

Immediate Exposure to TNF- α Activates Dendritic Cells Derived from Non-Purified Cord Blood Mononuclear Cells

Marzieh Ebrahimi^{1,3*}, Zuhair Mohammad Hassan¹, Jamshid Hadjati², Parisa Hayat⁴, Seyed Mohammad Moazzeni¹

¹Department of Immunology, Faculty of Medical Science, Tarbiat Modares University, ²Department of Immunology, School of Medicine, Tehran University of Medical Sciences, ³Department of Regenerative Medicine, Royan Institute for Stem Cell Biology and Technology, ⁴Cell and Molecular Research Center, Iran University of Medical sciences, Tehran, Iran.

ABSTRACT

Background: Tumor necrosis factor alpha (TNF- α) is a primary mediator of immune regulation and might be required in the early stages of DC development from CD34⁺ cells. However, details of optimal timing of exposure to TNF- α in DC development process in monocytes or non-purified hematopoietic cells are still lacking and clear benefits of this approach to the development of DCs remain to be validated. **Objective:** To evaluate the effect of early and late exposure to TNF- α on DC development from non-purified cord blood mononuclear cells. **Methods:** To define the effects of early exposure to TNF- α on cord blood mononuclear cells, we cultured UCB-MNC in the presence of SCF, Flt3L, GM-CSF and IL-4 for 14 days and matured them for an extra 4 days. TNF- α was added on day 0, 7 and 14 in TNF- α ⁺ group, and only on day 14 in TNF- α ⁻ group where it was used only as a maturation factor. **Results:** Immediate exposure to TNF- α was shown to: (1) enhance the survival of cells in the first week of culture; (2) produce mature DCs with higher maturation markers (CD80, CD83, CD86 and HLA-DR); and (3) increase secretion of IL-12 by mature DCs. In contrast, delayed exposure to TNF- α stimulate mature DCs with less purity producing a high level of IL-10 and a low level of IL-12. **Conclusion:** We developed a simple, easy and cost effective method to generate DCs from non-fractionating mononuclear cells in this study. Also we confirm the presence of a large number of functional DCs under inflammatory conditions, where local concentrations of TNF- α were high.

Keywords: Dendritic Cells, TNF- α , Cord Blood

*Corresponding author: Dr. Marzieh Ebrahimi, Department of Regenerative Medicine, Royan Institute for Stem Cell Biology and Technology, ACECR, P.O. Box 19395-4644, Tehran, Iran. Tel: (+) 98 2122518282 Fax: (+) 98, 212414532, e-mail: ebrahimi@royaninstitute.org

INTRODUCTION

Dendritic cells (DCs) comprise a connected system of bone marrow-derived leukocytes that are the primary antigen-presenting cells for the stimulation of naive T cells. DCs develop from hematopoietic stem/progenitor cells or CD14⁺ precursors and are thought to undergo discrete maturational stages. DCs at intermediate differentiation stages can be found in various non-lymphoid tissues, in which they are highly specialized in capturing and processing antigens (1,2). Two in vitro differentiation pathways for the development of DCs seem to exist, and these differ from each other in their relationship to in vitro granulomonopoiesis (3,4). One pathway is characterized by an intermediate monocytoïd cell stage (CD14⁺/c-fms⁺/CD1a⁻) with limited proliferation potential and the second pathway occurs via a distinct intermediate cell stage (CD14⁻/c-fms⁻/CD1a⁺) that possesses restricted DC differentiation potential. Birbeck granule (BG)-containing DCs resembling epidermal Langerhans cells seem to develop only from this latter pathway (4).

The key aspect in use of DCs in immunotherapy is identification of culture conditions as well as cell sources enabling the generation of large amounts of APCs in vitro. Among all investigated cytokines, TNF- α and GM-CSF play essential roles in DC developmental pathways (4). TNF- α is a known potent stimulator for immunologic response and is produced as a primary mediator of immune regulation and inflammatory response during the immune and host defence responses. Also, the monocyte/macrophage lineages are the predominant source of TNF- α , releasing it in response to a variety of biologic, chemical, and physical stimuli (5). Many studies have demonstrated that TNF- α might be required up to 72 h in the early stages of DC development from CD34⁺ cells in order to maintain a window of sensitivity to its priming effects (6) and many DC induction protocols involve exposure of CD34⁺ hematopoietic progenitor cells to TNF- α from the onset of DC induction (4,7,8). However, details of optimal timing of TNF- α exposure in DC development process are still lacking and clear benefits of this approach to the development of DCs remain to be validated. Two different sources can be used for the generation of DCs: proliferating CD34⁺ precursors (9) and non-proliferating CD14⁺ monocytes (10). While DCs from CD34⁺ cells require a prolonged culture and special cytokine setup in order to increase the small number of precursors, monocyte-derived DCs (moDCs) are easy to obtain after enrichment of monocytes by magnetic separation or adherence, followed by differentiation using GM-CSF and interleukin-4 (IL-4) (11), however use of monocytes has also some limitations such as production of a small number of DCs and it is impossible to proliferate monocytes or store them for a long time. Considering the predominant role of TNF- α in the inflammatory response and the presence of different kinds of cells in the inflammatory area, the present investigation intends to evaluate the effects of immediate and delayed exposure to TNF- α on the differentiation and maturation of non-purified cord blood mono nuclear cells into dendritic cells.

Because co-stimulatory molecules such as CD80, CD83 and CD86 are necessary for the activation of T lymphocytes and characterization of mature DCs (12,13), these markers were used to evaluate the maturation levels of DCs in our experiments. We used mixed leukocyte reaction (MLR) and cytokine assay to evaluate the activity of DCs produced. In essence, this study was aimed at further defining the relationship between cord blood monocytes and hematopoietic progenitor cells in DC maturation without necessarily

using expensive purification steps and in a model of inflammatory environment where many kinds of immune cells are present and TNF- α is secreted in high amounts.

MATERIALS AND METHODS

Specimen Collection and Sample Preparation. Specimens consisted of umbilical cord blood samples (n=10) from full-term deliveries via Cesarean section. Samples were taken from healthy subjects who tested negative for infectious diseases (HIV, HBV, HCV and syphilis) and were non-diabetic mothers. Following delivery, the cord was clamped and the venous blood was collected by a 50 cc syringe filled with 7 cc of acid citrate–dextrose A (ACD-A) (Sigma, St. Louis, MO) to prevent coagulation.

The anti-coagulated cord blood was diluted 1:5 with ethyl starch (HAES 10%, Free flex, SM 711202, Belgium) and the plasma rich leukocytes were separated after one hour and washed with phosphate buffered saline (PBS) containing 0.6% ACD-A. The cord blood mononuclear cells were isolated by means of Ficoll gradient centrifugation (1.077-1.08 g/cm², InnoTrain, H9L6114, Germany) and washed twice with PBS containing 0.6% ACD-A and 5% fetal calf serum (FCS, Gibco, 16141-09, Germany). The cell pellet was then resuspended in complete RPMI (10⁷ cell/ml) and the T cells were depleted by rosette method as previously described (14).

Cell Culture and Cytokines. Cells were plated at 1×10⁶ cells/well in 2 ml RPMI 1640 containing 10% FCS, 100U/ml penicillin and 100 μ g/ml streptomycin (Gibco,15070-063, Germany), 2 mM L-glutamine (Gibco, 250c-024, Germany), supplemented with human recombinant cytokines; 50ng/ml of flt3 ligand (R&D, 308-FK) and *c-kit* ligand (R&D, 255-SC), 100ng/ml GM-CSF (R&D, 215-GM) and 25ng/ml IL-4 (R&D, 204-IL-010). Cells were then cultured in 6-well plates (TPP, Germany) and incubated at 37°C in 5% CO₂. Medium was changed by semi depletion once a week.

TNF- α Exposure. Recombinant human tumor necrosis factor- α (R&D, 210-TA) was added to the culture plate in two different times. In the first group 3ng/ml TNF- α was added on the first day and another 3ng/ml dose on 7th day as a maintaining dose and later a 10ng/ml dose for maturation on the 14th day; the second group was given only a dose of 10ng/ml on the 14th day of culture for maturation. All experiments were run at least three times.

Determination of Dendritic Cell Phenotypes Using FACS Technique. The phenotype of dendritic cells at days 0, 7, 10, 14 and 18 was characterized using flowcytometry technique (FACS CaliburH, Becton-Dickinson, San Jose, California, USA). For this procedure, cells were harvested and collected by centrifugation and further processed on melting ice. Then the cells were diluted in a buffer solution (phosphate buffered saline (PBS) with 1% bovine albumin, pH 7.4) at a concentration of 10⁶ cells/ml. The cells were then labeled with monoclonal mouse anti-human antibodies against monocyte markers-CD14 (PE-TUK4, R0864, Dako, Denmark), the co-stimulatory molecules CD80 (hB7-1-PE, FAB1405, R&D) and CD86 (hB7-2-PE, FAB141P, R&D), the mature dendritic cell marker CD83 (FITC, MCA1582F, Serotec), MHC-II (FITC-EDU1, AHU0188, Biosource, USA), the hematopoietic stem cell marker CD34 (FITC, 130-081-001, MACS, Germany), and CD1a (FITC-NA1/34, F7141, Dako, Denmark), and CD11c (FITC-KB90, Dako, Denmark) with a mouse isotype control, and incubated at 4°C for 45 minutes. The cell phenotype was then analysed using FACS. The percentage

of fluorochrome-conjugated cells was calculated with Becton Dickinson FACScan software (Lysis II) or (winMDI). Developing DCs adhering to the plates were removed upon incubation of samples with cold PBS containing 0.1% EDTA until the attached cells started to detach (1-3 min).

Mixed Leukocyte Reaction Assays (MLR). 1×10^4 DCs pre-inactivated with 25 $\mu\text{g/ml}$ mitomycin-C (Sigma, USA) harvested from cultures were plated in U-bottom 96-well plates as stimulators together with 1×10^5 human peripheral lymphocytes as responders in a final volume of 200 μL . Culture media consisted of RPMI 1640 and 10% FCS. Cells were cultured for 5 days and pulsed with 1 μCi 3H-thymidine (Amersham, Sweden) per well during the last 18 hours of culture. 3H-thymidine incorporation was measured on a b-plate counter (Wallac, Gaithersburg, MD).

Cytokine Production Assay. For detection of IL-12 and IL-10 in culture supernatant, we used the HU-IL-12 and HU-IL-10 from Biosource ELISA kits, exactly as recommended by the manufacturer's instructions. Supernatants were collected either at the end of MLR or at the end of DC culture and stored frozen at -70°C prior to testing (< one month).

Statistical Analysis. The results are expressed as the mean \pm SD of at least three different experiments. Results were analyzed with the paired nonparametric Mann-Whitney test and $p < 0.05$ was considered significant

RESULTS

Morphologic Verification of DCs. The mononuclear cells were freshly isolated from human umbilical cord blood following T cell depletion using the rosette test. These cells contained approximately $1.5 \pm 1.4\%$ CD34⁺ and $27.6 \pm 12.3\%$ CD14⁺ cells. Cell preparation contained different large and small round floating cells and blood monocytes when they were stained with Wright-Giemsa and viewed under light microscope at day 0 of cell culture (Figure 1A). Maintaining the cells under the culture conditions, they were viewed again at days 7, 14 and 18. At day 7, approximately 70% of the cells in both groups had a nondescript appearance as small round floating cells similar to hematopoietic stem cells (HSCs) (Figures 1 B, C) and the remaining cells appeared as DC like cells. By day 14, the cells which were exposed to TNF- α at the onset of culture contained fewer cells resembling the HSCs (approximately 25–35% versus 45–60% in the second group). Some adherent colonies with the dendri form cells in their proximity appeared at day 10 and their number increased during the culture. More cells acquired the size and appearance of peripheral blood monocytes and DCs as well as large colonies depending on TNF- α exposure (Figures 1 D, E). DC morphology was also monitored after maturation. Both conditions produced cells with the typical morphology of DCs. They had a characteristic veiled appearance, with numerous cytoplasmic extensions radiating from the main cell body. However the amount of DC like cells was more abundant in TNF- α ⁺ groups than in the TNF- α ⁻ ones.

TNF- α appeared to enhance the expansion and the survival of the progenitor cells in the short term cultures. Immediate exposure to TNF- α on the first day caused a 6.1 ± 1.6 fold increase in cell numbers from day 0 to day 7, compared with 3.1 ± 1.4 fold increase in the cells which encountered a delay in TNF- α exposure. Although, it seemed that TNF- α decreased cell survival in long term cultures, TNF- α ⁺ cells demonstrated a 35.7% decrease in cell number at day 14, while cells in TNF- α ⁻ group showed a 35.17% increase

in cell number (Table 1). Overall, the peak of cell proliferation was day 7 in TNF- α ⁺ group and day 14 in TNF- α ⁻ group.

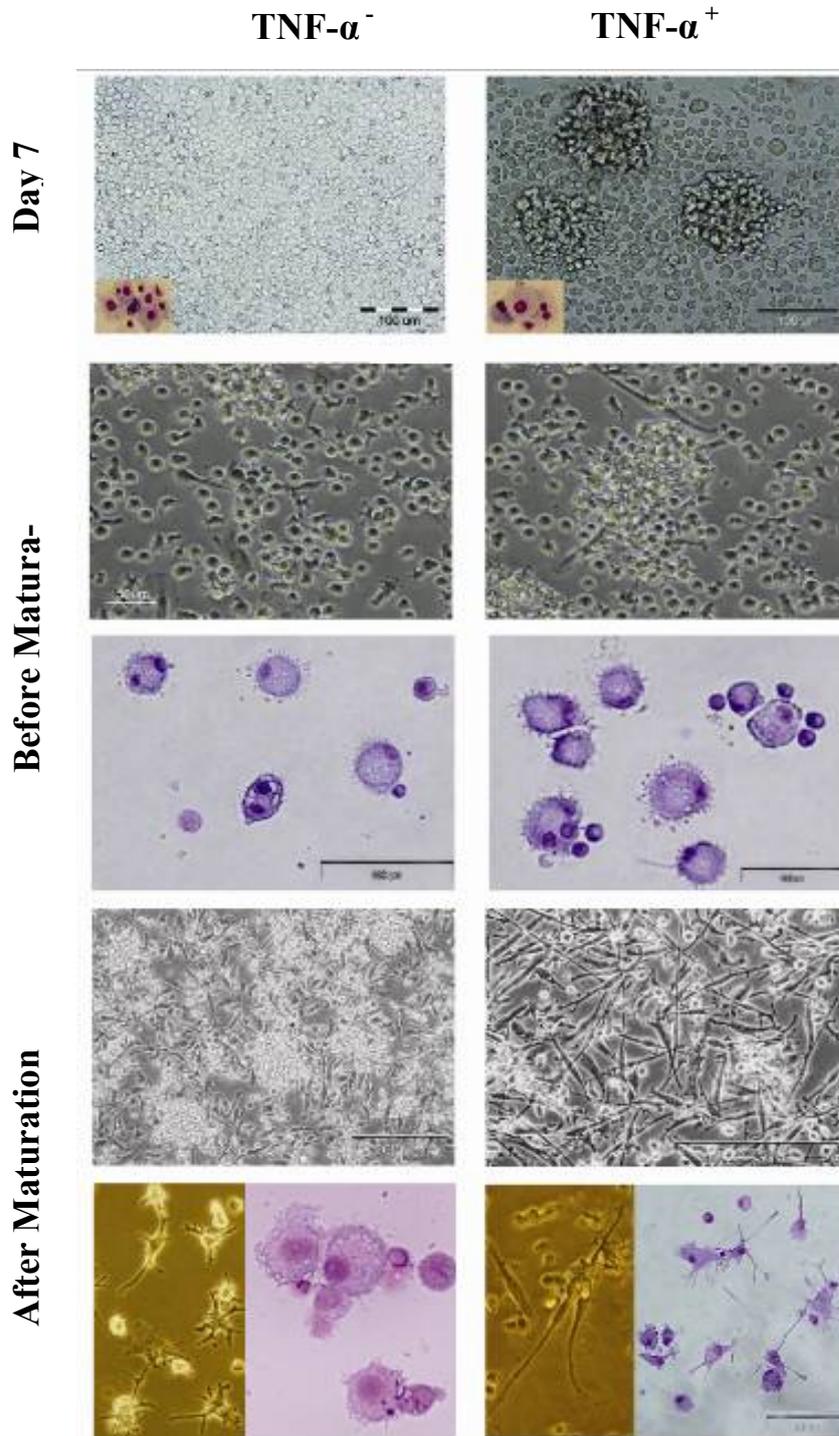


Figure1. Morphological characterization of DCs generated from non-fractionating cord blood dendritic cells. T cell depleted cord blood mono nuclear cells (Day 0) were cultured in presence of a combination of cytokines with TNF- α (TNF+ group) and without TNF- α (TNF- group). In Day 7 cells entered an expansion state, at Day 14, immature DCs were observed and at Day18, mature DCs were observed in the presence of 10 ng/ml of TNF- α .

Table 1. Effects of immediate and delayed exposure to TNF- α on the expansion of UCB-DCs.

Groups	Day 0 ($\times 10^6$)	Day 7 ($\times 10^6$)	Day 10 ($\times 10^6$)	Day 14 ($\times 10^6$)	Day 18 ($\times 10^6$)
TNF- α^-		1.95 \pm 0.66	5.84 \pm 2.49	19.44 \pm 5.39	21.63 \pm 8.59
	0.65 \pm 0.14	Median (1.87)	Median (5.2)	Median (19.21)	Median(19.16)
	Median (0.65)	Fold (3.1 \pm 1.4)	Fold(3.01 \pm 0.78)	Fold (4.2 \pm 1.64)	Fold(1.1 \pm 0.3)
TNF- α^+		3.89 \pm 0.65	11.06 \pm 2.75	17.45 \pm 6.07	13.25 \pm 6.12
	0.65 \pm 0.14	Median (3.84)	Median(10.06)	Medin(15.35)	Median(12.98)
	Median (0.65)	Fold (6.1 \pm 1.6)	Fold(2.8 \pm 0.4)	Fold (1.7 \pm 0.6)	Fold (0.65 \pm 0.18)

UCB-DCs were generated in the presence of SCF, Flt3L, GM-CSF, IL-4 plus TNF- α (TNF- α^+ group) or Only SCF, Flt3L, GM-CSF, and IL-4 (TNF- α^- Group). Total number of viable cells was measured using trypan blue dye exclusion and showed as Mean \pm SD.

TNF- α also had an effect on the number of DCs produced at day 18 of cell culture, as observed by light microscopy. Cells that were cultured in the presence of 3ng/ml TNF- α as a maintenance dose and matured in the presence of 10ng/ml TNF- α were only able to generate (13.25 \pm 6.12) $\times 10^6$ of the total cell population compared with (21.63 \pm 8.59) $\times 10^6$ cells in the second group that received TNF- α at a concentration of 10ng/ml as a maturation factor (Table 1).

Phenotypic Verification of DCs. Flow cytometry was used to measure the expression or absence of a panel of CD markers at days 0, 7, 14 and 18. Cells were positive for the surface antigens CD34 (%1.58 \pm 1.39), CD14 (%27.6 \pm 12.2), CD1a (%28.7 \pm 13.8), CD11c (34.8 \pm 25.1), CD80 (29.6 \pm 18.6), CD83 (34.5 \pm 17.3), CD86 (30.9 \pm 12.2) and HLA-DR (34.1 \pm 13.1) at day 0. The serial phenotypic shift of the cells in different conditions is illustrated in Figure 2. Flow cytometric data were also expressed as arithmetic means of the percent of positive cells for each marker (Figure 2). In both groups, the expression of some tested markers (CD80 and CD83) decreased significantly ($P < 0.05$) after seven days of culture, however the expression of CD14 and CD34 significantly increased in the presence of TNF- α (15 \pm 5.4%, 55.4 \pm 16.1% versus 8.9 \pm 3.6% and 37.8 \pm 15.6% in TNF- α^- group, respectively). After 14 days of cultures, the CD1a and CD11c positive cells increased and CD34 decreased significantly in both groups ($p < 0.05$ and $P < 0.01$, respectively). The decrease of CD34 $^+$ cells in TNF- α^+ group was significantly more than the TNF- α^- group (6.67 \pm 5.03 versus 23.13 \pm 8.26). It is interesting to note that CD14 positive cells increased when TNF- α was added to the culture media on day 0 (47.3 \pm 16.1% versus 13.97 \pm 8.64%). Low expression of DC mature markers (CD80, CD83 and CD86) was an indication of no significant mature DC development in both groups, however increase of CD1a and CD11c in both groups may be related to the development of immature DCs at day 14.

Early Exposure of TNF- α on Maturation of Non-Purified Cord Blood Cells. Analysis of CD1a and CD14 expression on UCB-MNCs cultured with immediate or delayed exposure to TNF- α revealed the presence of several phenotypically distinct immature cells. One possible explanation for this observation is that the cultures contained DCs at multiple stages of development. To test this hypothesis; the cultures were treated for an extra 4 days with TNF- α at a maturation dose (10ng/ml). TNF- α at this dose increased the expression of CD80, CD83 and CD86 on large numbers of cells cultured in the presence of TNF- α on day 0 ($P < 0.05$) (Figure 2), in addition, most of these cells displayed a DC morphology. While TNF- α treatment of cultures established without TNF- α in the first day with SCF, Flt3L, GM-CSF and IL-4 yielded low levels of CD80 (44.6%), CD83 (27.2%) and CD86 (49.0%) expression, not all cells expressed DC markers, and a significant number of CD1a and CD34 positive cells were also observed. This suggested that these cultures may contain a significant fraction of non-DC myeloid cells. This was

confirmed by light microscopic examination and Wright-Giemsa staining that revealed a number of myeloid lineage cells including neutrophils and macrophages at different stages of maturation and immature DCs, as well as progenitor cells (not shown).

Overall, the expression of CD11c, CD80, CD83, CD86 and HLA-DR on DCs were higher and CD14, CD34, CD1a were lower when TNF- α was added to culture media on day 0. However, absence of TNF- α during the culture and its later addition to the media for maturation caused the cells to maintain CD1a and even some maintained CD34 and demonstrated stem cell properties.

Ability of Dendritic Cells Generated in different Groups to Act as APC. To determine whether the putative DCs generated in two groups were able to activate T cells, mature DCs were harvested and tested in allogenic MLRs. As shown in Figure 3A, both types of DCs induced proliferation of the allogenic T cells and no significant difference was observed between the two groups ($P>0.05$). Immature DCs in TNF- α ⁺ group were much more potent stimulators in the MLRs than DCs cultured in the absence of TNF- α ($P<0.05$) (515.6 ± 751.2 compared with 1567 ± 684.4). This suggests that immature DCs produced in the presence of TNF- α may be qualitatively different from immature DCs formed without it.

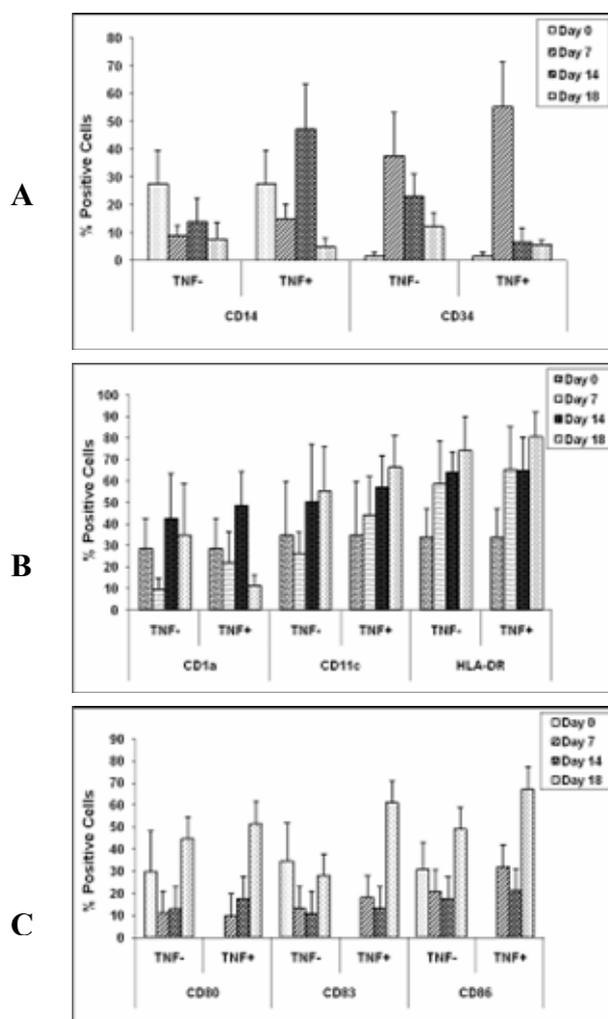


Figure 2. Phenotypic analysis of DCs by Flowcytometry. Panel (A) shows characteristics of DCs at day 0, Panel (B) shows characteristics of immature cells at day 14 in the two groups (TNF- versus TNF+), and panel (C) shows mature DCs in test groups at day 18. These histograms represent progressive phenotypic changes toward maturing DCs from a representative experiment.

IL-12 and IL-10 Production by DCs Cultured in Different Media. IL-12 produced by DCs is a heterodimeric cytokine that augments IFN- γ production by T cells and NK cells and drives the development of Th-1 T cell responses (15). However, IL-10 has a regulatory role in monocyte function and in DC maturation (16).

We assessed Production of IL-12p70 and IL-10 in immature and mature DC cultures in both groups. Results showed that IL-12 p70 was secreted by both types of mature DCs, even immature DCs also secrete it at a low level (Figure 3B). Our experiments showed that cells expanded in the presence of TNF- α contained a higher proportion of IL-12-secreting cells than TNF- α ⁻ group.

The production of IL-10 by DCs was analyzed by ELISA in the culture supernatants of DCs. Significantly greater levels of IL-10 were produced by immature DCs in TNF- α ⁻ group than in TNF- α ⁺ group. The matured cells in both groups and the DCs matured with LPS produced IL-10. In all groups, secretion of IL-10 was at a low level (<5 pg in mature and <15pg in immature DCs, respectively). These results were confirmed in three independent experiments (Figure 3C).

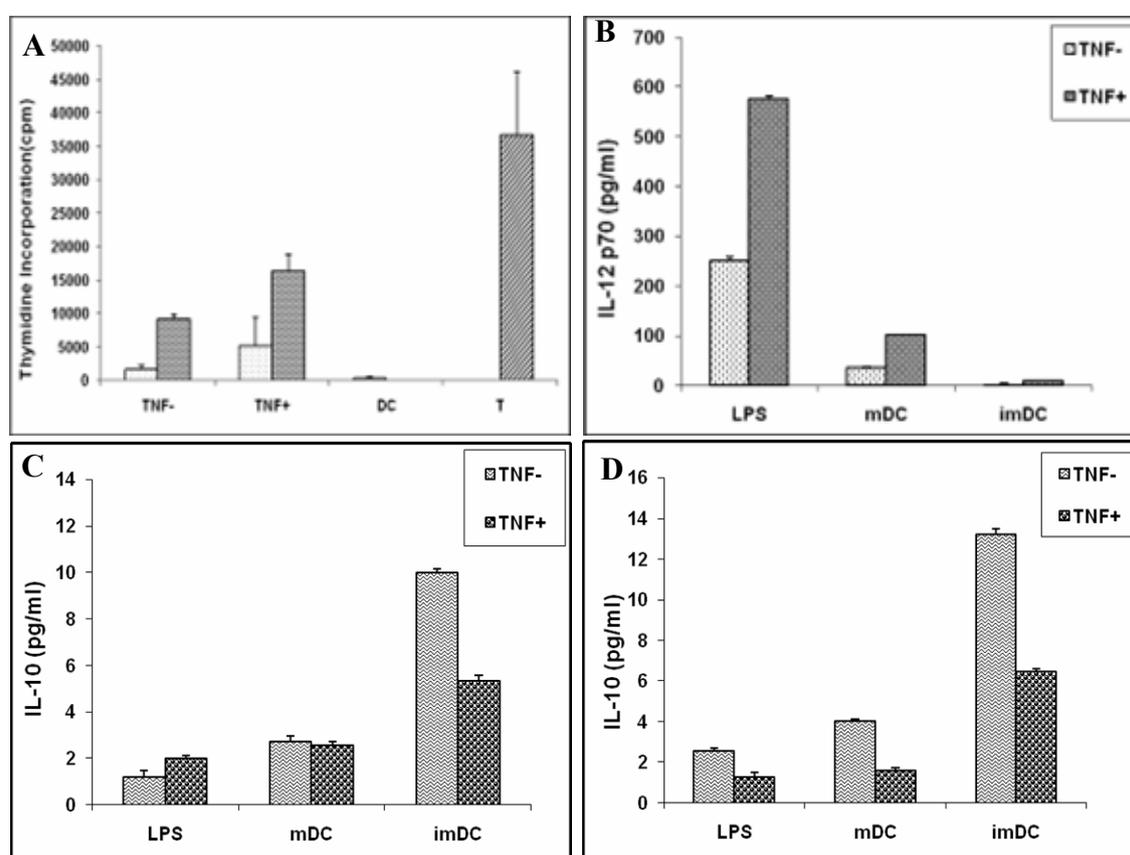


Figure 3. 1×10^4 pre-inactivated DCs as stimulators were cultured with 1×10^5 human peripheral lymphocytes as responders. The proliferation of T cells was measured by incorporation of [3H] thymidine at day 5. Unmanipulated DCs and T cells activated with PHA were used as negative and positive controls, respectively (A). Medium of DCs was screened for the presence IL-12p70 (B) and IL-10 (C) after maturation with TNF- α . DCs were harvested on day 18 and subsequently co-cultured with allogenic T cells for 5 days and the presence of IL-10 was estimated (D). Bacterial lipo polysaccharide (LPS) and immature DCs (imDC) were used as positive and negative controls, respectively.

IL-10 Production in MLR. T cell cytokine production was induced by the presentation of allogenic determinants on DCs in MLR. This assay discriminates among different populations of human APC, in particular among monocyte-derived DCs, which induce high levels of IFN- γ , and in plasmacytoid DCs, which induce high levels of IL-4 and IL-10 (17). The relative potencies of TNF- α ⁺ DC and TNF- α ⁻ DC to induce IL-10 in T cells were determined in three separate MLR. Both types of DCs induced IL-10-producing T cells (Figure 3D) at the same concentration ($p > 0.05$). In these experiments, immature DCs in TNF- α ⁻ group induced higher levels of IL-10-producing T cells than the immature DCs that received TNF- α ⁻ at day 0 ($p < 0.05$).

DISCUSSION

TNF- α is a primary mediator of the immune response produced in several pathologic inflammatory states, infections, cachexia, shock, autoimmune diseases, and organ transplant rejection. This factor has been widely used to support DC development. During the first days of culture, TNF- α in combination with GM-CSF is essential for the development of CD34⁺ cells in the DC pathway (9,18). The mechanism of its action includes upregulation of GM-CSF receptors on the developing cells, inhibition of granulocyte differentiation (19), induction of apoptotic events in the granulocyte compartment and generation of pure DC colonies (6,9,20,21). Also it can facilitate the phenotypic and morphologic development of the DCs. Many studies employ TNF- α at the beginning of CD34⁺ cell culture in combination with other cytokines to induce dendritic cells (20-22), or to use it as a maturation factor when DCs are derived from monocytes (12,23). However, it remains to be established whether exposure to TNF- α at different time periods may produce the most favorable results in obtaining optimal yields of DCs.

For immunotherapy, DCs were developed from magnetically purified cells that are very expensive, cause loss of the cells and are time consuming. In addition, few studies have produced DCs from mononuclear cells without purification. Therefore, in this report; we developed a simple and easy way to generate DCs from non-purified cord blood mononuclear cells exposed to rhSCf, rhFlt3L, rhGM-CSF and rhIL-4, and evaluated the immediate and delayed exposure to TNF- α during 18 days of culture.

In 1998, Santiago-Schwarz et al. showed that early removal of TNF- α provoked an expansion of monocytes (24), in our experience, cells cultured in GM-CSF and TNF- α strongly proliferate through the first seven days, and then proliferation slows down and differentiation increases during the next seven days confirming the results of Noirey et al (25). The biological effects of TNF- α may be either stimulatory or inhibitory (26,27), for instance Bryder et al. and Dybedal et al. observed that TNF- α negatively regulates self renewal in murine and human HSCs by enforcing myeloid differentiation (28,29). Although the exact mechanism associated with the increase in cell expansion upon early TNF- α exposure remains to be established, one explanation is the presence of other cells in our experiments which may inhibit the negative role of TNF- α on stem cell self renewal and may also induce proliferation of some cells. On the other hand TNF- α increases GM-CSF receptors and thus induces the survival of hematopoietic progenitor/stem cells and also promotes the amplification of early myeloid progenitors. Indeed it is possible that combination of SCF, Flt3L and GMCSF may promote the amplification of hematopoietic cells as well as myeloid progenitors that are then subjected to the

differentiated cells upon the presence of TNF- α during later periods as previously outlined (6,24). We, therefore, speculate that the priming of the MNCs with a combination of SCF, SCF, Flt3L, GM-CSF and IL-4 plus TNF- α at the onset of cell culture would maximize the self renewal of primitive progenitors and start the cells toward myeloid differentiation. Prolonged exposure to the danger signal, TNF- α , would slowly switch the cells to the pathway of dendritic cell development and stop the expansion and the cells enter the commitment pathway. Our immune phenotyping data confirm the above speculation. Because of using non-purified mononuclear cells, some cells were positive for DC markers in the first day of culture, but during the first week of culture, CD80, CD83 and CD86 positive cells decreased in number and CD34⁺ cells increased resulting in the expansion of progenitor/stem cells and induction of apoptosis in committed lineages. Upon continuous exposure to TNF- α , the percentages of CD14 and CD1a positive cells increased and the number of CD80, CD83 and CD86 positive cells decreased which were indications of the presence of immature DCs. Also we observed multiple phenotypically defined populations in both groups at different time points based on the expression of CD1a, CD14, and HLA-DR as well as other markers. In both groups we observed that CD1a⁺ CD14⁻, CD1a⁻ CD14⁺ and CD1a⁺CD14⁻ cells were fairly mature DCs, while the CD1a⁻ CD14⁺ cells were monocytes. The maturation of cells following exposure to TNF- α for an extra 4 days suggests that the CD1a⁺CD14⁻ cells may also be DC progenitors. In the peripheral blood, CD14⁺ progenitors have been identified that can yield both monocyte/macrophages and DCs under appropriate conditions (30,31).

According to Steinman and colleagues, the phenotypic characteristics of most mature DC population are CD14^{-dim} and (MHC Class II receptor, CD80, CD83 and CD86)^{++/bright} (32,33). In our experience delayed exposure to TNF- α resulted in a heterogeneous population containing a large number of CD14⁺ and CD1a⁺, and a small amount of CD80⁺, CD83⁺ and CD86⁺ cells which is considered as relatively immature DCs. Although mature DCs in both groups were able to promote T cell proliferation, but the cytokine secretion by the mature DCs showed significant differences between the two groups. Production of high amounts of IL-12 (a type 1 cytokine) by mature DCs following early exposure to TNF- α indicates a higher degree of maturation in the presence of TNF- α . However we have not yet examined the outcome of cross-talks between CD4⁺ T cells and our two types of DCs. Clearly, additional studies will be needed to resolve some of the issues raised by this study.

Xu et al (34) and Morrison et al (5) in separate studies demonstrated that delayed compared to early TNF- α exposure greatly enhances DC development from early CD34⁺ progenitor cells and also, the subsequent administration of TNF- α , although successful in producing more DCs, may be slightly inhibitory to the maturation of such DCs and result in expressing of less CD80 (5). Our observation does not confirm their data because we added TNF- α to the mixture of mononuclear cells but they used purified CD34⁺ cells. Also we used different cytokine combinations namely SCF, Flt3L and GM-CSF that induce cell expansion specially in myeloid cells and IL-4 was added for survival of myeloid progenitor cells, but they only used SCF in the first week of culture. GM-CSF was used continuously in the culture and IL-4 was added in the second week of cell culture. However this cytokine combination and also the time of TNF- α exposure needs to be tested in purified cells such as CD34⁺ or CD14⁺ cells to clarify crosstalks between cells during DC differentiation.

In summary, the local microenvironment is probably a significant factor in the

determination of the immune response and the formation of DCs and their activation state. Here, we described a simple inexpensive coculture system mimicking inflammatory conditions in some autoimmune diseases such as Lupus in which secretion of TNF- α increases in serum. We determined that the presence of inflammatory factors such as TNF- α is capable of profoundly influencing the outcome of DC production. In addition, we suggest that CD34⁺ cells in the cord blood mononuclear cell suspension in this microenvironment develops from CD14 pathway.

ACKNOWLEDGMENTS

We thank Ehsan Taghiabadi and Ehsan Jan Zamin for their technical help in cell culture and flow cytometry analysis. This work was supported by Tarbiat Modares University and Royan Institute.

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