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# The Ability of Precursory Monocytes (MO) to Differentiate Varies Among Individuals But Is Stable Over Time

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Study Design A
Data Collection B
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**Background:** The ability to generate dendritic cells (DCs) from precursory monocytes (MOs) was a breakthrough in the field of immunology. However, it is unknown whether the ability of MOs to differentiate into immature DCs (iDCs) differs across subjects or is time dependent. Given that the study of immune system function is gaining recognition in the field of clinical medicine, it is important to know how certain immunologic features vary over time.

**Material/Methods:** This study investigates how much individuals' MO-to-iDC differentiation potential changes over time. We estimated this potential by measuring the expression of an iDC marker (CD1a), cytokine secretion (interleukin [IL]-12p70), and the ability of IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) differentiation MOs to stimulate T cells. We collected MOs obtained from different subjects (n=17) at least 1 month apart. Furthermore, we investigated several variables (expression for cytokine receptors, timing, and emergence of DC-related transcriptional factor PU.1).

**Results:** The ability of MOs to become DCs under the influence of IL-4 and GM-CSF varied greatly between individuals (range of CD1a expression, 20–80%) but was stable over time (change of CD1a expression between sampling, ~5%). A similar pattern emerged when production of IL-12p70 was analyzed. The ability to stimulate T cells was variable and depended on the T-cell source. The ability of MOs to become iDCs was not linked to the surface expression of receptors for IL-4 and GM-CSF but rather to the activation of PU.1 in the precursory MO. It took 5 days for all committed MOs to become iDCs under *in vitro* influence of IL-4 and GM-CSF.

**Conclusions:** We concluded that the potential of MO to become iDC is an individual feature and depends on activation of PU.1.

**MeSH Keywords:** Dendritic Cells • Interleukin-4 • Lymphocyte Culture Test, Mixed • Monocytes • Receptors, Granulocyte-Macrophage Colony-Stimulating Factor

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## Background

Peripheral blood monocytes (MOs) are heterogeneous and pluripotent leukocytes [1]. Their main functions include phagocytosis, cytokine production, and the presentation of antigens to naive T cells [1–4]. One of the most important functions of MOs is their ability to differentiate into various cells, including activated MOs, specialized tissue macrophages, inflammatory macrophages, Langerhans cells (LCs), and dendritic cells (DCs) [1–4].

Initially, the origin of DCs was unclear [5]. After a seminal experiment by Inaba et al., a reliable source of DCs was discovered in peripheral blood MOs [6]. Other researchers quickly confirmed the importance of interleukin (IL)–4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in DC generation [6–8]. In today's standard experiments, peripheral blood MOs are stimulated with IL-4 and GM-CSF for 3 to 5 days [9]. During that time, MOs differentiate into immature dendritic cells (iDCs). iDCs are more capable of stimulating T cells; they also produce IL-12 and IL-15 and express a specific surface antigen-CD1a [10,11]. In the next step, maturation factors are added (lipopolysaccharide, tumor necrosis factor [TNF]-α, IL-6, prostaglandins, CD40 ligation, and others) with subsequent emergence of matured DCs (mDCs) able to fully process and present antigens to naive clone T cells [10,11]. mDCs are also an excellent source of IL-12 and IL-15, and they express CD83 while partially losing CD1a [10,11]. Both steps (MO→iDC followed by iDC→mDC) are necessary in the maturation process. This is the classic way to generate DCs from MOs, although alternative ways exist for MOs to become DCs or other cells [12–14].

These monocyte-derived dendritic cells (MODCs) were described as pivotal in several acute and chronic processes [9,15–17]. In trauma, burn injury, or sepsis, their rapid emergence from precursory MOs allows for the successful transformation of nonspecific innate response into acquired immunity [4,9,10]. The inability of MOs to become DCs (MO→DC) is related to unfavorable clinical outcomes secondary to a runaway and poorly controlled inflammatory process [11–13]. The rapid emergence of MODCs allows for effective control, since they emerge in a matter of days, in contrast to other DC-like cells [4,21]. For example, LCs take weeks to mature [3,10]. Consequently, there is an increasing emphasis on the role of MODCs in acute inflammation and its resolution [15,17,18]. However, the emergence of DCs is believed to be a highly individual process, resulting in large standard deviations of the control and study groups. Similar complaints seem to frequently arise in other immune system studies. However, no study has inquired into the stability of the potential of an individual's MOs to convert to iDCs over time. Here, we tested the hypothesis that MOs obtained from a particular individual will have an unchanged ability to differentiate into iDCs over a period of 3 months.

## Material and Methods

### Study population

We enrolled 17 healthy control subjects as a part of a larger study, on the immunologic effects of nonemergent coronary artery bypass grafting, in order to be processed as a positive control. There were 12 female and 5 male subjects. Mean age was 44.1±11.79 years.

Blood samples of 30 mL each were collected at least 3 months apart. Serum was stored, and mononuclear cells were separated out using the Ficoll gradient technique, and then using negative selection of MOs with DynalBeads® (Life Technologies, Grand Island, NY, USA), as described previously. We used 2 kits sequentially to isolate all MOs from the same production batch.

### Generation of iDCs from peripheral MOs

To generate iDCs, fresh MOs were incubated in X-VIVO 15™ Media with Gentamycin and Phenol Red (Lonza, Cohasset, MN, USA) supplemented with human IL-4 (Peprotech, Rocky Hill, NJ, USA) at 500 IU/mL and human GM-CSF (Peprotech) at 37°C 5% $CO_2$ in the dark. On day 3, 50% of the X-VIVO 15/10™ was replenished with fresh media along with 50% of the initial cytokine concentration. The cultures were terminated on day 5.

Considerable effort was made to standardize our experiments. We used previously aliquoted cytokines (stock solution of 10,000 IU/mL) from the same production batch dissolved in 1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA). The same batch of X-VIVO 15™ media (Lonza) was used throughout the study experiments. All cells were suspended at $10^6$ cells/mL and total working volume of media was 3 mL for differentiation condition. The cells were incubated on standard nonpyrogenic, individually packed, sterile 6-well plates (Sigma-Aldrich). The incubator was checked twice a year for accuracy of $CO_2$ measurement, and correct humidity was observed throughout. All pipettes were checked twice a year for accuracy.

The IL-4- and GM-CSF-stimulated MOs were harvested by a brief incubation in 1 mL of 10 mM EDTA buffered to pH 7.4 (Gibco, Grand Island, NY, USA) on ice, followed by the washout of all cells with phosphate buffered saline without calcium or magnesium (PBS w/o $Ca^{2+}$ or $Mg^{2+}$; Gibco). The procedure was repeated until fewer than 2 cells were seen under inverted microscope with a magnification of 10. Cells were counted twice with the Countess® Automated Cell Counter (Life Technologies),





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as per the manufacturer's instructions. Cells were suspended at $10^7$ live cells/mL in X-VIVO 15™ for further study.

Study personnel were kept unaware of what they were running, through the use of simple numerical values for experimental designs/stimulation. Only after completion of the experiments were all data combined and analyzed.

### Flow cytometry

A total of $10^5$ cells were incubated with FACS media (PBS w/o $Ca^{2+}$ or $Mg^{2+}$ with 0.01% sodium azide and 1% fetal bovine serum [FBS]) enriched with human TrueStain FcX™ (BioLegend, San Diego, CA, USA) for 15 minutes at 4°C in the dark. Then, cells were incubated with the antibodies for 30 minutes at 4°C in the dark with additional mixing at 15 minutes. Cells were washed twice in FACS media and resuspended in 100 µL of the 1% FlowFix (Polysciences, Warrington, PA, USA). The following antibodies were employed: CD1a (HI149; BioLegend), CD14 (Tuk4; Invitrogen, Grand Island, NY, USA), CD83 (HB15e; BD, San Jose, CA, USA), CD209, CD86 (clone IT2.2; BioLegend), CD116 (4H1; BioLegend), CD124 (G077F6; BioLegend).

Intercellular staining with PU.1 was done. αh PU.1 (clone Spi-1; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were conjugated with Dy488 (ThermoFisher, Rockford, IL, USA). Appropriate nonspecific immunoglobulin G were used as isotype control. After permeabilization with Wash Buffer (BioLegend), $10^5$ cells were stained intracellularly.

Cells were analyzed with an LSR™ (BD) or a FACSCalibur™ (BD) flow cytometer. At least $10^4$ cells were collected for each assessment. Duplets or dead cells were excluded by gating with forward and side scatter.

### Mixed lymphocyte reaction

A 1-way mixed lymphocyte reaction (MLR) was employed in this study. Responder allogeneic T cells were obtained from the Human Immunology Core at the University of Pennsylvania, from anonymous donors.

To a 96-well plate were added $2\times10^4$ harvested cells (unstimulated MOs or IL-4- and GM-CSF-stimulated MOs, depending on the experiment; stimulators/antigen-presenting cells [APCs]). Next, $2\times10^5$ T cells were added to the APCs and incubated for 1 hour in the X-VIVO 15™ Media with Gentamycin and Phenol Red (Lonza). The working media of 20 µL of Alamar Blue (LifeTechnologies) was subsequently added. Three sets of controls consisted of stimulator cells alone, T cells alone, and media mixed with Alamar Blue alone. Again, we used only 1 batch of Alamar Blue and X-VIVO 15™ media for all experiments.

After 18 hours of incubation, the level of absorbance was measured using the Opsys MR (Thermo Laboratories, Philadelphia, PA, USA) with Revelation software. The absorbance at 570 nm (with a reference filter at 630 nm) was measured. The degree of Alamar Blue reduction was calculated per manufacturer specification (Invitrogen). It has been shown previously that the degree of reduction correlates well with T-cell proliferation.

### Stimulation of IL-4- and GM-CSF-differentiated MOs

Next, $2\times10^5$ IL-4– and GM-CSF-differentiated MOs were suspended in 200 µL of X-VIVO 15™ media. Cells were stimulated with 1 µM ionomycin (ION) and 10 ng/mL phorbol-12 myristate 13-acetate (PMA). After 18 hours, 180 µL of supernatant were collected and frozen. Again, we used only 1 batch of X-VIVO 15™ media for all experiments. The biological agents were dissolved into stock solution using 1% BSA media from 1 batch and added to the cultures.

Supernatant levels of cytokines (IL-12p70, IL-15) were measured using a magnetic multiplex kit (Bio-Rad, Hercules, CA) per manufacturer's protocol and analyzed on the BioRad™ platform (Hercules, CA).

### iDC stimulation

Then, $2\times10^5$ isolated MOs were incubated in 180 µL X-VIVO 10™ media (BioWhittaker, Welkersville, MD, USA) combined with 20 uL of Alamar Blue (Life Technologies) with lipopolysaccharide (Sigma-Aldrich) at final concentration of 100 µg/mL for 18 hours. After incubation, cells were spanned down and supernatant was collected and stored at –80°C.

### Institutional review board approval

The institutional review board at the University of Pennsylvania, Philadelphia, PA, USA, approved this study. The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

### Statistical analysis

Parametric nature of the data was confirmed by the Levene and Shapiro-Wilk tests. *t* tests or Wilcoxon matched-pair tests were conducted, depending on the characteristics of the data. Analysis of variance was conducted for multiple group comparisons with a Bonferroni test for post hoc analysis. The data were flagged as significant if the 2-tailed hypothesis test result was $\leq 0.05$, unless otherwise specified. Statistica v8.0 (Statistica, Tulsa, OK, USA) was used.





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## Results

### MOs obtained from different individuals can differentiate into iDCs

First, we measured the level of CD1a, a specific marker of iDC. Becoming an iDC is the first necessary step for an MO in DC generation [11]. We found a significant variance in the%CD1a positive cells after 5 days of differentiation of MOs with IL-4 and GM-CSF (Figures 1A and 1C). However, when the same subjects were retested 2 to 5 months after their baseline estimate, a remarkable replication of the result was observed (%CD1a Baseline *vs.* After 3 months 66.64±17.41 *vs.* 68.17±17.9; Figure 1A, 1B). The variance between 2 time points was <5% and statistically insignificant. Additionally, we measured the production of IL-12p70 and IL-15 in the same individuals and the variance of the results was within 6% (Table 1). Finally, we checked the ability to stimulate T cells in MLR. This is the ultimate test judging the ability of MODCs, and all DCs, to perform their primary function: regulation of the immune response. Here the variance was significantly higher compared with differences in expression of CD1 or secretion of IL-12p70 and IL-15 by IL-4- and GM-CSF-stimulated MOs, but still below generally expected levels (%MO MLR 21±5%; Figure 1C). Additionally, the correlation between DC MLR and expression of CD1a was high and significant ($r$=0.878; Figure 1D)

Since we believed that part of the variance in DC MLR would result from different levels of HLA mismatch, we conducted an MLR using different IL-4- and GM-CSF-stimulated MOs with the same responder T cells, and measured the same T cells responding to IL-4- and GM-CSF-stimulated MOs obtained from different individuals [19–21]. We observed some variance with some T cells having very poor proliferative capacity regardless of the origin of the stimulatory IL-4- and GM-CSF-differentiated MOs (Figure 2).

The classic protocol of MO→iDC differentiation takes 5 days. However, other studies suggest that DCs can be generated in less time [4,11,12]. Therefore, we assessed the acquisition of DC markers over time. We found that significant variation existed in this respect but that all our subjects attained CD1a expression, or lost CD14 expression, by day 5 (Figure 3). This suggested that the ability of MOs in individuals to differentiate into iDCs is time-contextual.

### PU.1 expression correlates with ability to differentiate into iDCs under influence of IL-4 and GM-CSF but not surface expression of GM-CSF receptors, IL-4 receptors, or macrophage CSF (M-CSF) receptors, or preexisting ability of MOs to stimulate T cells

In the last step, we investigated possible reasons for the effective differentiation of MOs into iDCs under the influence of IL-4 and GM-CSF. First, we tested whether the preexisting ability of MOs to stimulate T cells correlates with positive IL-4 and GM-CSF differentiation. We found a negative, but statistically nonsignificant, correlation ($r$=-0.47; $P$=0.257; Figure 4A).

In our experimental design, we tested the ability of MO to differentiate using IL-4 and GM-CSF. Both cytokines are critical for successful MO-to-iDC differentiation, yet they rely on the respective receptors to induce the process. We found no significant variance in the percentage of positive cells and receptor density among our subjects with respect to IL-4 receptors and GM-CSF receptors (87±4.88% and 88.30±4.16%, $P$=ns; Table 2). Other cytokines (M-CSF) can be secreted by MOs, and affect the MO-to-iDC differentiation. However, the expression of M-CSF receptor was unaltered (Table 2). Furthermore, the correlation between percentage of positive cells and emergence of%CD1a cells after IL-4 and GM-CSF stimulation was receptor density mean fluorescence index (MFI) weak and nonsignificant (data not shown).

PU.1 is a critical factor in the emergence of DC under resting conditions [14]. So, we analyzed expression of PU.1 in the precursory MO using flow cytometry. We found a strong correlation between the frequency of MO positive for PU.1 and% of $CD1a^{(+)}$ cells ($r$=0.747; $P$=0.013) after IL-4 and GM-CSF differentiation (Figure 4B). MO from individuals with higher PU.1 levels had higher propensity to become DC under the influence of the cytokines.

## Discussion

The ability to generate large numbers of DCs from precursory MOs was a breakthrough in the field of immunology. Over time, the abnormalities of the process of MO transformation into DCs has been linked to many clinical conditions. Thus, the ability of MOs to become DCs under the influence of IL-4 and GM-CSF *in vivo* has the potential to become a clinical test. Hence, there is a need for a better characterization of this process.

We hypothesized that the effectiveness of the process of MO→iDC is stable over time. Therefore, we conducted a study using serial measurements of several markers of iDC emergence. We employed flow cytometry (CD1a, CD209), cytokine secretion (IL-12p70, IL-15), and functional measures (MLR) of the DC emergence since potential for MOs to differentiate into alternate cells is high [10,11,14]. We were able to determine that the ability of MOs to acquire the CD1a marker or to produce IL-12p70 was individually variable but stable over several weeks. The difference in acquisition of the CD1a marker between $t_0$ and $t_{+1}$ was small, with $P$=0.44. When we calculated confidence limits (assuming a confidence level of 0.8) for this data set, the confidence range was between -0.85 and





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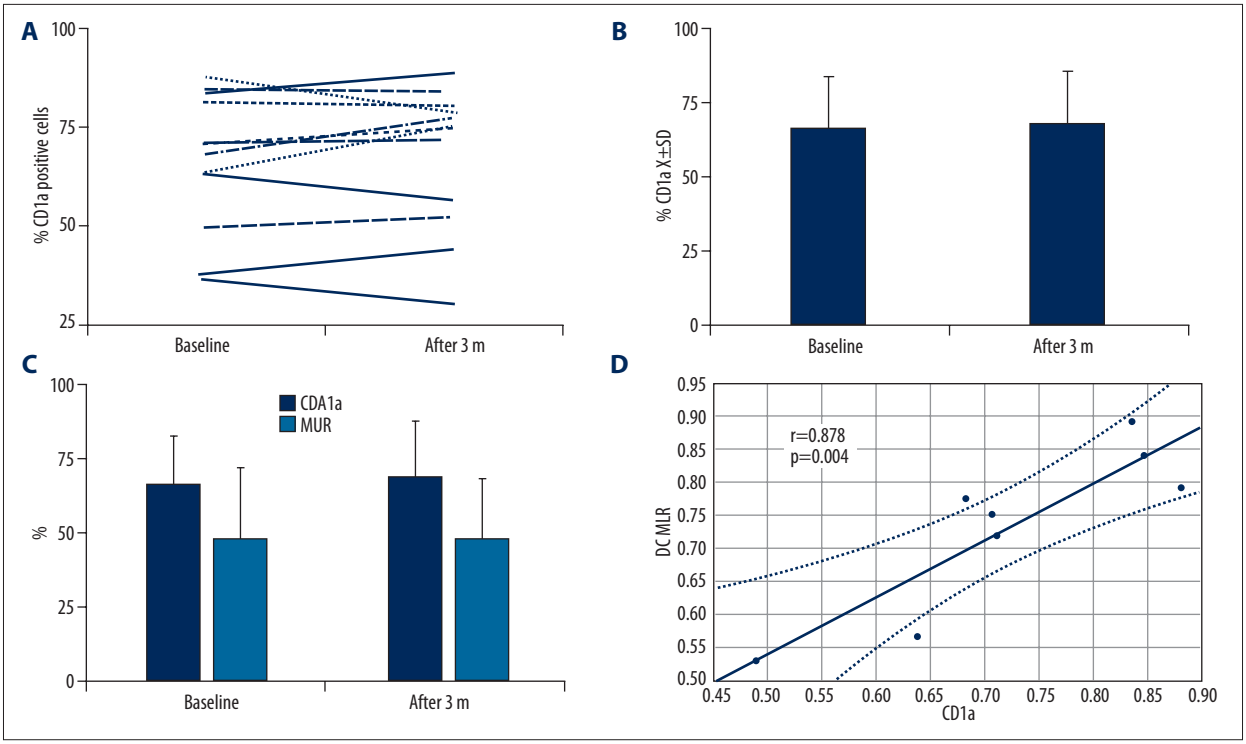
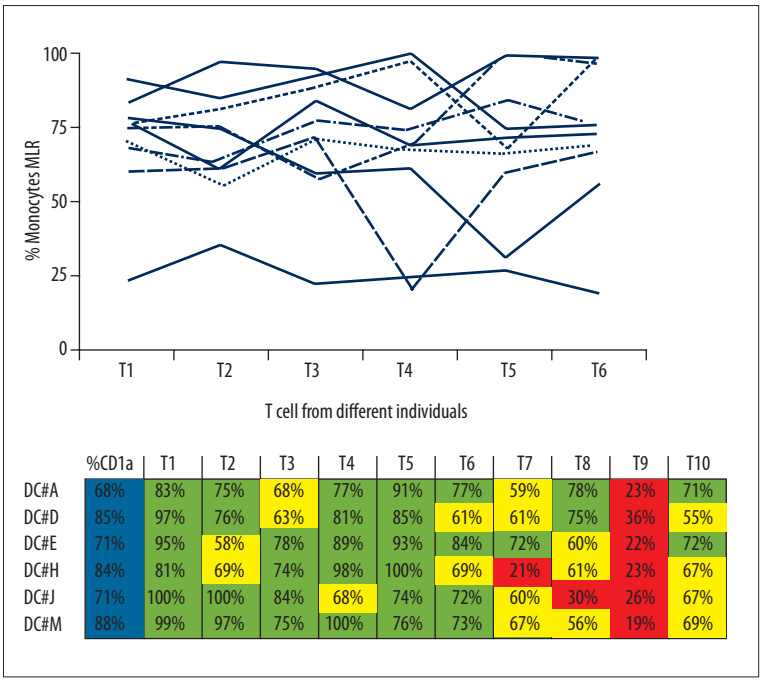



**Figure 1.** There was a strong interindividual variability in expression of CD1a (**A**), but there was no significant difference when the same individuals were compared after 3 months (**B**); there was no significant difference in the ability to stimulate T cells in mixed lymphocyte reaction (MLR) (**C**); there was a strong correlation ($r$=0.878) between CD1a expression and the ability of dendritic cells to stimulate T cells in MLR (**D**).




| | %CD1a | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DC#A | 68% | 83% | 75% | 68% | 77% | 91% | 77% | 59% | 78% | 23% | 71% |
| DC#D | 85% | 97% | 76% | 63% | 81% | 85% | 61% | 61% | 75% | 36% | 55% |
| DC#E | 71% | 95% | 58% | 78% | 89% | 93% | 84% | 72% | 60% | 22% | 72% |
| DC#H | 84% | 81% | 69% | 74% | 98% | 100% | 69% | 21% | 61% | 23% | 67% |
| DC#J | 71% | 100% | 100% | 84% | 68% | 74% | 72% | 60% | 30% | 26% | 67% |
| DC#M | 88% | 99% | 97% | 75% | 100% | 76% | 73% | 67% | 56% | 19% | 69% |

**Figure 2.** There was no significant difference in the ability to stimulate T cells from different individuals when they were compared with the monocytes from the same individual.





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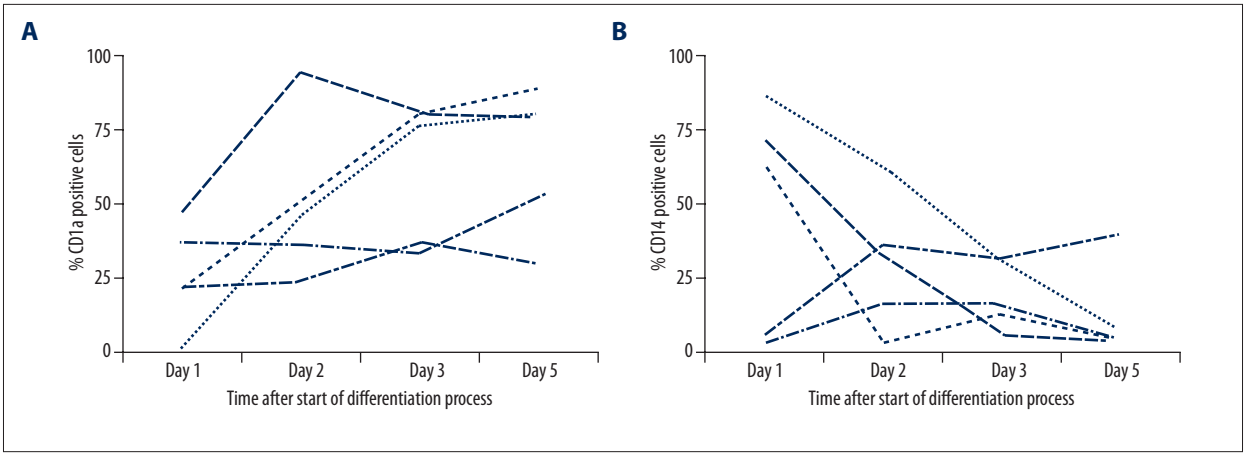


**Figure 3.** There was a strong interindividual variation in the ability either to gain CD1a markers (**A**) or lose CD14 markers (**B**) over 5 days of the differentiation process.

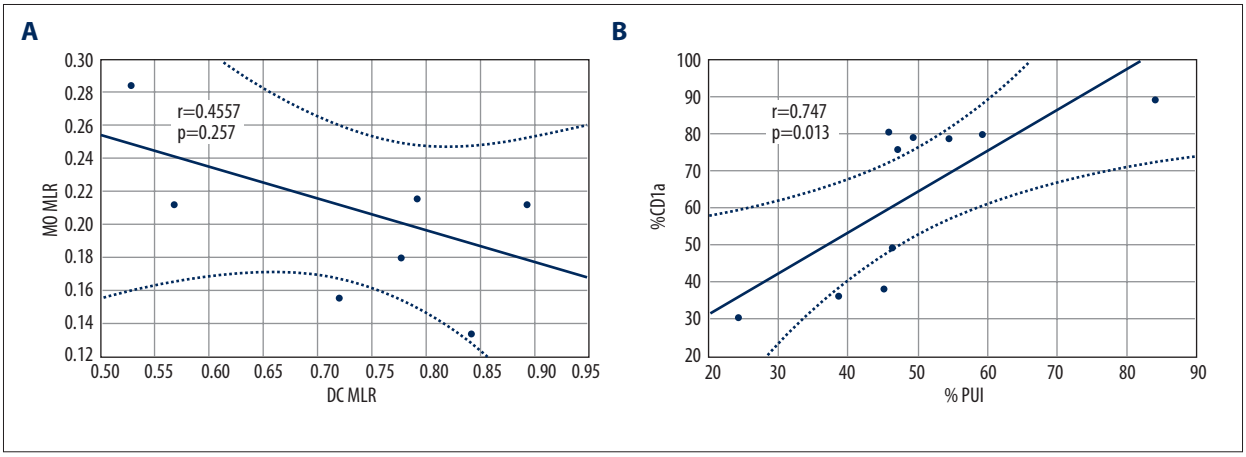


**Figure 4.** There was no correlation between preexisting ability of monocytes to stimulate T cells and differentiated dendritic cells (**A**). There was a strong correlation ($r$=0.747) between the expression of CD1a and PU.1 (**B**).

1.71, which is well below significant statistical value. When we performed a power analysis in order to estimate the number of individuals needed to show the difference between 2 time points, it turned out that we would have to study more than 1100 individuals to show the existence of this potential difference in CD1a level on IL-4- and GM-CSF-stimulated MOs over time. This clearly shows there is little, if any, variance between 2 time points in estimating the frequency of CD1a-positive cells in IL-4- and GM-CSF-differentiated MOs.

In order to increase the robustness of our findings, we employed a composite measure of DC emergence. Flow cytometry evaluated the CD1a marker – a gold standard for measuring the frequency of iDC. It is a highly selective marker, yet there are other measurements of iDC function that we assessed. IL-12p70 production is another hallmark of DC maturity [5]. IL-12p70 is critical for stimulation of the clones of the T cells if process selection has been completed. Again, when we compared $t_0$ and $t_{+1}$, no statistical change was detected in production of IL-12p70 between these 2 time points. Another marker is the ability of IL-4 and GM-CSF to stimulate T cells to proliferate [5]. This is a gold standard since MLR mimics the natural process of T cells selection by DC. We found significant variability over time, but that could be attributed to matching between T cells and stimulatory APCs. An MLR depends on the level of mismatch between APC and T cells because we used the allogeneic system. We showed this in practice because some of the T cells were poor responders regardless of the origin of APC. Use of the autogeneic system (both DCs and T cells are from the same individual) is dependent on time-related variability, and the MLR culture needs additional stimulus. Usually, vaccine antigen is used, but in our study we did not know the vaccination status of our donors [20,21].

Despite the high stability of the MO→DC process over time, it is apparent that there is a high degree of interindividual variability in MOs' ability to become iDCs. The expression of critical receptors for the MOàDC process did not correlate with





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CD1a. There was a strong correlation with the emergence of CD1a-positive MOs and activity of PU.1. This correlation is expected because PU.1 is a critical factor in the emergence of DCs from MOs. Its baseline level correlates with the degree of MO-to-DC commitment. However, PU.1 is a switchboard of several intracellular pathways [22] To pinpoint one that could be responsible for interindividual variability is beyond the scope of this study.

In the next step, we investigated the potential reason for interindividual variability in the MO?iDC process. The expression of critical receptors for IL-4 (CD124) and GM-CSF (CD116) was not significantly different across subjects and over time. We also measured the expression of receptors for M-CSF (CD115). CD115 is a marker of macrophages and can be used to measure the expression of M-CSF as both proteins are locked in positive feedback loop. One could hypothesize that in different individuals there are different numbers of macrophage-like cells, but our study did not show this as seen by MO MLR. M-CSF and IL-6 are negative regulators of the MO?iDC process. M-CSF can also be secreted in an autocrine fashion. So, it could negatively affect the process by interacting with its own receptor. However, we did not register any significant differences over time or significant correlations between the emergence of $CD1a^{(+)}$ IL-4 and GM-CSF cells and the expression of receptors for IL-6 or M-CSF. We did find a significant correlation between PU.1 and the emergence of CD1a. Though this correlation may not represent a cause-and-effect relationship, it is highly suggestive of PU.1's critical role in iDC emergence. PU.1 was previously shown to play a critical role in the emergence of DCs [22,23]. Our study suggests some interindividual variability in the expression of this transcription factor that is interlocked with an individual's ability to undergo MO→iDC process *in vitro*.

The process of MO→DC is very frequently studied in research, but suggestions were made to use the ability of MOs to become DCs in a clinical test [11,24–27]. However, a frequent problem with immunologic testing is significant variability secondary to technical aspects of measurements. In our study, we took a lot of precautions to minimize variability. All the cytokines we used came from 1 lot and were prediluted and stored in silicone-coated vials at –80°C until use. The medium used in this project was X-VIVO 15/10™ a proprietary, commercially available serum-free medium. In the classic experiments, FBS or fetal calf serum (FCS) is routinely used. It contains hard-to-predict and highly variable amounts of cytokine and biologically active components (e.g., transforming growth factor beta [TGF-β]). Routinely, such media are heat inactivated, but some cytokines are still active despite using heat to denaturate them. TGF-β is one such heat-resistant cytokine that is present in significant amounts in serum while having a negative influence on the MO→iDC process *in vitro* [16,28]. Therefore, using FBS or FCS introduces hard-to-predict variables because the source, processing, and biologic potency of FBS/FCS vary. CD1a is an exclusive marker for iDCs. However, the process of differentiation does not stop at iDC step; it progresses into maturity. mDCs, in contrast to iDCs, have varying expression of CD1a. That is why we see a gradual increase in the CD1a in our time-centered experiments. Introducing FBS/FCS can trigger the maturation process of the iDC, leading to variable CD1a expression [10,28]. Considering CD1a variability, we used another marker typical for DC. We used IL-12p70 secretion, a critical cytokine for T-cell stimulation, as a measure of the acquisition of DC proportion by MO, under the influence of IL-4 and GM-CSF [10,11,26]. In order to elicit this response, we used PMA and ION, in contrast to the bacterial pathogens used by others. PMA and ION are independent of the expression of PARP (poly[ADP-ribose] polymerase) receptors on the surface by direct activation of calcium influx and protein kinase C. Finally, we used MLR, but, predictably, we were able to establish that the level of T cell proliferation depends on major histocompatibility mismatch between IL-4 & GM-CSF differentiated MO and T cells [20,21].

This was a difficult study to conduct since we sought to show a lack of difference between immunological variables at two time points in the same individuals. This is very different objective than in majority of the regular scientific inquires. Vast majority of studies attempt to show the existence of a difference between two studied parameters. Here, we tried to show that there is no difference. This is a very different goal to achieve hence majority of statistical tests are aimed at showing the difference not providing proof of similarity.

## Conclusions

The ability of MO to differentiate into DC is highly variable between individual. However, this ability is remarkably stable over time. This stability depends on the expression of PU.1. Our study suggest that ability of MO to iDC differentiion under *in vitro* influence can be used as a clinical test considerinhigh stability of the variable.

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