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A novel kefir product (PFT) activates dendritic cells to induce CD4+T and CD8+T cell responses *in vitro*

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Abstract

Lactobacilli have been widely studied for their probiotic effects and have been reported to function as antiviral and anticancer agents. However, the underlying mechanisms via immune modulation are poorly understood. PFT is a freeze dried compound of *Lactobacillus kefiri* P-IF with a unique composition and functionality. In this study, we examined the potential stimulatory effects of two concentrations (50 μg and 100 μg/mL) of PFT on human monocyte-derived dendritic cell (DC) function *in vitro*. Results showed that PFT upregulated the expression of DC surface co-stimulatory and maturation markers CD80, CD86, and HLADR in a concentration dependent manner. PFT at 100 μg/mL markedly increased the secretion of IL-6, IL-10, TNF-α, and IL-1β by DCs. This concentration of PFT also stimulated the production of antiviral cytokines, IFN-α and IFN-λ(IL29) in DCs. Additionally, PFT at 100 μg/mL activated moDCs prime $CD4^+$T cells and significantly increased the levels of IL-10, IFN-γ, and TNF-α by 1.7, four, three-fold, respectively. Furthermore PFT-stimulated DCs were also effective in enhancing the cytotoxic potential of $CD8^+$T cells via the induction of Granzyme-B and upregulation of CD107a, and CD103 expression, a marker of resident/regulatory $CD8^+$T cells. These data suggest that PFT functions as a natural adjuvant for DC activation and thus may be used in DC-based vaccine strategies against viral infections and cancer.

Keywords

dendritic cells, CD8 T cells, PFT

Introduction

Probiotics are a natural class of immune modulators which might decrease the reliance on synthetic antimicrobials. Therefore, there is an urgent need to explore the prophylactic and therapeutic applications of probiotics. Lactic acid bacteria (LAB) are widely used as probiotics.[1] LAB strains present in many foods such as yogurt are normal components of intestinal microflora and help maintain a healthy balance of probiotic bacteria while reducing pathogenic bacteria.[2,3] In addition, LABs are frequently used as probiotics to favor some biological functions in the host. Our work[4] and that of others showed that LABs have the ability to exert antitumor and antiviral activity.[5–9] However the mechanisms underlying their effects have not been fully examined. The immune modulatory action of LAB is a possible mechanism by which LAB exerts its effects against cancer and viral infection. This is supported by the fact that LAB strains can induce adjuvant effects including enhancement of natural killer (NK) and cytotoxic T cell activities.[10–13] Earlier reports also suggest that treatment with LAB caused an increase in the number of IgA producing cells as well as an increase in the specific and total serum IgA titer.[14,15] Numerous studies have investigated the effect of LAB on dendritic

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cells (DCs). However, there is virtually nothing known regarding the effect of PFT on the immune system cells. PFT is a natural mixture composed primarily of *Lactobacillus kefiri* P-IF, a specific strain of *L. kefiri* with unique growth characteristics. Furthermore it also contains traces of three yeast strains, Kazachstania turicensis, Kazachstania unispora, and Kluyveromyces marxianus.[16] This unique composition of PFT is what is responsible for its enhanced immune modulatory activities compared to other LAB strains.

DCs are the professional antigen presenting cells (APCs) which bridge the innate and adaptive immunity because of their unique ability to sense pathogens and initiate immunity. These cells possess several sensors of pathogens known as pattern recognition receptors (PRRs). These include Toll like receptors (TLRs), Nod-like receptors, and C-type lectin receptors.[17,18] Depending on the microbial stimulus encountered, DCs secrete cytokines, chemokines, and prime naive T cells toward Th1, Th2, Treg, TH17, and T_{FH} cell responses.[19–22] DCs residing in the gut regularly encounter non-pathogenic organisms including *Lactobacillus* species[23,24] which may modulate DC properties, including their ability to activate specific immune responses at mucosal sites.[23–25] A balance of DC stimulation and tolerance after an encounter with *Lactobacillus* cells in the gut may be important to maintain the homeostasis required for symbiotic bacteria to perform their critical functions in host nutrition, intestinal permeability, and protection against foreign pathogenic microbes.[24] The present study was undertaken to examine the potential stimulatory effects of PFT on DC function as determined by modulating the expression of markers CD80, CD86, and HLA-DR, and the secretion of cytokines. In addition, the effects of PFT-treated DCs on priming $CD4^+$T cells to secrete cytokines, and the enhancement of cytotoxic reactivity of $CD8^+$T cells were also examined. The results indicate that PFT may function as a natural adjuvant for DC activation and may suggest its use in DC-based vaccine strategies against viral infections and cancer.

Materials and methods

Antibodies and reagents

The following anti-human antibodies were used: CD11c APC (Clone B-ly6), CD80 PE (Clone L307.4), CD86 PE (Clone 2331 (FUN-1)), HLADR PerCP (Clone L243 (G46-6)), CD4 PerCP (Clone SK3), CD8 PerCP, CD103 PE, CD107a PE, and Granzyme B Alexa 647 were all from BD Biosciences (San Jose, CA, USA). An isotype antibody was used as a negative control (BD Biosciences, San Jose, CA, USA). FACS analysis-flow cytometry was performed using FACScalibur (Becton-Dickenson, San Jose, CA, USA), and analyzed using FlowJo software (Tree Star, Inc.).

Probiotics fermentation technology (PFT) kefir grain product

PFT is a mixture that mainly contains (~90%) a freeze-dried form of heat-killed *L. kefiri* P-IF; it is a specific strain of LAB that has a unique DNA sequence and PET scans show a 99.6% homology with regular kefiries. PFT also contains ~2–3% of each bacterial strain, *Lactobacillus kefiri* P-B1, and three yeast strains, Kazachstania turicensis, Kazachstania unispora, and Kluyveromyces marxianus.[16] PFT was provided by Paitos Co., Ltd. Yokohama, Kanagawa, Japan.

Isolation and culture of monocyte-derived DCs

Monocyte-derived DCs were prepared essentially as described previously.[19,20] Briefly, peripheral blood mononuclear cells (PBMC) from normal healthy donors (approved by the Institutional Review Board [IRB], Charles Drew University) were separated over Ficoll-hypaque density gradient centrifugation. The cells were allowed to adhere to culture plates for 2 h. Non-adherent cells were subsequently removed. The adherent monocytes were cultured for 6 days under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, human granulocyte-macrophage colony stimulating factor (GM-CSF) at 50 ng/mL (Peprotech, Rocky Hill, NJ, USA), and 10 ng/mL recombinant human IL-4 (Peprotech). Half of the medium was replaced every 2 days with fresh medium and DCs were collected after 6 days. The purity of the DCs obtained was >95%. DCs were subsequently pulsed with PFT (50 and 100 μg/mL) for 24 h.

Peripheral blood DCs (Blood DCs) were isolated from PBMCs by PanDC isolation kits from Stem Cell Technologies (Vancouver, BC, Canada). Approximately 0.6 to 1 $\times 10^6$ PanDC were recovered from 100 $\times 10^6$ PBMCs.

DC phenotyping

The expression of cell surface markers was determined by flow cytometry. Briefly, gated $CD11c^+HLADR^+$DCs were analyzed for the expression of CD80, CD86, and HLADR with the appropriate antibodies supplied by BD Pharmingen (San Diego, CA, USA).

Cytokine production by DCs

Monocyte derived DCs were incubated with PFT (50 and 100 μg/mL) for 24 h. The supernatants were collected and stored at −70°C until analyzed. The cytokines IL-6, IL-10, TNF-α, IL-1β (BD Pharmingen) in the supernatants were measured by specific ELISA kits as per the manufacturer's protocol. Pan DC supernatant were collected and assessed for IL29 and IFNα by specific ELISAs (RnDsystems, Minneapolis, MN, USA).

DC-$CD4^+$T cells

Allogenic $CD4^+$T cells were purified by negative selection using a magnetic bead-based kit from Stem Cell Technologies. Allogenic $CD4^+$T cells were cultured with DCs that were stimulated with PFT (50 and 100 μg/mL) for 24 h as above. The DC-$CD4^+$T were co-cultured for 5 days in a U bottom 96-well plate. The ratio DC: $CD4^+$T cells was 1:5 (2×10^4: 1×10^5). At the end of 5 days the supernatant was collected and kept at −70°C. Cytokines IFN-γ, IL-10, and TNF-α were detected using a specific ELISA kit (BD Pharmingen).

Blood DC-$CD8^+$T cells

Allogenic $CD8^+$T cells were enriched by negative selection using a magnetic bead-based kit from Stem Cell Technologies. PFT-stimulated Blood DCs were cultured with $CD8^+$T cells at a 1:10 ratio in 96-well plates and kept for 7 days for $CD8^+$T cells to differentiate into cytotoxic and resident $CD8^+$T cells. After 7 days the supernatant was collected and cells were stained for the surface markers CD103 (tissue resident $CD8^+$T cells) and CD107a and intracellular Granzyme-B (cytotoxic $CD8^+$T cell) as per standard protocol by BD Biosciences.

Statistics

All of the experiments were repeated with samples from four to seven individual subjects. KrukerWallis (ANOVA) with post Dunn tests were used to calculate the statistical significance using Graph Pad Prism software. The level of significance was set at $P<0.05$.

Results

PFT induces maturation of moDCs

Monocyte derived DCs were incubated in the presence or absence of PFT for 24 h and the expression and density of maturation markers was determined by flow cytometry. A representative cytofluorograph is shown in Figure 1a. PFT caused an increase in the expression of DC surface co-stimulatory and maturation markers CD80, CD86, and HLADR. The magnitude of expression was dose dependent. Data in Figure 1b show the density of mean fluorescent intensity (MFI) of CD80, CD86, and HLADR in DCs at baseline values and the levels post-treatment with PFT. Treatment with PFT significantly ($P<0.01$) upregulated the expression of CD80 and CD86 markers as compared with untreated DCs.

PFT induces production of cytokines by moDCs

Data in Figure 2 show that PFT induced the production of cytokines IL-10, IL-6, TNF-α, and IL-1β in DCs. The effect of PFT was significant ($P<0.05$) except for IL-1β as compared to moDCs alone.

PFT activated moDCs prime $CD4^+$T cells and secrete IFN-γ, IL10, and TNF-α

Data in Figure 3 show that PFT-treated moDCs primed $CD4^+$T cells to secrete several cytokines: IFN-γ, IL10, and TNF-α. PFT treatment resulted in significant ($P<0.05$) increased levels of IFN-γ, IL10, and TNF-α as compared to DCs-$CD4^+$T cells alone. The secretion of cytokines increased as follows: IFN- γ by three- to four-fold, IL-10 by 1.5- to 1.7-fold, and TNF-α by three-fold.

PFT induces antiviral cytokines IFN-λ(IL29) and IFN-α in Blood DCs

The production of type I and type III interferons by DCs is one of the most potent of the antiviral actions exerted by DCs. Among the DC subsets, plasmacytoid DCs and circulating $CD141^+$ DCs are considered the primary producers of interferons. MoDCs can also produce interferons but at much lower

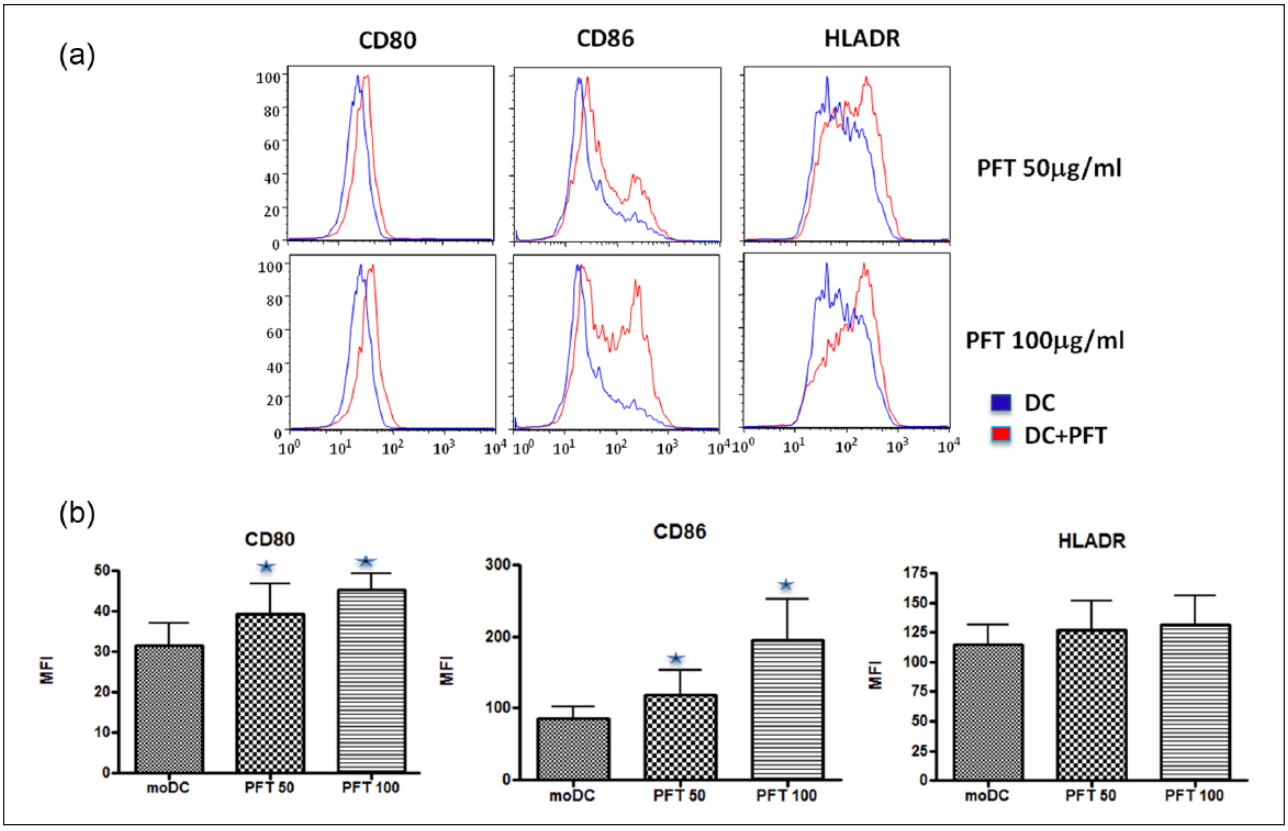

Figure 1. (a, b) Effect of PFT on moDC co-stimulatory and maturation markers CD80, CD86, and HLADR. Monocyte-derived DCs were treated for 24 h with PFT (50 and 100 µg/mL). Isotype antibody was used as a negative control. Expression of cell surface markers was determined by flow cytometry. (a) One representative cytofluorograph is shown from four individual experiments. (b) The density of mean florescent intensity (MFI) of CD80, CD86, and HLADR in DCs in the absence or presence of PFT. Data represent the mean ± SE of four experiments; ★P <0.01 as compared to DCs alone.

levels. Therefore, to determine whether or not PFT induces the production of antiviral cytokines, peripheral blood DCs were stimulated with PFT at 50 and 100 µg/mL for 24 h and production of IL-29 and IFN-α was determined by ELISA. Data in Figure 4 show that PFT treatment was effective in inducing secretion of IFN-λ(IL-29) and IFN-α (P <0.05).

PFT stimulated Blood DCs induce the expression of CD103 and CD107a, as well as Granzyme-B in CD8⁺T cells

Blood DCs stimulated with PFT for 24 h were cultured with allogenic CD8⁺T cells for 7 days. Subsequently, the cells were stained with CD103, a marker of resident/regulatory CD8⁺T cells, and CD107a as well as Granzyme-B, markers for cytotoxic T cells. Data in Figure 5 show that PFT activated DCs were efficient in upregulating the expression of CD103 and CD107a, as well as increasing the granular content of Granzyme-B in CD8⁺T cells.

Discussion

PFT is a natural mixture composed primarily of *Lactobacillus kefiri* P-IF. Results of the current study revealed the ability of PFT to activate human DCs as indicated by upregulation of the surface expression of co-stimulatory molecules CD80, CD86, and HLADR. In addition, PFT treatment stimulated DCs to secrete increased levels of cytokines such as interleukins (IL-6, IL-10, and IL-1β) and TNF-α. These data are in accordance with work by others who showed that other *Lactobacillus* species induced activation and maturation of myeloid and bone marrow DCs.[26,27] Furthermore, other studies with murine bone marrow derived DCs also reported production of significant levels of IL-6, TNF-α, and IL-10 post-treatment with LAB species.[28] Furthermore, *Saccharomyces boulardii* and *Bacillus subtilis* B10 also caused increased production of several cytokines including IL-1β and IL-10 from chicken bone marrow DCs.[27] On the other hand, other Lactobacillus species-exposed

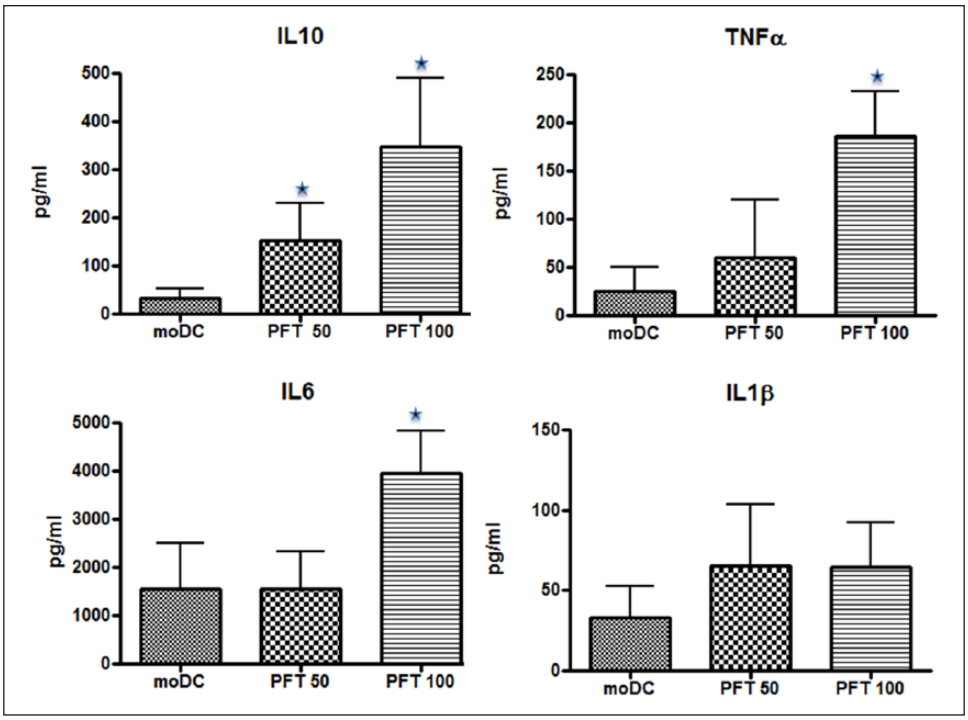

Figure 2. Effect of PFT on cytokine production by moDCs. Immature DCs were incubated with PFT (50 and 100 μg/mL) for 24 h. Supernatants were collected and the levels of IL-10, IL-6, TNF-α, and IL-1β were measured by specific ELISA kits. The data are the mean ± SD from seven individual experiments; ★P <0.05 as compared to DCs alone.

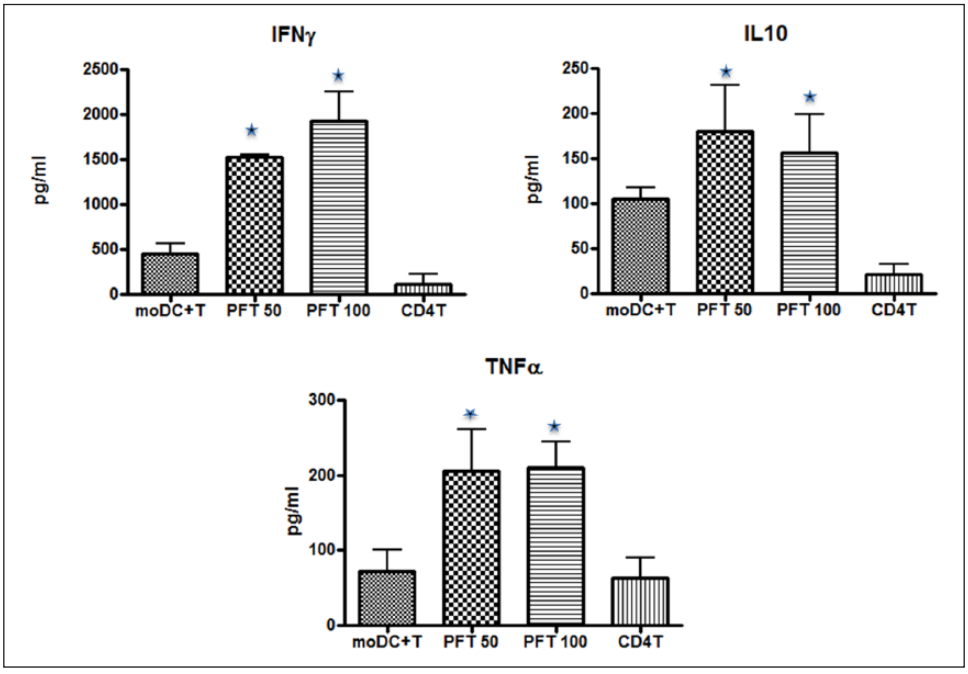

Figure 3. PFT stimulated DCs prime regulatory type CD4+T cells and secrete IFN-γ, IL-10, and TNF-α. The data are the mean ± SD from five individual experiments; ★P <0.05 as compared to DCs-CD4+T cells alone.

human myeloid DCs *in vitro* induced high levels of IL-12 and IL-18, but not IL-10,[26] suggesting that IL-10 production may depend on the LAB species. Earlier studies showed that several probiotics cell components are responsible for induction of IL-10 production. These include; genomic DNA from bifidobacteria or lactobacilli,[29] soluble or insoluble cell preparations from

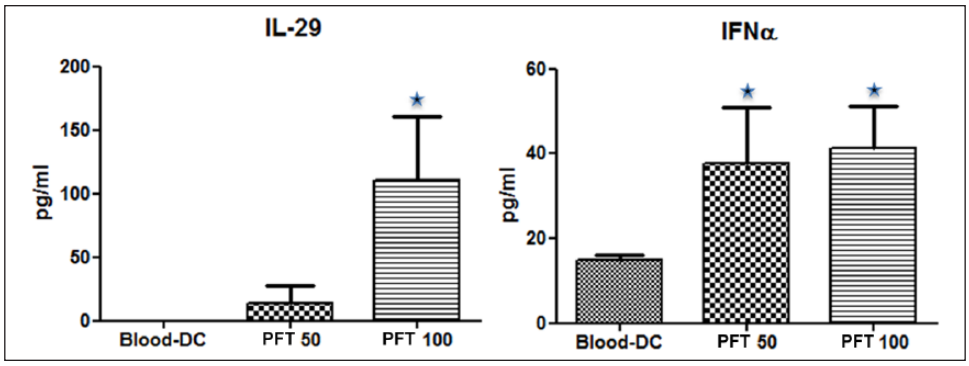

Figure 4. Blood DCs were stimulated with PFT at concentrations of 50 and 100 μg/mL for 24 h. Type I and type III interferons, IL29 and IFN-α, were detected by ELISA. The data are the mean ± SD from four individual experiments; ★P <0.05 as compared to DCs alone.

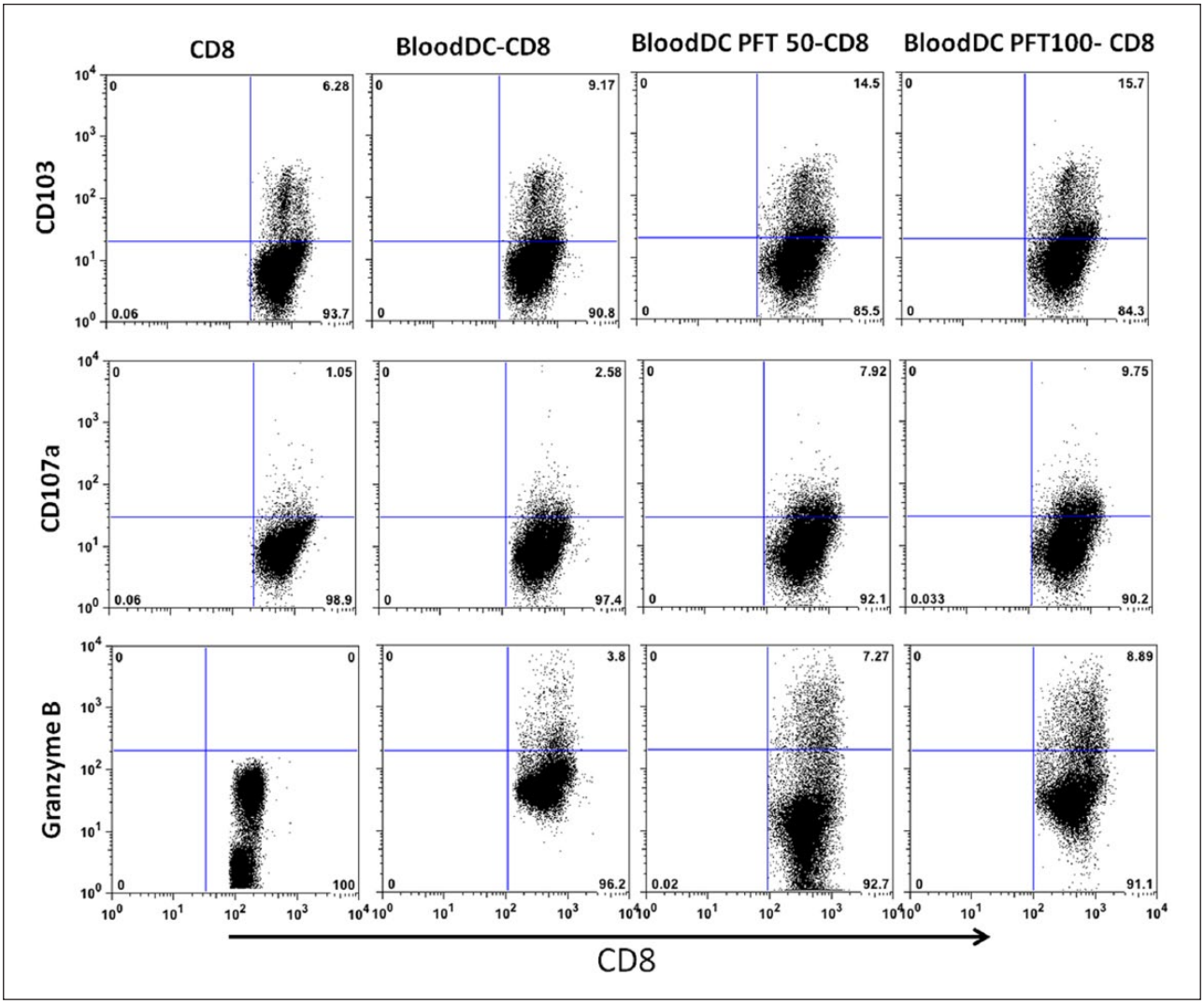

Figure 5. PFT stimulated DCs prime activated CD8+T cells. DCs were stimulated with PFT (50 and 100 μg/mL) for 24 h and then cultured with CD8+T cells for 7 days. CD8+T cells were stained with CD103, CD107a, and Granzyme-B. One representative experiment is shown from three individual experiments.

bifidobacteria,[30,31] and TA/LTA from lactobacilli.[32] LAB has demonstrated the ability to induce IL-10 production by different mechanisms. For example, S-layer protein A (SlpA) released from *Lactobacillus acidophilus* induces IL-10 production in DCs via a direct interaction of SlpA with DC-SIGN.[33] In the current study, PFT induces both pro- and anti-inflammatory cytokines from DCs.

The mechanism by which PFT induces its effect on DC is not fully elucidated. TLR-2 has been shown to be a signal transducer for cells activated by peptidoglycan, lipoteichoic acid, bacterial lipoprotein, and LPS.[34] Other *Lactobacillus* species upregulate expression of TLR-2 transcripts.[26] These data may suggest that PFT may deliver signals through TLR-2, thereby promoting the activation of DCs. Further studies also showed that the extracellular proteins secreted by *Bifidobacterium breve* C50 can interact with DCs via TLR-2.[35]

In this study, PFT-stimulated moDCs induced activation of $CD4^+$T which subsequently leads to production of IFN-γ, IL-10, and TNF-α. These data are in agreement with work by Mohamadzadeh et al. who showed that activation of moDCs by other *Lactobacillus*-species also induced proliferation of autologous $CD4^+$ and $CD8^+$ T cells and induced secretion of IFN-γ.[26]

Taken together, moDCs activated with PFT secrete increased levels of inflammatory TNF-α and IL-6 cytokines and anti-inflammatory cytokine IL-10. Additionally, these moDCs prime multifunctional $CD4^+$T cells to secrete IFN-γ, IL-10, and TNF-α.

Data of the current study shows interesting activity of PFT on Blood DCs, as indicated by its ability to activate them to secrete IFN-α and IL-29 and exhibits the profile of an antiviral, suggesting its possible role as an antiviral agent. Recent studies showed intake of yogurt fermented with *L. lactis* JCM5805 resulted in suppression in the risk of morbidity from the common cold, which was associated with the activation of human pDCs activity and increased levels of IFNs *in vivo*.[8]

IFN-λ (IL-29) is the main cytokine of the type III IFNs family produced in humans and its main biological function is antiviral activity at the mucosal surfaces. IFN-λ exerts several biological functions, such as induction of anti-proliferative and antitumor activities,[36,37] as well as immunomodulatory effects, including induction of proliferation of T cells.[38] Blood DCs also modulate $CD8^+$T cell responses via increased expression of markers of CTLs, CD107a, and Granzyme-B expression. $CD8^+$T cells expressing CD107a and Granzyme-B are hallmarks for cytotoxic T cells[39,40] which help the eradication of tumor cells and viral infected cells. Furthermore, $CD103^+$ $CD8^+$T cells are resident $CD8^+$T cells since they express CD103. $CD103^+$ $CD8^+$T cells display anticancer activity.[41] Recent studies by Wu et al. showed that $CD103^+$ $CD8^+$ mucosal T cells accumulate in tumors, and elicit an increase in cancer necrosis and prevent cancer progression *in vivo* in a humanized mouse model of breast cancer. $CD103^+$ $CD8^+$ mucosal T cells induced by DCs were also able to reject established cancer.[41]

Our results suggest that PFT functions as a natural adjuvant for DC activation and thus may be used in DC-based vaccine strategies against infections and cancer. Further research is needed to confirm the phenomenon.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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