

6. IMMUNOMODULATORY EFFECTS OF JACALIN ON THE PBMCs

6.1 INTRODUCTION

The effects of lectins on proliferation of cancerous cells and on the production of cytokines has been the focus of numerous studies. However, several levels of crosstalk occurs between the tumor cells and surrounding immunological microenvironment [325]. Hence, it is significant to shed light on the response of immune cells to lectin treatment so as to understand the effects of lectins on the modulation of immune responses. Henceforth, the immunomodulatory effects of jacalin on the PBMCs will be discussed in this chapter.

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 [326]. Plant lectins have long been recognized as immunomodulatory agents owing to their ability to recognize specific glycan moieties present on the surface of the immune cells [8]. They have been shown to modulate immune mechanisms including effector functions and inflammatory reactions. The *in vitro* as well as *in vivo* immunomodulatory effects of lectins have been extensively studied [327-329].

6.1.1 The tumor microenvironment (TME)

The TME refers to the tumor's surrounding and includes the rapidly proliferating tumor cells, the tumor stroma, blood vessels and other inflammatory cells [330]. 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 a few B cells [331]. The inflammatory cells in the TME either interfere with the tumor growth resulting in tumor regression or can facilitate tumor progression. The tumor cells, apart

from escaping the host immune surveillance, also actively modify the functions of the infiltrating effector cells resulting in an environment favorable for tumor growth and progression [332].

Peripheral blood mononuclear cells (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) tumor-specific immune responses through secretion of cytokines. The cytokines, regardless of their source, can stimulate or inhibit tumor growth [328]. Differential levels of cytokines in the peripheral blood are implicated in pathogenesis of numerous diseases including cancer. The changes in cytokine profile in response to a drug/compound will provide better knowledge as to whether the ensuing immune response will be detrimental or beneficial to the host.

6.1.2 Effects of lectins on the immune cells

Lectins are capable of cross-linking glycoproteins present on the immune cell surface, resulting in their proliferation and activation [333,334]. Such lectin induced activation of immune cells can trigger downstream signal transduction resulting in production of cytokines [8]. The secreted cytokines have been reported to be implicated in modulation of cancer cell growth and proliferation [335,336]. Unlike other plant lectins such as Con A and PHA 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 enhanced the biological significance of jacalin [337,338]. Previous studies on effects of jacalin on proliferation of PBMCs yielded contradictory results. Jacalin was initially reported to induce proliferation of T cells as well as B cell terminal differentiation [339]. In a later study, jacalin was reported to induce proliferation of T cells alone but inhibited production of immunoglobulins (Ig) by the B cells [340]. Among the T cell subsets, jacalin was shown to be a general T cell mitogen, inducing the proliferation of CD4⁺ and CD8⁺ T cells [341]. However, various other studies reported jacalin to specifically induce proliferation of CD4⁺ T helper (Th) cells

alone [246,259,342]. Interestingly, another study reported that the PBMCs did not possess any proliferative response to jacalin treatment [343]. 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 [344]. CD45, a protein tyrosine phosphatase is highly glycosylated and is considered as a major regulator of lymphocyte signaling [345].

Likewise, the production of interleukin-2 (IL-2) by jacalin-stimulated PBMCs 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 [259,346], another study reported IL-2 to be absent in the culture supernatants of jacalin-stimulated PBMCs. Further, IL-2 mRNA was also found to be absent, as determined by Northern blot analysis [347].

Such discrepancy in the mitogenic effects and binding specificity of jacalin may be attributed to the geographic variability [348]. 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 [343]. Thus, the immunomodulatory effects of purified jacalin demands further investigation.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Dihydrogen monosodium phosphate, disodium hydrogen phosphate, sodium chloride, MTT and sodium hydroxide were purchased from Sisco research laboratories (SRL, Maharashtra, India), Lymphoprep was purchased from Stem cell technologies, RPMI 1640 and DMEM powder were purchased from Himedia and prepared according to the manufacturer's protocol, FBS and trypsin-EDTA were purchased from Sigma-Aldrich Co.

6.2.2 Cell lines and Cell culture

K562 cells were obtained from NCCS, Pune and maintained in RPMI 1640, supplemented with 10% heat inactivated FBS and 1% antibiotics (penicillin, streptomycin) and HeLa cervical cancer cells were maintained in DMEM containing 10% heat inactivated FBS and 1% antibiotics (penicillin, streptomycin). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

6.2.3 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 PBMCs isolation. The blood was diluted with equal volumes of RPMI 1640 medium. 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 containing 10% FBS and 1% antibiotic (penicillin, streptomycin) and cultured at 37°C in a humidified atmosphere containing 5% CO₂.

6.2.4 MTT assay

The proliferative response of PBMCs to jacalin treatment was determined via the MTT tetrazolium assay. PBMCs, suspended in RPMI 1640 media, supplemented with 1% FBS and 1% antibiotics were seeded at a density of 3×10^3 cells/well into 96-well cell culture plates. The PBMCs were cultured in the presence of jacalin (500 to $7.8 \mu\text{g ml}^{-1}$). After the plate was incubated at 37°C for 24 h, 25 μl of MTT (5 mg ml^{-1} in PBS) was added to all the wells kept at 37°C for 4 h in dark. The formazan crystals allowed to solubilize in 100 μl of DMSO and left at RT for 30 mins.

6.2.5 Quantification of mRNA expression of cytokines in PBMCs

PBMCs were seeded at a density of 1×10^6 cells per well into a 6 well plate. Cells were then treated with 200 $\mu\text{g/ml}$ jacalin for 6 h, 12 h and 24 h, lysed with TRIzol reagent and RNA was isolated as described in chapter 4. 5 μg of total RNA was reverse transcribed to cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, CA), as per the manufacturer's protocol.

Quantitative real time PCR (qRT-PCR) was performed using Hi-Sybr Master mix (MBT074) as discussed previously in chapter 4. The list of primers are given in Table 6.1.

Table 6.1 List of primers used for quantification of mRNA expression of cytokines

Gene	Primer sequences (5'-3')	
	Forward primer	Reverse primer
Actin	GGACTTCGAGCAAGAGATGG	AGGAAGGAAGGCTGGAAGAG
IL-6	CCACCGGGAACGAAAGAGAA	TCTTCTCCTGGGGGTACTGG
TNF- α	TGCTTGTTCCCTCAGCCTCTT	GGTTTGCTACAACATGGGCT
IFN- γ	GGCAGCATCGCTTTAAACTC	GGAGGTGGGGGCTTTTATTA
TGF- β	GCAGAACCCAAAAGCCAGAGT	AGTTGGAGGTGCCATCAATACC
IL-10	GCCTAACATGCTTCGAGATC	CTCATGGCTTTGTAGATGCC

6.2.6 Effects of jacalin-prestimulated PBMCs on the viability of HeLa cells

PBMCs, suspended in DMEM containing 1% FBS and 1% antibiotics were seeded at a density of 1×10^4 cells in each well of a 12 well plate. 200 $\mu\text{g/ml}$ jacalin was added to the cells and incubated. After 6, 12, 24 and 48 h, the cells were collected, pelleted and the supernatant was discarded. The cell pellet was washed with sterile PBS, suspended in DMEM 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. 6 h, 12 h, 24 h and 48 h Jacalin-prestimulated and control PBMCs were added onto the adhered HeLa cells. MTT tetrazolium 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 jacalin-stimulated PBMCs were calculated accordingly.

6.2.7 Effects of jacalin-stimulated PBMC-conditioned medium on the viability of HeLa cells

PBMCs, suspended in RPMI 1640 containing 1% FBS and 1% antibiotics were seeded at a density of 1×10^4 cells in each well of a 12 well plate. 200 $\mu\text{g/ml}$ jacalin was added to the cells and incubated. After 12, 24 and 48 h, the CM from jacalin-stimulated and unstimulated PBMCs were collected and filtered using a 0.45 μm syringe filter prior to storage at -80°C . HeLa cells were suspended in serum-free DMEM, supplemented with 1% antibiotics and 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, they were treated with the 12 h, 24 h and 48 h jacalin-stimulated PBMC-conditioned medium, along with fresh DMEM 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 jacalin-stimulated PBMC-conditioned medium were calculated accordingly.

6.2.8 Effects of jacalin-stimulated PBMC-conditioned medium on the viability of K562 cells

PBMCs, suspended in RPMI 1640 containing 1% FBS and 1% antibiotics were seeded at a density of 1×10^4 cells per well into a 12 well plate. 200 $\mu\text{g/ml}$ jacalin was added to the cells and incubated. After 12, 24 and 48 h, the CM from jacalin-stimulated and unstimulated PBMCs were collected and filtered using a 0.45 μm syringe filter prior to storage at -80°C . K562 cells were seeded at a density of 3×10^3 cells/well into a 96-well cell culture plate and were treated with the 12 h, 24 h and 48 h jacalin-stimulated PBMC-conditioned medium, along with fresh RPMI 1640 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 jacalin-stimulated PBMC-conditioned medium were calculated accordingly.

6.2.9 Effects of jacalin-stimulated HeLa-conditioned medium on the viability of the PBMCs

HeLa cells were seeded at a density of 1×10^4 cells per well into a 12 well plate. 200 $\mu\text{g/ml}$ jacalin was added to the cells and incubated. After 12, 24 and 48 h, the CM from jacalin-stimulated and unstimulated HeLa 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, 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 jacalin-unstimulated HeLa-conditioned medium were considered as 100% cell growth and the viability of PBMCs cultured in jacalin-stimulated HeLa-conditioned medium were were calculated accordingly.

6.2.10 Effects of jacalin-stimulated K562-conditioned medium on the viability of the PBMCs

K562 cells were seeded at a density of 1×10^4 cells per well into a 12 well plate. 200 $\mu\text{g/ml}$ jacalin was added to the cells and incubated. After 12, 24 and 48 h, the CM from jacalin-stimulated and unstimulated 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 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 jacalin-unstimulated K562-conditioned medium were considered as 100% cell growth and the viability of PBMCs cultured in jacalin-stimulated K562-conditioned medium were expressed as a fraction of those cells.

6.3 RESULTS AND DISCUSSION

6.3.1 Effects of jacalin on the proliferation of PBMCs

When the effects of jacalin on the PBMC proliferation was analyzed, it was observed that jacalin up to a concentration of 200 $\mu\text{g/ml}$ had no effects while at higher concentrations, jacalin was found to stimulate the proliferation of PBMCs (figure 6.1). Also, in previous reports, 200 $\mu\text{g/ml}$ was shown to be the optimum jacalin concentration that induced remarkable difference in PBMC activation [342,346].

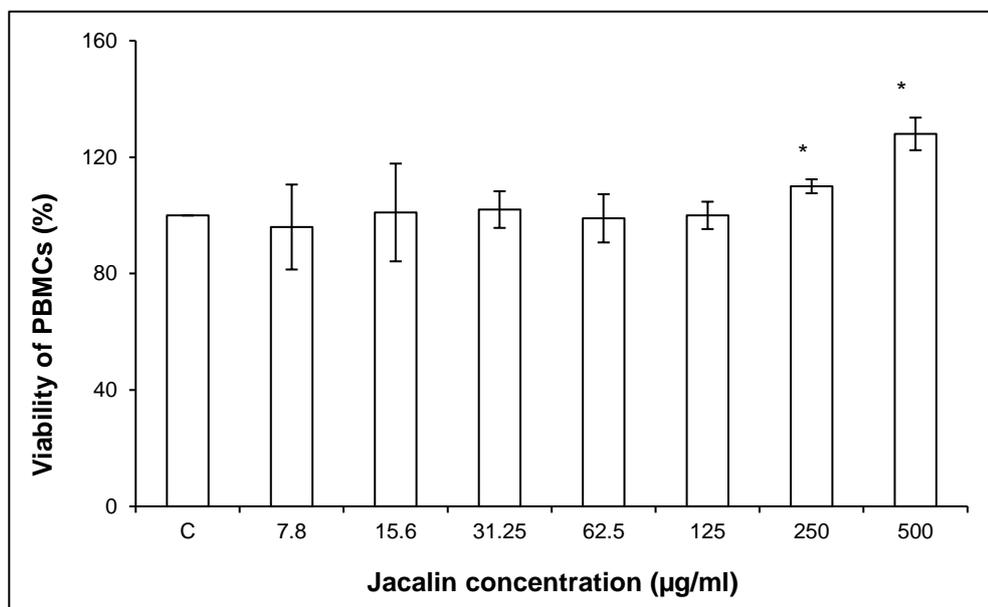


Figure 6.1 Effects of jacalin on the viability of PBMCs. PBMCs were cultured with various concentrations of jacalin (500-7.8 $\mu\text{g/ml}$). After 24 h, the viability percentage of the cells were determined by MTT assay. Untreated cells containing 1% FBS were regarded as 100% cell growth and the viability percentage of the treated cells were calculated accordingly. Error bars indicate the standard deviations. * $p \leq 0.05$.

6.3.2 Relative mRNA expression of cytokines in jacalin-treated PBMCs

Cytokines produced by infiltrating mononuclear cells can affect the immune surveillance and can modulate the response of immune cells towards tumor growth. Likewise, certain cytokines can also retard transforming 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 tumor 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 [figure 6.2a (i)]. Importantly, it has been documented that IL-6 in the tumor microenvironment can either procure tumor control by triggering anti-tumor T cell immune responses or promote tumor growth by increasing angiogenesis and conferring protection against immune surveillance [349]. However, no significant change in mRNA expression of TNF- α was observed in 6 h, 12 h and 24 h jacalin-treated PBMCs as compared to the respective controls [figure 6.2a (ii)].

Interestingly, a 42 fold increase in IFN- γ was observed in 6 h jacalin-treated PBMCs [figure 6.2a (iii)]. Increased production of IFN- γ by jacalin-stimulated PBMCs has been reported before [344,347]. However, the impact of the secreted IFN- γ is yet to be explored. IFN- γ is a signature Th1 cytokine that exerts significant antitumor activity. Also, it is known to exhibit antitumor effects by activating the antitumor T cells and by regulating the invasion of T cells into the tumor tissues [350,351].

While the mRNA expression of immunosuppressive cytokines IL-10 and TGF- β were analyzed, about 11 fold increase in mRNA expression of TGF- β was observed in in 12 h and 24 h jacalin-treated PBMCs [figure 6.2b (i)]. Nevertheless, no significant change in mRNA expression of IL-10 was

observed across the time points analyzed [figure 6.2b (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 [352]. Apart from inhibiting the functions of inflammatory cells, TGF- β is also known to selectively inhibit the proliferation of PBMCs without affecting the tumor cell growth. Further, TGF- β supports tumor growth by stimulating angiogenesis and suppressing the immune response in the TME [353,354].

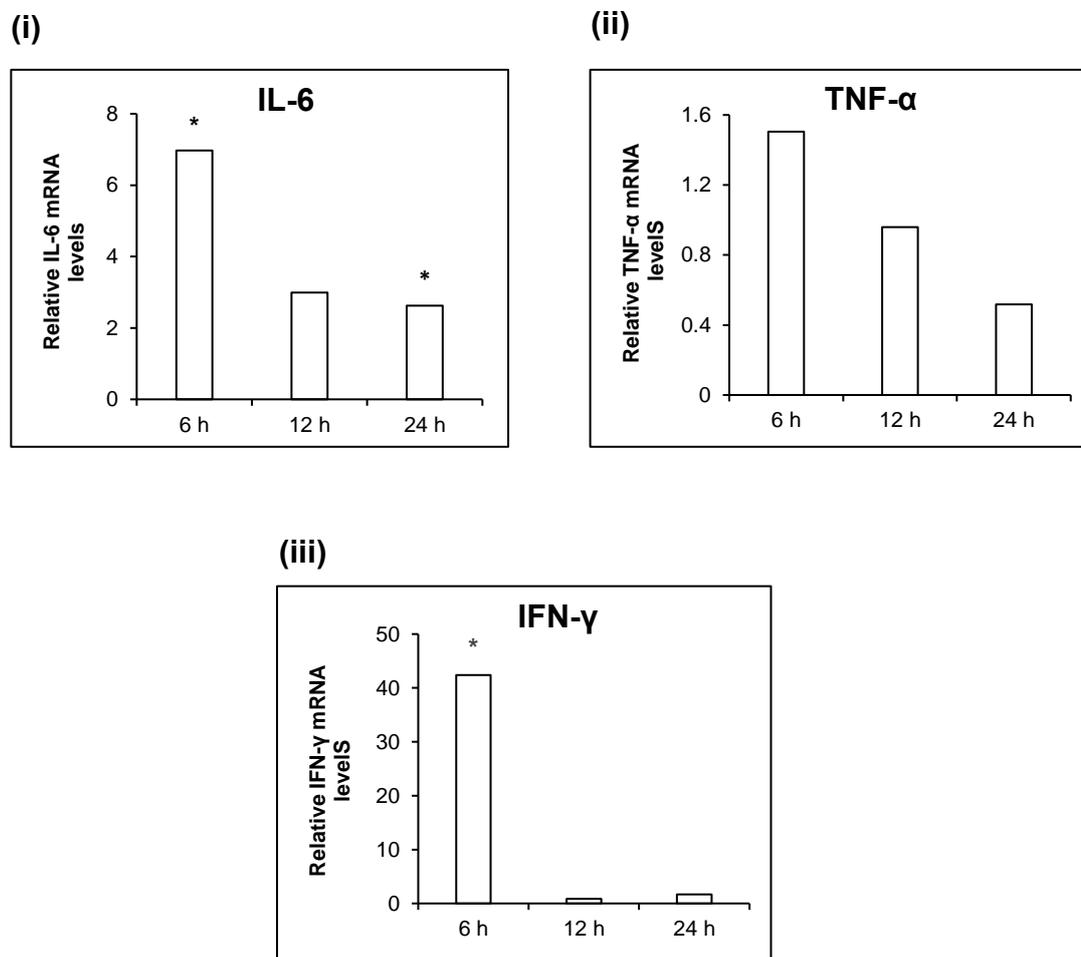


Figure 6.2a Relative mRNA levels of pro-inflammatory cytokines. PBMCs were cultured with 200 μ g/ml jacalin. After 3 h and 6 h of treatment, the mRNA expression of cytokines (i) IL-6 (ii) TNF- α and (iii) IFN- γ were analyzed. Bar graph denotes the fold changes of β -actin serves as the housekeeping gene. * $p \leq 0.05$.

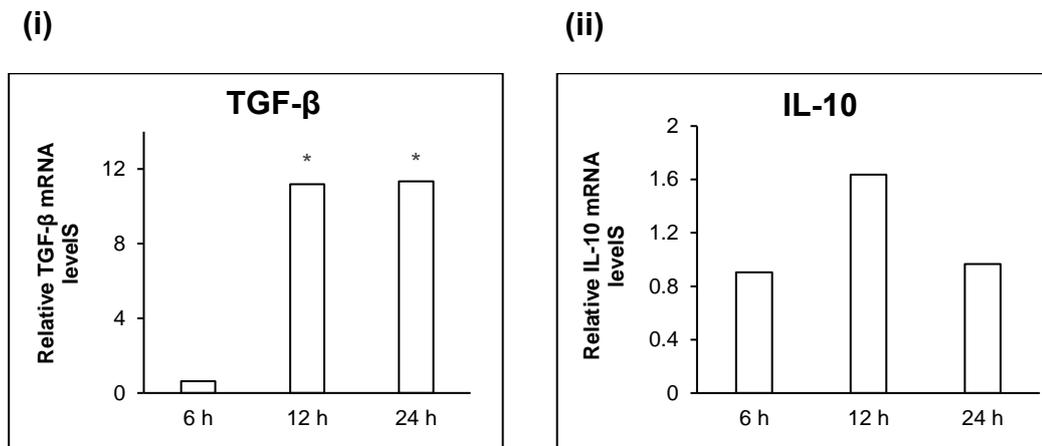


Figure 6.2b Relative mRNA levels of anti-inflammatory cytokines. PBMCs were cultured with 200 μg/ml jacalin. After 3 h and 6 h of treatment, the mRNA expression of cytokines (i) TGF-β and (ii) IL-10 were analyzed. Bar graph denotes the fold changes of β-actin serves as the housekeeping gene. *p≤0.05.

6.3.3 Effects of jacalin-prestimulated PBMCs on the viability of HeLa cells

The effects of jacalin-stimulated and unstimulated PBMCs on the HeLa cell proliferation were further assessed by directly co-culturing HeLa cells with jacalin-stimulated PBMCs. A significant decrease in cell proliferation was observed in HeLa cells that were directly cultured with the 6 h jacalin-stimulated PBMCs. In contrast, an increase in cell proliferation was observed when the HeLa cells were directly cultured with the 24 h jacalin-stimulated PBMCs (figure 6.3). But there was no significant change in proliferation of HeLa cells cultured in 12 h and 48 h jacalin-stimulated PBMCs. Based on these preliminary results, it may be postulated that jacalin initially polarizes the PBMCs to hinder the tumor growth. Nevertheless, it is possible that, prolonged contact with jacalin reoriented the PBMCs in favor of tumor growth.

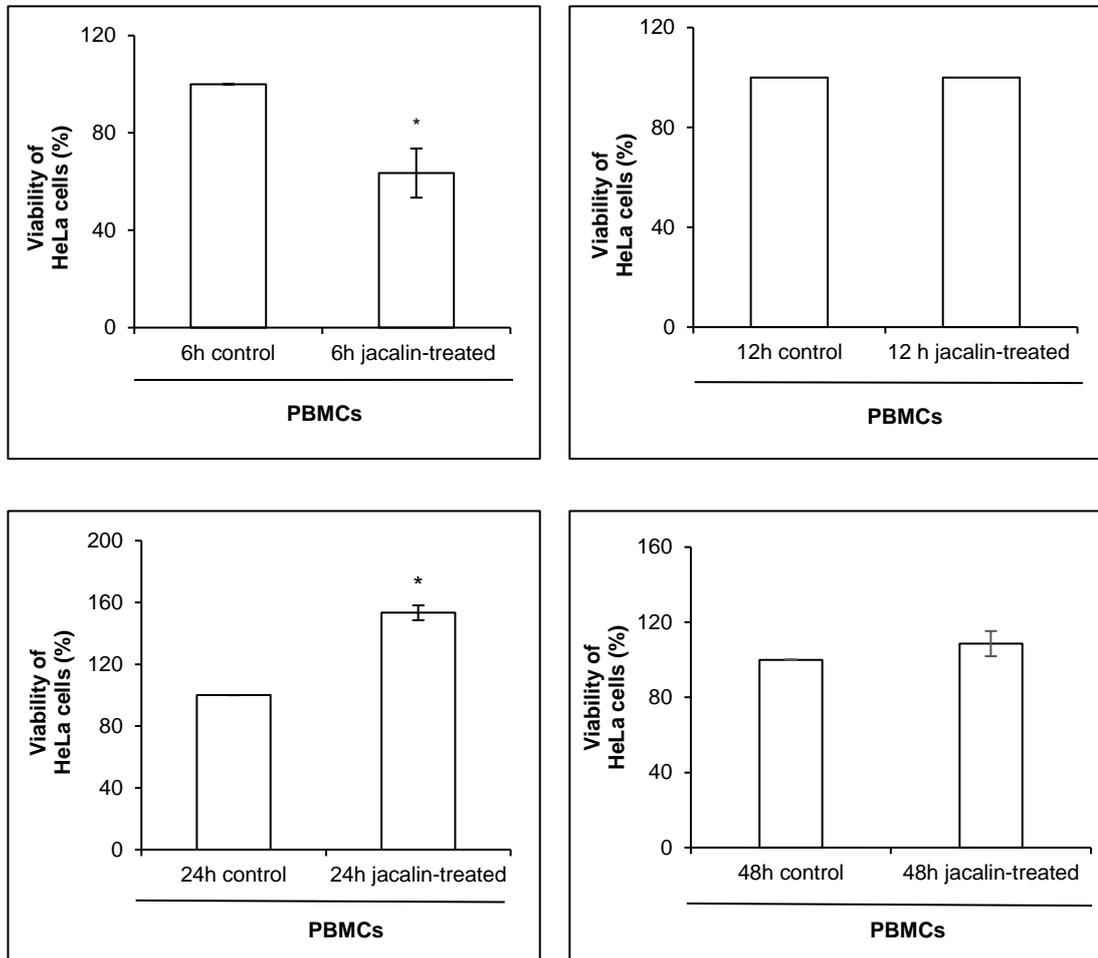


Figure 6.3 Effects of jacalin-stimulated PBMCs on the HeLa cell proliferation. Cell proliferation assay was done after 12 h of stimulating the HeLa cells with 6, 12, 24 and 48 h jacalin-stimulated PBMCs. Absorbance given by the HeLa cells cultured in jacalin unstimulated PBMCs were considered as 100% cell growth and the viability of cells cultured in jacalin-stimulated PBMCs were calculated accordingly. Error bars denote the standard deviations. * $p \leq 0.05$.

6.3.4 Effects of conditioned medium from jacalin-stimulated PBMCs on the viability of cancer cells

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 12 h jacalin-stimulated PBMC-conditioned medium was observed. However, no significant change in cell proliferation was observed in HeLa cells cultured in 24 h and 48 h jacalin-stimulated PBMC-conditioned medium (figure 6.4a).

Likewise, when the effects of jacalin-stimulated PBMC-conditioned medium on K562 cell proliferation was assessed, no significant change in cell proliferation was observed in K562 cells cultured in 12 h and 24 h jacalin-stimulated PBMC conditioned medium; however, a significant decrease in cell viability was observed in K562 cells cultured in 48 h jacalin-stimulated PBMC-conditioned medium as compared to the respective controls (figure 6.4b). As tumors actively down regulate the anti-tumor immune response with different strategies, the possibility of tumor cells neutralizing the effects of IFN- γ in the 12 h jacalin-stimulated PBMC conditioned medium cannot be ruled out.

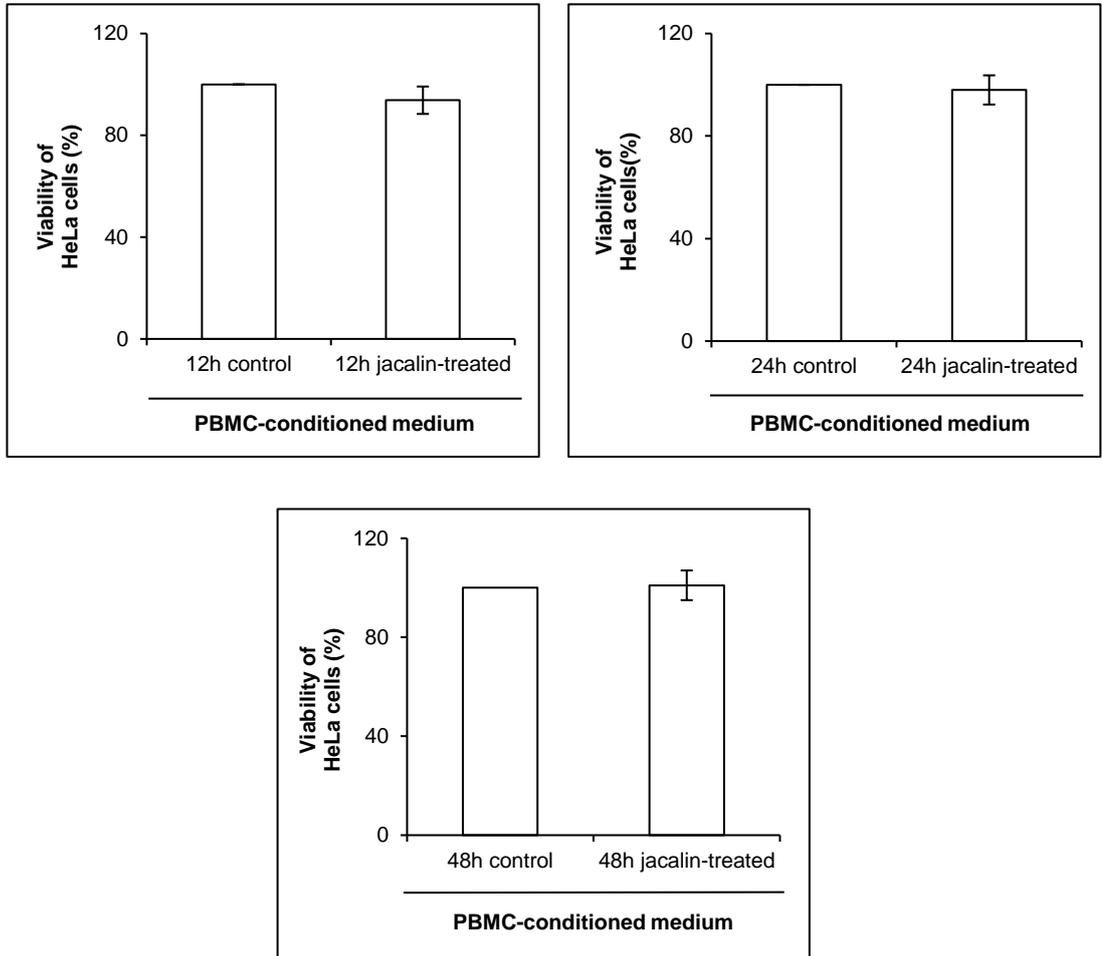


Figure 6.4a Effects of jacalin-stimulated PBMC-conditioned medium on the viability of HeLa cells. MTT assay was done after 24 h of stimulating the HeLa cells with 12 h, 24 h and 48 h jacalin-stimulated PBMC-conditioned medium. Absorbance given by HeLa cells cultured in jacalin-unstimulated PBMC-conditioned medium were considered as 100% cell growth and the viability of HeLa cells cultured in jacalin-stimulated PBMC-conditioned medium were calculated accordingly. Error bars denote the standard deviations. * $p \leq 0.05$.

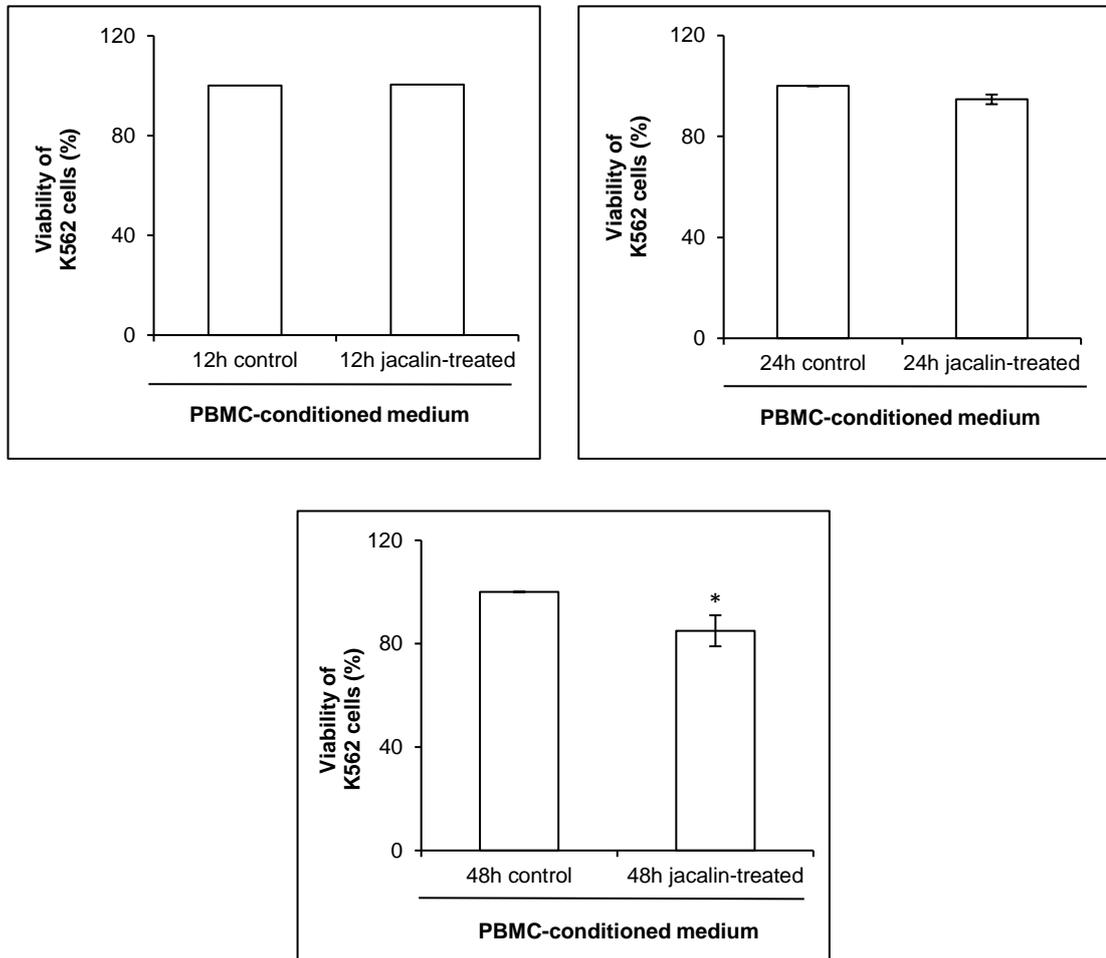


Figure 6.4b Effects of jacalin-stimulated PBMC-conditioned medium on the viability of K562 cells. MTT assay was done after 24 h of stimulating the K562 cells with 12, 24 and 48 h jacalin-stimulated PBMC-conditioned medium. Absorbance given by K562 cells cultured in jacalin-unstimulated PBMC-conditioned medium were considered as 100% cell growth and the viability of K562 cells cultured in jacalin-stimulated PBMC-conditioned medium were calculated accordingly. Error bars denote the standard deviations. * $p \leq 0.05$ as compared with control.

6.3.5 Effects of conditioned medium from jacalin-stimulated HeLa cells on the viability of PBMCs

As shown in figure 6.5a, a marked decrease in proliferation of PBMCs that were cultured in 24 h jacalin-stimulated HeLa-conditioned medium was observed, as compared with the cells cultured in jacalin untreated HeLa-conditioned medium. Likewise, while a slight decrease in proliferation was observed in PBMCs cultured in 12 h jacalin-stimulated HeLa-conditioned medium, no significant change in cell proliferation was observed in PBMCs cultured in 48 h jacalin-stimulated HeLa-conditioned medium, as compared with the cells cultured in the respective jacalin-unstimulated HeLa-conditioned medium.

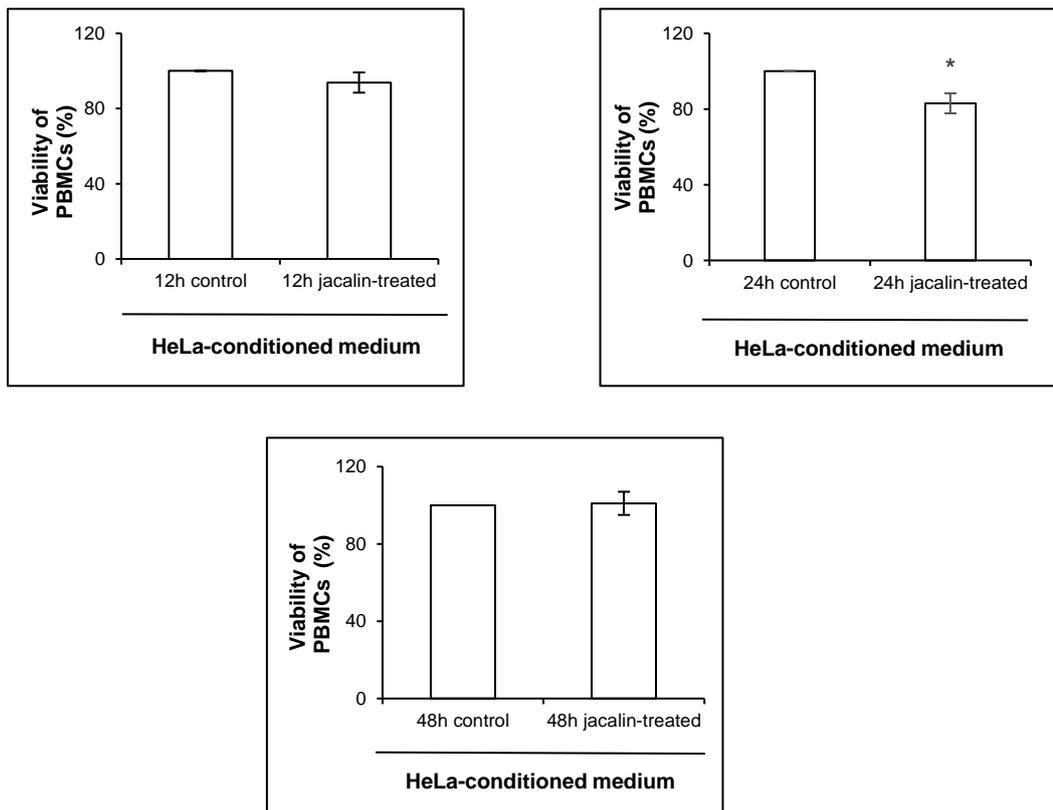


Figure 6.5 Effects of jacalin-stimulated HeLa-conditioned medium on the viability of PBMCs. MTT assay was done after 24 h of stimulating the PBMCs with the jacalin-treated HeLa-conditioned medium. Absorbance given by PBMCs cultured in jacalin-unstimulated HeLa-conditioned medium were considered as 100% cell growth and the viability of PBMCs cultured in jacalin-stimulated HeLa-conditioned medium were calculated accordingly. Error bars denote the standard deviations. * $p \leq 0.05$ as compared with control.

6.3.6 Effects of conditioned medium from jacalin-stimulated K562 cells on the viability of PBMCs

When the effects of jacalin-stimulated K562-conditioned medium on PBMC proliferation were assessed, a significant decrease in viability of PBMCs cultured in 48 h jacalin-stimulated K562-conditioned medium was observed, while, no remarkable change in proliferation was observed in PBMCs cultured in 12 h and 24 h jacalin-stimulated K562-conditioned medium (figure 6.5b).

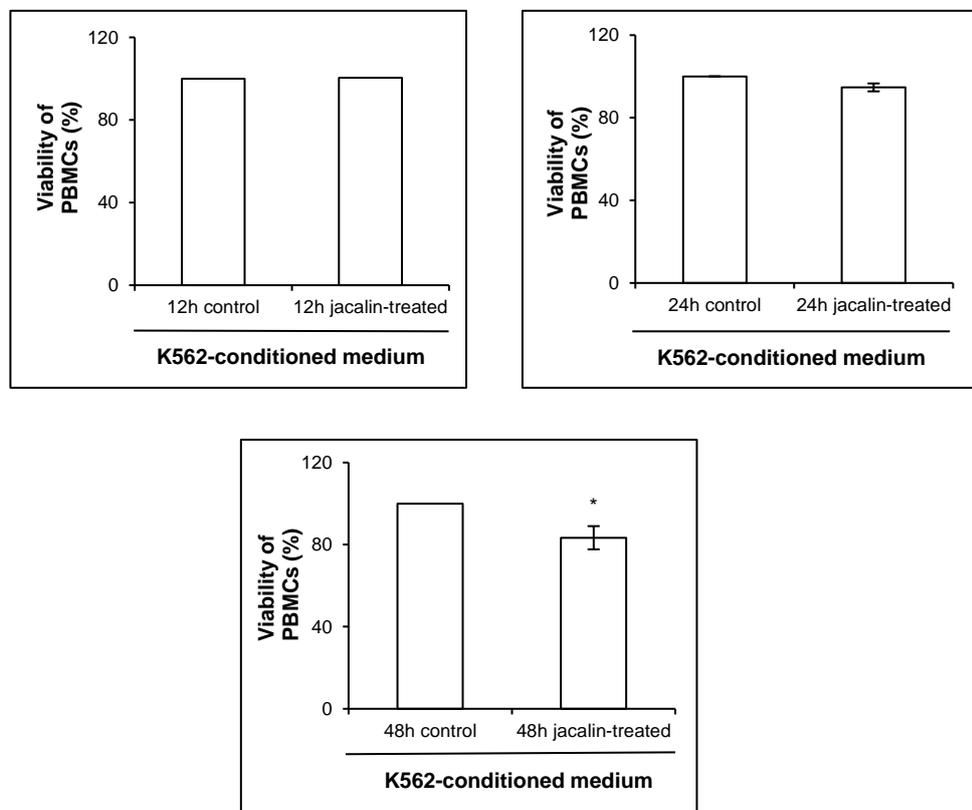


Figure 6.6 Effects of jacalin-stimulated K562-conditioned medium on the viability of PBMCs. MTT assay was done after 24 h of stimulating the PBMCs with the jacalin-treated K562-conditioned medium. Absorbance given by PBMCs cultured in jacalin-unstimulated K562-conditioned medium were considered as 100% cell growth and the viability of PBMCs cultured in jacalin-stimulated K562-conditioned medium were calculated accordingly. Error bars denote the standard deviations. * $p \leq 0.05$.

6.4 CONCLUSION

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. The current chapter provides a detailed account of the effects of jacalin on PBMC proliferation and cytokine production. 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 tumor-specific immune responses through secretion of cytokines. Also, as cytokines, regardless of their source can stimulate or inhibit tumor growth, the effects of jacalin-prestimulated PBMCs on the HeLa cell proliferation was assessed. While a significant decrease in cell growth was detected 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.