



## Research article

# Production of NOS2 and inflammatory cytokines is reduced by selected protein kinase inhibitors with partial repolarization of HL-60 derived and human blood macrophages



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## HIGHLIGHTS

- HL-60 derived and human blood macrophages are polarized by LPS+IFN $\gamma$  (M1) and IL-4 (M2), evidenced by polarization markers
- The expression of M1 polarization markers are decreased by protein kinase inhibitors without repolarization to M2 phenotype
- Both MAPK and JAK/STAT pathways are involved in complement-3b mediated phagocytosis, which was reduced by their inhibitors

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## ABSTRACT

JAK/STAT pathway plays a well-known role in macrophage polarization, but other signaling routes may also be involved. The aim of this study was to identify new signaling pathways and repolarize macrophages by selected protein kinase inhibitors. HL-60 derived macrophages were chosen as model cells and human blood macrophages were used for comparison. M1 and M2 polarization of HL60 derived and human blood macrophages was promoted by LPS + IFN $\gamma$  (LIF) and IL-4 treatments, respectively. In HL-60 derived macrophages, M1 polarization was mediated by Erk1/2 and p38 phosphorylation, while HSP27 phosphorylation was involved in M2 polarization. The inhibition of both MAPK and JAK/STAT pathways reduced the expression of NOS2, IP-10 and TNF $\alpha$ , IL-8 production was decreased by the inhibition of AMPK and PKD, the upstream kinase of HSP27. HSP27 phosphorylation was inhibited by NB 142, a PKD inhibitor. The expression of CD80 (M1 marker) was reduced by MAPK and JAK/STAT inhibitors, without increasing CD206 (M2 marker). On the other hand, CD206 was reduced by PKD and AMPK inhibitors, without increasing CD80 marker. Phagocytic capacity of HL-60 derived macrophages was higher in M1 macrophages and decreased by trametinib and a p38 inhibitor, while in human blood macrophages, where AT 9283, a JAK/STAT inhibitor also caused a significant decrease in M1 polarized macrophages, no difference was observed between M1 and M2 macrophages. Our results suggest that the repolarization of macrophages cannot be achieved by inhibiting their signaling pathways; nevertheless, the expression of certain polarization markers was decreased, therefore a “depolarization” could be observed both in M1 and M2 polarized cells. Selected protein kinase inhibitors of M1 polarization, decreasing NOS 2 and inflammatory cytokines may be potential candidates for therapeutic trials against inflammatory diseases.

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## 1. Introduction

Macrophages are important cells of the immune system and play crucial roles in inflammatory reactions. Macrophages are polarized during their differentiation into „classically” and „alternatively” activated M1 and M2 type macrophages, respectively [Martinez and Gordon, 2014; Murray, 2017]. This polarization is regulated by various cytokines, classified as Th-1 and Th-2 type cytokines [Mills et al., 2000]. M1 macrophages are involved primarily in acute inflammations, while M2 macrophages mediate anti-inflammatory reactions and chronic inflammations, frequently leading to neoplastic transformations [Mantovani, 2006; Mantovani and Sica, 2012].

The polarizing action of cytokines is mediated by cytokine receptors on the cell surface, followed by signaling pathways involving various protein kinases, finally resulting in activated transcription factors. These factors regulate the transcription of various genes and the produced proteins are responsible for the different properties of classical M1 and alternative M2 type macrophages. In previous studies several components of the M1 and M2 polarizing signaling pathways were described. In the case of M1 polarization, receptors for *Escherichia Coli* LPS, IFN $\gamma$  and GM-CSF were involved in ligand binding and the main signaling route is mediated by the JAK/STAT pathway (JAK 1/2 and STAT 1–3). In the case of M2 polarization, IL-4 and IL-13 cytokines are bound to their surface receptors, and the effects are mediated by other components of the JAK/STAT pathway (JAK 1/2/3 and STAT6) [Lawrence and Natoli, 2011; Tugal et al., 2013]. More recently, the role of other signaling pathways has also been observed, including the PI3K-Akt-mTOR axis [Vergadi et al., 2017], Notch [Lin et al., 2018], and MAPK pathways [Cheng et al., 2018].

The polarization of macrophages can be characterized by specific inflammatory markers. CD (cluster of differentiation) markers are frequently used to identify the polarization. Moreover, one of the most known markers of classically activated (M1) macrophages is NOS2 [Lawrence and Natoli, 2011]. Production of several Th-1 cytokines (e.g. IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-12) are also considered as characteristic markers of M1 macrophages. At the same time, arginase 1, another enzyme using L-arginine substrate is a marker of alternative (M2) activation of macrophages, at least in rodents, and IL-10 or TGF- $\beta$  are cytokines produced by M2 polarized cells [Hao et al., 2012].

Phagocytosis, one of the most known function of macrophages, has been demonstrated both in M1 and M2 macrophages, respectively [Liao et al., 2015]. According to some studies, M2 macrophages had higher phagocytic capacity, at least for antibody-opsonized particles [Atri et al., 2018]. The signaling routes of Fc-dependent phagocytosis was described in detail [Garcia-Garcia and Rosales 2002], but less data were available about complement-dependent phagocytic processes. According to an earlier review, Fc-dependent phagocytosis is related to proinflammatory processes, while complement-mediated phagocytosis is non-inflammatory [Aderem 2003]. Signaling routes include the role of integrins, Rho associated kinase (ROCK) and actin polymerization [Dupuy and Caron, 2008; Rosales and Uribe-Querol, 2017]. PI3K is also involved both in FcR and complement receptor (CR)-dependent phagocytosis, contributing to a Ca<sup>2+</sup>-signaling [Nunes and Demareux 2010]. Nevertheless, the involvement of additional signaling pathways may be supposed in the phagocytic response of macrophages.

The purpose of our study was to find kinase inhibitors to modify the polarization and function of macrophages, in order to influence inflammatory processes. We tried to detect new alternative signaling pathway(s) in macrophage polarization and to use various protein kinase inhibitors to change their polarization. HL-60 cell line was chosen as a suitable model, because it can be differentiated into macrophages by phorbol esters [Harris and Ralph, 1985 Aihara et al., 1991], and polarized by cytokines. Specific kinase inhibitors, added during the differentiation/polarization process, may help to study the role of various signaling pathways in the polarization. As a first orientation step, a protein kinase and a cytokine array were used to detect simultaneous

differences in the phosphorylation and cytokine patterns, using M1 and M2 specific cytokines (LPS + IFN $\gamma$  and IL-4, respectively). Based on these results, the occurrence of several M1-M2 polarization markers was examined both in the presence and absence of various kinase inhibitors, which can influence the previously detected signaling routes. We have also applied an alternative terminology for macrophages, using M<sub>LPS</sub> for macrophages treated with LPS + IFN $\gamma$  and M<sub>IL4</sub> for macrophages treated with IL-4. This idea was based on a recent publication discussing the problems of the M1-M2 treatment-marker correlations [Hoffman and Ponik, 2020].

In addition to the HL-60 derived macrophages, primary human macrophages differentiated from blood monocytes were also tested to compare our most marked findings obtained on HL-60 derived macrophages (including CD markers, NOS expression, inflammatory cytokine productions, phagocytic capacity).

## 2. Materials and methods

### 2.1. Materials

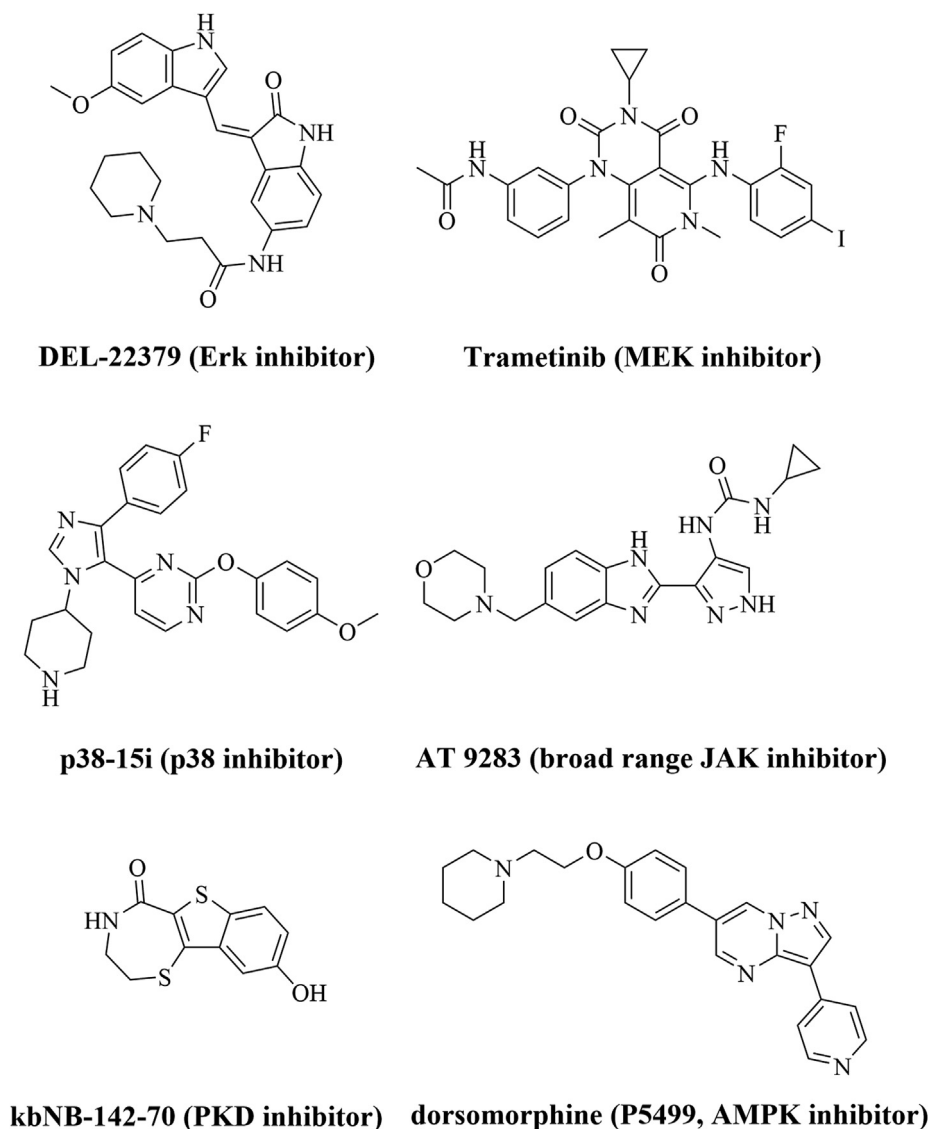
HL-60 cells were purchased from American Type Cell Collection (ATCC). Human monocyte-derived macrophages were isolated from buffy coat purchased from the Hungarian National Blood Transfusion Service. Monocytes were isolated and differentiated to macrophages with a slightly modified method of Meital et al., [2019]. Adherent cells after culturing in IMDM-5% FBS containing 50 ng/ml M-CSF for 6 days with a medium exchange on the third day were considered as macrophages.

Proteome Profiler Phosphorylation and Cytokine Arrays, anti-CD11b, anti-CD68, anti-CD80, anti-pSTAT3, anti-pSTAT6, anti-pHSP27 primary, NL493 and NL 557 fluorescent secondary antibodies were purchased from R & D Systems. ELISA kits (for IP-10, TNF $\alpha$ , IL-1 $\beta$ , IL-8, CCL2, RANTES), and recombinant IFN $\gamma$  and IL-4 were manufactured by Peprotech (USA). Protein kinase inhibitors were manufactured by Vichem Ltd (Budapest, Hungary), except trametinib (Focus Biomolecules, USA) and kbMB-142-70 (Tocris, England). <sup>3</sup>H-labeled L-arginine was purchased from American Radiochemical Company (ARC). Anti-NOS2, anti-arginase primary and HRPO-conjugated goat anti-mouse secondary antibodies were the products of BD Biosciences, while other antibodies, cell culture media and FBS were purchased from Thermo Fisher Hungary. Other chemicals were purchased from Merck/Sigma-Aldrich or from VWR Hungary.

The list of used kinase inhibitors was as follows (abbreviated symbols in brackets): trametinib, an inhibitor of MEK (Tra); DEL-22379, a direct inhibitor of Erk1/2 (abbreviated as DEL); a p38 inhibitor, 1-(Piperidin-4-yl)-4-(4-fluorophenyl)-5-[2-(4-methoxyphenoxy)-pyrimidin-4-yl]imidazole; (abbreviated as p38-15i) [Boehm et al., 2001]; AT9283 (AT) an inhibitor of JAK 2/3 and Aurora kinase A/B; kbNB 142-70, a PKD inhibitor (abbreviated as NB 142); dorsomorphine (or P5499), an inhibitor of AMPK (abbreviated as dorso). These inhibitors were used at 100 nM concentrations (1  $\mu$ M in phagocytosis study), which did not cause considerable cell toxicity even at 1  $\mu$ M in previously performed MTT viability tests (performed according to Merck/Sigma protocol), except trametinib (used at 10 nM final concentrations, found slightly toxic for HL-60 cells in a previous MTT cell viability test at higher concentration). The chemical structures of the inhibitors are shown in Figure 1.

### 2.2. Differentiation and activation of HL-60 cells into macrophages

HL-60 cells were cultured in RPMI 1640 containing 10 % FBS and an antibiotic-antimycotic mixture (1:100). They were differentiated into macrophages during 3 days with 500 nM PMA. Their further activation and polarization into M1 and M2 type macrophages has been achieved by adding LPS (40 ng/ml) + IFN $\gamma$  (20 ng/ml) and IL-4 (20 ng/ml), respectively, simultaneously with PMA. Kinase inhibitors were added on day 2 of differentiation, because most of the inhibitors may block cell



**Figure 1.** Chemical structures of the utilized protein kinase inhibitors.

adherence when added together with the cytokines. Cells were cultured in 5 % CO<sub>2</sub> at 37 °C.

Human blood derived macrophages were polarized for 2 days in IMDM-5% FBS with 100 ng/ml LPS and 20 ng/ml IFN $\gamma$  for M1 and with IL-4 (20 ng/ml) for M2 macrophages. Kinase inhibitors were added simultaneously with the polarizing agents.

### 2.3. Microscopical observations

#### 2.3.1. Immunofluorescence staining of CD68 and CD11b in HL-60 derived macrophages

HL-60 cells were seeded into eight well Ibidi®  $\mu$ -Slide microscopic slide ( $2 \times 10^4$  cells/well) and differentiated into macrophages as described in section 2.2. To demonstrate inducible expression of CD68 and CD11b, undifferentiated HL-60 cells were also investigated by this method. In this case HL-60 cells were seeded into Ibidi slides similarly to HL-60 cells for differentiation, but undifferentiated HL-60 cells were incubated in PMA-free cell culture medium. After 3 days of incubation, medium was removed only from differentiated (adhered) HL-60 cells. Differentiated cells were washed with phosphate buffered saline (PBS) and PBS was added into these wells. Ibidi slide was centrifuged (350 g, 4 min). From this step, differentiated and undifferentiated cells were

handled in the same way. Supernatant was removed from each well carefully, and PBS were added. Ibidi slide was centrifuged again (350 g, 4 min). PBS was removed carefully, and cells were fixed with 4% paraformaldehyde (PFA) for 20 min at 4 °C. PFA was removed and cells were washed with PBS. For blocking, Fc receptor binding inhibitor polyclonal antibody (Thermo Fisher Scientific) was used (1:50 ratio) in 5% bovine serum albumin (BSA) containing PBS, for 30 min at 25 °C. After removing blocking solution, CD11b and CD68 antibodies were added (1:100 dilution, 2 h, 25 °C) in PBS, except one well which was contained PMA treated (differentiated) HL-60 cells, these cells were used as negative control. After removing primary antibodies, CF™ 488A labelled anti-mouse secondary antibody (1:200) and 5 mM DRAQ5 (1:1000) were added in PBS into each well, and cells were incubated for 1 h at 25 °C. Cells were washed with PBS twice and finally PBS was added. Images were acquired with Zeiss Confocal LSM 710 microscope (Carl Zeiss AG, Oberkochen, Germany). Objective: Plan-Apochromat 63 $\times$ /1.40 Oil DIC M27. Pinhole: 1.99 AU. Laser Wavelength: 488 nm and 633 nm. Detection wavelength: 506–544 nm; 666–745 nm.

#### 2.3.2. Immunofluorescence staining of CD68 in human blood macrophages

HL-60 cells were seeded into eight well Ibidi®  $\mu$ -Slide microscopic slide and cultured as described in section 2.2. After removing medium,

cells were washed with PBS, fixed with 5 % paraformaldehyde (10 min, 25 °C), permeabilized with 0.2 % Triton-X-100 in PBS (10 min, 25 °C) and blocked with 10 mg/ml BSA (2 h, 25 °C). Anti-CD68 antibody was added (1:200 ratio, 16 h, 4 °C) in % BSA containing PBS. After removing primary antibody, Northern Lights™ 557-conjugated secondary antibody and 5 mM DRAQ5 (1:1000) were added in PBS (1 h, 25 °C). After washing with PBS twice, PBS was added and images were acquired with Zeiss Confocal LSM 710 microscope using the following parameters. Objective: Plan-Apochromat 63x/1.40 Oil DIC M27. Pinhole: 1,32 AU. Laser wavelengths: 543 and 633 nm, Detection wavelengths: 544–622 nm; 696–757 nm.

### 2.3.3. Immunofluorescence staining of CD80 and CD206 in HL-60 derived and human blood macrophages

HL-60 cells were seeded into eight well Ibidi®  $\mu$ -Slide microscopic slide and differentiated into macrophages as described in section 2.2. In the case of kinase inhibitor treatments, the final concentrations were 100 nM, except trametinib (10 nM). For immunofluorescence staining the same protocol was used as described in section 2.3.1., but in this experiment CD80 (mouse monoclonal) and CD206 (rabbit polyclonal) antibodies were added to cells, using 1:100 ratio. For secondary labelling, anti-mouse Alexa Fluor 594-conjugated (Jackson ImmunoResearch Inc., West Grove, PA, USA) and anti-rabbit Northern Lights™ 493-conjugated secondary antibodies were used simultaneously, in 1:200 ratio. The same method was used to label human blood macrophages. In HL-60 derived macrophages, double labelling has also been performed, in this case CD80 and CD206 primary antibodies and the corresponding secondary antibodies were added simultaneously, Imaging parameters: Zeiss Confocal LSM 710 microscope, plan-Apochromat 63x/1.40 Oil DIC M27 objective, Pinhole: 4,08 AU. Laser wavelengths: 488 nm, 543 nm and 633 nm. Detection wavelengths: 490–539 nm, 602–631 nm; 691–758 nm.

### 2.4. Flow cytometric analysis of CD68 and CD11b in HL-60 derived macrophages

HL-60 cells were seeded and cultured as described in section 2.2. Untreated HL-60 (suspension) cells were collected into 2 ml tubes, and centrifuged (350 g, 4 min). Supernatant was removed and 1 mM EDTA in PBS was added to the cells. Cells were incubated for 30 min at 37 °C. At the same time, supernatant of PMA differentiated HL-60 (adhered) cells were discarded, cells were washed with PBS and 1 mM EDTA in PBS was added to the cells. Cells were incubated for 30 min at 37 °C. Detached cells were suspended and transferred into 2 ml tubes. Then both non-differentiated HL-60 cells and differentiated cells were handled in the same way and rest of experiment was carried out on ice. Tubes were centrifuged (350 g, 4 min, 4 °C), supernatant was removed, 0.5 ml FACS buffer was added into the tubes and centrifuged again. Supernatant was removed again, and 100  $\mu$ l FC receptor blocking antibody (Thermo Fisher Scientific) was added (1:5 ratio) in FACS buffer for 30 min. After centrifugation supernatant was removed and 100  $\mu$ l CD68 or CD11b primary antibodies were added (1:100 dilution) in FACS buffer for 60 min. One sample both from differentiated and undifferentiated cells were not labeled by primary antibody which were used as negative control. After centrifugation supernatant was removed and 150  $\mu$ l CF™ 488A-conjugated secondary antibody (1:200 dilution) in FACS buffer was added for 45 min. During the last 15 min of secondary labelling, 10  $\mu$ l propidium iodide solution (5  $\mu$ g/ml final concentration) in FACS buffer was added into the tubes. After centrifugation, supernatant was removed, FACS buffer was added. After centrifugation, FACS buffer was removed, and FACS buffer was added again, samples were analyzed using Cytoflex flow cytometer. Data was evaluated using CytExpert software.

### 2.5. Orientation experiments with Proteome Profiler Array kits

In order to study the phosphorylation of signaling kinases, a first orientation experiment was carried out by using a Human Phospho

Kinase Array capable to detect and to compare phosphorylation differences for 46 different kinases. PMA-only treated controls, cells treated with LPS + IFN $\gamma$  (LIF) and IL-4 were compared in samples prepared from cell lysates.

Cytokines were detected in the supernatants of the same samples mentioned above using a Human Cytokine Array capable to detect and to compare differences of various cytokines. For both arrays, the spots on X-ray films were evaluated semi-quantitatively by ImageJ software, normalized to positive controls.

### 2.6. Quantitative measurement of CD80 and CD206 by cell based ELISA

Since the results of immunofluorescence studies could not give quantitative information about the CD marker levels, a cell based ELISA-method has been used for better comparison.  $8 \times 10^4$  HL-60 cells/well in 96 well plates were differentiated and treated as written above. Cells were then washed with PBS and fixed with 5 % formaldehyde. After blocking with 1 % BSA in PBS for overnight, anti-CD80 and anti-CD206 antibodies in 0.1 % BSA-PBS (1:200 dilution) were added for 24 h, and then washed four times with a washing buffer (0.05 % Tween-20, 0.1 % BSA in PBS). Then corresponding secondary antibody (anti mouse HRPO conjugated, 1:800 dilution) has been added to the samples for 2 h. After washing four times with the washing buffer, TMB (tetramethylbenzidine, Sigma) solution was added for 5–10 min, stopped with 1 M H $_2$ SO $_4$  and absorbances were measured at 450 nm. The background values caused by aspecific binding of secondary antibody to the cells was measured and subtracted from each sample.

HSP27 phosphorylation was also studied by this method, see the actual antibody dilutions in the Results.

### 2.7. Measurement of STAT phosphorylations by Western blotting

Since phosphorylation signals are not always stable for longer time, and were not different between M1 and M2 polarized HL-60 derived macrophages, they were studied during shorter polarization periods. Cells were treated with the corresponding polarizing cytokines for 4, 18 and 72 h, and then lysed in sample buffers. Proteins were separated on 8 % polyacrylamide gel followed by blot transfer [Müllner et al., 2002]. Both the phosphorylation and expression of STAT proteins were detected by using anti-phospho STAT3 and STAT6 rabbit and anti-STAT3 and STAT6 mouse monoclonal primary antibodies followed by HRPO-conjugated goat anti-rabbit and anti-mouse secondary antibodies, respectively. Spots were visualized by adding ELC reagent and exposing the membranes to X-ray films. The bands on X-ray films were evaluated by ImageJ software and the fractions of phosphorylated/non phosphorylated proteins were evaluated.

### 2.8. Measurement of NOS2 and arginase activities and protein expressions

$2 \times 10^5$  HL-60 cells were differentiated and cultured for 3 days as described above. Then enzyme activities were measured in their lysates as described earlier [Hrabák et al., 2006], in a „NOS measuring solution” containing 5 mM HEPES, 0.8 mM NADPH and 1  $\mu$ M BH $_4$ , using 60 min incubation time at 37 °C, by adding 20  $\mu$ M  $^3$ H-labeled L-arginine (ARC, final sp. act. 300 mCi/mmol). 20  $\mu$ l aliquots were applied to TLC plates and chromatographed in an eluent [Sessa et al., 1990]. Spots were visualized by ninhydrine and cut out, followed by their measurement in a Packard Trikarb 2100 TR liquid scintillation counter in Sigma Fluor Universal cocktail. Protein content of the same samples was determined by the Coomassie blue method [Bradford, 1976]. Since the products of NOS2 reaction are equimolar NO and citrulline, while arginase produces equimolar ornithine and urea, their specific activities were calculated from the radioactivity of formed citrulline and ornithine spots, respectively.

Western blot experiments were carried out as described elsewhere [Müllner et al., 2002]. The presence of the proteins was detected by using

anti-NOS2 and anti-arginase1 monoclonal primary antibodies followed by HRPO-conjugated goat anti-mouse secondary antibody. Spots were visualized by adding ELC reagent and exposing the membranes to X-ray films. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression was detected as a constitutive reference protein for NOS2 while  $\beta$ -actin for arginase (the molar masses of arginase is too close to GAPDH). The bands on X-ray films were evaluated by ImageJ software.

## 2.9. Quantitative determination of cytokine levels in cell supernatants with ELISA

The production of various pro-inflammatory and anti-inflammatory ( $M_{LIF}$  type and  $M_{IL4}$  type, respectively) cytokines was determined quantitatively by various ELISA kits according to the prescriptions of the manufacturer. The supernatants of HL-60 cell cultures were collected and stored at  $-80^{\circ}\text{C}$ , and 100  $\mu\text{l}$  aliquots were used for the determination.

## 2.10. Quantitative measurement of complement-dependent phagocytosis of macrophages

*Micrococcus (M.) luteus* cells were labeled with RITC and opsonized by human complement (1:4) for 30 min at  $37^{\circ}\text{C}$  and washed twice with PBS. Labeled bacteria were suspended in DMEM and  $5 \times 10^7$  bacteria were

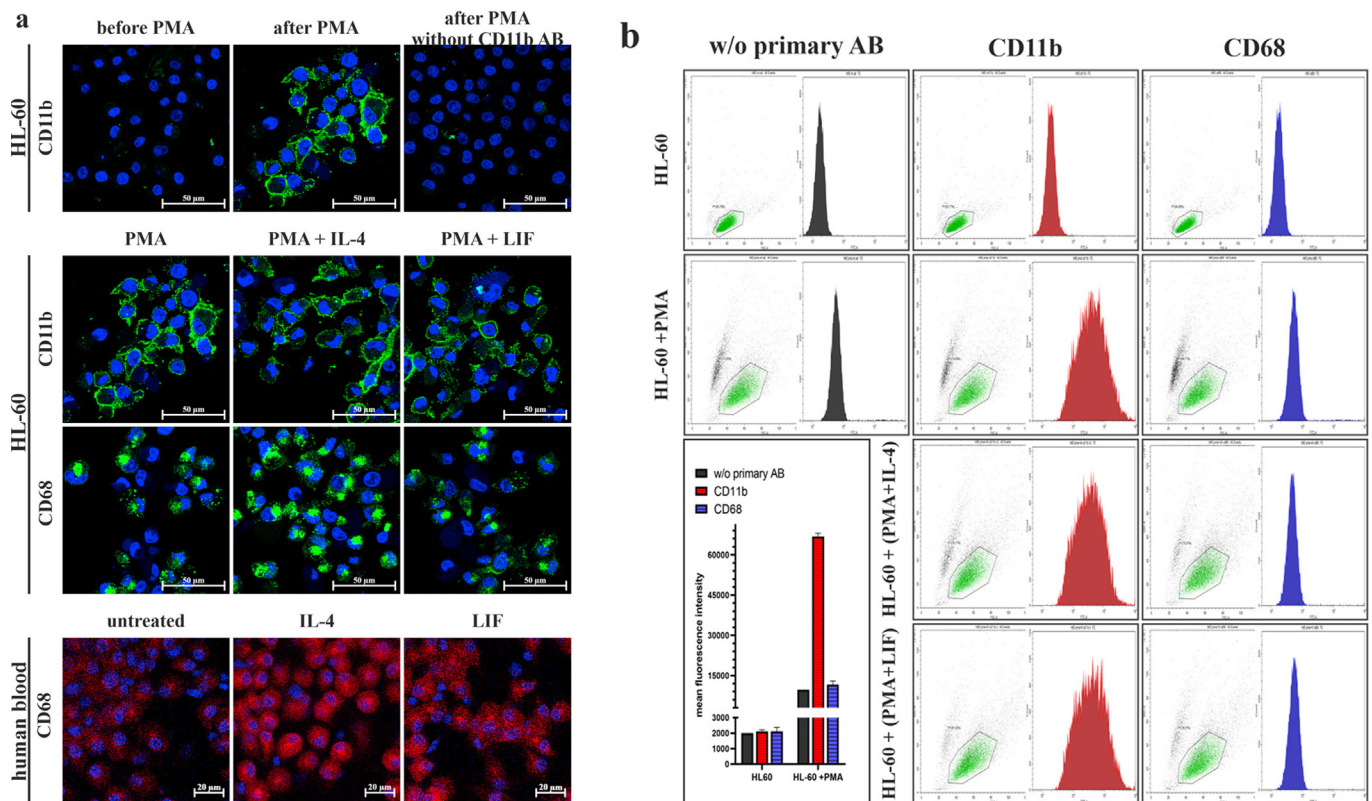
added to  $2 \times 10^5$  macrophages for 60 min on 24 well plates. Ingestion was stopped by adding 100  $\mu\text{l}$  NEM (2 mg/ml), washed twice by PBS (pH = 7.0), non-ingested bacteria removed by lysozyme treatment [Vray et al., 1980; Hrabák et al., 2008]. Finally, cells were solubilized in 1 ml 1 % Igepal detergent, fluorescence measured in a Thermo Varioskan Flash instrument, using 545 nm excitation and 590 nm emission wavelengths. The phagocytic index can be calculated by comparing the values to the fluorescence of standard bacterial suspensions, and is given as millions of ingested bacteria per  $10^5$  macrophages.

## 2.11. Statistical analysis

The mean values, SD and SEM values, one-way or paired ANOVA and posthoc tests were calculated with GraphPad software using Student t-tests. Differences of  $p < 0.05$  were considered as significant, with 95 % confidence level.

## 2.12. Ethical approval

The research was conducted in compliance with the principles stated in the Guide for the Care and Use of Laboratory Animals. The protocol regarding animal care was approved by the ethics committee of



**Figure 2.** Demonstration of macrophage character and morphological properties of PMA-differentiated HL-60 and human blood-derived macrophages. a: CD11b and CD-68 monocyte-macrophage markers were investigated by immunocytochemistry, using anti-CD11b anti-CD68 monoclonal primary antibodies and Northern Light 493 secondary antibody (green). Cell nuclei are counter-stained with DRAQ5 (blue). HL-60 derived cells: the expression of CD11b in PMA-treated HL-60 derived macrophages was much higher than in non-treated cells. Each PMA-treated cell population expresses CD68 and CD11b, but CD68 is localized intracellularly, while CD11b is mainly membrane-bound. Cell clusters and dendritic extensions were observed mainly in LPS + IFN $\gamma$ -treated (LIF, M1 polarized) samples. Human blood macrophages: untreated cells, IL-4 (M2 polarized) and LPS + IFN $\gamma$  (LIF, M1 polarized) treated cells all express CD68 marker (red). In this experiment, we used Northern Light 557 secondary antibody. b: Flow cytometric analysis of CD11b and CD68 in the plasma membrane of HL-60 cells and HL-60 derived macrophages. PI staining was used to exclude dead cells from the presented results, viable (PI negative) cells were marked with green color. Expression of CD11b (red) and CD68 (blue) were not detectable in non-treated HL-60 cells, compared to background control (w/o primary antibody) cells (gray). Morphological alteration of PMA treated HL-60 cells compared to their controls was clearly detectable on dot plots (cells marked with green). PMA treatment also induces expression of CD11b, however, elevated expression of CD68 was not detectable, compared to PMA-treated control cells (gray). Therefore, flow cytometry supports the different localization of CD11b and CD68 markers. IL-4 and LIF treatment did not cause any significant differences compared to PMA-treated controls.

Semmelweis University Regional and Institutional Committee of Science and Research Ethics.

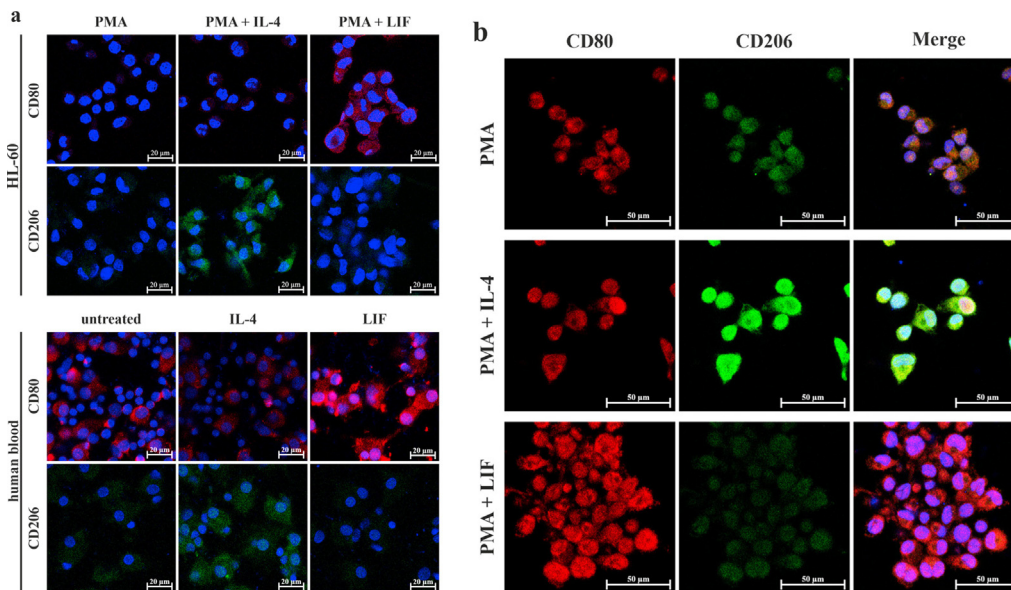
### 3. Results

#### 3.1. Differentiation and polarization of HL-60 cells

HL-60 cells were differentiated into macrophages with PMA, while their activation and polarization into M1 and M2 type macrophages has been achieved by LPS + IFN $\gamma$  (LIF) and IL-4, respectively. Although at different level, most cells were CD-68 positive, indicating their macrophage character, both in control and polarized samples, including HL-60 derived and human blood-derived macrophages (controls, LPS + IFN $\gamma$ , IL-4, Figure 2a). The morphology of the LPS + IFN $\gamma$ -treated HL-60 cells (M<sub>LIF</sub>) was slightly different from other macrophages: they developed branching extensions, creating cell clusters.

The macrophage character of HL-60 cells has been supported further by CD11b, a macrophage specific marker [Grolleau et al., 1999] (Figure 2a). Interestingly, when CD11b and CD68 was studied by flow cytometry, only CD11b marker was detected. This difference suggests, that CD68 marker is dominantly intracellular (see: uniprot.org/uniprotP31996), because its accessibility by the antibody may be due to the fixation of the cells, while CD11b is membrane-bound and could be detected in fixed cells by immunohistochemistry (Figure 2a) and on living cells by flow cytometry as well (Figure 2b).

M1 polarization of LPS + IFN $\gamma$  treated cells was confirmed by the higher expression of CD80, while M2 polarization of IL-4 treated cells was proven by the higher labeling of CD206 marker (Figure 3a). Nevertheless, both markers were detected both in control (PMA only) and in the opposite polarization, even if at a lower level, in HL-60 derived and primary blood macrophages as well. Therefore, the pattern of CD markers was similar both in HL-60 derived and human blood macrophages.



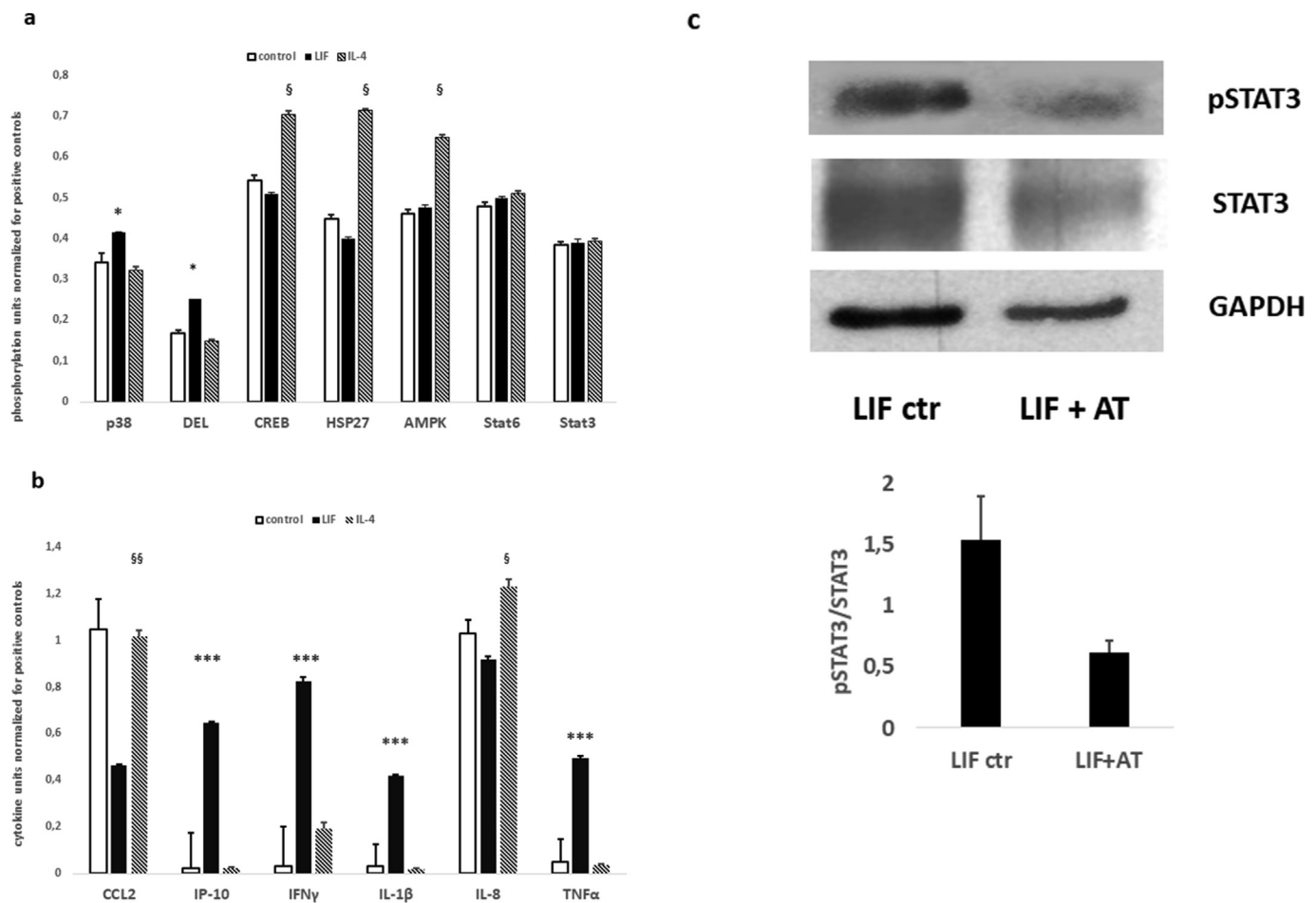
**Figure 3.** Identification of polarization markers in PMA-differentiated HL-60 cells and human blood-derived macrophage cells. a: Expression of CD80 as M1 polarization marker (labeled with red color) and CD206 as M2 polarization marker (labeled with green color) were investigated by immunohistochemistry. Cell nuclei were counter-stained with DAPI (marked with blue color). HL-60 derived macrophages: Intensive staining was observed for CD80 in LPS + IFN $\gamma$  treated (M1 polarized, LIF) cells, and for CD206 in IL-4 treated (M2 polarized) cells. Nevertheless, both CD80 and CD206 were poorly detectable in PMA-treated cells and in opposite polarization. Human blood macrophages: The most intensive staining was found for CD80 in M1 polarized (LIF) cells, while for CD206 in M2 polarized (IL-4) cells, but weaker signals were detectable in untreated and oppositely polarized cells as well. b: The previous experiment repeated by using double labeling. CD80 marker was labeled by a mouse monoclonal, CD206 marker by a rabbit polyclonal antibody. Secondary antibodies were Alexa Fluor 594 labeled anti-mouse (red) and NL 493 labeled anti-rabbit (green) antibodies. The predominance of CD80 on M1 and that of CD206 on M2 polarized cells has been supported, accompanied by a weaker expression of the opposite marker.

CD80 and CD206 markers were also studied by double staining. This experiment supports the dominant position of CD80 in M1 polarized and of CD206 in M2 polarized HL-60 cells, but both markers can be detected at lower levels in the opposite polarization (Figure 3b).

#### 3.2. Phosphorylation of various protein kinases and kinase substrates in differently polarized HL-60 derived macrophages

In order to obtain new information about signaling routes involved in macrophage polarization, a phosphokinase array has been used as a first orientation test. The phosphorylation patterns of control, LPS + IFN $\gamma$  treated (M1 or M<sub>LIF</sub>) and IL4 treated (M2 or M<sub>IL4</sub>) samples were compared. Most of phosphorylations were not or only slightly altered by the polarizing cytokines: nevertheless, Erk1/2 and p38 phosphorylations were higher in LPS + IFN $\gamma$  treated cells (M1), while CREB, AMPK $\alpha$ 2 and HSP27 were phosphorylated in a higher extent in IL-4 treated cells (M2) as shown in Figure 4a. Since the JAK/STAT pathway is considered as the most important signaling pathway for polarizing cytokines [Lawrence and Natoli, 2011; Tugal et al., 2013], it was surprising that neither STAT3, nor STAT6 phosphorylations were different between LPS + IFN $\gamma$  and IL-4 polarized, HL-60 derived macrophages (Figure 4a).

Since the phosphorylation signal is transient and does not persist, we performed an experiment, when polarizing agents were added for shorter periods, 18 h and 3 h. Neither STAT3 nor STAT6 did show significant differences between the IL-4 treated and LIF treated samples either during 18 or 3 h periods. Nevertheless, phosphorylation was well detected during any time period (see Supplement Figure 1 and Supplement Figure 2). When the M1 polarized HL-60 macrophages were treated with AT9283, a JAK1/2/3 inhibitor, the phosphorylation of STAT3 has been significantly reduced (Figure 4c, uncropped version in Supplement Figure 3). This may give an explanation for the inhibitory effect of AT9283 on NOS2 expression and cytokine production.



**Figure 4.** The most significant results of Proteome Profiler Experiments in HL-60 derived macrophages. The most significant phosphorylation changes (a) and the concomitant cytokine production alterations (b) in M1 (LPS + IFN $\gamma$ ) and M2 (IL-4) polarized HL-60 derived macrophages are shown. Units on y-axis were calculated as a ratio of the pixels of the sample divided by the pixels of a positive reference spot. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , where M1 samples were higher, § $p < 0.05$ , §§ $p < 0.01$ , where M2 samples were higher. c: STAT3 phosphorylation was studied by rabbit polyclonal anti-pSTAT3, mouse monoclonal anti-STAT3 and anti-GAPDH antibodies. The pSTAT3/STAT3 proportion was significantly reduced by AT 9283 JAK1/2/3 kinase inhibitor, supporting the role of JAK/STAT pathway in the M1 polarization of HL-60 derived macrophages. The uncropped version of (c) is shown as Supplement Figure 3.

### 3.3. Cytokine production in differently polarized macrophages tested with a human cytokine array

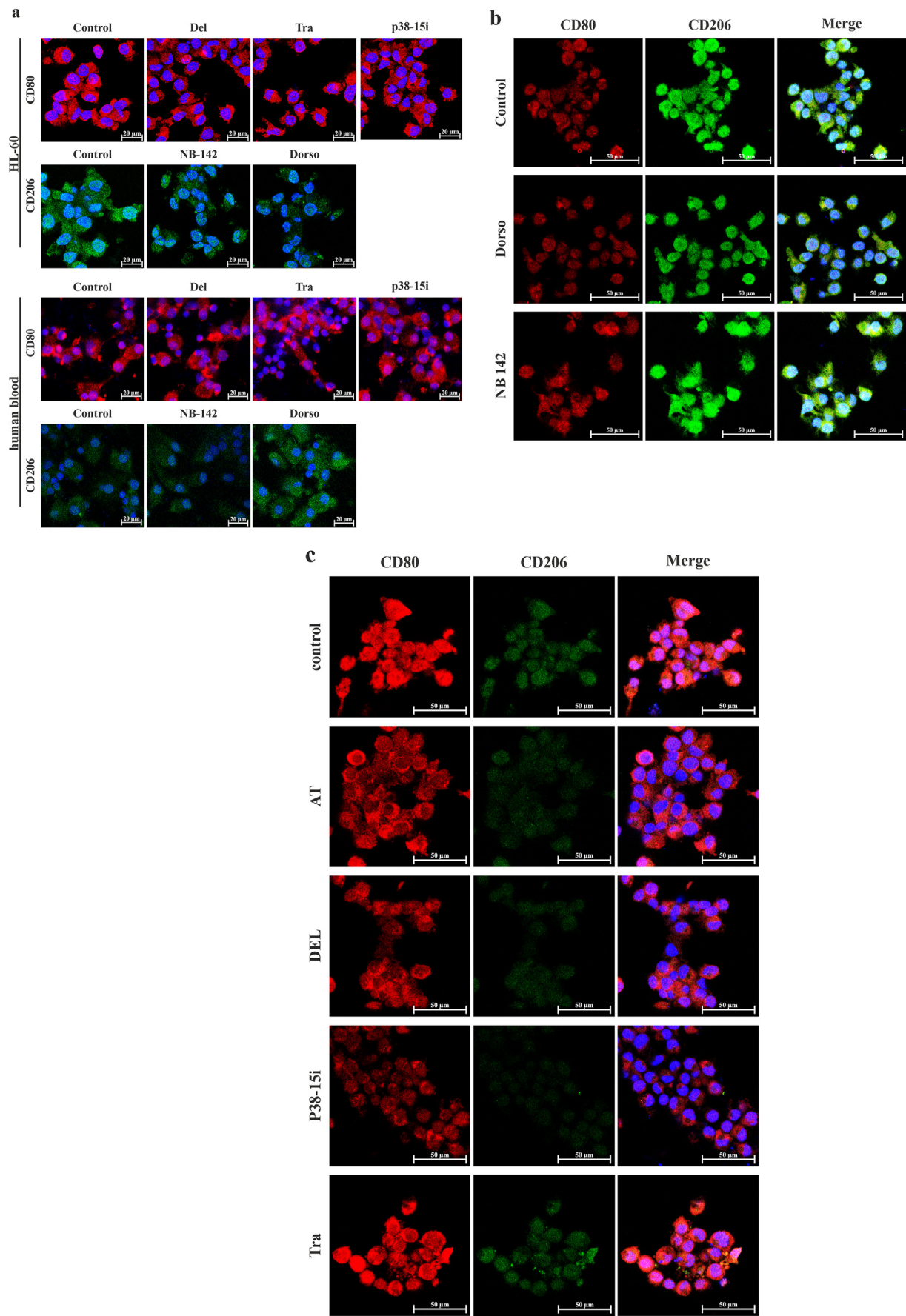
Cytokines are involved in macrophage polarization both as polarizing signals and polarization markers as well. Significant increase of the production of IP-10, IL-1 $\beta$  and TNF- $\alpha$  were detected in the supernatants of

LPS + IFN $\gamma$ -treated cells (high IFN $\gamma$  levels may be due to the IFN $\gamma$  added to the cultures for polarization). On the contrary, CCL2 (MCP-1) levels were not different in control and IL-4 treated samples, but were significantly lower in LPS-IFN $\gamma$ -treated cells. IL-8 levels were higher in IL-4-treated cells compared to LPS + IFN $\gamma$  treatment and to controls as well (Figure 4b). Other differences were also observed, but were not

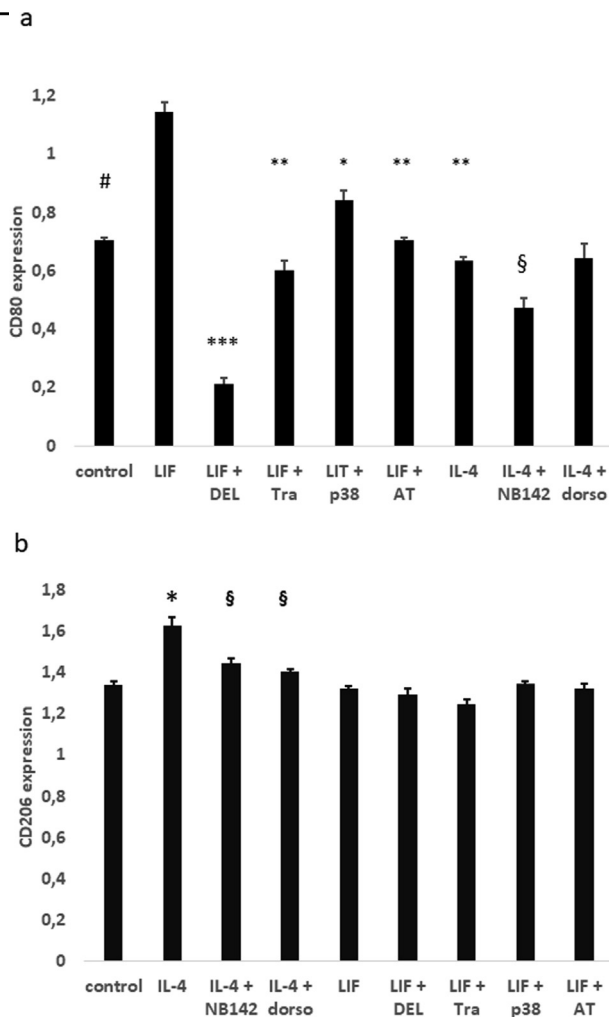
**Table 1.** The effects of protein kinase inhibitors on cell viability.

| Inhibitor     | Target kinase   | 10 nM                                       | 100 nM            | 1 $\mu$ M                                   |
|---------------|-----------------|---------------------------------------------|-------------------|---------------------------------------------|
| DEL 22379     | Erk             | 88.69 $\pm$ 1.08                            | 105.74 $\pm$ 6.11 | 94.25 $\pm$ 0.89<br>99.92 $\pm$ 6.71 (HBM)  |
| Trametinib    | MEK 1/2         | 95.12 $\pm$ 12.05<br>94.57 $\pm$ 6.35 (HBM) | 88.72 $\pm$ 9.60  | 79.30 $\pm$ 6.73*                           |
| p38-15i       | p38             | 117.12 $\pm$ 9.60                           | 106.21 $\pm$ 1.99 | 105.14 $\pm$ 1.99<br>26.3 $\pm$ 7.39 (HBM)  |
| AT 9283       | JAK 1/2/3       | 93.40 $\pm$ 3.84                            | 100.37 $\pm$ 6.34 | 93.93 $\pm$ 1.90<br>92.00 $\pm$ 5.84 (HBM)  |
| NB-142        | PKD             | 84.57 $\pm$ 2.24                            | 98.95 $\pm$ 3.74  | 102.72 $\pm$ 4.30<br>99.23 $\pm$ 7.34 (HBM) |
| Dorsomorphine | AMPK $\alpha$ 2 | 124.99 $\pm$ 1.21*                          | 109.84 $\pm$ 9.30 | 91.52 $\pm$ 2.40<br>124.9 $\pm$ 12.94 (HBM) |

$5 \times 10^4$  PMA-differentiated HL-60 cells were cultured for 72 h; inhibitors were added after 48 h and cell viability has been determined by MTT formazane test. Results are shown as mean  $\pm$  SEM.  $5 \times 10^4$  human blood derived macrophages (HBM) were cultured for 48 h with inhibitors; cell viability has been determined by MTT formazane test. Results are shown as mean  $\pm$  SEM. Cell viabilities are given in % of control (cells without inhibitors). \* $p < 0.05$ , significant differences from control.



(caption on next page)



**Figure 6.** The expression of CD80 (a) and CD206 (b) markers in HL-60 derived macrophages. Cell based ELISA was performed on fixed cells, primary antibodies were used in 1:200 (CD80) and 1:300 (CD206) dilution, secondary antibody (anti-mouse HRPO) was diluted in 1:800. TMB was used as peroxidase substrate, stopped by 2 N sulfuric acid. Values are given in absorbances measured at 450 nm, the aspecific binding of the secondary antibody was subtracted. CD80 is higher in M1 polarized cells and decreased with MAPK and JAK/STAT inhibitors. CD206 is higher in M2 polarized cells, decreased with a PKD and AMPK inhibitor. Repolarization was not observed. a) control compared to LIF  $^{*}p < 0.01$ , inhibitors compared to LIF  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$ , NB 142 compared to IL-4  $^{§}p < 0.01$ . b) IL-4 compared to control  $^{*}p < 0.05$ , NB 142 and dorso compared to IL-4  $^{§}p < 0.05$ .

significant, due to the low levels of cytokines or to the high standard deviation (data not shown).

### 3.4. Viability test to study the direct cytotoxicity of protein kinase inhibitors

In order to exclude the direct cytotoxicity of possible kinase inhibitors, cell viability has been tested at various concentrations by MTT

viability method. Table 1 shows that the selected inhibitors were not significantly toxic for the HL-60 cells even at 1  $\mu$ M concentrations, except trametinib (10 nM), which decreased the cellular protein content in Western blotting experiments even at 10 nM concentration. It is to be noted, that dorsomorphine caused a significant increase in viability at 10 nM, possibly due a higher oxidative metabolic rate in the cells leading to enhanced formazan production. Based on these results, DEL-22379, trametinib, p38-15i, AT9283, NB 142 and dorsomorphine were chosen to investigate their effects on most of the tested polarization markers.

The same kinase inhibitors were also tested for their direct cytotoxicity on blood-derived macrophages at 1  $\mu$ M concentration (except trametinib, at 10 nM). None of these drugs caused any significant cytotoxicity on primary blood macrophages.

### 3.5. The effect of protein kinase inhibitors on CD polarization markers

Since the phosphokinase array test suggested that the Erk 1/2 and p38 phosphorylations were increased in M<sub>LIF</sub> cells, 1  $\mu$ M DEL, p38-15i and 10 nM trametinib (MAPK pathway and p38 inhibitors) were tested for their effect on CD80 polarization marker. None of them changed the occurrence of this marker in M<sub>LIF</sub> cells. Similar experiments were performed on IL-4 treated cells, using NB-142 and dorsomorphine (PKD and AMPK $\alpha$ 2 inhibitors, respectively, at 1  $\mu$ M concentration). The expression of CD206 M2 marker was also not abrogated by these kinase inhibitors, although an insignificant reduction may be observed (Figure 5a). Similar results were obtained for human blood macrophages; in this assay the labeling of CD206 slightly increased in dorsomorphine-treated cells.

The previous experiment was repeated with double labeling. As shown in Figure 5b, kinase inhibitors did not cause a repolarization effect, i.e. the expression of CD206 is not increased, although a small decrease in CD80 labeling may be observed. Similarly, in M2 polarized samples, the corresponding kinase inhibitors caused only an insignificant CD206 reduction without the increase of CD80 marker (Figure 5c).

Since the immunohistochemical experiments produced only qualitative outcomes, a cell based ELISA test was used to obtain more comparable quantitative results for the effects of kinase inhibitors. HL-60 cells were polarized and treated with the selected kinase inhibitors.

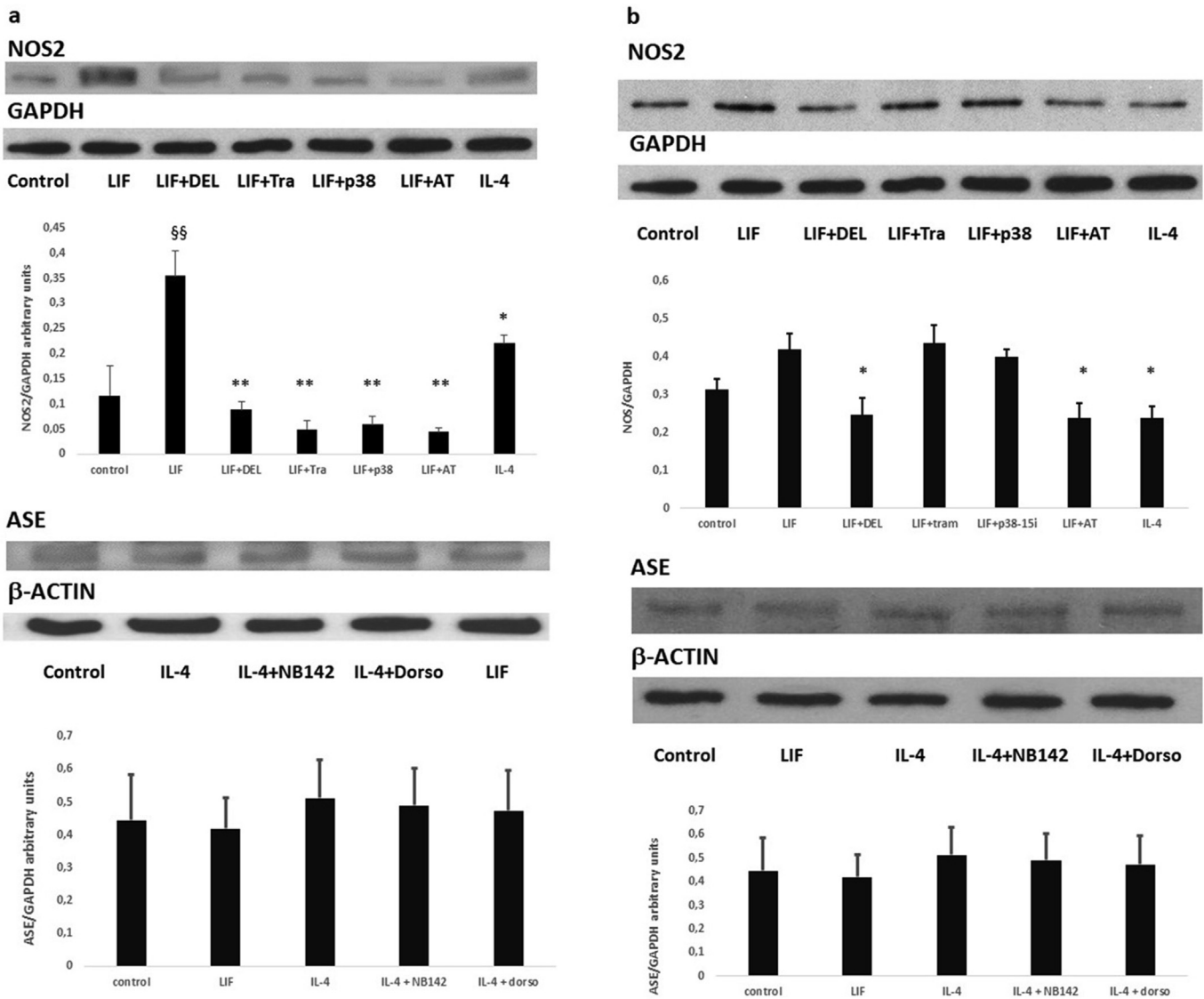
The expression of CD80 markers was substantially higher in M<sub>LIF</sub> cells, and it has been significantly decreased by the selected (MAPK, p38 and JAK/STAT) inhibitors. The lower occurrence of CD80 on IL-4 polarized cells has not been increased by the inhibitors of PKD and AMPK. Therefore, full repolarization could not be observed, only a partial “depolarization” (decreased CD80 expression) occurred.

CD206 markers were less markedly, but significantly higher on IL-4 polarized cells, compared to controls and LIF polarized cells. NB142 and dorsomorphine slightly, but significantly decreased the polarization, while kinase inhibitors of LIF did not cause any effect. Therefore, full repolarization did not occur, but IL-4 polarized cells partially lost their CD206 marker (Figure 6).

### 3.6. Comparison of NOS2 and arginase expressions and activities in HL-60 derived macrophages

The expression of NOS2, a widely accepted marker of M1 polarization has been detected by Western blotting. NOS2 expression was lower than

**Figure 5.** CD 80 and CD 206 differentiation marker expressions in HL-60 derived and human blood macrophages treated with various kinase inhibitors. a: CD80 (marked with red color) and CD206 (marked with green color) polarization markers were investigated by immunocytochemistry, after treating the M1 and M2 polarized macrophages with selected kinase inhibitors. M<sub>LIF</sub> (M1 polarized) samples were treated with 1  $\mu$ M DEL, Tra and p38-15i in the last 24 h during 72 h differentiation period. M<sub>IL4</sub> (M2 polarized) samples were treated with 1  $\mu$ M NB142 and dorsomorphine as described above. Untreated M1 and M2 polarized macrophages were used as positive controls. Cell nuclei were counter-stained with DRAQ5 (blue on merged images). b: Double labeling of M1 polarized (LIF-treated) cells. CD80 marker was labeled by a mouse monoclonal, while CD206 marker was labeled by a rabbit polyclonal antibody. Secondary antibodies were Alexa Fluor 594 conjugated anti-mouse (red) and NL 493 conjugated anti rabbit (green) antibodies. Kinase inhibitors did not cause repolarization, only a slight decrease of the CD80 marker labeling. c: Double labeling of M2 polarized (IL-4 treated) cells. CD80 marker was labeled by a mouse monoclonal, CD206 marker by a rabbit polyclonal antibody. Secondary antibodies were Alexa Fluor 594 conjugated anti-mouse (red) and NL 493 conjugated anti rabbit (green) antibodies. Kinase inhibitors did not cause repolarization, only a slight decrease of the CD206 marker labeling.



**Figure 7.** Expression of NOS2 and arginase1 (ASE) in HL-60 derived (a) and human blood (b) M1 and M2 macrophages. LIF = LPS + IFN $\gamma$  induction. a: Western blot experiment: NOS2 bands (upper samples), GAPDH bands (lower samples); The semi-quantitative evaluation of Western blotting: NOS/GAPDH values are shown, mean values  $\pm$ SEM of six experiments. NOS2 expression is higher in M1 polarization compared to control ( $^{**}p < 0.01$ ). NOS2 expression is higher in M1 compared to M2 polarization ( $^{*}p < 0.05$ ) and each tested inhibitor decreased NOS2 significantly ( $^{**}p < 0.01$ ). Each sample shows low arginase1 (ASE) expression: arginase bands (upper samples),  $\beta$ -actin bands (lower samples); The semi-quantitative evaluation of arginase Western blotting: ASE/ $\beta$ -actin values are shown, mean values  $\pm$ SEM (n = 3). No significant difference was found. The corresponding uncropped file is shown as Supplement Figure 4a. b: NOS2 bands (upper samples), GAPDH bands (lower samples); The semi-quantitative evaluation of Western blotting: NOS/GAPDH values are shown, mean values  $\pm$ SEM of five experiments. The difference between M1 and M2 polarized cells is significant, DEL and AT. inhibitors decreased NOS2 significantly ( $^{*}p < 0.05$ ), ASE expression is very low and not different in M<sub>LIF</sub> and M<sub>IL4</sub> cells. The corresponding uncropped file is shown as Supplement Figure 4b.

expected, nevertheless it was significantly higher in M1 polarized cells compared to M2 samples. Each tested kinase inhibitors (100 nM, except trametinib at 10 nM) significantly decreased the NOS2 expression in LPS + IFN $\gamma$  treated cells. Control (only PMA-treated) cells expressed NOS2 at lower level than other samples (Figure 7a, uncropped version in Supplement Figure 4a).

NOS2 specific activity was low, but detectable without significant differences between M1 and M2 cells. NOS2 specific activity was not significantly altered by Erk, MEK and p38 inhibitors (Table 2) suggesting that the inhibitors decreased the expression and not the activity of the enzyme. This explanation is supported by the lack of direct effects of kinase inhibitors on NOS2 activity (data not shown).

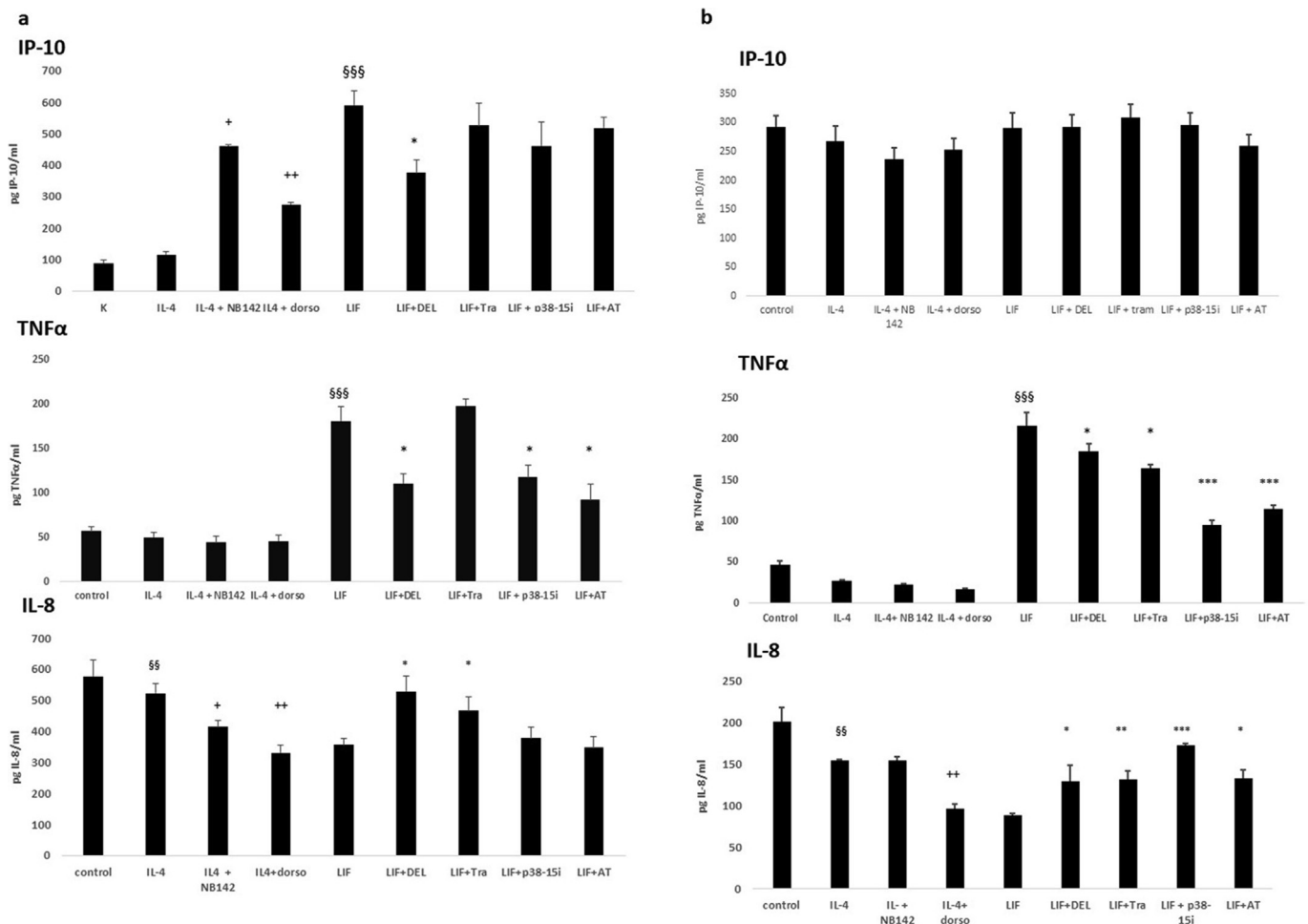
In human blood macrophages, NOS2 expressions were more marked, and were significantly higher in M1 polarized (LIF-treated) samples compared to M2 polarized (M<sub>IL4</sub>) cells. In human blood macrophages,

**Table 2.** NOS2 and ASE specific activities in various HL-60 derived macrophages.

| Sample, treatment        | NOS 2 activity mU/mg* protein | ASE activity mU/mg* protein |
|--------------------------|-------------------------------|-----------------------------|
| control (PMA only)       | 34.3 $\pm$ 7.1                | 17.0 $\pm$ 5.3              |
| LPS + IFN $\gamma$ (LIF) | 44.2 $\pm$ 16.6               | 22.2 $\pm$ 12.7             |
| LIF + DEL                | 50.4 $\pm$ 22.6               | 11.6 $\pm$ 6.1              |
| LIF + Tram               | 32.7 $\pm$ 14.1               | 8.5 $\pm$ 5.3               |
| LIF + p38-15i            | 36.1 $\pm$ 16.1               | 17.3 $\pm$ 12.3             |
| IL-4                     | 26.2 $\pm$ 7.5                | 10.7 $\pm$ 6.5              |
| IL-4 + NB-142            | 31.5 $\pm$ 16.5               | 25.2 $\pm$ 17.8             |

Values are given in mean  $\pm$  S.E.M. No significant differences were found ( $p > 0.05$ , ANOVA), either for NOS2 or ASE specific activity. n = 10, except control, IL-4 (n = 12) and LPS + IFN $\gamma$  (n = 16).

\* 1 mU = 1 nmol citrulline or ornithine per minute.



**Figure 8.** The effects of kinase inhibitors on the cytokine production of M1 and M2 polarized HL-60 derived (a) and human blood (b) macrophages. LIF = LPS + IFN $\gamma$  induction. Significant differences between sample pairs were calculated by Bonferroni posthoc t-test. a. HL-60 derived cells: IP-10 production is linked to M1 polarization (LIF, \$\$\$ $p < 0.001$ , ANOVA) and it was inhibited significantly by 100 nM DEL 22379 (Erk inhibitor, \* $p < 0.05$ ,  $n = 12$ ). At the same time, a significant increase of the low IP-10 production in M2 IL-4 macrophages was observed with 1  $\mu$ M NB142 (PKD inhibitor) and dorsomorphine (AMPK inhibitor), \* $p < 0.05$  and \*\* $p < 0.01$ , respectively,  $n = 8$ . TNF $\alpha$  production is also significantly higher in M1 polarized cells (LIF, \$\$\$ $p < 0.001$ , ANOVA), which could be inhibited significantly with DEL, p38 and AT (\* $p < 0.05$ ,  $n = 12$ ). IL-8 production is linked to M2 polarization (LIF, \$\$\$ $p < 0.001$ , ANOVA, IL-4 is significantly higher compared to LIF (\$\$ $p < 0.01$ ) and it was decreased significantly with NB142 (+ $p < 0.05$ ) and dorsomorphine (\*\* $p < 0.01$ ), while DEL and trametinib caused an increase of the lower level in M1 polarized cells (\* $p < 0.05$ ,  $n = 8$ ). b. Human blood macrophages: IP-10 production was not significantly different between M1 and M2 polarized human blood macrophages ( $p > 0.05$ , ANOVA) and kinase inhibitors did not show any effect. TNF $\alpha$  production is significantly higher in M1 polarized cells (LIF, \$\$\$ $p < 0.001$ ,  $n = 24$ , ANOVA), which could be inhibited significantly with 100 nM p38-15i and AT (\*\*\* $p < 0.001$ ) and less markedly by DEL and Tra (\* $p < 0.05$ ). IL-8 production is significantly higher in M2 polarized cells (IL-4, \$\$\$ $p < 0.01$ ,  $n = 18$ ), and dorsomorphine significantly reduced it (\*\* $p < 0.01$ ). On the other hand, LIF-treated samples are significantly lower than control and IL-4-treated cells (### $p < 0.01$ ) and the kinase inhibitors increased the IL-8 production (Tra and p38-15i \*\* $p < 0.01$ , DEL and AT \* $p < 0.05$ ).

DEL and AT decreased NOS 2 significantly (Figure 7b, uncropped version in Supplement Figure 4b), suggesting the role of both MAPK and JAK/STAT pathways in NOS expression.

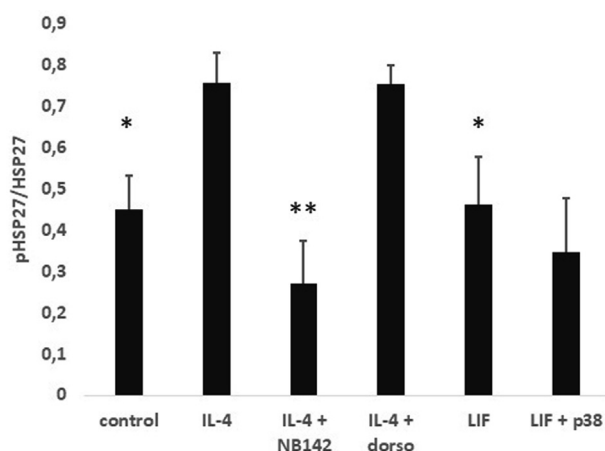
Both in HL-60 derived and human blood macrophages, arginase expression was poorly detected and not significantly different between M1 and M2 cells. Neither NB-142 nor dorsomorphine caused a significant change in IL-4 activated cells (Figure 7a,b). Arginase specific activities were also low and not altered by kinase inhibitors (Table 2).

### 3.7. Quantitative comparison of cytokine production of HL-60 derived and human blood macrophages

Cytokines were measured quantitatively by ELISA kits in the cell culture supernatants. Results are summarized in Figure 8. IP-10 levels

were significantly higher in LPS + IFN $\gamma$  treated HL-60 derived cells (Figure 8a), compared to other macrophages, in accordance with the orientation cytokine array (Figure 4b). Direct inhibition of Erk1/2 by DEL inhibitor caused a significant decrease in IP-10 indicating that Erk1/2 has an important role in its synthesis. On the contrary, the indirect Erk1/2 inhibitors (trametinib via MEK1/2, upstream kinase), the p38 inhibitor and AT 9283 caused only slighter (mathematically non-significant) decreases. IP-10 concentrations were not significantly different between M1 and M2 populations in human blood macrophages, and kinase inhibitors did not show any significant effect on them ( $p > 0.05$ , Figure 8b).

In HL-60 derived macrophages, IP-10 levels significantly increased in IL-4 treated samples when PKD or AMPK inhibitors (NB 142 and dorsomorphine, respectively) were added. This suggests that the inhibitory



**Figure 9.** The effect of kinase inhibitors on HSP27 phosphorylation. The phosphorylation of HSP27 was determined by cell based ELISA. Primary antibodies (anti-pHSP27, anti-HSP27 and anti-GAPDH) were used in 1:200 dilution, while anti-mouse secondary antibody was used in 1:800 dilution. The proportion of pHSP27/HSP27 is shown. IL-4 is significantly higher than control and LIF (\* $p < 0.05$ ), and NB-142 decreased its expression significantly (\* $p < 0.01$ ),  $n = 9$ .

effect of NB 142 and dorsomorphine partially shifts the M2 polarization towards M1. This effect could not be observed in human blood macrophages.

TNF $\alpha$  concentrations were also increased by LPS + IFN $\gamma$  treatment, compared to IL-4. The increased TNF $\alpha$  levels were significantly reduced by DEL (Erk-inhibitor), and p38-15i inhibitor. Moreover, the production of this cytokine was also reduced by AT 9283 (a JAK1/2/3 inhibitor, Figure 8a). Similarly to HL-60 derived macrophages, in human blood macrophages LIF treatment caused a significant increase of TNF $\alpha$  production compared to IL-4-treated macrophages. The inhibitor of p38-15i and AT (JAK inhibitor) decreased significantly the TNF $\alpha$  production of LIF-treated (M1 polarized) cells ( $p < 0.001^{***}$ ), while neither PKD nor AMPK inhibitor caused any significant effect (Figure 8b).

In contrast to the results predicted by the orientation cytokine array, the concentrations of IL-1 $\beta$ , and CCL2 measured by ELISA kits were not significantly different in LPS + IFN $\gamma$  and IL-4-treated samples, and none of the kinase inhibitors caused any effect on their expression (data not shown).

IL-8 levels were significantly higher in IL-4 treated HL-60 cells compared to other samples. Both NB 142 (an inhibitor of PKD) and dorsomorphine (an AMPK inhibitor) significantly decreased its production. The IL-8 level in LPS + IFN $\gamma$  treated HL-60 cells was significantly lower than in IL-4 treated cells. When trametinib and DEL were added, IL-8 levels increased significantly in previously LPS + IFN $\gamma$  treated cells (Figure 8a). Similar results were obtained in human blood macrophages, with the exception that NB-142 inhibitor did not decreased the IL-8 production. At the same time, each tested kinase inhibitor increased the IL-8 production of M1 polarized cells. Therefore, a partial polarization shift could be observed both in HL-60 derived and blood macrophages using various kinase inhibitors: from M2 to M1 by dorsomorphine and from M1 to M2 by DEL, Tra, p38 and AT.

### 3.8. Investigation of the role of HSP27 in the M2 polarization

The decreasing effects of a PKD inhibitor on IL-8 production raised the issue of the involvement of HSP27 protein, suggested by the kinase array (Figure 4a), because PKD is an upstream kinase of HSP27. Both the expression and the phosphorylation of HSP27 were investigated by cell based ELISA. Monoclonal anti-pHSP27 antibody (R & D Systems), anti-HSP27 and anti-GAPDH antibodies (ThermoFisher) were used in 1:200 dilutions, while anti-mouse secondary antibody was used in 1:800

dilutions (secondary antibody backgrounds were used for correction). These results showed that the phosphorylation of HSP27 was higher in IL-4 (M2) cells ( $p < 0.01$ ) compared to controls and LIF-polarized cells. The phosphorylation of HSP27 decreased significantly by the PKD inhibitor NB 142, but not by dorsomorphine (Figure 9). HSP27 phosphorylation of LIF-treated cells did not changed by p38-15i inhibitor. This finding supports the involvement of HSP27 phosphorylation in the IL-4 mediated M2 polarization of HL-60 derived macrophages.

### 3.9. Phagocytic activity of HL-60 derived macrophages

Phagocytic capacity of M1 polarized HL-60 derived macrophages was significantly higher than that of M2 cells ( $p < 0.01^{**}$ ). It was significantly reduced by 10 nM trametinib and 1  $\mu$ M p38-15i inhibitors ( $p < 0.05^*$ , Figure 10a). Phagocytic capacity of IL-4 polarized cells was not altered by the PKD inhibitor NB 142.

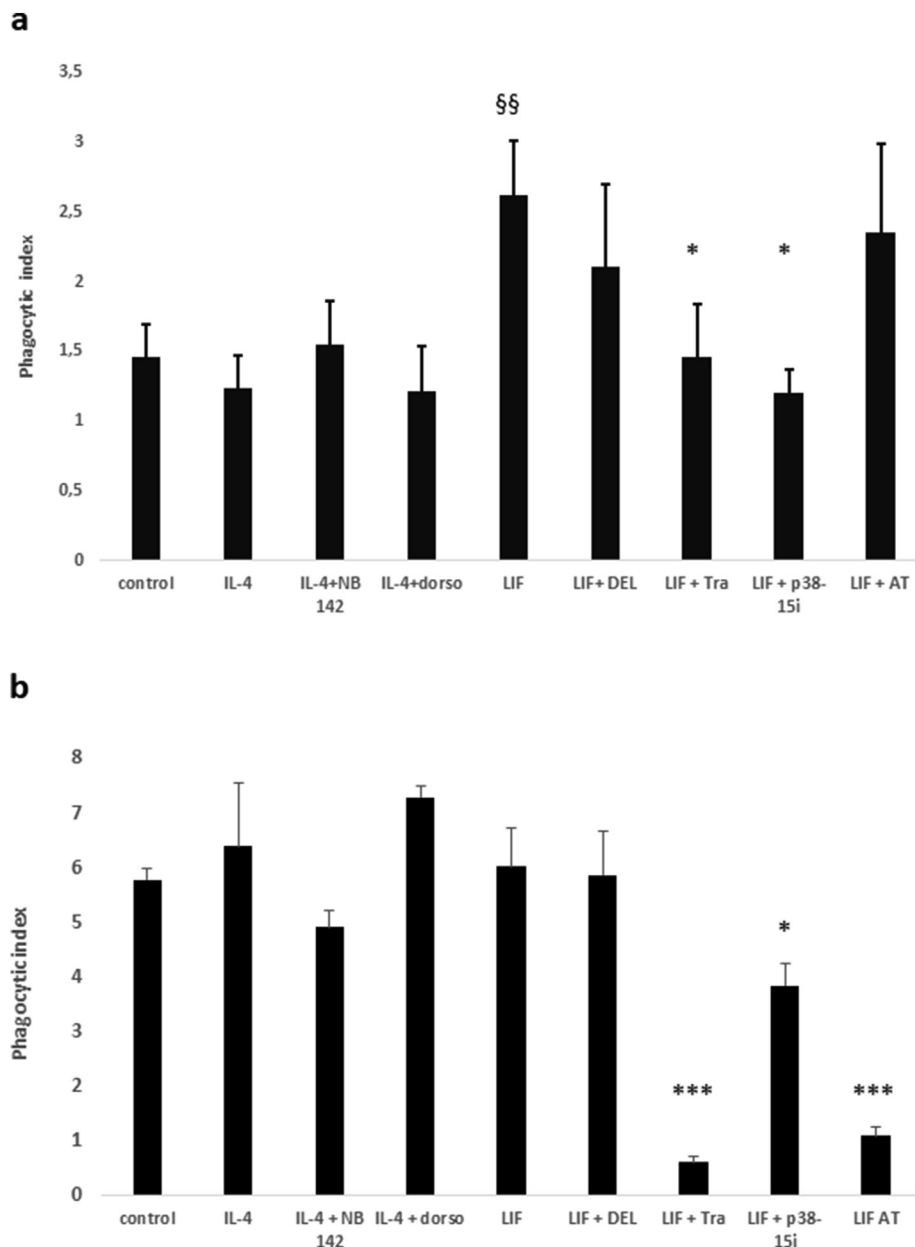
The phagocytic capacity of blood-derived macrophages was higher than that of HL-60 cells. Although the M1 and M2 polarized macrophages were not significantly different in phagocytic capacity, trametinib and AT caused a dramatic decrease of the ingestion of *M. luteus* bacteria ( $p < 0.001^{***}$ ). In addition, the inhibitory effect of p38-15i was also significant ( $p < 0.05^*$ ). For IL-4 polarized cells, none of the tested kinase inhibitors caused significant inhibition (Figure 10b).

Therefore, phagocytosis was decreased only in M1 polarized cells, by MAPK, p38 and JAK pathway inhibitors as well, suggesting the role of both MAPK and JAK/STAT pathways in the C3b-dependent bacterial phagocytosis.

## 4. Discussion

In this manuscript we studied a hypothesis if specific protein kinase inhibitors can modify the macrophage polarization by blocking signaling pathways mediating the polarizing effects. According to the previous studies, the opposite polarization by LPS + IFN- $\gamma$  and IL-4 are mediated by the JAK-STAT pathways, involving the phosphorylation of STAT 1, 2, 3 by JAK1/2 in M1 and STAT6 by JAK2/3 in M2 polarization, respectively [Murray, 2017; Mills et al., 2000; Mantovani, 2006; Mantovani and Sica, 2012]. Our first orientation test with phosphorylation and cytokine arrays (Figure 4) suggested the more important role of MAPK and p38 pathways in HL-60 derived macrophages. The significant increase of the phosphorylation of Erk1/2 and p38 kinases in LPS + IFN $\gamma$  treated (M1) macrophages was accompanied by a concomitant augmentation of the production of inflammatory cytokines (IP-10, TNF $\alpha$  and IL-1 $\beta$ ). Similar results were obtained in other recent investigations [Cheng et al., 2018; Jimenez-Garcia et al., 2015; Yang et al., 2016; Través et al., 2012; Zhang et al., 2019; Islam et al., 2018]. These results supported the involvement of the MAP-kinase pathway. Based on ELISA assays, the production of IP-10 and TNF $\alpha$  was reduced by DEL (Erk1/2 inhibitor) and by trametinib (MEK1/2-inhibitor). In addition, the level of TNF $\alpha$  has also been reduced by the p38-15i inhibitor, and AT9283, a JAK inhibitor (Figure 7). These findings suggest the involvement of both the MAP-kinase and the „canonical“ JAK-STAT pathways in the M1 polarization of HL-60 cell-derived macrophages. This suggestion was supported by the decreasing effect of AT 9283, a JAK inhibitor, on the phosphorylation of STAT3 (Figure 4c). The predominant role of MAPK pathway together with NF- $\kappa$ B in PMA-differentiated HL-60 derived macrophages has been described recently, based on cytokine patterns, in HL-60 cells [Islam et al., 2018]. The inflammatory cytokine pattern of human blood derived macrophages was similar, but somewhat different: while TNF $\alpha$  was significantly higher in M1 polarized cells compared to M2 macrophages, and p38-15i and AT significantly decreased it, IP-10 was not different in M1 and M2 polarized cells and was not reduced by the tested inhibitors.

NOS2 is considered as a typical marker of M1 polarization [Mantovani and Sica, 2012; Lawrence and Natoli, 2011; Martinez et al., 2008], and the glycolytic metabolism of M1 polarized macrophages can be



**Figure 10.** Phagocytic capacity of M1 and M2 polarized HL-60 derived macrophages and human blood derived macrophages. LIF = LPS + IFN $\gamma$  induction. HL-60 cells (a): Differences were significant (\* $p < 0.05$ , paired ANOVA,  $n = 14$ ). Differences between M2 (M<sub>IL4</sub>) and M1 (M<sub>LIF</sub>) macrophages are significant (§§ $p < 0.01$ ). The decreasing effect of p38-15i (1  $\mu$ M) and trametinib (10 nM, \* $p < 0.05$ ) on M<sub>LIF</sub> macrophages are also significant, using two-tailed t-test. Human blood derived macrophages (b): Differences were significant (\*\*\* $p < 0.001$ , paired ANOVA,  $n = 12$ ), but only trametinib (10 nM, \*\*\* $p < 0.001$ ), AT (\*\*\* $p < 0.001$ ) and p38-15i (\* $p < 0.05$ ) were significantly different compared to LPS + IFN $\gamma$  (LIF) treated samples, according to two-tailed t-test. Other inhibitors were applied at 1  $\mu$ M. No significant differences were found between M1 (LPS + IFN $\gamma$ ) and M2 (IL-4) polarized macrophages.

shifted to oxidative M2 type by NOS2 inhibitors [Van den Bossche et al., 2016]. More recently, similar observations were published: NO is responsible for the “break” in the mitochondrial TCA cycle and citrate accumulation in M1 polarization, while the lack of NO can maintain substantial levels of OCR and TCA cycle intermediates [Palmieri et al., 2020a]. Moreover, the pentose phosphate pathway, activated in M1 polarization, can provide NADPH for NOS, while NO and its derivatives cause damages of the components of the mitochondrial electron transport chain [Liu et al., 2021]. It was demonstrated earlier, that NO has dual regulatory effects on iNOS expression in RAW 264.7 cells. When the local concentration of NO is low in the early stages of inflammation, iNOS expression increases. High concentration of NO has the opposite effect, downregulating the proinflammatory response of macrophages. The biphasic activity of NO can facilitate both the initiation of a defense response against pathogenic stimuli and the termination of this response decreasing tissue damage [Connelly et al., 2001].

Although NOS2 expression was higher in M1 cells, compared to M2 macrophages, the expression level of NOS2 in HL-60 derived macrophages was lower than expected. This may be explained by epigenetic

effects: in human inflammatory macrophages, NOS2 gene has been silenced by the methylation of DNA in the promoter region, differently from murine cells [Gross et al., 2014]. In addition, similar effects were observed in other cells, including vascular endothelial cells [Chan et al., 2005], and a HUVEC cell line [Dreger et al., 2016]. The relationship between the DNA methylation of arginase and NOS genes caused by air pollution has been recently demonstrated in children’s asthma [Ji et al., 2021]. On the other side, the loss of CpG methylation in NF- $\kappa$ B enhancer elements of iNOS was found to be responsible for its induction in human chondrocytes [de Andrés et al., 2013].

In both macrophage types, NOS2 expression has been inhibited by the MAPK pathway inhibitors and AT 9283 JAK inhibitor as well (Figure 7). The lack of the effect of the same inhibitors on the specific activity of NOS suggests that the effect of the kinase inhibitors occurs at the level of the NOS2 expression, not directly on the enzyme activity.

Both in HL-60 derived and human blood macrophages, arginase specific activity and expression were very low and not significantly different in M1 and M2 polarization, without any effect of kinase

inhibitors. This finding supported that arginase is considered as an M2 marker only in rodent, but not in human macrophages [Röszer, 2015].

According to the phosphokinase array, the phosphorylation of HSP27, AMPK $\alpha$ 2 and CREB was higher in M2 polarized (IL-4 treated) cells. The involvement of CREB in the mediation of the prostaglandin E<sub>2</sub> effect in M2 polarization has been described recently in various species [Luan et al., 2015; Montero et al., 2016], but only sporadic data are available about the role of HSP27 in M2 polarization [Laudanski et al., 2007]. Since HSP27 is phosphorylated by protein kinase D, the inhibition of the latter may lead to a polarization shift from M2 to M1. The production of IL-8 is higher in M<sub>IL4</sub> macrophages, as shown by the cytokine array (Figure 4b) and reduced by NB 142 PKD inhibitor in M2 polarized cells. The involvement of HSP27 phosphorylation has also been supported by a cell based ELISA test. Dorsomorphine (an AMPK inhibitor) also reduced the production of IL-8 in IL-4 polarized cells (Figure 8); these data support the possible role of PKD and AMPK in M2 polarization [Sag et al., 2008; Li et al., 2015]. Moreover, IL-8 level has been increased in M1 cells treated with the inhibitors of MAPK pathway (DEL and trametinib), suggesting a partial shift to M2 polarization. Similar results were obtained in human blood macrophages, where dorsomorphine reduced the IL-8 production.

According to our results, C3b-dependent phagocytosis of *M. luteus* bacteria is significantly higher in M1 polarized cells in HL-60 derived, but not in human blood macrophages. This is in accordance with most studies, although certain authors described higher phagocytic capacity in M2 polarized cells [Garcia-Garcia and Rosales, 2002; Montero et al., 2016]. The phagocytic capacity of M1 macrophages has been significantly decreased by trametinib, and p38-15i in HL-60 derived cells (Figure 10a), while in human blood macrophages the inhibitory effect of AT was also observed, indicating the involvement of both the MAPK and JAK/STAT pathway in the C3b-dependent phagocytosis (Figure 10b).

The purpose of our studies was to modify the macrophage polarization with kinase inhibitors, influencing their signaling pathways. The repolarization of M2 macrophages would play an essential role in anti-cancer therapy (TAMs are M2 polarized cells [van Dalen et al., 2019; Mantovani et al., 2006]). Other authors could promote M2 polarization, which may be important to attenuate acute inflammations [Labonte et al., 2014; Tran et al., 2015] or to counteract the pro-inflammatory effects leading to obesity and Type 2 diabetes [Ren et al., 2019]. Nitric oxide regulates a metabolic reprogramming in M1 macrophages: NO not only impairs the mitochondrial respiratory system, but also blocks aconitase and pyruvate dehydrogenase directing the metabolism to glycolytic pathway, causing a shortage in TCA cycle metabolites. In this case, the anaplerosis of the cycle is dependent on glutaminolysis. On the other hand, this Warburg effect analogous process cannot be observed in NOS deficient macrophages [Palmieri et al., 2020b]. A more complex metabolism was described in tumor associated macrophages: NO blocks oxidative phosphorylation causing a higher rate of glycolysis and pentose phosphate pathway. This causes a cytotoxic effect of NO and ROS on the target tumor cells. However, in M2 polarization, which is characteristic of TAMs, the arginase pathway is predominant, resulted in the production of polyamines, which may be responsible for tumor proliferation together with an anti-inflammatory response [Nath and Kashfi, 2020]. Although TAMs are considered as M2 macrophages, their glycolytic rate is as high as M1 macrophages, which may be associated with angiogenesis and metastasis in certain tumors [Diskin and Pålsson-McDermott, 2019]. Nevertheless, only sporadic data are available about the trials of selective protein kinase inhibitors to modify the macrophage polarization [Chaudhuri, 2014; De Vries et al., 2019; Ding et al., 2015]. The involvement of the changes in p38-MAPK pathway (including Erk and p38) caused by the SARS-Cov2 infection in Vero 6 cells has recently been published [Bouhaddou et al., 2020]. Therefore, the inhibition of the production of inflammatory cytokines by protein kinase inhibitors may serve as a strategy against the cytokine storm (a suspected etiological factor in the fatal outcome of Covid-19 infections and other inflammatory processes). Nevertheless, this hypothesis requires further experimental studies.

It is to be noted that the results obtained for the same polarization markers in macrophages from various sources may be different. In addition, various markers of M1 and M2 polarized macrophages are varied independently, suggesting that the polarizing effects cannot be linked to only one signaling pathway, rather a more complicated network should be supposed, also involving species differences. A survey of macrophage polarization based on in vitro, in vivo and clinical data, also supports, that macrophage polarization is a more complex phenomenon than explicable by a few polarization markers or signaling pathways, rather a signaling network and a “switch M3 phenotype” can be supposed [Malyshev and Malyshev, 2015].

## 5. Conclusions

M1 and M2 polarization of both blood and HL-60 derived macrophages could be achieved by LPS + IFN $\gamma$  and IL-4 treatment. This was supported by the higher expression of M1-type (CD80, NOS2, inflammatory cytokines as IP-10 or TNF $\alpha$ ) and M2 type (CD206, IL-8) polarization markers in the corresponding cell population both in HL-60 and human blood derived macrophages. Involvement of p38 and MAPK pathways is as important as JAK/STAT pathway in LPS-IFN $\gamma$  polarized blood and HL-60 derived macrophages.

Selected protein kinase inhibitors, which are designed primarily for cancer therapy, can also act on specific signaling pathways in inflammatory processes, decreasing the expression of CD80 and NOS2 and the production of certain pro-inflammatory cytokines. Nevertheless, these inhibitors can cause only a partial “depolarization” (i.e. the decrease in certain polarization markers only), not a full repolarization from M1 to M2 phenotype or vice versa.

Phagocytic capacity, an essential function of macrophages was significantly higher in M1 polarized HL-60 derived cells, and the selected kinase inhibitors decreased the phagocytosis only in this polarization. This suggests the involvement of MAPK and JAK/STAT pathways in the mediation of C3b-dependent bacterial phagocytosis.

Although our hypothesis, that specific protein kinase inhibitors can repolarize macrophages via the inhibition of their signaling pathway has not been fully confirmed, but several markers of M1 polarization was reduced by MAPK, p38 and JAK/STAT inhibitors. These inhibitors may be potential candidates for therapeutical trials against inflammatory diseases.

## Declarations

### Author contribution statement

Gábor Bögel, József Murányi, András Hrabák: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Bálint Szokol, László Örfi: Contributed reagents, materials, analysis tools or data.

Zoltán Kukor: Analyzed and interpreted the data; Wrote the paper.

István Móra: Performed the experiments.

Tamás Kardon: Analyzed and interpreted the data.

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### Data availability statement

Data will be made available on request.

## Declaration of interests statement

The authors declare no conflict of interest.

## Additional information

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