Improved Function and Maturation of Dendritic Cells Stimulated by Recombinant pp65 Protein: *In vitro* Design

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ABSTRACT

Background: Stimulated dendritic cells (DCs) have been shown to be effective in the induction of specific immune cells. Also, the CMV pp65 plays an important role in CMV life cycle and immune recognition. **Objective:** To assess the effect of CMV pp65 on the maturity and function of dendritic cells. **Methods:** Splenic DCs were treated with non-cytotoxic concentrations of the pp65 and analyzed for MHC II, CD86, and CD40 expression by flow cytometry. Then, ROR-γ, GATA3, T-bet, and FOXP3 gene expression levels were evaluated in T cells co-cultured with DCs using Real time-PCR. Finally, the effects of pp65 on allogenic T-cell responses in mixed lymphocyte culture (MLR), and the release of cytokines were investigated by ELISA and flow cytometry. **Results:** The phagocytosis rate was significantly lower in the pp65-treated DCs than the non-stimulated DCs. There were significant differences in the raised level of CD40, CD86, and CCR7 in DCs as maturation markers. Furthermore, ROR-γ, and T-bet overexpression in T cells of the pp65-treated group compared with the non-stimulated group was observed. Significant differences were observed in the levels of IL-2, IL-6, IL-17, IL-22, TNF-α, and IFN-γ in pp65-stimulated groups compared with the non-stimulated DCs. **Conclusions:** The pp65-treated DCs can induce differentiation and functional activity of the cellular immune system, including Th17, and Th1, but not other major T-cell subsets such as Tregs, and Th2 population.

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Keywords: Cytomegalo Virus, Dendritic Cell, Function, Maturation, pp65

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INTRODUCTION

Cytomegalovirus (CMV), a genus of the Herpesviridae family, infects 50%-100% of the adults in different populations (1). CMV establishes a permanent latent asymptomatic infection that can be reactivated when the host's immune system is compromised (2). A persistence increase in the T cells during primary infection or viral reactivation often represent the host defense against the virus. This immune response interferes with viral strategies. In addition, CMV leads to get persistent asymptomatic infection, even in healthy individuals with acompetent immune system (3-5).

CMV is effectively suppressed by virus-specific T cells directed against a number of immunodominant proteins, such as pp65 and pp89, which are highly immunogenic in eliciting T-cell responses. In human immune response against CMV, tegument antigen of CMV (i.e., phosphoprotein pp65) has been identified as a major target antigen for CMV-specific T cells (6-8). The pp65 is an immunodominant antigen for CD4+ as well as CD8+ T-cell. Immune responses to CMV pp65leads to production of cytokines such as IFN and IL-2 (6,9). In this regard, DCs transduced with vector encoding pp65 protein are able to simultaneously expand cytotoxic T lymphocytes (CTL)(10,11). In addition, they activate CMV specific CD4+ T-helper cells (12). This approach could be available to patients of any human leucocyte antigen (HLA) haplotype.

Furthermore, dendritic cells (DCs) are specialized antigen-presenting cells that play a central role in cellular immunity and immunotolerance (13). The importance of the major histocompatibility complex (MHC) I and II genetic diversity that exists within the human population and the effects of differential immunogenicity between MHC molecules are referred to as immunodominance (14). Mature DCs up-regulate expression of the surface MHCs and of costimulatory molecules and are able to stimulate adaptive immune responses. DCs are critical in the activation of T cells, providing antigen presentation, costimulatory signals, and inflammatory cytokine stimulation to direct an effector T-cell response (15). DCs have been used in several studies as the sole therapeutic agents to stimulate immunity against infections and cancers (16,17). Given the unique capacity of DCs to initiate primary T-cell responses against pathogens and tumors, DC-based immunotherapy holds promise to trigger CMV-specific immune responses. Monocyte-derived DC pulsed with CMV protein has been used to stimulate T cells. The outcomes have shown that in an adoptive setting, they trigger an in vivo CMV-specific immune response (10,12).

During the acute phase, CMV can infect a variety of immune cells, including macrophages, DCs, hepatocytes, endothelial cells, and epithelial cells (18). More importantly, both innate and acquired immune systems are infected during CMV infection. The activity of Natural Killer (NK) and TCD8 cells is highly dependent on the function of DCs and macrophages (as antigen presenting cells) (19). Therefore, by determining the effect of various proteins on the maturity and function of DCs, the most appropriate protein can be used for CMV treatment. The present study aimed to assess the ability of CMV pp65 protein on the function and maturity of DCs. This study successfully determined that pp65 could stimulate the immune system in a proper way, suggesting that it is a potential candidate for application in CMV treatment.

MATERIALS AND METHODS

Animals and Ethics statement. Six- to 8-week-old female BALB/c and C57BL/6 mice were purchased from the Pasteur Institute of Iran. The whole study was approved by the Ethics Committee of Tehran University of Medical Sciences (Project No 9021409006). All efforts were made to minimize pain and suffering over the lifetime of the animal. If an animal was determined to be in overt pain/distress or appeared moribund, the animal was euthanized for humane reasons. Upon arrival, mice were housed in a standard polycarbonate cage. Standard pelleted rodent diet and bottled tap water were provided ad libitum. Ambient room temperature was controlled at 20-26℃ and at a relative humidity of 30-70%. Light cycles were set at 12 h on (06:00 to 18:00) and 12 h off (18:00 to 06:00) for the entire study period.

Recombinant pp65 antigen. CMV pp65 was commercially obtained from Jena Bioscience, GmbH, Germany (Cat No: PR-1251). This synthetic peptide is produced to Escherichia coli and is purified through the chromatographic technique. This peptide, which contains immunodominant regions and amino acids 297-510 (52.2 kDa), is fused to a 26 kDa GST-tag. It has to be noted that CMV pp65 is only for *in-vitro* use.

Separation of DCs and CD4+ T cell. DCs were isolated from the spleens of BALB/c mice in accordant with Moravej *et al.* (20). In brief, the spleens were chopped and digested with 1 mg/mL collagenase D (Roche, Germany). The cell suspensions were layered on Nycodenz (Axis-Shield) solution and then isolated using a positive selection magnetic adsorption cell sorting (MACS) technique by CD11c MicroBeads kit (Miltenyi Biotechnology, Germany), according to the manufacturer's instructions. CD4+ T-cells were isolated from draining lymph nodes of C57BL/6 mice using a negative selection MACS technique by CD4+ T-cell isolation Micro Beads kit (Miltenyi Biotechnology, Germany). The purity of the DCs and T cells isolated were evaluated by specific PE-conjugated anti CD11c antibodies and specific PE-conjugated anti-CD3 antibodies using flow cytometry analysis.

FITC-dextran phagocytosis assay. To evaluate the capacity for uptake of soluble antigens from the culture medium that is an indicator for assessing the phagocytic activity in isolated DCs, 6×10^5 DCs treated with the pp65 antigen were prepared in RPMI medium containing 10% FBS and 25 mM HEPES in two test tubes. Then, freshly-prepared FITC-Dextran (1 mg/ml) was added to the medium. Cells positive for FITC, which were identified as cells that had engulfed dextran, were placed at 4℃ and 37℃ for 1 hour or at 4℃ for the negative control. After incubation, the cells were washed by washing buffer twice and centrifuged at 350 g for 5 min at 24℃ prior to analysis by flow cytometry (FACSCalibur™; BD Biosciences). Data were analyzed using Flowjo software, version 7.6.2. Finally, data represent the difference in mean fluorescence intensity (MFI) of DCs after FITC-dextran uptake at 4℃.

Flow Cytometry analysis of treated-DCs. The pp65-treated DCs were analyzed for cell surface expression of multiple markers of maturation by flow cytometry. Cells were stained with antibodies against CD40, CD86, and MHC-II to study DCs surface markers, based on the manufacturer's protocols (Biolegend Company, USA). DCs surface markers were measured by flow cytometry using the following antibodies: PE-conjugated anti-CD11c antibodies, 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 response by mixed leukocyte reaction (MLR). *In vitro* cell proliferation was measured using 5-bromo-20-deoxyuridine (BrdU) by the commercial Cell Proliferation ELISA System (Tecan, Crailsheim, Germany). The pp65-treated DCs were inactivated with irradiation (30 Gy) and washed with PBS three times. A total of 10^4 pp65-treated DC cells (BALB/c), as stimulator cells, were co-cultured with 10^5 cells of allogeneic T cells (C57BL/6), as responder cells in flat-bottom 96-well culture plates. T cell proliferation ability in MLR was studied using the BrdU (Bromodeoxyuridine (5-bromo-2'-deoxyuridine)) test that was performed in the previous study. Briefly, after one-week culture incubation, BrdU labeling reagent was added and then the cells were harvested by centrifugation at 300 g for 10 min and were fixed in ethanol solution. Afterward, they were incubated with peroxidase-labeled anti-BrdU for 90 min. After three washes, tetramethyl-benzidine (TMB) was added and the mixtures were incubated until color development (15 min). Absorbance values were measured using an ELISA reader. Negative controls were allogeneic T-cells co-cultured with untreated DCs and culture medium alone was used as a control for nonspecific binding. After 48 hours, cultured T cells were harvested and washed once with PBS containing 0.5% BSA and 2 mM EDTA and resuspended in 2 ml. After 48 hours of incubation, the supernatants were collected for evaluation of cytokine levels.

Quantification of cytokine levels. MLR supernatants on 48 hours of culture were collected and stored at -20℃. Mouse Th1/Th2/Th17/Th22 13-plex Flow Cytomix Multiplex kit (eBioscience, USA) was designed for measuring mouse IL-1a, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, 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 analyzed cytokines were included in each reaction according to the kit instruction (ranging from 2.7 pg/mL-2000 pg/mL). Also, the level of IL-35 was assessed using the ELISA method.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Assay. Expression of transcriptional factors such as FoxP3, GATA3, ROR-γ, T-bet, and CCR7 genes mRNA was evaluated using real-time PCR. Briefly, the total RNAs (1 µg) were extracted using the TRIzol reagent (Invitrogen, USA), followed by cDNA synthesis kit (Takara, Japan). The standard curve was drawn for the genes from cDNA. Real-Time PCR efficiency was acceptable for all genes (95-95%). eEF1a1 was used as endogenous control and specific primers for this gene were designed using Allele ID software Version 7.5 (Table 1). Comparative real-time PCR using SYBR Green Supermix (Parsgenome, Tehran, Iran) was performed by real-time PCR machine (ABI step one plus, Applied Biosystems, USA). Finally, FoxP3, GATA3, ROR-γ, T-bet, and CCR7 relative expressions were determined using the equation $2^{-\Delta Ct}$.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software Inc California). Data are presented as mean ± standard deviation (SD) of the mean of at least three independent experiments. Statistical significance was determined using a multiple comparison t-test, one-way ANOVA, and post hock test. Moreover, two-way ANOVA analyses were performed depending on the number of comparatives. P-values less than 0.05 were considered to be statistically significant. The data are represented as mean ± SEM (n: the number of independent experiments (i.e., 4) and ns: non-significant).

Table 1. Specific primers used in this study.

Specific primers	Sequence
FoxP3	F: 5'- AATAGT TCCT TCCCAGAGT TCT TC -3' R: 5'- ATGGTAGAT T TCAT TGAGTGTCCT -3'
GATA3	F: 5'-TCTGGAGGAGGAACGCTAAT-3' R: 5'- CGGTTTCGGGTCTGGAT-3'
ROR-γ	F: 5'- CCATTGACCGAACCAGCC -3' R: 5'- GCCAAACTTGACAGCATCTC -3'
T-bet	F: 5'-AAC CGC TTA TAT GTC CAC CCA -3' R: 5'-TCT CCA TCA TTC ACC TCC ACG -3'
CCR7	F: 5'-GAGACAAGAACCAAAAGCACAG -3' R:5'- GGAAAATGACAAGGAGAGCCA -3'
eEF1a1a (Internal Control)	F:5'- AGTCGCCTTGGACGTTCTT-3' R: 5'- CCGATTACGACGATGTTGATGTG-3'

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

RESULTS

The isolated DCs and T cells characteristics. After their isolation, DCs and T cells were grown overnight at 37℃. The purity of DCs and T cells was respectively assayed by anti-CD11 and anti-CD3 antibodies with FACS. The presence of more than 95% of the surface markers showed a high purity of these cells (Figure 1).

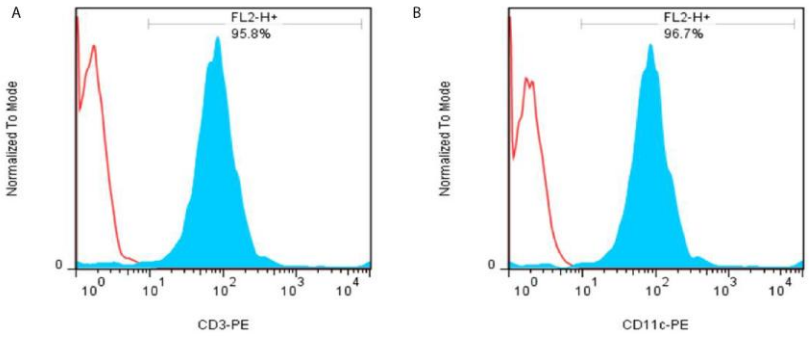

Figure 1. Isolated T cell and DCs were stained with antibodies against specific-surface markers (CD3 (A) and CD11c (B), respectively) and analyzed using flow cytometry. Histogram analysis of cell surface markers showed that the purity of isolated cells was more than 95% and they were not contaminated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

Effects of pp65 antigens on DCs phagocytosis. The take-up FITC-labeled dextran assay was used to assess the phagocytic ability of DCs. The difference in the mean fluorescence intensity (MFI) of phagocyted FITC-conjugated dextran by DCs in the pp65-pulsed group

was lower than the no treatment group and more than the positive control group (treatment with LPS) (12.86 ± 1.1 and 40.36 ± 1.45 vs. 10.1 ± 2.25; both p values <0.0001) (Figure 2).

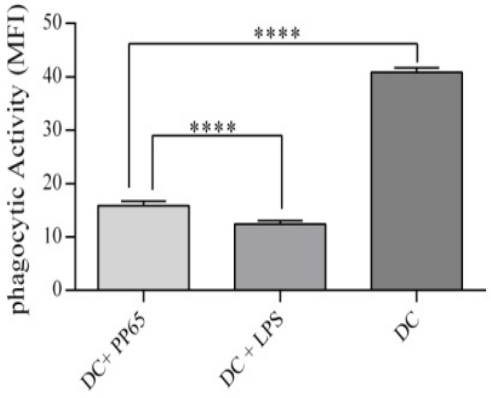

Figure 2. **Comparison of the mean fluorescence intensity (MFI) of phagocyted FITC-conjugated dextran by DCs.** the results of FITC-dextran particle phagocytosis show that two groups (i.e., pp65 and LPS-treated DCs) decreased their ability to take up antigen than immature DCs differently (p<0.0001), indicating that immature DCs from pp65-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).

DCs maturation abstained after pp65 treatment. DCs play an essential role in the initiation of the adaptive immune response. The expression of DCs surface antigens, CD40, CD86, MHC-II, and CCR7 molecules was investigated by flow cytometry and Real-time PCR on immature, LPS-, and pp65-treated DCs for determining levels of pp65-induced maturation of DCs. The mean expression levels of CD40 molecules were significantly increased in pp65-treated DCs as compared with cells obtained from non-treated DCs (47.14 ± 3.75 vs. 21.46 ± 2.1; p<0.0001) (Figure 3A). DCs treated with the pp65 antigen displayed an increase in the percentage of the CD86 in comparison without treatment group and LPS-treated group (61.1 ± 3.43 vs. 41.36 ± 1.1 and 45.12 ± 4.8; both p<0.0001) (Figure 3B). In contrast to the previous pattern, DCs treated with the pp65 antigen showed no increase in the MHC-II molecule compared with cells extracted from non-treated and LPS-treated DCs (3.1 ± 1.28 vs. 7.16 ± 2.6 and 47.25 ± 2.9; both p<0.0001) (Figure 3C). As expected, LPS-treated DCs did not exhibit an overexpressed increase in different groups. Expression of the CCR7 gene in pp65-treated DCs was investigated using Real-Time PCR. In the presence of the pp65 antigen, the mean expression of CCR7 mRNA was lower than LPS treated group but was higher than no-treated DCs (1.4 ± 0.8 vs. 1.8 ± 3.6 and 0.35 ± 1.5; both p<0.0001) (Figure 3D).

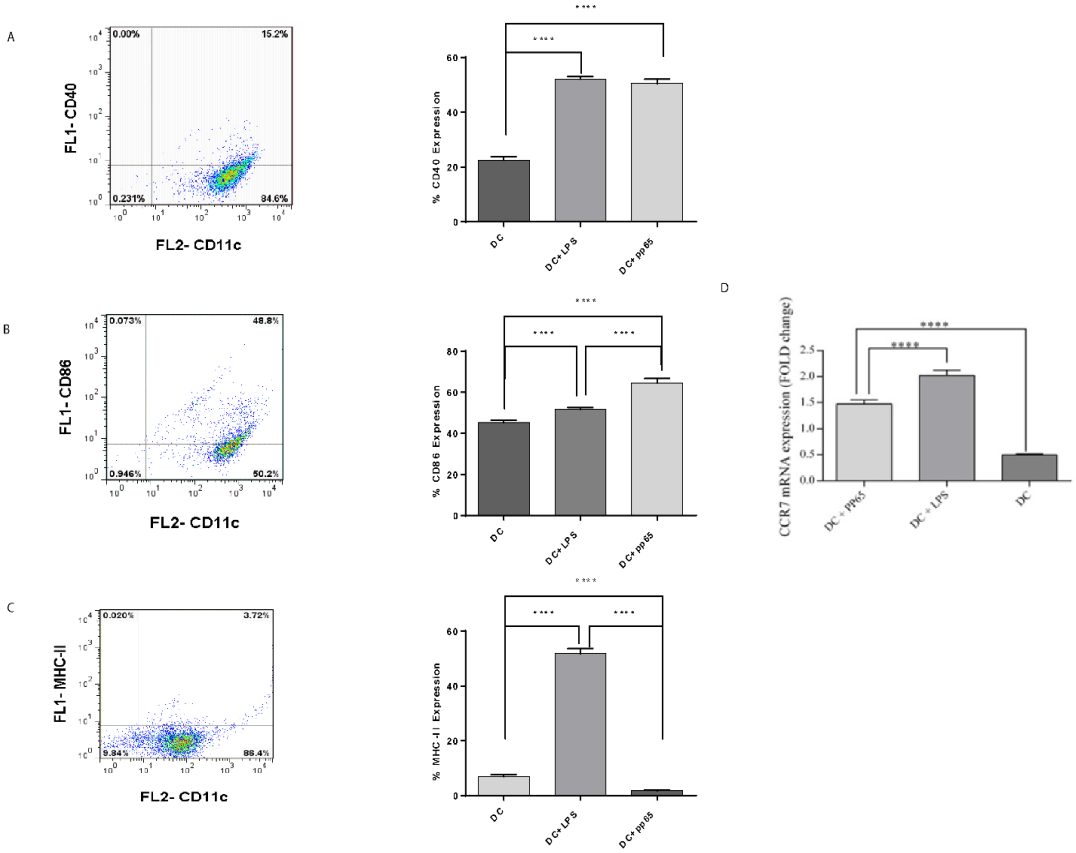

Figure 3. The phenotypic characterization of DCs. FACS analysis was performed with monoclonal antibodies against CD40, CD86, and MHC-II. A) pp65-treated DCs showed significantly higher expression levels of CD40 than the non-treated DCs. B) pp65-treated DCs show significantly higher CD86 expression level than the non-treated DCs. C) LPS-treated and no-treated DCs show significantly higher MHC-II expression level than the pp65-treated group. D) The effect of the pp65 antigen on the expression of CCR7 mRNA in DCs treated with the pp65 antigen investigated using Real-Time PCR. Expression of CCR7 mRNA was significantly lower and higher than the LPS-treated and no-treated group, respectively. 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).

The improved kinetics of pp65-incubated DCs functional maturation.

To further examine the role of induced-DC in T cell proliferation, we tested whether pp65-treated DCs can induce T cell proliferation compared to the absence of antigen. The proliferation of T cells compared to pp65-treated DCs showed a vast proliferation in pp65-induced DCs than only cultured. Also, when DCs were treated by pp65, a significant proliferation occurred in comparison to LPS-treated as a positive control, and negative control (1.13 ± 1.18 vs. 0.81 ± 0.31 and 1.21 ± 2.8; $p<0.05$ and $p<0.001$, respectively). This result indicated that Ag-induced DCs are able to uniquely stimulate T cell proliferation *in vitro* (Figure 4).

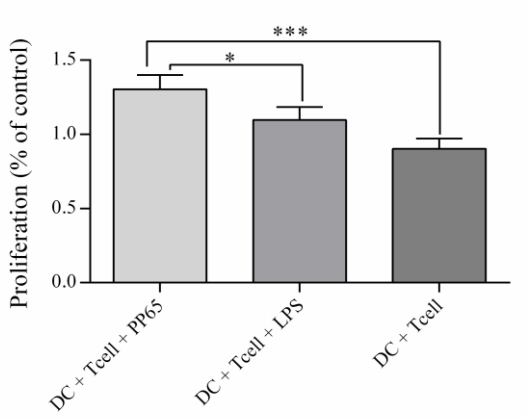

Figure 4. Effects of pp65-treated DCs on T-cell proliferation in MLR. The proliferation of alloreactive T-cells in MLR was measured using BrdU incorporation. Moreover, in pp65-treated DCs, T-cell proliferation was higher than T-cells co-cultured with non-stimulated DCs. The results present the mean ± SD of three independent experiments (***: $p<0.001$ and *: $p<0.05$).

Elevated expression levels of Foxp3, GATA3, ROR-γ, and T-bet mRNA as T cell proliferation indicator due to Ag-induced DCs. The expression levels of Foxp3, GATA3, ROR-γ, and T-bet genes were studied in MLR by Real-time PCR. Foxp3 expression in pp65-stimulated DCs co-cultured with T cells was observed to be different from non-stimulated DCs and LPS-treated DCs, co-cultured with T cell, and the second group expressed higher levels of Foxp3 (0.75 ± 0.8 vs. 0.96 ± 0.6 and 1.15 ± 0.5; $p<0.43$ and $p<0.38$, respectively) (Figure 5A). Gene expression of GATA3 in pp65-stimulated DCs cultured with T cell revealed higher level compared to DCs as a negative control. However, the expression of GATA3 in LPS-stimulated DCs showed a higher difference compared to pp65-stimulated DCs and negative control (1.15 ± 0.5 vs. 0.6 ± 0.1 and 1.3 ± 0.5; $p<0.25$ and $p<0.34$, respectively) (Figure 5B). In contrast to previous gene pattern, ROR-γ expression in pp65-treated DCs showed higher levels than LPS- and non-treated DCs probably due to the effect of pp65 Ag (1.8 ± 0.54 vs. 0.95 ± 0.24 and 0.4 ± 0.12; both $p<0.000.1$) (Figure 5C). As shown in Figure 5D, non-stimulated DCs cultured with T cell revealed declined expression of T-bet compared to pp65-treated DCs (0.5 ± 0.16 vs. 1.75 ± 0.67; $p<0.05$). In addition, LPS-stimulated DCs cultured with T cell showed decreased expression of T-bet compared to pp65-stimulated DCs (1.68 ± 0.26 vs. 1.75 ± 0.27; $p<0.32$).

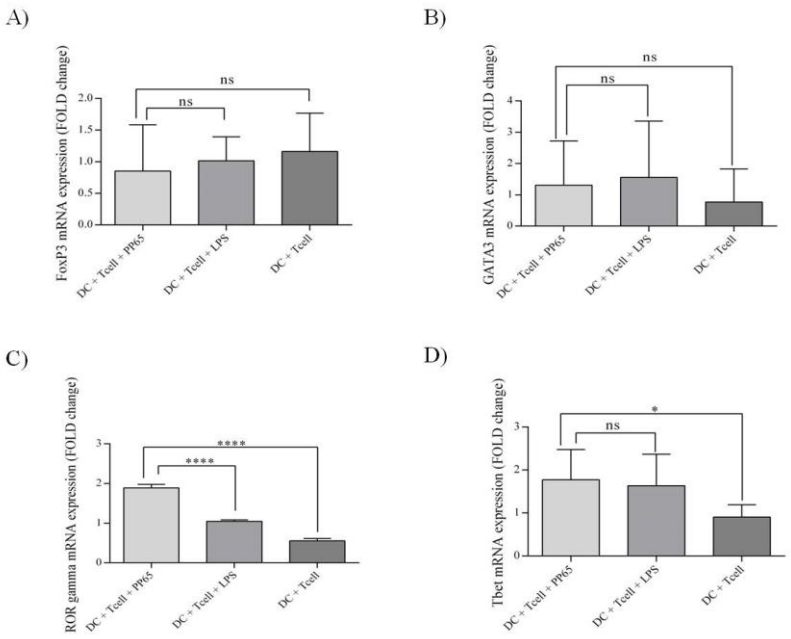

Figure 5. The mRNA expression of Foxp3, GATA3, ROR-γ, and T-bet genes in MLR. A) Foxp3 mRNA expression was not significantly lower in the treatment group with pp65 than the non-treated DCs and LPS-stimulated DCs group. B) Gene expression of GATA3 in pp65-stimulated DCs shows no significantly higher level than DCs as a negative control. C) ROR-γ expression in pp65-treated DCs shows a significantly higher level compared to LPS- and non-treated DCs. D) pp65-treated DCs represent a significantly higher expression of T-bet compared to non-stimulated DCs (Positive control: treatment with LPS; negative control: no treatment with antigens). The results show the mean ± SD in three independent experiments (ns: $p \geq 0.05$; *: $p<0.05$; and ****: $p<0.0001$).

Elevated proinflammatory Cytokine Production in contrast to indigenous behavior of cytokines. We assessed the immunostimulatory capacity to ensure that the higher levels of functional activation of DCs, when encountering exposed Ag, were accompanied by higher levels of functional maturation. Levels of different cytokines in the cell culture supernatant were determined on a 48-h culture in MLR. The raised secretion levels of IL-2, IL-17A, and IL-22 level were found in the pp65-treated DCs group than non-treated and LPS-treated DCs groups that have significant difference (101 ± 5.54 vs. 0.15 ± 0.05 and 56.4 ± 4.12; both $p<0.001$), (35.23 ± 3.54 vs. 0.15 ± 0.15 and 15.4 ± 2.12, both $p<0.001$), and (21.46 ± 3.24 vs. 7.25 ± 2.24 and 0.12 ± 0.05, respectively) (Figure 6A, Figure 6C, and Figure 6D). The IL-6 level was increased in the pp65-treated DCs group than non-treated-DCs group that indicated a significant difference (317 ± 5.54 vs. 0.15 ± 0.05; $p<0.001$) (Figure 6B). Different patterns of IL-6, IFN-γ, and TNF-α secretion were found in the pp65-treated DCs category compared to the non-treated DC group and LPS-treated group (302 ± 7.74 vs. 0.11 ± 0.05 and 285.4 ± 6.12, respectively) and (40 ± 3.54 vs. 0.11 ± 0.16 and 36.1 ± 3.12, respectively) that these differences observed were statistically significant, $p<0.001$) (Figures 6B and 6F). In comparison to other cytokines, IFN-γ showed a different pattern, suggesting that the LPS-treated group has a higher level than pp65-treated DCs and negative control (60 ± 2.74 vs. 0.18 ± 0.05 and 65.2 ± 5.12; both $p<0.001$) (Figure 6E). These results suggest that an immune response, characterized by a high level of proinflammatory cytokines,

may play a role in determining the outcome of the different T-cell responses. In comparison to other cytokines, IL-1a, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17F, IL-21, and IL-35 were found in undetectable levels that are shown in Figure S6.

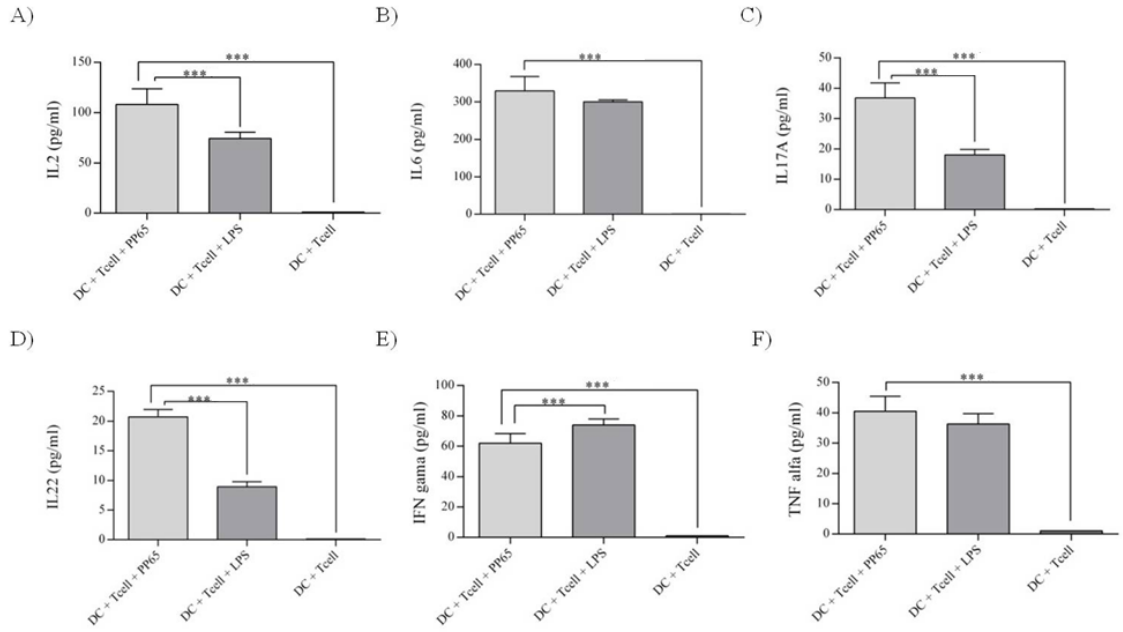

Figure 6. Kinetics of the inflammatory response by T cells co-cultured with and without pp65 and LPS. pp65-treated DCs co-cultures with T cell were established in different stimulation regimens (A-F). Finally, a cytokine release assay was performed using FACS from supernatants. The result show production of IL-4 (A), IL-10 (B), IL-13 (C), IL-5 (D), IL-21 (E), IL-9 (F), IL-1α (G), and IL-35 (H). The similar secretion pattern of the mentioned cytokines (in the previous sentence),is shown in the LPS-treated DCs category than no-treated and pp65- treated DCs group, (Fig 6 A-H) that the significant difference of undetectable levels of these cytokines was indicated in LPS-treated DCs group than two other groups. Data are presented as means ± SEM of at least three mice per group from one of two independent experiments, significant values as calculated by unpaired, and two-tailed Student's t-test (***: p<0.001).

DISCUSSION

In the present study, the effect of CMV pp65 recombinant protein was evaluated on the function and maturity of DCs. For this purpose, DCs were treated with the pp65 antigen and then the DC maturation was investigated by assessing the expression of the costimulatory molecule. We also assessed the ability of DCs to shift T-cell proliferation and differentiation. Our findings showed that DC phagocytosis, in the group treated with pp65, was lower than the negative control, demonstrating that the pp65 antigen may induce DC maturation. Our results were consistent with a previous study showing that CMV-infected mature DCs lead to decreased levels of cell surface markers required for phagocytosis (21).

We also assessed the costimulatory molecules such as CD40, CD86, and MHCII, and DC migration factor (i.e. CCR7). Our results showed that the presence of CD40 and CD86 molecules in the group treated with the pp65 antigen was significantly higher

than the negative control group but lower than the positive control group. It can be concluded that the pp65 antigen leads to the induction of DC maturation and proliferates T cells through their stimulation (22,23). This result was also reported in previous studies showing that DCs' proliferation and maturation increase after CMV infection (21,24).

A previous study showed that immature DCs have a lower number of costimulatory molecules, which is consistent with our findings (25). Wang *et al.* (2009) reported that deactivation of MCMV by UV radiation leads to decreased production of inflammatory cytokines and decreased expression of CD80 and CD86 molecules (26). In our study, untreated groups showed high phagocytic capacity, implying that the presence of immature cells in the untreated group. We found no increased levels of MHCII in the group treated with pp65 as compared with the non-treated group; this result is consistent the result of a previous study (9). The CCR7 molecule is considered to be the most important marker for DC migration to the lymph tissue to provide antigens for T cells (27). The results of our study showed that pp65, in addition to DC maturation with increased costimulatory molecules, led to the expression of DC migration molecules; in fact, the antigen improved the DCs maturation. This was parallel with the previous reports (46,47).

Previous studies have shown that the number of CD8+ cells containing memory and effector markers, such as CCR7, increases following CMV infection (28). In other studies, it was shown that the frequency of virus-specific CD8+ cells with the CCR7 marker increased in immunocompromised individuals (29). In this regard, Gibson (2015) showed that when PBMCs of children with congenital CMV infection were stimulated with the pp65 antigen, their pp65-specific CD4+ count was lower than those of adults (30). In addition, memory cells lacking CCR7 in these patients were less than those of adults. The reason for this result is the lack of cell exposure to the antigen. Therefore, it can be concluded that these cells underwent the maturity process when treated with the pp65 antigen. Our finding showed that the T-cell proliferation in the treatment group was higher than that in the non-treated group, showing that mature DCs caused by the pp65 antigen resulted in the proliferation of T cells; this result is consistent with that of Wagner *et al.* (31). The proliferation of T cells can be performed by the activity of DCs, following secretion of different cytokines from different T cells so that these CMV specific T cells included a mixture of CD4+ and CD8+ effectors and specific cytotoxicity correlated with IFN-γ production based on induced-pp65 DCs (6). To investigate this result, we assessed the ability of DCs to express transcription factor molecules in T subsets such as Th1, Th2, Th17, and Treg.

The FoxP3 transcription factor differentiates Treg (32). We showed that DCs treated with the pp65 antigen, along with T cells, were unable to increase the expression of the FoxP3 molecule in T cells, indicating no production of Treg cells. These data suggested that the pp65 antigen did not result in Treg induction and were unable to alter tolerogenic DCs that can inactivate T cells or convert them to Treg cells. Consistent with our findings, the results from a study conducted by Weinberg *et al.* showed that the activity of Treg cells is related to the production of FoxP3 (33). In this regard, Almanan *et al.* (2017) showed that these cells lead to persistent infection, and the lack of Treg induction after induction of immunity with dominant viral antigens is quite usual (34). Other studies showed that Treg was less effective at the beginning of CMV infection based on pp65-pulsed DCs study (35). Another study in 2014 reported that Treg-containing FoxP3 cells are found in the bone marrow of individuals after transplantation

with stem cells, indicating the role of immunotherapy of these cells (36). It can be concluded that DCs treated with pp65 could not differentiate into proliferative DCs producing more IL-10 but could differentiate T cells into Treg with more IL-10 levels. If pp65-pulsed DCs could induce CD8 T cells, however, antigen-binding CD8 T cells were still present and circulating pp65-specific IL-10 producers developed. In summary, the use of immature DCs led to silencing the effector T-cell functions and raising the induction possibility. Moreover, the silencing and recovery of effector T-cell function was associated with the appearance and then decline of peptide-specific IL-10 producers (23).

One study showed that the amount of GATA3-containing cells in the first two days of CMV infection was high but significantly decreased in the following days (37). Here, the reason for the lack of Th2 differentiation is the low concentration of pp65. Moreover, Duechting *et al.* revealed delayed activity of Th2 cells against CMV infection as compared with Th1 and Th17 cells (38). Consistent with that study, we showed that the level of Th2 cytokines was not detectable, and it might be better to examine the level of these cytokines at a longer time to infection in order to demonstrate the role of these cells in CMV infection.

ROR-γ and T-bet are Th17 and Th1 differentiation factors, respectively. Our results showed that DCs treated with pp65 plus T cells were able to increase the expression of ROR-γ and T-bet molecules in T cells, indicating that the main factor is the production of Th17 and Th1 cells. Thus, according to previous results, we indicated the role of Th17 and Th1 cells in CMV inflammatory immunity (39,40). We also showed an increase in the level of IFN-γ in the presence of pp65. Swedish scientists in 2005 showed that the inoculation of pp65 mRNA and the addition of pp65 to DCs lead to high-level production of IFN-γ; this result is consistent with the previous report based on a critical role for IFN-γ-producing CD4+ T cells in protection against CMV disease in primary immune responses (29, 41). Similar to our work, Gao *et al.* studied the effect of the pp65 gene on the CMV virus on DCs in mice and showed the production of IFN-γ from these cells (42). Our results suggest that the levels of TNF-α, IL-2, IL-6, IL-17A, and IL-22 increased in the suspension of MLR in the presence of pp65. Therefore, it can be concluded that the rate of this cytokine increased only in the presence of maturation stimuli such as LPS.

In addition, no increase was found in the levels of IL-4, IL-5, and IL-13. Th1 cells primarily produce IFN-γ and IL-2, whereas Th2 cells produce IL-4, IL-10, and IL-13, and IL-17 for Th-17 cells. Traditionally, these cytokines are measured within 6 h in flow cytometry. We showed that 48 h of stimulation is not sufficient to detect peptide-induced production of IL-4, IL-10, IL-35, IL-1α, IL-10, and IL-13. Hence, measurement of IL-4, IL-10, IL-5, and IL-13 producing Th2 cells requires more cultures for one week. Besides, frequency measurements of IL-9 and IL-35 and other cytokines secreting cells are necessary for this purpose. However, our data showed that 48 h is required to reveal the full frequency of protein antigen-specific Th1 and Th17 cells based on the increased levels of specific cytokines. Therefore, regarding raised levels of IL-2 and IFN-γ and also declined or undetectable IL-4 and IL-10 levels, it can be concluded that Th1 is induced by pp65-treated DCs.

Furthermore, the undetectable level of IL-35 may indicate a lack of their critical roles in CMV infection. This cytokine secreted by regulatory T-cells (Tregs) and CD8+ regulatory T cells (43) and also suppressed inflammatory responses of immune cells (44). IL-35 has selective activities on different T-cell subsets; it induces proliferation of

Treg cell populations but reduces the activity of Th17 cell populations (45). Nevertheless, our data are not sufficient for determining its role in this study.
Our data suggested that DC maturation and function are key targets for the regulation of cellular immunity, especially Th1 and Th17. Also, the induction of maturation in pp65-capturing DCs may promote the induction of T cells *in vitro*. Finally, the differentiation of some T-cell subsets such as Th2 and Treg may not occur. However, for elucidating the impact of specific pp65-impulsed DCs on immune cells, in-vivo studies are needed.

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