

RESEARCH PAPER

BAY 41-2272, a soluble guanylate cyclase agonist, activates human mononuclear phagocytes

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BACKGROUND AND PURPOSE

Phagocyte function is critical for host defense against infections. Defects in phagocytic function lead to several primary immunodeficiencies characterized by early onset of recurrent and severe infections. In this work, we further investigated the effects of BAY 41-2272, a soluble guanylate cyclase (sGC) agonist, on the activation of human peripheral blood monocytes (PBM) and THP-1 cells.

EXPERIMENTAL APPROACH

THP-1 cells and PBM viability was evaluated by methylthiazolotetrazolium assay; reactive oxygen species production by lucigenin chemiluminescence; gene and protein expression of NADPH oxidase components by qRT-PCR and Western blot analysis, respectively; phagocytosis and microbicidal activity by co-incubation, respectively, with zymosan and *Escherichia coli*; and cytokine release by ELISA.

KEY RESULTS

BAY 41-2272, compared with the untreated group, increased spreading of monocytes by at least 35%, superoxide production by at least 50%, and gp91^{PHOX} and p67^{PHOX} gene expression 20 to 40 times, in both PBM and THP-1 cells. BAY 41-2272 also augmented phagocytosis of zymosan particles threefold compared with control, doubled microbicidal activity against *E. coli* and enhanced the release of TNF- α and IL-12p70 by both PBM and THP-1 cells. Finally, by inhibiting sGC with ODQ, we showed that BAY 41-2272-induced superoxide production and phagocytosis is not dependent exclusively on sGC activation.

CONCLUSIONS AND IMPLICATIONS

In addition to its ability to induce vasorelaxation and its potential application for therapy of vascular diseases, BAY 41-2272 was shown to activate human mononuclear phagocytes. Hence, it is a novel pro-inflammatory drug that may be useful for controlling infections in the immunocompromised host.

Abbreviations

BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine; BHI, brain heart infusion broth; CFU, colony-forming unit; CGD, chronic granulomatous disease; DMSO, dimethyl sulfoxide; EPEC, enteropathogenic *Escherichia coli*; FHS, fresh human serum; IL-12p70, IL-12 subunit p70; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ODQ, 1H-(1,2,4) oxadiazole (4,3- α) quinoxaline-1-one; PBM, peripheral blood monocytes; PBMC, peripheral blood monocyte cell; PMA, phorbol myristate acetate; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase

Introduction

Phagocytes are responsible for the initiation of the immune response and their primary functions are the identification, ingestion (phagocytosis) and elimination of microorganisms, cellular debris and circulating immune complexes (Flannagan *et al.*, 2009). Phagocytes are very important not only as effector cells but also as regulators of inflammatory responses, by releasing cytokines and several other mediators (Aggelidou *et al.*, 2002). Primary defects in phagocytes result in the early onset of recurrent infections that lead to death if not properly managed (Samarghitean *et al.*, 2009).

Chronic granulomatous disease (CGD) is an example of defective phagocytes, where these cells are unable to produce reactive oxygen species (ROS), resulting in flawed microbial killing (Geha *et al.*, 2007). ROS are involved in phagocyte microbicidal mechanisms and are produced by the NADPH oxidase system, which consists of four main components, namely $p47^{PHOX}$ and $p67^{PHOX}$, found in the cytosol; and $p22^{PHOX}$ and $gp91^{PHOX}$, the so-called membrane cytochrome b_{558} . These components are encoded, respectively, by *NCF1*, *NCF2*, *CYBA* and *CYBB* genes (El-Benna *et al.*, 2005). IFN- γ therapy reduces the relative risk of severe infections in 70% in patients with CGD (Ezekowitz *et al.*, 1988; Kobayashi *et al.*, 2008); however, the mechanism involved in this reduction, initially attributed to positive regulation of iNOS (inducible NOS), has not been confirmed (Condino-Neto *et al.*, 1996). Therefore, other mechanisms have been investigated to explain the beneficial effects of IFN- γ in CGD (Condino-Neto and Newburger, 2000).

One of the most biologically relevant actions of NO is its binding to the haeme moiety in the soluble guanylyl cyclase (sGC), leading to intracellular accumulation of the second messenger molecule, cGMP, which, in turn, regulates numerous pathophysiological processes (Pacher *et al.*, 2007). There is extensive literature showing the relevance of NO in the microbicidal mechanisms against several intracellular pathogens in experimental models (Bogdan, 2001). In addition, cGMP has been shown to be a key messenger in many cellular immune processes, such as differentiation, chemotaxis, proliferation and release of soluble mediators (Kobialka and Gorczyca, 2001; Ciuman *et al.*, 2006).

NO-independent sGC activators have emerged as valuable tools for investigating the physiopathology of the NO-sGC-cGMP signalling pathway (Stasch *et al.*, 2001). One of most studied NO-independent sGC stimulator is BAY 41-2272, which is reported to act in synergism with NO to produce anti-aggregating, anti-proliferative and vasodilator effects (Evgenov *et al.*, 2007). Our group has recently demonstrated that BAY 41-2272 increases the production of cGMP, release of superoxide and *CYBB* gene expression in human THP-1 mononuclear cells (Oliveira-Junior *et al.*, 2007). These findings raise a new perspective for the development of new therapies for phagocyte defects and to treat infections by directly activating phagocytes and other cells of the immune system.

The aim of this work was to further evaluate the effects of BAY 41-2272 on the activation of human peripheral blood monocytes (PBM) and THP-1 cells. We specifically investigated the production of superoxide, expression of genes encoding components of the NADPH oxidase system, spread-

ing, phagocytosis, microbicidal activity and cytokine release by these cells, all important indicators of phagocyte function.

Methods

Isolation and culture of human PBM and THP-1 cells

Peripheral blood monocyte cells (PBMC) were obtained from healthy volunteers and were isolated by density-gradient centrifugation over Ficoll-Paque® (Sigma Chemical Co., St. Louis, MO, USA), followed by anti-CD16 antibody-labelled magnetic beads columns for PBM isolation (MACS Separator, Mylteni Biotec, Bergish Gladbach, Germany). The cells were washed three times with PBS and re-suspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA). THP-1 cells obtained from ATCC (Manassas, VA, USA) (human acute monocytic leukaemia cell line) were maintained as previously described (Tsuchiya *et al.*, 1980).

Cell viability

Cell viability was assessed using a method based on the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 5 mg·mL⁻¹) to formazan (Thomazzi *et al.*, 2005). Cells (1×10^6 cells·mL⁻¹, 100 μ L) were treated with either BAY 41-2272 (1.0, 3.0 and 10.0 μ M), phorbol myristate acetate (PMA; 30 and 300 nM) or dimethyl sulfoxide (DMSO, drug vehicle, 0.7%) for either 30 or 120 min at 37°C. Preliminary screening experiments were carried out to select effective drug concentrations not toxic to cells. Previous studies have demonstrated that concentrations of BAY 41-2272 (up to 10.0 μ M) are effective in inducing the production of cGMP and cAMP in mononuclear cells (Oliveira-Junior *et al.*, 2007).

Measurement of superoxide production

Superoxide anion production by PBM and THP-1 cells was measured as described previously (Hatanaka *et al.*, 2006). After a 48 h incubation with or without BAY 41-2272 (1.0 and 3.0 μ M) or IFN- γ (100 U·mL⁻¹), a lucigenin (1 mM)-preloaded cell suspension (1×10^6 cells·mL⁻¹, 100 μ L) was stimulated by adding PMA (30 nM) or IFN- γ (100 U·mL⁻¹). The chemiluminescence was monitored for 2 h using a microplate luminometer reader (EG&G Berthold LB96V, Bad Wildbad, Germany), and the results are expressed as relative light units (RLU). Peak levels of RLU were recorded to calculate the mean of the maximum superoxide production during the observation period.

CYBB, CYBA, NCF1 and NCF2 mRNA expression

mRNA was obtained after lysis of PBM and THP-1 cells (1×10^7 cells·mL⁻¹) previously treated for 48 h with IFN- γ (100 U·mL⁻¹) or BAY 41-2272 (1.0 and 3.0 μ M) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by reverse transcription with Super Script III RT and random hexamers (Invitrogen) to cDNA. For quantitative real-time PCR analysis, SYBR green MasterMix (Applied Biosystems, Foster City,

Table 1

Real-time PCR primers sequences of genes encoding components of NADPH system

Protein/gene	Forward primer	Reverse primer
p22phox (CYBA)	5'-atg tgg gcc aac gaa cag-3'	5'-gta ctc cag cag gca cac aa-3'
gp91phox (CYBB)	5'-ttg tgg aaa ccc tcc tat ga-3'	5'-aaa acc gca cca acc tct c.-3'
p47phox (NCF1)	5'-cct ctt tcc agt gca ttt aag g-3'	5'-gat gtg acg gat gaa ggt gtc-3'
p67phox (NCF2)	5'-cgg aca aga agg act gga ag-3'	5'-gga agt aag cca ctg cca ag-3'
β -actin	5'- tca ccg agc gcg gct-3'	5'-taa tgt cac gca cga ttt ccc-3'

CA, USA) was used in an ABI 5700 thermocycler. All results were normalized to human β -actin. Table 1 shows primers sequence used in the reactions.

p22^{PHOX}, p47^{PHOX} and p67^{PHOX} protein expression by Western blot

PBM were first treated with IFN- γ (100 U·mL⁻¹) or BAY 41-2272 (1.0 and 3.0 μ M) for 48 h. After removal of the medium, cells (1×10^7 cells·mL⁻¹) were washed in PBS and lysed in ice-cold lysis buffer [2% Triton X-100, 10 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM Na₃N, 2 mM EDTA] containing protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 45 min at 4°C. The soluble proteins (20 μ g) were separated by 7.5% SDS-PAGE, and the gel was electrophoretically transferred to nitrocellulose membrane (Hybond-ECL; Amersham, Buckinghamshire, UK) and then probed with monoclonal antibodies for p22^{PHOX}, p47^{PHOX}, p67^{PHOX} or β -actin, according to the method described by Chen *et al.* (2004). The immunoblots were labelled with horseradish-labelled anti-mouse IgG and detected via enhancement with chemiluminescence reagent. The total pixel intensity analyses of the blots were conducted by using ImageJ 1.44 (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; available at <http://rsb.info.nih.gov/ij/index.html>).

gp91^{PHOX} protein expression by flow cytometry

Gp91^{PHOX} expression in PBM (1×10^6 cells·mL⁻¹) treated with PMA (30 nM), IFN- γ (100 U·mL⁻¹) or BAY 41-2272 (1.0 and 3.0 μ M) for 48 h was assessed by using mAbs directed against an external epitope of gp91phox (7D5; Yamauchi *et al.*, 2001). Fluorescence was measured on a FACSCanto II flow cytometry (BD Biosciences, San Diego, CA, USA), and results are expressed as percentage of cells expressing gp91^{PHOX} and mean fluorescence intensity (MFI) values in arbitrary units (au).

Spreading assay

After a 1 or 48 h incubation with BAY 41-2272 (1.0 and 3.0 μ M), IFN- γ (100 U·mL⁻¹) or PMA (30 nM), PBM and THP-1 cells were labelled with a fluorescent dye, calcein-AM (Blease *et al.*, 1999). For spreading assays, 100 μ L of 1×10^6 cells·mL⁻¹ labelled cells were placed over microscope glass slides for 1 h. Analysis was performed using a phase-contrast microscope (Nikon, Tokyo, Japan) at 400 $\times$ magnification. The results are expressed as the percentage of adherent cells from a total of 200 cells.

Phagocytosis assay

After a 48 h treatment with or without BAY 41-2272 (1.0 and 3.0 μ M) or IFN- γ (100 U·mL⁻¹), PBM and THP-1 cells (1×10^6 cells·mL⁻¹) were incubated with zymosan (Sigma; 1×10^7 particles·mL⁻¹) for 2 h and stimulated by adding PMA (30 nM) or IFN- γ (100 U·mL⁻¹). Phagocytosing cells were defined on phase-contrast microscope (Nikon) as cells that internalized two or more particles. Results are expressed as a percentage of phagocytosing cells from a total of 200 cells.

Microbicidal activity

An enteropathogenic *Escherichia coli* (EPEC) strain was used for microbicidal assays. EPEC were cultured overnight in brain heart infusion broth (BHI – Fluka, Bucks, Switzerland) under shaking, at 37°C, and its concentrations were estimated photometrically (an optical density of 1.0 at 600 nm was equivalent to 1×10^8 bacteria·mL⁻¹). The CFU assay was performed as described previously (Bellinati-Pires *et al.*, 1995). A proportion of 1:10 of cells and EPEC, opsonized or not with fresh human serum (FHS), were mixed and incubated at 37°C under shaking for 30, 60, 90 and 120 min, after which cells were lysed with 1.5% Triton X-100. Recovered bacteria were re-suspended in BHI broth and plated on BHI agar for an overnight incubation at 37°C. The colonies were counted and the CFU number was converted to % bacteria survived (Bellinati-Pires *et al.*, 1995). Microbicidal activity was calculated as follows: $100 - [(CFU \text{ at time } t / CFU \text{ at time } 0) \times 100]$ and expressed as % of maximum microbicidal activity (minimum % bacteria survived).

Cytokine production

PBM and THP-1 cells were cultured at 1×10^6 cells·mL⁻¹ into 12-well plates treated with BAY 41-2272 (1.0 and 3.0 μ M) or IFN- γ (100 U·mL⁻¹) for 48 h. Supernatants were collected and assayed by ELISA for IL-12p70 or TNF- α release (BD Biosciences) as described previously (Pitzurra *et al.*, 2000).

BAY 41-2272 actions through sGC pathway

We performed a number of experiments using ODQ [100 μ M 1H-(1,2,4) oxadiazole (4,3- α) quinoxaline-1-one], a specific sGC inhibitor, to investigate the effects of BAY 41-2272 on superoxide production and phagocytosis mediated through the sGC pathway. We established two groups of treatments: (i) cells treated for 2 h together or not with PMA (30 nM), IFN- γ (100 U·mL⁻¹) or BAY 41-2272 (1.0 and 3.0 μ M); (ii) cells

Table 2

Effect of BAY 41-2272 on the viability of PBM or THP-1 cells

Cells	Treatment	MTT reduction (% control)	
		30 min	120 min
PBM	1.0 μ M BAY 41-2272	95.32 $\pm$ 5.41	104.30 $\pm$ 2.10
	3.0 μ M BAY 41-2272	98.62 $\pm$ 2.35	102.80 $\pm$ 0.44
	10.0 μ M BAY 41-2272	98.01 $\pm$ 3.34	99.12 $\pm$ 3.00
	3.0 μ M BAY 41-2272 + 100 U·mL ⁻¹ IFN- γ	72 $\pm$ 1.45	44 $\pm$ 2.65*
	0.7% DMSO	98.75 $\pm$ 2.87	96.07 $\pm$ 3.05
	30 nM PMA	94.03 $\pm$ 3.32	92.13 $\pm$ 2.72
	300 nM PMA	92.10 $\pm$ 0.38	68.45 $\pm$ 2.62*
THP-1	1.0 μ M BAY 41-2272	102.00 $\pm$ 5.20	101.40 $\pm$ 1.96
	3.0 μ M BAY 41-2272	98.05 $\pm$ 0.65	106.85 $\pm$ 2.62
	10.0 μ M BAY 41-2272	97.98 $\pm$ 3.06	105.78 $\pm$ 1.70
	3.0 μ M BAY 41-2272 + 100 U·mL ⁻¹ IFN- γ	78 $\pm$ 2.31	56 $\pm$ 0.20*
	0.7% DMSO	106.44 $\pm$ 2.07	102.05 $\pm$ 4.17
	30 nM PMA	98.45 $\pm$ 2.65	94.76 $\pm$ 3.10
	300 nM PMA	94.65 $\pm$ 2.54	84.33 $\pm$ 2.23*

Human PBM or THP-1 cell suspensions (2×10^6 cells·mL⁻¹) were incubated at 37°C for 30 min or 120 min with BAY 41-2272 (1.0–10.0 μ M) or PMA (30 or 300 nM). Each experiment was carried out in quintuplicate ($n = 5$).

The results are expressed as MTT reduction (% control, mean $\pm$ SEM).

*ANOVA, $P < 0.05$.

pretreated with ODQ together or not with BAY 41-2272 for 48 h before PMA or IFN- γ stimulation. In both treatments, ODQ was given 1 h before treatments to guarantee its binding and inactivation of sGC. After this, we performed the same protocols described above, and analysed superoxide release and phagocytosis.

Data analysis and statistical procedures

Results are expressed as mean $\pm$ SEM of n experiments. Statistical analysis was carried out using a two-way ANOVA followed by the Bonferroni post-test for multiple comparisons (Erceg-Hurn and Miroseovich, 2008). Graphpad Prism version 5.0 (GraphPad, San Diego, CA, USA) was used to perform statistical analysis; results were deemed significant if $P < 0.05$.

Drugs, chemicals reagents and other materials

Human IFN- γ was purchased from R&D Systems (Minneapolis, MN), and PMA from Sigma (St. Louis, MO, USA). BAY 41-2272 was provided by Pharma Research Center, Bayer AG (Wuppertal, Germany).

Results

BAY 41-2272 does not affect cell viability

Since a high rate of toxicity would not be an appropriate feature for a potential treatment, we evaluated the effects of BAY 41-2272 on cell viability. The MTT reduction assay showed that neither 30 nor 120 min exposure of human PBM

or THP-1 cells to BAY 41-2272 (1.0–10.0 μ M) caused any toxic effect (two-way ANOVA, $n = 6$, $P > 0.05$; Table 2). The vehicle used for BAY 41-2272 (0.7% DMSO) also does not affect cell viability.

BAY 41-2272 increases IFN- γ and PMA-induced superoxide production by human PBM and THP-1 cells

As ROS is known to have an important function in cell-mediated microbial death, we assessed whether BAY 41-2272 directly stimulated ROS production, as assayed by lucigenin-chemiluminescence. We used IFN- γ and PMA to stimulate ROS production and enable comparison between these treatments and BAY 41-2272 stimulation. BAY 41-2272 (1.0 and 3.0 μ M) increased the amount of superoxide released by PBM and THP-1 cells after direct stimulation for 2 h (two-way ANOVA, $n = 6$, $P < 0.05$) and also after 48 h with the 3.0 μ M dose. BAY 41-2272 primed the PBM and THP-1 cells so that both IFN- γ and PMA were more potent at releasing superoxide after stimulation for 48 h (two-way ANOVA, $n = 6$, $P < 0.05$; Figure 1A,B).

BAY 41-2272 augments the expression of CYBB and NCF2 genes

The activation of the NADPH oxidase system involves the phosphorylation of p47^{PHOX} and assembly of its components (El-Benna, 2005). In addition, regulation of the activity of the NADPH oxidase system also involves gene expression (Condino-Neto *et al.*, 1998). Therefore, we assessed whether BAY 41-2272 had an effect on CYBB, CYBA, NCF1 and NCF2

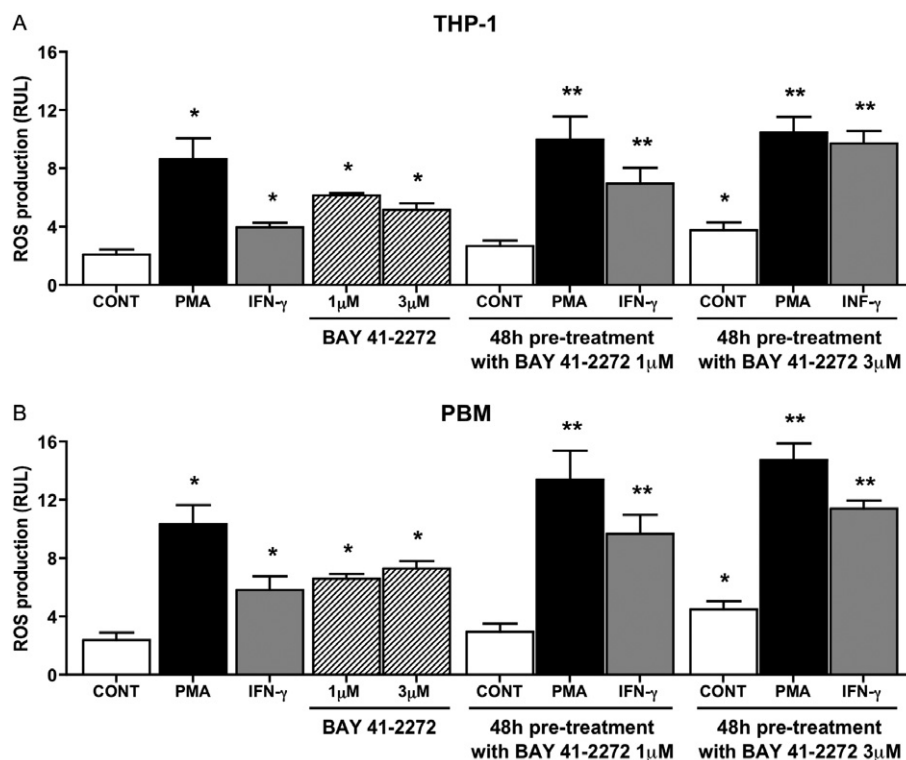


Figure 1

BAY 41-2272 primed THP-1 cells and PBM for a potent release of superoxide after IFN- γ or PMA stimulation. BAY 41-2272 treatment (1.0 or 3.0 μ M) for 2 and 48 h increases superoxide release in THP-1 (A) and PBM (B), as assessed by lucigenin-chemiluminescence assay, and was even more effective with subsequent stimulation with PMA (30 nM) or IFN- γ (100 U·mL⁻¹) for 2 h. Results are expressed in RLU as a mean $\pm$ SEM of maximum production ($n = 6$ healthy volunteers, in triplicate for PBM, or $n = 6$ independent experiments, in triplicate, for THP-1). CONT: no stimulus. * $P < 0.05$, compared with basal production; ** $P < 0.05$, compared with basal and BAY 41-2272 2 h treatment, by two-way ANOVA followed by Bonferroni test.

gene expression. Treatment of PBM and THP-1 cells with BAY 41-2272 for 48 h caused a significant increase in *CYBB* and *NCF2* expression, but not in *CYBA* and *NCF1*, both at 1.0 and 3.0 μ M (two-way ANOVA, $n = 6$, $P < 0.05$; Figure 2) compared with basal gene expression.

BAY 41-2272 increases p67^{PHOX} and gp91^{PHOX} protein expression

As we had demonstrated that BAY 41-2272 increased the levels of expression of *CYBB* and *NCF2*, we evaluated the protein expression of NADPH oxidase components. Our Western blot results confirmed that BAY 41-2272 increased the expression of p67^{PHOX} but no changes in protein expression of p22^{PHOX} and p47^{PHOX} were observed (two-way ANOVA, $n = 4$, $P < 0.05$; Figure 3A). Similarly, the flow cytometry of gp91^{PHOX} showed an increased expression of this protein in groups treated with BAY 41-2272 (two-way ANOVA, $n = 4$, $P < 0.05$; Figure 3B). These results show that gene and protein expression of these components was increased (Figure 2).

BAY 41-2272 increases spreading and phagocytosis

The early defence response of mononuclear phagocytes involves the ingestion of microorganisms and the generation of activation signals by these cells. BAY 41-2272 (1.0 and

3.0 μ M), treatment for 1 h or 48 h, increased the phagocytosis of zymosan induced by PBM and THP-1 cells (two-way ANOVA, $n = 6$, $P < 0.05$; Figure 4B,D). The same pattern of response was observed for spreading (Figure 4A,C).

BAY 41-2272 increases the bactericidal activity against *E. coli*

Phagocytosis and ROS generation by mononuclear phagocytes have been kinetically correlated with increased bactericidal activity (DeLeo *et al.*, 1999). Treatment with BAY 41-2272 (1.0 or 3.0 μ M) produced a significant increase in the bactericidal activity of PBM and THP-1 cells against EPEC (two-way ANOVA, $n = 6$, $P < 0.05$; Figure 5A,B). These results showed even higher significance for opsonized bacteria.

BAY 41-2272 increases the release of TNF- α and IL-12p70

Cytokine production is a major aspect of the immune response and they are involved in both effector and regulatory mechanisms, including mononuclear phagocytes (Geissmann *et al.*, 2008). Culturing PBM and THP-1 cells with BAY 41-2272 (1.0 and 3.0 μ M) for 48 h caused an increase in TNF- α and IL-12p70 production (two-way ANOVA, $n = 6$, $P < 0.05$; Figure 6).

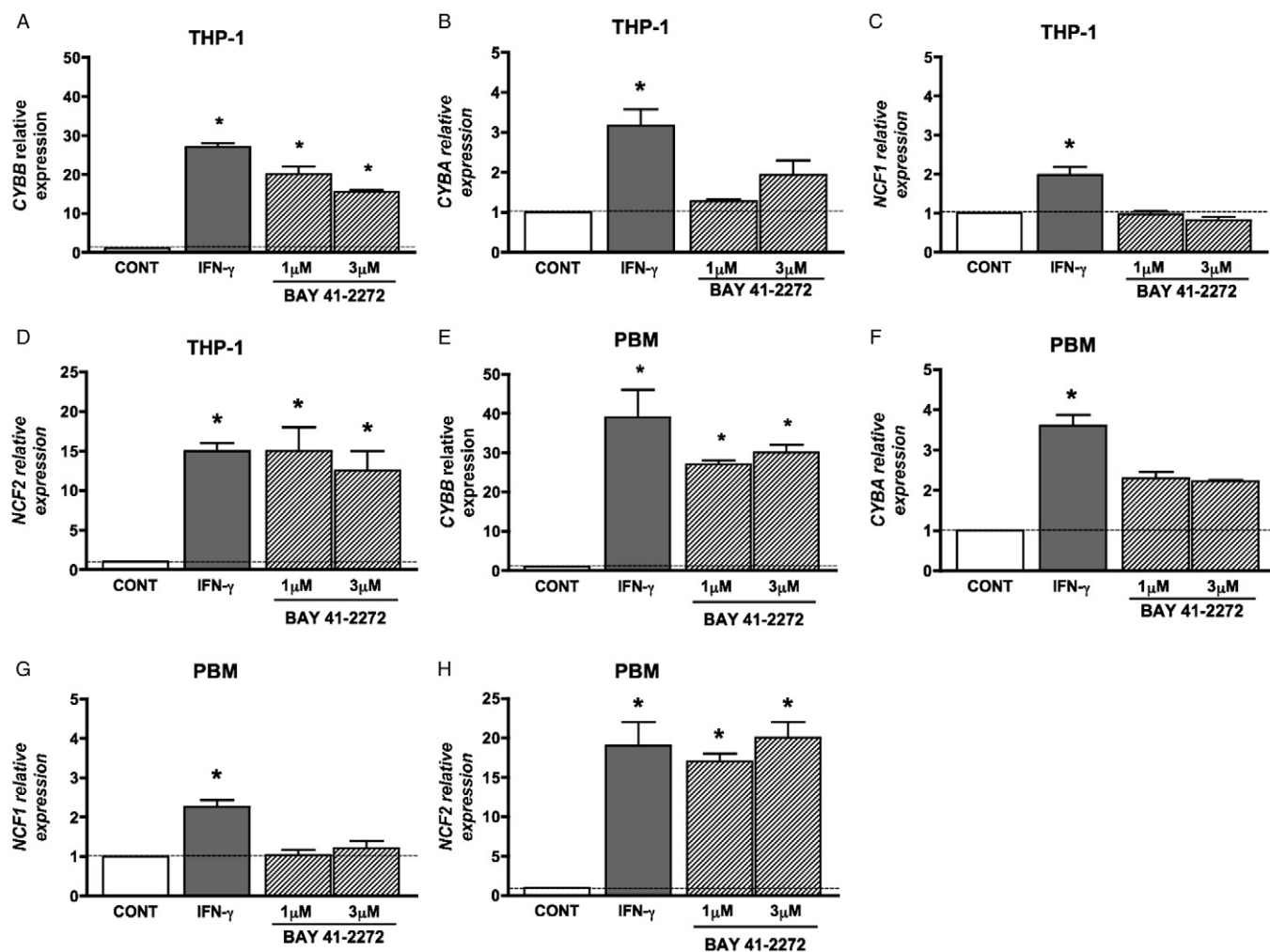


Figure 2

BAY 41-2272 increases *CYBB* (gp91^{PHOX}) and *NCF2* (p67^{PHOX}), but not *CYBA* (p22^{PHOX}) or *NCF1* (p47^{PHOX}), expression in THP-1 cells (A–B–C–D) and PBM (E–F–G–H). THP-1 (A–B–C–D) and PBM (E–F–G–H) treated with BAY 41-2272 (1.0 or 3.0 μ M) increases *CYBB* and *NCF2* expression levels after 48 h, quantified by qRT-PCR normalized to β -actin. BAY 41-2272 was less effective than IFN- γ (100 U·mL⁻¹) treatment for the same time. Dashed line represents expression of genes in untreated cells. Results are shown as mean $\pm$ SEM of expression relative to untreated group ($n = 6$ healthy volunteers, in triplicate for PBM, or $n = 6$ independent experiments, in triplicate for THP-1). * $P < 0.05$, compared with basal expression, by two-way ANOVA followed by Bonferroni test.

BAY 41-2272 produces effects on PBM and THP-1 cells through sGC pathway, specifically inhibited by ODQ

To clarify the pathways involved in the effects of BAY 41-2272, we inhibited sGC with ODQ to evaluate the specific interaction of the drug with this enzyme. We observed that treatment with ODQ did not result in a significant reduction of BAY 41-2272 effects on the parameters investigated, even when cells were treated with the higher dose of the compound (3 mM). With regard to the production of ROS (two-way ANOVA, $n = 6$, $P < 0.05$; Figure 7), the groups treated with BAY 41-2272 plus ODQ did not show a significant reduction in ROS production in any of the concentrations used. Even when we did the pretreatment for 48 h, in the absence or presence of ODQ, BAY 41-2272 still showed considerable effects on ROS production (two-way ANOVA, $n = 6$, $P < 0.05$;

Figure 7C,D). Similar results were found for phagocytosis (two-way ANOVA, $n = 6$, $P < 0.05$; Figure 8), using the same incubation protocol.

Discussion and conclusions

The present study clearly demonstrates that 2 or 48 h treatment with BAY 41-2272 (1.0 or 3.0 μ M) caused a marked increase in the activation of PBM and THP-1 cells, as demonstrated by induction of oxidative burst, increased phagocytosis and microbicidal activity, enhancement of IL-12p70 and TNF- α release, and also increased expression of *CYBB* and *NCF2* genes in these cells. These observations support the hypothesis that BAY 41-2272 activates mononuclear phagocytes and has the potential to be used in the control of infections in the immunocompromised host.

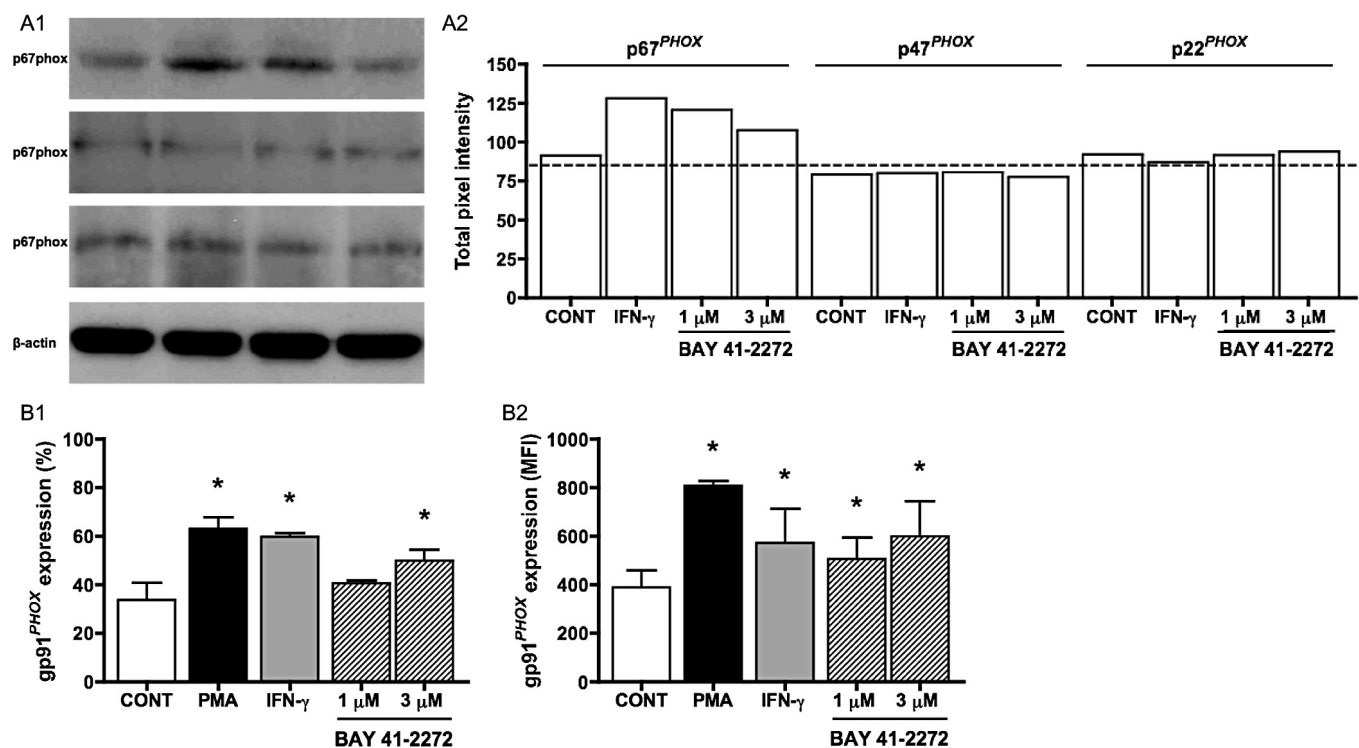


Figure 3

BAY 41-2272 increases p67^{PHOX} and gp91^{PHOX} protein expression, but not p22^{PHOX} and p47^{PHOX}. PBM (1×10^7 cells·mL⁻¹ for Western blot and 1×10^6 cells·mL⁻¹ for flow cytometry) treated with BAY 41-2272 (1.0 or 3.0 μM) increased p67^{PHOX} (A) and gp91^{PHOX} (B) expression after 48 h, as quantified by Western blot (normalized to β-actin) and flow cytometry, respectively. Cells were also treated with IFN-γ (100 U·mL⁻¹) and PMA (30 nM for flow cytometry). Dashed line represents basal expression normalized to β-actin expression. For Western blot, results are shown as a representative blot figure and as total pixel intensity of the blots. For flow cytometry, results are shown as mean ± SEM of percentage of cells expressing gp91^{PHOX} or mean ± SEM of MFI ($n = 4$ healthy volunteers, in triplicate). * $P < 0.05$, compared to basal protein expression, by two-way ANOVA followed by Bonferroni test.

ROS have an important function in cell-mediated microbial death and are an important indicator of monocytic cell activation (Huang *et al.*, 2008). We used IFN-γ (a receptor-mediated activator) and PMA (a direct PKC activator) to stimulate ROS production as positive controls and, after treatment with BAY 41-2272, to test its potential for priming cells. We observed that BAY 41-2272 induced a marked increase in superoxide production, which may be related to PKC stimulation of the sGC-cGMP signalling pathway (Khare *et al.*, 1994; Aggelidou *et al.*, 2002). PKC promotes 'phagocyte oxidase' phosphorylation and activation of the NADPH oxidase system (Ueyama *et al.*, 2007). Moreover, activation of PKC may explain the high response after just 2 h of treatment, since an increase in gene expression after this short time is not likely to occur for most genes, including the NADPH oxidase-related genes and proteins (Newburger *et al.*, 1988).

As extensively described, activation of the NADPH oxidase system involves phosphorylation and clustering of proteins in membranes, but the maintenance of the response depends on the expression of new NADPH oxidase components (Quinn *et al.*, 2006). Our results show that BAY 41-2272 markedly increases in the expression of *CYBB* and *NCF2*. Moreover, the protein expression of the NADPH oxidase components (p67^{PHOX} and gp91^{PHOX}) was also increased, confirm-

ing the data obtained by qRT-PCR. These results are similar to those found by Newburger *et al.* (1991), who showed an increase in the expression of these genes by up to 12 times after 24 h incubation of PBM with IFN-γ and also, similarly, Condino-Neto *et al.* (1998) found that IFN-γ increased the expression of *CYBB* in THP-1 cells. These results suggest that BAY 41-2272 stimulates not only signalling pathways but also important transcriptional factors related to the expression of *CYBB* and *NCF2* genes, such as STAB1, CDP (Hawkins *et al.*, 2001) and Pbx1, constitutively expressed in THP-1 (Skalnik and Neufeld, 1992; Eklund *et al.*, 2000); HAF-1 (Newburger *et al.*, 1994; Suzuki *et al.*, 1998); and PU.1/IRF1/ICSBP/CBP (Eklund and Kakar, 1999). Recently, Luengo-Blanco *et al.* (2008) reported that binding of NF-κB transcription factor to *CYBB* is important for NADPH oxidase function. Since there was a marked increased production of TNF-α, which is dependent on NF-κB activation (Ci *et al.*, 2008), we suggest that BAY 41-2272 may activate the signalling pathways involved in NF-κB activation.

In addition to ROS release, phagocytosis is an essential function that should be analysed in phagocytes. Signal pathways activated by BAY 41-2272 are involved in cGMP generation and are linked to morphological changes required for migration, phagocytosis and spreading (Borán and García, 2007). We showed that BAY 41-2272 increased phagocytosis

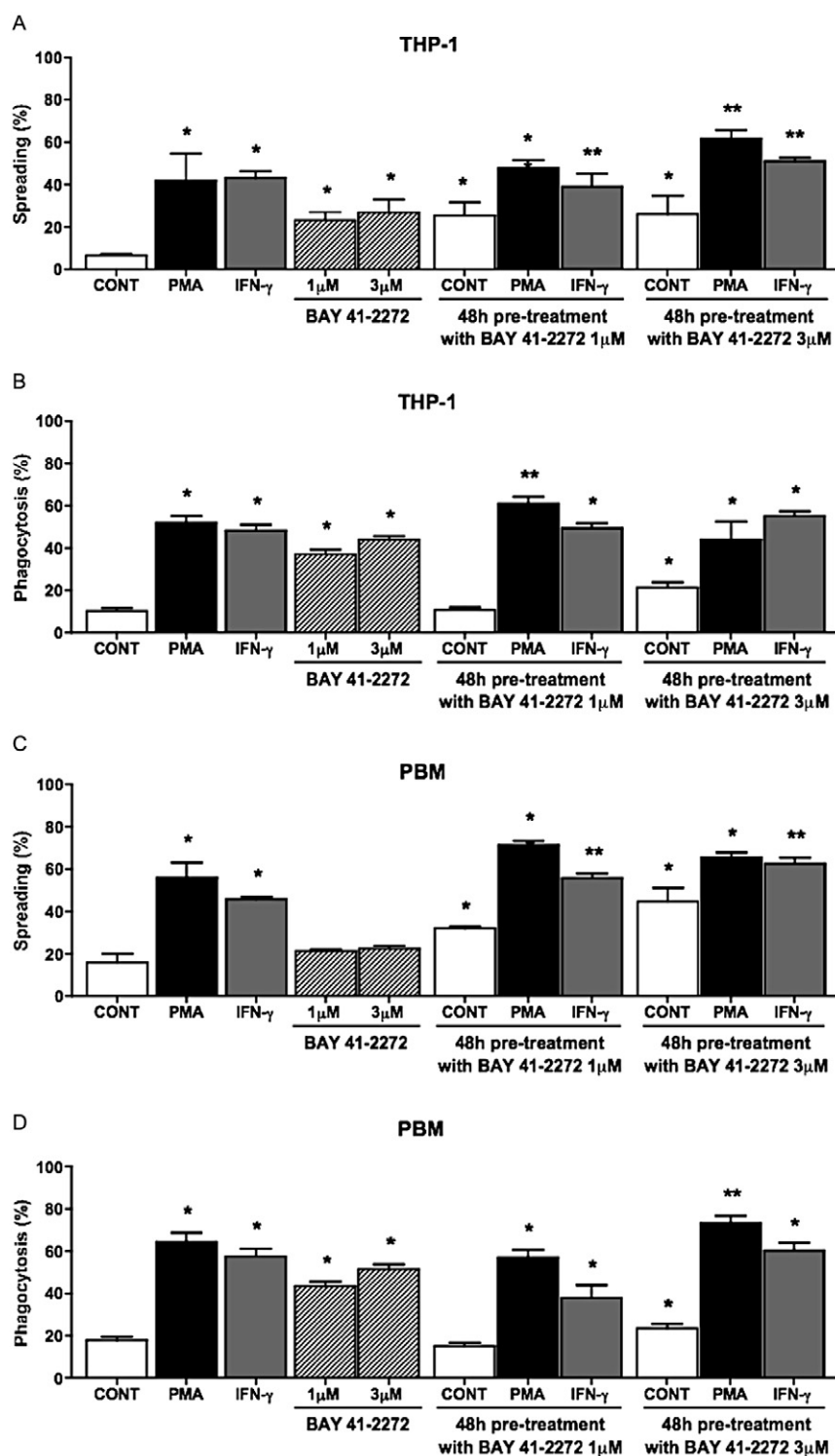


Figure 4

BAY 41-2272 increases spreading and phagocytosis of THP-1 cells and PBM. THP-1 (A, B) and PBM (C, D) (1×10^6 cells·mL⁻¹) pretreated with BAY 41-2272 for 48 h increased the spreading and phagocytic activity against zymosan. BAY 41-2272 (1.0 or 3.0 μ M) treatment was even more effective when PMA (30 nM) and IFN- γ (100 U·mL⁻¹) were added for 2 h after pretreatment. Number of spreading or phagocytosing cells is expressed as a percentage $\pm$ SEM of 200 total cells counted ($n = 6$ healthy volunteers, in triplicate for PBM, $n = 6$ independent experiments for THP-1). * $P < 0.05$, compared with basal (spreading or phagocytosis); ** $P < 0.05$, compared to cells not pretreated, by two-way ANOVA followed by Bonferroni test.

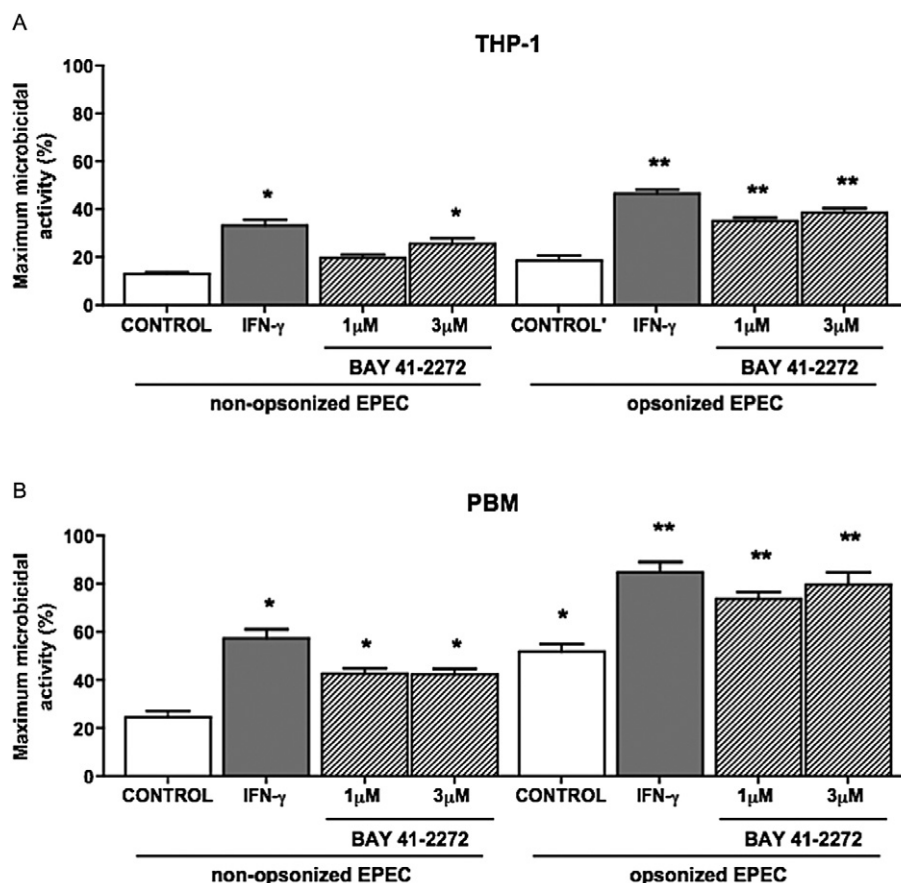


Figure 5

BAY 41-2272 increases microbicidal activity of THP-1 cells and PBM. Pretreatment of THP-1 (A) and PBM (B) (1×10^6 cells·mL⁻¹) with BAY 41-2272 (1.0 or 3.0 μ M) for 48 h increased their microbicidal activity against EPEC, assessed by CFU counting from recovering internalized bacteria after co-culture. As positive control, cells were treated with IFN- γ (100 U·mL⁻¹) for 48 h. Microbicidal activity is expressed as percentage $\pm$ SEM of maximum killing (minimum bacterial survival) ($n = 6$ healthy volunteers, in triplicate for PBM, $n = 6$ independent experiments, in triplicate for THP-1). * $P < 0.05$, compared with basal bacterial activity; ** $P < 0.05$, compared with cells in same treatment condition, cultured with bacteria opsonized or not, by two-way ANOVA followed by Bonferroni test.

and this can be explained by PKG activation, as this was recently shown by Bóran *et al.* (2008) to promote a rapid release and expansion of filaments in microglia cells and increase in the phagocytosis function after treatment with ANP (another sGC stimulator) and dibutyryl-cGMP (permeable analogue of cGMP).

The phagocytosis activity and oxidative burst by mononuclear phagocytes have been kinetically correlated with increased bactericidal activity. It is recognized that ROS are highly toxic to bacteria such as *E. coli* (Nauseef, 2007), and this increase is probably related to the amount of oxidative burst and phagocytosis. However, other important parameters such as acidification of phagosome pH and release of lysosomal enzymes and granules (Ismail *et al.*, 2002) have not yet been evaluated. Therefore, we cannot exclude a possible involvement of other factors such as the expression of surface molecules like Fc and complement receptors, making the phagocytosis more efficient and generating other signals that increase the phagocyte killing (Jongstra-Bilen *et al.*, 2003; Luo *et al.*, 2006; Rhein *et al.*, 2008).

All these effects may be related to cytokine production involved with cellular activation and enhancement of the immune response. According to our expectations, treatment with BAY 41-2272 generated an increase in IL-12p70 release, since activated monocytes and macrophages produced high levels of these cytokines (Murray *et al.*, 2006). BAY 41-2272 also promoted an increased production of TNF- α , strongly suggesting that this drug can activate MAPKs or NF- κ B signalling pathways (Igbal and Zaidi, 2008). In addition, TNF- α production is thought to act like an autocrine, signalling cell activation by acting on the TNF- α receptor in monocyte cells (Deering and Orange, 2006).

Since we observed a wide spectrum of functions activated in treated phagocytes, we wondered whether all these effects of BAY 41-2272 resulted from an effect involving the sGC cyclase pathway. We observed that treatment with ODQ, a sGC specific inhibitor, did not result in a significant reduction of BAY 41-2272-induced superoxide production and phagocytosis. These results show that the effects of the compound are not related only to the sGC

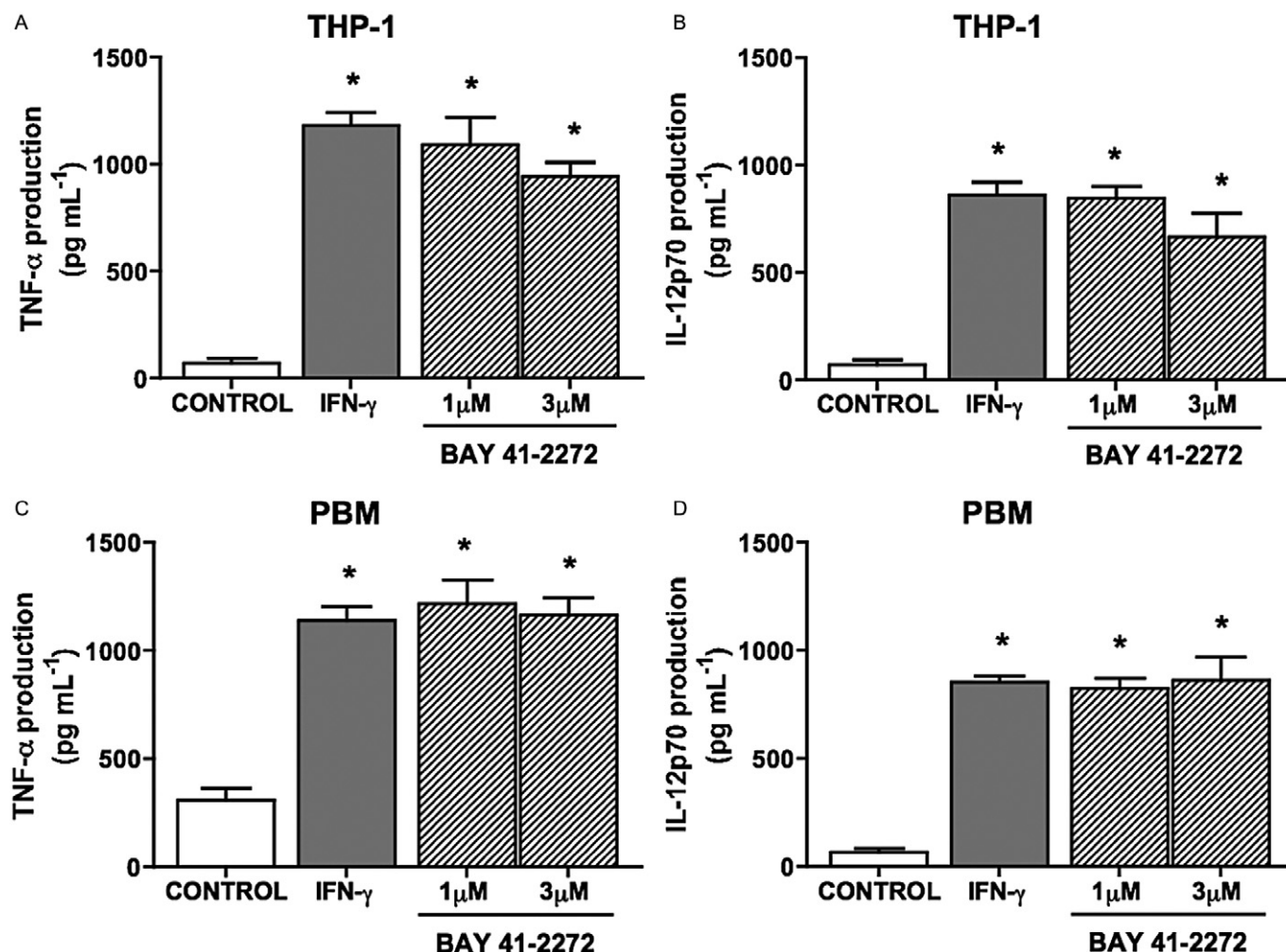


Figure 6

BAY 41-2272 increases the production of IL-12p70 and TNF- α by THP-1 cells and PBM. THP-1 (A,B) and PBM (C,D) cells (1×10^6 cells·mL⁻¹) pretreated with BAY 41-2272 (1.0 or 3.0 μ M) for 48 h increased TNF- α and IL-12p70 production. As positive control, cells were treated with IFN- γ (100 U·mL⁻¹) for 48 h. Results are expressed as mean $\pm$ SEM ($n = 6$ healthy volunteers, in triplicate for PBM, $n = 6$ independent experiments, in triplicate for THP-1). * $P < 0.05$, compared with basal cytokine production, by one-way ANOVA followed by Bonferroni post-test.

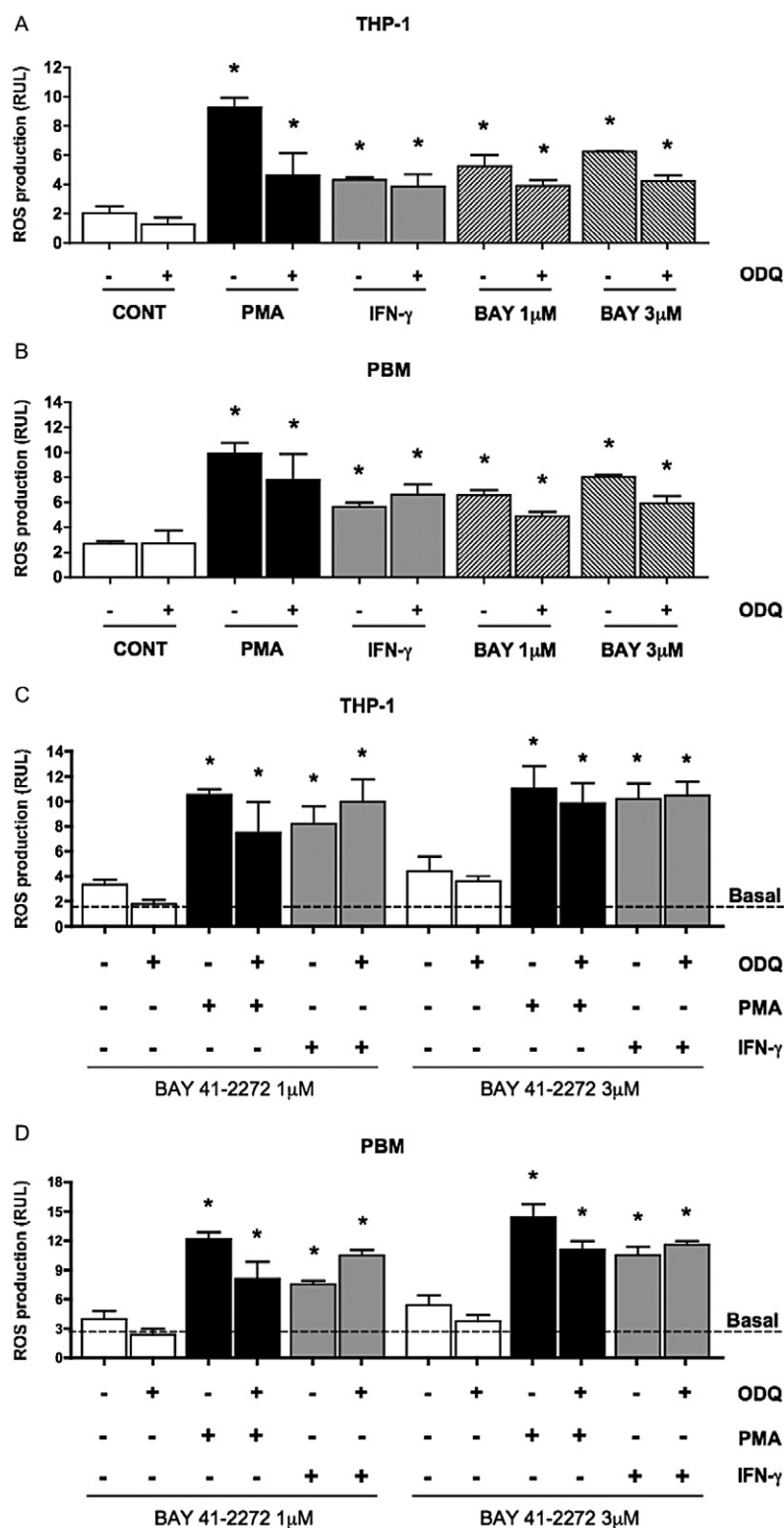
cyclase pathway, presenting activity on other signalling pathways.

Oliveira-Junior *et al.* (2007) showed that BAY 41-2272 is able to generate an increased production of cAMP, another important second messenger. Based on this, we suggest that other important pathways are activated by this compound. This increased production of cAMP could be due to a direct effect of the drug on the enzyme adenylate cyclase or an indirect effect through the production of cGMP. In addition to this hypothesis, Derbyshire *et al.* (2009) showed that the catalytic domains of GCs contain a catalytic and allosteric site capable of binding to both GTP and ATP nucleotides. This same group showed that the action of YC-1, a BAY 41-2272 analogue, leads to the production of cGMP and cAMP. This another alternative action of the drug, beyond the pathway involving sGC-cGMP-PKG.

Based on our results using ODQ and the results found by Koglin *et al.* (2002), showing BAY 41-2272 binding

sites at two isoforms of sGC, we started to perform an *in silico* analysis of proteins with potential binding sites for BAY 41-2272. In our initial analysis, we found four proteins with potential binding sites for this compound and functions related to differentiation/activation of myelomonocytic cells (data not shown). These results open a new way of thinking for BAY 41-2272 analysis and new pathways involved in the activation of human myelomonocytic cells.

In summary, our present study consistently demonstrated that BAY 41-2272 activates human mononuclear phagocytes and has a great potential to control infections. However, the cardiovascular effects of BAY 41-2272 limit its clinical application, and this should be addressed in future in *in vivo* experimental and pre-clinical studies. Moreover, additional studies to clarify the signalling pathways involved in phagocyte activation will help to elucidate and modulate the effects of BAY 41-2272 both in the cardiovascular and immune

**Figure 7**

BAY 41-2272 affects superoxide production in a manner not exclusively dependent on the sGC pathway. BAY 41-2272 treatment (1.0 or 3.0 μ M) for 2 h (A,B) and 48 h (C,D) increased superoxide release in PBM and THP-1 cells (1×10^6 cells·mL⁻¹), as assessed by lucigenin-chemiluminescence assay, even when sGC was specifically inhibited with ODQ (100 μ M). We also treated cells with PMA (30 nM) or IFN- γ (100 U·mL⁻¹) for 2 h, as controls. Dashed line represents expression of genes in untreated cells. Results are expressed in RLU as a mean $\pm$ SEM of maximum production ($n = 4$ healthy volunteers, in triplicate for PBM, or $n = 4$ independent experiments, in triplicate, for THP-1). * $P < 0.05$, compared with basal production by two-way ANOVA followed by Bonferroni test.

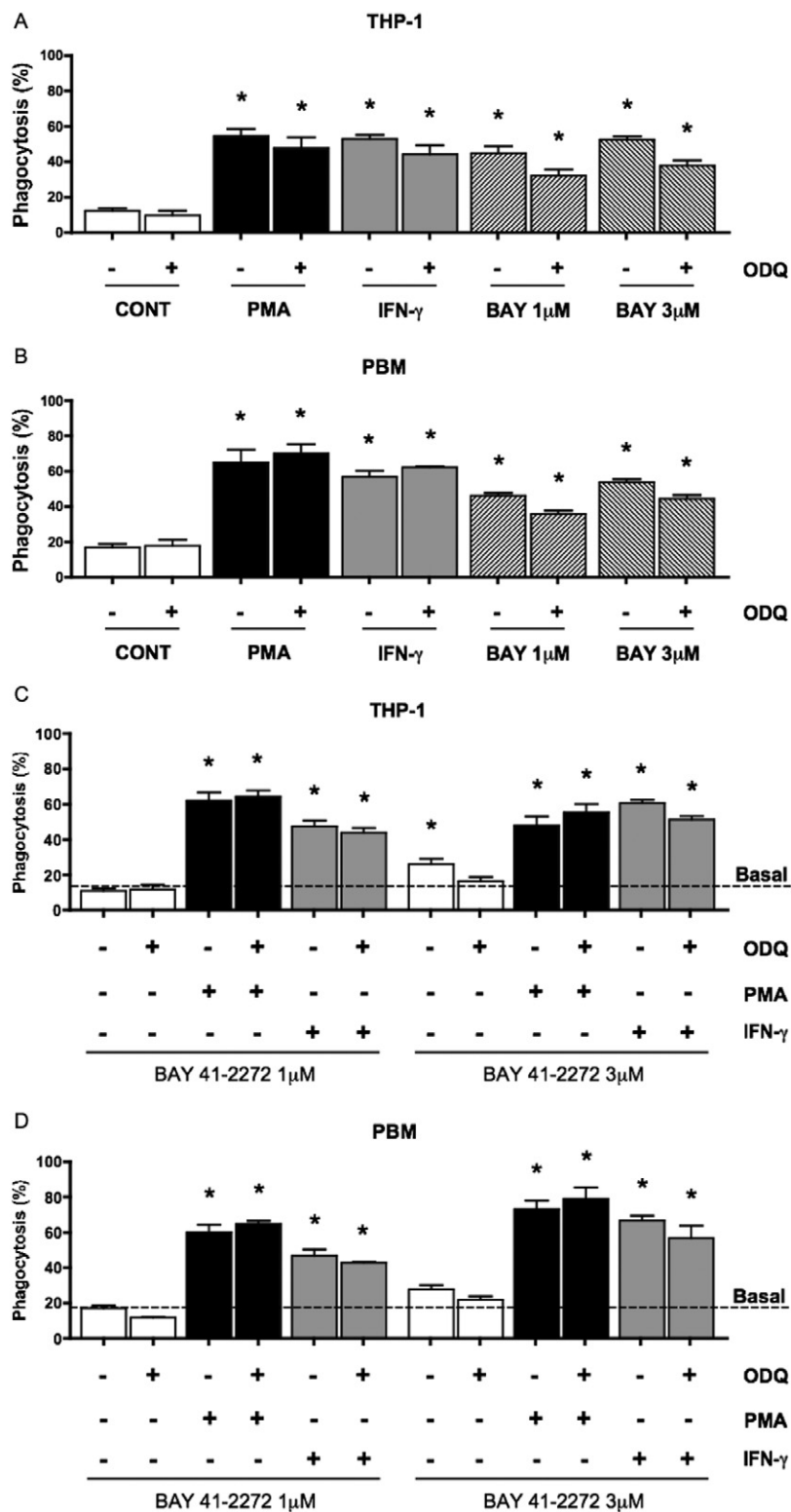


Figure 8

BAY 41-2272 affects phagocytosis in a manner not exclusively dependent on the sGC pathway. BAY 41-2272 treatment (1.0 or 3.0 μM) for 2 h (A,B) and 48 h (C,D) increases phagocytic activity of PBM and THP-1 cells (1×10^6 cells·mL⁻¹) against zymosan, even when sGC was specifically inhibited with ODQ (100 μM). We also treated cells with PMA (30 nM) or IFN- γ (100 U·mL⁻¹) for 2 h, as controls. Dashed line represents expression of genes in untreated cells. Number of phagocytosing cells is expressed as a percentage $\pm$ SEM of 200 total cells counted ($n = 4$ healthy volunteers, in triplicate for PBM, $n = 4$ independent experiments for THP-1). * $P < 0.05$, compared with basal spreading or phagocytosis; ** $P < 0.05$, compared to cells not pretreated cells, by two-way ANOVA followed by Bonferroni test.

systems. In addition, the effects of BAY 41-2272 in other immune cells, such as lymphocytes, are also needed to elucidate how this drug works and its potential applications for the immunocompromised host.

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Conflicts of interest

None.

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