

# Induction of tryptophan 2,3-dioxygenase expression in human monocytic leukemia/lymphoma cell lines THP-1 and U937

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**ABSTRACT:** Tumor-associated macrophages are immune cells with diverse functions in tumor development. Among other functions, they downregulate immune-mediated tumor rejection by depriving lymphocytes of nutrients. The essential amino acid tryptophan is metabolized by the enzymes indoleamine 2,3-dioxygenase 1 and tryptophan 2,3-dioxygenase (TDO). Indoleamine 2,3-dioxygenase 1 is expressed in a large number of human tumors, and inhibitors are in development to improve immunotherapy. Tryptophan 2,3-dioxygenase was also found in human tumors and preclinical working models confirmed its immunosuppressive power. We explored a potential expression of TDO by macrophages. This enzyme could be induced in two human cell lines, THP-1 and U937, by incubation with phorbol myristate acetate, lipopolysaccharide, and interferon gamma. Phorbol-myristate-acetate-mediated induction was inhibited by rottlerin, a protein kinase C inhibitor. In contrast to these monocytic cell lines, other cell lines or fresh human monocytes isolated from peripheral blood mononuclear cells and differentiated into proinflammatory or anti-inflammatory macrophages could not be induced to express TDO. Our results suggest that TDO might play an immunosuppressive role in human monocytic leukemias but not in untransformed macrophages.

**KEYWORDS:** Tryptophan 2,3-dioxygenase, PMA, PKC, monocyte, IFN-gamma, LPS

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## Introduction

Macrophages represent an important cell type implicated in all stages of tumor development. During tumor initiation, macrophages create a proinflammatory environment which causes tissue and DNA damage. In established tumors, macrophages stimulate tissue remodeling, angiogenesis, inhibit immune-mediated tumor rejection, and finally, favor metastases formation.<sup>1–3</sup>

One particular immunosuppressive mechanism of macrophages is nutrient starvation. Immunosuppressive macrophages express the enzyme arginase 1 which impairs T-cell function and proliferation by decreasing arginine and increasing urea concentrations in the tumor microenvironment.<sup>4,5</sup> Another example of nutrient starvation is the expression of the enzyme indoleamine 2,3-dioxygenase 1 (IDO1), catalyzing the first and rate-limiting step of tryptophan degradation. Indoleamine 2,3-dioxygenase 1 impacts lymphocyte proliferation and activity in a 2-fold way by nutrient deprivation and metabolite production and favors their differentiation to a regulatory T-cell phenotype.<sup>6–9</sup> In normal human tissues, IDO1 expression is mainly restricted to the placenta, where it is believed to favor feto-maternal tolerance<sup>10</sup> and to mature dendritic cells. In addition, IDO1 is induced by IFN $\gamma$  during inflammation<sup>11,12</sup> as a mechanism of retro-control of immune responses. Many human cancers also express IDO1 in order to escape immune-mediated tumor rejection.<sup>13,14</sup> Indoleamine

2,3-dioxygenase 1 expression is induced by IFN $\gamma$  in tumor cells and stromal cells of inflamed tumors, as an adaptive resistance mechanism.<sup>15</sup> Tumor cells may also express IDO1 in a constitutive manner, thereby mediating intrinsic resistance. Constitutive IDO1 expression is triggered in tumor cells by constitutively active COX2, producing prostaglandin which acts in an autocrine manner.<sup>16</sup> Those discoveries led to the synthesis of several small molecule inhibitors which are currently in clinical development.<sup>17</sup>

The enzyme tryptophan 2,3-dioxygenase (protein TDO encoded by gene *TDO2*), exerting the same enzymatic activity as IDO1, is expressed in the liver to regulate systemic tryptophan concentrations, in the murine placenta where it might contribute to feto-maternal tolerance and in tumors where it downregulates immune-mediated tumor rejection.<sup>8,18–21</sup> Enzymatically active TDO is also constitutively expressed by several human tumor cell lines like glioblastoma, colorectal, head and neck, and lung and gall-bladder carcinoma cell lines.<sup>18</sup> In addition, microarray data showed that *TDO2* mRNA was upregulated in the tumor-derived monocytic cell line THP-1 by differentiating the cells into a macrophage-like phenotype using phorbol 12-myristate 13-acetate (PMA).<sup>22</sup> This observation opens up the possibility that TDO might be induced in macrophages in tumors or other pathologies. In this report, we characterize the expression and enzymatic activity of TDO induced by PMA in two monocytic leukemia/lymphoma cell



lines, THP-1 and U937, by lipopolysaccharide (LPS) alone in THP-1 cells and by a combination of LPS and interferon gamma (IFN $\gamma$ ) in U937 cells. We also highlight the lack of TDO induction in human monocytes freshly isolated from peripheral blood mononuclear cells (PBMCs).

## Materials and Methods

### Compounds

PMA was purchased from Sigma (#P8139). Recombinant human IFN $\gamma$  was purchased from BD Biosciences (#554617). Lipopolysaccharide *Escherichia coli* O111:B4 was purchased from Sigma (#L2630). Rottlerin was purchased from Sigma (#R5648). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Sanofi (Leukine sargramostim). Recombinant human macrophage colony-stimulating factor 1 (M-CSF) was purchased from GenScript (#Z02001). Recombinant human interleukin-4 (IL-4) was produced at the Ludwig Cancer Research (Brussels, Belgium). Recombinant human IL-10 was purchased from R&D (#1064-IL). Ribomunyl was purchased from Pierre Fabre (France). Prostaglandin E2 (PGE2) was purchased from Sigma. Recombinant human tumor necrosis factor alpha (TNF $\alpha$ ) was purchased from PeproTech (#300-01A). Recombinant human transforming growth factor beta (TGF $\beta$ ) was purchased from R&D (#240-B).

### Cancer cell lines and in vitro induction of TDO

THP-1 and U937 cell lines were purchased from ATCC. Cells were plated in complete culture medium:  $0.016 \times 10^6$  cells in 96-well plates for the viability assay,  $0.2 \times 10^6$  cells in 12-well plates for real-time quantitative polymerase chain reaction (RT-qPCR; except from Figure 2:  $0.2\text{--}1 \times 10^6$  cells) and  $1.2 \times 10^6$  cells in 60-mm dishes for western blot. PMA (0.5–25 ng/mL), IFN $\gamma$  (50 ng/mL), LPS (1  $\mu$ g/mL), and rottlerin (5 nM) were added and cells were incubated for 48 hours at 37°C, 8% CO $_2$ .

### Isolation of human monocytes and in vitro induction of TDO

Peripheral blood mononuclear cells were isolated from human blood by lymphoprep centrifugation. Monocytes were enriched by negative selection using EasySep Human Monocyte Enrichment Kit without CD16 Depletion (Stemcell, #19058). Five million cells were plated in complete culture medium. PMA (1–20 ng/mL), IFN $\gamma$  (5 ng/mL), LPS (100 ng/mL), GM-CSF (100 ng/mL), M-CSF (100 ng/mL), IL-4 (20 ng/mL), IL-10 (10 ng/mL), ribomunyl (1  $\mu$ g/mL), PGE2 (500 ng/mL), TNF $\alpha$  (10 ng/mL), and TGF $\beta$  (4 ng/mL) were added, and cells were incubated for indicated durations at 37°C, 8% CO $_2$ .

### Western blot

The cells were lysed in Pierce Ripa buffer (Thermo Scientific, #89901) with Halt Protease and Phosphatase inhibitor Cocktail (Thermo Scientific, #78446); genomic DNA was then lysed by 15 seconds of sonication; the lysates were shaken for 30 minutes at 4°C, centrifuged for 10 minutes at 20000g; and the protein concentration of the supernatant was determined by Pierce Protein BCA Assay Kit (Thermo Scientific, #23225). The proteins were heated at 95°C for 5 minutes with a homemade loading buffer 6x (SDS 12%w/v, bromophenol blue 0.06%w/v, glycerol 47%, Tris 0.5 M pH 6.8, DTT 9.3% w/v). Twenty micrograms of proteins were loaded on NuPAGE Bis Tris 4% to 12% gels (Invitrogen #WG1402BOX) and separated by gel electrophoresis using MOPS running buffer (NuPAGE, #NP0001). Dry transfer was performed by iBlot (Thermo Scientific) using iBlot Gel Transfer Stacks, nitrocellulose, regular size (Thermo Scientific, #IB301001) and a transfer program of 7 minutes (1 minute at 20 V, 4 minutes at 23 V, and 2 minutes at 25 V). After blocking in phosphate-buffered saline (PBS) with 5% dry milk and 0.1% Tween 20 for 1 hour at room temperature, the membranes were incubated with the primary antibodies diluted in blocking buffer: mouse anti-TDO clone III at 1  $\mu$ g/mL (validated previously),<sup>23</sup> mouse anti-Vinculin clone hVIN-1 at 1:10000 (Sigma, #V9131) for 2 hours at room temperature or homemade mouse anti-IDO1 clone 4.16H1 at 2  $\mu$ g/mL<sup>14</sup> overnight at 4°C. After washing in PBS with 0.1% Tween 20, the membranes were incubated with horseradish peroxidase (HRP)-linked goat antimouse IgG antibody (BioLegend, #405306) at 1:2500 in blocking buffer for 1 hour at room temperature and washed again with PBS-Tween 20. The HRP was revealed using SuperSignal West Pico (Thermo Scientific, #1859674) and detected with Fusion Solo S (Vilber Lourmat). The intensity of the bands was quantified using Bio-1D software.

### Quantitative RT-PCR

RNA was extracted with NucleoSpin RNA (Macherey Nagel, #740955) according to the manufacturer's instructions. The RNA was quantified with a NanoDrop 2000c spectrophotometer (Thermo Scientific) and a defined amount of RNA was then retro-transcribed by the RevertAid RT Kit (Thermo Scientific, #K1691) according to the manufacturer's instructions. Quantitative polymerase chain reaction was performed with Takyon ROX Probe 2X MasterMix dTTP blue (Eurogentec, #UF-RPMT-B0701) in a StepOnePlus thermal cycler (Applied Biosystems) using the following program for *TDO2* and *IDO1*: 3 minutes at 95°C, then 40 cycles of 10 seconds at 95°C, and 1 minute at 60°C; for *EF1*: 3 minutes at 95°C, then 40 cycles of 3 seconds at 95°C, and 30 seconds at 60°C. The following primers were used for *TDO2*: CATGGCTGGAAAGAACTC (forward), CTGAAGTG

CTCTGTATGAC (reverse), TTTAGAGCCACATGG ATTTAACTTCTGGG (probe); for *IDO1*: GGTCAT GGAGATGTCCGTAA (forward), ACCAATAGAGA GACCAGGAAGAA (reverse), CTGTTCTTACTGCC AACTCTCCAAGAACTG (probe); for *EF1*: GCTTC ACTGCTCAGGTGAT (forward), GCCGTGTGGCA ATCCAAT (reverse), AAATAAGCGCCGGCTATGCCC CTG (probe). The probes were coupled to 5' FAM and 3' TAMRA. Standard curves were added for each gene.

### *Tryptophan and kynurenine quantification*

The cell culture supernatants were harvested and analyzed by high-performance liquid chromatography (HPLC) as previously described.<sup>18</sup>

### *Viability test*

Nineteen microliters of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) and 1  $\mu$ L of phenazine methosulfate (PMS) were added to the cells (100  $\mu$ L) which were then incubated for 3 hours and 30 minutes at 37°C. The absorbance was measured with a GloMax spectrophotometer (Promega) at 490 nm with reference at 600 nm.

## Results

### *TDO expression is induced by PMA in human monocytic cell lines*

To confirm that TDO is induced in the monocytic cell line THP-1 by PMA-mediated differentiation,<sup>22</sup> we treated two monocytic leukemia/lymphoma cell lines, THP-1 and U937, with increasing concentrations of PMA. As expected, both cell lines became adherent upon treatment with PMA and increased the expression of CD11c, CD14, TNF $\alpha$ , and *IL-1 $\beta$*  (Supplementary Figure 1).<sup>24–28</sup> We observed that *TDO2* mRNA and metabolically active protein were induced in a dose-dependent manner (Figure 1A to C). The viability of THP-1 cells, analyzed by the colorimetric MTS-PMS assay, was not affected, but U937 cells showed 50% of mortality upon differentiation with PMA (Figure 1D). Concerned about this high mortality rate, we analyzed apoptosis by propidium iodide (PI) and annexin V flow cytometry and obtained about 70% of double-negative, living cells (Supplementary Figure 2A). This discrepancy might be explained by reduced cell proliferation upon PMA treatment. The MTS-PMS assay measures the activity of dehydrogenase enzymes in metabolically active cells. As PMA-differentiated cells stop proliferating (Supplementary Figure 2B), the conversion rate of MTS is reduced compared to untreated cells. We treated other cell lines, like the human myeloblast cell line HL60, murine macrophage cell lines (J774, RAW 264.7, MF4/4) and human hepatocarcinoma cell lines (Huh-7 and Hep-G2), with PMA, but they did not induce TDO expression (data not shown).

### *Induction of TDO by PMA depends on cell density*

It is known that the cell density during culture can change the impact of PMA on the cell phenotype and cytokine expression of THP-1 cells.<sup>29</sup> We confirmed that the induction of TDO also depends on cell density because the induction of TDO decreased dramatically when the cell density of THP-1 and U937 cells increased (Figure 2).

### *The induction of TDO by PMA is PKC-dependent*

We next wondered which signaling pathway leads to the induction of TDO in those monocytic cell lines. As the protein kinase C (PKC) is a known target of PMA, we treated the cells with rottlerin, a PKC inhibitor which primarily inhibits PKC $\delta$ . When the cells were treated simultaneously with PMA and rottlerin, the induction of TDO was efficiently inhibited (Figure 3A to C). Although the viability of THP-1 cells was only mildly affected, the reduced expression of TDO in U937 could be biased by a high percentage of cell death upon rottlerin treatment (Figure 3D).

### *TDO expression is induced by LPS and IFN $\gamma$*

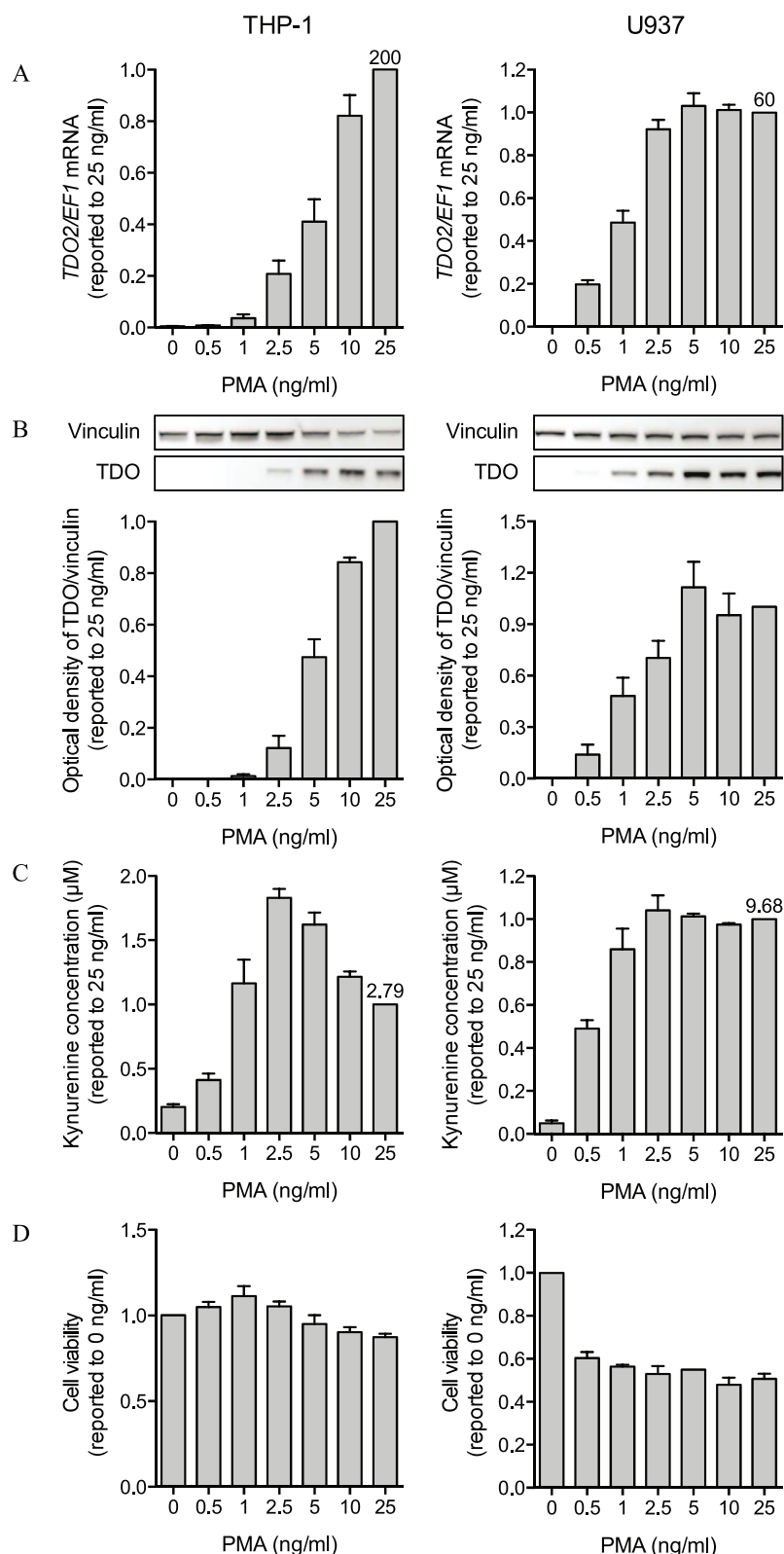
Beside PMA, monocytic cell lines can be differentiated and stimulated by LPS and IFN $\gamma$ . We observed that LPS induced TDO in THP-1 cells, whose expression was further increased by IFN $\gamma$ , whereas IFN $\gamma$  alone had little impact on TDO expression (Figure 4A to C). U937 cells needed the combined treatment of IFN $\gamma$  and LPS to strongly induce TDO, compared to their small response to LPS alone. As IFN $\gamma$  and LPS also induced *IDO1* (Figure 5), kynurenine was probably partially produced by *IDO1* and not exclusively by TDO in these conditions. THP-1 cells tolerated well the treatment with LPS and IFN $\gamma$ , whereas the viability of U937 cells was reduced to 65% (Figure 4D).

### *Treatments with other substrates*

It was previously reported that the expression of TDO increased in liver cells upon dexamethasone treatment, a synthetic glucocorticoid,<sup>30,31</sup> as well as in uterine stromal cells upon decidualization with progesterone and estrogen.<sup>32</sup> We treated THP-1 and U937 cells with dexamethasone, progesterone, and estrogen. None of those substrates induced TDO in these monocytic cell lines (data not shown).

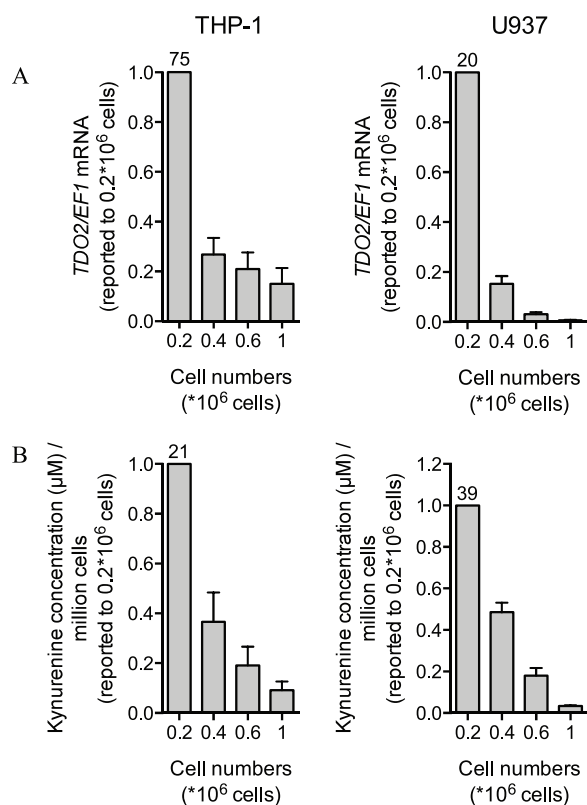
### *TDO is not expressed by normal monocytes and macrophages*

We finally studied the induction of TDO in normal monocytes or macrophages. If confirmed, this would support the hypothesis that TDO can represent an immunosuppressive mechanism exerted by macrophage infiltration in tumors and other pathologies. The differentiation of freshly isolated monocytes into a macrophage-like phenotype and their subsequent



**Figure 1.** Induction of TDO by PMA. THP-1 and U937 cells were treated for 2 days with PMA ranging from 0.5 to 25 ng/mL and *TDO2* mRNA, TDO protein, kynurenine production, and cell viability were analyzed in parallel. (A) *TDO2* mRNA was quantified by RT-qPCR. The graphs show the mean  $\pm$  SEM (reported to 25 ng/mL PMA) of three independent experiments. The mean value (*TDO2* transcripts per 2000 *EF1* transcripts) of the reference condition is indicated on top of the relevant bar. (B) 30 minutes before harvesting the cells, 300  $\mu$ M of tryptophan was added to the cell culture to stabilize the TDO protein. Tryptophan 2,3-dioxygenase and vinculin (loading control) were revealed by western blot and quantified. The graphs show the mean  $\pm$  SEM (reported to 25 ng/mL PMA) of three independent experiments. (C) The metabolite kynurenine was quantified in the supernatants by HPLC. The graphs show the mean  $\pm$  SEM (reported to 25 ng/mL PMA) of three independent experiments. The mean value ( $\mu$ M) of the reference condition is indicated on top of the relevant bar. (D) Cell viability was measured by an MTS-PMS assay. The graphs show the mean optical density  $\pm$  SEM (reported to the untreated condition) of three independent experiments. HPLC indicates high-performance liquid chromatography; mRNA, messenger ribonucleic acid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; PMA, phorbol 12-myristate 13-acetate; PMS, phenazine methosulfate; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean; TDO, tryptophan 2,3-dioxygenase.





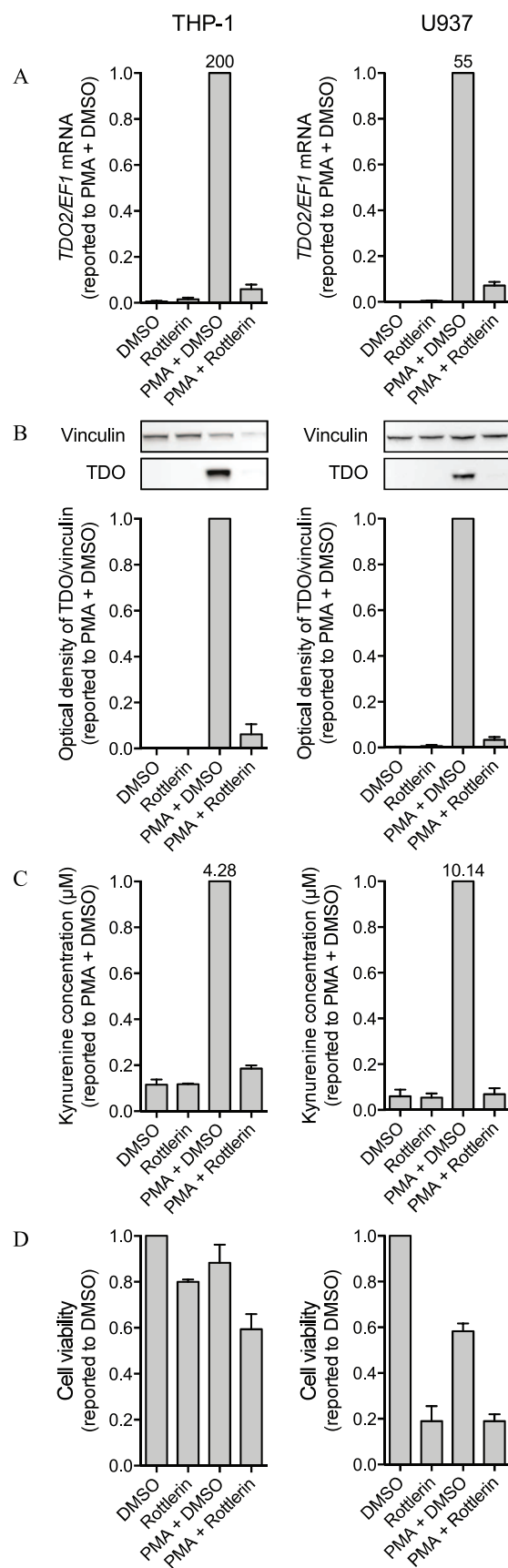
**Figure 2.** Impact of the cell density on the induction of TDO. THP-1 and U937 cells were plated in 12-well plates ranging from 0.2 to 1 × 10<sup>6</sup> cells per well and were treated for 2 days with 1 ng/mL of PMA. *TDO2* mRNA and kynurenine production were analyzed in parallel. (A) *TDO2* mRNA was quantified by RT-qPCR. The graphs show the mean ± SEM (reported to 0.2 × 10<sup>6</sup> cells) of three independent experiments. The mean value (*TDO2* transcripts per 2000 *EF1* transcripts) of the reference condition is indicated on top of the relevant bar. (B) The metabolite kynurenine was quantified in the supernatants by HPLC. The graphs show the kynurenine concentration divided by the corresponding cell number ± SEM (reported to 0.2 × 10<sup>6</sup> cells) of three independent experiments. The mean value of the reference condition is indicated on top of the relevant bar. HPLC indicates high-performance liquid chromatography; PMA, phorbol 12-myristate 13-acetate; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean; TDO, tryptophan 2,3-dioxygenase.

exposure to proinflammatory or anti-inflammatory cytokines and chemokines did not induce TDO expression (Table 1 and Supplementary Figure 3).

Taken together, we found that TDO expression can be induced in monocytic cell lines by PMA, LPS, and IFN $\gamma$ , but freshly isolated monocytes and other cell lines failed to induce TDO.

## Discussion

We found that TDO can be induced in THP-1 and U937 monocytic leukemia/lymphoma cell lines upon differentiation with PMA or a combination of LPS and IFN $\gamma$ . The level of protein TDO and its mRNA mostly correlated well, except for THP-1 cells treated with the combination of LPS and IFN $\gamma$ . In this condition, the mRNA was comparable to LPS treatment alone, but the protein level was lower in the presence of IFN $\gamma$  (Figure 4A and B). This might be explained by



**Figure 3.** Inhibition of the PKC pathway. THP-1 and U937 cells were treated for 2 days with 10 ng/mL of PMA, 5 nM of rottlerin or DMSO and *TDO2* mRNA, TDO protein, kynurenine production, and cell viability were

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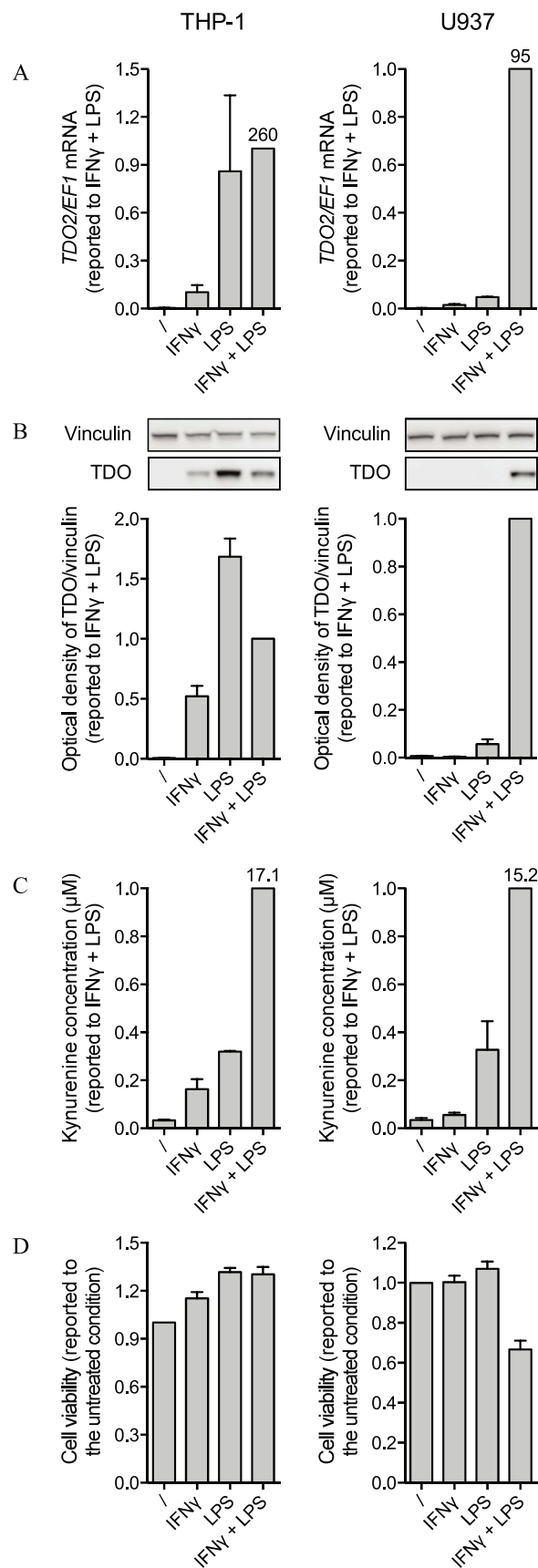
Figure 3. (Continued)

analyzed in parallel. (A) *TDO2* mRNA was quantified by RT-qPCR. The graphs show the mean  $\pm$  SEM (reported to PMA + DMSO) of three independent experiments. The mean value (*TDO2* transcripts per 2000 *EF1* transcripts) of the reference condition is indicated on top of the relevant bar. (B) 30 minutes before harvesting the cells, 300  $\mu$ M of tryptophan was added to the cell culture to stabilize the TDO protein, and TDO and vinculin (loading control) were revealed by western blot and quantified. The graphs show the mean  $\pm$  SEM (reported to PMA + DMSO) of three independent experiments. (C) The metabolite kynurenine was quantified in the supernatants by HPLC. The graphs show the mean  $\pm$  SEM (reported to PMA + DMSO) of three independent experiments. The mean value ( $\mu$ M) of the reference condition is indicated on top of the relevant bar. (D) Cell viability was measured by an MTS-PMS assay. The graphs show the mean optical density  $\pm$  SEM (reported to DMSO alone) of three independent experiments. DMSO indicates dimethylsulfoxide; HPLC, high-performance liquid chromatography; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; PMA, phorbol 12-myristate 13-acetate; PMS, phenazine methosulfate; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean; TDO, tryptophan 2,3-dioxygenase.

the complete degradation of tryptophan by IDO1 (data not shown), which is massively induced when combining LPS and IFN $\gamma$  (Figure 5). It was recently shown that the protein TDO is degraded in a proteasome-dependent manner in the absence of its substrate tryptophan, although the mRNA remains unaffected (Klaessens et al., in preparation).<sup>33</sup> Indoleamine 2,3-dioxygenase 1 was induced to a lesser extent in U937 cells (Figure 5). Tryptophan was not completely degraded (data not shown) and indeed, TDO protein and mRNA correlated well.

Tryptophan 2,3-dioxygenase expression and kynurenine production also correlated well, except for THP-1 cells treated with PMA (Figure 1A to C). Surprisingly, kynurenine concentrations decreased at PMA concentrations higher than 5 ng/mL (Figure 1C). We think that high PMA concentrations might induce downstream enzymes leading to the degradation of kynurenine.

We observed that TDO is probably induced by a PKC-mediated pathway (Figure 3 for PMA and data not shown for LPS and IFN $\gamma$ ), but we ignore the exact mechanism below PKC. Tryptophan 2,3-dioxygenase could be directly induced by this pathway or indirectly by the induction of new cytokines. For example, the differentiation of the cells leads to an increased expression and secretion of cytokines, like IL-1 $\beta$  and TNF $\alpha$  (Supplementary Figure 1),<sup>34,35</sup> which could induce TDO in an autocrine manner. Interestingly, D'Amato and co-workers found that triple-negative breast cancer (TNBC) cells induce TDO when losing attachment to the cell culture support.<sup>36</sup> These observations contrast with our results because nonadhering THP-1 and U937 cells start attaching to the cell culture support when differentiated to a macrophage-like phenotype with PMA or treated with LPS. On the contrary, we might confirm their results showing that TDO is induced by NF $\kappa$ B

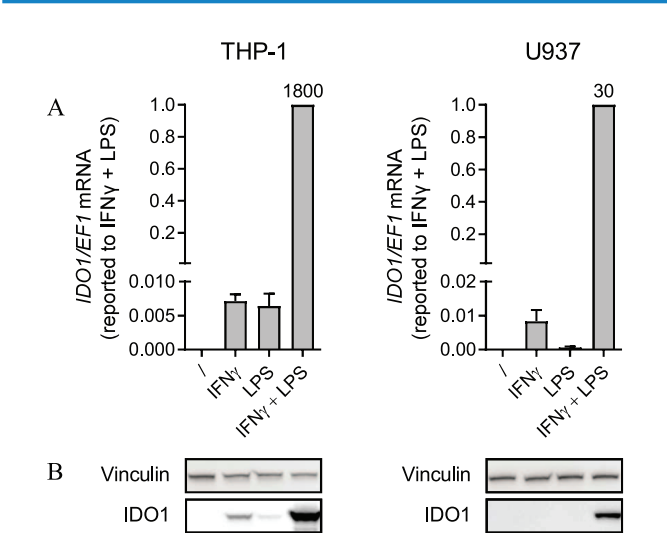


**Figure 4.** Induction of TDO by IFN $\gamma$  and LPS. THP-1 and U937 cells were treated for 2 days with 50 ng/mL of IFN $\gamma$  and/or 1  $\mu$ g/mL of LPS. *TDO2* mRNA, TDO protein, kynurenine production, and cell viability were analyzed

(Continued)

Figure 4. (Continued)

in parallel. (A) *TDO2* mRNA was quantified by RT-qPCR. The graphs show the mean  $\pm$  SEM (reported to IFN $\gamma$  + LPS) of three independent experiments. The mean value (*TDO2* transcripts per 2000 *EF1* transcripts) of the reference condition is indicated on top of the relevant bar. (B) 30 minutes before harvesting the cells, 300  $\mu$ M of tryptophan was added to the cell culture to stabilize the TDO protein, and TDO and vinculin (loading control) were revealed by western blot and quantified. The graphs show the mean  $\pm$  SEM (reported to IFN $\gamma$  + LPS) of three independent experiments. (C) The metabolite kynurenine was quantified in the supernatants by HPLC. The graphs show the mean  $\pm$  SEM (reported to IFN $\gamma$  + LPS) of three independent experiments. The mean value ( $\mu$ M) of the reference condition is indicated on top of the relevant bar. (D) Cell viability was measured by an MTS-PMS assay. The graphs show the mean optical density  $\pm$  SEM (reported to the untreated condition) of three independent experiments. HPLC indicates high-performance liquid chromatography; LPS, lipopolysaccharide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; PMA, phorbol 12-myristate 13-acetate; PMS, phenazine methosulfate; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean; TDO, tryptophan 2,3-dioxygenase.



**Figure 5.** Induction of IDO1 by IFN $\gamma$  and LPS. THP-1 and U937 cells were treated for 2 days with 50 ng/mL of IFN $\gamma$  and/or 1  $\mu$ g/mL of LPS. *IDO1* mRNA and IDO1 protein were analyzed in parallel. (A) *IDO1* mRNA was quantified by RT-qPCR. The graphs show the mean  $\pm$  SEM (reported to IFN $\gamma$  + LPS) of three independent experiments. The mean value (*IDO1* transcripts per 2000 *EF1* transcripts) of the reference condition is indicated on top of the relevant bar. (B) 30 minutes before harvesting the cells, 300  $\mu$ M of tryptophan was added to the cell culture, and IDO1 and vinculin (loading control) were revealed by western blot. LPS indicates lipopolysaccharide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of mean; TDO, tryptophan 2,3-dioxygenase.

signaling<sup>36</sup> because PMA,<sup>37</sup> LPS, and IFN $\gamma$  activate NF $\kappa$ B signaling. We did not test this hypothesis due to high cell mortality upon NF $\kappa$ B inhibition. In our experiments, the induction of TDO was specific for THP-1 and U937 cell lines and could not be observed in other tumor-derived myeloid cell lines. In addition, it contrasted with the lack of TDO expression in freshly isolated monocytes. In line with this last observation, we described recently the expression profile of TDO in solid tumors and showed that TDO is

Table 1. Differentiation of freshly isolated monocytes.

2 DAYS	
PMA 0, 0.5, 1, 2.5, 5 ng/mL	
5 DAYS	
GM-CSF	
GM-CSF + IFN $\gamma$ + LPS	
GM-CSF + IL-4	
GM-CSF + IL-4 + PGE2	
M-CSF	
M-CSF + IL-4	
M-CSF + IL-10	
M-CSF + ribomunyl + LPS	
IL-10 + IL-4	
PMA 5, 20 ng/mL	
5 DAYS	THEN 2 DAYS
GM-CSF	/
	IFN $\gamma$ + LPS
GM-CSF + IL4	/
	LPS
	Ribomunyl
M-CSF	/
	IL-4
	Ribomunyl + LPS
	IL-10
	TGF $\beta$
	TNF $\alpha$
	IFN $\gamma$ + LPS

The table lists all tested differentiation protocols and treatments of freshly isolated human monocytes. Cells were isolated from peripheral blood mononuclear cells and treated with either one single dose of cytokines for 2 or 5 days or differentiated for 5 days and subsequently treated with cytokines for 2 other days. The corresponding *TDO2* and *IDO1* RT-qPCR results and kynurenine quantifications are shown in Supplementary Figure 3. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; TGF, transforming growth factor; TNF, tumor necrosis factor.

mostly expressed by tumor-associated pericytes or vascular smooth muscle cells and, in some rare cases, in tumor cells surrounding TDO-positive vessels.<sup>23</sup> We did not observe TDO expression in tumor-associated macrophages. Therefore, the induction of TDO seems to be restricted to tumoral monocytic cell lines. Other human tumor lines are known to express TDO,

including glioblastoma lines and lines of colorectal, head and neck, and lung and gall-bladder carcinomas.<sup>18</sup> Expression of TDO in mouse tumor cells allows them to resist immune rejection.<sup>8,18</sup> It remains to be determined whether TDO expression in differentiated THP-1 and U937 cells also provides them the ability to resist immune attack.

Given the widespread use of THP-1 and U937 cells, our report of TDO induction in these lines by PMA or LPS and IFN $\gamma$  might help interpreting the results of experiments performed with these lines. In addition, a mechanistic study of TDO induction in these lines could help characterizing the regulation of TDO expression in normal and pathological contexts.

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## Author Contributions

Conception and design: DH, LP and BJVdE

Methodology: DH, LP and BJVdE

Acquisition of data: DH, VS

Writing: DH and BJVdE

Study supervision: BJVdE

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## Supplemental material

Supplemental material for this article is available online.

## REFERENCES

- Yang M, McKay D, Pollard JW, Lewis CE. Diverse functions of macrophages in different tumor microenvironments. *Cancer Res.* 2018;78:5492-5503.
- Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *J Immunol.* 2008;180:2011-2017.
- Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol.* 2010;11:889-896.
- Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood.* 2007;109:1568-1573.
- Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol.* 2005;5:641-654.
- Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol.* 2010;185:3190-3198.
- Terness P, Bauer TM, Rose L, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med.* 2002;196:447-457.
- Opitz CA, Litzenburger UM, Sahm F, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature.* 2011;478:197-203.
- Munn DH, Sharma MD, Baban B, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity.* 2005;22:633-642.
- Munn DH, Zhou M, Attwood JT, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science.* 1998;281:1191-1193.
- Dai W, Gupta SL. Regulation of indoleamine 2,3-dioxygenase gene expression in human fibroblasts by interferon-gamma. *J Biol Chem.* 1990;265:19871-19877.
- Chon SY, Hassanain HH, Pine R, Gupta SL. Involvement of two regulatory elements in interferon-gamma-regulated expression of human indoleamine 2,3-dioxygenase gene. *J Interferon Cytokine Res.* 1995;15:517-526.
- Uytendhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med.* 2003;9:1269-1274.
- Theate I, van Baren N, Pilotte L, et al. Extensive profiling of the expression of the indoleamine 2,3-dioxygenase 1 protein in normal and tumoral human tissues. *Cancer Immunol Res.* 2015;3:161-172.
- Spranger S, Spaepen RM, Zha Y, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Sci Transl Med.* 2013;5:200ra116.
- Hennequart M, Pilotte L, Cane S, et al. Constitutive IDO1 expression in human tumors is driven by cyclooxygenase-2 and mediates intrinsic immune resistance. *Cancer Immunol Res.* 2017;5:695-709.
- Brochez L, Chevolet I, Kruse V. The rationale of indoleamine 2,3-dioxygenase inhibition for cancer therapy. *Eur J Cancer.* 2017;76:167-182.
- Pilotte L, Larrieu L, Stroobant V, et al. Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. *Proc Natl Acad Sci U S A.* 2012;109:2497-2502.
- van Baren N, Van den Eynde BJ. Tryptophan-degrading enzymes in tumoral immune resistance. *Front Immunol.* 2015;6:34.
- Kanai M, Funakoshi H, Takahashi H, et al. Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. *Mol Brain.* 2009;2:8.
- Tatsumi K, Higuchi T, Fujiwara H, et al. Induction of tryptophan 2,3-dioxygenase in the mouse endometrium during implantation. *Biochem Biophys Res Commun.* 2000;274:166-170.
- Kohro T, Tanaka T, Murakami T, et al. A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. *J Atheroscler Thromb.* 2004;11:88-97.
- Hoffmann D, Dvorakova T, Stroobant V, et al. Tryptophan 2,3-dioxygenase expression identified in human hepatocellular carcinoma cells and in intratumoral pericytes of most cancers. *Cancer Immunol Res.* 2019; Dec 5 [Epub ahead of print]. doi:10.1158/2326-6066.CIR-19-0040.
- Daignault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS ONE.* 2010;5:e8668.
- Chen RF, Wang L, Cheng JT, Yang KD. Induction of IFN alpha or IL-12 depends on differentiation of THP-1 cells in dengue infections without and with antibody enhancement. *BMC Infect Dis.* 2012;12:340.
- Garcia A, Serrano A, Abril E, et al. Differential effect on U937 cell differentiation by targeting transcriptional factors implicated in tissue- or stage-specific induced integrin expression. *Exp Hematol.* 1999;27:353-364.
- Wang W, Alpert E. Downregulation of phorbol 12-myristate 13-acetate-induced tumor necrosis factor-alpha and interleukin-1 beta production and gene expression in human monocytic cells by human alpha-fetoprotein. *Hepatology.* 1995;22:921-928.
- Zhao YL, Liu WW, Liu W, et al. Phorbol ester (PMA)-treated U937 cells cultured on type I collagen-coated dish express a lower production of pro-inflammatory cytokines through lowered ROS levels in parallel with cell aggregate formation. *Int Immunopharmacol.* 2018;55:158-164.
- Aldo PB, Craveiro V, Guller S, Mor G. Effect of culture conditions on the phenotype of THP-1 monocyte cell line. *Am J Reprod Immunol.* 2013;70:80-86.
- Danesch U, Hashimoto S, Renkawitz R, Schutz G. Transcriptional regulation of the tryptophan oxygenase gene in rat liver by glucocorticoids. *J Biol Chem.* 1983;258:4750-4753.
- Kheolamai P, Dickson AJ. Liver-enriched transcription factors are critical for the expression of hepatocyte marker genes in mES-derived hepatocyte-lineage cells. *BMC Mol Biol.* 2009;10:35.
- Li DD, Gao YJ, Tian XC, et al. Differential expression and regulation of Tdo2 during mouse decidualization. *J Endocrinol.* 2014;220:73-83.
- Lewis-Ballester A, Forouhar F, Kim SM, et al. Molecular basis for catalysis and substrate-mediated cellular stabilization of human tryptophan 2,3-dioxygenase. *Sci Rep.* 2016;6:35169.
- Akeson AL, Schroeder K, Woods C, Schmidt CJ, Jones WD. Suppression of interleukin-1 beta and LDL scavenger receptor expression in macrophages by a selective protein kinase C inhibitor. *J Lipid Res.* 1991;32:1699-1707.
- Schwende H, Fitzke E, Ambs P, Dieter P. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *J Leukoc Biol.* 1996;59:555-561.
- D'Amato NC, Rogers TJ, Gordon MA, et al. A TDO2-AhR signaling axis facilitates anoikis resistance and metastasis in triple-negative breast cancer. *Cancer Res.* 2015;75:4651-4664.
- Peters RT, Liao SM, Maniatis T. IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. *Mol Cell.* 2000;5:513-522.