Identification and Characterization of Novel Immunomodulatory Bursal-derived Pentapeptide-II (BPP-II)*

Received for publication, June 27, 2011, and in revised form, December 7, 2011 Published, JBC Papers in Press, December 19, 2011 DOI 10.1074/jbc.M111.273854

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Background: The bursa of Fabricius is the acknowledged central humoral immune organ. Results: Novel bursal-derived BPP-II regulates expressions of immune-related gene and exerts immunomodulatory functions and activates p53 expression with strong antiproliferation on tumor cells.

Conclusion: BPP-II integrates linking between humoral immunity and cell-mediated immunity, including antitumor.

Significance: This might be the first sign of mechanisms involved in humoral immune system.

The bursa of Fabricius, the acknowledged central humoral immune organ, plays a vital role in B lymphocyte differentiation. However, there are few reports of the molecular basis of the mechanism on immune induction and potential antitumor activity of bursal-derived peptides. In this paper, a novel bursal-derived pentapeptide-II (BPP-II, MTLTG) was isolated and exerted immunomodulatory functions on antibody responses in vitro. Gene microarray analyses demonstrated that BPP-II regulated expression of 2478 genes in a mouse-derived hybridoma cell line. Immune-related gene ontology functional procedures were employed for further functional analysis. Furthermore, the majority of BPP-II-regulated pathways were associated with immune responses and tumor processes. Moreover, BPP-II exhibited immunomodulatory effects on antigen-specific immune responses in vivo, including enhancement of avian influenza virus (H9N2 subtype)-specific antibody and cytokine production and modification of T cell immunophenotypes and lymphocyte proliferation. Finally, BPP-II triggered p53 expression and stabilization and selectively inhibited tumor cell proliferation. These data identified the multifunctional factor, BPP-II, as a novel biomaterial representing an important linking between the humoral central immune system and immune induction, including antitumor. Information generated in this study elucidates further the mechanisms involved in humoral immune system and represents the potential basis of effective immunotherapeutic strategies for treating human tumors and immune improvement.

Two separate differentiation pathways for lymphocytes are established, one for T (thymic) lymphocytes and the other for antibody-secreting B (bone marrow and bursal) lymphocytes. In mammals and human, the B cell-differentiating organ equivalent to the T cell-differentiating thymus has not been defined (1, 2). The bursa of Fabricius (BF) is the acknowledged central humoral immune organ (3), which is vital to B differentiation and antibody production (4). B cell is named after "bursal-derived lymphocyte." Therefore, BF provides an invaluable model for studies on the basic immunology of mammals and human.

Various immunomodulatory peptides have been isolated from BF. Bursin, a specific molecule for the differentiation of B cells (5), selectively induced avian B cells, but not avian T cells, from their precursors in vitro (1, 2) and promoted immunoglobulin switching from IgM to IgG (6). Bursal septapeptide-II (BSP-II, TPSGLVY) induced various immune responses in vivo and regulated tumor cells proliferation (7). Bursopentin was proved to induce B lymphocyte proliferation through activating various pathways, such as MAPK and NF-κB signals (8). Also, bursal septapeptide-I and bursal pentapeptide (BPP)-I have antiproliferative effects on the tumor cells and the initiation of p53 expression, an important tumor suppressor (9, 10).

However, the molecular basis and potential mechanisms by which BF stimulates and regulates immune response are not fully understood. Therefore, it is important to study the mechanisms and cellular basis of active peptides derived from BF on basic immunology. In this paper, a novel bursal-derived immune-inducing BPP-II was isolated, and the induced downstream signaling pathways and biological consequences were investigated using gene microarrays to characterize the potential mechanisms by which BF functions in immunity and tumorigenesis. Also, BPP-II exerted significant immunomodulatory effects on both humoral and cellular-mediated immune responses. It was demonstrated that BPP-II activated the tumor suppressor p53 expression with strong antiproliferation on tumor cells, thus providing an insight into the link between the humoral central immune system and immune induction.
including antitumor. These data indicated the potential basis of immune induction and immunotherapeutic strategies for the treatment of cancer and immune improvement.

**EXPERIMENTAL PROCEDURES**

*Mouse and Cell Lines*—BALB/c female mice (6–8 weeks old, 17–21 g) were obtained from Yang Zhou University (Yangzhou, China). All of the animal experimental procedures were performed in accordance with the institutional ethical guidelines for animal experiments. Hybridoma cells (1H5F9 strain, IgG1 κ subtype antibody) (10), were cultured with RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS; Invitrogen) at 37 °C with 5% CO2. Tumor cell lines MCF-7 and HeLa and normal cell lines CEF, BHK21, MDBK, and Vero were cultured with DMEM supplemented with 10% FBS at 37 °C with 5% CO2.

*Isolation and Identification of BPP-II Derived from BF*—Bursal peptide was purified from avian BF by reversed-phase (RP) high performance liquid chromatography (HPLC), according to methods described previously (7–10) with some slight modifications. Briefly, a BF extract prepared by homogenization and centrifugation was ultrafiltered (lower than 1000 Da) for 48 h at 4 °C and filtered (0.22 μm) and analyzed using a 4.6 × 250-mm SinoChrom ODS-BP RP-HPLC affinity column (Elite) with a linear gradient of acetonitrile (2–100%) and monitored at 220 nm. The elution was collected and analyzed using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) (Bruker). The bursal-derived peptide was synthesized with purity >97.8%.

*Hybridoma Cell Treatment*—Hybridoma cells (10⁶ cells/ml) were prepared in 96-well plates and treated with or without BPP-II (20, 0.2, and 0.02 μg/ml). After 48 h, the viability was determined with the MTT reagent (Sigma) (11, 12), and the supernatant antibody titers were determined by ELISA method (7).

*cDNA Microarray and Microarray Data*—Total RNA was harvested from 0.2 μg/ml BPP-II-treated hybridoma cells using TRIzol reagent (Invitrogen) according to the instructions provided by the manufacturer. RNA was amplified, labeled, and hybridized with microarrays and analyzed using the Agilent G2505B microarray scanner. The resulting data were analyzed by the Agilent GeneSpring GX software (version 11.0) system, a knowledge-based system of computer algorithms (13), and the microarray data sets were normalized in GeneSpring GX using the Agilent FE one-color scenario (mainly median normalization). Differentially expressed genes were identified through fold-change screening. GO analysis and Pathway Analysis were performed on this subset of genes.

*Semiquantitative RT-PCR Analysis*—RNA was prepared from BPP-II-treated hybridoma cell using the TRIzol reagent. The primer pairs can be found in supplemental Table S1, and regulated genes were estimated using a One Step SYBR® PrimeScript® RT-PCR kit (Takara, Shiga, Japan).

*Immunization and Detection Protocols*—The immunomodulatory roles of BPP-II were investigated in female BALB/c mice (6–8 weeks old), as reported previously (7), in which mice were immunized intraperitoneally with a 0.2-ml inactivated avian influenza virus (AIV, H9N2 subtype) antigen containing 10, 50, and 250 μg/ml in the presence or absence of BPP-II on days 0 and 14, respectively. PBS was used as a negative control, and AIV/H9N2 vaccine served as a positive control. The sera were collected on the 14th and 28th days to detect the antigen-specific antibody responses (IgG, IgG1, and IgG2a) by ELISA method (7), respectively, and sera were collected on 7th day after the final immunization for measurement of IL-4 and IFN-γ cytokines using ELISA kits (R&D). Also, T cell immunophenotyping of spleen lymphocytes from immunized mice was performed by three-color flow cytometry analysis (BD, LSR) using mixtures of specific anti-mouse mAbs CD3, CD4, and CD8 labeled with PE, FITC or PE-Cy5. Spleen cells were isolated from immunized mice and stimulated for 48 h as described previously (7) to measure cell viability by MTT incorporation (11, 12). Additionally, the isolated splenocytes were restimulated with 10 μg/ml BPP-II or AIV antigen, and supernatant antibody and cytokine production was measured after 48 h as described previously (7).

*p53 Luciferase Activity and Expression*—The wild-type p53 Vero cell line was transfected with the indicated p53-Luc plasmids containing the luciferase reporter gene cloned under the control of p53-binding DNA sequences using a Lipofectamine 2000/DNA conjugate according to the manufacturer’s instructions (Invitrogen). After 24 h, the transfected cells were stimulated with BPP-II (0.02–20 μg/ml) for 24 h. Also, the transfected Vero cells were preincubated for 2 h with the p53 inhibitor 20 μM α-pifithrin (14) and treated with 0.2 μg/ml BPP-II for 22 h. The p53 relative luciferase activity was assayed using a Dual Luciferase Reporter Assay system (Promega).

Nontransfected Vero cells were treated with or without BPP-II (0.02–20 μg/ml) for 24 h. Cells were treated with 1 μg/ml doxorubicin (Sigma) which has been reported to induce p53 expression (15), in parallel as a positive control. The cells were lysed with cell culture lysis reagent (Promega), and protein samples were collected. Western blotting was performed as described previously (16) using mouse anti-human p53 (DO-1; Santa Cruz Biotechnology), mouse anti-human β-actin (AC-15; Sigma), and rabbit anti-human Bax (N-20; Santa Cruz Biotechnology), to detect protein expression of p53 and Bax, in which Bax is a key component of apoptotic cascades (17). Vero cells were treated with or without 2 μg/ml BPP-II for 16 h and subsequently exposed to 100 μg/ml cycloheximide (Sigma), and incubated for the indicated periods at 37 °C.

*Various Cell Line Treatments with BPP-II*—Tumor cells MCF-7 and HeLa were added to 96-well flat-bottomed microtiter plates (100 μl/well of 2 × 10⁵ cells/ml) and were stimulated with or without BPP-II (0.04–50 μg/ml). Also, four normal cell lines (BHK21, CEF, MDBK, and Vero) were treated with or without BPP-II at concentrations that ranged from 0.4 to 50 μg/ml for 48 h. The cell proliferation was measured, using a standard MTT-based method (11, 12), and data were statistically analyzed with SPSS software.

*Statistical Analysis*—Results were expressed as means ± S.D. The statistical significance of the observed differences was analyzed by t tests or one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. A p value <0.05 was considered to be a significance level.
RESULTS

Isolation and Characterization of BPP-II—A relatively abundant novel polypeptide was isolated by RP-HPLC from avian BF with an elution time of 21.40 min (Fig. 1A). Characterization of the peptide termed BPP-II with a sequence of MTLTG was identified by MALDI-TOF analysis (Fig. 1B). The chemical formula (Fig. 1B) and the titration curve (Fig. 1C) were analyzed by DNASTAR. The isoelectric point of BPP-II is 5.55 with a negative charge of 0.09. D, synthetic BPP-II was assessed on an RP-HPLC affinity column with a linear acetonitrile gradient (gray line).

Alignment of the amino acid sequence to BPP-II was carried out using all five possible linearized peptides as query sequences in the National Center for Biotechnology Information nonredundant and Expressed Sequence Tags data bases. Screening of the identical polypeptide sequences identified revealed homology with bactericidal/permeability-increasing protein-like 3 (BPIL3) and sterile/H9251 motif domain containing 8 (SAMD8) in Gallus gallus (supplemental Table S2). Furthermore, high level homology was identified (one amino acid discrepancy) with proteins including MHC class I antigen and several factors in ubiquitin-conjugating enzyme E2 variants (supplemental Table S2). The synthetic BPP-II was identical to natural BPP-II as determined by RP-HPLC (Fig. 1D). Therefore, these data confirmed the structure of natural BPP-II.

BPP-II-stimulated Antibody Production of Hybridoma Cell—Hybridoma cell antibody and proliferative responses were determined following BPP-II treatment for 48 h. Increased antibody responses were observed after treatment with a range of BPP-II concentrations (0.02, 0.2, 2, and 20 μg/ml) (Fig. 2A). Enhanced cell viability was detected only at 0.02 μg/ml BPP-II (Fig. 2B). These results demonstrated the regulation of BPP-II on antibody responses in vitro.

Overview of Gene Expression Patterns—cDNA microarray systems have been used widely to investigate gene expression patterns and functional classification and to identify the specific suppression of the humoral immune response in insects (18). In this paper, to elucidate the inducing effect of BPP-II on immune cells, hybridoma cells treated for 4 h with 0.2 μg/ml BPP-II were compared with PBS-treated control hybridoma cells by mouse cDNA microarray analysis. BPP-II-associated changes (≥1.5-fold) in gene expression (1770 genes up-regulated; 708 genes down-regulated) are summarized in sup-
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Gene Ontology Functional Analyses of Regulated Gene Expression—Genes differentially expressed between the BPP-II-treated and PBS control groups were categorized according to Gene Ontology (GO) (19). Given the fact that BPP-II has a broad range of effects on immune response, it is conceivable that there might be multiple gene transcriptional changes caused by BPP-II. FlyBase orthologs of mouse genes from BPP-II-induced hybridoma that were differentially expressed were used for GO enrichment analyses of immune related genes (Fig. 3 and supplemental Table S5). Analysis revealed 63 up-regulated and down-regulated genes involved in immune-related processes, including immunity, T cell activation and proliferation, B cell-mediated immunity, and cytokine production, were present, and common genes are represented in the intersections. More information about the involved genes is provided in supplemental Table S4.

Gene expression related to B cell-mediated immunity was induced by BPP-II, including STAT6, Fas, protein-tyrosine phosphatase, receptor type, C (Ptprc), Ptpn6, protein kinase Cδ (Prkcd), interferon regulatory factor 7 (Ifi7), and CD55 antigen (Cd55) (Fig. 3 and supplemental Table S5). It was identified that various genes are involved in T cell-related cellular processes, including T cell activation, differentiation, and proliferation. Also, the induced gene Fyn is involved in both T cell receptor binding and T cell receptor complex, respectively.

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II-treated splenocytes from immunized mice than that of PBS control, respectively (supplemental Fig. S1). Also, the significantly increased productions of IL-4 and IFN-γ were observed in spleen cell cultures from immunized mice after AIV stimulation (supplemental Fig. S1B) compared with PBS control. These results suggested that BPP-II exerts the immunomodulatory functions in vitro.

To assess T cell immunophenotyping and lymphocyte proliferation response through BPP-II treatment, spleen lymphocytes were prepared at the 7th day after the second immunization. T cell populations were increased in mice that received AIV antigen and BPP-II, compared with those of mice receiving AIV antigen alone (Fig. 5C). Splenic lymphocyte proliferation, measured using the MTT method (11, 12), was significantly greater in mice immunized with AIV antigen and 50 μg/ml BPP-II compared with AIV antigen alone (Fig. 5D).

**BPP-II Treatment Activated p53 Luciferase Activity and p53 Expression**—It was observed that the expressions of genes involved in p53 signal pathway was regulated at the transcriptional level after BPP-II treatment (supplemental Table S6). To determine the effect of BPP-II on p53, Vero cells were transfected with the p53 luciferase (p53-Luc) reporter plasmid encoding 14 tandem repeats of the p53 consensus binding sites (Stratagene; La Jolla, CA), and luciferase activity was assayed with or without BPP-II treatment (Fig. 6A). Western blotting analysis revealed dose-dependent up-regulation of p53 and target Bax protein expression by BPP-II treatment (Fig. 6C and D). However, the expressions of p53 mRNA in Vero cells were not affected by BPP-II treatment (data not shown). These data suggested that BPP-II induces p53 activity and the expression of p53 protein.

To test whether accumulation of p53 was dependent on the regulation of protein stability, BPP-II- and PBS-treated Vero cells were treated with 100 μg/ml cycloheximide, an inhibitor of protein biosynthesis in eukaryotic cells. The p53 protein decay was monitored by Western blot analysis (Fig. 6E). The half-life of p53 in PBS-treated Vero cells (~0.5 h) was noticeably shorter than in BPP-II-treated Vero cells (Fig. 6E). In contrast, there was no significant difference in the stability of β-actin between the PBS- and BPP-II-treated cells. These results indicated that p53 protein was stabilized in BPP-II-treated Vero cells.

**Inhibition of Tumor Cell Proliferation by BPP-II Treatment**—It was observed that BPP-II exerted a dual dose-dependent effect on tumor cell proliferation measured by MTT assay (11, 12). MCF-7 cell proliferation was inhibited by 18.8, 11.1, and 3.6% following BPP-II treatment at 50, 20, and 5 μg/ml, respectively, whereas proliferation was increased by 1.87, 21.6, and 18.44% following BPP-II treatment at 1, 0.2, and 0.04 μg/ml, respectively (Fig. 7A). HeLa cell proliferation following BPP-II treatment at 50, 20, and 5 μg/ml was lower than that of the control groups by 29.0, 17.9, and 9.6%, respectively (Fig. 7A), whereas proliferation was enhanced by 13.0, 42.57, and 31.86%
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**FIGURE 6.** BPP-II induced increase in p53 activity, with enhanced p53 protein and Bax protein expression. A, increase in p53 luciferase activity after BPP-II treatment. Vero cells were transfected with p53 Luc and pRL-TK plasmid. After 24 h, the transfected Vero cells were treated with or without BPP-II for 24 h to determine p53 luciferase activity. Data are presented as mean ± S.D. (error bars). Statistical significance is indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared with control without BPP-II treatment (Control). B, inhibition on BPP-II-induced p53 luciferase activity. The transfected Vero cells were preincubated with α-pifithrin for 2 h and then stimulated with or without BPP-II for 22 h to detect the level of p53 luciferase activity. Data are presented as mean ± S.D. Statistical significance is indicated as follows: **, p < 0.01 compared with BPP-II treatment (BPP-II). C, expression levels of p53 and Bax protein. Nontransfected Vero cells were stimulated with or without BPP-II for 24 h, and the p53 and Bax protein were determined by Western blot analysis. D, intensity of the protein expression in Western blots measured by densitometer and normalized to the untreated control (Control). Data are presented as mean ± S.D. Statistical significance is indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared with control without BPP-II treatment (Control). Doxorubicin (Dox) was used as a positive control. E, stabilization of p53 in BPP-II-treated Vero cells. Vero cells were treated with or without BPP-II at 2 μg/ml for 16 h and subsequently exposed to 100 μg/ml cycloheximide (CHX). The cells were collected at the indicated times (hours) post-treatment (hpt) and subjected to Western blot analysis using the indicated antibodies.

**FIGURE 7.** Inhibition on tumor cell proliferations after BPP-II treatment. Tumor cells MCF-7 and HeLa, and normal cells BHK21, CEF, MDBK, and Vero were treated with or without BPP-II for 48 h to determine the tumor cell proliferations (A) and normal cell proliferations (B) by MTT array. Data are presented as mean ± S.D. (error bars). Statistical significance is indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001, compared with control without BPP-II treatment (Control).
following BPP-II treatment of 1, 0.2, and 0.04 μg/ml, respectively.

To identify potential selective antiproliferation on tumor cell of high BPP-II concentrations, the effects BPP-II treatment on proliferation of four normal cell lines (CEF, BHK21, Vero, and MDBK) were investigated. The results showed that 5 μg/ml BPP-II significantly enhanced CEF cell proliferation by 26.34% (p < 0.05), and BPP-II significantly induced MDBK cell proliferation by 26.22, 54.93, 69.69, 88.32, and 45.03% in a dose-dependent manner over the range from 0.4 to 50 μg/ml (5 and 10 μg/ml, p < 0.001; 2 and 50 μg/ml, p < 0.01; 0.4 μg/ml, p < 0.05), compared with that of control without BPP-II treatment (Fig. 7B). However, there was no significant inhibition was observed in cell proliferation of BPP-II-treated BHK21 or Vero cells at reachable concentrations (Fig. 7B). These results suggested that no inhibition of proliferation of the four normal cell lines was observed following BPP-II treatment at experimentally achievable concentrations.

DISCUSSION

Here, we report identification of inducing immune response and characterization of the profile of gene expression regulated after BPP-II treatment, a new isolated biological peptide from the humoral central immune organ, BF. These will only be selectively discussed.

In this study, a new bursal pentapeptide (BPP-II), MTLTG is the first reported (Fig. 1). BPP-II was found to be homology with various proteins in G. gallus (supplemental Table S2), which are related to the innate immune system, including defense against bacterial, viral, and fungal infections (20), ubiquitination process (21), and antitumor (22, 23). These proteins are conserved in various species, including chicken, human, chimpanzee, dog, mouse, and rat. Identification of functional homology proteins will provide elucidation of the function of BF in experimental study and clinical application. However, the potential inducing functions of BPP-II in various immune responses remain to be defined.

For sharing characteristic of B lymphocyte-secretory antibody, hybridoma cell was used as immunocyte model to study further the potential molecular basis of the regulation of BPP-II on immune responses (supplemental Table S3). The results of microarray analysis showed BPP-II regulated numerous genes involved in various immune signaling (supplemental Table S6), which provided an opportunity to survey the potential mechanism of BPP-II on immune responses. The key question is: could all of the involved genes of activated signals in concert cause the observed regulation in immune-related signaling? To consider how BPP-II signaling converges on immune signaling, we will briefly consider the roles of genes and their possible roles in activation of immune signaling.

Microarray analysis demonstrated that BPP-II regulated expression of various genes involved in T cell activation (Fig. 3 and supplemental Table S5). Furthermore, immunization experiments revealed that BPP-II altered the T cell phenotype of immune responses (Fig. 5C), which suggested involvement of BPP-II in T cell signaling. Key steps during T cell activation are the activation of Ras and Rho family GTPase, which are also important targets for the products of PI3K. Pip5k1c regulates the adhesion through facilitating RhoA GTPase and integrin activation by a Rho guanine nucleotide exchange factor 12 (Arhgef12) (24, 25), which was up-regulated after BPP-II treatment (supplemental Table S4). Furthermore, Pip5k1c(r) contributes to the critical second messenger inositol trisphosphate and diacylglycerol (26). The up-regulation of CACNB4 expression and down-regulation of CACNA1I expression following BPP-II treatment (supplemental Table S4) suggest that BPP-II affects the second messenger Ca$^{2+}$, leading to immune responses on effector cells (27, 28). Optimal T cell activation requires Ca$^{2+}$/calcineurin-NFAT signaling in concert with Ras/MAPK activation. These results indicated that BPP-II might participate the second messenger inositol trisphosphate pathway through voltage-dependent calcium channel to alter T cell activation.

JAK-STAT pathway plays vital roles on T cell activation, cell cycle, proliferation, and differentiation of effector cells. Regulation of various genes encoding interleukins and interleukin receptors that are involved in the JAK-STAT signaling pathway was observed following BPP-II treatment in hybridoma cells (supplemental Table S6). The potential role of BPP-II on Th1 and Th2 type cytokine responses was further confirmed in immunization experiments (Fig. 5B and supplemental Fig. S1B). The family of SOCS genes, including CISH, induced by STATs, were markedly induced in BPP-II–treated hybridoma (supplemental Table S4). It was predicted that that up-regulation of CISH would normally result in down-regulation of STAT3 and STAT5, and as expected, protein inhibitor of activated STAT3 (Pias3) was increased after BPP-II exposure. However, STAT4, STAT5α, STAT5b, and STAT6 factors which are involved in T cell activation and differentiation, proliferation, and B cell–mediated immune response, and cytokine–mediated signaling (Fig. 3 and supplemental Table S5), were up-regulated after BPP-II treatment. STATs are activated by tyrosine phosphorylation in response to cytokines and mediate many of their functional responses (29). Therefore, it was speculated that BPP-II regulates JAK–STAT signaling by STATs through various negative-feedback mechanisms, leading to T cell activation and various immune responses.

BF is the primary site of B cell lymphopoiesis in birds and is critical for antibody production and normal development of B lymphocytes accompanied by expression of a series of cell surface molecules and modulation by regulatory factors (3, 4, 29). In humoral immune responses, B cell differentiation and development are well characterized. In the bone marrow, the differentiation from pro-B cells to immature B cells can be defined by several surface antigens. Immature B cells are stimulated to become mature B cells that circulate in the peripheral blood as naive B cells. In the peripheral lymphoid tissues, naive B cells differentiate into memory B cells, or plasma cells (30). However, little is known about the differentiation of B cells in response to BPP-II treatment. In this paper, it was observed that BPP-II stimulated antibody production in immunized mice and splenocytes from immunized mice (Fig. 5A and supplemental Fig. S1A), and BPP-II treatment induced the down-regulation of Msho2 and the up-regulation of Feer1a, which are subunits of the high affinity IgE receptor (31) involved in B cell–mediated immunity, regulation of humoral immune responses, and IgE
receptor activity (Fig. 3 and supplemental Table S5). Enhancement of B cell receptor cross-linking involves wide involvement of the secondary messenger Ca$^{2+}$ and simultaneous activation of multiple serine kinase pathways (32, 33). BPP-II regulated the expression of the CACNB4 and CACNAI1 sub-units of calcium channels, suggesting that BPP-II contributes to B cell receptor signaling through the regulation of calcium channels. These results indicated that BPP-II might modulate humoral immune responses; however, further research is required to elucidate the role and underlying mechanism of BPP-II in B cell differentiation.

BPP-II is a simple structured, low molecular weight peptide, which belongs to the hapten family of molecules (Fig. 1). It has been reported that hapten molecules used in isolation are poorly immunogenic vaccines (34). Therefore, it was found that mice immunized with BPP-II alone did not induce significant response (data not shown). However, the simple structural features of BPP-II do not influence its adjuvant activity. It was proved that BPP-II itself could induce antibody and cytokine production by splenocytes from immunized mice (supplemental Fig. S1), and microarray analysis showed that BPP-II stimulation regulated expressions of genes involved in T cell activation, humoral immune response, and cytokine production, suggesting that BPP-II might induce strong humoral and cellular immune responses in vitro through various immune-related signal activations. Furthermore, BPP-II functions as an immunomodulatory factor to induce AIV-specific immune responses (Fig. 5). These results suggested that BPP-II might be an immunomodulatory factor and could induce antigen-dependent humoral and cellular immune responses.

In the immune system, signal transduction pathways are functionally important for the appropriate development of properly selected T and B lymphocytes as well as in controlling responses to antigen by more mature cells (35, 36). These results illustrated the profound molecular basis of the regulation of BPP-II on the humoral immune and cellular immune responses.

The potential linking between biomaterials from the humoral central immune organ and antitumor immunity have yet never been elucidated. In this paper, expressions of various genes involved in various cancer pathways and apoptosis were shown to be regulated by BPP-II treatment (supplemental Table S6). It has been reported that the dysregulated expression of the CsF1r proto-oncogene is important for the resultant tumors survival in B cell-derived lymphoma cells (37). The Fas/ Fas ligand (FasL) system is a key signaling transduction pathway of apoptosis in cells and tissues and plays an important role in immune privilege (38). Up-regulations of CSF1R and Fas suggest that BPP-II might contribute to antitumor defense (supplemental Table S4). Apoptosis plays an important role in homeostasis and tissue development (39). Up-regulation of Perp (TP53 apoptosis effector) and antiapoptotic molecule Bcl2LI (40) following BPP-II treatment indicates dual functional roles in apoptosis.

The tumor suppressor p53 protein plays a crucial role following DNA damage (41). In this paper, BPP-II was shown to be identical to SAMD8. Sterile a motif (SAM) domains present in p63 and p73 family members, homologs of the tumor suppressor p53, might mediate negative regulation of p53-like activity (22). Homologous ubiquitin-conjugating enzyme E2 to BPP-II (supplemental Table S2), also known as ubiquitin-carrier proteins, participate in the ubiquitination process (21, 42). Ubiquitination plays a key role in regulating the stabilization and expression of p53 (43), and although the precise mechanisms are not fully understood, in posttranslational modification (44). It has been reported that various factors involved in the ubiquitination modification pathway influence p53 regulation. p53 ubiquitination and degradation are more complex, and MdmX, HAUSP, ARF, COP1, Pirh2, and ARF-BP1 continue to this pathway (43, 44). The results proved that BPP-II regulated gene expressions of the p53 signal pathway (supplemental Table S6) and enhanced p53 activity and expression (Fig. 6). However, the factors involved in BPP-II-stimulated p53 regulation remain to be elucidated, which will be major aspects in our subsequent research.

It was observed that high doses of BPP-II (5–50 μg/ml) selectively inhibited growth of tumor cells, but not normal cells (Fig. 7) by the MTT method. As an indicator of cell metabolic viability (11), the MTT assay represents a useful tool in estimating cell number and viability (12, 45). Clonogenic cell survival assay is used to assess reduction of the potential of individual tumor cells to form colonies (46). Flow cytometry provides a powerful and versatile approach to the measurement of cell death by the addition of various fluorescent nonvital DNA dyes (47). These three methods demonstrate different approaches to the analysis of cell proliferation and death. In this paper, BPP-II-mediated regulation of proliferation in normal and tumor cells was analyzed by the MTT method. In our further works, the function of BPP-II on proliferation or cell death by flow cell cytometry sorting or clonogenic assay will be investigated.

In this paper, it was found that although BPP-II induced p53 and Bax (Fig. 6), no inhibition effect on Vero cell viability was observed after BPP-II treatment (Fig. 7B). Apoptosis is tightly controlled by multiple conserved genes, including the Bcl-2 and caspase families. Bcl-2 protein suppresses the apoptotic death programs, whereas Bax (Bcl2-associated X protein) is an apoptosis-inducing protein (48), and the ratio of these two proteins determines cell fate following an apoptotic stimulus (49). Furthermore, Bcl-2 exhibits caspase-inhibiting activity downstream or aside from cytochrome c release to prolong cell survival after Bax induction (50). However, the effects of BPP-II on Bcl-2 protein and caspase proteins in Vero cells are unclear. Vero cell is derived from African green monkey kidney cell and exhibits characteristics different from tumor cells. It can be speculated that Vero cells share some regulation of antiapoptotic pathways, accounting for the observation that Vero cell viability was not significantly affected by BPP-II treatment, despite the observed increases in p53 and Bax protein levels. This phenomenon requires further investigation.

BPP-II was observed to play an antiproliferative role in tumor cells (Fig. 7). What is the probable effect of BPP-II as an adjuvant on antitumor immune responses? Tumors, unlike infectious organisms, do not induce effective innate immunity, and antitumor vaccines require adjuvant for efficacy (51). The competitive interaction between tumors and the immune system is highly complex (52). Prevention of tumor recurrence depends
on the induction of a stably maintained population of specific CD8⁺ cytotoxic effector T cells (53, 54) that recognize antigen in the context of MHC class I expressed by the “target” cell (23, 55). In this paper, BPP-II was shown to exhibit similarity with MHC class I antigens (supplemental Table S2). BPP-II stimulation altered the CD8⁺ T cell phenotype (Fig. 5C) and reduced tumor cell proliferation (Fig. 7A). These results might provide information regarding adjuvant potential of BPP-II on antitumor immune responses in vivo. Our subsequent works will be an essential elucidation of the immunomodulatory functions of BPP-II in the induction of antitumor immune responses.

BF is the acknowledged central humoral immune organ responsible for B cell difference (3). These results suggest that BPP-II is a bursal-derived potential immunomodulatory peptide. Although the function of BPP-II in the immune induction requires further elucidation, information generated in this study elucidates the link between humoral and cellular-mediated immune responses, including antitumor, and provides some novel insights on the research of the humoral central immune system and on the effective immunotherapeutic strategies for treating human tumors and immune improvement.

Acknowledgment—We thank KangChen Bio-tech Inc. (China, Shanghai) for performing microarray analysis.

REFERENCES

1. Audhya, T., Kroon, D., Heavner, G., Viamontes, G., and Goldstein, G. (1986) Tripeptide structure of bursin, a selective B cell-differentiating hormone of the bursa of fabricius. Science 231, 979–999
2. Brand, A., Gilmour, D. G., and Goldstein, G. (1976) Lymphocyte-differentiating hormone of bursa of fabricius. Science 193, 319–321
3. Fred, D. (2008) in Avian Immunology (Fred, D., Bernd, K., and Karel, A. S., eds) 1st Ed., pp. 1–12, Elsevier Academic Press, New York
4. Ratcliffe, M. I. (2006) Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development. Dev. Comp. Immunol. 30, 101–118
5. Lassila, O., Lambris, J. D., and Gisler, R. H. (1989) Role for Lys-His-Gly-NH₂ in avian and murine B cell development. Cell Immunol. 122, 319–328
6. Baba, T., and Kita, M. (1977) Effect of extracts of the bursa of Fabricius on IgG antibody production in hormonally bursectomized chickens. Immunology 32, 271–274
7. Feng, X., Su, X., Wang, F., Wei, J., Wang, F., Cao, R., Zhou, B., Mao, X., Zheng, Q., and Chen, P. (2010) Identification of an immunological and potential immunomodulatory characterization of TPSGLVY, a novel bursal seaptide isolated from the bursa of Fabricius. Peptides 31, 1562–1568
8. Li, D. Y., Geng, Z. R., Zhu, H. F., Wang, C., Miao, D. N., and Chen, P. Y. (2011) Immunomodulatory activities of a new pentapeptide (Bursopen) from the chicken bursa of Fabricius. Amino Acids 40, 505–515
9. Feng, X., Liu, T., Wang, F., Cao, R., Zhou, B., Zhang, Y., Mao, X., Chen, P., and Zhang, H. (2011) Isolation, antiproliferation on tumor cell and immunomodulatory activity of BSP-I, a novel bursal peptidase from chicken humoral immune system. Peptides 32, 1103–1109
10. Feng, X., Liu, Q. T., Cao, R. B., Zhou, B., Wang, F. Q., Deng, W. L., Qiu, Y. F., Zhang, Y., Ishag, H., Ma, Z. Y., Zheng, Q. S., and Chen, P. Y. (2011) A bursal pentapeptide (BPP-I), a novel bursal-derived peptide, exhibits antiproliferation of tumor cell and immunomodulator activity. Amino Acids doi:10.1007/s00726-011-0961-8
11. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63
12. Du, L., Lyle, C. S., Obey, T. B., Gaarde, W. A., Miu, J. A., Bennett, B. L., and Chambers, T. C. (2004) Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: evidence that mitotic Bcl-2 phosphorylation is JNK-independent. J. Biol. Chem. 279, 11957–11966
13. Chernov, A. V., Zolotovskaya, S., Golubkov, V. S., Wakeman, D. R., Snyder, E. Y., Williams, R., and Strongin, A. Y. (2010) Microarray-based transcriptional and epigenetic profiling of matrix metalloproteinases, collagens, and related genes in cancer. J. Biol. Chem. 285, 19647–19659
14. Walton, M. L., Wilson, S. C., Hardcastle, I. R., Mirza, A. R., and Workman, P. (2005) Evaluation of the ability of pifithrin-α and -β to inhibit p53 function in two wild-type p53 human tumor cell lines. Mol. Cancer Ther. 4, 1369–1377
15. He, L., He, X., Lim, L. P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A. L., Linsley, P. S., Chen, C., Lowe, S. W., Cleary, M. A., and Hannon, G. J. (2007) A microRNA component of the p53 tumor suppressor network. Nature 447, 1130–1134
16. Qiu, Y., Shen, Y., Li, X., Liu, Q., and Ma, Z. (2008) Polyclonal antibody to porcine p53: a new tool for studying the p53 pathway in a porcine model. Biochem. Biophys. Res. Commun. 377, 151–157
17. Yamaguchi, H., Chen, J., Bhalla, K., and Wang, H. G. (2004) Regulation of Bax activation and apoptotic response to microtubule-damaging agents by p53 transcription-dependent and -independent pathways. J. Biol. Chem. 279, 39431–39437
18. Pal, S., St Leger, R. J., and Wu, L. P. (2007) Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in Droso phila melanogaster. J. Biol. Chem. 282, 8969–8977
19. Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, A. M., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., and Sherlock, G. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25, 25–29
20. Gorr, S. U., Sotsky, J. B., Shelar, A. P., and Demuth, D. R. (2008) Design of bacteria-agglutinating peptides derived from parotid secretory protein, a member of the bacterial/permeability increasing-like protein family. Peptides 29, 2118–2127
21. Hegde, A. N. (2004) Ubiquitin-proteasome-mediated local protein degradation and synaptic plasticity. Prog. Neurobiol. 73, 311–357
22. Thanos, C. D., and Bowie, J. U. (1999) p53 Family members p63 and p73 are SAM domain-containing proteins. Proteins Sci. 8, 1708–1710
23. Del-Val, M., and López, D. (2002) Multiple proteases process viral anti gens for presentation by MHC class I molecules to CD8⁺ T lymphocytes. Mol. Immunol. 39, 235–247
24. Xu, W., Wang, P., Petri, B., Zhang, Y., Tang, W., Sun, L., Kress, H., Mann, T., Shi, Y., Kubes, P., and Wu, D. (2010) Integrin-induced PIP5K1C kinase polarization regulates neutrophil polarization, directionality, and in vivo infiltration. Immunity 33, 340–350
25. Deng, L., Sugira, R., Ohta, K., Tada, K., Suzuki, M., Hirata, M., Nakamura, S., Shuntoh, H., and Kuno, T. (2005) Phosphatidylinositol-4-phosphate 5-kinase regulates fission yeast cell integrity through a phospholipase C-signaling concept. J. Biol. Chem. 280, 27561–27568
26. Lecompte, O., Poch, O., and Laporte, J. (2008) PtdIns5P regulation through evolution: roles in membrane trafficking? Trends Biochem. Sci. 33, 453–460
27. Lawrence, M. C., Naziruddin, B., Levy, M. F., Jackson, A., and McGlynn, K. (2011) Calcineurin/nuclear factor of activated T cells and MAPK signaling induce TNF-α gene expression in pancreatic islet endocrine cells. J. Biol. Chem. 286, 1025–1036
28. Miokoshba, K. (2007) IP3 receptor/Ca²⁺ channel: from discovery to new signaling concepts. J. Neurochem. 102, 1426–1446
29. Arakawa, H., and Buerstedde, J. M. (2004) Immunoglobulin gene conversion: insights from bursal B cells and the DT40 cell line. Dev. Comp. Immunol. 28, 27561–27568
30. Lecompte, O., Poch, O., and Laporte, J. (2008) PtdIns5P regulation through evolution: roles in membrane trafficking? Trends Biochem. Sci. 33, 453–460
31. Lecompte, O., Poch, O., and Laporte, J. (2008) PtdIns5P regulation through evolution: roles in membrane trafficking? Trends Biochem. Sci. 33, 453–460
IgG-mediated in vivo responses. *Immunity* **8**, 517–529

32. Beurel, E., and Jope, R. S. (2008) Differential regulation of STAT family members by glycosyn thase kinase-3. *J. Biol. Chem.* **283**, 21934–21944

33. Xu, W., Nair, J. S., Malhotra, A., and Zhang, J. I. (2005) B cell antigen receptor signaling enhances IFN-γ-induced STAT1 target gene expression through calcium mobilization and activation of multiple serine kinase pathways. *J. Interferon Cytokine Res.* **25**, 113–124

34. Chappey, O., Debray, M., and Niel, E. (1994) Association constants of monoclonal antibodies for hapten: heterogeneity of frequency distribution and possible relationship with hapten molecular weight. *J. Immunol. Methods* **172**, 219–225

35. Niño, H., and Clark, E. A. (2002) Regulation of B cell fate by antigen-ubiquitination. *Annu. Rev. Immunol.* **20**, 945–956

36. Lanzavecchia, A. (1990) Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* **8**, 773–793

37. Lamprecht, B., Walter, K., Kreher, S., Kumar, R., Hummel, M., Lenze, D., Köcher, K., Bouhlel, M. A., Richter, J., Soler, E., Stadhouders, R., Jöhrens, K., Wurster, K. D., Callen, D. F., Harte, M. F., Giefing, M., Barlow, R., Stein, H., Anagnostopoulos, I., Janz, M., Cockerill, P. N., Siebert, R., Dörken, B., Böni, C., and Mathas, S. (2010) Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. *Nat. Med.* **16**, 571–579

38. Nagata, S., and Golstein, P. (1990) The Fas death factor. *Science* **267**, 1449–1456

39. Igney, F. H., and Krammer, P. H. (2002) Death and anti-death: tumor resistance to apoptosis. *Nat. Rev. Cancer* **2**, 277–288

40. Vikstrom, I., Debray, M., and Niel, E. (1994) Association constants of monoclonal antibodies for hapten: heterogeneity of frequency distribution and possible relationship with hapten molecular weight. *J. Immunol. Methods* **172**, 219–225

41. Saville, M. K., Sparks, A., Xirodimas, D. P., Wardrop, J., Stevenson, L. F., Bourdon, J. C., Woods, Y. L., and Lane, D. P. (2004) Regulation of p53 by the ubiquitin-conjugating enzymes UbcH5B/C in vivo. *J. Biol. Chem.* **279**, 42169–42181

42. Lee, J. T., and Gu, W. (2010) The multiple levels of regulation by p53 ubiquitination. *Cell Death Differ.* **17**, 86–92

43. Brooks, C. L., and Gu, W. (2006) p53 ubiquitination: Mdm2 and beyond. *Mol. Cell* **21**, 307–315

44. Du, J., Daniels, D. H., Ashbury, C., Venkataraman, S., Liu, J., Spitz, D. R., Oberley, L. W., and Cullen, J. J. (2006) Mitochondrial production of reactive oxygen species mediates dicumarol-induced cytotoxicity in cancer cells. *J. Biol. Chem.* **281**, 37416–37426

45. Moody, T. W., Mantey, S. A., Pradhan, T. K., Schumann, M., Nakagawa, T., Martinez, A., Fuselier, J., Coy, D. H., and Jensen, R. T. (2004) Development of high affinity camptothecin-bombesin conjugates that have targeted cytotoxicity for bombesin receptor-containing tumor cells. *J. Biol. Chem.* **279**, 23580–23589

46. Schmid, I., Krall, W. J., Uittenbogaart, C. H., Braun, J., and Giorgi, J. V. (1992) Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry* **13**, 204–208

47. Ranger, A. M., Malynn, B. A., and Korsmeyer, S. J. (2001) Mouse models of cell death. *Nat. Genet.* **28**, 113–118

48. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609–619

49. Wurster, K. D., Callen, D. F., Harte, M. F., Giefing, M., Barlow, R., Stein, H., Anagnostopoulos, I., Janz, M., Cockerill, P. N., Siebert, R., Dörken, B., Böni, C., and Mathas, S. (2010) Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. *Nat. Med.* **16**, 571–579

50. Rosenberg, S. A., Yang, J. C., and Restifo, N. P. (2004) Cancer immunotherapy: moving beyond current vaccines. *Nat. Med.* **10**, 909–915

51. Adam, J. K., Odhav, B., and Bhoola, K. D. (2003) Immune responses in cancer. *Pharmacol. Ther.* **99**, 113–132

52. Mandapathil, M., Hilldorfer, B., Szczepanski, M. J., Czystowska, M., Szajnik, M., Ren, J., Lang, S., Jackson, E. K., Gorelik, E., and Whiteside, T. L. (2010) Generation and accumulation of immunosuppressive adenosine by human CD4+CD25+FOXP3+ regulatory T cells. *J. Biol. Chem.* **285**, 7176–7186

53. Riese, M. J., Grewal, J., Das, J., Zou, T., Patil, V., Chakraborty, A. K., and Koretzky, G. A. (2011) Decreased diacylglycerol metabolism enhances ERK activation and augments CD8+ T cell functional responses. *J. Biol. Chem.* **286**, 5254–5265

54. Grommé, M., and Neefjes, J. (2002) Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways. *Mol. Immunol.* **39**, 181–202