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ORIGINAL ARTICLE

BP8, a novel peptide from avian immune system, modulates B cell developments

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Abstracts The bursa of Fabricius (BF) is the key humoral immune organ unique to birds, and is critical for early B-lymphocyte proliferation and differentiation. However, the molecular basis and mechanisms through which the BF regulates B cell development are not fully understood. In this study, we isolated and identified a new bursal peptide (BP8, AGHTKKAP) by RP-HPLC and MALDI-TOF–MS. BP8 promoted colony-forming pre-B formation, bound B cell precursor, regulated B cell development in vitro as well as in vivo, upstream of the EBF-E2A-Pax5 regulatory complex and increased immunoglobulin secretion. These data revealed a bursal-derived multifunctional factor BP8 as a novel biomaterial which is essential for the development of the immune system. This study elucidates further the mechanisms involved in humoral immune system and has implications in treating human diseases.

Keywords Bursal peptide (BP8) · Immunomodulatory function · B cell development

Introduction

The bursa of Fabricius (BF) is the key humoral immune organ unique to birds (Davison et al. 2011), and is critical for early B-lymphocyte proliferation and differentiation (Cooper et al. 1966; Glick et al. 1956; Lydyard 1976). Two separate differentiation pathways exist for lymphocytes; one for T (thymic) lymphocytes and the other for B [bone marrow (BM) and bursal] lymphocytes. However, in mammals, adult B lymphocytes develop in bone marrow (BM), equivalent to the T-cell-differentiating thymus, has not yet been fully understood (Hardy and Hayakawa 2001). Due to the fact that birds and mammals evolve from a common reptilian ancestor more than 200 million years ago and have inherited many common immunological systems (Davison et al. 2011), the BF therefore provides an invaluable model for studying basic immunology.

The BF contains several biologically-active factors, and a high dose of chicken BF extract was shown to enhance blastogenic responses to T-cell mitogens (Murthy and Ragland 1992). Bursin is a candidate for the specific factor responsible for B cell differentiation (Olli et al. 1989). It selectively stimulates the differentiation of avian B cells, rather than that of T cells, from their precursors in vitro (Audhya et al. 1986; Brand et al. 1976), and promotes immunoglobulin (Ig) switching from IgM to IgG (Baba and Kita 1977). Bursal anti-steroidogenic peptide is responsible for synchronizing B cell division during embryogenesis and the neonatal period (Moore et al. 2003). Bursal septpeptide (BSP)-I and BSP-II can induce both humoral and cellular immune responses in vivo (Feng et al. 2010, 2011), and bursopentin (BP5) was shown to induce B-lymphocyte proliferation by various signaling pathways (Li et al. 2011). Bursal pentapeptide (BPP)-I exerts immune-inducing functions, including antitumor responses (Feng et al. 2012), while bursal peptide (BP)11 was found to regulate B cell development and antigen-specific immune responses (Liu et al. 2012).

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However, the molecular basis and potential mechanisms through which BF regulates B cell development are not fully understood, and there is therefore a need to study the biologically-active factors derived from BF. In this study, we isolated and purified a new bursal peptide (BP8), and investigated various biological functions of BP8 in terms of colony-forming unit (CFU) pre-B formation, B cell development and immunoglobulin (Ig) production.

Materials and methods

Animals

BALB/c female mice (4–6 weeks old, 17–21 g) and C57/BL6 mice (4–6 weeks old, 17–21 g) were obtained from Yang Zhou University (Yangzhou, China). Twenty-one-day old chickens were purchased from Qinglongshan Farm (Nanjing, China). All the animal experimental procedures were performed in accordance with the institutional ethical guidelines for animal experiments.

Isolation and identification of peptides from BF

BP was purified from avian BF extract by reverse-phase-high-performance liquid chromatography (RP-HPLC), as described previously (Liu et al. 2012; Tang et al. 1999), with slight modifications. Briefly, BF tissue was homogenized in 0.1 M phosphate buffer solution (pH 6.0), and then centrifuged at 5,000×*g* for 10 min at 4 °C. The supernatant (BF extract) was lyophilized and dissolved in 0.1 M phosphate buffer solution with protease inhibitors (1 mM phenylmethanesulfonylfluoride; 1 μg/ml leupeptin; 1 μg/ml aprotinin), and then dialyzed (less than 10,000 Da) for 48 h at 4 °C. Following filtration through a 0.22-μm filter, BF extract was fractionated by using a 2.6 × 100-cm Sephadex G-50 gel filtration column (Superfine, Amersham Biosciences, USA). The sample peaks were pooled and subjected to chromatography by using a 4.6 × 250-mm SinoChrom ODS-BP RP-HPLC affinity column (Elite, China) in a linear gradient of acetonitrile (2–100 %), with monitoring at 220 nm. The eluted samples were collected and analyzed by using MALDI-TOF–MS (Bruker, Germany). The chemical formula, titration curve and the isoelectric point were analyzed by DNAStar software.

Peptide synthesis

BP8 (AGHTKKAP), analogs of BP8 (A2, AAHTKKAP; A3, AGATKKAP; A4, AGHAKKAP; A5, AGHTAKAP; A6, AGHTKAAP; A8, AGHTKKAA), BP8-scrambled (THKAPAGK), fluorescein isothiocyanate (FITC)-BP8 and fluorescein isothiocyanate (FITC)-BP8-scrambled were synthesized by Shanghai Taishi Bioscience Company (Taishi Bioscience Co, China) and analyzed by HPLC and electrospray ionization tandem mass spectrometry to confirm that the purity was higher than 95 %. The synthesized BP8 was dissolved in water. BP8 was tested by using the E-Toxate Limulus LPS detection kit (Sigma Chemical Co, USA.) to exclude lipopolysaccharide contamination.

CFU pre-B assay and systematic SAR analysis of BP8

BM cells (1×10^6 cells/ml) from C57/BL6 mice were suspended in Iscove's Modified Dulbecco's Medium containing 1 % methylcellulose, 2 mM L-glutamine, 10 % fetal calf serum, 50 μM 2-mercaptoethanol, 0.1 g/l streptomycin, 105 U/ml penicillin, and IL-7 (10 ng/ml), and then treated with and BP8 (1–40 μg/ml), BP8 analog (40 μg/ml) (both added immediately before plating), then mixed with methylcellulose, and plated in 35-mm culture dishes (1 ml/dish), BP8-scrambled (40 μg/ml) was used as a control peptide. The plates were incubated at 37 °C in 5 % CO_2 for 7 days. Colonies containing at least 40 cells were counted, and CFU pre-B cells were counted as described previously (Fine et al. 1994).

Flow cytometry of B cell development

BM cells (1×10^6 cells/ml) from C57/BL6 mice were mixed with BP8 (40 μg/ml) or BP8-scrambled (40 μg/ml) in the presence of IL-7 (10 ng/ml), then plated in 35-mm culture dishes (1 ml/dish), maintained for 7 days. Single-cell suspensions were collected. Red blood cells were lysed with ammonium chloride solution, and the remaining cells were stained with phycoerythrin (PE)-Cy5 anti-B220 (RA3-6B2), PE-CD43 (eBioR2/60) and PE-Cy7 anti-IgM (II/41), purchased from eBioscience (USA). B cell progenitors were defined by the presence of the following antibody combinations: pro-B cells (B220+IgM−CD43+), pre-B cells (B220+IgM−CD43−), iMB/MB (B220+IgM+CD43−). FITC-BP8 (40 μg/ml) and FITC-BP8-scrambled (40 μg/ml) were also used to detect the binding of BP8 to BM cells. Samples were maintained at 4 °C in the dark and analyzed by flow cytometry (Cytomics FC500 MPL flow cytometry system, Beckman Coulter, USA). A total of 10, 000 cells were analyzed for each sample.

In vivo experiments

Five C57/BL6 mice (6-week-old, female) were injected intravenously with 1 mg/kg BP8 or BP8-scrambled in 0.1 % bovine serum albumin (BSA)/saline per day for 7 days. At 72 h after the final dosing, BM cells were

collected from each mouse for CFU pre-B and flow cytometry assays of B cell precursors, as described previously.

Evaluation of effects of BP8 on the antibody secretion in cultures initiated from BM cells

Day 4^{IL-7} BM cells were plated with IL-7 (10 ng/ml) in combination with BP8 (40 μg/ml), or BP8-scrambled (40 μg/ml). Supernatants were harvested on day 7, and IgM and IgG were detected using ELISA kits (Lengton, China) according to the manufacturer's instructions.

Evaluation of effects of BP8 on the antibody secretion in mice and chickens

The roles of BP8 in the formation of Ig-secreting cells were examined in 6–8 week-old BALB/c female mice, as previously reported (Le Bon et al. 2001). Mice were immunized twice intraperitoneally with 0.2 ml AIV (Avian influenza virus) inactivated antigen with 40 μg/ml BP8 on days 0, and 14, respectively. Mice were immunized twice intraperitoneally at 2-week intervals with the same volume containing AIV inactivated antigen as a positive control. Mice immunized with BP8-scrambled were used as a negative control. Sera were collected on days 14 and 28 to detect antigen-specific antibody responses (IgG) by ELISA, as described previously (Liu et al. 2012).

The immunomodulatory functions of BP8 were confirmed in 21-day-old chickens immunized with AIV vaccine. Chickens were divided randomly into groups (10 chickens per group) and immunized subcutaneously on day 1, followed by boosting on day 14, with 0.5 ml AIV vaccine and 1 mg/kg BP8 or BP8-scrambled, respectively. Control animals were immunized with either sterile PBS or AIV vaccine alone. At 2 weeks after the first and second immunizations, serum samples were collected randomly for determination of the hemagglutination-inhibition (HI) antibody titers, as described previously (Oveissi et al. 2010).

FACS cell sorting

BM cells were cultured with IL-7 (10 ng/ml) for 4 days (day 4^{IL-7}) as described above. On day 4, cells were labeled with appropriate antibodies and sorted using a in-house service center equipped with a MoFlo XDP cell sorter and Summit 4.1 software to >98 % purity. proB (B220+IgM−CD43+), preB (B220+IgM−CD43−), and IM/MB (B220+IgM+CD43−) were stained using PE-Cy5 anti-B220 (RA3-6B2; eBioscience), PE-CD43 (eBioR2/60; eBioscience), PE-Cy7 anti-IgM (II/41; eBioscience), and FITC-anti-IgD (11-26c; eBioscience).

Real-time polymerase chain reaction (PCR) to examine PU.1, Pax5, EBF1 and E2A expression in B cell progenitors

Total RNA was isolated using TRIzol Reagent (Invitrogen, USA). DNA-free total RNA (100 ng) was subjected to cDNA synthesis using a PrimeScript® RT Reagent Kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Real-time PCR was performed using a Light Cycler instrument (Eppendorf, Germany) in a total volume of 20 μl with a SYBR® Premix Ex Taq™ (Perfect Real Time) kit (TaKaRa Bio Inc.) according to the manufacturer's instructions. The following primers were used: E2A (forward, 5′-GTCCTGGGTGGATGATGAAC-3′; reverse, 5′-CATCCCTGCTGTAGCTGTCA-3′); EBF1 (forward, 5′-CCAACTCACCCTATGCCATT-3′; reverse, 5′-GCAAGGTCGGTGATTTTGTT-3′); Pax5 (forward, 5′-CAGCAAAATTCTTGGCAGGT-3′; reverse, 5′-TGCTGTGTGAACAGGTCTCC-3′); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, 5′-GTCAACGGATTTGGTCGTATT-3′; reverse, 5′-GATCTCGCTCCTGGAAGATGG-3′).

Statistical analysis

Results were expressed as mean ± SD. The statistical significance of the observed differences was analyzed by two-sided *t* tests, unless otherwise specified. A P value < 0.05 was considered to be statistically significant.

Results

Isolation and characterization of BP8

Natural bursal extract was divided into three peaks by Sephadex G-50 gel filtration (Fig. 1a). The eluted samples from peak I were further purified by RP-HPLC (Fig. 1b) and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF–MS). The molecular weight of this bursal sample was 808.9 (m/z), and the amino acid sequence was AGHTKKAP. The chemical formula (Fig. 1c) and titration curve (Fig. 1d) are analyzed by DNAStar. The isoelectric point of BP8 is 10.0 with a negative charge of 2.07. This sequence has not been reported previously. This bursal peptide was designated as BP8. The amino acid sequence of BP8 was aligned with the National Center for Biotechnology Information Non-redundant and Expressed Sequence Tags databases (http://blast.ncbi.nlm.nih.gov/). BP8 was found to be homologous to various functional proteins in *Gallus gallus*, including stimulated by retinoic acid gene 6 homolog

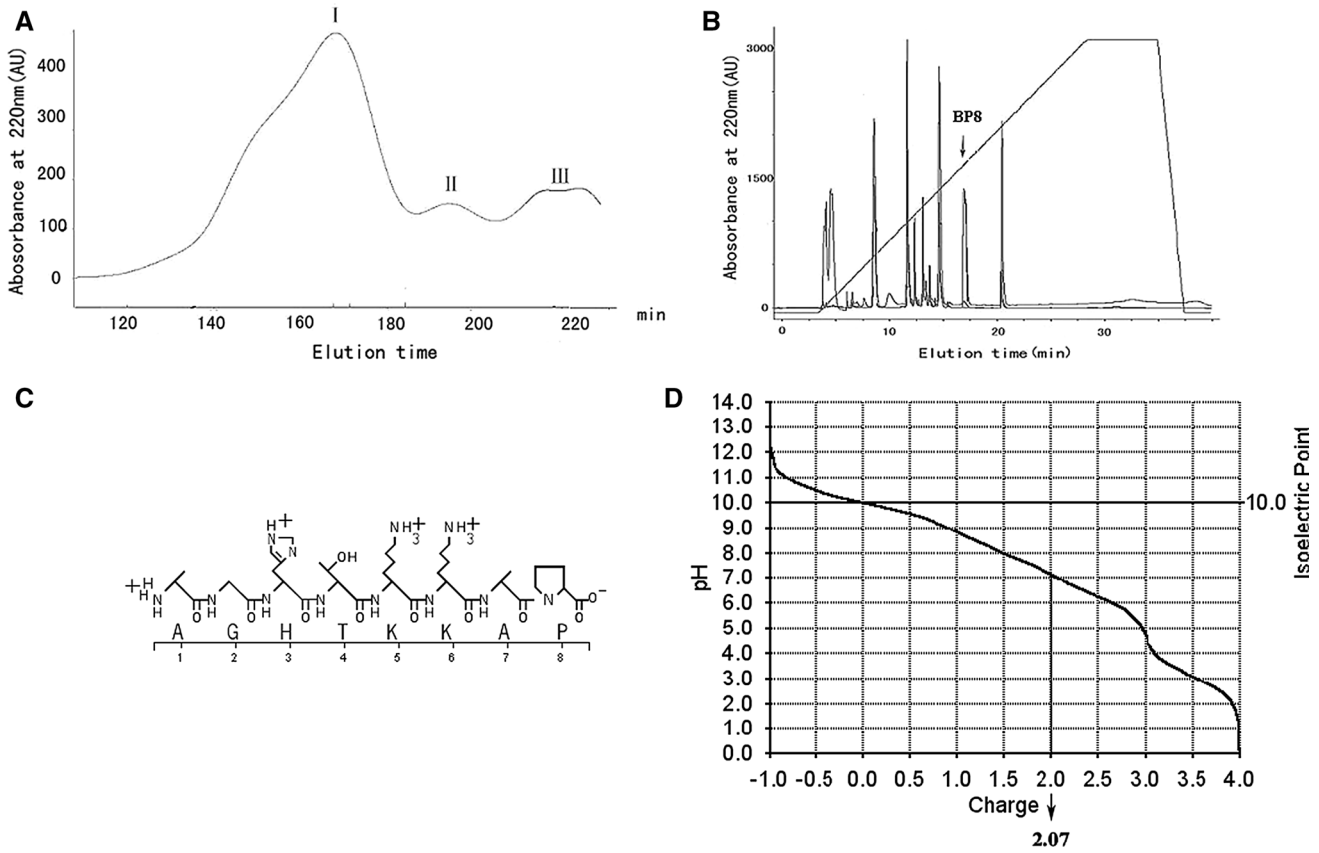

Fig. 1 Purification of bursal peptide BP8. **a** Bursal peptide extract was fractionated on a Sephadex G-50 gel filtration column. **b** Peak I was further purified by RP-HPLC on a 4.6 × 250-mm SinoChrom ODS-BP RP-HPLC affinity column equilibrated in aqueous 0.1 % TFA, and developed with a linear acetonitrile gradient (*gray line*). The retention peak of BP8 was eluted in the *arrow-marked* peak. **c** The molecular structure of BP8. BP8 is composed of AGHTKKAP, and was analyzed by DNAStar. **d** The titration curve of BP8. The isoelectric point of BP8 was 10.0 with a negative charge of 2.07

(*Gallus gallus*) (STRA6, NP_001280131), with maximum homology to BP8, and to various human and mouse proteins, such as BC040756 protein, partial (*Mus musculus*) (GI: 26252087) and hCG1780696, isoform CRA_a, partial (Homo sapiens) (GI:119600906), suggesting that these species also express this peptide.

BP8 binds B cell precursor

The BF provides the environment supporting the differentiation of B-lineage cells. Bursin and cytokines are soluble factors that promote the proliferation and differentiation of B cells (Armitage et al. 1995; Brand et al. 1976). As a novel peptide from the avian immune system, we wanted to know the effects of BP8 on B cells. Using a FITC derivative of BP8, we found that Pro-B cells, pre-B cells and Immature/mature B cells (iMB/MB) demonstrated high levels of BP8 binding (Fig. 2e–g). In contrast, other cells did not bind BP8, as expected (data not shown).

BP8 promotes CFU pre-B formation

CFU pre-B formation assays were used to investigate the roles of BP8 in B cell progenitor development. BM cells (1×10^6 cells/ml) from C57/BL6 mice were plated in methylcellulose supplemented with interleukin (IL)-7 (10 ng/ml), with BP8 or BP8-scrambled for 7 days. With the absence of IL-7, only one or no colonies were formed, while BP8 promoted CFU pre-B formation in a dose-dependent manner at concentrations ranging from 1 to 40 μg/ml, suggesting that BP8 has the potential to promote the formation of CFU pre-B (Table 1).

To study the structure–activity relationship of BP8, systematic structure–activity-relationship (SAR) analysis was used, by which the amino acid in BP8 was individually replaced by L-alanine. As was shown in Table 1, analysis of these peptides revealed that the mutation of fourth, fifth, sixth and eighth amino acids reduced the CFU pre-B forming activity, indicating that they were crucial for BP8 activity (Table 1).

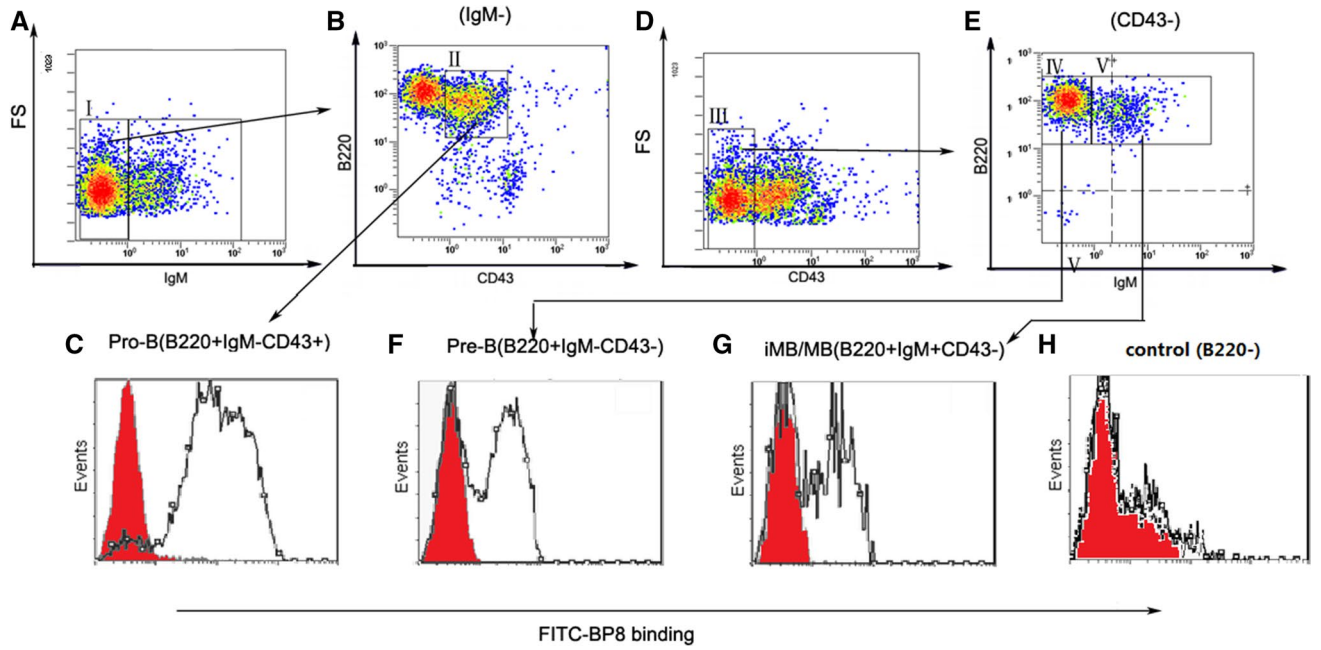

Fig. 2 BP8 binds to B cells. BM cells were cultured with IL-7 for 4 days (day 4^{IL-7}) to enrich B cells. Binding of BP8 to B cells was detected by FACS using FITC-BP8. FITC-BP8-scrambled was used as control. Traces for FITC-BP8 (*bold line*) and FITC-BP8-scrambled (*shaded*) are shown. Results shown are from one of three experiments. **a** B cells were analysis for expression of IgM, Regions I: IgM- (**b**) B cells were analysis for expression of CD43 and B220, Regions II: Pro-B cells (B220+IgM−CD43+) (**c**) B cells were analysis for expression of CD43, Region III: CD43- (D) B cells were analysis for expression of IgM and B220, Region IV: Pre-B cells (B220+IgM−CD43−); Region V: iMB/MB cells (B220+IgM+CD43−) (**e**) Binding of BP8 to Pro-B cells (**f**) Binding of BP8 to Pre-B cells (**g**) Binding of BP8 to iMB/MB cells (**h**) Binding of BP8 to B220- cells

Table 1 Amino acid sequences and CFU-Pre-B values of BP8 analogs

Peptide	Sequence	CFU-pre-B[a]
BP8 (40ug/ml)	AGHTKKAP	82.00 ± 7.54**
BP8 (5ug/ml)	AGHTKKAP	68.33 ± 2.52**
BP8 (1ug/ml)	AGHTKKAP	53.67 ± 3.21*
[A2]BP8 (40ug/ml)	A**A**HTKKAP	85.00 ± 6.24**
[A3]BP8 (40ug/ml)	AG**A**TKKAP	78.00 ± 5.29**
[A4]BP8 (40ug/ml)	AGH**A**KKAP	65.00 ± 5*
[A5]BP8 (40ug/ml)	AGHT**A**KAP	49.00 ± 2.64*
[A6]BP8 (40ug/ml)	AGHTK**A**AP	48.67 ± 5.51*
[A8]BP8 (40ug/ml)	AGHTKK**AA**	38.00 ± 3.46*
BP8-scrambled	THKAPAGK	35.67 ± 4.04
Control	BSA protein	34.67 ± 1.53

CFU-pre-B colony-forming pre-B formation

* $P < 0.05$, ** $P < 0.01$ compared with control

[a] Results shown are mean ± SD of measurements

BP8 modulates B cell development

To examine the effect of BP8 on the development of B cell progenitors, BM cells isolated from C57/BL6 mice were cultured in the presence of IL-7 (10 ng/ml) with BP8 or BP8-scrambled (40 μg/ml) for 7 days, and B cell differentiation was then assessed by flow cytometry. BP8 treatment increased the percentages of pre-B ($P < 0.05$) and iMB/MB cells ($P < 0.05$) in the cultures (Fig. 3).

To determine if BP8 had a similar effect on B cell development in vivo, mice were injected with BP8 or BP8-scrambled daily for a total of 7 days. BM cells were collected 72 h after the final injection for CFU pre-B and fluorescence-activated cell sorting (FACS) assays. In agreement with the in vitro results, BM cells from BP8-treated mice showed a significant increase in CFU pre-B colony formation (Fig. 4a). In addition, BP8 treatment also increased the levels of pre-B and iM/MB cells (Fig. 4c, d). These results confirmed that BP8 affected B cell development, and supported/proved the hypothesis that BP8 accelerates the maturation of B cell progenitors.

BP8 regulates the expression of Pax5, EBF1 and E2A in B cell progenitors

Given that B cell progenitors continue to mature when cultured in vitro, we investigated the effects of BP8 exposure on B cell progenitor gene expression. BM cell cultures were treated with BP8 or BP8-scrambled for 24 h and then sorted into proB, preB, and iMB/MB cell populations.

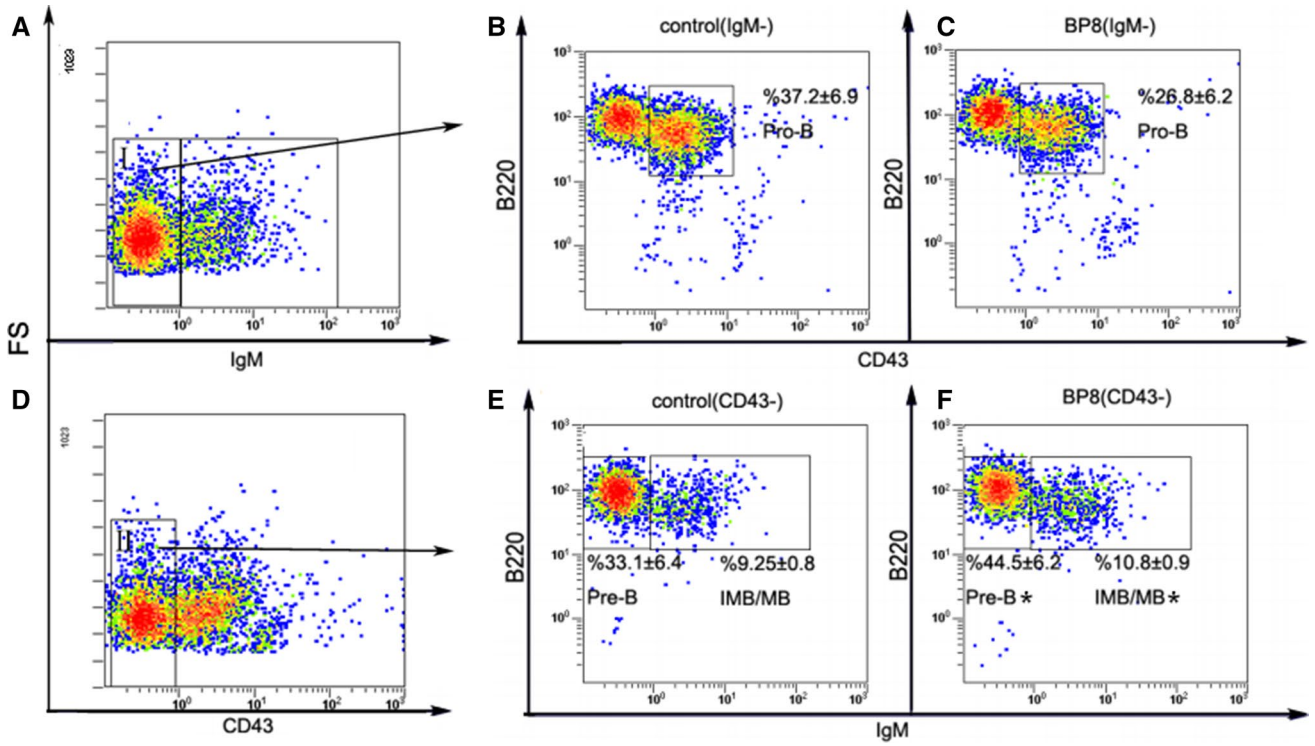

Fig. 3 BP8 regulation of B cell development. BM cells were cultured in the presence of IL-7 (10 ng/ml) with BP8 (40 μg/ml) or BP8-scrambled (40 μg/ml) for 7 days. The cell population was then analyzed by staining with antibodies against B220, IgM, CD43, and IgD, followed by FACS analysis. **a** B cells were analysis for expression of IgM, Regions I: IgM- (**b**) Pro-B cells (B220+IgM−CD43+) of control (**c**) Pro-B cells (B220+IgM−CD43+) treated with BP8 (**d**) B cells were analysis for expression of CD43, Regions II: CD43- (**e**) Pre-B cells (B220+IgM−CD43−) and iMB/MB cells (B220+IgM+CD43−) of control (**f**) Pre-B cells (B220+IgM−CD43−) and iMB/MB cells (B220+IgM+CD43−) treated with BP8. $*P < 0.05$, compared with the BP8-scrambled. Data were expressed as the mean ± SD of three experiments in duplicate

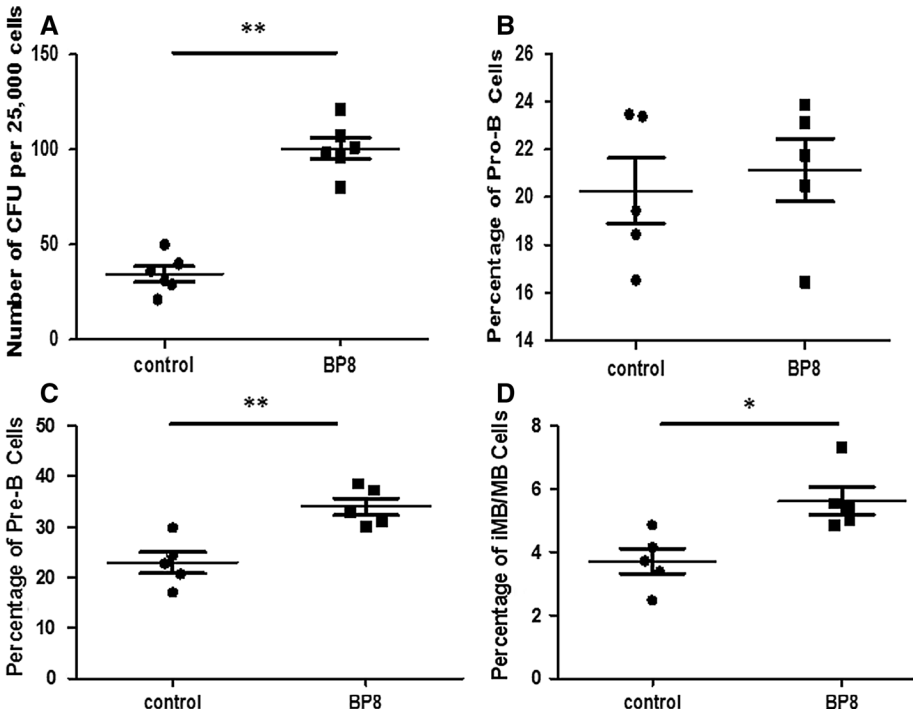

Fig. 4 In vivo BP8 experiments. Five C57/BL6 mice (6-week-old, female) were injected intravenously with 1 mg/kg BP8 or 1 mg/kg BP8-scrambled (Control) in 0.1 % BSA/saline per day for 7 days. At 72 h after the final dosing, BM cells were collected from each mouse for pre-B CFU and flow cytometry assays of B cell precursors. Pre-B CFU (*top left*); percentage of pro-B cells (*top right*); percentage of pre-B cells (*bottom left*); percentage of iM/MB (*bottom right*). Data were expressed as the mean ± SD in duplicate. $*P < 0.05$, $**P < 0.01$

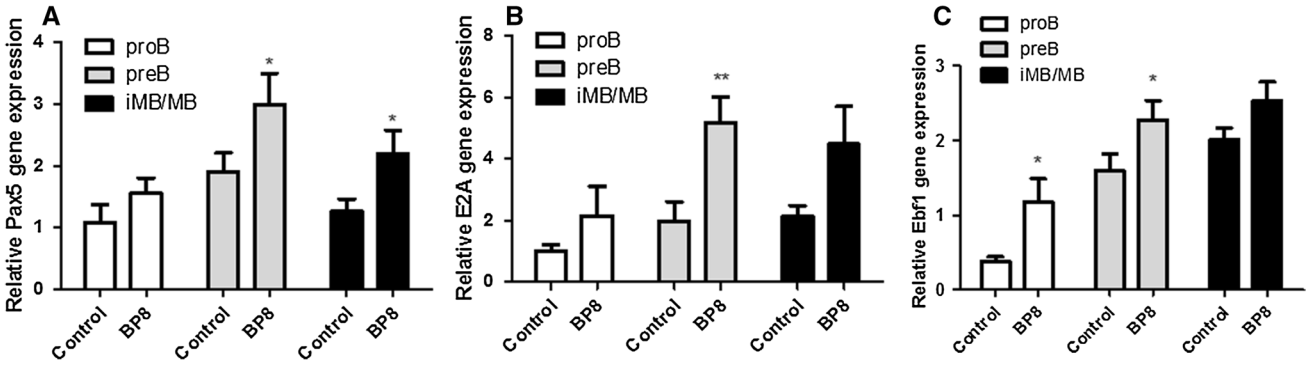

Fig. 5 BP8 regulates gene expression of Pax5, Ebf1 and E2a in B cell progenitors. BM cells were grown in IL-7. On day 3, BP8 (40 μg/ml) or BP8-scrambled (40 μg/ml) was added for 24 h to half of the culture. Cells were sorted on day 4 into pro-B (B220+CD43+IgM−), pre-B (B220+CD43−IgM−), and immature/mature B(B220+CD43−IgM+) cells, and total RNA was extracted. Real-time PCR analysis using PU.1, Pax5, Ebf1 and E2a -specific primers were performed on cDNA as described in Materials and Methods. Figures show normalized values from a single experiment and fold change over untreated from two independent experiments (*right panel*). **a** Relative expression of Pax5, **b** Relative expression of Ebf1, **c** Relative expression of E2a

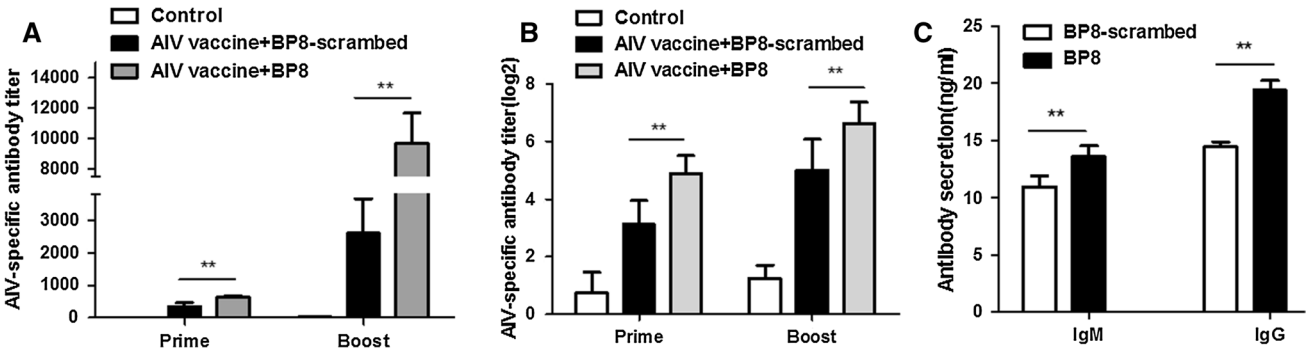

Fig. 6 BP8 regulates Ig secretion. **a** BALB/c mice were immunized with BP8 and AIV vaccine, following the prime-boost vaccination program (days 0 and 14), respectively. Sera were collected on day 14 after two immunizations to detect antibody (IgG) titers by ELISA. Data from samples immunized with AIV vaccine and BP8-scrambled were used as a basic control. $*P < 0.05$, $**P < 0.01$. Data are shown as mean ± SD and are fully representative for the individual mice tested. **b** Chickens were immunized twice with BP8 and AIV vaccine, following the prime-boost vaccination program (days 0 and 14), respectively. The induction of AIV-specific antibodies was measured by HI assay. Data from samples immunized with AIV vaccine and BP8-scrambled were used as a basic control. $*P < 0.05$, $**P < 0.01$. Data are shown as mean ± SD and are fully representative for the individual mice tested. **c** Day 4^{II-7} BM cells were plated with IL-7 in combination with BP8, or BP8-scrambled. Supernatants were harvested on day 7, and ELISA was performed as described in Materials and methods. Results are representative of three independent experiments. Data were expressed as the mean ± SD of three experiments in duplicate. $*P < 0.05$, $**P < 0.01$

We observed significant differences in gene expression between progenitor populations treated with BP8 prior to sorting. The transcription of Pax5 transcription was increased in preB and iMB/MB cells (preB, $P < 0.05$; iMB/MB, $P < 0.05$) (Fig. 5a), the transcription of E2A transcription was increased in preB cells (iMB/MB, $P < 0.01$) (Fig. 5b), while and EBF1 was increased by BP8 in the proB and preB populations (Fig. 5c).

BP8 promotes immunoglobulin secretion

Bursin is known to induce plasma-cell differentiation and Ig secretion (Olli et al. 1989). To determine the role of BP8 in immunoglobulin secretion in mouse immunization, we compared antibody levels in mice immunized and boosted with avian influenza virus (AIV) vaccine and BP8. As expected, serum levels of anti-AIV antibodies at the 2nd week after the first and second immunization in mice immunized with AIV vaccine and 1 mg/kg BP8 were significantly increased (Fig. 6a). We also compared antibody levels in chicken immunized and boosted with avian influenza virus (AIV) vaccine and BP8. Chickens were injected with synthetic AIV vaccine and 1 mg/kg BP8 or BP8-scrambled, antibody IgG profiles were assessed in sera from all groups at first immunization and boost immunization (Fig. 6b). Antibody levels were significantly

increased in chickens immunized with BP8 at first immunization and second immunization compared with chickens immunized with BP8-scrambled ($P < 0.01$). In vitro, to test whether BP8 was able to drive the differentiation of BM B cell progenitors into Ig-secreting cells, we plated day 4IL-7 BM cells with IL-7 and BP8 (40 μg/ml) or BP8-scrambled (40 μg/ml). IL-7 was included in the culture to allow the continued expansion of pro-B cells. Supernatants were harvested on day 7 and analyzed by enzyme-linked immunosorbent assay (ELISA) as described in Materials and methods. Results showed that the presence of BP8 markedly increased IgM and IgG production (Fig. 6c). These results suggested that BP8 regulates immunoglobulin secretion in vitro and vivo, and confirmed the hypothesis that BP8 accelerates the maturation of B cell.

Discussion

The BF is a vital lymphoid organ in the immune system of birds, and plays a primary role in the development and function of B-lymphocytes. Birds and mammals evolved from the same vertebrate ancestor, and therefore share some common immune properties (Brand et al. 1976), including the possession of a thymus for T-cell-differentiation. However, the organ responsible for B cell-differentiation in mammals has not yet been identified (Glick et al. 1956). The BF provides the environment which can support the differentiation of B-lineage cells. Bursin and cytokines are soluble factors that promote the proliferation and differentiation of B cells (Armitage et al. 1995; Brand et al. 1976). Studies of the immunological properties of molecules in the avian BF are therefore important for deepening our understanding of the immune system in an evolutionary perspective.

The current study provides the first report of a novel bursal peptide (BP8) from the avian immune system. We studied the role of BP8 in B cell development by CFU pre-B cell assay, and found that BP8 promoted IL-7 induced CFU pre-B formation. The B cell progenitors responding to IL-7 were most likely cells at the pre-, pro-, or C stage, as described by Hardy and his co-workers (Hardy et al. 1991; Li et al. 1993). The results of the current study therefore suggest the inducing roles of BP8 on B cell development.

Then we found that BP8 bound pro-B, pre-B, and iMB/MB populations, suggesting that B cells possess BP8 receptors. Furthermore, the role of BP8 in B cell development has been studied, and we found that the percentages of pre-B and iMB/MB cells increased when BM cells were stimulated with BP8 in vitro. In support of these in vitro observations, similar results were obtained in vivo by intravenous injection of C57/BL6 mice (6 weeks old, female) with BP8 for 7 days.

BP8 also promoted the differentiation of BM B cell progenitors into Ig-secreting cells in vitro, with or without anti-CD40. Then, we verified the potential effect of BP8 on promotion of antibody responses in immunized mice and chickens in vivo. These results suggest that BP8 acts as a modulator of B cell maturation and Ig-secretion.

EBF1 is an essential specification factor for B cell development. Mice lacking EBF1 fail to express most B cell genes (Lin and Grosschedl 1995). Retrovirus-mediated expression of EBF1 in HSCs skews their differentiation towards the B cell lineage, suggesting EBF1 appears sufficient to activate the B cell-lineage gene program (Zhang et al. 2003). Similar to EBF, E2A regulates the expression of genes activated as part of the early B cell program (Hagman and Lukin 2005). E2A regulates expression of genes activated as part of the early B cell program, including Igll1 (λ5), Vpreb1 (Vpre-B), Cd79a (mb-1), Cd79b (B29), Rag1, and Cd19, and is absolutely required for early B cell development (Hagman and Lukin 2005). Pax5 is known to be induced by EBF and E2A. Pax5 is a multifunctional transcriptional regulator that is expressed at a remarkably stable amount throughout the B cell lineage, from the pro-B cell stage until its down-regulation in plasma cells (Fuxa et al. 2004). In the absence of Pax5, B cell development is arrested at the early pro-B cell (or pre-BI) stage of differentiation characterized by expression of many B cell-specific transcripts and D-J_H rearrangements at the Igh locus (Nutt et al. 1998). Interestingly, the present study demonstrated that BP8 increased EBF, E2A and Pax5 expression. It is likely that BP8 acts upstream of the EBF-E2A-Pax5 regulatory complex to modulate B-lineage cell specification, commitment, and differentiation. This may partly explain the mechanism whereby BP8 promotes IL-7-induced B cell development.

Compared to the peptides from Bursa of Fabricius previously published, such as BSP-I which reduced MCF and Hela tumor cells proliferation, enhanced antitumor factor p53 luciferase activity and protein expression, improved the antibody production, stimulating cytokines IL-4 and IFN-γ (Feng et al. 2011), and BSP- II which regulated the antibodies titers, levels of cytokines IL-4 and IFN-γ, spleen cell lymphocyte proliferation, and the T-lymphocyte subtype composition, and enhanced DT40 cell viability (Feng et al. 2010), BP8 could also regulated the humoral immune, including promoting immunoglobulin secretion. What is more, BP8 could regulate the B cell development in vitro and vivo. B cells are derived from hematopoitic stem cells (HSCs) in a process by which cells progressively develop B cell traits while repressing traits of other lineages; The stepwise differentiation of lymphocytes from HSCs is controlled in part by signaling pathway activated by various cytokines produced in the local microenvironment, which regulated cell survival, proliferation and maturation. As a

new peptide which regulated B cell development, including promoting CFU pre-B formation, regulating B cell development, upstreaming of the EBF-E2A-Pax5 regulatory complex, and promoting Ig secretion, BP8 have applications as a new reagent for regulating immunopharmacological uses.

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