

Bee venom modulates murine Th1/Th2 lineage development

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Abstract

Administration of bee venom (BV) elicits anti-inflammatory, anti-nociceptive and anti-allergic effects in various animal models. This study was designed to evaluate the direct effects of BV on helper T cell activities and on Th1/Th2 lineage development using both in vitro and in vivo conditions. In the Th1 skewed condition, BV increased the expression of IFN- γ mRNA and enhanced the expression of T-bet on purified CD4⁺ T cells from splenocytes of BALB/c mice. On the other hand, BV treatment did not alter the expression of IL-4 or GATA-3 in a Th2 driven environment. To elucidate the effects of BV on Th1/Th2 lineage development under in vivo conditions, BV was given by intraperitoneal injection to BALB/c mice. It significantly increased the CD4⁺ T cell population and enhanced IFN- γ expression, while IL-4 transcripts were not altered upon in vivo activation using an anti-CD3 antibody injection. Taken together, these results imply that BV induces Th1 lineage development from CD4⁺ T cells by increasing the expression of a Th1-specific cytokine, IFN- γ . In addition, this result may be mediated by inducing a Th1-specific transcription factor, T-bet.

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1. Introduction

Bee venom consists of several biologically active agents such as phospholipase A₂, apamin,

adolapin, melittin and mast cell degranulating peptide [1]. Melittin and phospholipase A₂ are the two major ingredients thought to be responsible for inducing irritation and the well known allergic reaction associated with bee stings. Several studies demonstrated that BV administration in the cat or rat animal model produced prolonged and tonic nociceptive responses [2–7]. Additionally, this analgesic effect seems to be significantly enhanced by BV injection into specific anatomical

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locations, so called acupoints [8]. A recent report about BV's nociceptive effects showed that BV treatment also elicits a significant anti-inflammatory effect on cases of Freund's complete adjuvant-induced arthritis in rats [9]. Interestingly, BV has been widely used for the treatment of allergic disorders, including asthma and allergic rhinitis. Known as immunotherapy (IT), this procedure involves the subcutaneous injection of an allergen to which the patient is sensitized. Successful IT has been associated with decreased Th2 cytokine secretion and increased levels of Th1 cytokines in allergen-specific peripheral T cells [10,11]. However, the precise immunological responses and mechanisms of the helper T cell lineage commitment phase (caused by BV) are largely unknown.

Naive CD4⁺ T cells are divided into two subpopulations, termed Th1 and Th2, according to differences in their cytokine expression profiles [12]. The functional differences distinguishing the Th subsets are due to the activities of their secreted cytokines. IFN- γ , secreted from Th1 cells, is known to induce differentiation of naïve CD4⁺ T cells into Th1 cells and to inhibit the proliferation of Th2 cells [13]. On the other hand, IL-4 and IL-10, secreted from Th2 cells, are known to induce the differentiation of naive CD4⁺ T cells into Th2 cells and to also inhibit the function of Th1 cells [14,15]. These distinct subsets of helper T cells are responsible for specific immune functions; Th1 cells contribute to cell-mediated inflammatory immunity, while Th2 cells are responsible for humoral responses [16,17]. The Th1/Th2 classification has been useful in relating the overall patterns of cytokine production to clinical outcomes in a variety of pathological states [18]. It has been demonstrated that the Th1/Th2 cell response shifts to a predominantly Th1 cell response in autoimmune diseases, causing IFN- γ production from Th1 cells to increase in those diseases [19]. In order to better understand these pathogenic advances in Th1/Th2 deviation, the development of specific agents affecting helper T cell differentiation has drawn special attention in the last decade.

The present study investigated the possible immune modulatory effects of BV on Th1/Th2 lineage development both in vivo and in vitro.

2. Materials and methods

2.1. Mice

Eight-week-old female BALB/c mice were purchased from Taconic, Korea and maintained in temperature and humidity controlled, pathogen-free conditions at Kyunghee University.

2.2. Antibodies

The antibodies used were anti-CD3 ϵ mAb (145-2C11), anti-CD28 mAb (37.51), anti-IL-4, anti-IL12, FITC-conjugated anti-CD4⁺, recombinant IL-2 (rIL-2), rIL-12, rIL-4 (BD Bioscience, San Diego, CA, U.S.A.) and magnetic cell sorting CD4⁺ (L3T4) microbeads (Mitenyi Biotec, Bergisch, Germany).

2.3. Splenocytes preparation and CD4⁺ T cell isolation

Splenocytes were prepared by disrupting the spleen with a syringe in complete medium (RPMI 1640 with 10% fetal bovine serum, 1% penicillin–streptomycin and 10 mM Hepes). After a 10 min centrifugation at 300 $\times$ g to separate debris, the cells were washed in RPMI medium, followed by lysis of erythrocytes using ammonium chloride reagent (BD Bioscience, San Diego, CA, U.S.A.). The cells were then counted and viability was determined by trypan blue exclusion. Subsequently, CD4⁺ T cells were isolated from splenocytes by positive selection using MACS CD4⁺ (L3T4) microbeads (Mitenyi Biotec, Bergisch, Germany), as directed by the manufacturer. Briefly, spleen cells were resuspended in 90 μ l of medium per 10⁷ total cells. 10 μ l of MACS CD4⁺ microbeads were added and incubated for 15 min at 4 °C. After adding a sufficient amount of medium, cells were centrifuged for 10 min at 300 $\times$ g and the supernatant was removed. The cell pellet was resuspended in 500 μ l of medium. A positive selection column was chosen and placed in the magnetic field of the MACS separator (Mitenyi Biotec, Bergisch, Germany). After applying the cell suspension onto the column, it was removed from the separator and placed on a collection tube. The positively fractionated cells were flushed out of the column using the plunger supplied.

2.4. Survival and proliferation assay

To determine the survival and proliferation capacity of the splenocytes and CD4⁺ T cells, the Cell Titer 96™ non-radioactive cell proliferation assay (Promega, Madison, WI, U.S.A.) was utilized according to the directions provided by the manufacturer. Cells were plated at a density of 4×10^5 cells/well in a total volume of 100 μ l. For activation of CD4⁺ T cells, cells were placed onto anti-CD3 coated plates, followed by the addition of anti-CD28 Ab and cultured in medium containing BV (0, 0.01, 0.1, 1 or 10 μ g/ml). The cells were then incubated for 48 h at 37 °C in 5% CO₂. For the T cell and B cell proliferation assay using BV injected mice, BV resolved in PBS was intraperitoneally administered to BALB/c mice at a dose of 1 mg/kg for 3 consecutive days. Control mice were treated with the same amount of PBS. For T cell activation, splenocytes (5×10^5) were incubated in wells previously coated with anti-CD3 ϵ antibody (1 μ g/ml). Lipopolysaccharide (LPS) was added at a concentration of 20 μ g/ml for B cell activation and assayed for proliferation as described above.

2.5. In vitro Th1 and Th2 cell polarization

CD4⁺ T cells (3×10^6 cells/ml) were resuspended in a complete medium (RPMI 1640 with 10% fetal bovine serum, 1% penicillin–streptomycin and 10 mM Hepes), then activated with plate-bound 10 μ g/ml anti-CD3 (BD Bioscience, San Diego, CA, U.S.A.) and 2 μ g/ml anti-CD28 (BD Bioscience, CA, U.S.A.) for 24 h in the presence or absence of BV (10 ng/ml). Naïve CD4⁺ T cells were incubated with 5 ng/ml rIL-12 (BD Bioscience, San Diego, CA, U.S.A.), 10 μ g/ml anti-IL-4 (BD Bioscience, CA, U.S.A.) and 50 U/ml rIL-2 for Th1 differentiation. For Th2 cell differentiation, 10 ng/ml rIL-4 (BD Bioscience, San Diego, CA, U.S.A.) and 10 μ g/ml anti-IL-12 (BD Bioscience, San Diego, CA, U.S.A.) were added to the culture medium in the presence of 50 U/ml of rIL-2. Under both culture conditions, the volume of the medium containing the corresponding antibodies and cytokines was doubled on day 3, then incubated for another 4 days. On day 7, cells were harvested, washed and restimulated (at 10^6 cells/ml) with plate-bound 10 μ g/ml anti-CD3 (BD Bioscience, San Diego, CA, U.S.A.)

and 2 μ g/ml anti-CD28 (BD Bioscience, San Diego, CA, U.S.A.) for 24 h.

2.6. Isolation of RNA, cDNA synthesis and real-time PCR analysis

CD4⁺ T cells and Th1/Th2 cells were lysed in 500 μ l of Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and the total RNA was isolated according to the manufacturer's protocol. Integrity of the RNA was confirmed using denaturing agarose gel electrophoresis (data not shown) and the concentration of total RNA was quantified by determining the optical density at 260 nm (OD260). The total RNA was used, and reverse transcription was performed by mixing 2 μ g of RNA, 7.5 μ l DEPC-DW with 1 U Dnase I and 10 $\times$ Dnase I reaction buffer. The solution was incubated for 15 min at room temperature. One microliter of 25 mM EDTA was added to the tubes and heated for 10 min at 70 °C. To this, 0.5 μ g oligo (dT) primer was added, then incubated at 70 °C for 5 min. A solution containing 5 μ l of M-MLV 5 $\times$ reaction buffer, 1 μ l of 10 mM dNTPS, 20 U of RNase inhibitor and 5.5 μ l of DEPC-DW was added, and the tubes were incubated for 2 min at 25 °C. The tubes then received 200 U of M-MLV reverse transcriptase and were incubated for 50 min at 42 °C. Finally, the tubes were heated for 15 min at 70 °C. The samples were stored at –20 °C until further use.

Real-time quantitative PCR was performed using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.), employing SYBR Green I as the dsDNA-specific binding dye for continuous fluorescence monitoring. Amplification was carried out in a total volume of 25 μ l containing 2.5 μ M of each cytokine-specific primer (Table 1), 2 $\times$ PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and 2 μ l of 1:4 diluted cDNA. The PCR reactions were cycled 40 times through denaturation (95 °C, 15 s) and annealing (60 °C, 1 min). Data was analyzed using GeneAmp 5700 SDS software (Applied Biosystems, Foster City, CA, U.S.A.).

2.7. In vivo T cell activation

BV resolved in PBS was intraperitoneally administered in doses of 1 mg/kg for 3 consecutive days.

Table 1
Sequences of primer used for real-time RT-PCR

Gene	Oligonucleotide sequence
GAPDH	RV: 5'-GGC ATG GAC TGT GGT CAT GA-3' FW: 5'-TTC ACC ACC ATG GAG AAG GC-3'
IL-4	FW: 5'-ACA GGA GAA GGG ACG CCA T-3' RV: 5'-GAA GCC CTA CAG ACG AGC TCA-3'
IFN- γ	FW: 5'-TCA AGT GGC ATA GAT GTG GAA-3' RV: 5'-TGG CTC TGC AGG ATT TTC ATG-3'
T-bet	FW: 5'-GCC AGG GAA CCG CTT ATA TG-3' RV: 5'-GAC GAT CAT CTG GGT CAC ATT-3'
GATA-3	FW: 5'-GAA GGC ATC CAG ACC CGA AAC-3' RV: 5'-ACC CAT GGC GGT GAC CAT GC-3'

Control mice were treated with the same amount of PBS. On day 4, mice were injected intravenously with a single dose of anti-CD3 ϵ mAb (4 μ g in 0.1 ml of PBS). Mice were sacrificed 1 h after this injection. The spleen was removed immediately and stored at -80°C for cytokine analysis by real-time PCR.

2.8. Flow cytometric analysis

Splenocytes from control and BV injected BALB/c mice (1×10^6 cells) were incubated at 4°C with fluorescein or phycoerythrin-conjugated antibodies (BD Bioscience, San Diego, CA, U.S.A.) and analyzed using a FACScan (Beckton Dickinson) flow cytometer. Staining and analysis were performed on at least three animals of each group, and had similar results.

2.9. Statistical analysis

Statistical analyses of the data were performed by independent sample *t*-tests using SPSS 11.0.1 for Windows (SPSS Inc., Chicago, IL, USA). All experiments were repeated at least three times and comparable results were found each time. Values are depicted as mean $\pm$ S.E.M.

3. Results

3.1. BV direct influences on CD4 $^{+}$ T cell activities

To test whether CD4 $^{+}$ T cells alone can respond to BV, CD4 $^{+}$ T cells were isolated using a magnetic anti-

CD4 $^{+}$ antibody from mice splenocytes. Sorted CD4 $^{+}$ T cells were plated onto anti-CD3 immobilized wells followed by the addition of anti-CD28 Ab, and then cultured in the presence or absence of BV. BV treatment did not significantly alter the proliferation rate of CD4 $^{+}$ T cells up to a concentration of 1 μ g/ml (Fig. 1).

Subsequently, in order to investigate the direct effects of BV on Th1/Th2-specific cytokine gene expressions, we performed real-time RT-PCR using RNAs from activated CD4 $^{+}$ T cells. In the Th1 skewed condition, the transcription of IFN- γ was increased significantly, while IL-4 expression was not dramatically altered under Th2 polarized condition (Fig. 2). Similarly, the major transcription factor responsible for inducing Th1 lineage programming, T-bet, was strongly induced upon BV treatment. This suggests that BV may modulate Th1 development by mediating T-bet (Fig. 2). Conversely, the expression of GATA-3, a transcription factor controlling Th2 lineage commitment, was not influenced by BV in the Th2 skewed condition.

Together, these results strongly suggest that BV selectively induces Th1 cell lineage development via an up-regulation of T-bet. In addition, this effect was mediated by directly influencing CD4 $^{+}$ T cells, without any antigen presenting cells.

To investigate the possible active compound conferring helper T cell lineage development

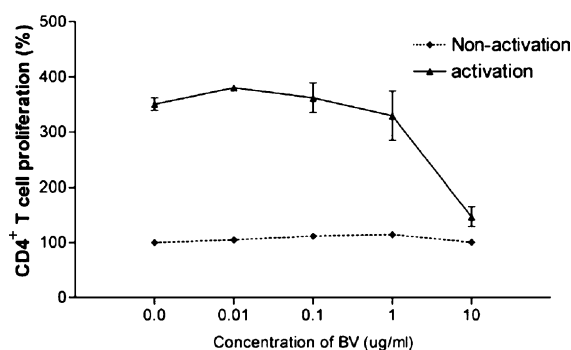


Fig. 1. The effect of BV on sorted CD4 $^{+}$ T cell proliferation. CD4 $^{+}$ T cells were isolated from BALB/c mice splenocytes. Anti-CD3/anti-CD28 stimulated CD4 $^{+}$ T cells and unstimulated CD4 $^{+}$ T cells were cultured for 48 h in a medium containing various concentrations of BV (0, 0.01, 0.1, 1 and 10 μ g/ml). Cell survival was quantified by their ability to reduce the tetrazolium dye MTS. Values are depicted as mean $\pm$ S.E.M.

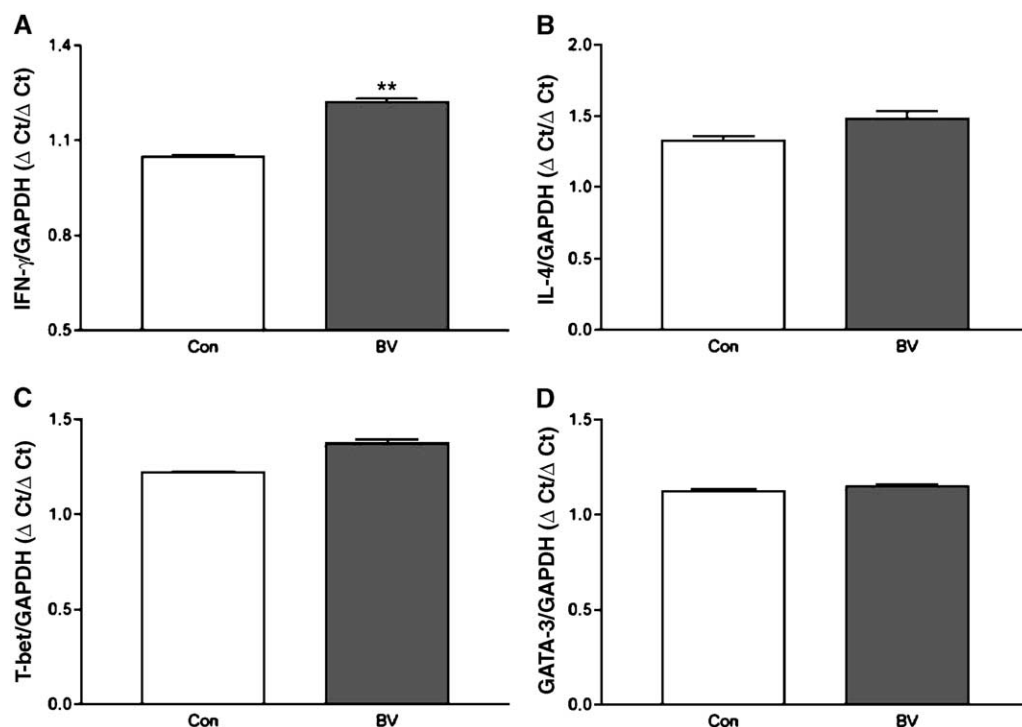


Fig. 2. The effect of BV on IFN- γ , IL-4, T-bet and GATA-3 mRNA expression in Th1/Th2 skewed conditions by real-time PCR analysis. Naïve CD4⁺ T cells were incubated with rIL-12 and anti-IL-4 Ab for Th1 differentiation (A and C). For Th2 cell differentiation, rIL-4 and anti-IL-12 Ab were added to the culture medium in the presence of rIL-2 (B and D). The cells were then incubated in the absence or presence of 10 ng/ml BV. In both culture conditions, double the original medium volume containing the corresponding antibodies and cytokines in the absence or presence of BV was added on day 3, then incubated for another 4 days. On day 7, cells were harvested, washed and re-stimulated with plate-bound anti-CD3 and anti-CD28 for 24 h. Total RNA was isolated and real-time PCR was performed as described in Materials and methods. The amount of gene expression was depicted as delta-CT value and the data normalization was performed by CT of GAPDH. (A) The amount of IFN- γ expression was determined in the Th1 skewed condition. (B) The amount of IL-4 expression in the Th2 skewed condition. (C) The amount of T-bet expression was determined in the Th1 skewed condition. (D) The amount of GATA-3 expression in the Th2 skewed condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ analyzed by the independent sample two-tailed *t*-test.

contained in BV, the effects of PLA₂ on cytokine production were examined in Th1 or Th2 polarized conditions. The results demonstrated that PLA₂ decreased the production of both IFN- γ and IL-4 in Th1/Th2 skewed conditions, respectively (Fig. 3). These data suggest that PLA₂ is not the major active compound in conferring selective Th1/Th2 immune modulatory effects contained in BV.

3.2. Effect of BV on cytokine secretion in anti-CD3 antibody injected mouse

Another set of experiments investigated the effect of BV on in vivo Th1/Th2 lineage development.

When anti-CD3 antibody is administered in vivo, T cell dependent cytokine production is rapidly induced without a predominance of either Th1 or Th2 type cells [20]. BV was injected intraperitoneally for 3 consecutive days and, on day 4, anti-CD3 mAb was intravenously injected. Correlating with the in vitro results, BV treated mice showed significantly increased levels of IFN- γ production in the spleen, while IL-4 expression was not dramatically altered (Fig. 4). The B cell populations and the LPS-induced proliferation of B cells were not changed by BV treatment. The T cell response was also not dramatically changed, while the ratio of CD4⁺/CD8⁺ T cell population tended to increase (Fig. 5).

4. Discussion

The present study demonstrated that BV enhances the Th1 cell-dominated immune response by increasing the expression of IFN- γ mRNA, without altering the Th2 cell response in both in vitro and in vivo conditions.

The imbalance of Th1/Th2 type responses plays an important role in the development and perpetuation of a number of immune disorders such as allergies, systemic lupus erythematosus (SLE) and rheumatoid arthritis [19]. While this differentiation program can be influenced by antigen concentration or through

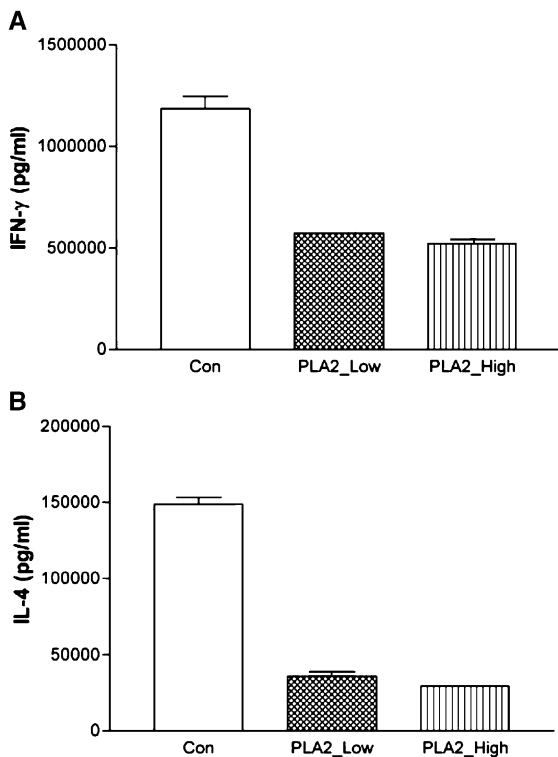


Fig. 3. The effects of PLA₂ on IFN- γ and IL-4 cytokine production from Th1/Th2 cells. Naïve CD4⁺ T cells were incubated with rIL-12 and anti-IL-4 Ab for Th1 differentiation (A). For Th2 cell differentiation, rIL-4 and anti-IL-12 Ab were added to the culture medium in the presence of rIL-2 (B). The cells were then incubated in the absence or presence of 0.1 or 1 μ g/ml of PLA₂ (PLA2_low and PLA2_high, respectively). CD4⁺ T cells were activated with plate-bound 10 μ g/ml anti-CD3 ϵ mAb (BD Bioscience, San Diego, CA, U.S.A.) and 2 μ g/ml anti-CD28 Ab (BD Bioscience, CA, U.S.A.). The amounts of IFN- γ (A) and IL-4 (B) in the culture medium were quantitated using ELISA.

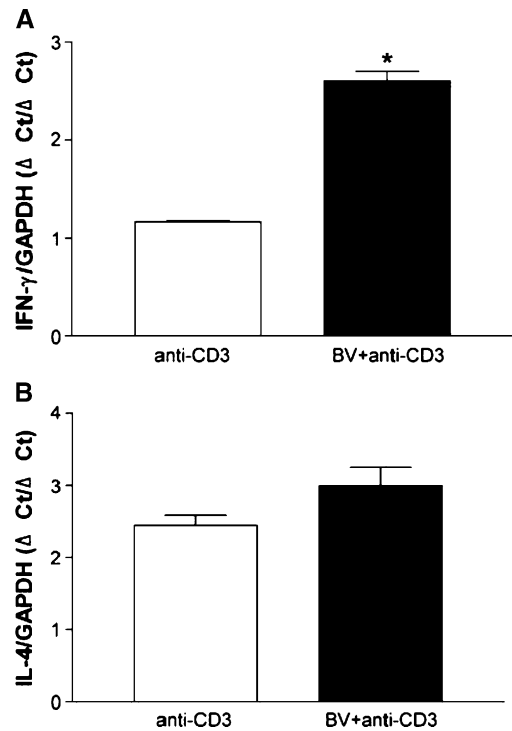


Fig. 4. The effect of BV on IFN- γ and IL-4 mRNA expression in BV injected BALB/c mice by real-time PCR analysis. BALB/c mice were intraperitoneally injected with BV resolved in PBS (closed bar) or PBS only (open bar) for 3 consecutive days. On day 4, mice were injected intravenously with a single dose of anti-CD3 ϵ mAb (4 μ g in 0.1 ml of PBS). Total RNA was isolated from the spleen. Real-time PCR was performed to determine IFN- γ (A) and IL-4 (B) expression. The amounts of gene expression were depicted as the delta-CT values and data normalization was performed by CT of GAPDH. * P <0.05, ** P <0.01, *** P <0.001 analyzed by the independent sample two-tailed t -test.

ligation of co-stimulatory molecules, the most potent regulators of Th cell differentiation are undoubtedly cytokines [18,21,22]. The presence of IL-4 promotes differentiation into Th2 cells and suppresses Th1 cells, whereas IFN- γ drives precursor Th cells to differentiate into Th1 cells and decreases Th2 cells [23,24].

In this study, we utilized a highly purified CD4⁺ T cell source to investigate the direct effects of BV on helper T cells. The results showed that BV increased the expression of IFN- γ and T-bet on purified CD4⁺ T cells in a Th1 condition, while the amount of IL-4 and GATA-3 mRNA were not altered in a Th2 driven environment. Also, BV did not behave as an antigenic stimulator (or toxin) on sorted CD4⁺ T cells (Figs. 1

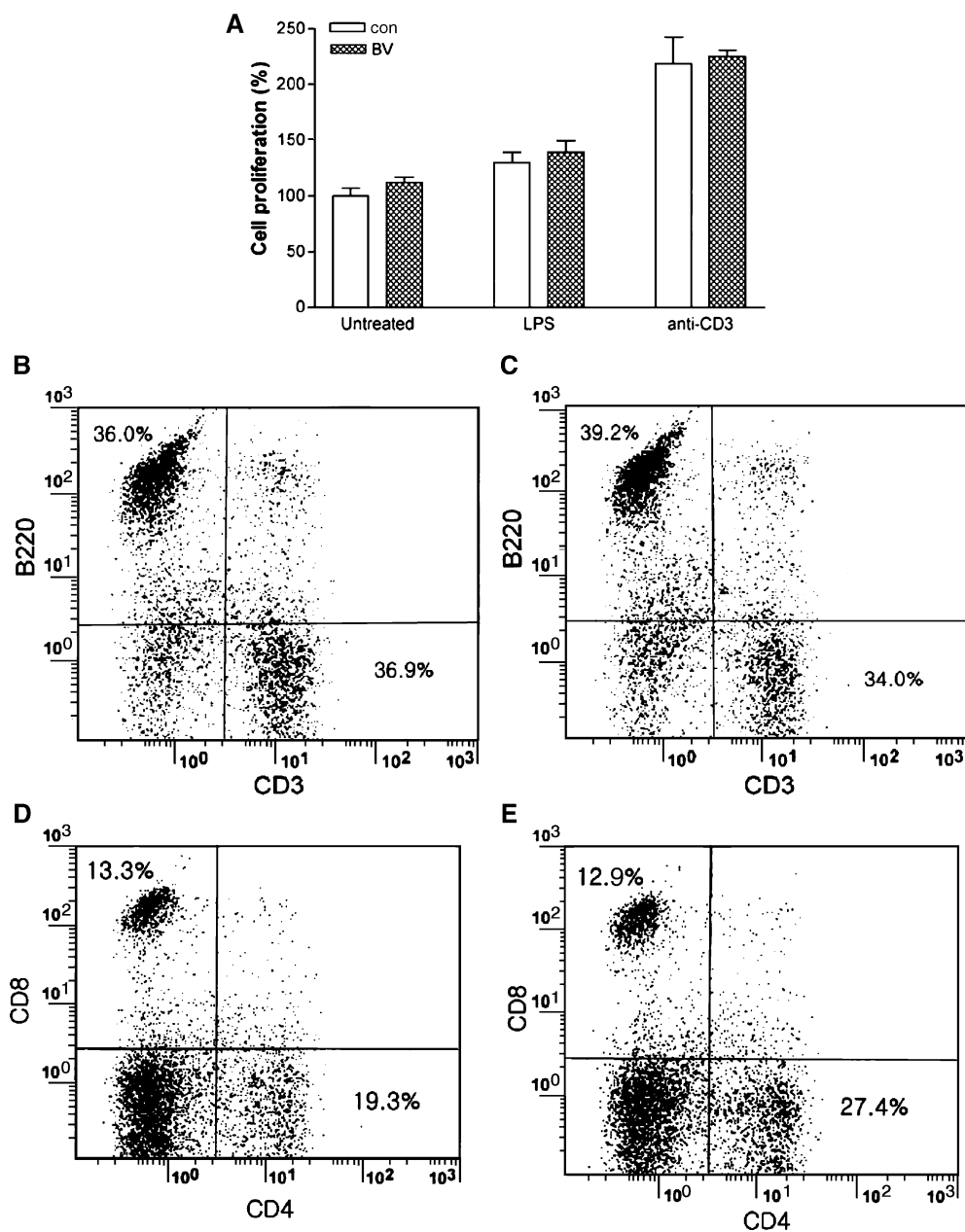


Fig. 5. Proliferation and flow cytometric analysis of splenocytes from BV injected BALB/c mice. (A) BALB/c mice were intraperitoneally injected with BV resolved in PBS (hatched bar) or PBS only (open bar) for 3 consecutive days. Splenocytes were isolated and incubated with plate-bound anti-CD3 antibody or lipopolysaccharide (LPS) as indicated. The proliferation assay was performed as described in Materials and methods. In flow cytometric analysis, splenocytes from BALB/c were stained with fluorescein- (abscissa) or phycoerythrin- (ordinate) conjugated antibodies to murine surface markers as indicated. (B, D) Mice were injected with BV for 3 days; (C, E) mice were injected with PBS only. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ analyzed by the independent sample two-tailed t -test.

and 2). These results imply that BV does affect the helper T cell responses without antigen presenting cells (APCs). Interestingly, the *in vivo* effect of BV on the T cells was significantly increased compared with the *in vitro* data, suggesting that APCs also play a role in BV-mediated T cell responses. Taken together, these results imply that BV modulates Th1/Th2 differentiation without distorting the general activities of CD4⁺ T cells. However, it should be noted that the strain used in this study, BALB/c, tends to develop overwhelming Th2 responses, so that we cannot guarantee that the observations made here will occur in the general genetic background. Future studies should be performed to support the inferences drawn from this report by using other strains that have a tendency to develop strong Th1 responses, such as C57BL/6.

Currently, several transcription factors have been identified that control the differentiation of Th1/Th2 cells; GATA-3 and c-Maf are involved in the Th2 transition, while T-bet is unregulated in Th1 cells. Ectopic expression of T-bet enhances endogenous IFN- γ production and induces polarized Th2 cells to redirect into Th1 cells [13]. Thus, these results strongly suggest that T-bet initiates Th1 lineage development from naïve Th cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs. Moreover, T-bet knock-out mice manifest multiple physiological and inflammatory features characteristic of asthma, suggesting that T-bet deficiency itself induces allergic asthma in the absence of allergen [25]. In this study, BV strongly induces the expression of T-bet while GATA3 expression remains unchanged. These results imply that the anti-allergic effect of BV may arise from the direct regulation of helper T cell lineage programming via T-bet regulation.

The role of other transcription factors associated with helper T cell development such as c-Maf, Stat6 and Stat4 in the presence of BV should be investigated to elucidate the detailed mechanisms of BV's action on Th1/Th2 lineage development. These projects are currently underway. Since the present study focused only on the initial stage of helper T cell development, it should be determined whether BV can divert Th2 committed cells into Th1 cells and address the therapeutic effects of BV in a pathological condition.

In conclusion, this study demonstrates that BV directly influences CD4⁺ T cell immune activity without APCs and that BV improves Th cell responses, from a Th2-dominant to a Th1-dominant pattern, by increasing expression of INF- γ via an up-regulation of T-bet expression.

Acknowledgements

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References

- [1] Lariviere WR, Melzack R. The bee venom test: a new tonic-pain test. *Pain* 1996;66:271–7.
- [2] Chen J, Luo C, Li HL. The contribution of spinal neuronal changes to development of prolonged, tonic nociceptive responses of the cat induced by subcutaneous bee venom injection. *Eur J Pain* 1998;2:359–76.
- [3] Chen J, Luo C, Li H, Chen H. Primary hyperalgesia to mechanical and heat stimuli following subcutaneous bee venom injection into the plantar surface of hindpaw in the conscious rat: a comparative study with the formalin test. *Pain* 1999;83:67–76.
- [4] Chen J, Chen HS. Pivotal role of capsaicin-sensitive primary afferents in development of both heat and mechanical hyperalgesia induced by intraplantar bee venom injection. *Pain* 2001;91:367–76.
- [5] Chen HS, Chen J. Secondary heat, but not mechanical, hyperalgesia induced by subcutaneous injection of bee venom in the conscious rat: effect of systemic MK-801, a non-competitive NMDA receptor antagonist. *Eur J Pain* 2000;4:389–401.
- [6] Chen HS, Chen J, Sun YY. Contralateral heat hyperalgesia induced by unilaterally intraplantar bee venom injection is produced by central changes: a behavioral study in the conscious rat. *Neurosci Lett* 2000;284:45–8.
- [7] Kwon YB, Kang MS, Kim HW, Ham TW, Yim YK, Jeong SH, et al. Antinociceptive effects of bee venom acupuncture (apipuncture) in rodent animal models: a comparative study of acupoint versus non-acupoint stimulation. *Acupunct Electrother Res* 2001;26:59–68.
- [8] Kwon YB, Lee JD, Lee HJ, Han HJ, Mar WC, Kang SK, et al. Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. *Pain* 2001;90:271–80.
- [9] Kwon YB, Kim JH, Yoon JH, Lee JD, Han HJ, Mar WC, et al. The analgesic efficacy of bee venom acupuncture for knee osteoarthritis: a comparative study with needle acupuncture. *Am J Chin Med* 2001;29:187–99.
- [10] Akdis CA, Akdis M, Blesken T, Wymann D, Alkan SS. Epitope-specific T cell tolerance to phospholipase A2 in bee

- venom immunotherapy and recovery by IL-2 and IL-15 in vitro. *J Clin Invest* 1996;98:1676–83.
- [11] Rolland J, O’Hehir R. Immunotherapy of allergy: anergy, deletion, and immune deviation. *Curr Opin Immunol* 1998;10: 640–5.
- [12] Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone: I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348–57.
- [13] Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000;100:655–69.
- [14] Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell: IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989;170: 2081–95.
- [15] Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. *J Immunol* 1990; 145:3796–806.
- [16] Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 2001;292:1907–10.
- [17] Das J, Chen CH, Yang L, Cohn L, Ray P, Ray A. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat Immunol* 2001; 2:45–50.
- [18] Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996;383:787–93.
- [19] Rao A, Avni O. Molecular aspects of T-cell differentiation. *Br Med Bull* 2000;56:969–84.
- [20] Scott DE, Gause WC, Finkelman FD, Steinberg AD. Anti-CD3 antibody induces rapid expression of cytokine genes in vivo. *J Immunol* 1990;145:2183–8.
- [21] Constant SL, Bottomly K. Induction of Th1 and Th2 CD4⁺ T cell responses: the alternative approaches. *Annu Rev Immunol* 1997;15:297–322.
- [22] O’Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998;8: 275–83.
- [23] Rincon M, Flavell RA. T-cell subsets: transcriptional control in the Th1/Th2 decision. *Curr Biol* 1997;7:R729–32.
- [24] Swain SL. T-cell subsets. Who does the polarizing? *Curr Biol* 1995;5:849–51.
- [25] Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, et al. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 2002;295:336–8.