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Unique and conserved functions of B cell-activating factor of the TNF family (BAFF) in the chicken

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Abstract

The chicken represents the best-characterized animal model for B cell development in the so-called gut-associated lymphoid tissue (GALT) and the molecular processes leading to B cell receptor diversification in this species are well investigated. However, the mechanisms regulating B cell development and homeostasis in GALT species are largely unknown. Here we investigate the role played by the avian homologue of B cell-activating factor of the tumor necrosis factor family (BAFF). Flow cytometric analysis showed that the receptor for chicken B cell-activating factor of the tumor necrosis factor family (chBAFF) is expressed by mature and immature B cells. Unlike murine and human BAFF, chBAFF is primarily produced by B cells both in peripheral lymphoid organs and in the bursa of Fabricius, the chicken's unique primary lymphoid organ. *In vitro* and *in vivo* studies revealed that chBAFF is required for mature B cell survival. In addition, *in vivo* neutralization with a decoy receptor led to a reduction of the size and number of B cell follicles in the bursa, demonstrating that, in contrast to humans and mice, in chickens BAFF is also required for the development of immature B cells. Collectively, we show that chBAFF has phylogenetically conserved functions in mature B cell homeostasis but displays unique and thus far unknown properties in the regulation of B cell development in birds.

Introduction

B cell development in most domestic animals takes place in gut-associated lymphoid tissues (GALTs). This was clearly shown for rabbits (1, 2) and sheep (3), and more recently for cattle (4, 5) and pigs (6). The most intensively studied model organism for B cell development in the GALT is the chicken (7) in which B cells differentiate in a unique organ, the bursa of Fabricius. Studies performed mainly in chickens indicate that the GALT pathway for B cell differentiation differs fundamentally in key aspects from the paradigm established in humans and mice, but seems to be more of a rule than an exception in domestic species.

In the human and mouse bone marrow, lymphopoesis from progenitor cells to B cells is a livelong process [reviewed in (8) and (9)]. In contrast, chicken B cell development occurs in defined temporal steps known as pre-bursal, bursal and post-bursal phases (10). During the pre-bursal stage of development, hematopoietic precursor cells derived from the intra-embryonic mesenchyme commit to the B cell lineage and colonize the bursal anlage (11). Commitment to the B cell lineage is manifested by Ig gene rearrangement during a window of ~10 days in embryonic development and initially takes place in the embryonic yolk sac. Subsequently, these committed cells spread to blood, spleen and various lymphoid organs (12, 13). This small number of pre-bursal stem cells migrates to the bursa, where only cells with productive Ig gene rearrangement undergo proliferative expansion (14). Furthermore, B cell development in the bursal environment triggers the diversification of the B cell receptor (BCR) by gene conversion (15, 16). Beginning around hatch and continuing until bursa involution, immature bursal B cells with functional and diversified surface Igs start to emigrate to the periphery and distribute to the secondary lymphoid organs (17, 18). In striking contrast to humans and mice where bone marrow B cell development is maintained for the entire life, the bursa of Fabricius involutes with sexual maturity. Thus, the peripheral B cell compartment in chickens is maintained by a small pool of long-living, self-renewing B cells, the so-called post-bursal stem cells (19).

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While the molecular events of BCR diversification in the bursa are well understood, very little information exists on signals that control death, survival and developmental checkpoints in the avian B cell compartment in general. Extensive studies using retroviral gene transfer *in vivo* have shown that the cytoplasmic domains of the Ig$\alpha\beta$ heterodimer are as efficient as the endogenous complete BCR in supporting the early stages of B cell differentiation (20, 21). However, for further development and emigration from the bursa, an intact surface IgMR complex is necessary. Of note, only a small percentage of the newly generated cells emigrates while the vast majority of bursal B cells dies by apoptosis (22–24). In addition to B cell-intrinsic factors, signals derived from the bursal microenvironment have been postulated to exist. These include adhesion molecules which may support homing and emigration of B cells in and from the bursa (25) and soluble factors. The soluble tripeptide bursin was described as a factor which may act on the appearance of surface IgM^+ cells during bursa ontogeny (26, 27). Recently, we were able to identify chicken B cell-activating factor of the tumor necrosis factor family (chBAFF), the chicken homologue of mammalian B cell-activating factor of the tumor necrosis factor family (BAFF/BLyS), as a potent B cell-specific cytokine (28).

In mammals, members of the tumor necrosis factor (TNF) family and their receptors are well known as important regulators of B cell development and function (29, 30). BAFF is a relatively new member of the TNF family of cytokines and plays a major role in peripheral B cell survival and maturation in humans and mice. Deregulated, increased BAFF production is associated with autoimmune disorders like systemic lupus erythematosus, rheumatoid arthritis and Sjörgens syndrome (31–33). On the contrary, a lack of BAFF leads to severe immunodeficiency as shown in BAFF-deficient mice (34) and in cultured human cells (35). In mice, two distinct sources of BAFF have been identified. Radiation-resistant stroma cells constitutively express BAFF (36), and monocytes, macrophages, dendritic cells (37, 38) and neutrophils (39) produce BAFF in response to other cytokines. While the constitutive BAFF pool controls the size of the peripheral B cell pool (36, 40), the inducible fraction seems to be required for the accumulation of additional B cell populations, for example, at sites of infection (41, 42). It was also shown that neoplastic B cells can be a source of BAFF (43, 44) but so far it is widely accepted that normal human and murine B cells do not produce BAFF (44, 45).

BAFF can bind three receptors of the TNFR family, B cell maturation antigen (BCMA); transmembrane activator, calcium modulator and CAML interactor (TACI) and BAFFR. Interestingly, BCMA and TACI can also bind a proliferation-inducing ligand (APRIL), another member of the TNF family, while BAFFR exclusively binds to BAFF. BAFFR is expressed by all peripheral B cells (46, 47) and additionally by a small T cell subset (35) where it is able to co-stimulate T cell activation (35). Interaction with BAFFR is essential for peripheral B cell survival as well as the formation and differentiation of marginal zone B cells (48). BCMA surface expression is restricted to germinal centers and plasma cells and mediates long-term survival of plasma cells (49). TACI is primarily expressed on T2 and MZ B cells, and current functional data suggest that TACI may serve as a negative regulator of B cell activation. From intensive studies in mice and humans, the generally accepted concept emerged that BAFF solely acts on mature peripheral B cell, while early stages of human and murine B cell development in the bone marrow are BAFF independent (50). In the chicken genome, APRIL, BCMA and TACI appear to be absent (51), leaving birds with just BAFF and BAFFR homologues.

As previously reported, chBAFF also acts as survival factor for peripheral B cells *in vitro* and *in vivo* (28). Intriguingly, in the chicken this cytokine is primarily expressed in the bursa of Fabricius (28, 52) and expression of receptors for chBAFF could be detected on all chicken B cells, including immature bursal B cells. Here we extend our previous studies and demonstrate that BAFF has phylogenically conserved functions on peripheral chicken B cells, and we further demonstrate unique BAFF properties in this model species with GALT-dependent B cell generation.

Methods

Animals

Fertilized eggs of the M11 (B^{x2x}/B^{x2x}) white leghorn chicken line were obtained from the Institute for Animal Science Mariensee (53) and hatched at the Institute of Animal Physiology, Munich, Germany. Birds were housed under conventional conditions in groups of up to 10 birds. All animal experiments were authorized by the Regierung von Oberbayern (registration number 209.1/211-2531-46/03)

Cells

Chicken splenocytes, cells of the bursa of Fabricius and the cecal tonsils were separated by density gradient centrifugation on Ficoll–Paque (Amersham Pharmacia Biotech, Freiburg, Germany) after tissue dissociation. PBLs were prepared by slow-speed centrifugation as previously described (54). Cells were maintained in RPMI 1640 medium with Glutamax (GIBCO, UK) supplemented with 10% FCS at 40°C in a CO_2 (5%) incubator.

Chemical bursectomy

To deplete B cells from bursal follicles, chicks were injected intramuscularly with 300 µl of a 1% solution of cyclophosphamide in 0.9% NaCl on days 1, 2, 3 and 4 after hatch. On day 10, the treated animals were killed and tissue samples were collected for RNA preparation and histology.

RNA isolation and northern blot analysis

RNA was derived from various organs and from isolated cells using TRIZOL™ reagent (GIBCO, Karlsruhe, Germany) according to the manufacturer's protocol. The indicated amounts of RNA were size fractionated by electrophoresis through an agarose gel containing 4% formaldehyde using standard procedures before transfer onto a nylon membrane. For hybridization, cDNAs containing the complete ORFs of chBAFF (28) and chicken GAPDH (chGAPDH) (55) were digested with *Pst*I/*Eco*RI and *Not*I/*Xho*I, respectively. [^{32}P]-radiolabeled cDNA probes were generated using the High Prime DNA labeling kit (Roche Diagnostics, Mannheim,

Germany) and hybridization was performed with QuickHyb® Hybridization Solution (Stratagene, Heidelberg, Germany).

In situ hybridization

chBAFF-specific PCR products coding for the extracellular part of chBAFF were cloned between the *Eco*RI and *Hin*dIII restriction sites of vector pGEM®-4Z (Promega, Mannheim, Germany). Purified plasmids were digested with *Eco*RI and *Hin*dIII for sense and antisense riboprobes, respectively. Radiolabeled RNA probes were prepared from the resulting linearized plasmids using T7 and SP6 polymerases (Stratagene) and $\alpha[^{33}P]$UTP. Cryostat sections from bursal tissue of 10 μm thickness were fixed for 5 min in 4% buffered formaldehyde, washed twice in PBS, treated for 7 min at room temperature (RT) with 24 U ml^{-1} of Pronase K in TE buffer (Roche Diagnostics), washed again twice with PBS and incubated for 10 min in 0.25% acetic acid anhydride solution. After further washes in PBS, sections were postfixed in 4% buffered formaldehyde (5 min, RT), washed again, dehydrated in an ascending alcohol series and air-dried. After addition of 75 μl hybridization solution [250 ng ml^{-1} probe, 50% formamide, 20% dextran sulfate, 10 mM EDTA, 1 mg ml^{-1} of yeast tRNA (Invitrogen, Karlsruhe, Germany), 10 mM dithiothreitol (Applichem, Darmstadt, Germany), 200 mM NaCl, 10 mM Tris–HCl pH 7.5, 5 mM EDTA, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 0.1% w/v SDS, 0.1% w/v Ficoll 400, 0.1% w/v polyvinylpyrrolidone, 20 units ml^{-1} of heparin], sections were sealed with Hybri-well press seal-hybridization chambers (Sigma, Taufkirchen, Germany) and incubated in a humidified chamber overnight at 55°C. Unbound probes were removed by two washes in 2× saline–sodium citrate (SSC), followed by a 30-min RNAseA digestion (20 μg ml^{-1} in 0.5 M NaCl/10 mM Tris/1 mM EDTA) at 37°C and further two washes in 2× SSC, 1× SSC, 0.5× SSC for 5 min each and a high stringent wash in 0.1× SSC for 30 min at 60°C. After a dehydrating step in an ascending alcohol series, slides were air-dried and processed for autoradiography with NTB-2 Emulsion, D-19 Developer and Fixer (Kodak, Stuttgart, Germany). Finally, sections were counterstained with Mayer's Hematoxylin (Sigma), dehydrated one more time and mounted in Eukitt® mounting medium (Fluka, Buchs, Switzerland).

Histology

Bursal tissue was embedded in Tissue-Tek® (Sakura Finetek, Heppenheim, Germany), snap frozen in liquid nitrogen and stored at −80°C. Cryostat sections (7 μm thick) were mounted on Super Frost®Plus slides (Nunc, Wiesbaden, Germany), air-dried and fixed in cold acetone for 10 min. For immunohistochemical staining, sections were rehydrated in PBS and blocked for 20 min with 10% normal horse serum in PBS. This was followed by an overnight incubation with the anti-chicken Bu1 antibody AV20 (0.5 μg ml^{-1} in PBS-T/SBA, Birmingham, AL, USA) at 4°C and incubations with biotinylated horse anti-mouse Ig (7.5 μg ml^{-1} in PBS/1% BSA, Vector Laboratories, Burlingame, CA, USA) for 30 min at RT and StreptABComplec/HRP (DakoCytomation GmbH, Hamburg, Germany) according to the manufacturers' protocol. Peroxidase activity was developed using 3,3′diaminobenzidine-tetrahydrochloride (Sigma). Subsequently, sections were counterstained with Mayer's Hematoxylin (Sigma), gradually dehydrated with graded alcohol and mounted in Eukitt® mounting medium (Fluka).

To visualize successful B cell depletion in cyclophosphamide-treated birds, cryostat sections were prepared as described above and subjected to a standard hematoxylin and eosin staining.

Polyclonal antiserum

To generate a polyclonal antiserum against chBAFF, a rabbit was immunized with affinity-purified recombinant His-tagged chBAFF, expressed in *Escherichia coli* (28). The IgG fraction of the serum was purified by affinity chromatography on a Protein A–Sepharose column (Amersham Pharmacia Biotech) and used for western blotting.

Cell lysis and western blotting

For cell lysis, organs were minced through a steel sieve immediately after extraction. The resulting cell suspension was sedimented on ice for 10 min whereupon the supernatant was centrifuged. The cell pellet was re-suspended in cold PBS with Ca++ and Mg++ (DPBS). Cells were counted and washed again in DPBS. All cells were lysed for 45 min on ice in lysis buffer (1 ml per 5×10^7 cells) [150 mM NaCl, 50 mM Tris–Cl pH 7.4, 1 mM EDTA, 1% Triton X-100 and a protease inhibitor mixture (complete, Roche Diagnostics)]. The nuclear and insoluble components were removed by a 16 000 × *g* centrifugation step for 30 min at 4°C and the supernatant was used for western blotting. Purified Flag–chBAFF or cell lysates were diluted in 2× Laemmli buffer and separated on 12% SDS-PAGEs under reducing conditions. Proteins were transferred to nitrocellulose by semidry blotting as described (56). Membranes were blocked with 4% skim milk and incubated with HRP-conjugated mouse anti-Flag M2 (Sigma) or 5 μg ml^{-1} of rabbit anti-chBAFF followed by HRP-coupled goat anti-rabbit IgG (Jackson, Cambridgeshire, UK). Membranes were developed with the ECL western blotting detection reagent (GE Healthcare, Buckinghamshire, UK).

In vitro survival assay

Cells were cultured in standard RPMI 1640 medium supplemented with Glutamax and 10% FCS at 5×10^6 cells per well in 24-well plates at 40°C. Supernatants of 293 cells stably transfected with either the Flag–chBAFF expression construct or with an empty vector (28) were added to the culture medium at a final concentration of 5%. The frequency of living B cells [light chain (L chain)$^+$/propidium iodide (PI)$^-$] was analyzed by flow cytometry as described below. Absolute numbers of viable cells were determined by trypan blue exclusion and absolute numbers of viable B cells were calculated from these data.

Splenic and bursal B cell preparations of >95 and 99% purity, respectively, were obtained by magnetic cell sorting on MACS separation columns LS (Miltenyi Biotec GmbH, Germany) using a chicken Ig L chain-specific antibody (Southern Biotechnology Associates, Birmingham, AL, USA), anti-mouse Ig–FITC (Sigma) and anti-FITC microbeads (Miltenyi Biotec GmbH).

To obtain B cell-free preparations from splenic leukocytes (<1.5% L chain$^+$ cells), cells were stained alike and depletion was done using MACS separation columns LD (Miltenyi Biotec GmbH).

Cell proliferation

For cell proliferation assays, 5×10^5 bursal or splenic lymphocytes per well were cultured in 96-well plates in the presence or absence of 5% chBAFF-containing 293 supernatant. Polyclonal stimulation was achieved by the addition of 10 ng ml^{-1} phorbol myristate acetate (PMA) (Sigma). After 24 h, cells were pulsed with [^{3}H]thymidine ([^{3}H]TdR) and harvested 16 h later.

Flow cytometry

Staining of the cells for flow cytometric analysis was performed according to standard procedures. Cells were stained with mAbs against IgM (M1) (57), L chain (Southern Biotechnology Associates), Bu1(58) or a mixture of anti-TCRγδ (TCR1), TCRα/vβ1 (TCR2) and TCRα/vβ2 (TCR3) (59) followed by an anti-mouse Ig–FITC conjugate (Sigma).

Receptor binding of chBAFF was shown as described recently (28). Briefly, after staining with mAb AV20 and an anti-mouse–FITC conjugate, cells were incubated with Flag-chBAFF-containing 293 supernatants and normal mouse serum, followed by staining with biotinylated anti-Flag mAb (M2) (Sigma) and streptavidin–PE (Southern Biotechnology Associates).

To demonstrate the ability of the soluble human B cell maturation antigen (hBCMA)-Fc construct to block the interaction of chBAFF with its receptor, different concentrations of hBCMA-Fc were pre-incubated with chBAFF-containing supernatants for 20 min at RT. Subsequently, these preparations were used to stain chBAFFR-positive cells as described above.

Dead cell discrimination for *in vitro* survival assays was done through staining with PI (2.5 μg ml^{-1}). Analysis was performed with a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using Cell Quest Plus software.

In ovo and post-hatch neutralization of chBAFF

hBCMA-Fc consisting of the signal peptide of IgG1 heavy chain, amino acid residues 2–54 of hBCMA and the Fc domain of human IgG1 was expressed in 293 cells and submitted to Protein A affinity purification (60). For *in ovo* neutralization of chBAFF activity, fertilized eggs were injected with 100 μg of hBCMA-Fc (1 mg ml^{-1} in PBS) intravenously (i.v.) on day 15 of embryogenesis as described (61). On day 18, embryos were extracted and organs were subjected to immunohistochemistry.

To block chBAFF activity after hatch, 1-day-old chicks were given intra-peritonial (i.p.) injections of 300 μg of hBCMA-Fc. For one experimental approach, this treatment was repeated on days 5 and 8. On day 10, spleens, cecal tonsils and bursae were removed and the spleen/body weight index and the bursa/body weight index were calculated. For a second set of experiments, 1-day-old chicks were injected once a day over a period of 5 days and analyzed on day 6. Lymphocytes from spleens and cecal tonsils were separated on Ficoll (Amersham Pharmacia Biotech) and subjected to analysis by flow cytometry. In order to obtain sufficient numbers of cecal tonsil lymphocytes, organs from all birds in each group were pooled prior to lymphocyte isolation.

Results

BAFF is expressed early during B cell ontogeny

In our earlier work we have shown that chBAFF is mainly expressed in the bursa of Fabricius and that BAFFRs are found on all B cells including bursal B lymphocytes. To follow up on this initial finding, BAFF expression was investigated by northern blot analysis of tissue samples from spleen, bursa, liver and thymus using a radiolabeled PCR fragment derived from the BAFF sequence. Tissue samples were obtained from day 10, 14 and 18 embryos and birds at the age of 1 day (Fig. 1A). No cytokine expression was found in tissues from day 10 embryos. On embryonic day (ED) 14, weak BAFF expression could be detected in the bursa but not in the spleen and liver tissue. A strong up-regulation of BAFF gene expression was observed in the bursa between EDs 14 and 18 which further increased until hatch (D1). In the spleen, clearly detectable BAFF signals were not found until D1. No BAFF expression was observed in the thymus at any time point. After hatch, a similar expression pattern was found with a strong BAFF signal in the bursa, a weak signal in the spleen and no expression in the thymus (data not shown).

To obtain evidence for expression of the BAFF protein in the bursa, we developed a BAFF-specific polyclonal antiserum by repeated immunization of a rabbit with purified *E. coli*-derived protein. Binding specificity was confirmed by western blot analysis. The chBAFF antiserum recognized purified Flag-tagged chBAFF with a similar reactivity pattern as the Flag-epitope-specific M2 mAb (Fig. 1B). Next, cell lysates derived from bursa, spleen and thymus were subjected to SDS-PAGE, transferred to nitrocellulose and probed with the chBAFF antiserum. As expected from the gene expression studies, BAFF protein was readily detectable in bursal lysates and, with a much weaker signal, in spleen samples but not in thymic control lysates (Fig. 1C). Collectively, these experiments demonstrated that BAFF is expressed as a mature protein in the bursa of Fabricius and in the spleen.

BAFF is primarily expressed by B lymphocytes

To characterize the cell types expressing BAFF in the chicken, we next performed *in situ* hybridization studies on bursal tissue sections. Cytokine expression was found throughout bursal follicles and to some extent in the interfollicular connective tissue (Fig. 2). While staining was seen in both cortex and medulla of bursal follicles, the stronger signal was seen in the medulla. These results indicated that, in contrast to mammals, chickens may express BAFF primarily in B lymphocytes. To further address this hypothesis, we used the chemical bursectomy technique with cyclophosphamide to selectively deplete B cells from the bursa, leaving the bursal stroma intact. Successful bursectomy was achieved in all birds as revealed by histology, demonstrating the loss of organized follicular structures after cyclophosphamide

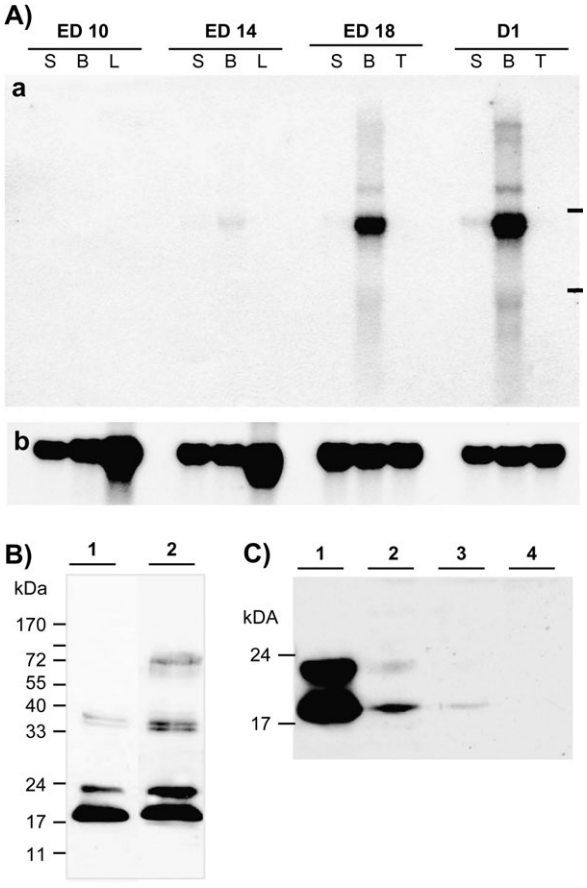

Fig. 1. Early chBAFF expression during B cell ontology. (A) RNA from spleen (S), bursa (B), liver (L) and thymus (T) was isolated at the indicated time points of embryonic development (EDs 10–18) and from 1-day-old chicks (D1), respectively. chBAFF cDNA (28) was used to probe a northern blot containing 10 μg of RNA per lane (a). Reprobing of the membrane was performed with chGAPDH cDNA (55) to compare the relative amounts of RNA per lane (b). The gel positions of the 28S rRNA and 18S rRNA are indicated. (B) A total of 1 μg of Flag–chBAFF per lane was subjected to SDS-PAGE under reducing conditions and probed by western blotting with either Flag-specific mAb M2 (lane 1) or chBAFF antiserum (lane 2). The prominent bands at 18 and 23 kDa represent the Flag-tagged protein. Minor reactivity with anti-Flag at 36 kDa most likely represents Flag–BAFF aggregates. The reactivity found with the anti-chBAFF serum at 72 kDa can be attributed to an unspecific reactivity of the antiserum. (C) The chBAFF antiserum was used to analyze cell lysates from bursa (lane 2), spleen (lane 3) and thymus (lane 4) by western blotting. Recombinant Flag–chBAFF served as positive control (lane 1).

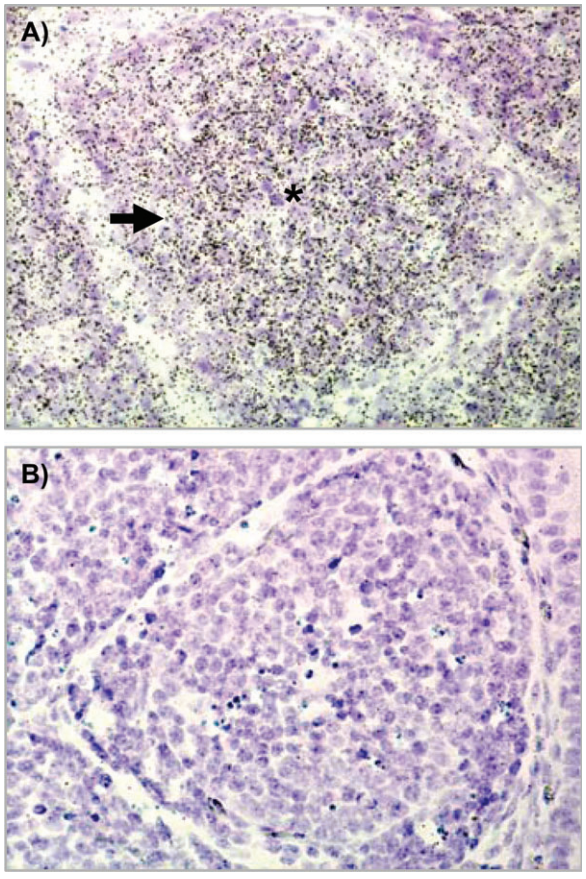

Fig. 2. *In situ* hybridization shows chBAFF expression in bursa follicles. Bursa cryostat sections from 6-week-old birds were hybridized with [^{33}P]-labeled antisense (A) and sense probes (B) for chBAFF and counterstained with hematoxylin. The asterisk indicates the follicle medulla; the arrow marks the follicle cortex; 40-fold magnification.

treatment (Fig. 3A). Northern blot analysis of individual bursal and splenic tissue samples showed strong BAFF expression in bursa samples and clear but significantly weaker expression in spleens of untreated birds. No cytokine mRNA could be demonstrated in the tissues of B cell-depleted animals (Fig. 3B). As demonstrated by chGAPDH hybridization, the comparison of total organ samples results in unequal RNA loading of the gels. However, an analysis performed with comparable amounts of total RNA (Fig. 3C) yielded the same conclusion that no BAFF mRNA could be detected in B cell-depleted animals while an intense signal was found in control animals.

Expression analysis was next performed on isolated cell populations. B cells were purified from bursa and spleen samples by positive sorting with magnetic beads. In addition, splenic lymphocyte preparations were depleted from B cells by the same technique (<1% B cells). chBAFF expression in these preparations was again determined by northern blot analysis (Fig. 4). As expected, a signal was found in the bursal control sample and an even stronger signal was found in RNA prepared from positively sorted bursal B cells. Furthermore, chBAFF mRNA was found in total spleen samples and splenic leukocyte preparations. Importantly, a clear cytokine expression signal could be detected in purified splenic B cells, while B cell-depleted preparations gave no signal. These data strongly indicate that BAFF is primarily expressed by B lymphocytes and to a much lesser degree, if at all, by the stromal tissue of the bursa and the spleen.

BAFFR is expressed early in B cell development

Since eukaryotic recombinant chBAFF shows avid binding to B cells, we next analyzed expression of BAFFRs on B lymphocytes from different developmental stages (Fig. 5). Pre-bursal, bursal and post-bursal B lymphocytes were identified by their expression of the B cell-specific antigen Bu1 with

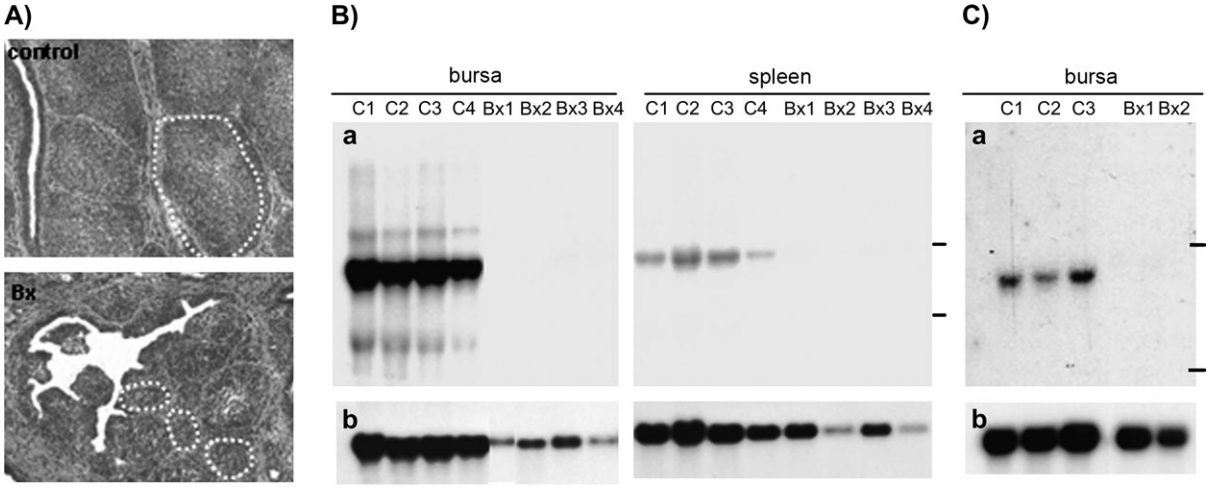

Fig. 3. Chemical bursectomy abrogates chBAFF expression. B cell depletion in young chicks was achieved by the chemical bursectomy method using cyclophosphamide. (A) To control successful bursectomy, hematoxylin and eosin staining from cryostat sections of bursa from all treated (Bx) and control birds was performed. A representative data set is shown where clearly developed B cell follicles in the control and remnants of follicular connective tissue in bursectomized birds are marked with a dashed line. (B) RNA samples from bursa and spleens of four control (C1–C4) and four bursectomized (Bx1–Bx4) birds representing a total organ equivalent were subjected to northern blot analysis using a chBAFF-specific cDNA probe (a). Bars indicate the gel positions of the 28S and 18S rRNA. A chGAPDH-specific cDNA probe was used to estimate the relative amounts of loaded RNA (b). (C) Likewise, northern blotting with 10 μg of bursa RNA per lane from control (C1–C3) and bursectomized (Bx1–Bx2) birds was performed using probes for chBAFF (a) and chGAPDH (b).

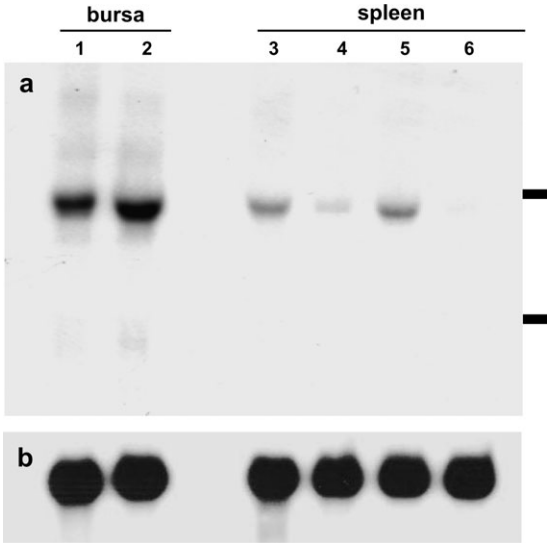

Fig. 4. chBAFF is expressed by B cells. (a) Expression analysis was done by northern blotting with a chBAFF-specific cDNA probe using 10 μg of RNA each from entire organs or isolated cell populations: complete bursa (1), purified bursal B cells (>99.5% purity) (2), complete spleen (3), splenic leukocytes obtained by density centrifugation (4), purified splenic B cells (>96% purity) (5), B cell-depleted splenic leukocytes (<1.5% B cells) (6). The gel positions of the 28S and 18S rRNA are marked by black bars. (b) The RNA integrity and equal loading was controlled by hybridization with a chGAPDH-specific probe.

the mAb AV20. AV20$^+$ pre-bursal stem cells were detectable in ED 10 spleens but did not show BAFFR expression. The frequency of pre-bursal B cells increased during the next 4 days of embryogenesis and these cells became weakly positive for BAFFR. At the same time, B cells became detectable in the early bursal anlage and showed a comparable BAFFR expression level to the splenic pre-bursal cell population. BAFFR expression significantly increased during the following days until hatch. At ED 18 and later time points, all AV20$^+$ cells in the bursa were positive for BAFFR and after hatch AV20$^+$ cells in the spleen showed the same intensity of BAFFR expression.

chBAFF regulates peripheral B cell homeostasis

Previously, we have shown by *in vitro* studies that chBAFF is a potent survival factor for splenic B lymphocytes. To investigate if this functional property applies to peripheral B cells, in general, we isolated lymphocytes from spleens and cecal tonsils, one of the few organized secondary lymphoid structures in the chicken, and cultured these cells in the presence or absence of chBAFF. Cytokine treatment led to a significant increase in viable B lymphocyte numbers in spleen cell cultures as expected from our earlier work (Fig. 6A). This effect was even more pronounced in cultures of cecal tonsil lymphocytes and B cells purified from spleens by magnetic cell sorting (>95% B cells). The survival factor activity was also observed when cell proliferation of splenic B lymphocytes was induced by PMA. A comparison of proliferative activities in chBAFF-treated and control cultures (Fig. 6B) after 40 h of incubation showed a significant increase of [^{3}H]TdR uptake in cultures with the cytokine from an average of 754 counts per minute (c.p.m.) (medium) and 33.643 c.p.m. (PMA) up to 2348 c.p.m. (BAFF) and 64.737 c.p.m. (PMA + BAFF). These data confirm our previous observations and support the concept that BAFF is an important regulator of B cell homeostasis in secondary lymphoid organs in general.

To investigate the functional relevance of chBAFF *in vivo*, we made use of our earlier observation that this avian

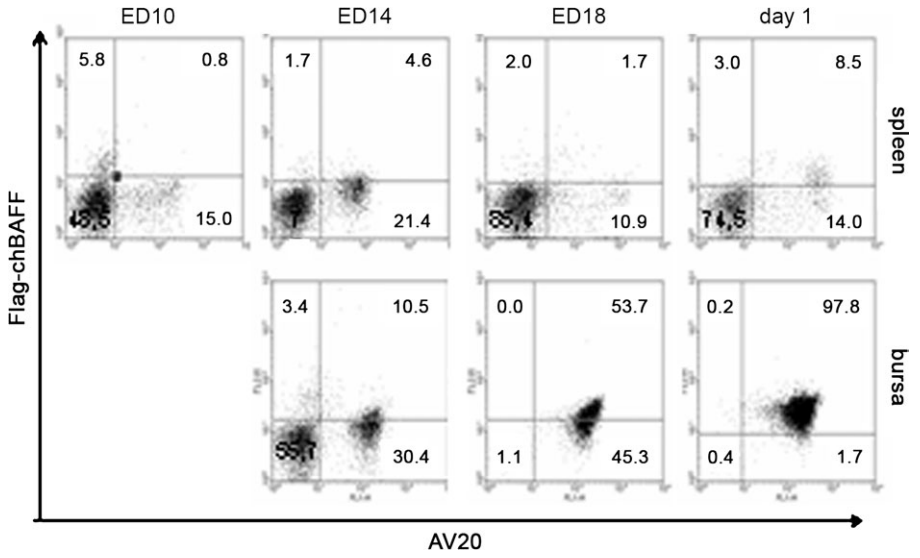

Fig. 5. chBAFFR is expressed early during embryogenesis. Leukocytes from spleen (upper panel) and bursa (lower panel) were isolated at different times of embryogenesis (EDs 10–18) and on the day of hatch (D1). Binding of Flag–chBAFF to AV20^{+}B cells was analyzed by flow cytometry.

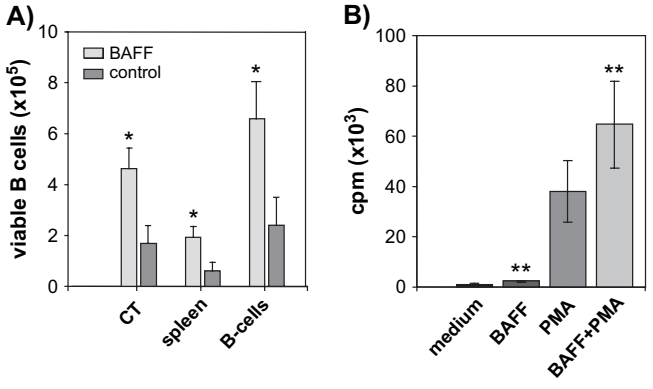

Fig. 6. chBAFF promotes the survival of peripheral B cells *in vitro*. (A) Leukocytes from cecal tonsils (CT) and spleen, as well as purified splenic B cells, were cultured in the presence or absence of 5% conditioned medium containing Flag–chBAFF. After 48 h, the absolute number of viable B cells was determined by a combination of trypan blue exclusion (absolute number of viable cells) and FACS analysis (frequency of viable B cells). (B) A total of 1×10^5 splenocytes per well were cultured with or without 5% of Flag-chBAFF-containing medium in the presence or absence of 10 ng ml^{-1} of PMA. After 24 h, cells were pulsed with [^{3}H]TdR and harvested 16 h later. Data represent mean ± SD of three independent experiments (*$P \leqslant 0.05$, **$P \leqslant 0.01$, *t*-test).

cytokine strongly binds to hBCMA in a receptor–ligand interaction ELISA (28). To further demonstrate that BCMA can block the binding of chBAFF to its receptor on chicken cells, we pre-incubated chBAFF with increasing concentrations of hBCMA-Fc prior to the staining of the BAFFR on B cells. Addition of 2.5 μg ml^{-1} of hBCMA-Fc completely blocked chBAFF interaction with its receptor (Fig. 7A). We therefore used hBCMA-Fc as a soluble decoy receptor to neutralize the biological activity of chBAFF in young chicks. Five 1-day-old birds per group received either hBCMA-Fc in PBS at a dose of 300 μg per animal i.p. and additional treatments 3 and 6 days later or PBS alone. The birds were killed on day 10 after hatch. hBCMA treatment did not affect animal health and body weights. However, spleen weights were reduced from an average of 110 mg in the controls to 66 mg in hBCMA-Fc-treated birds (Table 1).

Spleens and cecal tonsils were analyzed for the relative numbers of B cells and T cells by flow cytometry. hBCMA-Fc treatment significantly reduced the frequency of B cells in the spleens and in the cecal tonsils in comparison with mock-treated birds (Fig. 7B and C). As a consequence, the relative numbers of T cells increased in hBCMA-Fc-treated animals in both tissues. The reduced number of splenic B cells in the absence of functional BAFF in young chickens clearly demonstrates the important role of BAFF in the homeostasis of mature B cells.

chBAFF promotes survival of immature bursal B cells

The analysis of BAFF and BAFFR expression revealed the existence of both the cytokine and its receptor at very early stages of chicken B cell development. This observation suggested a new role for chBAFF during maturation of bursal B cells. To further address this hypothesis, chBAFF was added to cultures of bursal lymphocytes. Although the addition of chBAFF could not abrogate the gradual loss of viable cells, it induced a significant delay of bursal B cell death (Fig. 8A). The bursa of Fabricius is a highly proliferating tissue and pulsing of unstimulated bursal lymphocytes with [^{3}H]TdR immediately after cell isolation yielded incorporation values between 20 000 and 40 000 c.p.m. However, this proliferating activity decreased rapidly with time of culture (data not shown). The presence of BAFF in these cultures caused a markedly higher proliferation rate after 40 h (Fig. 8B).

To verify these effects on immature B cells *in vivo*, we again took advantage of the neutralizing ability of hBCMA. In the initial experiment with i.p. application of 300 μg of

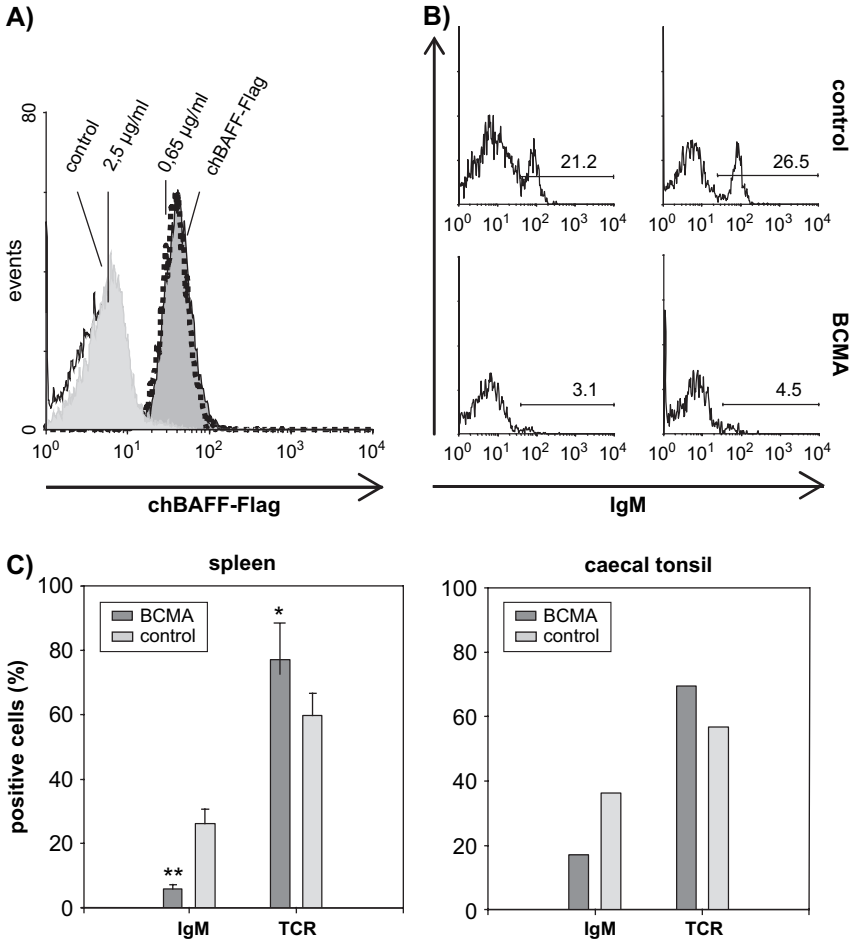

Fig. 7. Neutralization of chBAFF *in vivo* decreases peripheral B cell frequencies. (A) Medium containing Flag–chBAFF was incubated with the indicated concentrations of hBCMA-Fc and subsequently used to analyze binding to chBAFFRs on bursal B cells by flow cytometry. Staining with control supernatant and Flag–chBAFF without hBCMA served as controls. (B and C) Beginning on the day of hatch, four chicks per group received i.p. injections with 300 µg of hBCMA-Fc per animal or PBS once each day over a period of 5 days. On day 6, spleens and cecal tonsils were analyzed by flow cytometry. (B) Splenocytes from control (upper panel) and hBCMA-treated birds (lower panel) were stained against surface IgM. Two representative FACS profiles per group are shown. (C) B and T cell frequencies in spleens and cecal tonsils were determined by staining with antibodies against Bu1 (AV20) and the TCR (using a combination of TCR1, TCR2 and TCR3). Data represent means ± SD ($n = 4$). Cecal tonsils from each group were pooled prior to lymphocyte isolation (*$P \leq 0.05$, **$P \leq 0.01$, *t*-test).

Table 1. Influence of chBAFF neutralization on spleen and bursa weight

	Body weight (g)	Spleen weight (mg)	Bursa weight (mg)	Spleen/body weight index (mg g^{-1})	Bursa/body weight index (mg g^{-1})
hBCMA	65.6 ± 7.1	66.0 ± 55.7	249.3 ± 138.4	1.18 ± 1.5	6.9 ± 3.9
Control	70.7 ± 1.9	110.0 ± 87.8	323.0 ± 158.0	3.0 ± 2.5	8.6 ± 4.5

Five animals per group were injected with either 300 µg hBCMA-Fc or PBS i.v. on days 2, 5 and 8. On day 10, spleen and body weights were determined, and the spleen/body weight index of each animal was calculated. Mean values ± SDs are given for each group.

hBCMA as described above, a trend toward a reduction in bursal weights from a mean of 323–249 mg was observed (Table 1), although this decrease was not statistically significant. In order to neutralize chBAFF activity at a very early stage of B cell development, we used the i.v. injection route *in ovo* with 100 µg of hBCMA per egg on day 15 of embryogenesis. When bursae of hBCMA-Fc-treated and control animals were taken from day 18 embryos, immunohistochemical staining with the B cell marker AV20 revealed an obvious decrease in the number of B cell follicles due to BAFF neutralization (Fig. 9A). To quantify this effect, the number and area of AV20-positive B cell follicles were determined. hBCMA treatment *in ovo* reduced the number of B cell follicles from an average of 129 in control bursae

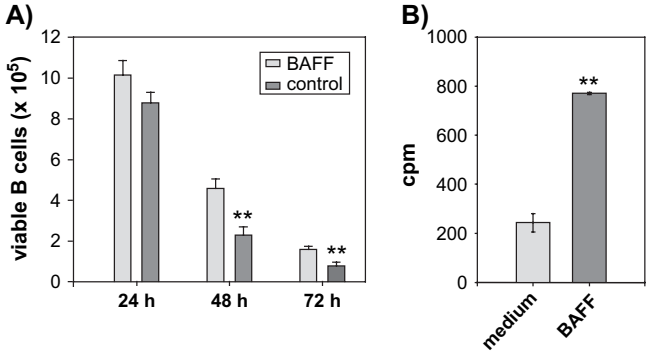

Fig. 8. chBAFF decelerates death of bursal lymphocytes in culture. (A) Bursal lymphocytes were cultured in the presence or absence of 5% chBAFF-containing medium. At the indicated times, the number of viable B cells in these preparations was determined by combining trypan blue exclusion and FACS analysis. (B) A total of 1×10^5 bursal lymphocytes per well were cultured with or without 5% Flag-chBAFF-containing medium. After 24 h, cells were pulsed with [^{3}H]TdR and harvested 16 h later. Data represent mean ± SD of three independent experiments (**$P \leq 0.01$; *t*-test).

to 63. Likewise, the mean follicle area in hBCMA-treated bursae was significantly smaller than that in untreated controls (40 versus 65 mm^2) (Fig. 9B).

Discussion

The immune system of most domestic animals differs from that of the common immunological model organism, the mouse, in a variety of aspects. This is particularly true for birds which use a unique organ, the bursa of Fabricius, for B cell differentiation (18, 62, 63). The availability of EST databases and the recent publication of the chicken genome sequence (64, 65) have finally provided the information required to investigate the phylogeny, structure and function of the avian immune system (65). Sequence information from the animal genome projects have now to be translated into functional data to allow studies on the host response to pathogens and to understand the evolutional differences among higher vertebrates. Here we further characterize the biological properties of the recently discovered avian homologue of BAFF and show that this cytokine has conserved functions *in vitro* and *in vivo*, as well as unique properties which have not been described in mammals thus far. Although mammalian BAFF is part of a complex system containing the two ligands BAFF and APRIL and the three receptors BAFFR, TACI and BCMA, the situation in birds seems to be simpler in this respect. Indeed, so far no homologues of APRIL, BCMA and TACI can be detected in the chicken genome (51), leaving birds with a single ligand–receptor pair consisting of chBAFF and chBAFFR, the homologue of the mammalian BAFFR/BR3.

Previous northern blot analysis had shown that chBAFF is expressed in the spleen of mature birds (28, 38), as expected from data obtained in mice (50, 66). Surprisingly, the highest expression levels were found in the bursa of Fabricius, the bone marrow equivalent of birds. This observation contrasts with findings in mice and humans where BAFF is

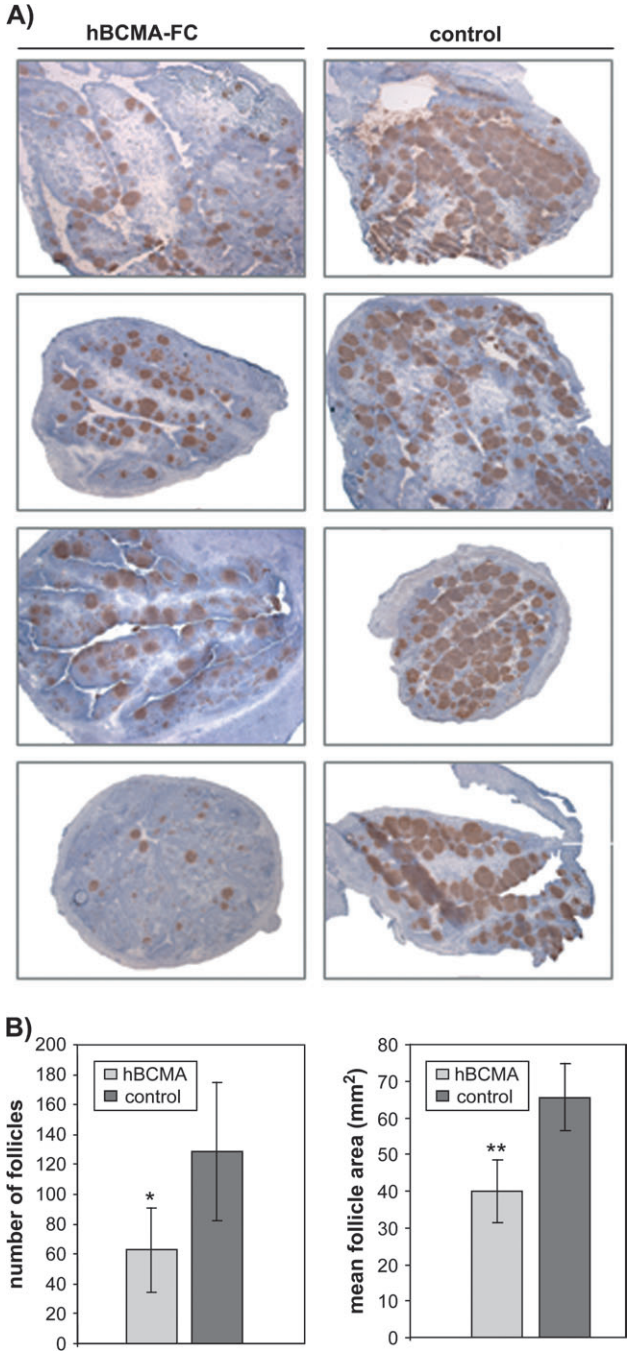

Fig. 9. *In ovo* neutralization of chBAFF interferes with embryonic B cell development. (A) On day 15 of embryogenesis, fertilized eggs were i.v. injected with 100 μg of hBCMA-Fc (at 1 mg ml^{-1} in PBS). On day 18, embryos were extracted and bursae were subjected to immunohistochemical staining for the B cell marker Bu1 followed by counterstaining with hematoxylin. Bursae of Fabricius from four treated and four untreated animals are shown. (B) Number of follicles (left) and mean follicle area (right) from the sections shown in (A) was determined using the AXIO-Vision Rel.4.5. software (Zeiss). Data represent mean ± SD (*$P \leq 0.05$, **$P \leq 0.01$, *t*-test).

essentially absent from the bone marrow and is not required for early B cell differentiation (47, 50). To further address this discrepancy, we performed ontogenic studies which showed

that chBAFF is first expressed in the bursa when pre-bursal stem cells home into this organ around EDs 10–14 (11). As the number of bursal B cells expands, chBAFF expression is strongly up-regulated. Importantly, no cytokine expression is observed in peripheral lymphoid organs prior to B cell emigration form the bursa which starts around the day of hatch (17). mRNA expression correlates well with the expression of the chBAFF protein, which was detectable in bursa and, at lower levels, in spleen lysates. The simultaneous appearance of chBAFF and B cells in the bursa and spleen is an indication that chBAFF might be primarily expressed by B cells.

This hypothesis was confirmed by *in situ* hybridization studies on bursal tissue sections. In agreement with a previous report (52), staining was observed throughout the bursal follicles with a slightly higher expression level in the medulla, arguing against a restricted expression by dendritic or other myeloid cells, which is characteristic for mice and humans (37, 67). Selective depletion of proliferating lymphocytes in the bursa using cyclophosphamide (68) also showed that the elimination of B cells was associated with the loss of chBAFF mRNA expression. Interestingly, an identical result was obtained for chBAFF expression in the spleen. Finally, chBAFF message was found in control tissue samples and highly enriched B cell preparations from bursa and spleen but was completely absent in spleen cell preparations depleted from B cells. Collectively, these studies provide strong evidence that B cells are the main source of chBAFF, which is in striking contrast with humans and mice, where BAFF expression by B cells is only seen in malignant cells (36, 43, 44, 67). Since the BAFF system has not been investigated in other domestic mammals, it is unclear whether BAFF expression by B cells is unique to birds or may also be found in other species in which B cells develop in the GALT.

The presence of high levels of chBAFF mRNA and protein in the bursa suggests a unique role for this cytokine in B cell development in birds. This is supported by chBAFFR expression in immature chicken B cells, which can be detected by indirect fluorescence staining with the soluble recombinant Flag–BAFF protein and flow cytometric analysis (28). Pre-bursal B cells in ED 10 spleens do not bind BAFF, but as these cells colonize the bursal anlage (EDs 10–14), BAFFR is weakly expressed on peripheral B cell progenitors and on early bursal B cells. BAFFR expression level on bursal lymphocytes further increases with bursal maturation, while the few remaining pre-bursal stem cells in the spleen remain BAFFR negative. The next wave of BAFFR-expressing B cells in peripheral lymphoid tissues is observed when mature B cells start to emigrate from the bursa to peripheral secondary lymphoid tissues.

In mice, BAFF acts as a central mediator in the homeostasis of mature B cell subsets (34, 40), mainly through the modulation of cell survival (66, 69–72). This function seems to be evolutionary conserved, since the addition of recombinant chBAFF to cultures from different secondary lymphoid organs significantly delays the otherwise rapid cell death. The effect of chBAFF on B cell survival is more pronounced on purified B cells, indicating that chBAFF acts directly on mature B cells and not through the activation of other cells in the mixed cultures. Stimulation with chBAFF does not directly induce B cell proliferation (28) but seems to delay apoptotic cell death of unstimulated B cells. This cytokine activity is more clearly seen after polyclonal expansion of splenic B cells with PMA stimulation, in which setting BAFF most likely rescues cells from activation-induced cell death (73).

Further elucidation of BAFF function in the chicken was approached through *in vivo* neutralization of cytokine activity. hBCMA-Fc was used for this purpose, as chBAFF cross-reacts with the mammalian receptors TACI, BCMA and BAFFR (28) and as hBCMA-Fc was able to prevent binding of chBAFF to B cells in a dose-dependent manner. Attempts to inactivate endogenous chBAFF *in vivo* by repeated injections of the soluble decoy receptor decreases the spleen/body weight index, though high variability prevents a statistically significant conclusion. This variability is probably due, at least in part, to the fact that in the chicken system outbred animals have to be used. Importantly, inactivation of endogenous chBAFF led to a significant reduction of relative B cell numbers in the peripheral lymphoid organs of all treated animals. Though either an absolute increase in T cells or an absolute decrease in B cells could explain the alteration in the B/T cell ratio, the latter corresponds much better with the decreased spleen/body weight index and the known function of BAFF in mammals, where BAFF activity was inhibited either with a decoy receptor (74) or through BAFFR gene knockout (34, 75). We conclude that the function of BAFF in the homeostatic control of the mature B cell compartment is conserved in birds and mammals. However, homeostatic control of the peripheral B cell pool in birds must differ from that of mammals in certain details, as birds display a lifelong requirement for B cell maintenance in the periphery as a consequence of bursa involution (19). Unfortunately, the lack of suitable markers for the differentiation of mature B cells subsets in chickens hinders a more detailed analysis of the target populations responsive to BAFF. As BAFF binding was found on all B cells isolated from the spleen or cecal tonsils (28), it is, however, likely that BAFF is required for all mature chicken B cells.

Our observation that purified immature bursal B cells also express BAFFR and respond to BAFF *in vitro* by enhanced survival and thymidine incorporation strongly indicates an additional role for chBAFF in early B cell development. The bursa to body weight index decreases in birds with severe depletion of peripheral B cells. Although this index was slightly decreased in birds treated with BCMA Ig, the difference was not significant and histological examination of the bursal tissue did not reveal obvious morphological differences between treated and untreated groups, suggesting either that BAFF is not essential for immature B cells or that the treatment protocol might have been insufficient to neutralize the high amounts of BAFF in the bursa. We favor the second hypothesis, because administration of BCMA-Fc *in ovo* at ED 15, when B cell expansion in the bursa has just begun, efficiently reduced the number of B cell follicles in comparison to sham-treated embryos. A major regulating mechanism for developmental processes in the bursa is apoptosis (76) and it has been shown that both pro- and anti-apoptotic Bcl-2 family members like Bax, Bcl-2 and Nr13 play a role in this process (77, 78). Since BAFF-induced

B cell survival in humans and mice is mainly mediated by the up-regulation of anti-apoptotic and down-regulation of pro-apoptotic Bcl-2 family members (48, 71), chBAFF might also interfere with these pathways to regulate the balance between cell death and survival in immature chicken B cells. Taken together, these data indicate an important function of chBAFF in bursal B cell development. The dependence of immature B cells for BAFF-mediated signals in a primary lymphoid organ is so far unique to chicken. Indeed, B cell development in the bone marrow of mice proceeds efficiently in the absence of BAFF (47, 50).

Based on these results, we conclude that BAFF has maintained its function in the homeostatic control of the mature B cell compartment since the divergence of mammals and birds some 350 million years ago (79). Our unexpected observation that BAFF is also involved in early B cell development in birds fits two models. In the first one, the evolution of the bursa (the avian B cell differentiation organ) is linked to the gain of a new function for BAFF. Until recently, it was believed that B cell development in a gut-associated lymphoid organ was unique to birds (80). However, with the demonstration that domestic mammals such as sheep and rabbits rely on B cell development in the GALT, this concept was revised (2). Therefore, a second model may be proposed in which BAFF is an important cytokine for species experiencing B cell development in the GALT, but that the BAFF dependence of immature B cells may have been lost during switch of B cell development from the GALT to the bone marrow. Comparative studies on the BAFF system in additional species should help to clarify these hypotheses.

Acknowledgements

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Abbreviations

APRIL	a proliferation-inducing ligand
BAFF	B cell-activating factor of the tumor necrosis factor family
BCMA	B cell maturation antigen
BCR	B cell receptor
chBAFF	chicken B cell-activating factor of the tumor necrosis factor family
chGAPDH	chicken GAPDH
c.p.m.	counts per minute
DPBS	PBS with Ca++ and Mg++
ED	embryonic day
GALT	gut-associated lymphoid tissue
hBCMA	human B cell maturation antigen
[^{3}H]TdR	[^{3}H]thymidine
i.p.	intra-peritoneal
i.v.	intravenous
L chain	light chain
PI	propidium iodide
PMA	phorbol myristate acetate
RT	room temperature
SSC	saline–sodium citrate
TACI	transmembrane activator, calcium modulator and CAML interactor
TNF	tumor necrosis factor

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