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The Efficacy of Coriandrum Sativum, Anethum Graveolens, And Linum Usitatissimum Essential Oil Nanoemulsions On Human Dendritic Cells

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ABSTRACT

Dendritic cells (DCs) are specialized antigen-presenting cells that orchestrate the innate and adaptive immune response. The aim of this study was to prepare nanoemulsions and investigate their role in controlling DCs phenotype expression, apoptosis, and cytokine secretion. Three nanoemulsions (NEs) were produced by mixing essential oils, surfactants, and water with droplet sizes of NEs formulations in the range of 25-62nm. DCs were generated in vitro from peripheral blood mononuclear cells of healthy donors, followed by stimulation with lipopolysaccharide (LPS), Vitamin D and NEs. DCs morphology was determined using a bright-field inverted microscope. Phenotype characteristics and apoptosis were investigated using flow cytometry, and ELISA technique to determine IL-10 and IL-12 (p70) production. NEs showed significantly lower expression of maturation marker CD83 compared to LPS which stimulated DCs. Also, costimulatory molecules CD80 showed higher expression and CD86 showed significantly lower expression compared with unstimulated DCs. In addition, NEs showed a non-significant effect on the viability and apoptosis of DCs at high concentrations, beside secretion of significantly low levels of IL-12 (p70) and high levels of IL-10. In conclusion, the result suggested that NEs may have a tolerogenic effect on Dendritic cells.

Key words: *Dendritic cells, Nanoemulsion, Essential oils, Immune tolerance.*

INTRODUCTION

Various plant components may have the ability to affect the activity of DCs (innate and adaptive immune cells [1-3]) including essential oils, which are secondary metabolites of aromatic plants [4]. These essential oils are synthesized, stored and released by different structures that occur in leaves, stems, flowers, roots, and seeds with specific attributes to plants, and they are known to exhibit low toxicity to mammals [5]. EOs are hydrophobic compounds, thus they have low solubility range in water which limited use in food, beverages, and other products. The problem of solubility can be solved by encapsulating plant oils into the emulsion system, which is called microemulsion or Nanoemulsions [6]. Nanoemulsion is a heterogeneous mixture that usually consists of two immiscible liquids, one is water and the other is oil, and the droplet size ranges from 5-200 nm. An interfacial tension exists between the two liquids and can be stabilized by adding surfactants which are chemical compounds highly soluble in at least one of the liquid phases [7]. Depending on its composition there are three types of nanoemulsions: oil in water, water in oil, multiple emulsion (oil in water in oil; water in oil in water). Nowadays, nanoemulsions can be used in cell culture technology, cancer therapy, and controlled drug delivery [8]. The immune system is fundamental for survival as it protects the body from pathogens, viruses, bacteria, and parasites that cause diseases through immune responses that mediated by a variety of cells and soluble molecules secreted by these cells [9]. Dendritic cells are antigen-presenting cells that act as a bridge

between innate and adaptive immune response by stimulating naïve T-cell, either by induction of immunity or tolerance. They perform the distinct function of antigen capture in one location and antigen presentation in another [10]. Nanoparticles can be engineered and designed to either target or avoid interactions with the immune system. An interaction between a nanoparticle and the immune system is considered desirable when it may lead to various beneficial medical applications, such as vaccines or therapeutics for inflammatory and autoimmune disorders [11].

MATERIALS AND METHODS

Extraction of essential oils

Plants seeds were oven-dried for 24 h at 70°C. Then, dried seeds were grinded into a fine powder and essential oils were extracted from 600 g dried and powdered seeds by Hexane at 65-70°C using Soxhlet extractor. The resulting essential oils were stored at -20°C in sealed brown glass vials until use.

Preparation of nanoemulsion

Water in oil NEs were prepared by mixing 1ml from distilled water, 500 µl from previously extracted essential oils and 100 µl from each non-ionic surfactant (tween 80, span 20), except for *Linum usitatissimum* (200 µl of tween 80). The mixtures were sealed and placed in boiling water up to 90-100°C in the water bath until slightly opaque nanoemulsion was formed similar to the color of their extracted essential oils. Different concentrations of nanoemulsions (35, 50, and 100 ppm) were prepared for each plant oil.

Physical characterization of nanoemulsion

All prepared NEs were diluted with media (DMEM) and centrifuged at 4500 rpm for 15 min. Then**,** droplet size and zeta potential ofnanoemulsions were obtained at 25°C and analyzed using the Zetasizer Nano ZS analyzer.

Generation of human monocyte-derived DCs (MoDCs)

Fresh blood (50 ml) was collected from healthy human volunteers into LH vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by diluting blood 1:1 in HBSS containing 2 ml EDTA, followed by density gradient centrifugation on Lymphoprep. PBMCs or Buffy coat layer was removed and washed three times in HBSS containing 2 ml EDTA and resuspended in complete medium RPMI 1640. To obtain monocytes, PBMCs were incubated in 12-well plates for 2 hours in 5% CO_2 humidity at 37°C, then non-adherent lymphocytes were removed. Adherent monocytes were differentiated into immature MoDCs by culturing for 7 days in complete medium supplemented with 800 U/ml of rh GM-CSF and 500 U/ml of rh IL-4. On the seventh day, DCs maturation was stimulated by adding nanoemulsions and incubated at 37°C.for 24 h in 5% CO_2.

Monocytes viability assay

Adherent monocytes were resuspended into complete medium RPMI 1640 with 50 µl of each NEs concentrations (35, 50, and 100 ppm) and incubated for 24 h in 5% CO_2 humidity at 37°C. Cells were collected and resuspended in 0.5 ml of PBS, and 10 µl of the cell suspension was stained with 10 µl of 0.04 % trypan blue for counting and determining of cell viability by using a hemocytometer chamber. Cell viability was determined using an inverted microscope (Nikon Eclipse Ti, Tokyo, Japan) supplemented with digital camera software (NIS-Elements F 3.2, Nikon, Tokyo, Japan) and the results were expressed as the percentage of survived cells.

Flow Cytometry analysis

Human DCs were stained with fluorescein isothiocyanate (FITC) human monoclonal antibodies (CD14, CD83, CD80, CD86, and HLA-DR). Then, cells were incubated in the refrigerator at 4°C in the dark for 30 min. DCs were then washed twice with PBS and analyzed by flow cytometry to determine the phenotyping of DCs. Apoptosis of DCs was also determined by staining cells with annexin V incubation reagent. Cells were incubated in dark on ice for 15 min at room temperature, then washed and analyzed by flow cytometry.

Cytokine analysis

Cytokines levels of IL-10 and IL-12 (p70) from DCs culture supernatant were determined using ELISA kits (Biolegend) according to the manufacturer's protocol.

Statistical analysis

All data were expressed as mean ± standard deviation ($\bar{x}$ ± SD). Statistical analysis was performed with one-way analysis of variance (ANOVA) and pairwise t-test using MegaStat and Microsoft Excel. The statistically significant difference was considered when *p*-value <0.05.

RESULTS AND DISCUSSION

Physical characterization of nanoemulsion

Zetasizer Nano (ZS) analyzer was used to identify the Droplet size (Z-average), polydispersity index (PDI) (homogeneity or quality of the dispersion) and Zetapotential(electrophoretic mobility)of the nanoemulsions formulations. Droplet size was in the range between 25-62 nm. All NEs have negative zeta potential values as shown in **Table 1**. Neem oil, tween 20, and distilled water were successfully optimized with a mean droplet size of 67.85 nm [12]. A mixture of surfactants provides better effectiveness than one.The droplet size increases as the amount of water increases and decreases with the amount of surfactant due to the increase in the interfacial area and the decrease in the interfacial tension [13]. It was clear that the amounts of components used in the preparation of NEs affect the droplet size of the nanoparticles. Non-ionic surfactants havehydrophilic and lipophilic molecules that balance the size and strength of these opposing molecules; this is called a hydrophilic-lipophilic balance number [14].

Table 1: The Z-average diameters, PDIs, and zeta potential measurements of nanoemulsion formulations expressed as $\bar{x}$ ± SD.

Oil Nanoemulsions	Z-average diameter (nm)	PDI	Zeta potential (mV)
***Linum usitatissimum* Nanoemulsion**	38.30 ± 8.28	0.216	-0.267 ± 0.048
***Coriandrum sativum* Nanoemulsion**	55.25 ± 6.13	0.111	-2.360 ± 2.740
***Anethum graveolens* Nanoemulsion**	28.67 ± 3.13	0.109	-2.170 ± 2.130

Monocytes viability assay

Trypan blue stain 0.04% assay was used to determine the viability of human monocytes after the cells were treated with different concentrations of nanoemulsions for 24 hoursas shown in Table 2. These results indicated that the viability of monocytes decreased as the nanoemulsion concentration increased, therefore, the lowest concentration (35 ppm) of three NEs was used for the next experiments on DCs. Trypan blue stain is negatively charged and interacts with damaged membrane cells [15]. Viable cells have an intact cell membrane, so they do not take in the stain from their perimeter. On the other hand, non-viable cells do not have an intact and functional membrane so they do take up the stain from their surroundings. These results indicate the ability to distinguish between viable and non-viable cell, since the former is unstained, bright, small, and round, while the latter are stained, dark, and swollen [16].

Table 2: Percentage of human monocytes viability treated with different concentrations of NEs for 24 h, expressed as $\bar{x}$ ± SD.

Nanoemulsion concentrations	% Cell viability for 24 h
Control	94.6±3.0
L6 (35 ppm)	91.4±1.7
L7 (50 ppm)	78.1±1.0
L8 (100 ppm)	20.7±6.0
C6 (35 ppm)	89.7±1.4
C7 (50 ppm)	83.3±1.6
C8 (100 ppm)	74.8±2.0
A6 (35 ppm)	87.6±2.1
A7 (50 ppm)	82.0±2.1
A8 (100 ppm)	28.3±5.3

L: *Linum usitatissimum* oil, C: *Coriandrum sativum* oil, A: *Anethum graveolens* oil

Effect of nanoemulsions on Dendritic cells phenotyping

The effect of different NEs on the DCs surface marker expression is demonstrated in Table 3. Most DC cultures showed down-regulation of monocyte marker CD14 expression, and this was proof that monocytes were differentiated into DCs. CD14 is a marker for monocytes whose expression decreases gradually during DC differentiation from monocytes to immature DCs as a result of the addition of GM-CSF and IL-4 into their growth medium [17]. The up-regulation of HLA-DR was observed in all cultures upon stimulation. HLA-DR is an MHC class II cell surface receptor. It is a marker for immune stimulation to present antigens, normally expressed on professional antigen-presenting cells (APCs) like DCs. Zheng and colleagues [18] reported that mature DCs stimulated with LPS showed up-regulation of MHC class II. CD83 is a surface marker that differentiates immature and mature human DCs. The exposure of immature DCs to LPS enhanced the expression of DC83 in comparison to unstimulated DCs, while NEs suppress the maturation of DCs even in the presence of LPS. Tomić and colleagues [19] indicated that gold nanoparticles (GNPs) impaired significantly the LPS-induced expression of CD83 and CD86 by DCs. CD80 is a co-stimulatory molecule expressed on APCs and known to provide efficient costimulation along with CD86. CD80 was highly expressed upon exposure to LPS, vitamin D, and NEs in comparison to unstimulated DCs. CD86 is a co-stimulatory molecule expressed on APCs and is a more important costimulator of T-cell activation. CD86 was highly expressed when stimulated with LPS in comparison to unstimulated DCs. Also, NEs+LPS expressed high levels of CD86 compared to LPS, while NEs alone showed low regulation of CD86 expression.

CD80 and CD86 are molecules on the DC surface that can interact with CD28 on T-cells, and they provide a potent costimulatory-signaling pathway for T-cell proliferation and cytokine production. Although CD80 and CD86 are both costimulatory molecules required for sufficient antigen presentation. The proliferation of T-cells, CD80, and CD86 have opposing functions through CD28 and CTLA-4 (CD152) on Treg. CD28 is a key activator, whereas CTLA-4 is a definitive attenuator of T-cell responses. CD86 promotes T-cell activation to induce immune response upon attachment to CD28, and CD80 induce Treg to tolerate undesirable immune responses as in transplantations and autoimmune disease upon attachment with CTLA-4 [18-20]. Verdijk and colleagues [21] found that LPS-stimulated DC exhibited a typical mature phenotype compared to unstimulated cells, as evidenced by the expression of maturation marker CD83, and costimulatory molecules CD80 and CD86. Stimulation by 1,25(OH)2D3 can arrest the differentiation and inhibit maturation of mature DCs, resulting in decreased expression of maturation markers CD80, CD86, and retention of antigen uptake [22]. Similarly, Piemonti *et al.* [23] stated that analysis of D3-DC showed decreased levels in CD86 expression, also, after exposure to LPS, D3-DC was unable to up-regulate CD83 as well as the molecules involved in antigen presentation (MHC II, CD80, and CD86). These results suggested that NEs alone were unable to induce full DC maturation and may drive partial or uncompleted DC maturation, which presented with low expression of some maturation markers. However similar expressions of markers between Vit D treatment and NEs treatment indicated the possibility of tolerogenic effects of these particles. Further studies are required to confirm the possibility of tolerogenic effects of the particles, especially by functional studies. The influence of other factors such as the NEs composition and difference in zeta potential might also affect the biological activity of NEs, since different surface charges of NEs may cause varied physical interactions with cellular components [24].

Table 3: Percentages of Dendritic cells surface markers' expression upon stimulation with 3 different nanoemulsions at concentration 35 ppm.

Marker	Unstimulated DCs (%)	Dendritic cells stimulation (%)							
		LPS	Vit D	L6	L6+LPS	C6	C6+LPS	A6	A6+LPS
CD14	1.4±0.5	0.4±0.2	1.3±1.3	11.0±4.7	6.1±4.5	0.6±0.4	0.8±0.7	0.8±0.7	1.9±2.5
CD83	2.6±0.8	62.1±4.9	2.5±0.3	16.3±2.3	18.4±2.7	2.7±0.5	23.6±1.4	2.5±0.6	10.3±0.6
CD80	76.6±6.4	98.0±0.9	82.7±4.9	93.1±1.8	93.0±5.7	89.6±3.9	89.8±1.5	84.8±2.6	86.4±1.4
CD86	16.3±3.1	94.0±2.0	29.1±5.2	48.9±0.1	86.5±1.6	36.2±2.4	92.8±3.4	46.2±1.1	91.5±3.0
HLA-DR	98.7±1.2	99.7±0.2	97.9±2.3	99.1±1.1	97.8±2.6	99.1±1.0	99.2±0.3	97.9±1.4	98.8±1.0

Unstimulated DCs: Dendritic cells + medium only, LPS: lipopolysaccharide (DCs positive control stimulator), Vit D: Vitamin D (DCs negative control stimulator), L6: *Linum usitatissimum* oil at 35 ppm, C: *Coriandrum sativum* oil at 35 ppm, A: *Anethum graveolens* oil at 35 ppm.

Apoptosis of Dendritic cells

Annexin V allows the identification of cell surface changes that occur during the early apoptotic process by flow cytometry. The effect of NEs on DCs apoptosis is shown in Table 4**.** Dendritic cell (DC) apoptosis is an important event that regulates the balance between tolerance and immunity through multiple pathways and defects in DC apoptosis can trigger autoimmunity [25]. Camptothecin (CAM) is a topoisomerase inhibitor that has been shown to induce apoptosis and cell death in a variety of cell types [26], and it was used as a positive control for apoptosis. Our results showed that most unstimulated DCs were viable and not apoptotic in comparison to DCs treated with CAM. Hingoraniet *et al.* [26] indicated that in the absence of Camptothecin, the majority (79.98%) of untreated stimulated PBMCs were viable and non-apoptotic (Annexin V–PI–); with the increasing doses of Camptothecin, there was an increase in the (Annexin V+PI+) late apoptotic cells' population accompanied by a lesser increase in the (Annexin V+PI–) early apoptotic cells' population. DCs also treated with LPS and different NEs concentrations showed low apoptotic and no necrotic effect on DCs comparable to unstimulated DCs. This may be due to preparation of NEs from natural component "EOs and water". Also, Non-ionic surfactants are relatively less toxic than their ionic counterparts [27]. Anti-apoptotic Bcl-2 protein is normally highly expressed in immature DCs, but is down-regulated in mature DCs [25]. This would explain that LPS-induced DC maturation showed apoptotic effect more than the NEs, since NEs did not induce maturation to DCs as seen in marker CD83.This experiment considered as cytotoxicity assay on DCs and showed that NEs had no toxicity on DCs. Although, that increased concentrations of NEs led to slight decrease in the viability of DCs. It is clear that NEs did not induce apoptosis nor necrosis to these cells. This may be beneficial for using NEs to generate or suppress immune response mediated by DCs.

Table 4: Percentages of viable, early apoptotic, late apoptotic and necrotic DCs upon stimulation with different concentrations of three nanoemulsions expressed as $\bar{x} \pm SD$.

Marker	Unstimulated DCs (%)	Dendritic cells stimulation (%)										
		LPS	CAM	L6	L7	L8	C6	C7	C8	A6	A7	A8
Necrosis	0.8±0.2	0.7± 0.6	1.1± 0.3	0.7± 0.6	0.4± 0.3	0.5± 0.5	0.8± 0.2	0.6± 0.3	0.7± 0.6	0.6± 0.2	0.8± 0.1	0.7± 0.6
Viable cells	74.7±2.8	72.1± 2.6	57.3± 6.1	82.5± 2.9	75.6± 5.1	63.8± 1.2	84.8± 2.9	76.6± 5.4	76.3± 2.0	84.1± 1.2	74.3± 2.6	69.4± 0.0
Late apoptosis	12.4±3.5	19.6± 1.3	26.9± 2.7	8.1± 1.4	9.9± 0.3	12.0± 0.7	8.0± 2.1	10.6± 4.5	10.1± 5.5	8.5± 1.6	10.1± 2.3	12.9± 0.0
Early apoptosis	9.2±0.1	7.7± 0.4	21.9± 1.4	6.5± 0.3	10.0± 3.2	9.5± 3.1	3.8± 0.5	7.6± 0.4	8.9± 3.0	4.7± 0.2	5.0± 0.6	7.5± 0.1

CAM: (Camptothecin) positive control for apoptosis, L6,7,8: *Linum usitatissimum* oil at different concentrations (35, 50, and 100 ppm), C6,7,8: *Coriandrum sativum* oil at different concentrations (35, 50, and 100 ppm), A6,7,8: *Anethum graveolens* oil at different concentrations (35, 50, and 100 ppm).

Dendritic cells cytokines secretion

Cytokines are proteins produced by various types of cells including immune cells in response to activation. DCs produce IL-12 (p70) and IL-10 cytokines upon stimulation with LPS, Vit D, and NE concentrations. The amount of cytokines secreted by DCs was measured using the ELISA technique shown in Tables 5 and 6. IL-10 is an anti-inflammatory cytokine that inhibits the capacity of monocytes and macrophages to present antigen to T-cells via an inhibitory effect on the expression of MHC II, costimulatory molecules such as CD80 and CD86 and therefore down-regulates the expression of IL-1, IL-6, IL-8, IL-12, and TNF-α [28]. IL-12 (p70) is a pro-inflammatory cytokine produced by monocytes, macrophages, and dendritic cells in response to bacterial products such as lipopolysaccharide (LPS), intracellular pathogens or upon interaction with activated T-cells. IL-12 is essential for the differentiation, proliferation, and maintenance of T-cell responses and activation [29]. Our cytokines analysis revealed that LPS individually could significantly promote DCs to secrete elevated levels of IL-10 and IL-12 (p70) in comparison to control unstimulated DCs; we found that they produced low levels of IL-10 and IL-12. Corinti *et al*. [30] found that immature monocyte-derived DCs released low but sizeable amounts of IL-10; but after stimulation with LPS, DCs secreted high levels of IL-10. Another study by Abadi *et al.* [31] revealed that resting DCs make large amounts of IL-12 after stimulation with LPS. Vit D-treated DCs secrete significantly high levels of IL-10 compared to unstimulated DCs and low levels of IL-12

(p70) compared to LPS-stimulated DCs. Barragan *et al.* [22] indicated that expression of maturation markers CD80 and CD86 were inhibited, along with decreased IL-12 and up-regulation of IL-10 production after DCs were stimulated with 1,25(OH)2D3. This was also revealed in our present results. Similarly, 1,25(OH)2D3 inhibits the presentation of soluble antigens and affects IL-12 production by DC [20]. Moreover, the secreted levels of IL-10 and IL-12 by DCs treated with NEs and NEs+LPS were evaluated. All NEs and NEs+LPS treated DCs secret significantly low levels of IL-12 compared to LPS and high levels of IL-10 compared to unstimulated DCs. These results may be functionally prove the possibility that NEs have tolerogenic effect on DCs, which appeared when used in combination with LPS to induce maturation. Interestingly, NEs were unable to induce maturation or IL-12 production even in the presence of LPS.

Table 5: Levels of IL-10 and IL-12 (p70) secreted by DCs upon stimulation with different NE concentrations expressed as $\bar{x} \pm SD$

Interleukins	Unstimulated DCs (pg/ml)	Dendritic cells stimulation (pg/ml)									
		LPS	Vit D	L6	L7	L8	C6	C7	C8	A6	A7	A8
IL-10	44.5±0.7	247.3 ±3.5	102.3 ±2.8	85.0 ±2.8	81.3 ±3.5	86.0 ±2.8	88.5 ±2.5	87.5 ±3.5	101.5 ±4.0	93.3 ±1.5	84.0 ±5.0	111.0 ±2.1
IL-12(p70)	13.0±1.4	397.3 ±14.1	12.7 ±0.6	13.3 ±0.6	14.7 ±0.6	16.0 ±1.0	16.7 ±1.5	17.3 ±2.1	22.7 ±2.1	20.0 ±1.0	26.7 ±1.5	27.0 ±4.4

L6,7,8: *Linum usitatissimum* oil at different concentrations (35, 50, 100 ppm), C6,7,8: *Coriandrum sativum* oil at different concentrations (35, 50, 100 ppm), A6,7,8: *Anethum graveolens* oil at different concentrations (35, 50, 100 ppm).

Table 6: Levels of IL-10 and IL-12 (p70) secreted by DCs upon stimulation with different NE concentrations and LPS expressed as $\bar{x} \pm SD$.

Interleukins	Unstimulated DCs (pg/ml)	Dendritic cells stimulation (pg/ml)									
		LPS	Vit D	L6+ LPS	L7+ LPS	L8+ LPS	C6+ LPS	C7+ LPS	C8+ LPS	A6+ LPS	A7+ LPS	A8+ LPS
IL-10	44.5±0.7	247.3 ±3.5	102.3 ±2.8	92.3 ±0.7	87.5 ±0.7	85.5 ±3.5	101.0 ±1.5	94.5 ±6.4	102.5 ±9.2	95.3 ±2.1	108.3 ±5.1	97.5 ±4.6
IL-12(p70)	13.0±1.4	397.3 ±14.1	12.7 ±0.6	15.3 ±2.3	13.0 ±2.6	17.0 ±2.6	62.3 ±7.1	18.3 ±0.6	18.7 ±1.5	28.0 ±5.3	18.7 ±3.2	26.7 ±6.7

L6,7,8: *Linum usitatissimum* oil at different concentrations (35, 50, and 100 ppm), C6,7,8: *Coriandrum sativum* oil at different concentrations (35, 50, and 100 ppm), A6,7,8: *Anethum graveolens* oil at different concentrations (35, 50, and 100 ppm).

CONCLUSION

As a conclusion from different experiments that performed on dendritic cells, we can conclude that these nanoemulsions may have a tolerogenic effect on DCs through down-regulation of maturation marker CD83 and what considered as immunogenic marker CD86 and up-regulation of CD80 as a tolerogenic marker. Also, the elevated levels of secretion of anti-inflammatory IL-10 cytokine, which inhibits the immunogenic activity of DCs and lower the secreted levels of pro-inflammatory IL-12 (p70) cytokine, makes DCs act as tolerogenic cells. Low apoptotic effect of NEs on DCs may be due to the presence of anti-apoptotic Bcl-2 protein that is normally highly expressed in immature DCs, since NEs showed down-regulation of maturation marker CD83. So, these NEs can be useful to induce Treg to tolerate undesirable immune responses as in transplantations and autoimmune diseases. Based on this study, it is recommended to establish further *in vitro* researches on NEs immunomodulatory effects on DCs (tolerogenic and immunogenic) in order to discover more about the immunogenicity pathways of NEs affecting DCs.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

1 ,25-(OH)2D3: 1,25-dihydroxyvitamin D3; **Bcl-2**: B-cell lymphoma 2; **CAM:** Camptothecin; **CD:** Cluster of differentiation; **CTLA-4:** Cytotoxic T-lymphocyte-associated protein 4; **DCs:** Dendritic cells; **DMEM:** Dulbecco's Modification of Eagles Medium; **ELISA:** Enzyme-linked immunosorbent assay; **HBSS:** Hanks' Balanced Salt Solution; **HLA-DR:** Human Leukocyte Antigen-antigen D Related; **IL:** Interleukin; **LPS:** Lipopolysaccharides; **PBMCs:** Peripheral blood mononuclear cells; **RPMI 1640:** Roswell Park Memorial Institute 1640 medium; **T-cell**: T lymphocyte; **Treg:** Regulatory T-cell; **Vit D:** Vitamin D.

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