Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

In vitro Studies to Determine the Effect of Boeravinone B on Human Dendritic Cells

Praveen Kumar Posa Krishnamoorthy, Sivanandham Muthukumaran

Department of Biotechnology, Sri Venkateswara College of Engineering, Sriperumbudur, Tamil Nadu, India

Submitted: 29-12-2017 Revised: 15-02-2018 Published: 14-08-2018

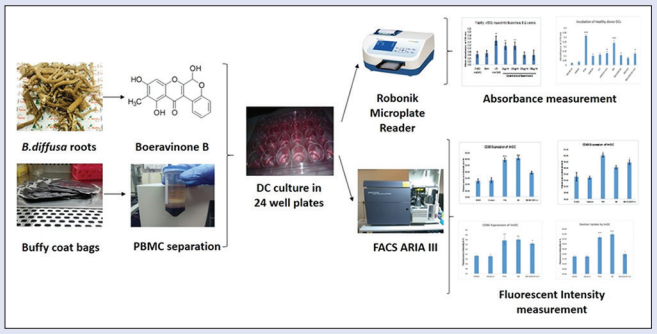
ABSTRACT

Background: Boeravinone B (BB), a therapeutic biomarker isolated from roots of *Boerhavia diffusa*, possesses immunomodulatory properties. **Objective:** In the present study, we investigated cytotoxicity and immunomodulatory effect of BB on human dendritic cells (DCs). **Materials and Methods:** In this study, buffy coat of human healthy volunteers was collected and the monocytes were separated. Effect of various concentrations of BB on DCs from induced differentiation of monocytes was studied on day 7 using ELISA microplate reader. The effect of BB on the immature DCs generated in the presence and absence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin4 (IL4) was also noted. The binding of BB with the DC maturation markers CD80, CD83, and CD86 including antigen uptake were evaluated using fluorescent-activated cell sorter (FACS). **Results:** Viability of DCs was not affected at BB concentration of 100 μg/ml. Phytohemagglutinin (PHA), BB showed increase in DC expression of CD80 expression up to 6.7% ($P < 0.001$) and 7.27% ($P < 0.001$) and increase in antigen uptake up to 7.67% ($P < 0.001$) and 8.87% ($P < 0.001$) when compared with dimethyl sulfoxide control. PHA and Boeravinone B supplemented with GM-CSF and IL4 showed increase of CD83 expression up to 6.86% ($P < 0.01$) and 4.63% ($P < 0.05$). However, PHA and BB showed increase of CD86 expression up to 6.6% ($P < 0.01$) and 6.73% ($P < 0.01$) when compared with control. **Conclusion:** The results justify that BB induced differentiation of monocytes into mature DCs.

Key words: Boeravinone B, dendritic cells, enzyme-linked immunosorbent assay, fluorescent activated cell sorter

SUMMARY

- The novel report in this research is exploring about the effect of Boeravinone B (BB) on maturation of human dendritic cells (DCs) and antigen uptake of human DCs
- Purified BB effectively matured DCs on day 7.



Abbreviations used: DCs: Dendritic cells, ELISA: Enzyme-linked immunosorbent assay, GMCSF: Granulocyte-macrophage colony-stimulating factor, IL4: Interleukin4, PHA: Phytohemagglutinin, CD: Cluster of differentiation, DMSO: Dimethyl sulfoxide, FACS: Fluorescent activated cell sorter.

Correspondence:

Mr. Praveen Kumar Posa Krishnamoorthy,
Department of Biotechnology,
Sri Venkateswara College of Engineering,
Sriperumbudur - 602 117, Tamil Nadu, India.
E-mail: praveenpk@svce.ac.in

DOI: 10.4103/pm.pm_625_17

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Dendritic cells (DCs) are highly specialized antigen presenting cells with the unique capacity to promote and regulate primary immune responses.[1] DCs have the ability to activate naive T-cells which in turn initiate the adaptive immune response but that depends on their maturation.[2] Mature DCs exhibit reduced phagocytic activity and increased expression of MHC and costimulatory molecules and secrete large amounts of immunostimulatory cytokines.[3]

Cytokines play a key role in modulation of DC-associated immune responses. Cell-to-cell communication (cellular "crosstalk") is maintained through cytokine networks.[4] In immunodeficiency or autoimmune diseases, these networks undergo imbalance. Autoimmune disorders are emerging noncommunicable diseases and there are more than 80 different types of autoimmune disorders affecting approximately 100 million people worldwide.[5] According to the World Health Organization, the antibiotics will not be effective beyond 2020 for communicable diseases because of the rapid emergence of multidrug-resistant strains.[6] Novel treatments include herbal medicines, essential oils, phage therapy, cowpathy, and cytokine therapy was being used for the treatment of communicable and noncommunicable diseases.[7] Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts.[8]

Boerhavia diffusa (*Nyctaginaceae* family) is an herbaceous perennial medicinal plant, native of India and Brazil.[9] The root of *B. diffusa* contains alkaloids (punarnavine), rotenoids (boeravinones A-J), aminoacids, flavonoids, eicosanoic, stearic, β-sitosterols, tetracosanoic, and ursolic acids.[10,11] In our earlier study, phytochemical characterization of aqueous

Cite this article as: Posa Krishnamoorthy PK, Muthukumaran S. *In vitro* studies to determine the effect of boeravinone B on human dendritic cells. Phcog Mag 2018;14:465-70.

and alcoholic extracts of *B. diffusa* roots are carried out and identified the presence of compounds such as flavonoids, saponin, protein, carbohydrate, phenols, alkaloids, glycosides, and isoflavonoids.[12] Furthermore, studies were performed in the laboratory to isolate, purify, and characterize Boeravinone B (BB), a rotenoid from roots of *B. diffusa*.[13]

BB, a bioactive marker compound from *B. diffusa*, has a therapeutic effect for treating rheumatoid arthritis, osteoarthritis, acute myoskeletal disorders, spondylosis, tendonitis, atherosclerosis, systemic lupus erythematosus, and psoriasis in mammals.[14] In another aspect of the invention, it is also used for treating pain from various organs in mammals.[14] The immunomodulation characteristics of BB are being examined widely to evade the undesirable effects on autoimmune diseases. Boeravinone E, another compound from *B. diffusa*, was having binding affinity with granulocyte–macrophage colony-stimulating factor receptor (GM-CSFR) and tumor necrosis factor alpha (TNF-α) receptor, and hence, it could be used as an adjuvant for rheumatoid arthritis cure by DC therapy.[15]

In vitro studies have also suggested the immunomodulatory potential in roots of *B. diffusa*. Ethanolic extract of root inhibited T-cell mitogen phytohemagglutinin and concanavalin A-stimulated proliferation of human peripheral blood mononuclear cells (PBMCs).[16] However, also *in vivo* immunostimulatory activity of *B. diffusa* alkaloidal fraction without any *in vitro* effect was observed.[17] Bd-I (eupalitin-3-O-beta-D-galactopyranoside) inhibited the production of interleukin (IL)-2 at the protein and mRNA transcript levels (phytohemagglutinin-stimulated) and TNF-α production (lipopolysaccharide induced) in human PBMCs. Bd-I was also shown to block the activation of DNA binding of nuclear factor-(kappa) B (NF-kB) and transcription factor AP-I (activating protein-1) as reported.[18]

The number of DCs and the modulation of the expression of CD80 and CD86 costimulatory molecule are among several factors that inhibit the immunological functions of DCs.[19] Blocking antibodies targeting surface costimulatory molecules CD80 or CD86 weakened intercellular interactions and dampen T-cell activation, highlighting the amplificatory roles of CD80/86 in regulating APC: T-cell interactions and T-cell functional activation. The variable strength of mechanical forces between DC: T-cells and B: T-cell interactions were not solely dependent on differential APC expression of CD80/86 since DCs were superior to B-cells in promoting strong interactions with T-cells even when CD80 and CD86 inhibited were reported.[20] Hence, the present study aims to investigate the effect of BB on the maturation of human DCs by checking the expression of CD80/CD83/CD86 markers including antigen uptake.

MATERIALS AND METHODS

Preparation of RPMI complete and granulocyte macrophage colony-stimulating factor medium

To the 100 ml, sterile RPMI-1640 medium with L-glutamine and phenol red (Invitrogen, USA), 10 ml of sterile heat-inactivated fetal bovine serum (FBS) (Invitrogen, USA) and 1 ml of penicillin/streptomycin antibiotic solution (10×) (Himedia, India) were added and mixed well.[21] The medium was then filtered through the 0.22-μm filter membrane (Pal Life sciences, USA) connected to a vacuum suction pump. To prepare the complete media containing FBS, the FBS bottle stored at −20°C was brought to room temperature and then decomplemented by placing in 56°C water bath for 30 min. It was then transferred to the centrifuge tube and spun at 2000 rpm for 10 min to remove any sediment. 500 μg of GM-CSF (Emgrast-M, India) was reconstituted in 1 ml sterile distilled water. 50 μl of the reconstituted GM-CSF was added to 500 ml of RPMI media. 5 ml of the RPMI media containing GM-CSF was then diluted further in 500 ml of RPMI medium.

Separation of peripheral blood mononuclear cells from human blood

Buffy-coat blood sample bags of 12 healthy human volunteers were collected from VHS blood bank, Taramani. Blood was diluted with double the volume of phosphate-buffered saline (PBS) (Himedia, India) and taken into a sterile centrifuge tube using sterile pipettes and gently mixed to prevent hemolysis. Diluted blood was carefully layered on to 10 ml Ficoll-Hypaque (Himedia, India) taken in 50 ml centrifuge tube. The tubes were centrifuged in a cooling centrifuge (REMI, India) for 30 min at 1700 RPM. After centrifugation, buffy-coat containing PBMC's was aspirated and taken into a fresh tube and cell count was performed by Trypan blue assay using hemocytometer.[22] Buffy coat was diluted with PBS and centrifuged at 800 RPM for 10 min to remove platelets. This procedure was repeated twice.

Isolation of dendritic cells by plastic adhesion method

To the above pellet, RPMI media were added and placed in the CO_2 incubator at 37°C and 5% CO_2 supply for 2 h. After 2 h incubation, the supernatant with nonadherent floating cells (lymphocytes) were removed.[23] The adherent cells attached with plastic surface (mainly monocytes) were washed three times with PBS.

Incubation of dendritic cells with boeravinone B

After removing the nonadhering cells, 24-well Petri plates containing 1×10^7 cells were taken in 10 ml of RPMI-1640 DC complete medium complemented with 50 ng/ml of rHuman GM-CSF and 100 ng/ml of rHuman IL-4 (Invitrogen, USA). The 24-well plate was filled with approximately 24 ml of the media. Approximately, 10 mg of purified BB (92% HPLC pure) obtained from roots of *B. diffusa* in our laboratory was dissolved in 3 ml of dimethyl sulfoxide (DMSO) (Merck, India) and were further diluted in 100 ml of PBS. 100 μl of above prepared BB solution were then incubated with imDCs on day 4 in the CO_2 incubator at 37°C and 5% CO_2 supply for 3 days. Cell proliferation and cytotoxicity were measured through 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Viability of boeravinone B-treated dendritic cells

Viability of Human DCs was investigated through MTT assay and measured through absorbance at 570 nm by ELISA microplate reader. DCs were cultured in the presence of BB (0–500 μg/ml) on 3rd day for 4 days. Later, the phenotype of DCs was cultured in various culture conditions in the presence and absence of BB, GM-CSF, and IL-4 and absorbance at 570 nm were compared and measured on 7th day using ELISA microplate reader. Lipopolysaccharide (1 μg/ml)-treated DCs was used as positive control and DMSO-treated DCs was taken as negative control in entire experiments.

Cryopreservation

The remaining cells were placed in cold storage for future usage. This was done by adding 5 ml of RPMI and 10% FBS and 5 ml of FBS along with 2 ml of DMSO in tubes. The tubes were then vortexes well. The mixture was transferred to vials. They were placed at − 80°C for 1 day.

Immunostaining

Immunostaining of DCs was carried out by the following method for human healthy volunteer samples.[19] The following mAbs were used for fluorescent-activated cell sorter (FACS) analysis: Mouse Anti-Human CD80-PE (BD Biosciences, USA), Mouse Anti-Human CD83-FITC (BD Biosciences, USA), and Mouse Anti-Human CD86-FITC

(BD Biosciences, USA). Endocytic activity was measured by incubating cells for 1 h with Dextran-FITC (1 mg/ml) were purchased from Sigma-Aldrich (Steinheim, Germany) at 37°C. 10 µl of antibodies were added to 100 µl of RPMI-1640 medium containing 10^6 cells/ml imDC cultured in the presence of BB supplemented with GM-CSF and IL4 and in the presence of BB alone. Same protocol was maintained for phytohemagglutinin (PHA)-treated imDCs and DMSO-treated imDCs, which were used as positive and negative control for the experiment. Later, the imDC suspension was incubated at room temperature for 1 h in a place protected from light. After incubation, the cells were taken in tubes and was centrifuged at 1000 rpm for 5 min. The supernatant was decanted. The imDC were subsequently washed two times with PBS (Himedia, India) containing 0.2% BSA (Himedia, India) and 0.1% sodium azide (Himedia, India). The resulted cell pellet was resuspended in 500 µl of PBS with 0.1% p-formaldehyde and stored at 4°C, protected from light for a minimum of 1 h and maximum 24 h. Immunostaining was thus performed on 7th-day culture of immature DCs with cell surface maturation markers CD80-PE, CD83-FITC, and CD86-FITC including Dextran-FITC. Corresponding percentage fluorescence intensity was measured. Cells were analyzed on a FACSAria-III flow cytometer (BD Biosciences, Heidelberg, Germany). Data were analyzed using CellQuest (BD Biosciences) and FlowJo (Tree star, San Carlos, CA) software.

Statistical analysis

Statistical significance between the test and control was calculated conjunction with a one-way ANOVA by *post hoc* Tukey's honestly significant difference test. The results were expressed as mean ± standard deviation for triplicates carried out in three experiments. $P < 0.05$ were considered statistically significant.

RESULTS

Viability of Boeravinone B-treated dendritic cells

Initially, viability of peripheral blood mononuclear cells was checked through hemocytometer and was found to be 95.77%. BB shown [Figure 1] added at concentrations of 10–100 µg/ml did not affect DC viability, although viability was affected at higher concentrations [Figure 2]. However, BB at concentrations of 10 µg/ml and 100 µg/ml had significantly higher ($P < 0.01$) absorbance when compared with negative control (DMSO) on human DCs and so this concentration was used as safe dose of BB for the further experiments on DCs. Similar studies were performed with the effect of ginsan on mice DCs[24] and *Z. tenuior L.* extract on mice DCs.[25]

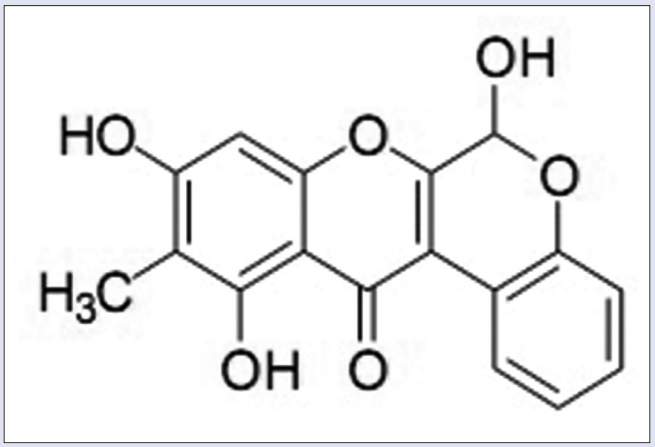


Figure 1: Chemical structure of Boeravinone B

DCs pulsed in PHA and BB showed highly significant ($P < 0.001$) absorbance when compared to negative control or DMSO-treated DCs [Figure 3]. However, DCs grown in GM-CSF supplemented with IL-4 and BB-treated DCs grown in GM-CSF supplemented with IL-4 showed slightly significant ($P < 0.05$) absorbance. Later, the surface expression of costimulatory molecules (CD80, CD83, and CD86) and dextran uptake are checked with BB-treated DCs grown in GM-CSF supplemented with IL-4 using FACS. Similar studies were performed with DCs cultured in either GM-CSF alone or in GM-CSF supplemented with IL-4.[26]

CD80/CD83/CD86 analysis

On day 7, the blood cells drawn from healthy volunteers buffy-coat bags had shown DC morphology and showed a higher expression of CD11c and a reduced expression of CD14, indicating that the monocytes were differentiated into DCs (data not shown). Figure 4 represents the %fluorescence intensity of CD80-PE as CD80 expression in Human healthy volunteers. It was observed a significant increase of 7.27% in CD80 expression when imDC treated with BB when compared with imDC treated with DMSO ($P \leq 0.001$). PHA as a positive control displayed 6.7% increase in CD80 expression when compared with DMSO ($P \leq 0.001$). In addition, BB supplemented with GM-CSF and IL4 in RPMI1640 medium showed increase of CD80 expression up to 2.6%. Figure 5 represents the %fluorescence intensity of CD83-FITC as CD83 expression in imDC of healthy human volunteers. When compared with DMSO-treated control imDCs, PHA-stimulated imDCs showed increase of 6.86% in CD83 expression ($P \leq 0.01$). BB-treated imDC showed 3.03% increase of CD83 expression but not statistically significant when compared with DMSO-treated imDC. In addition, BB supplemented with GM-CSF and IL4-treated imDC displayed 4.63% increase in CD83 expression when compared with DMSO-negative control imDC ($P < 0.017$). Figure 6 represents the %fluorescence intensity of CD86-FITC as CD86 expression in healthy human volunteer's imDC. PHA and BB showed significant expression of 6.6% and 6.73% increase of CD86 expression in imDC when compared with DC cultured in medium ($P < 0.002$). These results indicate BB and PHA treatment upregulated the CD86 expression on imDC from healthy donors. However, BB supplemented with GM-CSF and IL4 showed significant increase of CD86 expression up to 5.1% ($P < 0.018$).

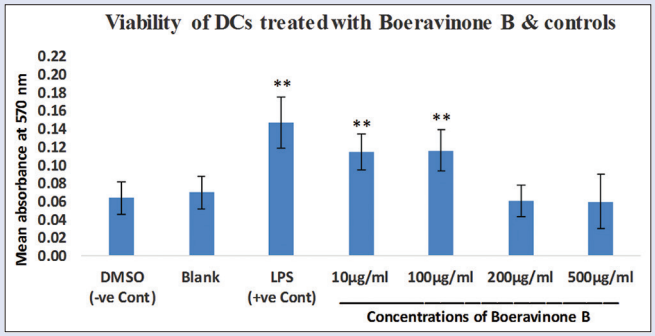


Figure 2: Viability of dendritic cells treated with different concentrations of Boeravinone B (10-500 µg/ml), dimethyl sulfoxide as negative control, and lipopolysaccharide at 1 µg/ml as positive control. The results show mean ± standard deviation of three experiments performed in triplicate. The maximum viability of dendritic cells incubated with Boeravinone B was not affected up to 100µg/ml. Significant differences between treatments and control are shown as $P < 0.01$ (**)

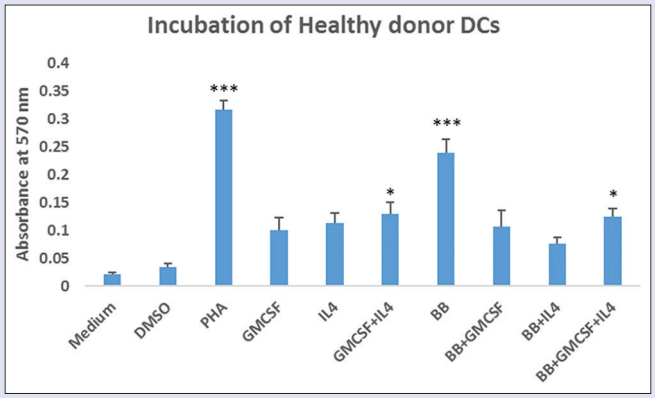


Figure 3: Viability of dendritic cells treated with Boeravinone B, granulocyte-macrophage colony-stimulating factor, interleukin 4, and controls. When compared with dimethyl sulfoxide-treated dendritic cell or control dendritic cell, phytohemagglutinin, and Boeravinone B-treated dendritic cells showed significant differences on maximum viability of dendritic cells and was shown as $P < 0.001$(***). Similarly, dendritic cells cultured in granulocyte-macrophage colony-stimulating factor and interleukin 4 and in Boeravinone B supplemented with granulocyte-macrophage colony-stimulating factor and interleukin 4 in RPMI medium showed significant differences on maximum viability of dendritic cells and was shown as $P < 0.05$(*). The results shown as mean ± standard deviation of three experiments performed in triplicate

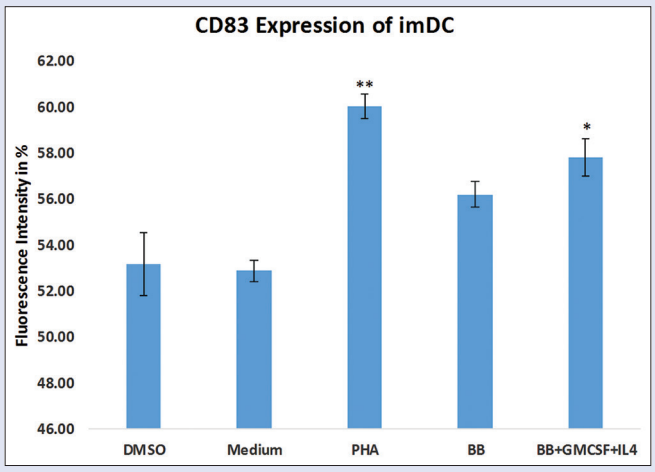


Figure 5: CD83 expression in Immature dendritic cell. When compared with control, phytohemagglutinin-treated imdendritic cell showed significant difference on increase of CD83 expression up to 6.86% and was shown as $P < 0.01$ (**). Similarly, Boeravinone B (BB) supplemented with granulocyte-macrophage colony-stimulating factor and interleukin 4 in RPMI medium showed significant difference on increase of CD83 expression up to 4.63% and was shown as $P < 0.05$ (*). However, Boeravinone B showed increase of CD83 expression up to 3.03%; Data represented are mean ± standard deviation; $n = 3$

Antigen uptake assay

Antigen uptake property of immature DC is vital for adaptive immunity. Figure 7 represents the mean fluorescence intensity of dextran-FITC as percentage of dextran uptake capacity of imDC cultured in the presence of BB or BB supplemented with GMCSF and IL4 in medium. There was a 7.67% and 8.87% significant increase of dextran uptake in human healthy volunteer's imDC when treated with PHA ($P < 0.001$) and BB ($P < 0.001$), when compared with DMSO-treated imDC. These results suggest that

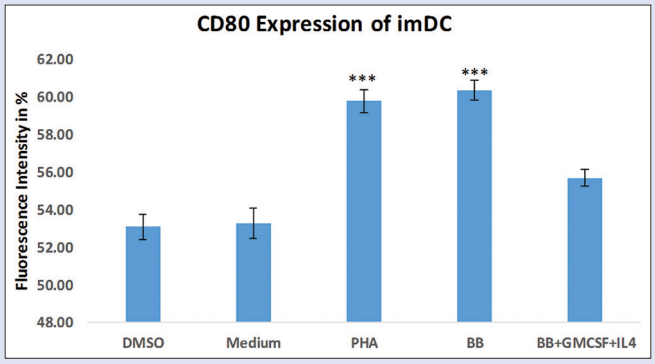


Figure 4: CD80 expression in Immature dendritic cell. When compared with dimethyl sulfoxide-treated imdendritic cell or control, phytohemagglutinin, and Boeravinone B-treated imdendritic cell showed significant differences in increase of CD80 expression up to 6.7% and 7.27% and was shown as $P < 0.001$(***). However, Boeravinone B supplemented with granulocyte-macrophage colony-stimulating factor and interleukin 4 showed an increase of CD80 expression up to 2.6%; Data represented are mean ± standard deviation; $n = 3$

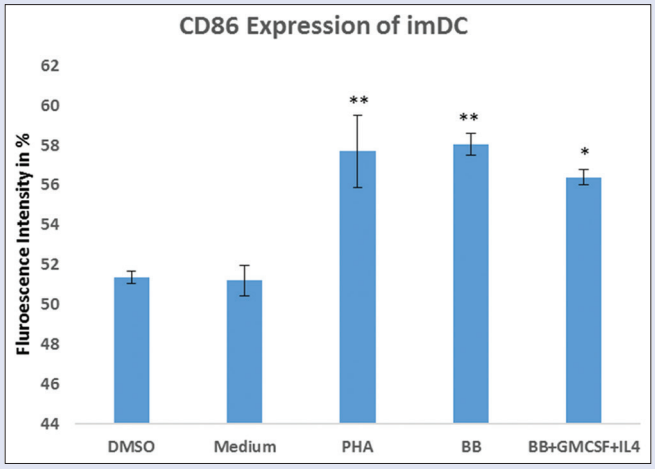


Figure 6: CD86 expression in Immature dendritic cell. When compared with control, phytohemagglutinin and Boeravinone B-treated imdendritic cell showed significant differences on increase of CD86 expression up to 6.6%, 6.73% and was shown as $P < 0.01$(**). However, Boeravinone B supplemented with granulocyte-macrophage colony-stimulating factor and interleukin 4 showed significant difference on increase of CD86 expression upto 5.1% and was shown as $P < 0.05$(*); Data represented are mean ± standard deviation; $n = 3$

mean fluorescence intensity was increased after treatment with both PHA- and BB-treated imDC indicating increase in endocytic activity, as shown by increased dextran uptake, strongly suggests that treatments of these compounds leads to the maturation of DCs. Figure 8 depicts the protocol indicating stimulation of IL-4, GM-CSF with BB -treated isolated monocytes differentiate into mature DCs on the 7th day. Similar studies are mentioned in protocols for generation of immunogenic DCs from monocytic precursors.[27]

DISCUSSION

BB is a rotenoid from *B. diffusa*, a well-known medicinal herb. In this study, the immunomodulatory effects of BB on the DCs have been elucidated. Several studies showed that natural agents such as

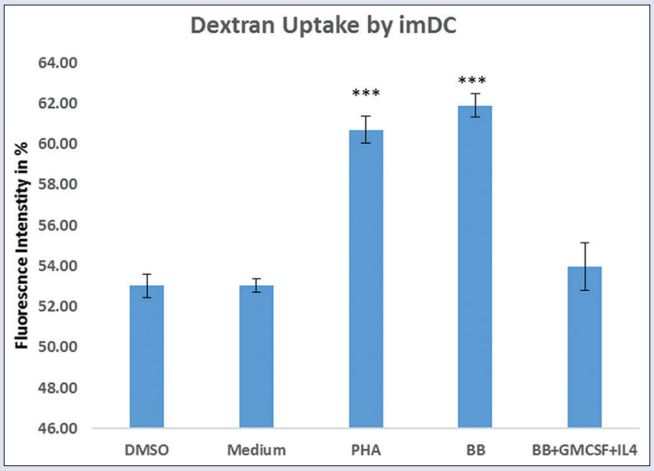


Figure 7: Dextran uptake assay in imDC. When compared with control, phytohemagglutinin and Boeravinone B-treated imdendritic cell showed significant increase of dextran uptake up to 7.67% and 8.87% and was shown as $P < 0.001$ (***). However, BB supplemented with granulocyte-macrophage colony-stimulating factor and interleukin 4 showed increase in dextran uptake upto 0.94%; Data represented are mean ± standard deviation; $n = 3$

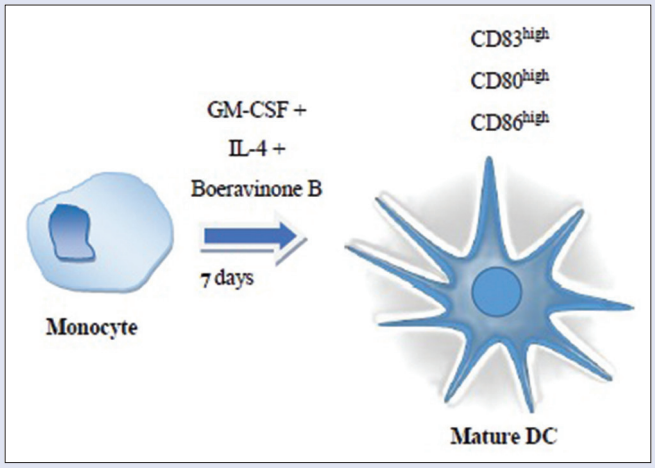


Figure 8: Generation of mature dendritic cell from monocytic precursor

plants and its compounds can modulate DCs activity.[7,28-30] Human CD14+ leukocytes in blood acquire the phenotype and function of DC when cultured in GM-CSF and IL-4.[31] In the present study, the effect of BB on the phenotypic maturation and function of DCs were examined. The expression of CD80, CD83, and CD86 are important costimulatory and maturation markers on DCs and have critical roles in antigen presentation and T-cell activation.[24,25] Our results showed that the BB modulate the percentage expression fluorescence intensity of CD80, CD83, CD86, and dextran uptake from human PBMCs through FACS. Similar studies were carried out in identifying DC subsets and assessment of DCs from whole blood samples but through 6-color flow cytometry.[32]

Few small molecular inhibitors such as flavonoids like BB have also been developed which are capable of binding with high affinity to IL-4Rα, and therefore, can be very effective in blocking IL-4 mediated responses.[33] A study on different signalling pathway of DCs was reported that ERK regulates DC survival, whereas NF-kB is responsible for DC maturation in their study by PHA-induced DC activation.[34] It is evident that the maturation of DCs altered when treated with BB through statistically significant reports of expression of CD80, CD83, and Dextran uptake when compared with negative control, which implies BB upregulates the NF-kB pathway. Similar type of studies was carried out in mice using methanolic extract of *Echinacea purpurea* by characterization with high-density CD83 expression, an excellent marker for DC maturation.[35] Investigations have been reported that when compared with untreated imDC from healthy donors, PPD or *Mycobacterium bovis* BCG-treated imDC showed higher expression of CD80, CD86, and dextran uptake.[19]

A plant-derived diterpenoid, taxol provides DCs with survival signal throughthe maturation of DCs by an enhanced expression of MHC class II.[36] It can also be inferred that BB upregulates ERK pathway because of the survival of DCs after treatment of this compound. The studies ascertains that under in *in vitro* experiments from Human PBMCs have proved that BB is promoting the maturation of monocyte differentiated DCs by significantly high expression of CD80, a DC costimulation marker and dextran uptake.

CONCLUSION

This study demonstrates that treatment of BB promotes human blood DC maturation shown by the maturation markers. Further studies are required to explore the beneficial use of BB as a drug or drug adjuvant for immunomodulation, disease prevention, and therapy with minimal side effects at lowest cost.

Acknowledgements

The authors express sincere thanks to the management of SVCE to carry out primary research in SVCE, Sriperumbudur, by receiving financial support through project intramural grant and valuable consistent guidance provided by Secretary and Professor, Department of Biotechnology, SVCE and further support extended through peer mentoring by Dr. S. Prabhu, Associate Professor, Department of Biotechnology, SVCE. The authors also express earnest thanks to Prof. Luke Elizabeth Hanna, Scientist "E" and Sivasankaran from NIRT, Chennai, for providing the flow cytometry facility.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Dauer M, Obermaier B, Herten J, Haerle C, Pohl K, Rothenfusser S, *et al.* Mature dendritic cells derived from human monocytes within 48 hours: A novel strategy for dendritic cell differentiation from blood precursors. J Immunol 2003;170:4069-76.
2. Kasajima-Akatsuka N, Maeda K. Development, maturation and subsequent activation of follicular dendritic cells (FDC): Immunohistochemical observation of human fetal and adult lymph nodes. Histochem Cell Biol 2006;126:261-73.
3. Schnurr M, Then F, Galambos P, Scholz C, Siegmund B, Endres S, *et al.* Extracellular ATP and TNF-alpha synergize in the activation and maturation of human dendritic cells. J Immunol 2000;165:4704-9.
4. Theresa L. Whiteside. Introduction to cytokines as targets for Immunomodulation. Cytokines in Human Health. Totowa, NJ: Humana Press Inc.; 2007. p. 1-15.
5. Cooper GS, Bynum ML, Somers EC. Recent insights in the epidemiology of autoimmune diseases: Improved prevalence estimates and understanding of clustering of diseases. J Autoimmun 2009;33:197-207.
6. Dhama K, Chakraborthy S, Tiwari R. Panchgavya therapy (Cowpathy) in safeguarding health of animals and humans: A review. Res Opin Anim Vet Sci 2013;3:170-8.

7. Dhama K, Saminathan M, Jacob SS, Singh M, Karthik K, Amarpal TR, *et al.* Effect of immunomodulation and immunomodulatory agents on health with some bioactive principles, modes of action and potent biomedical applications. Int J Pharmacol 2015;11:253-90.
8. Sandhya B, Thomas S, Isabel W, Shenbagarathai R. Ethnomedicinal plants used by the Valiyan community of Piranmallai Hills (Reserved forest), Tamil Nadu, India. A pilot study. Afr J Tradit Complement Alternat Med 2006;3:101-14.
9. Das S. Antimicrobial activity study of ethanolic extract of *Boerhaavia diffusa* whole plant. Int J Pharm Life Sci 2012;3:2006-9.
10. Ashok Kumar CK, Tejasri M, Satheesh Kumar D, Ramya M, Revathi K, Avinash Kumar Reddy G. A review on anti-oxidants. Int J Innov Drug Discov 2012;2:98-114.
11. Rajpoot K, Mishra RN. *Boerhaavia diffusa* roots (Punarnava mool) – Review as Rasayan (Rejuvenator/Antiaging). Int J Res Pharm Biomed Sci 2011;2:1451-60.
12. Praveen Kumar PK, Swarnalakshmi M, Sivanandham M. Phytochemical analysis of *Boerhaavia diffusa*, *Emblica officinalis*, *Terminilia chebula*, *Terminilia bellirica*, *Withania somnifera*. World J Pharm Res 2015;4:1747-56.
13. Praveen Kumar PK, Sivanandham M, Akila M, Prabhu S. Isolation, purification and characterization of Boeravinone B from *Boerhaavia diffusa* linn. Int Res J Pharm 2017;8:140-4.
14. Katiyar CK, Kanaujia A, Duggar R, Yadav SS, Sharma N, Ray A, *et al.* Standardized bioactive herbal extracts. EP 2139504 A1; 2010.
15. Praveen Kumar PK, Sivanandham M, Karthick J, Sivagami U. *In silico* analysis of binding of Boeravinone E with GMCSFR and TNFαR. Int J Biol Ecol Environ Sci 2013;2:103-7.
16. Mehrotra S, Mishra KP, Maurya R, Srimal RC, Singh VK. Immunomodulation by ethanolic extract of *Boerhaavia diffusa* roots. Int Immunopharmacol 2002;2:987-96.
17. Mungantiwar AA, Nair AM, Shinde UA, Dikshit VJ, Saraf MN, Thakur VS, *et al.* Studies on the immunomodulatory effects of *Boerhaavia diffusa* alkaloidal fraction. J Ethnopharmacol 1999;65:125-31.
18. Pandey R, Maurya R, Singh G, Sathiamoorthy B, Naik S. Immunosuppressive properties of flavonoids isolated from *Boerhaavia diffusa* linn. Int Immunopharmacol 2005;5:541-53.
19. Manickam A, Sivanandham M. *Mycobacterium bovis* BCG and purified protein derivative-induced reduction in the CD80 expression and the antigen up-take function of dendritic cells from patients with cervical cancer. Eur J Obstet Gynecol Reprod Biol 2011;159:413-7.
20. Lim TS, Goh JK, Mortellaro A, Lim CT, Hämmerling GJ, Ricciardi-Castagnoli P, *et al.* CD80 and CD86 differentially regulate mechanical interactions of T-cells with antigen-presenting dendritic cells and B-cells. PLoS One 2012;7:e45185.
21. Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, *et al.* Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 2004;53:1602-9.
22. Fuss IJ, Kanof ME, Smith PD, Zola H. Isolation of whole mononuclear cells from peripheral blood and cord blood. Curr Protoc Immunol 2009;7-1.
23. Seager Danciger J, Lutz M, Hama S, Cruz D, Castrillo A, Lazaro J, *et al.* Method for large scale isolation, culture and cryopreservation of human monocytes suitable for chemotaxis, cellular adhesion assays, macrophage and dendritic cell differentiation. J Immunol Methods 2004;288:123-34.
24. Kim MH, Byon YY, Ko EJ, Song JY, Yun YS, Shin T, *et al.* Immunomodulatory activity of Ginsan, a polysaccharide of *Panax ginseng*, on dendritic cells. Korean J Physiol Pharmacol 2009;13:169-73.
25. Azadmehr A, Latifi R, Mosalla S, Hajiaghaee R, Shahnazi M. Immunomodulatory effects of *Ziziphora tenuior* L. extract on the dendritic cells. Daru 2014;22:63.
26. Tjoa BA, Bosch ML. Generation of dendritic cells from monocytic dendritic precursor cells with GMCSF in the absence of additional cytokines. US 2013/0273654 A1; 2013.
27. Hubo M, Trinschek B, Kryczanowsky F, Tuettenberg A, Steinbrink K, Jonuleit H, *et al.* Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. Front Immunol 2013;4:82.
28. Naga Preethi N, Rajeshwari P. An overview on immunomodulators. Int J Curr Pharm Clin Res 2014;4:108-14.
29. Aldahlawi AM. Modulation of dendritic cell immune functions by plant components. J Microsc Ultrastruct 2016;4:55-62.
30. Cheng JH, Lee SY, Lien YY, Lee MS, Sheu SC. Immunomodulating activity of *Nymphaea rubra roxb.* extracts: Activation of rat dendritic cells and improvement of the T (H) 1 immune response. Int J Mol Sci 2012;13:10722-35.
31. Kiertscher SM, Roth MD. Human CD14+ leukocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GMCSF and IL4. J Leukoc Biol 1996;59:208-18.
32. Della Bella S, Giannelli S, Taddeo A, Presicce P, Villa ML. Application of six-color flow cytometry for the assessment of dendritic cell responses in whole blood assays. J Immunol Methods 2008;339:153-64.
33. Ul-Haq Z, Naz S, Mesaik MA. Interleukin-4 receptor signaling and its binding mechanism: A therapeutic insight from inhibitors tool box. Cytokine Growth Factor Rev 2016;32:3-15.
34. Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. J Exp Med 1998;188:2175-80.
35. El-Ashmawy NE, El-Zamarany EA, Salem ML, El-Bahrawy HA, Al-Ashmawy GM. *In vitro* and *in vivo* studies of the immunomodulatory effect of *Echinacea purpurea* on dendritic cells. J Genet Eng Biotechnol 2015;13:185-92.
36. Joo HG. Altered maturation of dendritic cells by taxol, an anticancer drug. J Vet Sci 2003;4:229-34.