Immunomodulatory Effects of Jacalin, A Dietary Plant Lectin on the Peripheral Blood Mononuclear Cells (PBMCs)

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Research Article

Keywords: tumor microenvironment, tumor growth and invasion, peripheral blood mononuclear cells

Posted Date: August 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-731785/v1

Abstract

The tumor microenvironment that refers to the tumour's surroundings is a key modulator of tumor growth and invasion. The tumour derived signals are known to downregulate the anti-tumor effects of the effector cells present in the TME. Thus, the cross talk between the tumor cells with the surrounding immune cells helps in evading the tumor surveillance as well as aiding in tumor growth and proliferation. Hence, knowledge regarding the effects of drugs/compound on the tumor-stromal interactions are gaining importance. In the present study, the effects of jacalin, a dietary lectin on the proliferation and cytokine production of peripheral blood mononuclear cells (PBMCs) are investigated. Jacalin was shown to act as a mitogen of PBMCs, the key cytokine secreting immune cells. Also, jacalin initially induces increased mRNA expression of pro-inflammatory cytokine IFN-γ; however prolonged stimulation of PBMCs resulted in increased expression of anti-inflammatory cytokine, mainly TGF-β. Further, 6 h jacalin prestimulated PBMCs (Jac-PBMCs) were shown to inhibit HeLa cell proliferation while 24 h (Jac-PBMCs) were found to favor tumor growth. Thus, it may be postulated that while jacalin initially polarizes the PBMCs to hinder the tumor growth, after a stipulated time point, interaction of jacalin with PBMCs can lead to an immunosuppressive TME that may probably assist in tumor growth and progression.

Introduction

The TME refers to the tumour's surrounding and includes the rapidly proliferating tumour cells, the tumour stroma, blood vessels and other inflammatory cells[1]. Immune cells present in the TME include the effectors of innate immunity such as polymorphonuclear leukocytes, macrophages, mast cells, and natural killer cells as well as the cells of the adaptive immunity such as the dendritic cells, T lymphocytes and few B cells [2]. The inflammatory cells in the TME either interfere with the tumour growth resulting in tumour regressionor can facilitate tumour progression. The tumour cells, apart from escaping the host immune surveillance, also actively modify the functions of the infiltrating effector cells resulting in an environment favourable for tumour growth and progression[3].

PBMCs that include the lymphocytes (T cells, B cells, and natural killer cells), monocytes and the dendritic cells form an integral part of the TME. PBMCs can modulate (inhibit or stimulate) tumour-specific immune responses through secretion of cytokines. The cytokines, regardless of their source, can stimulate or inhibit tumour growth [4]. Differential levels of cytokines in peripheral blood have been implicated in pathogenesis of numerous diseases including cancer.The changes in cytokine profile in response to a drug/compound will provide additional knowledge as to whether the ensuing immune response will be detrimental or beneficial to the host.

Lectins are carbohydrate-binding proteins that are capable of cross-linking glycoproteins present on the immune cell surface, resulting in their proliferation and activation[5, 6]. Such lectin induced activation of immune cells can trigger downstream signal transduction resulting in production of cytokines. [7]. Thesecreted cytokineshave been reported to be implicated in modulation of cancer cell growth and proliferation [8, 9].

Jacalin is a 66,000 Da, tetrameric two chain lectin isolated from jackfruit seeds. It exhibits specificity towards the tumour specific disaccharide Thomsen–Friedenreich antigen (Galβ1-3GalNAc). Jacalin is primarily a β sheet carbohydrate-binding protein with a wide range of applications[10]. Previous reports on effects of jacalin on proliferation of PBMCs bestowed contradictory results' for instance, while jacalin was initially reported to induce proliferation of T cells as well as B cell terminal differentiation [11],in a later study, jacalin was reported to induce proliferation of T cells alone but inhibited production of immunoglobulins (Ig) by the B cells [12]. Among the T cell subsets, jacalin was shown to be a general T cell mitogen, inducing the proliferation of $CD4^+$ and $CD8^+$ T cells [13]. However, various other studies reported jacalin to specifically induce proliferation of CD4 + T helper (Th) cells alone[14–16]. Interestingly, another study reported that the PBMCs did not possess any proliferative response to jacalin treatment[17]. More recently, jacalin was reported to induce apoptosis of B cells by binding to CD45, that is exclusively expressed on the surface of the lymphocytes [18]. CD45, a protein tyrosine phosphatase is highly glycosylated and is considered as a major regulator of lymphocyte signalling[19].

Likewise, the production of interleukin-2 (IL-2) by PBMCs stimulated with jacalin also remains controversial. IL-2 is required for growth and proliferation of T cells and further for its differentiation into effector cells. While there are reports to show that jacalin induced PBMCs to secrete IL-2[15, 20], another study reported IL-2 to be absent in the culture supernatants of jacalin-stimulated PBMCs. Further, IL-2 mRNA was alsofound to be absent, as determined by Northern blot analysis[21]. Such discrepancy in the mitogenic effects and binding specificity of jacalin may be attributed to the geographic variability [22]. Also, it has to be reckoned that few of the studies involved use of jackfruit seed crude extract (JCE) rather than the pure lectin. The JCE includes a mixture of proteins and more specifically a mannose binding lectin which may have contributed to the contradictory reports[23]. Thus, the immunomodulatory effects of purified jacalin demands further investigation. The purpose of this study was to evaluate the effects of jacalin on PBMC proliferation and cytokine production*in vitro*. Further, the direct and indirect impact of jacalin stimulated PBMCs on the cancer cell growth has been explored.

Materials And Methods

Materials

Dihydrogen monosodium phosphate, disodium hydrogen phosphate, sodium chloride and sodium hydroxide were purchased from Sisco research laboratories (Maharashtra, India), Lymphoprep was purchased from Stem cell technologies, Roswell park memorial institute (RPMI) 1640and DMEM powder were purchased from Himedia and the medium were prepared according to the manufacturer's protocol, Fetal bovine serum (FBS) and trypsin-EDTA were purchased from Sigma-Aldrich Co. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from SRL.

Purification of jacalin

Jacalin was purified from jackfruit seeds by affinity chromatography using epichlorohydrin-activated guar gum as the affinity matrix [24]. The activity of the lectin was checked by its ability to agglutinate the

red blood cells (RBCs) and the purity was analysed by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Cell line and Cell culture

K562 cells were obtained from National Centre For Cell Science (NCCS), Pune and maintained in RPMI 1640 medium, supplemented with 10 % foetal bovine serum and 1% antibiotics (penicillin, streptomycin) and HeLa cervical cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1 % antibiotics (penicillin, streptomycin). The cells were maintained at 37°C in a humified atmosphere containing 5% CO_2.

Isolation of Peripheral blood mononuclear cells (PBMCs)

5 ml of peripheral venous blood of a normal donor was collected in a heparinized tube and kept at RT for about 2 h prior to isolation of PBMCs. The blood was diluted with equal volumes of PBS. The diluted blood was then carefully layered onto 5 ml of Lymphoprep and centrifuged at 1700 rpm for 40 mins at 4°C, with brake off. After removing and discarding the upper plasma layer, the interface of mononuclear cells were carefully collected in a fresh tube. The cells were then washed twice with PBS and once with non-complemented RPMI 1640. Finally, the cells were suspended in RPMI 1640 supplemented with 10% FBS and 1% antibiotic (penicillin, streptomycin) and cultured at 37°C in a humified atmosphere containing 5% CO_2.

MTT assay

The effects of jacalin on viability of PBMCs were evaluated using the MTT tetrazolium assay, which involves the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to produce formazan crystals [25]. Cells suspended in RPMI 1640 medium, supplemented with 1% FBS and 1% antibiotics were seeded at a density of 3 x 10^3 cells/well into 96-well cell culture plates. The cells were then treated with decreasing concentrations (500 to 7.8 µg ml^{-1}) of jacalin, in triplicate and incubated for 24 h at 37°C. Thereafter, 25 µl of MTT (5 mg ml^{-1} in PBS) was added to each well and incubated for 4 h in dark at 37°C. After 4 h, the formazan crystals were solubilized in 100 µl of DMSO and incubated at room temperature for 20 mins. Absorbance was measured at 570 nm in multimode plate reader (Model Victor X3, Perkin Elmer). Absorbance given by untreated cells were considered as 100% cell growth.

Quantification of mRNA expression of cytokines in PBMCs

PBMCs were seeded at a density of 1x10^6 cells per well into a 6 well plate. Cells were then treated with 200µg/ml jacalin for 6 h, 12 h and 24 h, lysed with TRIzol reagent and RNA was isolated as described. Briefly, 200 µl of chloroform was added to the lysed cells, mixed well by inversion and the tubes were centrifuged at 20,817g for 10 mins. The upper aqueous layer was transferred into a fresh tube and equal volumes of isopropanol was added. The tubes were incubated at -80°C for about 2 hrs. To precipitate the RNA, the tubes were centrifuged at 20,817g for 10 mins.After washing twice with 70 % ethanol, the RNA pellet was suspended in 20 µl of diethylpyrocarbonate (DEPC) water. 5 µg of total RNA was reverse

transcribed to cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, CA), as per the manufacturers protocol.

Quantitative real time PCR (qRT-PCR) was performed using SYBR green master mix (Hi-Sybr Master mix, MBT074) under cycling conditions as follows : 95°C for 2 min, 40 cycles of annealing and extension at 55°C for 45 secs followed by melt curve analysis in the real time PCR machine (Model CFX96, BioRad). The samples were run in triplicate with appropriate no template control (NTC) and the threshold cycle (Ct) values recorded. β-actin was used as the endogenous control.

Effects of jacalin-prestimulated PBMCs (Jac-PBMCs) on viability of HeLa cells

PBMCs were seeded at a density of $1x10^4$ cells per well into a 12 well plate and were treated with 200 µg/ml jacalin for 6 h, 12 h, 24 h and 48 h. After the respective time points, the cells were collected, pelleted and the supernatant was discarded. The cell pellet was washed with sterile PBS, suspended in RPMI 1640 and stored at -80°C until further use. HeLa cells were seeded at a density of 3×10^3 cells/well into 96-well cell culture plates. Jacalin prestimulated and control PBMCs were added onto the adhered HeLa cells. Cell proliferation assay was done after 12 h of stimulation. Absorbance given by HeLa cells cultured in jacalin unstimulated PBMCs were considered as 100% cell growth and the viability of cells cultured in Jac-PBMCs were expressed as a fraction of those cells.

Effects of jacalin-stimulated PBMC-CM (Jac-PBMC-CM) on the viability of HeLa and K562 cells

PBMCs were seeded at a density of $1x10^4$cells per well into a 12 well plate and were treated with 200 µg/ml jacalin for 12 h, 24 h and 48 h.The CM from 12 h, 24 h and 48 h jacalin stimulated and unstimulated PBMCs were collected and filtered using a 0.45 µm syringe filter prior to storage at-80°C. HeLa cells, suspended in serum free DMEM, supplemented with 1% antibiotics were seeded at a density of 3×10^3 cells/well into a 96-well cell culture plate. After the cells were allowed to adhere, theywere treated with the 12 h, 24 h and 48 hJac-PBMC-CM, along with fresh medium in 1:1 ratio. Cell proliferation assay was done after 24 h of stimulation. Absorbance given by HeLa cells cultured in jacalin unstimulated PBMC-conditioned medium were considered as 100% cell growth and the viability of cells cultured in Jac-PBMC-CM were expressed as a fraction of those cells.

Likewise, to assess the effects of Jac-PBMC-CM on the viability of K562 cells, K562 cells were seeded at a density of $3x10^3$ cells/well into a 96-well cell culture plate and were treated with the 12 h, 24 h and 48 h Jac-PBMC-CM, along with fresh medium in 1:1 ratio. Cell proliferation assay was done after 24 h of stimulation. Absorbance given by K562 cells cultured in jacalin unstimulated PBMC-conditioned medium were considered as 100% cell growth and the viability of cells cultured in Jac-PBMC-CM were expressed as a fraction of those cells.

Effects of jacalin-stimulated cancer cell-conditioned medium on the viability of the PBMCs

HeLa cells or K562 cells were seeded at a density of $1x10^4$ cells per well into a 12 well plate and were treated with 200 µg/ml jacalin for 12 h, 24 h and 48 h. The CM from 12 h, 24 h and 48 h jacalin-stimulated and unstimulated HeLa cells and K562 cells were collected and filtered using a 0.45 µm syringe filter prior to storage at -80°C. PBMCs, seeded at a density of 3×10^3 cells/well into a 96-well cell culture plate were treated with the 12 h, 24 h and 48 h jacalin-stimulated HeLa-conditioned medium or with the 12 h, 24 h and 48 h jacalin-stimulated K562-conditioned medium along with fresh RPMI 1640 in 1:1 ratio. Cell proliferation assay was done after 24 h of stimulation. Absorbance given by PBMCs cultured in respective jacalin-unstimulated cancer cell-conditioned medium were considered as 100% cell growth and the viability of PBMCs cultured in jacalin-stimulated cancer cell-conditioned medium were expressed as a fraction of those cells.

Real time data analysis

The expression of the cytokines analysed were expressed as fold change ($2^{-\Delta\Delta Ct}$). The fold change was calculated with respect to cells that were allowed to grow under the same condition, in the absence of jacalin. β actin was used as the normalizing control.

Fold change = $2^{-\Delta\Delta Ct}$

Where, $\Delta\Delta Ct = (C_{t,\,Target} - C_{t,Actin})_{treated} - (C_{t,\,Target} - C_{t,Actin})_{untreated}$

Treated – refers to cells treated with jacalin for 6 h, 12 h or 24 h

Untreated – refers to cells that were allowed to grow in the absence of jacalin

[34].

Results And Discussion

Induction or suppression of the immune system using immunomodulatory agents is gaining importance as an approach in the treatment of cancer and other infectious diseases [26]. Plant lectin have long been recognized as immunomodulatory agents owing to their ability to recognize specific glycan moieties present on the surface of the immune cells [7]. The in vitro as well as in vivo immunomodulatory effects of lectins have been extensively studied [27–29].

Unlike other plant lectins such as Concanavalin A and phytohaemagglutinin that are general T cell mitogens, jacalin was known to selectively induce proliferation of $CD4^+$ cells. Likewise, the unique property of specifically binding to IgA1 subclass further enhancedthe biological significance of jacalin[30, 31]. In the present study, the immunomodulatory effects of jacalin on cytokine production from PBMCs

isolated from normal donor has been investigated. As jacalin is a dietary plant lectin, it was deemed significant to study the effects of jacalin on PBMCs, the key cytokine-secreting immune cells. It was anticipated that the cytokine expression profiles will reveal whether jacalin polarizes the immune cells in favour of or against tumorigenesis. When the effects of jacalin on the PBMC proliferation was analysed, it was observed that jacalin up to a concentration of 200 µg/ml had no effects, while at higher concentrations, jacalin was found to stimulate the proliferation of PBMCs (Fig. 1). In previous reports, 200 µg/ml was shown to be the optimum jacalin concentration that induced remarkable difference in PBMC activation [14, 20].

Cytokines produced by infiltrating mononuclear cells can affect the immune surveillance and can modulate the response of immune cells towards tumour growth. Likewise, certain cytokines can also retard tumour growth and proliferation. As cytokines such as IL-6, IFN-γ, TNF-α, TGF-β and IL-10 act as inflammatory mediators in modulating the growth of tumour cells, the mRNA expression of these cytokines in jacalin-stimulated PBMCs were analyzed.

IL-6 and TNF- α are multifunctional cytokines that are involved in malignant transformation and inflammation process. In the current study, a 7 fold increase in mRNA levels of IL-6 was observed in 6 h jacalin-treated PBMCs. However, the mRNA expression of IL-6 was found to decline to 2.9 fold and 2.6 fold in 12 h and 24 h jacalin-treated PBMCs, respectively [Fig. 2a (i)].Importantly, it has been documented that IL-6 in the tumour microenvironment can either procure tumour control by triggering anti-tumour T cell immune responses or promote tumour growth by increasing angiogenesis and conferring protection against immune surveillance[32]. However, no significant change in mRNA expression of TNF-α was observed in 6 h, 12 h and 24 h cells as compared to the respective controls[Fig. 2a (ii)].

Interestingly, a 42 fold increase in IFN-γ was observed in 6 h jacalin treated PBMCs and was found to decline to normal in 12 h and 24 h jacalin treated cells [Fig. 2a (iii)]. Increased production of IFN- γ by jacalin stimulated PBMCs has been reported before [21, 18]. However, the impact of the secreted IFN- γ is yet to be explored. IFN-γ is a signature Th1 cytokine that exerts significant antitumour activity. It is known to exhibit antitumour effects by activating the antitumour T cells and by regulating the invasion of T cells into the tumour tissues[33, 34].

While the mRNA expression of immunosuppressive cytokines IL-10 and TGF- β were analysed,about 11 fold increase inmRNA expression of TGF- β was observed in in 12 h and 24 h jacalin treated PBMCs [Fig. 2b (i)]. No significant change in mRNA expression of IL-10 was observed across the time points analysed[Fig. 2b (ii)]. While IL-10 is known to suppress T cell proliferation, the level of TGF- β has been found to be increased in advanced carcinomas and is positively correlated with cancer progression[35]. Apart from inhibiting the functions of inflammatory cells, TGF- β is also known to selectively inhibit the proliferation of PBMCs without affecting the tumour cell growth. Further, TGF- β supports tumour growth by stimulating angiogenesis and suppressing the immune response in the TME [36, 37].

The effects of jacalin stimulated and unstimulated PBMCs on HeLa cell proliferation were further assessed by directly co culturing HeLa cells with Jac-PBMCs. A significant decrease in cell proliferation

was observed in HeLa cells that were directly cultured with the 6 h Jac-PBMCs. Interestingly, an increase in cell proliferation was observed when the HeLa cells were directly cultured with the 24 h Jac-PBMCs (Fig. 3). But there was no significant change in proliferation of HeLa cells cultured in 12 h and 48 h Jac-PBMCs. Based on these preliminary results, it may be postulated that jacalin initially polarizes the PBMCs to hinder the tumour growth. Nevertheless, prolonged contact with jacalin reoriented the PBMCs in favour of tumour growth.

To assess the indirect effects of jacalin stimulated PBMCs on cancer cell growth, PBMCs were stimulated with 200 µg/ml jacalin for different time points and the CM were used to stimulate the cancer cells. A slight decrease in proliferation of HeLa cells, cultured in 12h Jac-PBMC-CM was observed. However, no significant change in cell proliferation was observed in HeLa cells cultured in 24 h and 48 h Jac-PBMC-CM (Fig. 4). Likewise, when the effects of Jac-PBMC-CM on K562 cell proliferation was assessed, no significant change in cell proliferation was observed in K562 cells cultured in 12 h, 24 h and 48 h Jac-PBMC-CM (Fig. 5). As tumours actively down regulate the anti-tumour immune response with different strategies, the possibility of tumour cells neutralizing the effects of IFN-γ in the 12 h PBMC-CM cannot be ruled out.

As diet plays a pivotal role in fortifying immune responses, it is important to gain knowledge about the immunomodulatory effects of lectins, which forms a formidable part of our daily diet. In the present study, the effects of jacalin on PBMC proliferation and cytokine production were studied. Further, the effects of jacalin-prestimulated PBMCs on cancer cell proliferation have been investigated. When the PBMCs were treated with jacalin for a shorter time point, increased mRNA expression of pro-inflammatory cytokine IFN-γ was observed. However, prolonged stimulation of PBMCs resulted in increased expression of anti-inflammatory cytokine, mainly TGF-β. PBMCs are known to modulate tumour-specific immune responses through secretion of cytokines. Also, as cytokines, regardless of their source can stimulate or inhibit tumour growth, the effects of jacalin-prestimulated PBMCs on the HeLa cell proliferation was assessed. While a significant decrease in cell proliferation was observed in HeLa cells that were directly cultured with the 6 h jacalin-stimulated PBMCs, an increase in cell proliferation was observed when the HeLa cells were directly cultured with the 24 h jacalin- stimulated PBMCs. Besides, CM obtained from the jacalin-treated PBMCs had no substantial effect on the viability of cancer cells. As jacalin is a dietary plant lectin, this observation can have particular significance under *in vivo* conditions.

Declarations

Funding: Not applicable.

Conflicts of interest/Competing interests: The authors declare no competing interests.

Availability of data and material: Not applicable

Code availability: Not applicable

Ethics approval:

This study was approved by the Institutional ethical committee of B.S.A.C.I.S.T. (BSAU: REG-OFF: 2015/01SLS).

Authors' contributions

SJ and NA conceived and designed the methodology and research. LV, AKB conducted the experiments and analyzed the data. LV wrote the manuscript and SJ, NA analyzed the final results. All the authors read and approved the manuscript.

Consent to participate: Not applicable.

Consent for publication: Not applicable.

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Figures

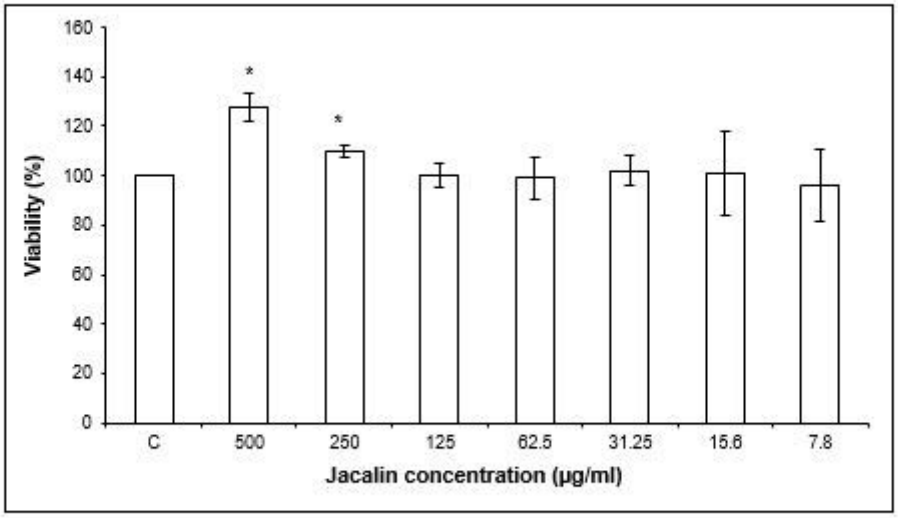

Figure 1

Effects of jacalin on viability of peripheral blood mononuclear cells. Cells were incubated in the presence of various concentrations of jacalin for 24 h and the viable cells were estimated by MTT assay. Cells without treatment were considered as 100% viable and the viability of the cells were expressed as a fraction of untreated cells. Data represent the average of three parallel wells and one of the three independent experiments. Error bar represent standard deviations. *P<0.05 compared with control.

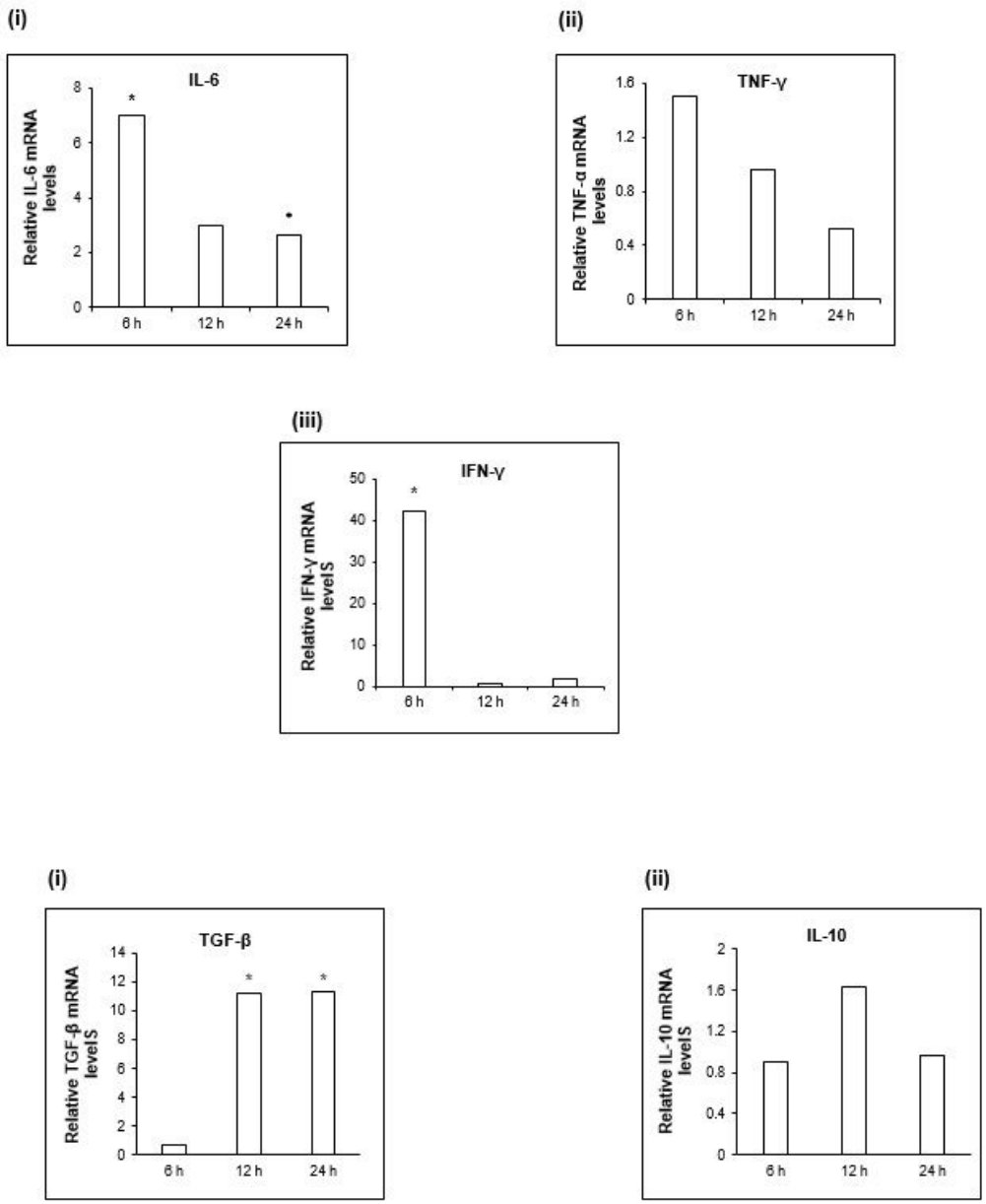

Figure 2

2aReal time mRNA expression of pro inflammatory cytokines. PBMCs were treated with jacalin (200 µg/ml) for 6 h, 12 h and 24 h and the relative mRNA levels of the cytokines IL-6, TNF-α and IFN-γ, were analyzed with respect to the untreated control. Bar graph represent the fold change of mRNA levels quantified by normalization to the β-actin as an internal control. Error bars on the graph indicate the standard deviation from triplicate wells. * $p<0.05$. Fig. 2b Real time mRNA expression of anti-

inflammatory cytokines. PBMCs were treated with jacalin (200 µg/ml) for 6 h, 12 h and 24 h and the relative mRNA levels of the cytokines IL-10 and TGF-β were analyzed with respect to the untreated control. Bar graph represent the fold change of mRNA levels quantified by normalization to the β-actin as an internal control. Error bars on the graph indicate the standard deviation from triplicate wells. * p<0.05.

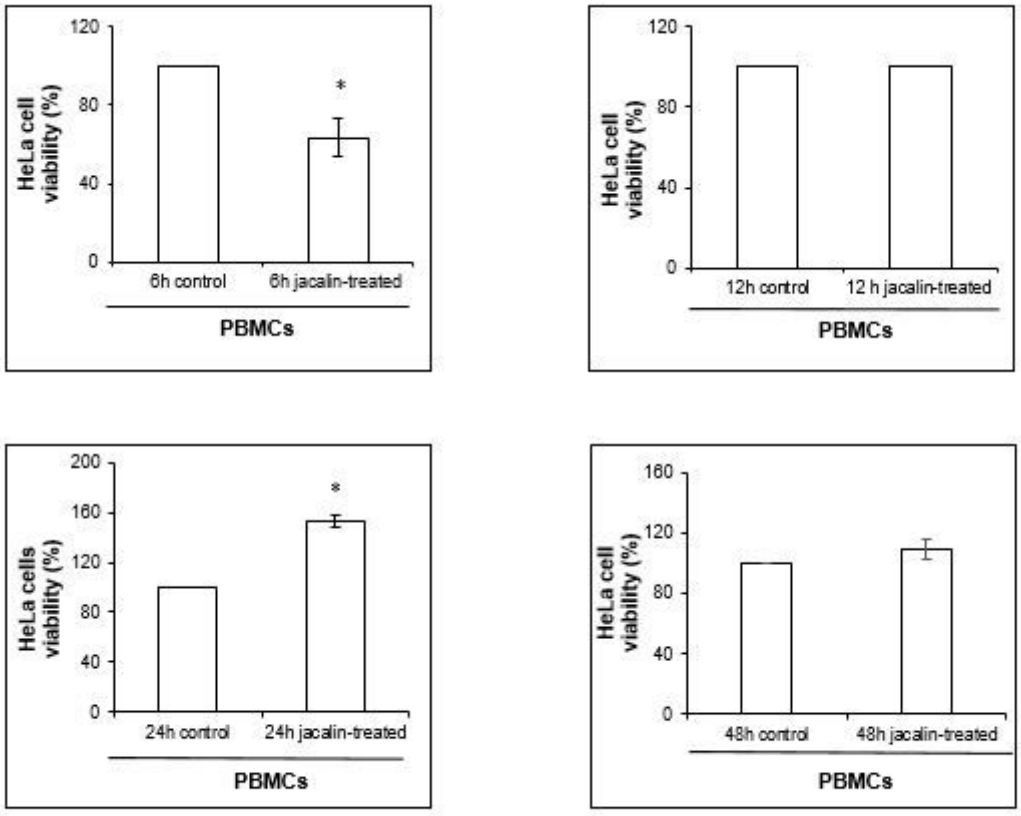

Figure 3

Effects of Jac-PBMCs on HeLa cell proliferation. PBMCs were treated with jacalin for 6, 12, 24 and 48 h, cells were pelleted and suspended in fresh media. HeLa cells were treated with the the jacalin stimulated and unstimulated PBMCs. Cell proliferation assay was done after 12 h of stimulation. HeLa cells treated with the respective control PBMCs were taken as control and the viability of HeLa cells cultured in Jac-PBMCs were expressed as a fraction of the control cells.

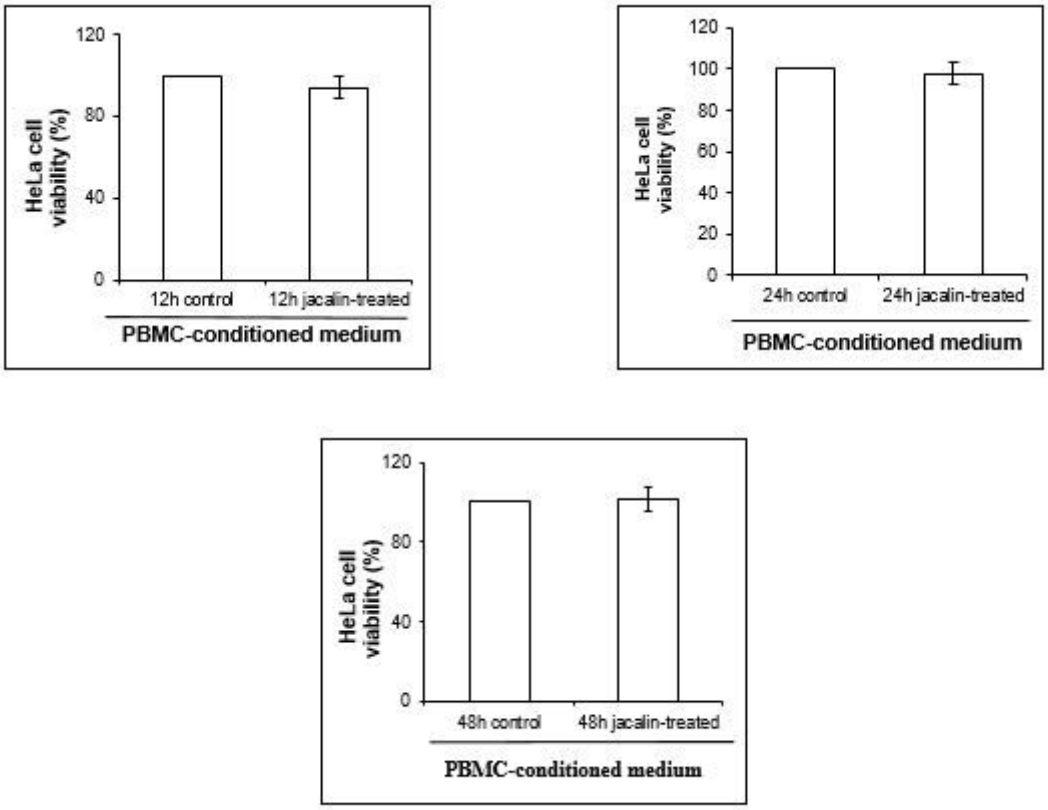

Figure 4

Effects of Jac-PBMC-CM on HeLa cell proliferation. PBMCs were treated with jacalin for 12, 24 and 48 h, CM were collected and filtered. HeLa cells were treated with the CM, diluted with fresh media in 1:1 ratio. Cell proliferation assay was done after 24 h of stimulation. HeLa cells cultured in CM collected from jacalin untreated PBMCs were taken as control and the viability of cells cultured in Jac-PBMC-CM were expressed as a fraction of control cells.

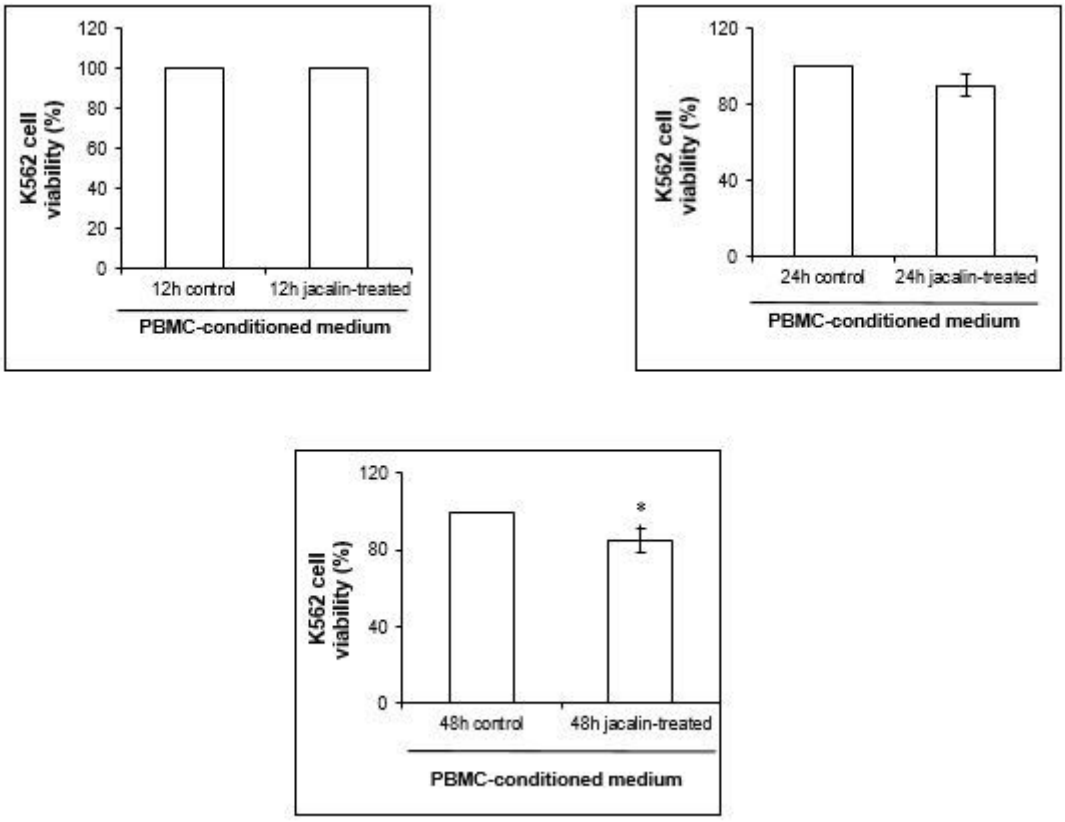

Figure 5

Effects of Jac-PBMC-CM on K562 cell proliferation. PBMCs were treated with jacalin for 12, 24 and 48 h, CM were collected and filtered. K562cells were treated with the CM, diluted with fresh media in 1:1 ratio. Cell proliferation assay was done after 24 h of stimulation. K562 cells cultured in CM collected from jacalin untreated PBMCs were taken as control and the viability of cells cultured in Jac-PBMC-CM were expressed as a fraction of control cells.