

Pharmacological modulation of dendritic cell function by anti-inflammatory dexamethasone but not by GSH depletion

Bassim I Mohammad *PhD*

Date Submitted: 20/4/2016

Date Accepted: 30/5/2016

Address for Correspondence:

Dr. Bassim I Mohammad, College of Pharmacy, University of Al-Qadissiyah, Iraq.

Email: jumabassim@yahoo.co.uk

Abstract

The principal function of dendritic cells (DCs) is to acquire antigens from the environment and present them to naïve T-cells to initiate an immune response. DCs also provide potent co-stimulatory signals to T-cells through expression of co-stimulatory receptors such as CD40 and CD86. Anti-inflammatory and other drugs that deplete intracellular glutathione (GSH) can affect the immune response. However, it is unclear whether these drugs can modulate DC function. In this study, we evaluated the effects of the anti-inflammatory drug dexamethasone and the GSH lowering compound as buthionine sulfoximine (BSO) on DC function. Mouse bone marrow derived dendritic cells (BMDCs) were generated and treated with dexamethasone or BSO. Expression of the co-stimulatory molecules MHCII, CD86 and CD40 was quantified by flow cytometry. Endocytic and phagocytic capacity of DCs was measured by dextran uptake and necrotic cell phagocytosis respectively. We found that dexamethasone reduces the expression of co-stimulatory molecules both in immature and mature DCs. Dexamethasone had a marginal increase in the endocytic capacity of iDCs but caused a decrease in phagocytic function. Reducing GSH levels did not affect co-stimulatory molecules expression, endocytic and phagocytic capacity of iDCs. We conclude that anti-inflammatory corticosteroids could modulate the immune response by down regulating DC co-stimulatory molecule and by inhibition of phagocytosis. Further work is needed to examine the ability of dexamethasone treated DCs to stimulate T-cell activation. These findings have implications for DC based immunotherapies and in transplant tolerance.

Key words: Dexamethasone, GSH, Co-stimulation, phagocytosis, endocytosis

INTRODUCTION

Dendritic cells (DCs) represent a group of bone marrow derived cells with a characteristic ability of antigen phagocytosis, processing and presentation to T-cells, associated with the initiation of immune responses¹. DCs are present in two main forms; immature DCs (iDCs) which are located in the peripheral tissue where they are in contact with external environment such as skin and mucosa; these cells are highly phagocyte and possess low surface expression of MHCII and co-stimulatory molecules (CD86 and CD40)²⁻³. When the iDCs subjected to antigenic stimulation (like microbial products or cytokines of the innate immunity) they started to exhibit cytoskeleton rearrangement and

morphological changes include high expression of MHCII and other co-stimulatory molecules (CD86 and CD40) and form what is called mature DCs (mDCs)⁴. mDCs are powerful antigen presenting cells that have the ability to induce T-cells differentiation and proliferation associated with starting the immune response and this needs the presence of high MHCII as well as co-stimulatory surface molecules on DCs in addition to cytokine release from DCs⁵. When the mDCs present low levels of co-stimulatory molecules, they will be unable to stimulate T-cells proliferation and this condition known as tolerogenic DCs⁶.

Various pathological conditions and chemical substances influence DCs phagocytosis and cell surface

co-stimulatory molecules presentation hence, they interfere with their primary function and could alter the immune response. In this research, we aimed to study the function of DCs in two different conditions; first when DCs treated with corticosteroid anti-inflammatory drug (dexamethasone). Anti-inflammatory corticosteroids are well known for their immune suppression effects and widely used for allergic diseases and prevention of graft rejection. It has been suggested that these drugs target DCs to render them tolerogenic⁷⁻⁹. The effect of corticosteroids on DC phagocytosis is not well established and this is needed to be studied along with the changes in the co-stimulatory molecules. On the other hand, GSH depletion can be caused by a number of drugs like anti-inflammatory drugs e.g. ibuprofen or by alkylating agents like cisplatin and melphalan¹⁰⁻¹² or by chemicals like buthionine sulfoximine (BSO)¹³ which is an inhibitor of γ -glutamylcysteine synthetase (γ -GCS) and as a result decreases glutathione levels¹⁴. We preferred to use BSO because it causes a profound global inhibition of GSH within the cell. Glutathione (GSH) is a ubiquitous tripeptide of glutamate, cysteine and glycine and it is vital in many metabolic functions of the cell especially detoxifications of free radicals and exogenous toxins and establishment of intracellular redox state¹⁵. However, could GSH depletion affect immune system? Does the depletion of GSH affect DC co-stimulatory molecules expression and phagocytic capacity? This is not well explored and this is what we aimed to study.

PATIENTS AND METHODS

All reagents were of analytical grade and purchased from Sigma-Aldrich, Poole, UK unless stated otherwise. C57BL/6J mice were used in the study and bred under pathogen free conditions.

Generation of DCs from bone marrow cells (BMDCs)—Dendritic cells were generated from bone-marrow precursors as described in Lutz et al¹⁶. Briefly, bone-marrow cells were flushed from femora and tibiae of mice. Cells were washed and cultured in complete medium supplemented with 10 ng/ml GM-CSF at 3×10^6 cells/plate in 10 ml microbiological petri dishes. Cultures continue for 7 days, the media was changed on day 3. iDCs were ready to use by day 7 while DC maturation can be obtained by adding LPS (1×10^{-6} g/ml) to DC petri dishes at day 6. Dexamethasone at three different doses; 1×10^{-6} , 1×10^{-7} and 1×10^{-8} M was added to the dishes on day 6, while 500×10^{-4} M of BSO was added to BSO treated DCs also at day 6.

GSH/Bradford analysis—BMDCs were centrifuged and resuspended in 10 mM HCl. Cell supernatants were assayed for glutathione (GSH) according to Vandeputte

C et al¹⁷ in microtitre plates. Total protein content was determined by a Bradford assay and the ratio of GSH to protein content determined¹⁷.

Flow cytometric assay of maturation marker—BMDCs at a density of 1×10^6 cells in 100 μ l culture media per FACS tube were used and then washed with Stain buffer (2% FBS and 2 M EDTA in Sheath Fluid) and incubated either with anti-CD40 antibody, anti-CD86 antibody or anti-MHCII antibody with or without anti-CD11c plus stain buffer in the dark on ice for 30 mins. Unlabeled cells were used as control. After incubation, cells were washed and resuspended in 700 μ l sheath fluid and analysed on a flow cytometer. Mature BMDCs for dexamethasone assay were generated by overnight (at day 6) incubation with lipopolysaccharide LPS at a concentration of 1×10^{-6} g/ml.

Dextran-FITC endocytosis—Phagocytic ability was determined using Dextran particles as surrogate to antigen particles according to Sallusto F et al¹⁸. Briefly, BMDCs were harvested and resuspended at a density of 1×10^6 cells in 100 μ l complete medium and then incubated with 0.5 μ g/ml FITC-Dextran [40,000 MW] (Invitrogen, Paisley, UK) for indicated time points at 37°C or 4°C (as negative control) and washed twice in stain buffer and resuspended in 700 μ l sheath fluid. Cells were analysed on a Coulter Epics XL flow cytometer and data acquired on SYSTEM II software package (Backman Coulter, UK).

Phagocytosis of necrotic cells—Jurkat cells were maintained in complete media and snap freeze in liquid nitrogen at a density of 20×10^6 cell/ml for few seconds. Frozen cells were then thawed in a 37°C water bath and cells that have taken up trypan blue were counted. More than 70% of cells were necrotic. Necrotic cells were co-cultured with 2×10^6 cells/well (24-well plate) plate adherent DCs at a ratio of 2:1 for 2 hours at 37°C under 5% CO₂ while a control plate remain on ice. Cells were harvested and washed in stain buffer prior to analysis by flow cytometry.

Statistics—Raw data obtained were analyzed using the unpaired t test. p values < 0.05 were considered to be statistically significant.

RESULT

Dexamethasone decreases expression of MHCII in immature and mature DCs—Flow cytometry was used to measure the changes in the expression of co-stimulatory molecules in control iDCs and dexamethasone treated iDCs. Figure (1A) shows a dose dependant decrease in the expression of MHCII molecules upon dexamethasone treatment. The highest reduction is observed by the dose of 1×10^{-6} M

dexamethasone. Treatment of iDCs with LPS results in DC maturation that can be observed by increased expression of MHCII and co-stimulatory molecules including CD86 and CD40. Figure (1B) shows a decrease in the expression of MHCII molecule in mDCs with dexamethasone treatment comparing to untreated mature DCs. The reduction is highly significant ($p < 0.05$) at the high dose of dexamethasone (1×10^{-6} M). Indicating that dexamethasone has the ability to reduce the maturity of DCs and subsequently affecting their ability to initiate proper immune response.

Dexamethasone decreases expression of CD86 in immature and mature DCs—A proper immune response will require, in addition to adequate MHCII expression, high co-stimulatory molecules expression. Therefore, we next inspect the effect of dexamethasone treatment on CD86 expression. There were decreased in the expression of CD86 molecules in iDCs treated with dexamethasone comparing to untreated iDCs as Figure (2A) shows. In a similar manner to iDCs, dexamethasone treatment decreases expression of CD86 in mDCs as shown in Figure (2B). Again the significant reduction ($p < 0.05$) was observed with the highest dose of dexamethasone (1×10^{-6} M). Such reduction in the expression of co-stimulatory molecules indicates that dexamethasone interferes with the process of DC maturation.

Dexamethasone decreases expression of CD40 in immature and mature DCs—Flow cytometric analysis of iDCs treated with dexamethasone demonstrate that the proportion of iDCs that express high levels of CD40 was significantly low after treatment with all doses of dexamethasone comparing to untreated iDCs as Figure (3A) shows. Furthermore, similar reduction was seen in the expression of CD40 molecules in mDCs treated with all doses of dexamethasone comparing to untreated mDCs as shows in Figure (3B).

Dexamethasone enhances dextran uptake by DCs—Endocytosis is an important step in DC biology and it is a function of mannose receptor and dextran particles used to assess this ability. Dexamethasone causes increase in the uptake of dextran and this is expected as it was known that increase maturation is associated with decrease endocyte ability and vice versa (Figure 4).

Dexamethasone reduces DC phagocytosis—DCs use phagocytosis to remove endogenous antigens (like dying cells via apoptosis or necrosis) and exogenous antigens (infectious agents)¹⁹. Dexamethasone decreased the ability of iDC to phagocyte necrotic Jurkat cells (Figure 5) which seems to be a dose dependent decrease.

GSH depletion can be achieved by BSO in DCs—Redox potential is vital to maintain normal cell growth and survival, and chemicals altered redox potential will have an impact on cell functions. GSH is one of cytoprotective tools used by the cells to maintain normal redox state. Drugs causing depletion in GSH have been used widely and their effects on cell function. BSO was found to cause decrease in GSH levels as seen in Figure (6).

Lowering GSH does not affect co-stimulatory molecules expression of DCs—DCs treated with BSO shows no changes in the expression of co-stimulatory molecules in comparison to untreated DCs as shown in Figure (7)

Lowering GSH does not affect endocytic capacity of DCs—BSO treated DCs has the same capacity of untreated one to endocytosis and this means that depletion GSH has no consequence on endocytosis as shown in Figure (8).

GSH depletion does not affect on DC phagocytosis—BSO treated DCs show no changes in phagocytic capacities in compare to untreated cells and this suggests that GSH has no effect on DC phagocytosis as shown in Figure (9).

DISCUSSION

Dendritic cells play an important role in the immune system and they represent the connection between innate and adaptive immunity³. Depending on their maturation state, co-stimulatory expression and cytokines release; DCs can be immune-stimulatory or immune-regulatory, both these functions rendering them vital in vaccine development and treatment of graft rejection²⁰. Their ability to phagocyte antigens, processing them and present them to T-cells is the corner stone in DCs function and factors interrupt with these processes have great attention.

We observed that dexamethasone causes decrease in co-stimulatory molecules presentation on DCs which is a dose dependant decrease; this will produce a condition known as tolerogenic DCs. In this condition the DCs are unable to present sufficient co-stimulatory molecules on their surface and this is mean even if the DCs engulfed an antigen, they are unable to prime T-cells because of low co-stimulatory molecules presentation. This finding is similar to previous results obtained by Adorini et al⁸ and Canning et al²¹ and the explanation for that could be reached by considering the mechanism of action of dexamethasone which involved nucleus receptor interaction²², a condition may affect transcription factors regulated the co-stimulatory molecules expression. But how this changing in the co-stimulatory expression could affect the phagocytic ability of these DCs? We

found that adding dexamethasone, at different doses to DCs, causing impeding in their ability to phagocyte necrotic cells; this could be due to the inhibition of phagocytic receptors on iDCs due to dexamethasone treatment and this may be one of mechanism for which corticosteroids known as immunosuppressive agents. This effect may explain why patients on corticosteroids therapy at a high risk of infection due to interruption of normal DCs function.

On the other hand dexamethasone treated iDCs showed an enhancement in the dextran uptake and this may suggest a sparing effect on mannose receptors responsible for endocytosis. Implication of this may explain the role of DCs in prevention of graft rejection or in treatment of autoimmune diseases as the DCs could endocytose exogenous or endogenous antigens but not causing full immune response activation. DCs treated with dexamethasone can be used in immunotherapeutic approach of treatment in asthma and other allergic diseases. Further works in this field may involve DCs cytokine levels measurement because it is known that DCs secrete group of cytokines like TNF- α , IL-10, TGF- β and IL-12 and these are vital along with co-stimulatory molecules for the initiation of T-cell priming²²⁻²³. In same direction assessing the ability of dexamethasone treated DCs to induce T-cell proliferation is another aspect need to be evaluated. Measuring surface presentation of the phagocytic and mannose receptors in dexamethasone treatment could explain why such difference between phagocytic and endocytic process was observed.

Dexamethasone causes apoptosis in T lymphocytes and macrophages²⁴ and this is could be the case also in DCs, so that these DCs are in their apoptotic stage and their features of low co-stimulatory molecules expression and defective phagocytosis may be an early stage of apoptotic cell death.

Previous studies demonstrated that GSH depleted DCs expressed normal or just above normal levels of surface co-stimulatory molecules²⁵. We found no effect of lowering GSH by BSO on the co-stimulatory molecules presentation and neither on endocytic or phagocytic capacities of DCs as shown in figures 7, 8 and 9 respectively. These results suggested that GSH has no role in DCs maturation and function however; these results need to be supported by future work on GSH SiRNA and hence; we can confirm the exclusion of GSH role. However, the cells adapted different mechanisms to withstand adverse oxidative condition and one of these is the antioxidants cytoprotective genes and enzymes (mainly through Nrf2 pathway). This pathway will help to maintain the cell to function well in such an environment.

The ability of DCs (treated with dexamethasone or BSO) to stimulate T-cells proliferation, a work needed to be undertaken in future, will be of great relevance for the role of DCs in control of immune response to various pathological conditions like engulfment and presentation of self antigens in systemic lupus syndrome or in the development of new vaccine as DC therapy.

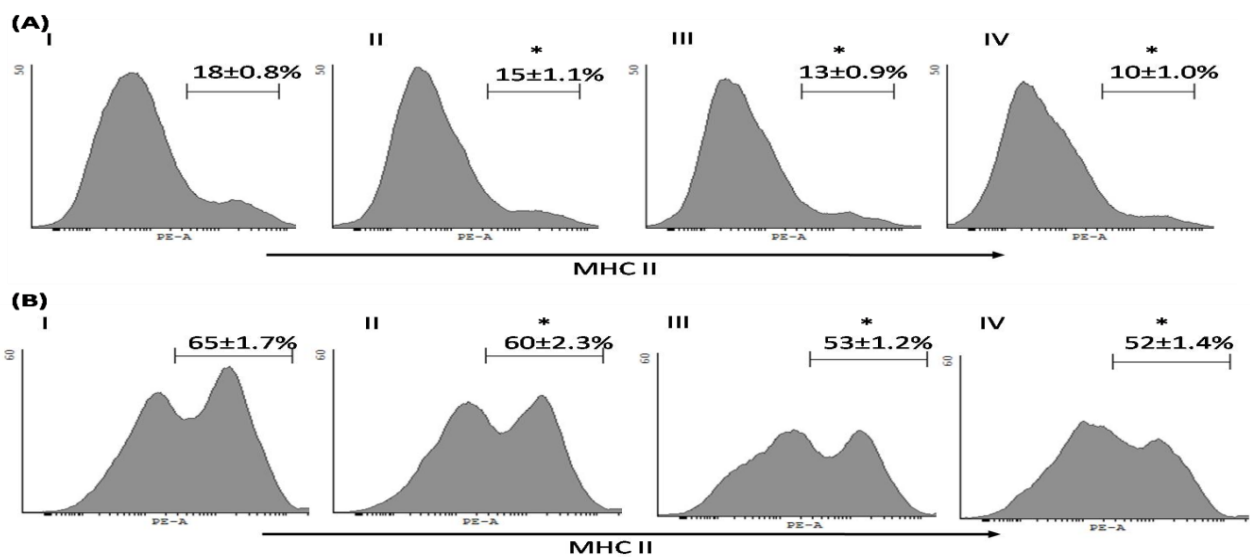


Figure (1): Decreased MHCII molecule expression in iDC (A) and mDC (B) treated with dexamethasone. Co-stimulatory molecules expression determined by flow cytometry. The percentage of iDCs expressing high levels of co-stimulatory receptors is indicated above the marker. I-untreated DCs, II-1x10⁻⁸M, III-1x10⁻⁷M, IV-1x10⁻⁶M dexamethasone treated DCs. Representative Histograms with data derived from five independent experiments are presented as average percentage \pm S.D.* p<0.05.

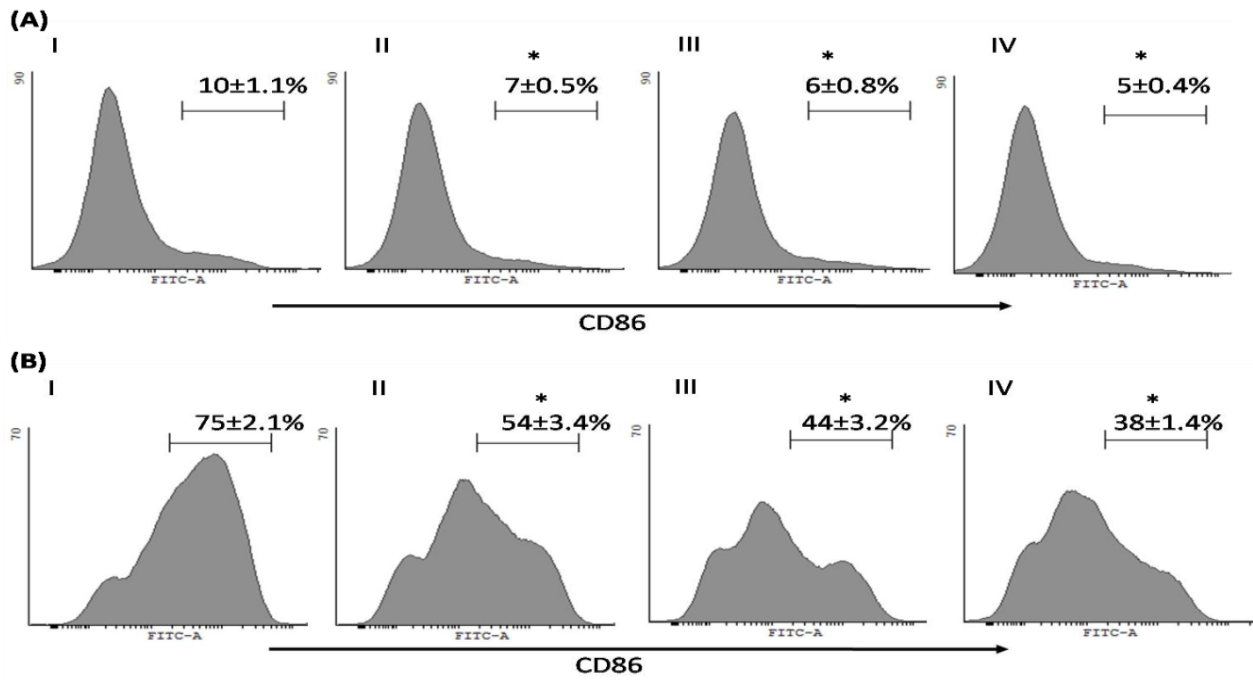


Figure (2): Decreased CD86 molecule expression in iDC (A) and mDC (B) treated with dexamethasone. Co-stimulatory molecules expression determined by flow cytometry. The percentage of iDCs expressing high levels of co-stimulatory receptors is indicated above the marker. I-untreated DCs, II- $1 \times 10^{-8} \text{M}$, III- $1 \times 10^{-7} \text{M}$, IV- $1 \times 10^{-6} \text{M}$ dexamethasone treated DCs. Representative Histograms with data derived from five independent experiments are presented as average percentage \pm S.D.

* $p < 0.05$.

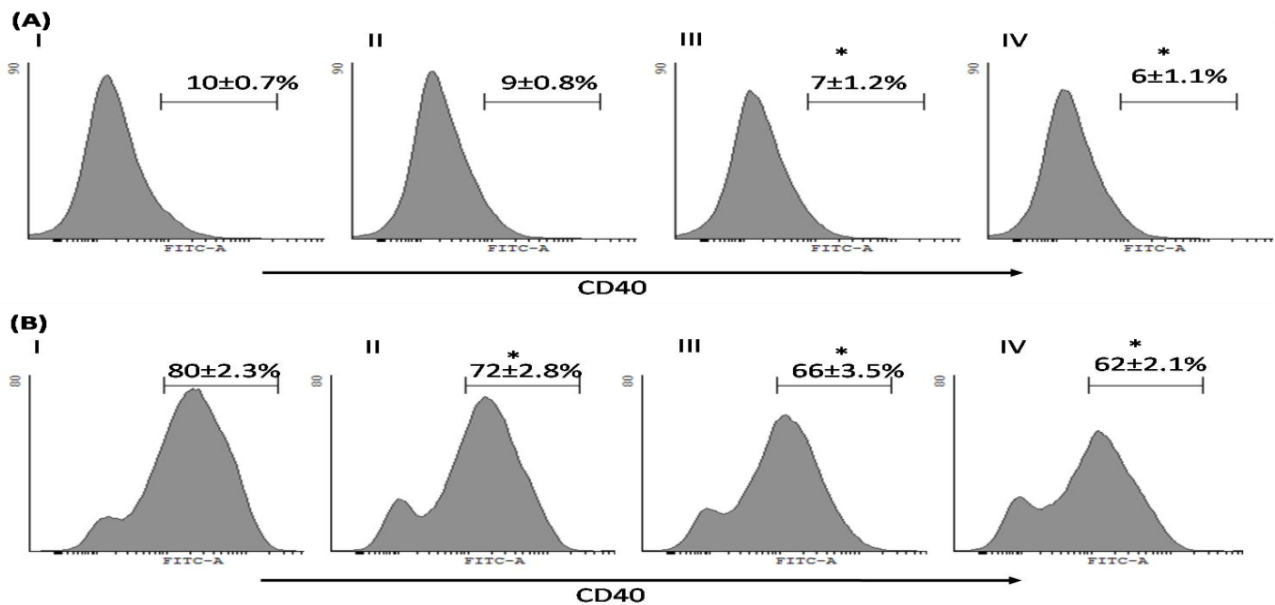


Figure (3): Decreased CD40 molecule expression in iDC (A) and mDC (B) treated with dexamethasone. Co-stimulatory molecules expression determined by flow cytometry. The percentage of iDCs expressing high levels of co-stimulatory receptors is indicated above the marker. I-untreated DCs, II- $1 \times 10^{-8} \text{M}$, III- $1 \times 10^{-7} \text{M}$, IV- $1 \times 10^{-6} \text{M}$ dexamethasone treated DCs. Representative Histograms with data derived from five independent experiments are presented as average percentage \pm S.D. * $p < 0.05$.

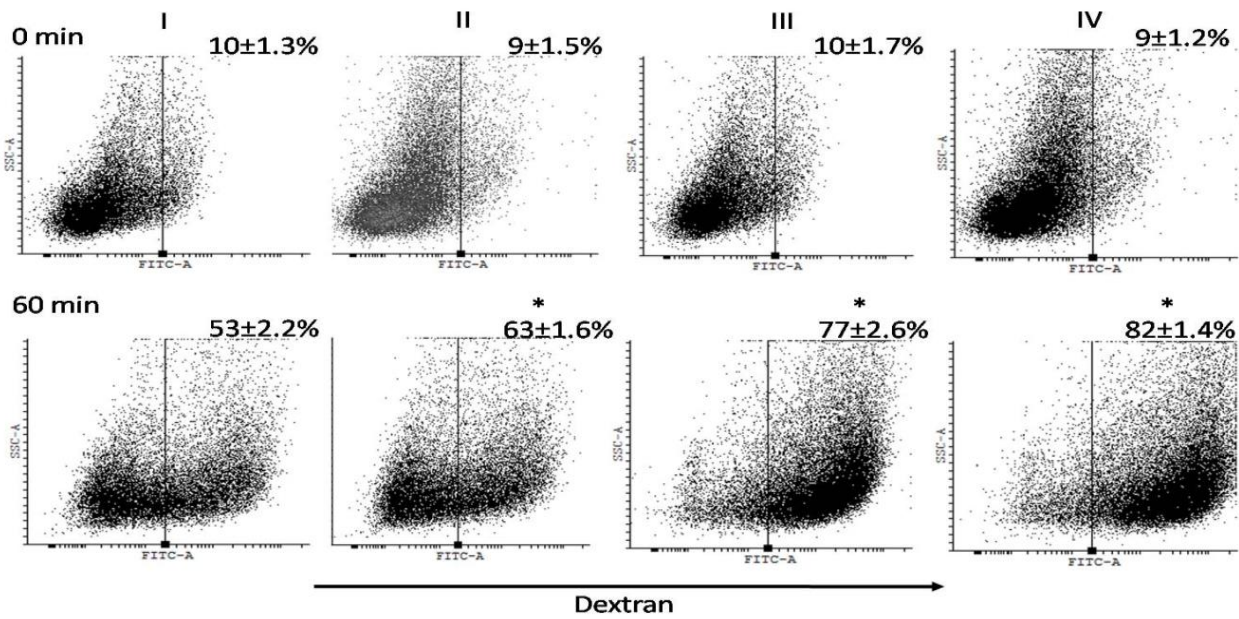


Figure (4): Increased uptake of dextran by dexamethasone treated iDC. Flow cytometric analysis of endocyte ability of DCs with dexamethasone at different doses or untreated iDCs express by dextran uptake from 0 minute to 60 minutes. I-untreated DCs, II-1x10⁻⁸M, III-1x10⁻⁷M, IV-1x10⁻⁶M dexamethasone treated DCs. Dot plots with data derived from five independent experiments are presented as average percentage ± S.D. * p<0.05.

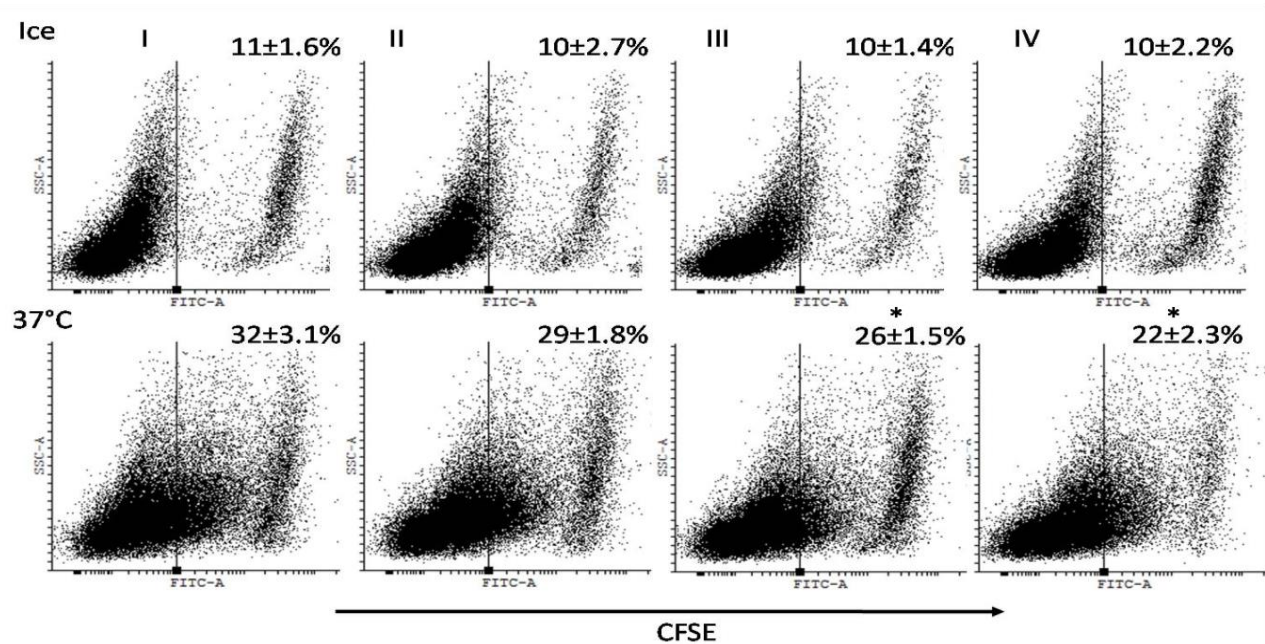


Figure (5): Reduction in phagocytic ability of DCs with dexamethasone treatment. Flow cytometric analysis of phagocytosis of DCs with dexamethasone at different doses or untreated DCs at 0 and 37°C. I-untreated DCs, II-1x10⁻⁸M, III-1x10⁻⁷M, IV-1x10⁻⁶M dexamethasone treated DCs. Dot plots with data derived from five independent experiments are presented as average percentage ± S.D. * p<0.05.

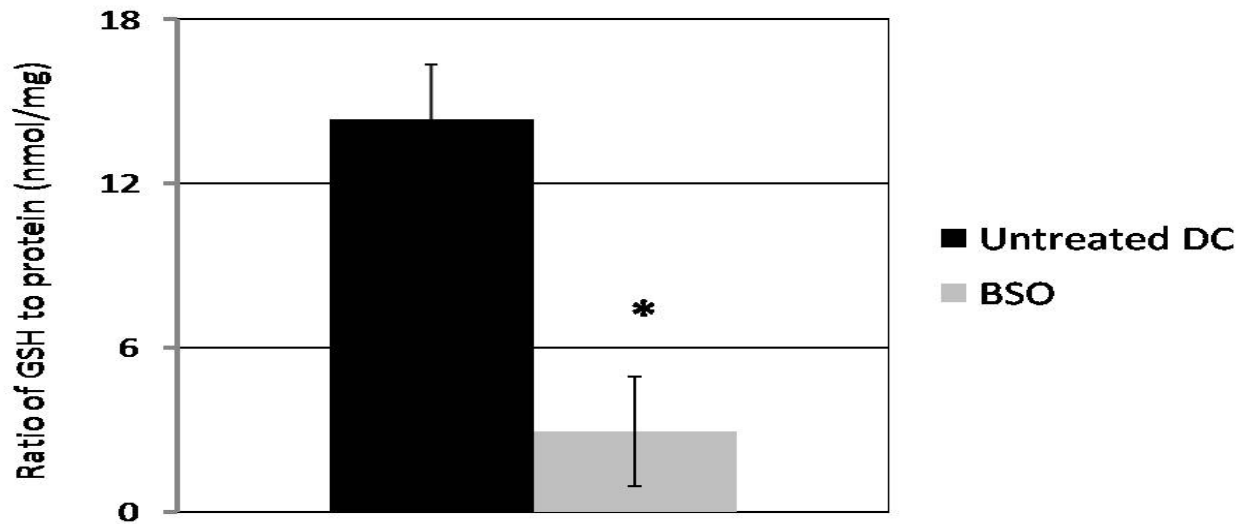


Figure (6): GSH levels are lower in BSO treated DCs. Untreated iDCs or treated with BSO (500×10^{-6} M) for 24 h. Data are presented as average percentage \pm S.D. Statistical significance was tested by unpaired Student's t test (*, $p < 0.05$). Data are derived from five independent experiments. * $p < 0.05$.

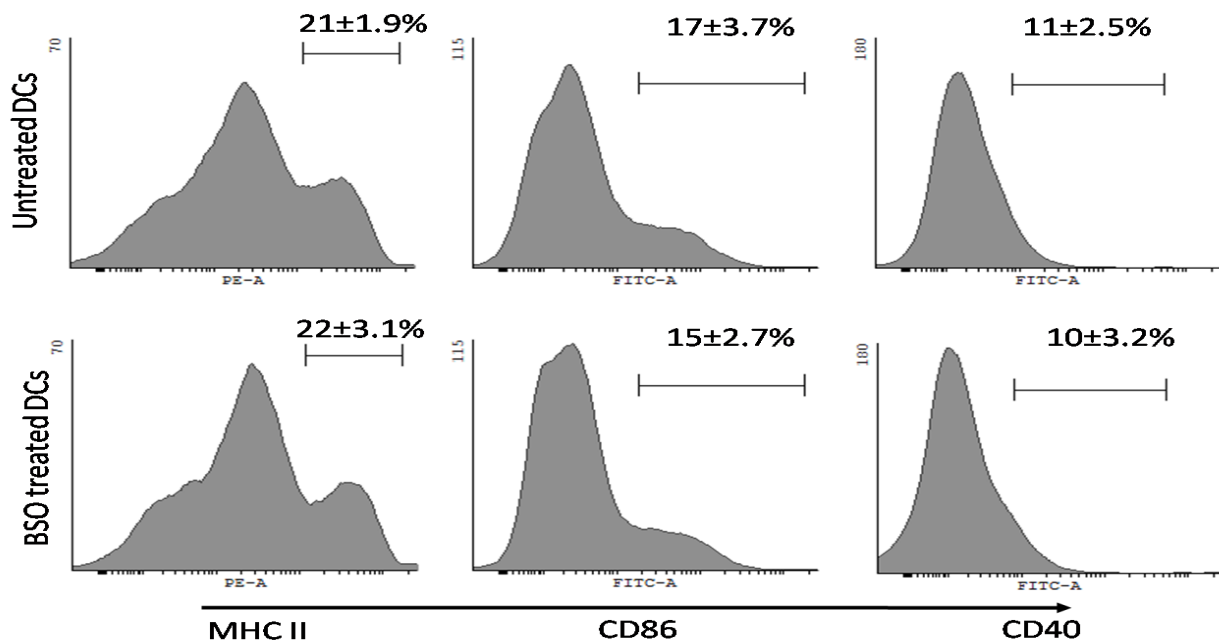


Figure (7): No difference in the expression of co-stimulatory molecules in GSH depleted DCs. Co-stimulatory molecules expression determined by flow cytometry. The percentage of iDCs and BSO (500×10^{-6} M) treated DCs expressing high levels of co-stimulatory receptors (MHCII, CD86 and CD40) is indicated above the marker. Histograms with data derived from five independent experiments are presented as average percentage \pm S.D.

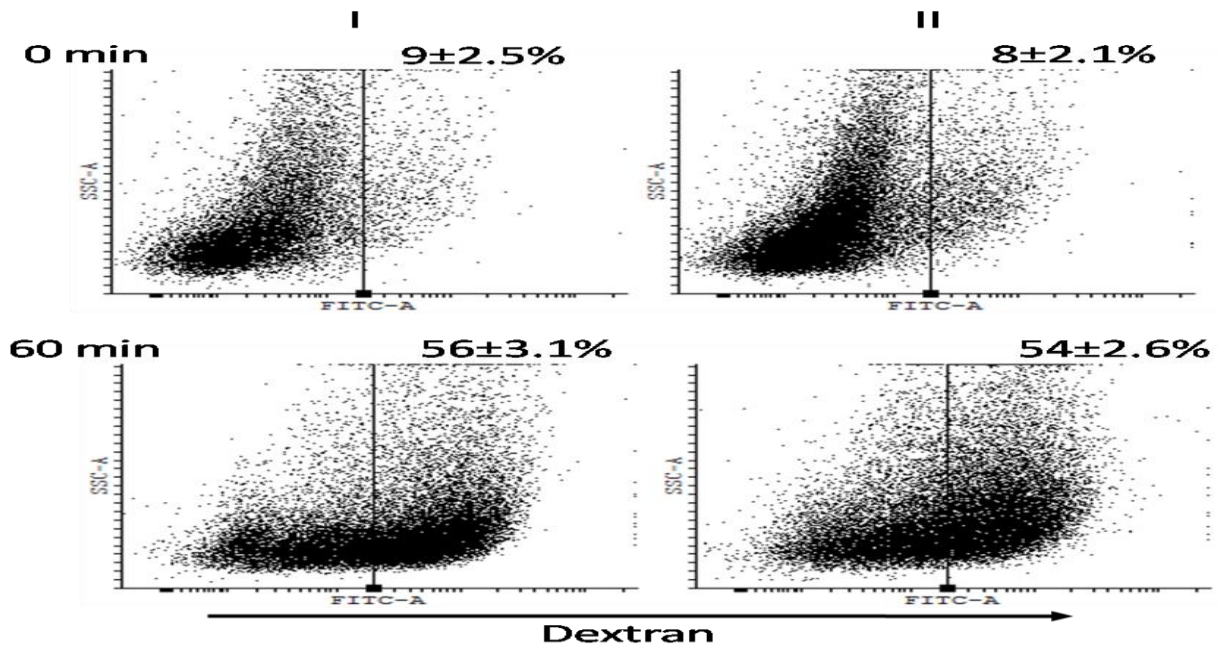


Figure (8): No change in dextran uptake in GSH depleted DCs. Flow cytometric analysis of endocytosis by (I) iDCs and (II) BSO (500×10^{-6} M) treated iDCs express by dextran uptake from 0 minute to 60 minutes. Dot plots with data derived from five independent experiments are presented as average percentage \pm S.D.

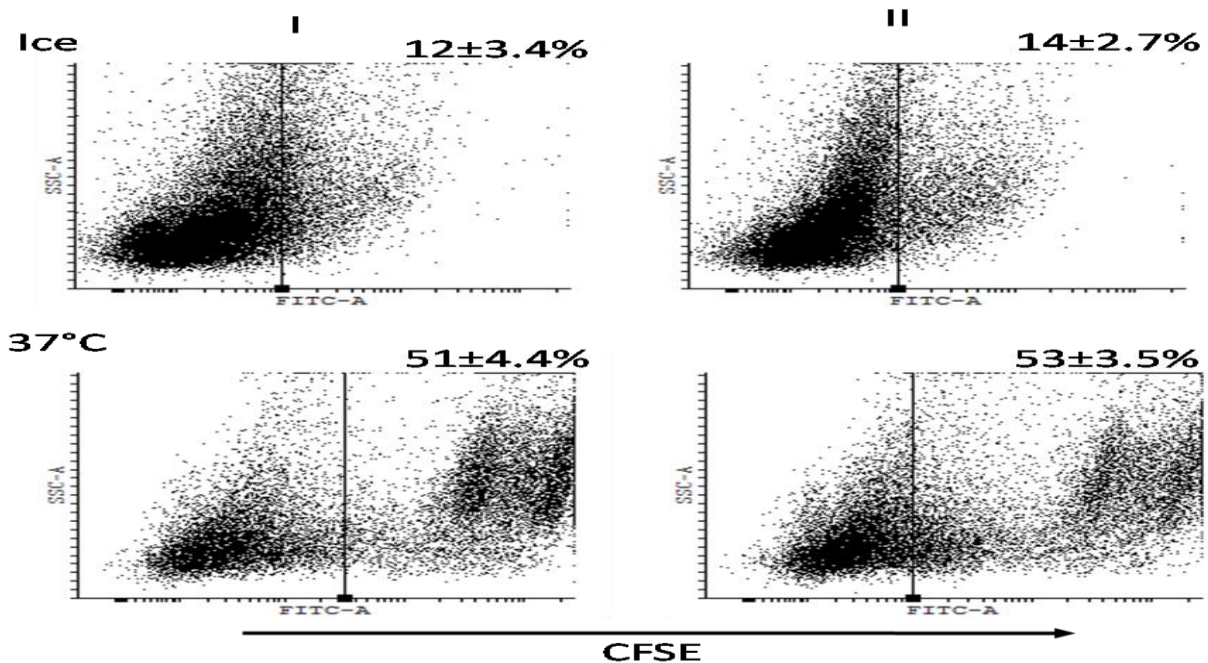


Figure (9): Phagocytosis does not affected by depletion in DCs GSH. Flow cytometric analysis of phagocytosis by untreated (I) or BSO (500×10^{-6} M) treated (II) iDCs after incubation of necrotic cells with iDCs for 2 hours either at ice or 37°C. Dot plots with data derived from five independent experiments are presented as average percentage \pm S.D.

REFERENCES

- 1- Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol.* 1991;9:271-96.
- 2- Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, et al. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med.* 1997 Jan 20;185(2):317-28.
- 3- Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. *Physiol Rev.* 2002 Jan;82(1):97-130.
- 4- Cella M, Engering A, Pinet V, Pieters J, Lanzavecchia A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature.* 1997 Aug 21;388(6644):782-7.
- 5- Langenkamp A, Casorati G, Garavaglia C, Dellabona P, Lanzavecchia A, Sallusto F. T cell priming by dendritic cells: thresholds for proliferation, differentiation and death and intraclonal functional diversification. *Eur J Immunol.* 2002 Jul;32(7):2046-54.
- 6- Buckland M, Lombardi G. Aspirin and the induction of tolerance by dendritic cells. *Handb Exp Pharmacol.* 2009(188):197-213.
- 7- Vieira PL, Kalinski P, Wierenga EA, Kapsenberg ML, de Jong EC. Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential. *J Immunol.* 1998 Nov 15;161(10):5245-51.
- 8- Adorini L, Giarratana N, Penna G. Pharmacological induction of tolerogenic dendritic cells and regulatory T cells. *Semin Immunol.* 2004 Apr;16(2):127-34.
- 9- Matasic R, Dietz AB, Vuk-Pavlovic S. Dexamethasone inhibits dendritic cell maturation by redirecting differentiation of a subset of cells. *J Leukoc Biol.* 1999 Dec;66(6):909-14.
- 10- Jain A, Singhai AK. Nephroprotective activity of *Momordica dioica* Roxb. in cisplatin-induced nephrotoxicity. *Nat Prod Res.* 2010 May;24(9):846-54.
- 11- Zhang J, Ye Z, Lou Y. Metabolism of melphalan by rat liver microsomal glutathione S-transferase. *Chem Biol Interact.* 2005 Apr 15;152(2-3):101-6.
- 12- Liang Q, Sheng Y, Ji L, Min Y, Xia Y, Wang Z. Acetaminophen-induced cytotoxicity on human normal liver L-02 cells and the protection of antioxidants. *Toxicol Mech Methods.* 2010 Jun;20(5):273-8.
- 13- Takahashi K, Tatsunami R, Oba T, Tampo Y. Buthionine sulfoximine promotes methylglyoxal-induced apoptotic cell death and oxidative stress in endothelial cells. *Biol Pharm Bull.* 2010;33(4):556-60.
- 14- Drew R, Miners JO. The effects of buthionine sulphoximine (BSO) on glutathione depletion and xenobiotic biotransformation. *Biochem Pharmacol.* 1984 Oct 1;33(19):2989-94.
- 15- Meister A, Anderson ME. Glutathione. *Annu Rev Biochem.* 1983;52:711-60.
- 16- Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* 1999 Feb 1;223(1):77-92.
- 17- Vandeputte C, Guizon I, Genestie-Denis I, Vannier B, Lorenzon G. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol Toxicol.* 1994 Dec;10(5-6):415-21.
- 18- Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med.* 1995 August 1, 1995;182(2):389-400.
- 19- Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. *Cell.* 2010 Mar 5;140(5):619-30.
- 20- Woltman AM, van Kooten C. Functional modulation of dendritic cells to suppress adaptive immune responses. *J Leukoc Biol.* 2003 Apr;73(4):428-41.
- 21- Canning MO, Grotenhuis K, de Wit HJ, Drexhage HA. Opposing effects of dehydroepiandrosterone and dexamethasone on the generation of monocyte-derived dendritic cells. *Eur J Endocrinol.* 2000 Nov;143(5):687-95.
- 22- He XK, Wu XL, He XJ, Li B, Hu YX. Dexamethasone impairs the differentiation and maturation of murine dendritic cells by Toll-like receptor 4-nuclear factor-kappaB pathway. *Chin Med J (Engl).* 2010 Feb;123(3):344-50.
- 23- Xia CQ, Peng R, Beato F, Clare-Salzler MJ. Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity. *Scand J Immunol.* 2005 Jul;62(1):45-54.
- 24- Talaber G, Boldizsar F, Bartis D, Palinkas L, Szabo M, Berta G, et al. Mitochondrial translocation of the glucocorticoid receptor in double-positive thymocytes correlates with their sensitivity to glucocorticoid-induced apoptosis. *Int Immunol.* 2009 Nov;21(11):1269-76.
- 25- Bergamo P, Maurano F, D'Arienzo R, David C, Rossi M. Association between activation of phase 2 enzymes and down-regulation of dendritic cell maturation by c9,t11-conjugated linoleic acid. *Immunol Lett.* 2008 May 15;117(2):181-90.