Original Article

J Vet Sci 2015, 16(4), 501-507 · http://dx.doi.org/10.4142/jvs.2015.16.4.501

JVS

Isolation and immunomodulatory activity of bursal peptide, a novel bursal peptide from the chicken bursa of Fabricius

Xiao-Dong Liu[1], Yingjuan Qian[2], Yong-Sam Jung[2,*], Pu-Yan Chen[2,*]

[1]*College of Animal Science and Veterinary Medicine, Qingdao Agricultural University, Qingdao 266109, China*

[2]*Division of Key Lab of Animal Disease Diagnosis and Immunology of China's Department of Agriculture, Nanjing Agricultural University, Nanjing 210095, China*

The bursa of Fabricius (BF), which is unique to birds, serves as the central humoral immune organ and plays a significant role in B lymphocyte differentiation. In this study, a new bursal peptide (BP-IV) was isolated from BF, which promoted colony-forming unit pre-B formation and regulated B cell differentiation. BP-IV also exerted immunomodulatory effects on antigen-specific immune responses via both humoral and cellular immunity in chicken and mice that had been immunized with inactivated avian influenza virus (AIV; H9N2 subtype), including enhancing AIV-specific antibody and cytokine production. The results of this study provided novel insights into the use of a potential candidate reagent for B cell development and future immuno-pharmacological use.

Keywords: B cell development, bursal peptide, immunomodulatory experiment, immunomodulatory function

Introduction

The bursa of Fabricius (BF), which serves as the primary humoral immune organ and is unique to birds [5], is critical to early B-lymphocyte proliferation and differentiation [4,8,14]. The process of B-cell maturation was elucidated in birds. Thus, the "B" refers to the BF. However, similar to the T-cell-differentiating thymus, the B-cell-differentiating organ has not yet been identified in mammals [3]. As a result, the BF serves as a unique model for studying basic immunology.

Avian species are unique in that they possess the primary lymphoid organ, BF, which is required for the differentiation of B cells [10]. Specifically, the differentiation and development of avian B cells in the BF is the primary contributor to production of B cells and provides key insights into their expansion. During myeloid differentiation, transcription factor PU.1 is required for formation of the earliest myeloid and lymphoid progenitor cells [9]. The ablation of PU.1 in mice leads to the complete absence of B cells and macrophages, suggesting that expression of the PU.1 gene is tightly controlled during hematopoietic differentiation [15,19]. However, the molecular mechanisms leading to appropriated PU.1 levels are not fully understood.

Several biologically active factors have been reported to be contained in the BF. Specifically, high doses of chicken BF extract have been used to induce blastogenic responses to T-cell mitogens [17]. As a specific factor, bursin is responsible for B-cell differentiation [11]. Specifically, it selectively stimulates the differentiation of avian B cells, rather than that of T cells, from their precursors *in vitro* [1,3], and promotes immunoglobulin (Ig) switching from IgM to IgG [2]. Bursal anti-steroidogenic peptide is responsible for synchronizing B-cell division during embryogenesis and the neonatal period [16]. Bursopentin (BP5) has been shown to induce B-lymphocyte proliferation via various signaling pathways [12]. Additionally, bursal peptide (BP) 11 was found to regulate B-cell development and antigen-specific immune responses [13].

Although the molecular basis and mechanisms of action of the aforementioned peptides from BF have been well characterized, there should be additional BF peptides regulating B-cell development and immune response. A new bursal peptide (BP-IV) was isolated from BF using reversed-phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS), presenting different biological effects such as promotion of colony-forming unit (CFU) pre-B formation and B cell differentiation and exertion of immunomodulatory function on antigen-specific immune

Received 6 Nov. 2014, Revised 4 Apr. 2015, Accepted 2 Jun. 2015

*Corresponding authors: Tel/Fax: +86-25-84396335; E-mails: ysjung@njau.edu.cn (YS Jung), puyanchennj@163.com (PY Chen)

pISSN 1229-845X
eISSN 1976-555X

responses in chickens and mice.

Materials and Methods

Animals

C56BL/6 mice and BALB/c (4–6 weeks old, 18–21 g) were obtained from Yang Zhou University (Yangzhou, China). The 21-day old fertilized eggs of White Leghorn chickens (LSL) were purchased from Qinglongshan farm. The guidelines of the Animal Care and Ethics Committee of Nanjing Agricultural University (approval no. 200709005) were followed for all animal experiments.

Isolation and identification of peptides from BF

The bursa of 1,000 fertilized eggs of White Leghorn chickens were collected, after which the peptides were purified from the bursa by RP-HPLC, as previous described [13,21]. The elutions were collected and the novel peptides were analyzed using MALDI-TOF-MS (FLEX series; Bruker, Germany). Bursal peptide (KNEVEEEAKTP) and scrambled peptide (VEPAEEKTENK) were synthesized by Shanghai Taishi Bioscience (China).

CFU pre-B assay

The CFU pre-B assay was performed as previously described [7]. Briefly, bone marrow (BM) cells (1×10^6 cells/mL) from C56BL/6 mice were suspended in Iscove's Modified Dulbecco's Medium containing 1% methylcellulose, 2 mM L-glutamine, 10% fetal calf sera, 50 μM 2-mercaptoethanol, 0.1 g/L streptomycin, 105 U/mL penicillin, and interleukin (IL)-7 (10 ng/mL). The BM cells were then treated with the IL-7 (10 ng/mL) and BP-IV or BP-IV-scrambled, mixed with methylcellulose (both added immediately before plating), and plated in 35 mm culture dishes, then incubated at 37°C under 5% CO_2 for 7 days. The CFU pre-B formation was then determined. Colonies consisting of at least 100 cells were determined as CFU pre-B formation.

Flow cytometry

BM cells (1×10^6) from C56BL/6 mice were maintained with BP-IV or BP-IV-scrambled (25 μg/mL) in the presence of IL-7 (10 ng/mL) for 7 days. The cells were then 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 ($B220^+IgM^+CD43^-$). Samples were analyzed using the Cytomics FC500 MPL flow cytometry system (Beckman Coulter, USA) and Cytomics FC500 Flow Cytometry CXP software. A total of 10,000 cells were analyzed for each sample. The cells were also sorted using a MoFlo XDP cell sorter and the Summit 4.1 software to > 98% purity.

Real-time polymerase chain reaction (RT-PCR)

Total RNA of isolated pro-B cells, pre-B cells and immature/mature (iMB/MB) cells were isolated using TRIzol reagent (Invitrogen, USA). DNA-free total RNA (100 ng) was then subjected to cDNA synthesis using the PrimeScript RT reagent Kit (Takara Bio, Japan) according to the manufacturer's instructions. Additionally, RT-PCR was performed using a SYBR Premix Ex Taq (Perfect Real Time) kit (Takara Bio) in accordance with the manufacturer's protocols. The following primers were used: PU.1 (forward, 5′-GCATCTGGTGG GTGGACAA-3′; reverse, 5′-TCTTGCCGTAGTTGCGCAG-3′); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, 5′-GTCAACGGATTTGGTCGTATT-3′; reverse, 5′-GATCTCGCTCCTGGAAGATGG-3′) [13].

Animal experiment

Sixty 21 day-old chickens that had been immunized with AIV vaccine (AIV, H9N2 subtype) (10^7 $TCID_{50}$/0.1 mL) were used to evaluate the immunomodulatory functions of the BP-IV. Chickens (10 chickens per group) were divided to six groups and immunized subcutaneously on day 1, then boosted on day 14 with AIV vaccine at 0.5 mL per dose (10^7 $TCID_{50}$/0.1 mL) along with or without BP-IV (0.2, 1 and 5 mg/kg). Control animals were subsequently immunized with AIV vaccine or BP-IV-scrambled (1 mg/kg). At day 7 after the second immunization, the sera of immunized chickens were collected and the cytokines of IL-4 and IFN-γ were measured using commercial avian cytokine ELISA kits (R&D Systems, USA). At 2 weeks after the first and second immunizations, the sera of immunized chickens were collected. Samples were tested for hemagglutination-inhibition (HI) antibody as previously described, and the immunized peripheral lymphocyte proliferation was measured by standard MTT assay [13].

Forty BALB/c female mice (6–8 weeks old) were used to evaluate the immunomodulatory roles of the BP-IV. Mice were intraperitoneally immunized with a 0.2 mL AIV vaccine (10^7 $TCID_{50}$/0.1 mL) containing 1 mg/kg BP-IV or BP-IV-scrambled on days 0 and 14, respectively, and the sera were collected at 2 weeks after the first and second immunization. The antigen specific antibody responses (IgG) were tested using the enzyme-linked immunosorbent assay (ELISA) method as previously described [13]. At day 7 after the final immunization, mice cytokine ELISA kits (R&D Systems) were used to detect the cytokine levels of IL-4 and IFN-γ according to the manufacturer's instructions. The spleen cells were then isolated from immunized mice. Red blood cells were lysed using ammonium chloride solution, and the remaining cells were stained with the specific mAbs CD3, CD4, and CD8 for immunophenotyping of spleen T lymphocytes, which was conducted using a BD FACS Calibur flow cytometer (BD

Biosciences, USA). A total of 10,000 cells were analyzed for each sample.

Statistical analysis

Results were expressed as the means ± SD. Duncan's multiple range test was used to identify differences among groups. Analyses were conducted with the SPSS 16.0 software (SPSS, USA), p value < 0.05 was considered to be statistically significant.

Results

Isolation and characterization of novel peptide BP-IV

The supernatant of avian BF extract was purified by RP-HPLC, then analyzed by MALDI-TOF-MS. BP-IV was eluted in the arrow-marked peak (panel A in Fig. 1). The molecular weight of this bursal sample was 1273.34 (m/z), and the amino acid sequence was KNEVEEEAKTP. This sequence has not been reported previously, and was designated as BP-IV (panel B in Fig. 1). The amino acid sequence of BP-IV was aligned with the National Center for Biotechnology Information Non-redundant and Expressed Sequence Tags databases. The results showed that BP-IV was homologous to various proteins in *Gallus gallus*, including calcipressin-1, which is essential to the immune system and T cell activation. BP-IV was also homologous to various human and mouse proteins, such as NEFH protein (human) and BCL2-like 13 (mouse).

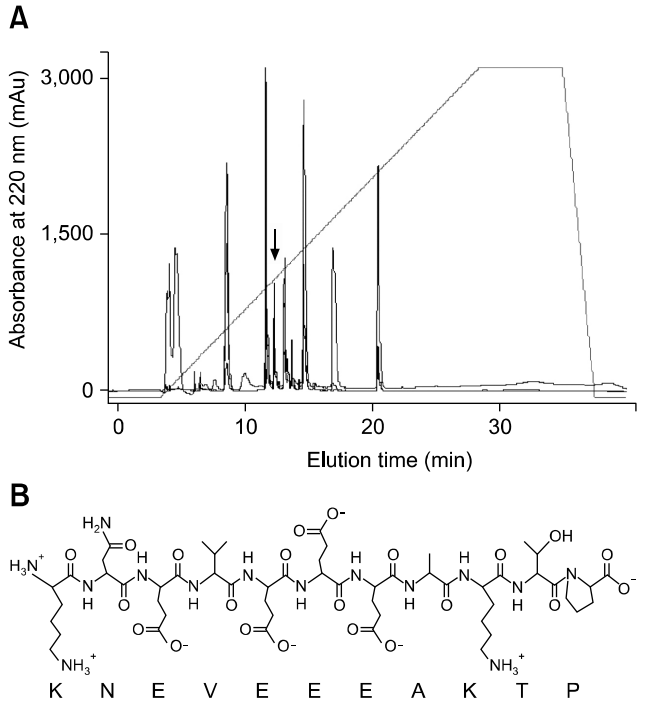

Fig. 1. Purification of bursal peptide BP-IV. (A) The bursal extracts from healthy young chickens were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a 4.6 × 250 mm SinoChrom ODS-BP (Eliteplc, China) RP-HPLC affinity column equilibrated in aqueous 0.1% TFA and developed with a linear acetonitrile gradient (from 0.5% to 50%). BP-IV was eluted in the arrow-marked peak. (B) BP-IV is composed of KNEVEEEAKTP, and was analyzed by DNA Star.

BP-IV promotes CFU pre-B formation and modulates B-cell development

CFU pre-B formation assays were used to investigate the roles of BP-IV in B-cell progenitor development. We found that BP-IV promoted CFU-pre-B formation in a dose-dependent manner at concentrations ranging from 1 to 25 μg/mL (1 μg/mL, $p < 0.05$; 5 μg/mL, $p < 0.05$; 25 μg/mL, $p < 0.05$) when compared to the control and BP-IV-scrambled, suggesting that BP-IV has the potential to promote the formation of CFU pre-B (panel A in Fig. 2). Furthermore, BM cells isolated from C56BL/6 mice were cultured in the presence of IL-7 (10 ng/mL) with BP-IV (25 μg/mL) for 7 days, after which B-cell differentiation was assessed by flow cytometric analysis. The percentages of iMB/MB cells in the cultures were increased by BP-IV treatment ($p < 0.05$), and the percentages of pro-B cells were decreased by BP-IV treatment ($p < 0.05$) (panel B in Fig. 2).

To investigate the functional role of BP-IV on B cell development, RT-PCR was employed to analyze the gene expression of BM cells after BP-IV treatment. Given that B-cell progenitors continue to mature when cultured *in vitro*, we investigated the effects of BP-IV exposure on B-cell progenitor gene expression. BM cell cultures were treated with BP-IV for 24 h, then sorted into pro-B, pre-B, and iMB/MB cell populations (panel C in Fig. 2). We observed significant differences in gene expression between progenitor populations treated with BP-IV or BP-IV-scrambled prior to sorting. Among major hematopoietic regulators, we found that PU.1 gene expression was significantly suppressed by BP-IV treatment in the pro-B, pre-B and iMB/MB cell populations (panel D in Fig. 2). The current study demonstrated that BP-IV can inhibit the transcriptional activation of PU.1, which may partly explain the mechanism by which BP-IV promotes IL-7-induced B-cell development.

Immunomodulatory characteristics of BP-IV on chicken as the host animal model experiment

Since BP-IV was obtained from chicken bursa of Fabricius, it led us to examine the effects of BP-IV on immune responses. Chickens were injected with BP-IV and AIV vaccine and the levels of antibodies were measured (panels A–C in Fig. 3). Antibodies from chicken immunized with BP-IV and AIV vaccine increased significantly ($p < 0.05$), with the levels of antibodies from chickens immunized with AIV vaccine and

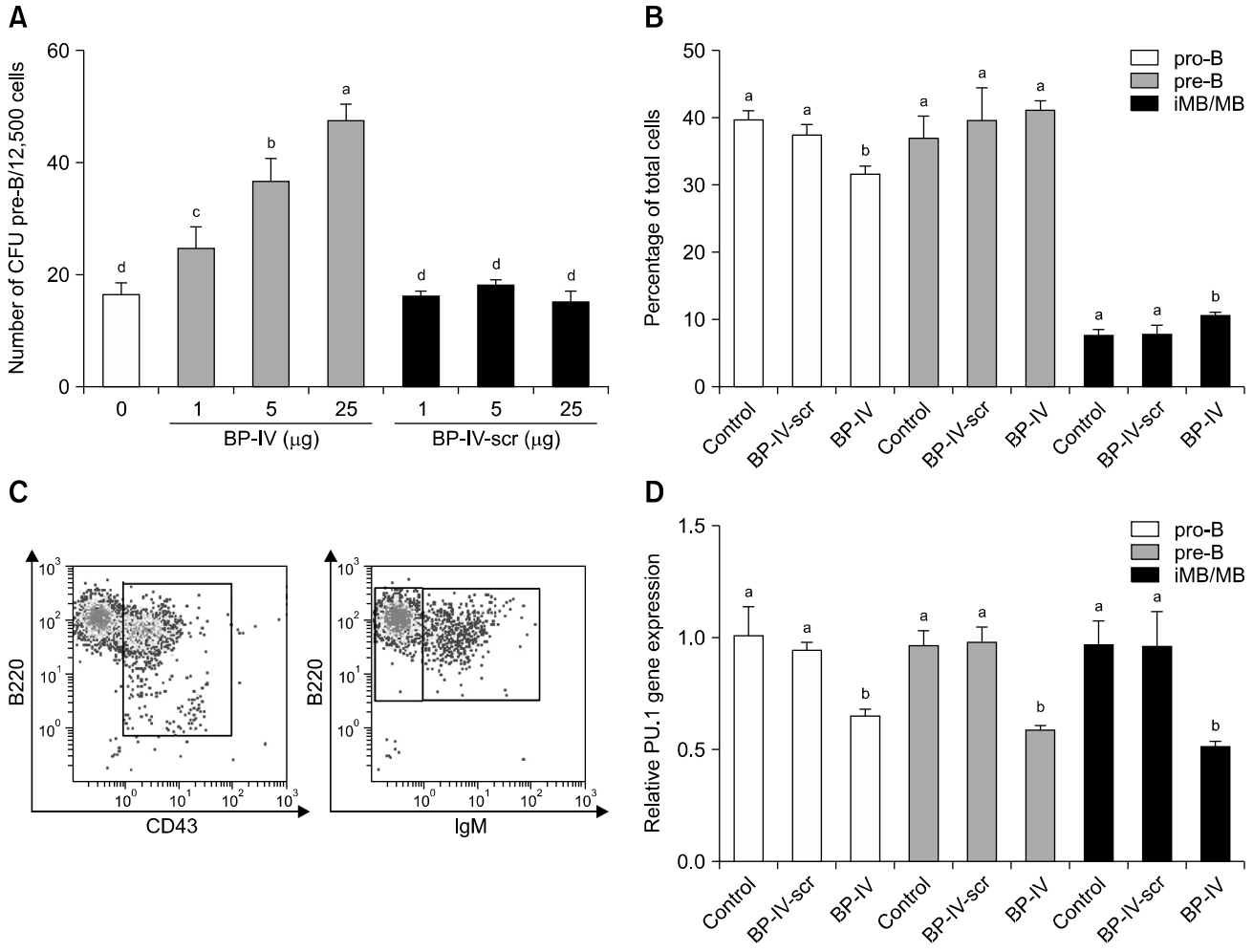

Fig. 2. BP-IV modulates B cell development. (A) Bone marrow cells were plated in 35-mm dishes at 2.5×10^4/mL in methycellulose medium. Various concentrations of BP-IV and BP-IV-scrambled (from 1 to 25 μg/mL) were added to the culture. Control cells (0 μg/mL) were treated with BSA (1 μg/mL). The cells were cultivated for 7 days, after which the produced colonies (CFU pre-B) were scored. All experiments were repeated three times independently. Values with different letters in the same column (a-d) are significantly different ($p < 0.05$). Data were expressed as the means ± SD (n = 3). (B) BM cells were cultured in the presence of IL-7 (10 ng/mL) with BP-IV or BP-IV-scrambled (25 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. The proportion of each population was defined as the percentage of the entire cell population. All experiments were repeated independently three times. Values with different letters in the same column (a-d) are significantly different ($p < 0.05$) from each other. Data were expressed as the means ± SD. (C) Relative PU.1 gene expression. BM cells were grown in IL-7. On day 3, BP-IV or BP-IV-scrambled (25 μg/mL) was added to half of the culture for 24 h. Cells were sorted on day 4 into pro-B ($B220^+CD43^+IgM^-$), pre-B ($B220^+CD43^-IgM^-$), and immature/mature (iMB/MB) cells ($B220^+CD43^-IgM^+$). (D) Total RNA was extracted. Real-time PCR analysis was performed on cDNA using PU.1-specific primers as described in the materials and methods. Figures show normalized values from a single experiment and fold-change over untreated from three independent experiments. scr, scrambled.

BP-IV (5 mg/kg) being highest among the three concentrations (panel A in Fig. 3). These results suggested that BP-IV promotes AIV-specific immunoglobulin secretion *in vivo*.

To determine if BP-IV enhances a specific cytokines response, IL-4 and IFN-γ cytokines were measured by the ELISA method. When compared to the control (chickens that received AIV vaccine), IL-4 and IFN-γ production increased significantly in response to the two immunizations with BP-IV (IL-4: 0.2, 1, and 5 mg/kg, $p < 0.01$; IFN-γ: 0.2, 1, and 5 mg/kg, $p < 0.01$) (panel B in Fig. 3).

Lymphocyte proliferation was measured as an indicator of cell immunity and immune cell function. The results showed that the proliferation of lymphocytes from immunization with BP-IV and AIV vaccine was higher than in chickens that

received AIV vaccine control (panel C in Fig. 3). These results suggest that the bursal-derived peptide BP-IV could induce Th1 and Th2 type cytokine immune responses.

Immunomodulatory characteristics of BP-IV on immunized mice

We next examined the biological activity of BP-IV on the

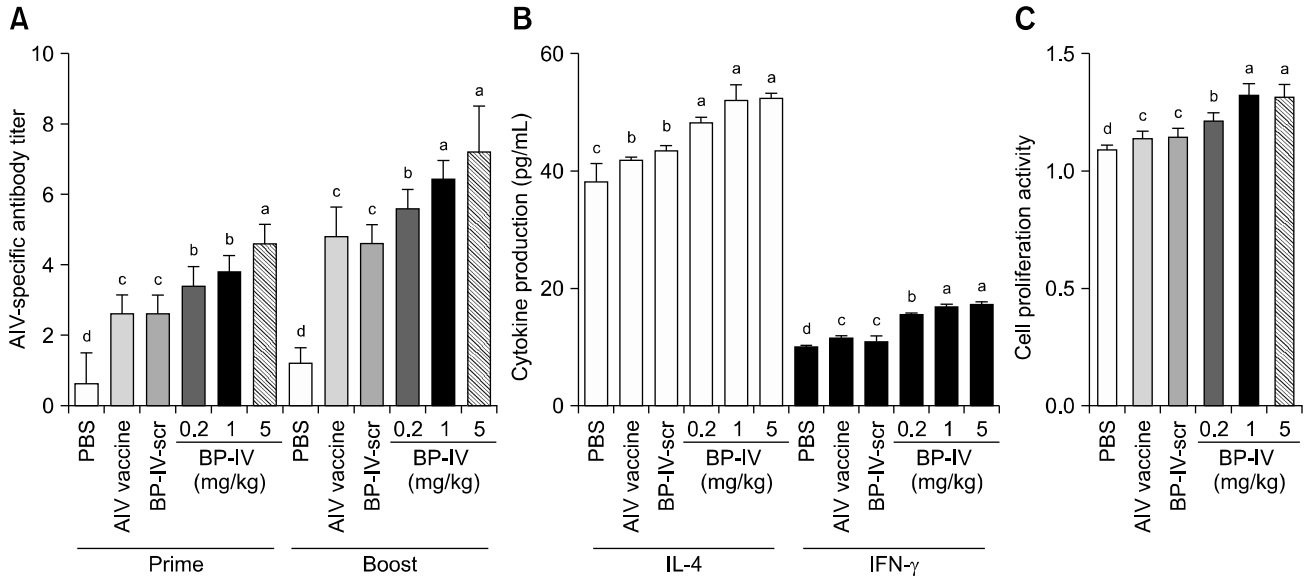

Fig. 3. Immunomodulatory roles of BP-IV on chicken immunization regimen. Chickens were twice immunized with BP-IV or BP-IV-scrambled and avian influenza virus (AIV) vaccine. (A) The induction of AIV-specific antibodies was measured by hemagglutination inhibition assay. (B) The sera were collected from the immunized chickens to determine IL-4 and IFN-γ cytokine production by enzyme-linked immunosorbent assay (ELISA). (C) Lymphocytes were prepared from the blood of immunized chickens, then stimulated with AIV antigen for 48 h to detect cell viability by MTT incorporation. All experiments were repeated independently three times. Values with different letters in the same column (a-d) are significantly different ($p < 0.05$) from each other. Data were expressed as the means ± SD (n = 10) from two independent experiments. PBS, phosphate buffered saline.

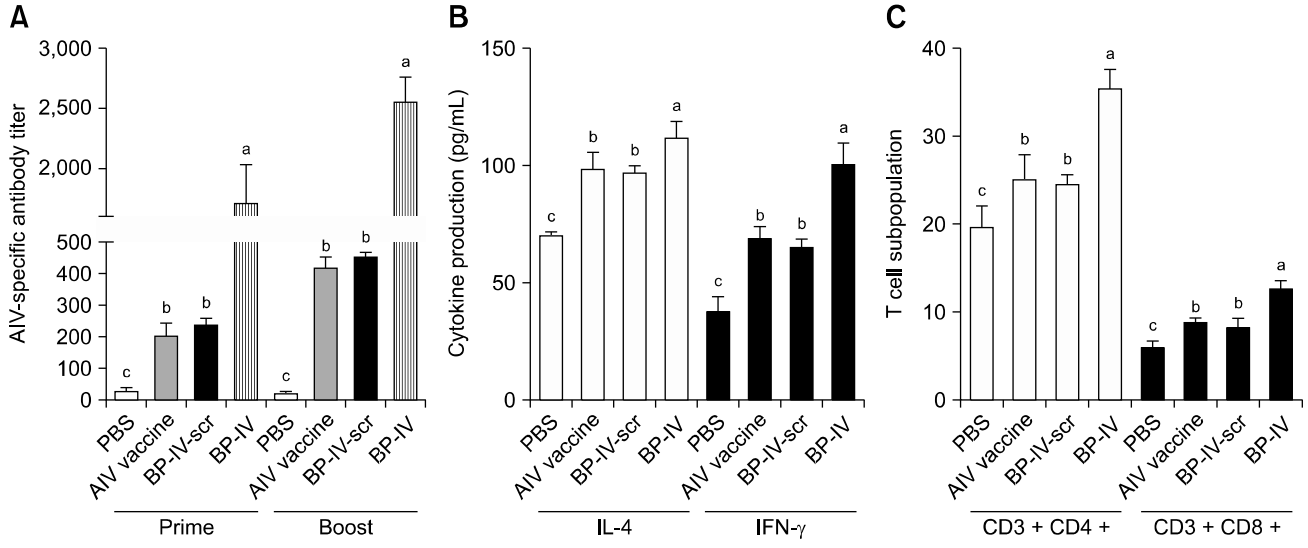

Fig. 4. Immunomodulatory roles of BP-IV on mouse immunization regimen. Mice were immunized with BP-IV or BP-IV-scrambled and AIV vaccine following the prime-boost vaccinations programs (days 0 and 14), respectively. (A) Sera were collected on day 14 after the two immunizations for use in antibody (IgG) titers by ELISA. (B) On day 7 after the second immunization, sera were collected to detect cytokine IL-4 and IFN-γ. (C) T cell immunophenotyping was expressed as the T-cell subtype percentages by flow cytometry. All experiments were repeated independently three times. Values with different letters in the same column (a-d) are significantly different ($p < 0.05$) from each other. Data were expressed as the mean ± SD (n = 5) from two independent experiments.

mice prime–boost immunization regimen to confirm the results of the chicken model. Anti-AIV antibodies of the sera at two weeks after the first and second immunization from mice immunized with AIV vaccine and 1 mg/mL BP-IV were significantly increased (panel A in Fig. 4; $p < 0.05$). The endogenous cytokine levels of IL-4 and IFN-γ in mice immunized with AIV vaccine were also measured. As shown in panel B of Fig. 3, IL-4 and IFN-γ cytokines were significantly increased in mice immunized with AIV vaccine and 1 mg/mL BP-IV (panel B in Fig. 4; $p < 0.05$).

The effects of BP-IV on the immunophenotyping of splenic T lymphocytes were also assessed. The results showed that the percentage of CD3+CD4+T cells ($p < 0.01$) and CD3+CD8+ T cells ($p < 0.05$) increased significantly in mice immunized with AIV vaccine and BP-IV (panel C in Fig. 4). Taken together, these results showed that BP-IV has strong immunomodulatory effects in the humoral and cellular response to AIV vaccine in a mouse model.

Discussion

There are many different types of short peptides with immunomodulatory activity that can be used in specific clinical applications. For example, primary/secondary immune deficiencies and autoimmune disorders have been treated with the immunomodulatory peptide Thymopentin [20]. Bursin is a candidate for the specific factor responsible for B-cell differentiation [11]. In this study, we found that BP-IV has immunomodulating activities and exhibits strong immunomodulatory function in antigen-specific humoral and cellular immune responses. Moreover, BP-IV can regulate B cell differentiation, which integrates an important linking between humoral and cell-mediated immunity, including B cell development. The immunological impact of BP-IV on cell-mediated immunity and B cell development has provided numerous insights into specific immunotherapeutic strategies for enhancing immune responses and protective immunity.

As a novel peptide from the avian immune system, this peptide led us to examine the effects of BP-IV on B cell differentiation. To the best of our knowledge, birds and mammals evolved from a common reptilian ancestor and have many common immunological systems. The mouse model has been extensively employed to detect the biological effects of BP-IV. Myeloid differentiation and B-cell-fate can be determined by PU.1 expression level [18,22]. Additionally, low concentrations of PU.1 protein have been reported to induce a B-cell fate, while high concentrations blocked B-cell development [6]. Several PU.1 knockout/mutant mouse strains have been generated and shown various phenotypic changes. PU.1 knockout mice showed an obvious lack of T and B cells [15,19]. These results indicate that BP-IV might modulate B/T cell-mediated immune responses, including antibody generation, cytokine production, and T cell maturation. Our previous study showed that BP5 can activate B cell proliferation directly and activate T cell proliferation indirectly, regulating both humoral immunity and cellular immunity [11]. BP11 also exerted immunomodulatory function on antigen-specific immune responses. However, BP11 could only increase IL-4 cytokine production [13]. When compared to BP5, BP11 and other adjuvants, such as oil and aluminum adjuvants, the biologically active peptide BP-IV acts as an adjuvant, activating humoral immunity and cellular immunity.

In summary, BP-IV was successfully isolated from the bursa of Fabricius through RP-HPLC and MALDI-TOF-MS methods and exhibited strong immunomodulatory function. Additionally, analysis of CFU pre-B formation showed that BP-IV could regulate B cell differentiation. These results suggest that the potential activity to regulate B cell differentiation indicates that BP-IV might act as an immunomodulatory factor, and therefore be useful as an agent for developing effective immunopharmacological strategies.

Acknowledgments

This work was supported by a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the National Special Research Programs for Non-profit Trades, Ministry of Agriculture (no. 200803015), and the International S&T Cooperation Program of China (ISTCP) (no. 2014DFR30980). The funders played no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of Interest

There is no conflict of interest.

References

1. **Audhya T, Kroon D, Heavner G, Viamontes G, Goldstein G.** Tripeptide structure of bursin, a selective B-cell-differentiating hormone of the bursa of Fabricius. Science 1986, **231**, 997-999.
2. **Baba T, Kita M.** Effect of extracts of the bursa of Fabricius on IgG antibody production in hormonally bursectomized chickens. Immunology 1977, **32**, 271-274.
3. **Brand A, Gilmour DG, Goldstein G.** Lymphocyte-differentiating hormone of bursa of Fabricius. Science 1976, **193**, 319-321.
4. **Cooper MD, Peterson RDA, South MA, Good RA.** The functions of the thymus system and the bursa system in the chicken. J Exp Med 1966, **123**, 75-102.
5. **Davison F, Kaspers B, Schat KA, Kaiser P.** Avian immunology. pp. 15-20, Academic Press, New York, 2011.
6. **DeKoter RP, Singh H.** Regulation of B lymphocyte and

macrophage development by graded expression of PU.1. Science 2000, **288**, 1439-1441.
7. **Fine JS, Macosko HD, Grace MJ, Narula SK.** Influence of IL-10 on murine CFU-pre-B formation. Exp Hematol 1994, **22**, 1188-1196.
8. **Glick B, Chang TS, Jaap RG.** The bursa of Fabricius and antibody production. Poult Sci 1956, **35**, 224-225.
9. **Klemsz MJ, McKercher SR, Celada A, Van Beveren C, Maki RA.** The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. Cell 1990, **61**, 113-124.
10. **Lassila O.** Emigration of B cells from chicken bursa of Fabricius. Eur J Immunol 1989, **19**, 955-958.
11. **Lassila O, Lambris JD, and Gisler RH.** A role for Lys-His-Gly-NH_2 in avian and murine B cell development. Cell Immunol 1989, **122**, 319-328.
12. **Li DY, Geng ZR, Zhu HF, Wang C, Miao DN, Chen PY.** Immunomodulatory activities of a new pentapeptide (Bursopentin) from the chicken bursa of Fabricius. Amino Acids 2011, **40**, 505-515.
13. **Liu XD, Feng XL, Zhou B, Cao RB, Li XF, Ma ZY, Chen PY.** Isolation, modulatory functions on murine B cell development and antigen-specific immune responses of BP11, a novel peptide from the chicken bursa of Fabricius. Peptides 2012, **35**, 107-113.
14. **Lydyard PM, Grossi CE, Cooper MD.** Ontogeny of B cells in the chicken. I. Sequential development of clonal diversity in the bursa. J Exp Med 1976, **144**, 79-97.
15. **McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA.** Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J 1996, **15**, 5647-5658.
16. **Moore RW, Caldwell DY, Berghman LR, Caldwell DJ, McElroy AP, Byrd JA, Hargis BM.** Effect of bursal anti-steroidogenic peptide and immunoglobulin G on neonatal chicken B-lymphocyte proliferation. Comp Biochem Physiol C Toxicol Pharmacol 2003, **134**, 291-302.
17. **Murthy KK, Ragland WL.** Effect of thymic extract on blastogenic responses of chickens. Poult Sci 1992, **71**, 311-315.
18. **Nagamura-Inoue T, Tamura T, Ozato K.** Transcription factors that regulate growth and differentiation of myeloid cells. Int Rev Immunol 2001, **20**, 83-105.
19. **Scott EW, Simon MC, Anastasi J, Singh H.** Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994, **265**, 1573-1577.
20. **Singh VK, Biswas S, Mathur KB, Haq W, Garg SK, Agarwal SS.** Thymopentin and splenopentin as immunomodulators. Current status. Immunol Res 1998, **17**, 345-368.
21. **Tang YQ, Yuan J, Österbay G, Österbay K, Tran D, Miller CJ, Ouellette AJ, Selsted ME.** A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated α-defensins. Science 1999, **286**, 498-502.
22. **Zou GM, Wu W, Chen J, Rowley JD.** Duplexes of 21-nucleotide RNAs mediate RNA interference in differentiated mouse ES cells. Biol Cell 2003, **95**, 365-371.