EFFECT OF VARIOUS AGENTS ON THE DIRECTION OF THP-1 CELL DIFFERENTIATION

Svetlana V. Zubova[1], Yaroslav V. Radzyukevich[1], Sergey V. Grachev[1,2], Isabela R. Prokhorenko[1]
[1]FSBIS Institute of Basic Biological Problems Russian Academy of Sciences,
Institutskaya st. 2, Pushchino, Moscow Region, 142290 Russia
[2]Federal State Autonomous Educational Institution of High Professional Education
I.M. Sechenov's First Moscow State Medical University of Russian Healthcare Ministry, Trubetskaya st. 8, Moscow, 119991 Russia

UTICAJ RAZLIČITIH AGENASA NA SMER THP-1 ĆELIJSKE DIFERENCIJACIJE

Svetlana V. Zubova[1], Jaroslav V. Radžukevič[1], Sergej V. Gračev[1,2], Izabela R. Prokhorenko[1]
[1]FSBIS Institut za osnovne biološka istrazivanja, Ruska akademija nauka,
Institutskaia st. 2, Pushchino, Moskva, 142290 Rusija
[2] Prvi Moskovski državni medicinski univerzitet I.M. Sechenov, Trubetskaia ul. 8, Moskva, 119991 Rusija

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ABSTRACT

The ability of physiological (1α,25-dihydroxyvitamin D3, retinoic acid) and non-physiological (various LPS) agents and their combinations to influence the direction of pro-monocytic THP-1 cell differentiation was studied.

The differentiating activity of the agents was evaluated by the expression and the ratio of surface receptors (TLR4, CD11b, and CD14) as well as by the change in THP-1 cell phagocytic activity of different degree of differentiation by Flow cytometry.

The THP-1 cell differentiation by VD3 was shown to lead probably to the formation of classical monocytes.

Summarizing we can conclude that VD3 induces the THP-1 cells differentiation with the formation of classical monocytes and the sequence of 1α, 25-dihydroxyvitamin D3 and non-toxic LPS R. capsulatus PG causes the THP-1 cells differentiation with the formation of inflammatory or intermediate monocytes.

Keywords: *THP-1 cells, vitamin D3, retinoic acid, lipopolysaccharides, receptor expression, phagocytosis*

SAŽETAK

Ispitivama je sposobnost fiziološkog (1alfa, 25-dihidroksivitamina D3, retinoinska kiselina) i ne-fizioloških (različitih LPS) agenasa i njihove kombinacije i uticaj na smer promonocita i diferencijaciju ćelija THP-1.

Diferencijalna aktivnost agenasa je prikazana odnosom površinskih receptora (TLR4,CD11b i CD14), kao i promenom THP-1 ćelije i fagocitne aktivnosti pri cemu je razlicit stepen diferencijacije odredjen protocnom citometrijom.

Rezultati su pokazali da je doslo do diferencijacije ćelija THP-1 pomoću VD3 i verovatno do formiranja klasičnih monocita.

Sumirajući možemo zaključiti da VD3 indukuje diferencijaciju ćelija THP-1 sa formiranjem klasičnih monocita i sekvence 1a, 25-dihidroksivitamina D3 kao i da netoksični LPS R. capsulatus PG uzrokuje diferencijaciju THP-1 ćelije sa formiranjem inflatornog ili međuproizvoda -monocita.

Ključne reči: *TNR-1 ćelije, vitamin D3, retinoinska kiselina, lipopolisaharidi, ekspresija receptora, fagocitoza*

ABBREVIATIONS

CD — cluster of differentiation;
CR3 — complement receptor;
MHC II — major histocompatibility complex,
MD-2 — myeloid differentiation protein 2;
MyD88 — myeloid differentiation protein;
PMA — phorbol myristate acetate;
TLR4 — toll-like receptor 4;
TRIF — adaptor protein;
VDR — vitamin D receptor.

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Corresponding author: Zubova V. Svetlana,
FSBIS Institute of Basic Biological Problems Russian Academy of Sciences,
Institutskaya st. 2, Pushchino, Moscow Region, 142290 Russia; Fax: (4967)33-05-32, e-mail: zusvet@rambler.ru

INTRODUCTION

THP-1 is a line of acute monocytic leukemia cells and good model system for studying differentiation mechanisms and TLR4-mediated cellular response to bacterial lipopolysaccharides (LPS, endotoxins) (1). THP-1 cells express some typical monocyte surface receptors such as TLR4, CD11b, CD14, receptors to Fc-fragment of immunoglobulins and C3b component of complement (2-4).

To obtain a convenient model system of the innate immune cell response to various infectious agents THP-1 cells are differentiated into monocytes or macrophages. During differentiation both physiological agents are used, as vitamin D3 (VD3) and retinoic acid (RA), and nonphysiological agents, as LPS and PMA (5, 6).

LPS as non-physiological agents can induce differentiation of leukemia cells by themselves and also reinforce the differentiating activity of some physiological agents (7, 8). The toxic LPS *E. coli* are shown to reinforce the CD14 and CD11b receptors expression on THP-1 cells differentiated by vitamin D3 (9).

The differentiation is characterized by a change in various cell parameters such as cell morphology, TLR4, CD11b, and CD14 receptors surface expression (4, 10), as well change in the functional activity under various stimuli including LPS (11) compared to the undifferentiated phenotype.

The CD14 was described initially as a marker of monocytes differentiation. CD14 is expressed on 10-15% of the THP-1 cells with activation of expression during the differentiation (12). Thus the increase of CD14 expression on a cell surface is a good marker for cell maturation (13).

CR3 (β_2-integrin, CD11b) is another classical marker of monocytic differentiation. The CD11b/CD18 is expressed in mature myeloid cells and is widely used as an early marker of monocyte differentiation (14). This complex is expressed predominantly on macrophages as a marker of differentiation and provides adhesion (15-17).

Macrophages can be distinguished from monocytes directly in culture by the following strict criteria: change of the cell shape, increase of the cytoplasm volume, and adhesion cell capacity (18).

The populations of macrophages and monocytes are heterogeneous. Based on the primary monocyte markers (CD14 and CD16 receptors) expression the monocytes are divided into classical, inflammatory, and non-classical subpopulations. The classical monocytes with $CD14^{+}CD16^{-}$ phenotype form about 90% of all blood monocytes and are phagocytes (19, 20). Their phagocytic activity is higher than that of macrophages (18). Inflammatory monocytes ($CD14^{++}CD16^{+}$) have a low phagocytic activity but are highly active concerning IL-1β and TNF-α induction in response to LPS (21). Non-classical monocytes ($CD14^{+}CD16^{++}$) synthesize IL-1β and TNF-α in response to DNA and RNA and participate in the development of autoimmune diseases such as rheumatoid arthritis (21). These monocytes express a large number of MHC II and CD32 molecules and are closest to mature tissue macrophages (19, 22). In blood it is difficult to distinguish these monocyte subpopulations, because the development of inflammation is accompanied by a consistent differentiation of classical monocytes to the non-classical subpopulation (23). During the differentiation of monocytes to macrophages the expression of the primary monocyte CD14 marker is decreased. Thus the phagocytic activity seems to lower during the differentiation of monocytes from classical to the non-classical subpopulation.

The discovery of the heterogeneity of the monocyte and macrophage populations caused new studies of the structure and functions of these cells (20, 24-27). However, the literature search showed no data on the subtypes of monocytes obtained from the D3-differentiated THP-1 cells.

An essential feature of the cell differentiation stage is the CD11b/CD14 receptors ratio. The dominance of one of these receptors can indicate the direction of THP-1 cell differentiation. One of the criteria of phagocyte physiological activity is their ability for phagocytosis. The preliminary results of the work are published in the Proceedings of the Conference (28) and patent application (29).

This was aimed to evaluate the ability of physiological (VD3 and RA) and non-physiological (various LPS) agents and their combination to influence the direction of THP-1 cell differentiation. The differentiation stage was evaluated by the expression and the ratio of surface receptors (TLR4, CD11b, and CD14) as well as by the cell phagocytic activity under various differentiating agents.

MATERIALS AND METHODS

Materials

Retinoic acid, 1α,25-dihydroxyvitamin D3, PBS, endotoxin-free cell culture medium RPMI-1640, supplemented with 25 mM HEPES, $NaHCO_3$, L-glutamin-penicillin-streptomycin solution was obtained from Sigma (USA). FBS Standard was obtained from Hyclone (USA). Cell Staining Buffer, Human TruStain FcX™, RBS Lysis buffer was obtained from Biolegend (USA). BODIPY FL conjugate *E. coli* (K-12 strain) BioParticles, *E. coli* BioParticles opsonizing reagent, Trypan blue in citrate-balanced salt solution, pH=4.4 was obtained from Invitrogen (USA).

Toxic LPS *Escherichia coli* 055:B5, *Salmonella enterica* serotype Typhimurium was obtained from Sigma (USA). The non-toxic LPS was isolated from the phototrophic bacterium of the strain *Rhodobacter capsulatus* PG deposited in VKM IBPM RAS № B-2381D (RF) by phenolic extraction as described previously (30).

Alexa Fluor 488-labeled anti-human CD284 (TLR4) Clone HTA125 (eBioscience, USA), anti-human CD14 Clone HCD14, Anti-Human CD11b Clone ICRF44 (BioLegend, USA) monoclonal antibodies (mAb) and isotype matched mouse IgG2a k isotype Crtl (FC) Clone MOPC-173, IgG1 k isotype Crtl (FC) Clone MOPC-21 (BioLegend, USA) controls were used.

THP-1 cell cultivation and differentiation

THP-1 cell line from the ATCC®TIB™202 collection (USA) were cultivated in RPMI 1640 media, containing 2 mmol/l of L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% inactivated FBS in CO_2-incubator (Jouan, France) at 37°C and in humidified air containing 5% (v/v) CO_2. The cells viability determined by the trypan blue staining averaged 94%.

The THP-1 cells (10^6 cells/ml) in RPMI 1640 media containing antibiotics and 10% serum were differentiated by either 10^{-7} M VD3, 10^{-7} M RA, or combination of these two differentiation-stimulating agents of the same concentrations; 500 ng/ml LPS *E. coli*, *S. enterica*, or *R. capsulatus* PG for 72 h at 37°C and in 5% (v/v) CO_2. To study the combined VD3 and LPS action on cell differentiation the THP-1 cells pre-differentiated by VD3 for 72 h were differentiated additionally for 24 h by LPS *S. enterica* or *R. capsulatus* PG. The cells viability averaged 92%.

Expression of TLR4, CD11b, and CD14 receptor on the THP-1 cells and whole blood monocyte

After incubation with the corresponding differentiating agents THP-1 cells were separated from the cultivation media by centrifugation and resuspension in Cell Staining Buffer (10^6 cells/100 μl). To eliminate the non-specific linking of antibodies to the studied receptors Human TruStain FcX buffer was added to each sample and incubated for 10 min at room temperature and then the corresponding mAb (5 μl) was added and incubated in the dark for 30 min at 4°C. The cells were washed twice with Cell Staining Buffer and resuspended in 400 μl of the same buffer.

The blood from healthy volunteers (mean age 25±2 years) of both sexes was collected into tubes (BD, UK) with heparin sodium (17 U/ml) in clinical conditions. Written informed consent was provided by each volunteer. Blood samples (100 μl) were incubated with 5 μl of the corresponding mAb in the dark for 30 min at 4°C. Then erythrocytes were lysed by RBS Lysis buffer and remaining leucocytes were washed twice with Cell Staining Buffer and resuspended in 400 μl of the same buffer.

The receptor expression of the THP-1 cells and monocytes was measured as mean fluorescence intensity (MnI) cells were gated on the basis of FS-SS using EPICS XL-MCL Flow cytometer (Beckman Coulter, United States). At least 6000 events were collected for each experimental variant.

Phagocytic activity of the THP-1 cells

BODIPY FL conjugate *E. coli* (K-12 strain) BioParticles (6×10^9 cells/ml PBS) are used to determine the phagocytic activity of THP-1 cells of various differentiation degrees. The bacteria were opsonized for 1 h at 37°C by opsonizing reagent and added to THP-1 cells of different differentiation degree with the cell:bacteria ratio equal to 1:6. The samples were incubated for 30 min at 37°C. The external fluorescence was extinguished by cold trypan blue solution washed twice by cold Cell Staining Buffer and resuspended in 400 μl of the same buffer.

The phagocytic activity of the cells was measured as mean fluorescence intensity (MnI) of THP-1 cells were gated on the basis of FS-SS using EPICS XL-MCL Flow cytometer (Beckman Coulter, United States). At least 6000 events were collected for each experimental variant.

Statistical analysis

The results are presented as averages with standard errors. The significance of the differences between the sample mean values was assessed by paired Student t-test. The Flowing Software, Microsoft office Excel 2010 (Attestat plug-in), and OriginPro 7.5 were used for statistical analysis and graphical presentation of the data.

RESULTS

Expression of TLR4, CD11b, and CD14 receptors on THP-1 cells.

Results of the influence of physiological (VD3 and RA) and non-physiological (various LPS) agents on TLR4, CD11b, and CD14 expression on THP-1 cells are presented as relative units compared to receptor fluorescence (MnI) of the control undifferentiated THP-1 cells taken as 1 and in Figures 1 and 2.

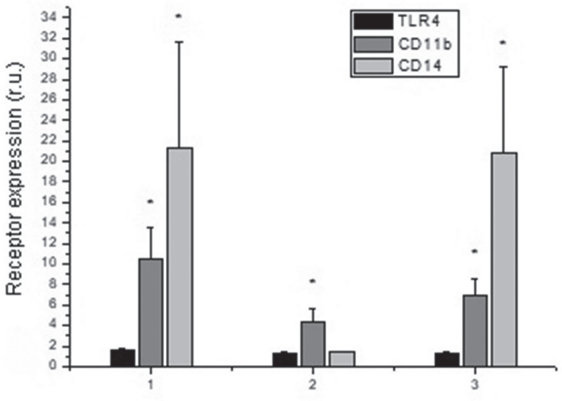

Figure 1. Change in the TLR4, CD11b, and CD14 receptors expression on the surface of THP-1 cells differentiated by 1. VD3, 2. RA, 3. VD3 and RA (n=6). *P<0.05 vs undifferentiated cells.

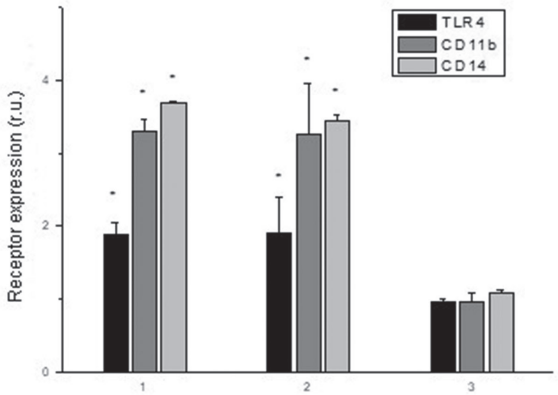

Figure 2. Change in the TLR4, CD11b, and CD14 receptors expression on the surface of THP-1 cells differentiated by 1. LPS *E. coli*, 2. LPS *S. enterica*, 3. LPS *R. capsulatus* PG (n=6). *P<0.05 vs undifferentiated cells.

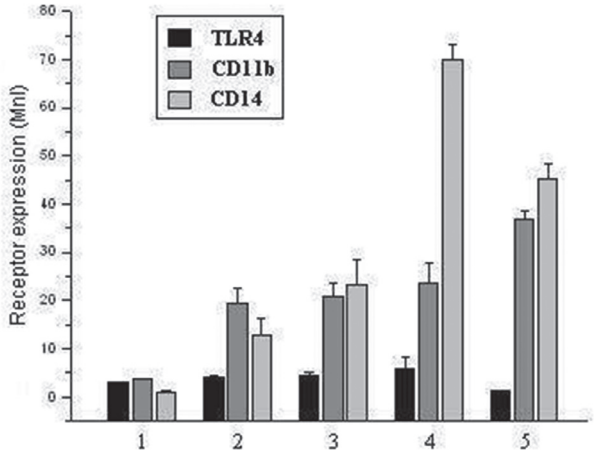

Figure 3. Figure 3. TLR4, CD11b, and CD14 receptor expression on THP-1 cells 1. undifferentiated, 2. VD3-differentiated, 3. VD3- and *R. capsulatus* LPS-differentiated, 4. VD3- and *S. enterica* LPS-differentiated, 5. human blood monocytes (n=6).

We have founded that VD3 and RA had virtually no influence on the TLR4 expression on the THP-1 cells but significantly activated the CD11b expression (in 10 and 4 times, respectively) (Fig. 1).

The combined use of VD3 and RA caused a decrease in CD11b expression compared to the VD3. A notable change in the CD14 expression was observed under the VD3 action. A weaker influence of RA compared to VD3 on the studied receptor expression can be explained by the retinoids action on the myeloid cell lines with the induction of mostly granulocyte formation. The results show that VD3 is the best of the THP-1-differentiating physiological agents since it caused the highest expression of receptors marking the differentiation into monocytes and macrophages.

Furthermore, we studied the influence of toxic LPS *E. coli* and *S. enterica* and of non-toxic LPS *R. capsulatus* PG on TLR4, CD11b, and CD14 receptor expression (Fig. 2). The figure shows that *E. coli* and *S. enterica* endotoxins induced the similar changes in the expression of the studied receptors on the cell surface: the TLR4 amount increased approximately twice and the CD11b and CD14 amount is increased 3-4 times as compared to that in undifferentiated THP-1 cells. Non-toxic LPS *R. capsulatus* PG did not show a significant influence on the expression of the receptors.

In this study we examined the influence of endotoxin *S. enterica* and the non-toxic LPS *R. capsulatus* PG on chang-

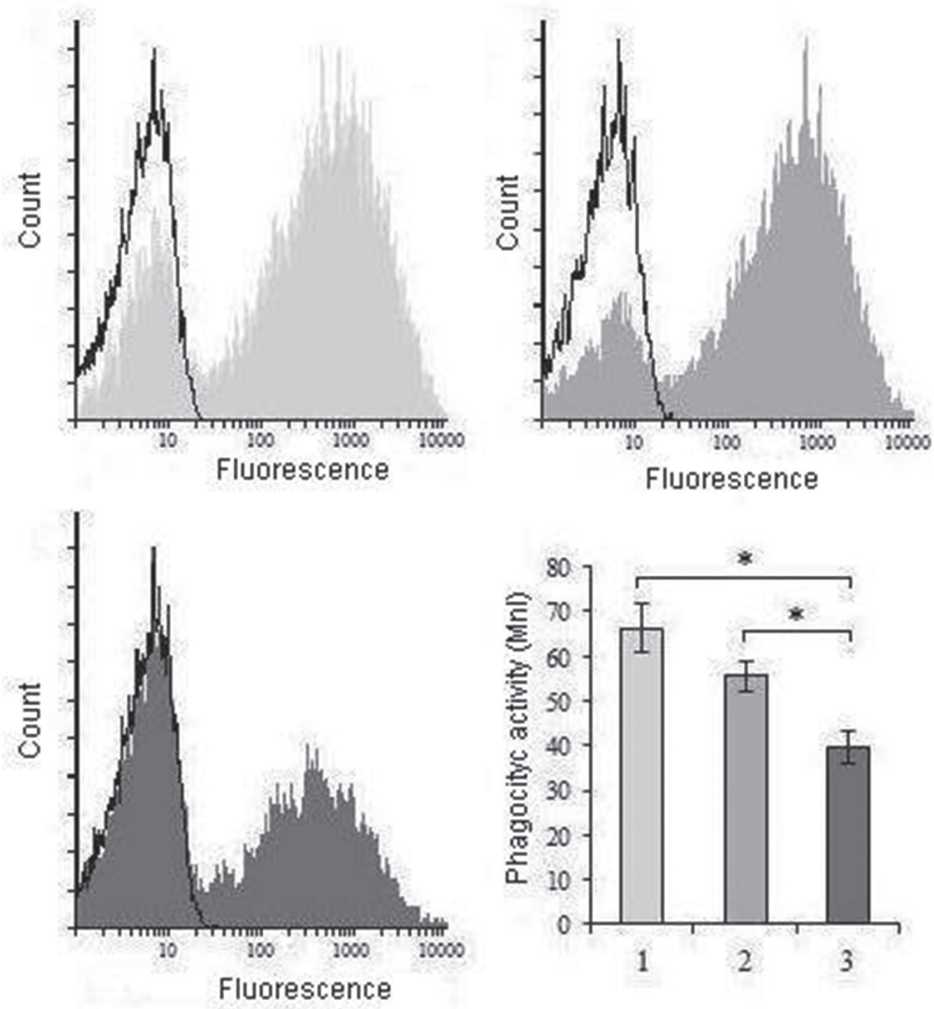

Figure 4. Phagocytic activity of THP-1 cells *a)* undifferentiated, *b)* VD3-differentiated, *c)* VD3- and *R. capsulatus* LPS-differentiated, *d)* average values of the phagocytic activity (n=6). 1. undifferentiated, 2. VD3-differentiated, 3. VD3- and *R. capsulatus* LPS-differentiated. Unshaded area – isotypic control; shaded area – fluorescence of phagocytic cells. *$P<0.05$ between experimental variants.

ing of surface receptor level of the pre-differentiated THP-1 cells by VD3 (Fig. 3). The results show that the THP-1 cells are differentiated by VD3 with the predominance of CD11b receptors expression. Against the backdrop of VD3 both the toxic LPS *S. enterica* and the non-toxic LPS *R. capsulatus* PG changed the ratio of receptor expression to increasing the CD14 level. Concerning the number and ratio of the receptors none of the differentiation agent versions used was sufficient to cause a complete differentiation of THP-1 cells in normal monocytes.

To assess the direction of THP-1 cell differentiation by various agents their CD11b/CD14 receptor ratio was compared with that in the human whole blood monocytes. The decrease in the CD11b/CD14 receptor ratio indicates the direction of THP-1 cells differentiation into monocytes. Table 1 shows that the receptors ratio closest to that on the human whole blood monocytes (0.81 ± 0.05) was observed under the combined action of VD3 and LPS *R. capsulatus* PG on THP-1 cells (0.89 ± 0.33).

Phagocytic activity of THP-1 cells

Phagocytic activity *of THP-1 cells* was determined by absorption of BODIPY FL conjugate *E. coli.* THP-1 cells differentiated by VD3 and additionally differentiated by LPS *R. capsulatus* PG were compared to control undifferentiated cells using the Flow cytometry. The results of cell phagocytic activity measured by the average intensity of fluorescence (MnI) of the cells are presented in Fig. 4 and the data show that LPS *R. capsulatus* PG reduce the phagocytic activity of THP-1 cells previously differentiated by VD3.

DISCUSSION

The promonocyte cells of the THP-1 line are widely used as a model system for the study of monocytic line cells (monocytes and macrophages) responses to various stimuli. To obtain the cells closest to the native ones THP-1 cells are differentiated using various agents and investigated by Flow cytometry using the surface labeling (18, 27). The most commonly used agents are $1,25(OH)_2D3$ and PMA. The study of THP-1 cell differentiation based on morphology, adhesion and phagocytosis ability, loss of proliferation, and expression of CD11b and CD14 showed that PMA stimulates THP-1 differentiation into macrophage-like cells and VD3 causes differentiation into monocytic cell type (5, 13, 18). However the question remains whether the differentiated THP-1 cells are close to the native monocytes and macrophages.

The receptors we investigated play the principal role in the cellular immune responses on endotoxins and are the primary markers of differentiation. TLR4 is identified as a signal molecule essential to the LPS recognition (32). CD14 is considered as receptor transporting LPS to the TLR4/MD-2 complex (33, 34). The TLR-stimulated active CD11b integrin is shown to be involved in MyD88 and TRIF signaling pathways with subsequent inhibition of TLR-signaling in the innate immune responses (35). LPS activate a signal transduction from the TLR4-MD2/CD14/CD11b receptor complex through some kinases to the nuclear factor NF-κB (36). NF-kB-dependent transcription response of differentiated THP-1 cells to LPS is similar to that of the human mononuclear cells (37). The $1,25(OH)_2D3$ is known to modulate the responses of the human monocyte line cells through the NF-kB-dependent activation of the anti-inflammatory target genes (9).

As promonocytes THP-1 cells can differentiate to monocytes (with increased CD14 expression) and macrophages (with increased CD11b expression). Each of the differentiation stages: promonocyte → monocyte → macrophage is characterized by a change in the TLR4, CD14, and CD11b receptor ratios. To determine the THP-1 cells maturity and differentiation degree we assessed the CD11b/CD14 receptor ratio on the cell surface.

The results show that under the VD3 action both the non-toxic LPS *R. capsulatus* PG and the toxic LPS *S. enterica* changed the CD11b/CD14 receptor ratio to increase the CD14 level (Fig. 3, Table 1) which confirms the literature data (9). The non-toxic LPS *R. capsulatus* PG has a weak differentiating capacity but increases the pro-differentiating activity of VD3 in monocyte-like cells. Neither endotoxins nor VD3 nor a combination of the agents cause a complete differentiation of THP-1 cells in native monocytes (Fig. 3). The receptor ratio closest to those in monocytes is observed after the combined action of VD3 and LPS *R. capsulatus* PG (Table 1).

Expression of CD16 is absent on the undifferentiated THP-1 cells (38). The change in the CD11b and CD14 receptor expression on VD3-differentiated THP-1 cells as well as the phagocytic activity allowed us to discover a significant increase in the CD14 level (Fig. 1) and a slight decrease in phagocytic activity (Fig. 4) possibly indicating the formation of the classical monocytes of $CD14^{+}CD16^{-}$ phenotype.

The THP-1 cells differentiation by combination of VD3 and non-toxic LPS *R. capsulatus* PG caused a significant decrease in phagocytic activity and a further increase of CD14 level on the THP-1 cells. Activation of CD14 expression simultaneously with reduction the phagocytic activity of the THP-1 cells differentiated by the combination of VD3 and LPS *R. capsulatus* PG suggests the formation of a phenotype of intermediate or inflammatory $CD14^{++}CD16^{+}$ monocytes involved in response to bacteria and viruses.

It is known that neither CD11b nor CD14 are NF-κB target genes (39). However our findings show that the VD3 and LPS increase the expression of these receptors on the surface of THP-1 cells. This conclusion is also confirmed by the literature data on the mRNA pool increase correlating quantitatively with the CD14 and CD11b protein expression in response to these agents (9, 40). It is possible that the expression of these receptors is regulated through other nuclear factors and alternative receptors to LPS (5). Studies of recent years have shown that LPS in addition to the NF-kB activation also

Table 1. The CD11b/CD14 receptors ratio on the THP-1 cells and human whole blood monocytes (n=6)

Options	CD11b/CD14 ratio
Undifferentiated THP-1 cells	3.42±0.56
THP-1+LPS *R. capsulatus*	4.03±0.38
THP-1+VD3	1.51±0.55
THP-1+VD3+LPS *R. capsulatus*	0.89±0.33
Human monocytes	0.81±0.05

induce activation of PU.1 (41), which is considered to be the central regulator of TLR4 expression (42, 43). PU.1 is also an essential modulator of signals transduction from VDR in the monocytic line cells (44). VD3 induces the expression of the CD11b receptors through the activation of the PU.1/C-Jun transcription factors and the CD14 receptors expression through CEBP/Sp-1 (45). An additional differentiating action of LPS *R. capsulatus* PG on the VD3 background can be due to an increased synthesis of PU.1 which is the principal transcription factor of monopoiesis.

Conclusion

Summarizing we can conclude that VD3 induces the THP-1 cells differentiation with the formation of classical monocytes and the sequence of 1α, 25-dihydroxyvitamin D3 and non-toxic LPS *R. capsulatus* PG causes the THP-1 cells differentiation with the formation of inflammatory or intermediate monocytes.

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