch recombination, a process impaired in $TACI^{-/-}$ mice (reviewed in [46]) which may explain the ability of innate B cells to produce autoantibody isotypes such as IgG2b/c in a T-independent but TLR-dependent manner [16]. Moreover, a recent article has shown interaction of TACI with MyD88, a signalling element used by numerous TLRs, but as mentioned above MyD88 expression in B cells is necessary for disease [47]. Finally, we have shown that TLR signalling strongly up-regulates the expression of TACI [16]. Collectively, these results suggest a potentially important role of TACI and innate receptors in the T-independent production of autoantibodies by $TACI^{high}$ B cells such as B1 B cells. Inhibition of BAFF in the clinic does not eliminate all B cells [48], therefore, one aspect of the efficacy seen with BAFF inhibitors in the clinic may potentially lie in their ability to block BAFF binding and signalling through TACI.

In conclusion, BAFF-mediated autoimmunity is a powerful process that relies on the function of key innate B cells, and the studies here point in particular to B1 B cells, rather than typical B cells from organised immune structures. It will be important to identify similar innate B cells in humans as they may represent the most important subset to target in next generation therapies, should BAFF inhibition prove effective in subsets of SLE patients. Such a strategy would enable selective depletion of a potential pathogenic cell type and preservation of normal B cells important for adaptive immune responses.

Conflicts of interest

The authors declare having no conflict of interest.

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Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.jaut.2010.12.002.

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