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**ORIGINAL ARTICLE**

# Effect of Cytomegalovirus Recombinant Phosphoprotein 150 (pp150) on Function and Maturation of Murine Dendritic Cells: an *In-Vitro* Study

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## ABSTRACT

**Background:** Tegument protein pp150 of *cytomegaloviruses* (CMVs) plays a vital role in all stages of viral life cycle, representing the most important tegument protein candidate for HCMV treatment. However, the exact role of pp150 in immune regulation is yet to be elucidated. **Objective:** To examine the effects of pp150 on the maturity and function of murine dendritic cells (DCs). **Methods:** Maturity status (CD40, CD86, and MHC-II expression) and phagocytic capacity of DCs (dextran uptake assay) were characterized. Gene expression profiles of ROR-γ, GATA-3, T-bet, and FOXP-3 as well as the protein expression of INF-γ (Th1), IL-4 (Th2), IL-35 (Treg), IL-17A (Th17), IL-22, TNF-α, IL-6, and IL-2 were evaluated in T cells co-cultured with DCs. **Results:** A significant increase in CD40, CD86, and CCR7 expression and a reduction in the phagocytosis rate were observed in pp150-stimulated DCs compared with unstimulated DCs. T cells co-cultured with stimulated DCs showed higher expressions of ROR-γ, IL-6, IL-2, IL-17A, IL-22, and TNF-α. **Conclusion:** Despite improvements in maturity status, pp150-stimulated DCs does not seem to be able to induce Th1 or Th2 immunity. In fact, Th17 and its mediators, IL-17A and IL-22, might be the main inflammatory factors involved in pp150-stimulated DC's action mechanism. However, it is necessary to conduct further investigations to corroborate these observations.

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**Keywords: Dendritic Cell Maturation, Cytomegalovirus, pp150**

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## INTRODUCTION

Human *Cytomegalovirus* (HCMV) belongs to the *Herpesviridae* family, which persistently infects individuals and entails deaths in patients following solid organ transplantations (1). In high risk individuals, HCMV is able to have a lifelong persistence through controlling the immune system owing to evasion strategies interfering the host's immune response. In these patients, primary HCMV infections are generally asymptomatic with virus latently persisting throughout life (2). As a persistent pathogen, HCMV employs a variety of strategies to prevent or postpone the induction of particular immune responses. Among these, conditions such as acute phase refer to the ability of HCMV to infect and induce a variety of immune cells, including monocytes, macrophages, endothelial cells, epithelial cells, and dendritic cells (DCs) as professional antigen-presenting cells (APCs) (3-5). More importantly, both innate and acquired immune systems are involved during HCMV infections. Natural killer (NK) cells have a key role in HCMV infection through recognizing and killing the infected cells. The activity of NK and T $CD8^+$ cells is highly dependent on the function of DCs and macrophages (6). DCs play a crucial part in inducing and regulating adaptive immune responses. Virus infection induces the maturation of DCs, allowing them to professionally present antigens to T cells to prime and activate immune responses (7). Nevertheless, HCMV interferes with host immune responses through damaging a variety of DC functions, exerting a variety of mechanisms, including maturation inhibition (8,9). Based on previous studies, CMV infection could down-regulate antigen presentation through MHCs I and II as well as the lower T-cell-inducing ability of infected DCs, presumably interfering with cytokine and chemokine production (10-12). It was shown that $CD8^+$ cytotoxic T cells (CTLs) and T helper (Th), as antigen-specific T cells induced by infected or peptide-pulsed DCs, were able to efficiently induce $CD8^+$ and $CD4^+$ mediated responses, thereby providing an important strategy to stimulating T cells (13,14). Accordingly, these cells have a central role in inhibiting lethal infections and controlling CMV. T cells generated by stimulation can also play a major role in controlling CMV and preventing subsequent lethal infections. HCMV tegument proteins have a pivotal part in all stages of viral life cycle, including entry, gene expression, immune escape, assembly, and release. The phosphoprotein pp150 is the most important tegument protein candidate for HCMV treatment (15), hence the need to utilize peptides for stimulating either $CD8^+$ or $CD4^+$ T cells (15-17). It is further suggested that the presentation efficiency of antigens such as HCMV pp150 antigen could depend on the early acquisition of viral antigens by immature DC (iDC) presentation. In addition, more mature DCs result in stronger T cell responses. An appropriate approach to properly activating such cells is the proper stimulation of DCs. Of note, HCMV tegument proteins, including pp150, were demonstrated to be a potential target for CMV treatment. In the present study, we investigated whether DCs stimulated with CMV pp150 peptide are able to expand the function and maturity of DCs.

## MATERIALS AND METHODS

**Animals and Ethics.** Ethics Committee of Tehran University of Medical Sciences approved all the protocols used in this study (Project No 9021409006). Six-8 week

female C57BL/6 and BALB/c mice were purchased from Pasteur Institute of Iran. All mice were housed in a humidity- and temperature-controlled room under a 12 h/12 h light/dark cycle and a standard pelleted diet and bottled tap water. Additionally, all experiments were carried out in accordance with these approved protocols.

**Separation of DCs and CD4$^+$ T Cell**. We isolated DCs from the spleens of BALB/c mice in accordance with Moravej *et al.* (18). In brief, the spleens were chopped and digested using 1 mg/mL collagenase D (Roche, Germany), and cell suspensions were layered on Nycodenz (Axis-Shield) solution; the cells were then isolated by a positive selection magnetic adsorption cell sorting (MACS) procedure using the CD11c Micro Beads kit (Miltenyi Biotechnology, Germany), based on the manufacturer's protocols. CD4$^+$ T-cells were isolated from the draining lymph nodes of C57BL/6 mice by a negative selection MACS method through the use of CD4$^+$ T-cell isolation Micro Beads kit (Miltenyi Biotechnology, Germany). DC and T cell purity was assessed using specific PE-conjugated anti-CD11c and anti-CD3 antibodies via flow cytometry analysis.

**Recombinant pp150 Antigen.** CMV pp150 was commercially obtained from Jena Bioscience, GmbH, Germany (Cat No: PR-1252). Generated in *Escherichia coli* and purified using a chromatogeraphic technique, this synthetic peptide contains immunodominant regions in amino acids 1011-1048. It is important to note that this peptide is for *in-vitro* use only.

**Dextran Uptake Test.** To assess the ability for soluble antigen uptake as an indicator of phagocytic activity, DCs ($6 \times 10^5$ cell/mL) treated with the pp150 antigen (100 ng/mL) for 1 hour were prepared in RPMI medium (Gibco, USA) consisting of 10% FBS (Gibco, USA) and 25 mM HEPES (Gibco, USA) in 2 test tubes. Afterwards, freshly-prepared FITC-Dextran (Sigma, USA) (1 mg/ml) was added. Cells positive for FITC were identified as cells engulfing dextran and placed at 4˚C and 37˚C for 1 hour or at 4˚C for the negative control. Following incubation, cells were washed twice and centrifuged at 350 g for 5 minutes at 24˚C and then examined by flow cytometry (FACSCalibur™; BD Biosciences). Results were analyzed by Flowjo software (Flowjo, Treestar Inc., Ashland, OR, USA), version 7.6.2. The following formula was applied to measure FITC-Dextran loaded cells: Actual Uptake = MFI of cells incubated at 37°C – MFI of cells incubated at 4˚C. Lastly, data presented the difference in mean fluorescence intensity (MFI) of DCs following FITC-dextran uptake at 4˚C.

**Flow Cytometry Analysis of Treated DCs.** Using flow cytometry, DCs treated with pp150 were analyzed for cell surface expression of markers showing maturation (19). Cells were stained using antibodies for CD40, CD86, and MHC-II to study DC surface markers, based on the manufacturer's protocols (Biolegend company, USA). The surface markers of DCs were measured using flow cytometry by the following antibodies: PE-conjugated anti-CD11c antibody, FITC-conjugated anti-CD86 antibody, FITC-conjugated anti-MHC-II antibody, and FITC-conjugated anti-CD40 antibody for DCs.

**Analysis of T cell proliferation and allogenic responses by mixed leukocyte reaction (MLR).** *In vitro* cell proliferation was evaluated by 5-bromo-2'-deoxyuridine (BrdU) employing the commercial Cell Proliferation ELISA System (Tecan, Crailsheim, Germany). The pp150-treated DCs were inactivated using irradiation (30 Gy), washed using PBS. pp150-treated DCs ($10^4$ cells/mL), and co-cultured with $10^5$ cells of allogeneic T cells (C57BL/6), as responder cells in 96-well plates. T cell proliferation was studied utilizing the BrdU method, as mentioned in the previous study

(20). Briefly, after 1 week, the cultivated cells were subjected to BrdU through the addition of 10 ml of BrdU solution to the wells; the incubation was continued for more than 18 hours. Next, media were removed, the cells were fixed, and DNA was denatured to enhance the accessibility of the incorporated BrdU for detection using antibodies. After washing, peroxidase-conjugated anti-BrdU antibodies were added to each well and the plate was incubated at room temperature for 90 minutes. Following three washes, tetramethyl-benzidine (TMB) substrate was added and the mixtures were incubated for 15 minutes. Ultimately, sample absorbance was analyzed in a microplate reader at 450 nm and 690 nm reference wavelengths. Negative controls were allogeneic T-cells co-cultured with untreated DCs, and culture medium alone was applied as control for nonspecific binding. DCs stimulated with 100 ng/ml LPS were applied as a positive control in media containing DMSO (1%) with no other compounds. The proliferation index (PI) was calculated through dividing the absorbance of the treated cells by one of the controls. After 48 hours, cultivated T cells were harvested, washed once with PBS comprised of 0.5% BSA (Invitrogen, USA) and 2 mM EDTA, and resuspended in 2 ml. Following 48-hour incubation, supernatants were collected to assess the cytokine levels.

**Quantification of Cytokine Levels.** MLR supernatants were collected after 48-hour culture and stored at -20˚C. Mouse Th1/Th2/Th17/Th22 13-plex Flow Cytomix Multiplex kit (eBioscience, USA) was designed to measure mouse IL-1a, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17A, IL-21, IL-22, TNF-α, and IFN-γ in an immunoassay analyzed on a flow cytometer (FACS Calibur, BD, USA) according to the manufacturer's protocols. Standard curves for all the cytokines were included in each reaction according to the kit instructions (ranging from 2.7 pg/mL-2000 pg/mL). Additionally, the level of IL-35 was assessed based on the ELISA method.

**Real-Time Quantitative Polymerase Chain Reaction (qPCR) Assay.** Expression of transcriptional factors such as FoxP-3, GATA-3, ROR-γ, T-bet, and CCR7 genes mRNA was evaluated using real-time PCR. Briefly, total RNAs were extracted using the TRIzol reagent (Invitrogen, USA), followed by cDNA synthesis kit (Takara, Japan). To determine PCR efficiency, cDNA was diluted and real time was carried out, which revealed that Real Time PCR efficiency was acceptable for all genes (>95%). eEF1a1 was employed as an endogenous control and specific primers for these genes were designed using Allele ID software Version 7.5 (Table 1).

**Table 1. Specific primers used in this study.**

| Specific primers | Sequence |
|---|---|
| FoxP3 | F: 5'- AATAGT TCCT TCCCAGAGT TCT TC -3'<br>R: 5'- ATGGTAGAT T TCAT TGAGTGTCCT -3' |
| GATA3 | F: 5'-TCTGGAGGAGGAACGCTAAT-3'<br>R: 5'- CGGTTTCGGGTCTGGAT-3' |
| ROR-γ | F: 5'- CCATTGACCGAACCAGCC -3'<br>R: 5'- GCCAAACTTGACAGCATCTC -3' |
| T-bet | F: 5'-AAC CGC TTA TAT GTC CAC CCA -3'<br>R: 5'-TCT CCA TCA TTC ACC TCC ACG -3' |
| CCR7 | F: 5'-GAGACAAGAACCAAAAGCACAG -3'<br>R:5'- GGAAAATGACAAGGAGAGCCA -3' |
| eEF1a1[a] (as Internal Control) | F:5'- AGTCGCCTTGGACGTTCTT-3'<br>R: 5'- CCGATTACGACGATGTTGATGTG-3' |

F: Forward, R: Reverse; a: Elongation factor 1-alpha 1 (eEF1a1).

Comparative real-time PCR using SYBR Green supermix (Parsgenome, Tehran, Iran) was conducted through the use of real-time PCR machine (ABI step one plus, Applied Biosystems, USA). Finally, FoxP3, GATA3, ROR-γ, T-bet, and CCR7 relative expression were determined using equation $2^{-\Delta\Delta Ct}$.

**Statistical Analysis.** Statistical analyses were conducted by GraphPad Prism version 6.00 (GraphPad Software Inc colifornia). Results are presented as mean ± standard deviation (SD) of the mean of a minimum of three independent experiments. To compare the variables, statistical significance was specified by a multiple comparison t-test or one-way ANOVA. P-values less than 0.05 were considered to be significant.

## RESULTS

### Characteristics of Isolated DCs and T Cells.

Following their isolation, DCs and T cells were grown overnight at 37˚C. The purity of DCs and T cells was assayed using anti-CD11c and anti-CD3 with FACS. The presence of more than 95% surface markers indicated a high purity of these cells (Figure 1).

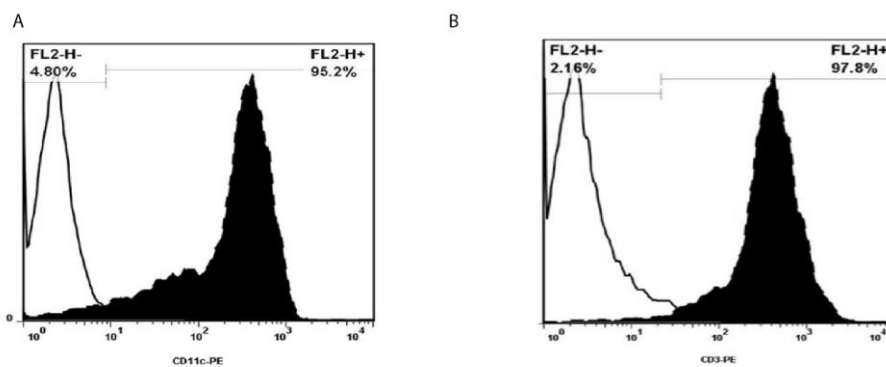


**Figure 1. Isolation of DCs and T cells.** Isolated DCs and T cells were stained using antibodies against specific-surface markers (CD11c (A) and CD3 (B), respectively), which were analyzed using flow cytometry. Histogram analysis of cell surface markers indicates that the purity of isolated cells is more than 95%, and they are not contaminated by other cell lineages (specific antibodies: dashed histograms; isotype control antibodies: filled histograms).

### Effects of pp150 on the Phagocytosis of Treated DCs.

Evaluation of the phagocytosis capacity of the pp150-treated DCs showed that immature DCs could efficiently take up antigens and lose this capacity upon maturation. As expected, the results of FITC-dextran particle phagocytosis using immature DCs obtained from pp150- and LPS-treated DCs showed that DCs diminished their capacity to take up the antigen following LPS induction; this indicates that immature DCs belonging to pp150-treated group internalized comparable amounts of the antigen (18.86 ± 1.1 and 10.86 ± 1.45 vs. 38.14 ± 2.25) (Figure 2).

### pp150-treated DCs lead to DCs Maturation.

DCs play an essential role in the initiation of adaptive immune response. Levels of pp150 induced the maturation of DCs when incubated with exposed or unexposed pp150. Expression of CD40, CD86, MHC-II, and CCR7 surface molecules on DCs was investigated by flow cytometry and real-time PCR.

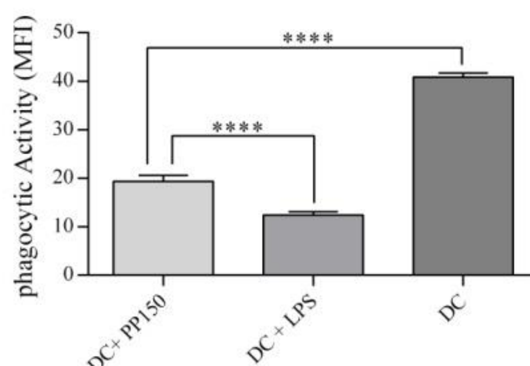


**Figure 2. Comparison of the mean fluorescence intensity (MFI) of phagocyted FITC-conjugated dextran by DCs.** The results of FITC-dextran particle phagocytosis showed that the two groups, pp150 and LPS-treated DCs, decreased their capability to take up antigen than immature DCs, did differ (p<0.0001), indicating that immature DCs from pp150-treated group internalized comparable amounts of antigen (p<0.0001). Cumulative data represent the mean+ SD between the two groups (Student's t-test) and derived from three independent experiments (****: p<0.0001).

A significant increase was detected in the expression profiles of CD40 marker and CD86 molecules in pp150-treated DCs as compared with cells obtained from non-treated DCs (13.16 ± 2.1 vs. 5.34 ± 2.19 and 32.26 ± 3.1 vs. 18.45 ± 1.39, respectively) (Figure 3A and 3B). In addition, the mean expression levels of MHC class II molecules significantly increased in pp150-treated DCs in comparison with cells obtained from non-treated DCs (5.82 ± 1.26 vs. 3.21 ± 0.45). Furthermore, the MFI of dendritic cells treated with pp150 was lower than that of untreated dendritic cells. The figure shows that MFI of dendritic cells treated with LPS was significantly higher than that of dendritic cells treated with pp150 (Figure 3C). As expected, LPS-treated DCs exhibited an overexpressed increase in maturation markers. The expression of the CCR7 gene in pp150-treated DCs was investigated using real-time PCR. The relative expression of CCR7 mRNA in pp150-treated DCs was higher than the non-treated group (1.24 ± 1.21 vs. 0.41 ± 0.48) (Figure 3D).

### Impact of pp150 on T Cell Proliferation in MLR.

To further examine the role of induced DCs in T cell proliferation, we determined whether pp150-treated DCs could stimulate T cell proliferation in culture compared to the absence of antigen. When DCs were cultured alone for 5 days, the proliferation of naïve T cells did not differ from pp150-treated DCs (73.24 ± 5.25 vs. 103.41 ± 4.31). On the contrary, a vast proliferation occurred in pp150-induced DCs compared with the only cultured ones. In contrast, when DCs were treated with pp150, a significant proliferation was generated unlike treatment with LPS as a positive control. Furthermore, in the presence of LPS, T-cell proliferation was significantly lower than pp150-treated DCs in MLRs (103.41 ± 4.31 vs. 101.21 ± 2.01) (Figure 4). This result indicates that Ag-induced DCs were uniquely able to stimulate T cell proliferation *in vitro*.

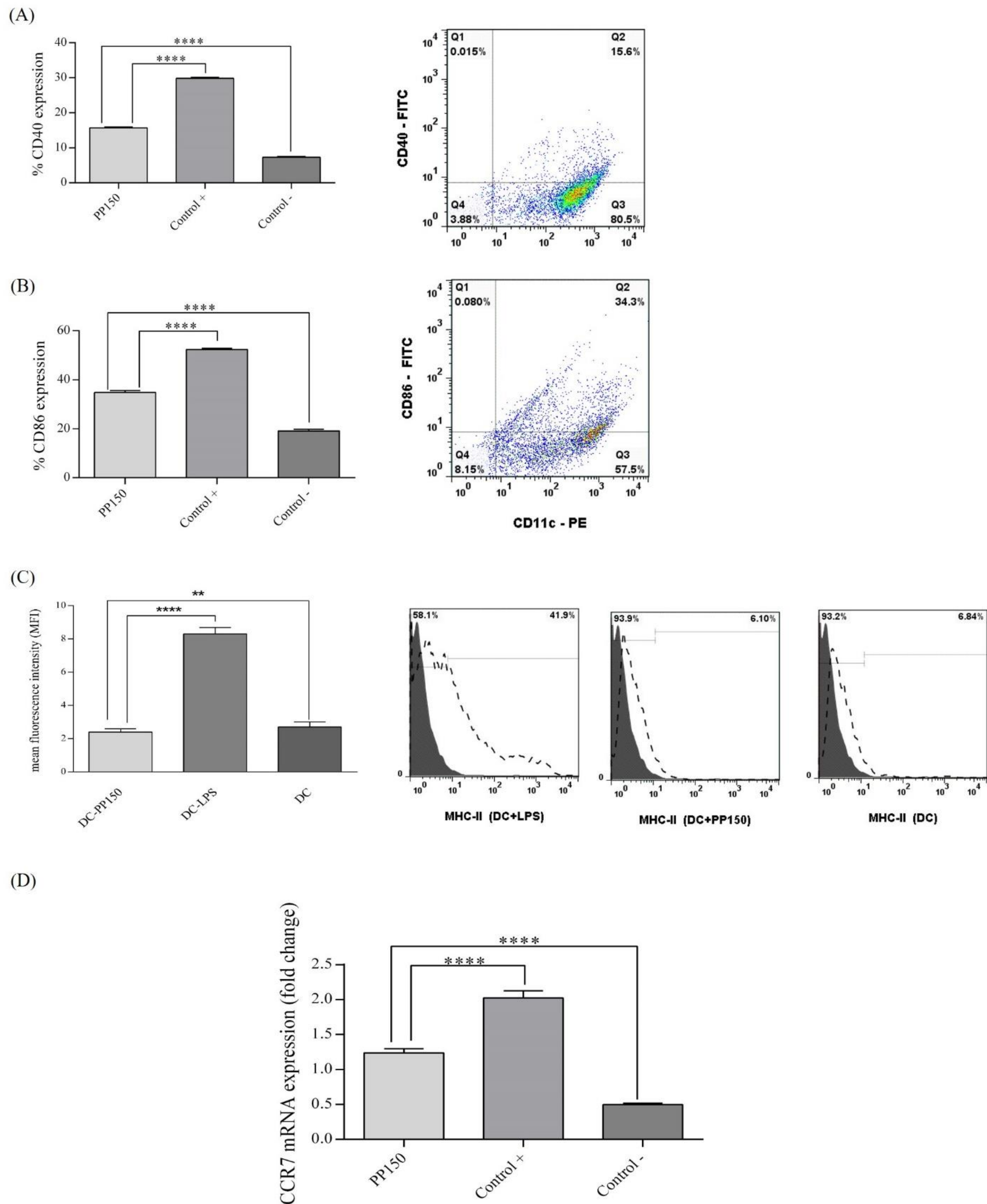


**Figure 3. The phenotypic characterization of DCs. FACS analysis was performed with monoclonal antibodies against CD40, CD86, and MHC-II.** A-B) pp150-treated DCs show significantly expressed CD40 and CD86 higher levels than the non-treated DCs (DCs). C) MFI of dendritic cells treated with pp150 is lower than that of untreated dendritic cells. The figure shows that MFI of dendritic cells treated with LPS is significantly higher than that of dendritic cells treated with pp150. D) The effect of the pp150 antigen on the expression of CCR7 mRNA in DCs treated with the pp150 antigen investigated by real-time PCR. Expression of CCR7 mRNA is significantly lower than the LPS-treated group. Positive control: treatment with LPS; negative control: no treatment with antigens. The results represent the mean ± standard deviation of three independent experiments (****: $p<0.0001$ and **: $p<0.01$).

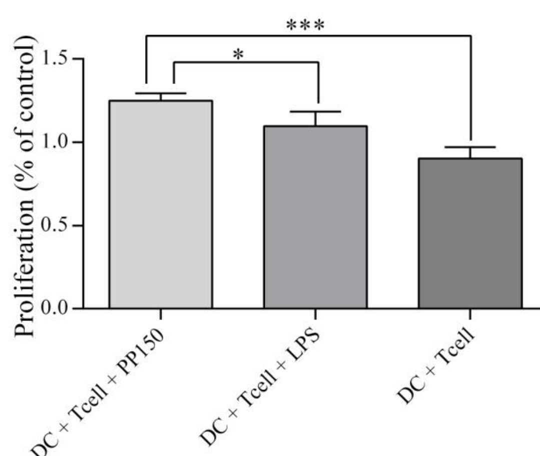


**Figure 4. Effects of pp150-treated DCs on T-cell proliferation in MLR.** Proliferation of alloreactive T-cells in MLR was assessed by BrdU incorporation. In the presence of LPS, T-cell proliferation was lower than pp150-treated DCs in MLRs. Moreover, in pp150-treated DCs, T-cell proliferation was higher than DCs co-cultured with T cell (no-treated with pp150). The results present the mean ± SD of three independent experiments (***: $p<0.001$ and *: $p<0.05$).

**Immunomodulatory Effects of pp150-treated DCs on Treg and Th17 Cells in Contrast to Th1 and Th2.**

The expression levels of Foxp3, GATA-3, ROR-γ, and T-bet genes were studied in MLR by Real-time PCR. Foxp3 expression levels in pp150-stimulated DCs cultured with T cell were significantly different from non-stimulated DCs cultured with T cell (0.71 ± 0.31 vs. 1.07 ± 0.67). In addition, Foxp3 expression in LPS-stimulated DCs was similar to pp150-stimulated DCs (0.82 ± 1.06 vs. 0.71 ± 2.31) (Figure 5A). Gene expression profiles of GATA-3 in pp150-stimulated DCs cultured with T cell revealed higher levels compared to DCs as a negative control. The expression of GATA-3 in LPS-stimulated DCs revealed a non-significant difference with LPS-stimulated DCs (0.85 ± 2.1 and 1.34 ± 2.42 vs. 0.79 ± 1.86, respectively) (Figure 5B). In contrast to the previous gene patterns, higher levels of ROR-γ expression were found in pp150-treated DCs compared with LPS- and untreated DCs influenced by pp150 Ag (1.75 ± 2.7 vs. 1.04 ± 1.12 and 0.41 ± 1.46, respectively) (Figure 5C). As shown in Figure 5D, non-stimulated DCs cultured with T cell revealed the reduced expression of T bet compared to pp150-treated DCs (0.7 ± 0.34 vs. 0.68 ± 0.11). In addition, LPS-stimulated DCs cultured with T cell had increased T bet expressions in comparison to pp150-stimulated DCs (1.6 ± 0.34 vs. 0.68 ± 0.11).

**Elevated Secretion of IL-2, IL-6, and IL-17A and Blockage of other pro-inflammatory Cytokines.**

We assessed the immunostimulatory capacity to ensure higher functional activation levels in DCs when encountering exposed Antigen. Levels of different cytokines in cell culture supernatant were determined after 48 hours of culture in MLR. A similar pattern of IL-2, IL-6 and IL-17A secretion was found in the pp150-treated DC category compared with untreated DCs group with a statistically significant difference (71.85 ± 4.1, 120.34 ± 6.42 and 22.64 ± 5.42 vs. 0.87 ± 2.46 pg/ml, respectively).

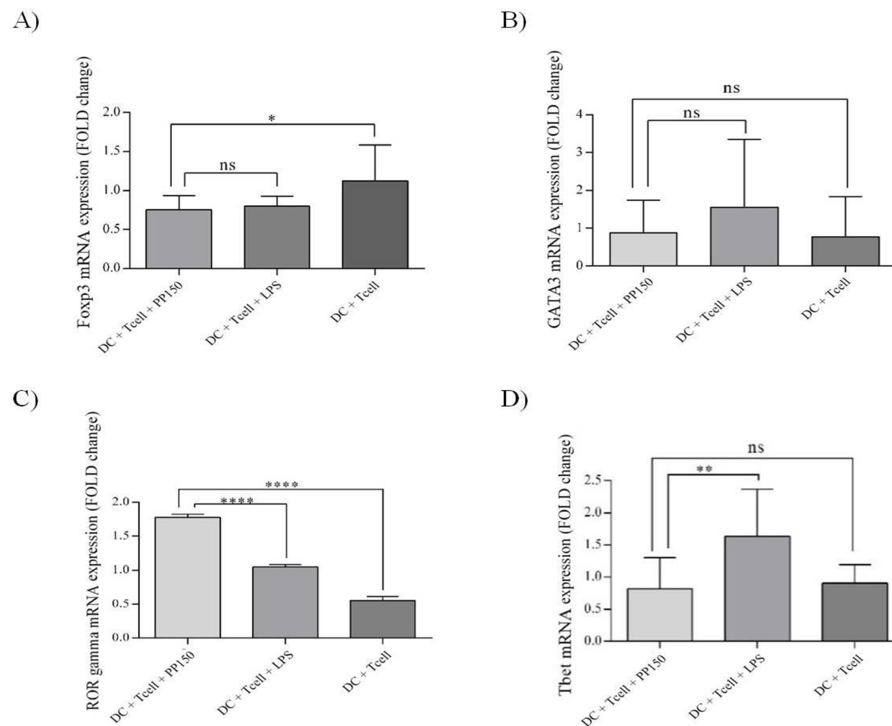


**Figure 5. The mRNA expression of Foxp3, GATA3, ROR-γ, and T-bet genes in MLR.** A) Foxp3 mRNA expression is significantly lower in the treatment group with pp150 than the non-treated DCs control, but this level is similar to LPS-stimulated DCs group. B) Gene expression of GATA3 in pp150-stimulated DCs is not significantly higher than DCs as a negative control. C) ROR-γ expression in pp150-treated DCs is significantly higher than LPS- and non-treated DCs. D) As shown, non-stimulated DCs cultured with T cell do not have a significantly higher T bet expression compared to pp150-treated DCs. (Positive control: treatment with LPS; negative control: no treatment with antigens). The results show the mean ± SD in three independent experiments (*: $p<0.05$; **: $p<0.01$ and ****: $p<0.0001$).

In addition, elevated levels of IL-17A were detected in the same group compared to LPS-treated DCs groups and in contrast to other cytokines with a different pattern (Figure 6 A-C). Various patterns of IL-22, IFN-γ, and TNF-α secretion were observed in the pp150-treated DC category as compared with untreated DCs group (8.35 ± 4.1 vs. 0.81 ± 0.86, 22.64 ± 5.42 vs. 38.82 ± 2.59 pg/ml, respectively). Upregulated IFN-γ levels existed in the same group compared to LPS-treated DCs groups and contrary to other cytokines with a different pattern (Figreu 6 D-F). Noteworthy, unlike pro-inflammatory cytokines, IL-22 and IL-17A only exhibited a pattern of pp150-treated DC category similar to LPS- and untreated DCs groups where these cytokines were upregulated in this cell culture system. These results suggested that an immune response, characterized by high levels of pro-inflammatory cytokines, might be a parameter for determining the outcome of different T-cell subset responses. With respect to other cytokines, IL-1a, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17F, IL-21, and IL-35 indicated undetectable levels. The cytokines had elevated levels in the LPS-treated DCs group compared to pp150-treated and untreated DCs groups (8.35 ± 4.1 vs. 0.81 ± 0.86, 22.64 ± 5.42 vs. 38.82 ± 2.59, respectively).

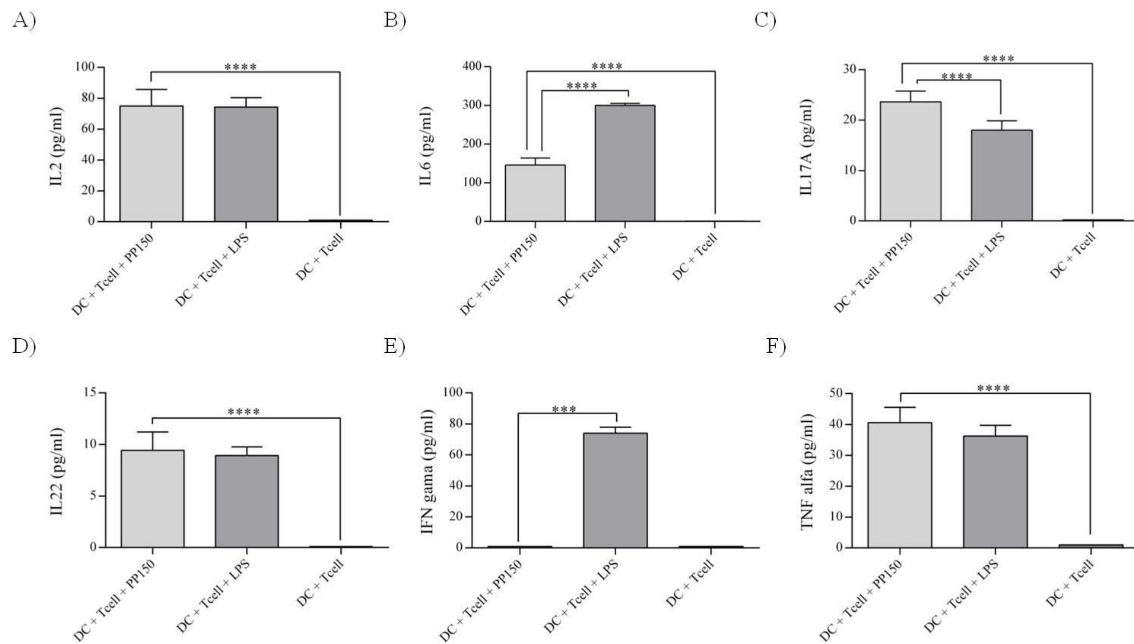


**Figure 6. Kinetics of inflammatory response by T cell co-cultured with and without pp150, LPS.** pp150-treated DCs co-cultured with T cell were established in different stimulation regimens (A-F). Finally, cytokine release assay was performed using an immunoassay analyzed on a flow cytometer from supernatants. Figures indicate the production of IL-2 (A), IL-6 (B), IL-17AA (C), IL-22 (D), IFN-γ (E), and TNF-α (F). A similar pattern of IL-2, IL-6 and IL-17AA secretion is shown in the pp150-treated DCs category compared with non-treated DCs group, indicating a statistically significant difference. Also, there are elevated levels of IL-17AA in the same group in comparison to LPS-treated DCs groups, which is in contrast to other cytokines with a different pattern (A-C). The various patterns of IL-22, IFN-γ, and TNF-α secretion are shown in the pp150-treated DCs category compared with non-treated DCs group. The same group has upregulated levels of IFN-γ compared to LPS-treated DCs groups, contrary to other cytokines with a different pattern (D-F). Also, as noted, IL-4 and IL-10 levels are undetectable (data not shown). Data are presented as means ± SEM of at least three mice per group from one of two independent experiments; significant values were calculated by unpaired, two-tailed Student's t test ;(****: $p<0.0001$; ***: $p<0.001$).

## DISCUSSION

This study evaluated the effect of HCMV pp150 recombinant protein on the function and maturity of DCs. To this end, DCs were primarily treated with pp150 antigen, and their maturation was then evaluated using co-stimulatory molecule expression. We further assessed the capacity of DCs to shift the proliferation and differentiation of T cells. Our findings showed that DC phagocytosis in the group treated with pp150 was lower than the negative group. Generally, relatively immature DCs have to not only phagocytose antigens, undergo a coordinated alteration in the expression of MHC, chemokine receptors, adhesion, and co-stimulatory molecules, but also migrate from the peripheral tissue to the draining lymph nodes (21). Therefore, pp150-treated DCs must have a higher phagocytic ability compared with mature DCs because this event is a maturation marker which also shows that the pp150 antigen could induce DC maturation. Additionally, there was a decrease in the cell surface markers required for

viral antigen uptake. Our findings are consistent with a previously-published study where CMV-infected mature DCs reduced the levels of cell surface markers required for phagocytosis (22). We also investigated the co-stimulatory molecules such as CD40, CD86 and MHC-II, and CCR7, as DC migration factor, in lymphoid tissues. Our results revealed that the presence of CD40 and CD86 molecules in the group treated with the pp150 antigen was significantly higher than the negative group and lower than the positive group. It can be concluded that the pp150 antigen results in the induction of DC maturation and proliferates T cells through their stimulation (9,10). Additionally, the increased number of these co-stimulatory molecules indicated DC activation, leading to the production of mature and active DCs; this was indicated in previous studies which reported that DCs increased following CMV infection (22,23). Moreover, there is a general consensus that T cell co-stimulatory molecules, including CD40, CD80 and CD86, and MHC classes I and II are downregulated on the surfaces of immature DCs following HCMV infection; however, this effect possibly depends on the infection time, which is consistent with pp150-treated immature DCs in our study (24,25). Previous studies have suggested that immature DCs have fewer co-stimulatory molecules, also in line with our findings (26). Wang *et al.* (2009) proved that MCMV either induces inflammatory cytokines or positively regulates CD80 and CD86 on mature DCs (27). Notably, the exposure of cells to the NF-κB peptide inhibited MCMV infection and reduced the induction of cytokines as well as CD80 and CD86 molecules (22). In the current study, MHC-II molecule levels increased in the group treated with pp150 as compared with the non-treated group. It can be concluded that increased levels of MHC-II in the group treated with the pp150 antigen is independent of IFN-γ. This is in accordance with a previous study carried out by Takei *et al.* (28) and inconsistent with the results of Heise *et al.* (29). Mobilization of DCs in to lymphatic vessels requires cytokine stimulation and induction of the chemokine receptor CCR7. The CCR7 molecule is considered as the most important marker for DC migration to lymph tissue to provide antigens for T cells (30). Our results showed that CCR7 expression was higher in the treatment group. This indicates that in addition to DC maturation with increased co-stimulatory molecules, pp150 led to the expression of DC migration molecules to lymphoid tissues; in fact, this event improved the DC maturation (22) owing to the increased level of costimulatory molecules in DCs and the reduced phagocytic capacity with pp150 antigen treatment. Our results also showed that the T cell proliferation in the treatment group was higher than no-treatment group. This finding indicated that mature DCs resulting from treatment with pp150 might lead to T cell proliferation, which is in line with the results of Wagner *et al.* (27). T cell proliferation can be carried out by both the activity and confrontation of DCs and the secretion of different cytokines through different types of T cells. For this, we studied the ability of DCs to express transcription factor molecules such as Th1, Th2, Th17, and Treg. The FoxP3 transcription factor is the Treg differentiation factor (31) and regulatory T cells (Treg) were shown to contribute to the immune-mediated control of acute MCMV infection (32). In our study, DCs treated with the pp150 antigen were unable to augment the expression of FoxP3 in T cells; however, pp150-treated group and the non-treated group were significantly different regarding the FoxP3 expression. Such findings suggested that the pp150 antigen might not result in Treg induction and not be able to change tolerogenic DCs that can inactivate or convert T cells to Treg cells. In agreement with our results, Weinberg *et al.* reported that the activity of Treg cells was related to the production of FoxP3 (33). Of note, Almanan *et al.* (2017)

observed latent CMV reactivation was controlled by regulatory T cells, hence their regulatory role in HCMV infection. Treg is not generally induced after immunity induction with dominant viral antigens (34). Another study revealed that Treg was less effective at the beginning of HCMV infection (35). A study conducted in 2014 showed that Treg cells containing FoxP3 cells existed in the bone marrow of individuals following transplantation with stem cells, implying the role of these cells in immunotherapy (36). It can be concluded that DCs treated with viral antigens failed to differentiate in to proliferative DCs that yield further IL-10 and can differentiate T cells into Treg with more IL-10. The lack of Th2 immunity induction as with IL-4 secretion after exposure to pp150 implies that pp150 could not induce differentiation, GATA3 expression, activation of Th2 cells, and IL-4 production. A study reported a large number of GATA3-containing cells in the first two days of HCMV infection but significantly reduced numbers in the following days (37). A study performed by Duechting *et al.* in 2017 showed the delayed activity of Th2 cells against CMV infection as compared with Th1 and Th17 cells (38). Nonetheless, the reason for the absence of Th2 cell differentiation is the low concentration of pp150. Consistent with that study, our research revealed that the level of Th2 cytokines was not detectable; also, it might be better to examine the level of these cytokines over time periods longer than the infection so as to demonstrate their role in CMV infection. The ROR-γ factor is the Th17 differentiation factor (39). Prior reports have indicated that Th17 plays a major role in inflammatory events such as immunological responses mediated by DCs (40-42). Our results showed that DCs treated with pp150 co-cultured with T cells were able to augment ROR-γ expression in T cells in MLR, implying that Th17 production is the main factor. On the other hand, elevated IL-22 levels, as selectively expressed by Th17 cells, play a crucial role in organizing several different tissues, could be the major role of Th17 in induced immunity using pp150-treated DCs (43). These findings showed that pp150 led to Th17 induction, indicating the possible role of Th17 cells in HCMV inflammatory immunity (44). Interestingly, no increase was found in T-bet expression in the presence of pp150, suggesting that pp150 could not induce Th1 cells; moreover, no increase was observed in the IFN-γ cytokine levels in the presence of pp150; our results revealed that the level of TNF-α, IL-2, IL-6, IL17A, and IL-22 increased in DCs treated with T cells. Th1 cells generate IL-2 and IFN-γ, but the low levels of Th1-type cytokines, particularly IFN-γ upon induction with CMV-pp150 antigens, were related to reduced cell-mediated immunity to HCMV (45). Because Th1 responds by producing IFN-γ, it is critical in the defense against viral infection; however, based on our finding, the role of Th1 producing IFN-γ needs to be described in the prevention of HCMV infection. Based on our findings, it can be concluded that treatment of DCs with pp150 results in the phenotypic and functional maturation of DCs. In addition, pp150 could induce the differentiation and functional activity of the Th17-related cellular immunity, which is better characterized by in-vivo studies. Despite the role of IFN-γ as a major Th1 response, treatment of DC with pp150 could induce IFN-γ production; Accordingly, these data could not support the hypothesis that DCs treated with pp150 induce IFN-γ by Th1 regulation.

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