International Immunopharmacology 77 (2019) 105976

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

BAY 41-2272 inhibits human T lymphocyte functions

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ARTICLE INFO

Keywords:
BAY 41-2272
cGMP
sGC
Human T lymphocytes

ABSTRACT

BAY 41-2272 increases guanosine 3′, 5′-cyclic monophosphate (cGMP) levels by stimulating soluble guanylate cyclase (sGC). In this study, we evaluated the effect of BAY 41-2272 on human T lymphocyte functions. Pretreating T cells for 24 h with BAY 41-2272 at 3 μM and 30 μM, followed by activation with 90 nM phorbol myristate acetate (PMA), inhibited interferon-gamma (IFN-γ) production, with 3 μM and 30 μM BAY causing 16.5-fold and 12.1-fold inhibition, respectively, compared to PMA alone ($p < 0.05$, one-way ANOVA followed by Tukey's test). We also observed suppressive effects on the expression of CD69, with 30 μM BAY causing 3.55-fold lower expression than PMA/ionomycin ($p < 0.001$ one-way ANOVA followed by Tukey's test), and T-bet, with 30 μM BAY causing 1.47-fold lower expression than PMA/ionomycin ($p < 0.05$, one-way ANOVA test followed by Tukey's test). Additionally, T lymphocyte proliferation was reduced 2.13-fold and 4.3-fold, respectively, by 3 μM BAY and 30 μM BAY compared to PMA/ionomycin ($p < 0.01$, $p < 0.001$, one-way ANOVA followed by Tukey's test). BAY 41-2272 inhibits human T lymphocyte function and may be explored as an immunomodulatory drug in patients with autoimmune/inflammatory diseases and lymphoproliferative syndromes.

1. Introduction

T lymphocytes ($CD3^+$) comprise functionally diverse populations, and the best characterized populations are helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) [1]. $CD4^+$ T cells are functionally classified according to their receptors, cytokine production patterns, and expression of specific transcription factors. Following activation, targeting of the effector response to the major lymphocyte subtypes occurs, which involves Th (T helper) 1, Th2, Th17 or Treg (regulatory T) cells as well as memory $CD4^+$ T cells. Stimulation of $CD8^+$ T cells through the T cell receptor complex induces proliferation and differentiation in cytolytic T lymphocytes (CTLs), which undergo apoptosis after performing their effector functions, and memory $CD8^+$ T cells, which are generated in small quantities and act during subsequent exposure to the same antigen [2].

Failure of the effector functions of T cells leads to various diseases, such as recurrent infections and autoimmune diseases, as well as general immunological dysregulation. Despite advances in characterization and diagnosis, these diseases remain a clinical challenge. Recently, THP-1 cells and peripheral blood monocytes were shown to become activated when treated with BAY 41-2272, resulting in increased intracellular levels of guanosine 3′, 5′-cyclic monophosphate (cGMP) and 3′,5′-cyclic adenosine monophosphate (cAMP); superoxide anion (O_2^-) production; expression of *CYBB* and *NCF2*; phagocytic responses and microbicidal function; and TNF-α and IL-12p70 secretion. These findings suggest that BAY 41-2272 and/or its pathway may modulate the immune system and help to control infection in immunocompromised individuals [3,4].

BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo [3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine), which is derived from pyrazolopyrine, was developed as a nitric oxide (NO)-independent activator of soluble guanylate cyclase (sGC). The activation of sGC by NO leads to the accumulation of cGMP, which regulates different aspects of cellular function by interacting with several kinases, ion channels and phosphodiesterases (PDEs) [5,6]. In the immune response, cGMP is involved in cell differentiation, chemotaxis and cell proliferation, as

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https://doi.org/10.1016/j.intimp.2019.105976
Received 1 April 2019; Received in revised form 12 September 2019; Accepted 12 October 2019
Available online 13 November 2019
1567-5769/ © 2019 Published by Elsevier B.V.

well as the release of soluble mediators [7].

Recently, our group found an immunomodulatory effect of BAY 41-2272 on human neutrophils [8]. In addition, we have already described the role of this drug in monocytes and THP-1 [3,4]. In this study, we investigated the effect of BAY41-2272 on T lymphocytes. The effect of the NO-sGC-cGMP pathway on T lymphocytes has been extensively studied. Exogenous NO inhibits proliferation or even leads to death among T cells. However, small amounts of NO promote survival and differentiation among T cell subpopulations [9]. Moreover, sGC activation by low levels of NO raises cGMP levels and selectively induces the expression of interleukin-12 receptor (IL-12Rβ2) rather than interleukin-4 receptor (IL-4R) in T cells [10]. Increased intracellular cGMP concentrations have also been shown to cause calcium influx and interleukin-4 (IL-4) production [11], and NO induces a specific Treg cell profile ($CD25^+$, $CD27^+$, $FOXP3^-$, $GITR^+$, T-bet^{low}, $GATA3^{high}$) that is not similar to the profile of natural Treg cells [12]. Considering that BAY 41-2272 has been shown to be effective in activating phagocytic and mononuclear cells and the potential of the sGC pathway in the T cell response, our hypothesis is that in vitro treatment with this drug may have a modulatory effect on these cells. Thus, we evaluated the potential of BAY 41-2272 and its pathway to act as a tool for modulating the functions of human T lymphocytes.

2. Materials and methods

2.1. Source of lymphocytes

Lymphocytes were obtained from the peripheral blood of healthy volunteers and processed according to the protocols approved by the Institutional Ethics Committee, Ministry of Health of Brazil. Blood aliquots were fractionated by Ficoll-Hypaque density gradient centrifugation (density = 1.077 g/mL; GE Healthcare, Sweden). The obtained mononuclear cells (PBMCs) were plated in 6-well flat-bottom plates in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C and 5% CO_2 under endotoxin-free conditions (< 10 pg/mL) for 2 h. Nonadherent lymphocytes were collected, treated with the drugs used in the present study, and labeled with anti-CD3, anti-CD4, and anti-CD8 antibodies [13].

Lymphocytes were treated with BAY 41-2272, phorbol myristate acetate (PMA) or dimethyl sulfoxide (DMSO; Sigma, St. Louis, USA) for 48 h, and cell viability was evaluated using LIVE/DEAD® Fixable Violet Dead Cell Stain (Thermo Fisher Scientific Inc., Massachusetts, USA) and an Applied Biosystems Attune® NxT flow cytometer (Thermo Fisher Scientific Inc., Massachusetts, USA). This result was confirmed by ANNEXIN V (BD Biosciences, San Diego, USA). In both approaches, the live cell region was determined using forward (FSC) versus side (SSC) scatter parameters, and 50,000 events were collected for each sample. FlowJo software (Tree Star Inc., Oregon, USA) was used for data analysis.

2.2. Treatments

Lymphocytes were divided into three treatment groups: **1.** no pretreatment; **2.** pretreatment with BAY 41-2272 (0.1–30 μM) (Bayer, Wuppertal, Germany), 8Br-cGMP (100 μM), 8Br-cAMP (100 μM), or S-nitroso-N-acetylpenicillamine (SNAP; 1 μM); and **3.** pretreatment with ODQ [1H-(1,2,4) oxadiazol (4,3-alpha) quinoxaline-1-one] (100 μM) (Sigma, St. Louis, USA) followed by BAY 41-272 or SNAP (Supplementary Fig. 1).

Lymphocytes were incubated with ODQ (100 μM) for 30 min prior to treatment, and drugs were added 24 h prior to the experiment. After pretreatment, lymphocytes were incubated with PMA (90 nM) (Sigma, St. Louis, USA) and ionomycin (500 ng/mL) (InvivoGen, San Diego, USA), and IFN-γ production and the expression of CD69, CD25, T-bet, FOXP3 and RORγT were evaluated.

2.3. Cytokine production

Lymphocytes were pretreated and subsequently stimulated for 24 h. Supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) to detect IFN-γ, IL-4, IL-10, IL-2, IL-17 and TNF-α according to the "Human IFN-γ ELISA Set-BD OptEIA", "Human IL-4 ELISA Set-BD OptEIA", "Human IL-2 ELISA Set-BD OptEIA", "Human IL-17 ELISA Set-BD OptEIA", "Human TNF-α ELISA Set-BD OptEIA" and "Human IL-10 ELISA Set-BD OptEIA" (BD Biosciences, San Diego, USA) kits, respectively [13].

2.4. Expression of surface markers and transcription factors

The expression of surface markers and transcription factors in lymphocytes ($2x10^5$ cells/mL) treated with BAY 41-2272, 8-Br-cGMP, 8Br-cAMP, SNAP, ODQ, PMA or ionomycin was evaluated using antibodies against CD3, CD4, CD8, CD69, CD25, T-bet, GATA3, FOXP3 and RORγT. To detect transcription factors, cells were fixed and permeabilized according to the Transcription Factor Buffer kit (BD Bioscience, San Diego, USA). Fluorescence was measured by flow cytometry (Attune NxT, Life Technologies, California, USA), and the results for each marker are expressed as the percentage of cells and the median fluorescence intensity (MFI).

2.5. Lymphoproliferation

Cells were treated with the dye carboxyfluorescein succinimidyl ester (CFSE; 5 mM) (Invitrogen, Oregon, USA) according to the manufacturer's protocol. After labeling with CFSE, the cells were treated with BAY 41-2272, 8-Br-cGMP, 8-Br-cAMP, SNAP, ODQ or phytohemagglutinin (1%) for 5 days, and the samples were analyzed by flow cytometry [13].

2.6. cGMP measurement

The production of cGMP by lymphocytes ($2x10^5$) treated with BAY 41-2272, SNAP or PMA for 10 min was assessed according to the Cyclic GMP EIA kit (Cayman Chemical, No 581021, Ann Arbor, Michigan). Absorbance was measured at 412 nm, and cGMP concentrations are expressed in pmol/mL [14].

2.7. Statistical analysis

The results are expressed as the mean and standard deviation of N experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. The analyses were performed in GraphPad Prism version 5.0 (GraphPad Software Inc.), and the significance level adopted was 5%, with $p < 0.05$.

3. Results

3.1. BAY 41-2272 decreases the secretion of IFN-γ, IL-4, and IL-10 by human T lymphocytes

We first evaluated whether BAY 41-2272 is toxic to cells by examining the effects of the drug on cell viability. The concentrations of DMSO employed to dilute BAY 41-2272 (from 0.005% to 0.01%) and the doses of BAY 41-2272 used in the present work did not affect cell viability (Supplementary Fig. 2A). To confirm cell viability, an Annexin V test was performed (Supplementary Fig. 2A). Cytokine production is an important indicator for the differentiation and responses of lymphocytes. Initially, we utilized ELISA to determine the secretion of IFN-γ, TNF-α, IL-2, IL-4, IL-10, and IL-17 after treatment with BAY 41-2272. We observed that BAY 41-2272 alone did not induce IFN-γ, TNF-α, IL-2, IL-4, IL-10, or IL-17 secretion by lymphocytes (Supplementary Fig. 3).

Because treatment with BAY 41-2272 did not induce IFN-γ

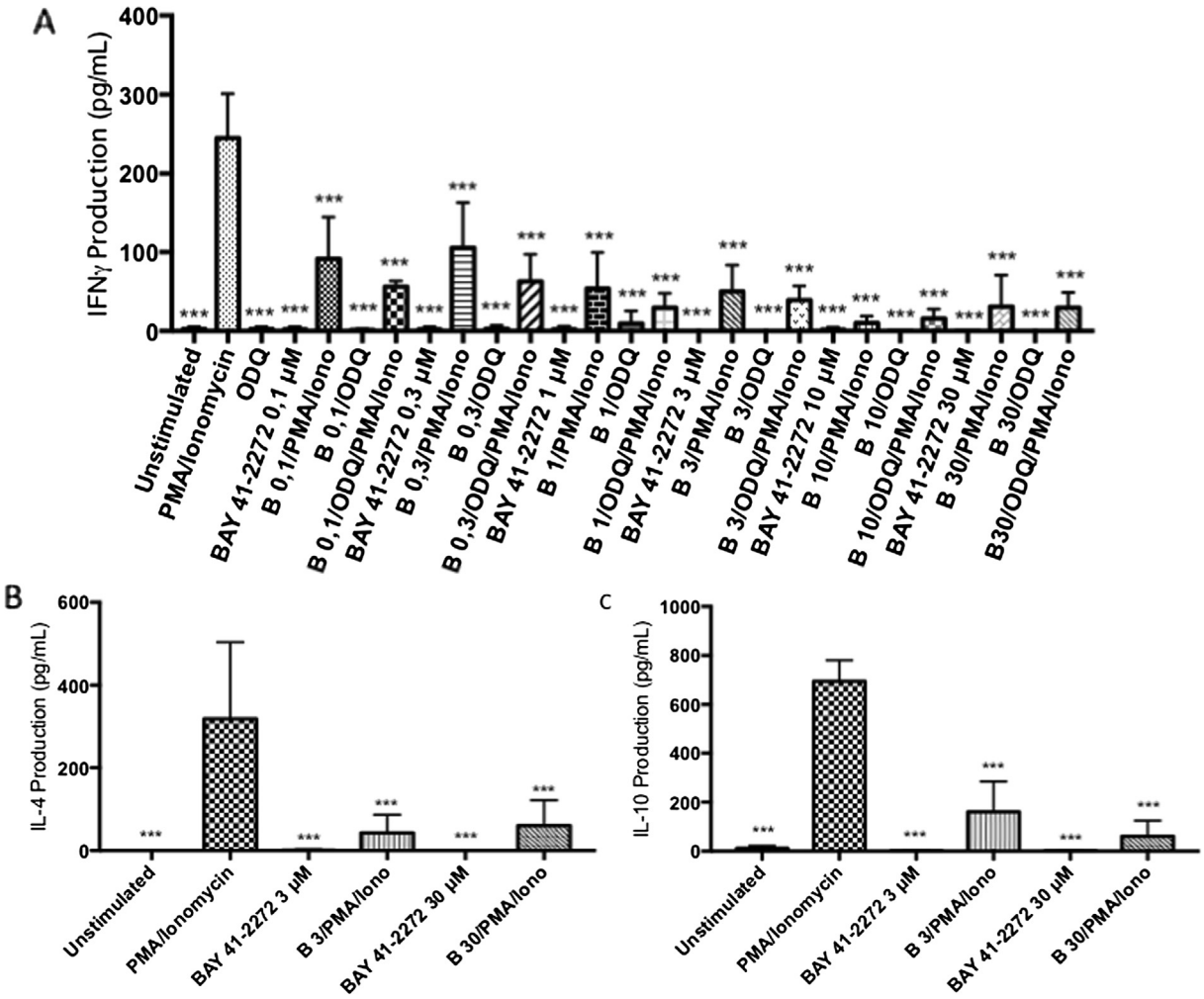

Fig. 1. Release of cytokines by lymphocytes after pretreatment with BAY 41-2272 and subsequent activation with PMA. Peripheral blood lymphocytes were divided into two groups, one of which was pretreated with ODQ (100 µM) for 30 min. After the two groups were incubated with increasing doses of BAY 41-2271 (3 µM, 30 µM) for 24 h, the cells were then stimulated with PMA (90 nM) or ionomycin (500 ng/mL) for 24 h. The culture supernatant was then collected for IFN-γ (A), IL-4 (B), and IL-10 (C) measurement. N = 3, median and maximum, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the nonstimulated control group at 48 h, ANOVA followed by Tukey's test; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the untreated control group stimulated with PMA, ANOVA followed by Tukey's test.

secretion, we evaluated whether BAY 41-2272 exhibits priming potential in lymphocytes. To this end, we performed pretreatment with BAY 41-2272 for 24 h and subsequent activation with PMA, followed by evaluation of IFN-γ, IL-2, IL-4, IL-10, TNF-α, and IL-17 secretion. In addition to the above-described treatments, we added ODQ, which inhibits the sGC pathway. It was observed that pretreatment with BAY 41-2272 significantly inhibited both the activation and release of IFN-γ, IL-4, and IL-10 in lymphocytes when compared to the post-activated control with PMA (90 nM), but it did not inhibit the release of IL-2, IL-17, and TNF-α. However, treatment with ODQ did not interfere with IFN-γ secretion (Fig. 1).

3.2. BAY 41-2272 inhibits CD69 expression

An inhibitory role of BAY 41-2272 was observed when cells were pretreated for 24 h before stimulation with PMA, and we next examined the effect of BAY 41-2272 pretreatment plus subsequent stimulation with PMA/ionomycin on CD69 expression. We found no inhibitory effect on CD69 expression in the $CD3^+CD8^+$ T lymphocyte population pretreated with 3 µM or 30 µM BAY 41-2272 or 8-Br-GMP or in the $CD3^+CD4^+$ T lymphocyte population pretreated with 3 µM BAY 41-2272 or 8-Br-cGMP (Fig. 2A and Supplementary Fig. 5). However, we observed a statistically significant decrease in CD69 expression after stimulation with PMA/ionomycin in the $CD3^+CD4^+$ T lymphocyte population pretreated with 30 µM BAY 41-2272 compared to the stimulated control group without pretreatment (Fig. 2B). In addition, treatment with sGC, ODQ and W45 inhibitors did not restore CD69 expression, suggesting that BAY 41-2272 at a dose of 30 µM has an inhibitory function independent of sGC. We also evaluated the effect of SNAP and 8Br-cAMP on CD69 expression. Although we did not observe induction of CD69 expression after stimulation with BAY 41-2272, SNAP, 8Br-cGMP or 8Br-cAMP alone (Fig. 2C), pretreatment with 30 µM BAY 41-2272 and 1 µM SNAP inhibited

Since the results demonstrated an inhibitory profile of BAY 41-2272 in lymphocytes, we evaluated the ability of this drug to activate these cells through CD69 expression, a classical marker of the early stages of activation. However, treatment with BAY 41-2272 alone did not induce CD69 expression in either $CD3^+CD4^+$ T cells or $CD3^+CD8^+$ T lymphocytes compared to the nonstimulated control group (Supplementary Fig. 4).

3.3. BAY 41-2272 inhibits CD25 expression

Our previous results suggested an inhibitory effect of BAY 41-2272

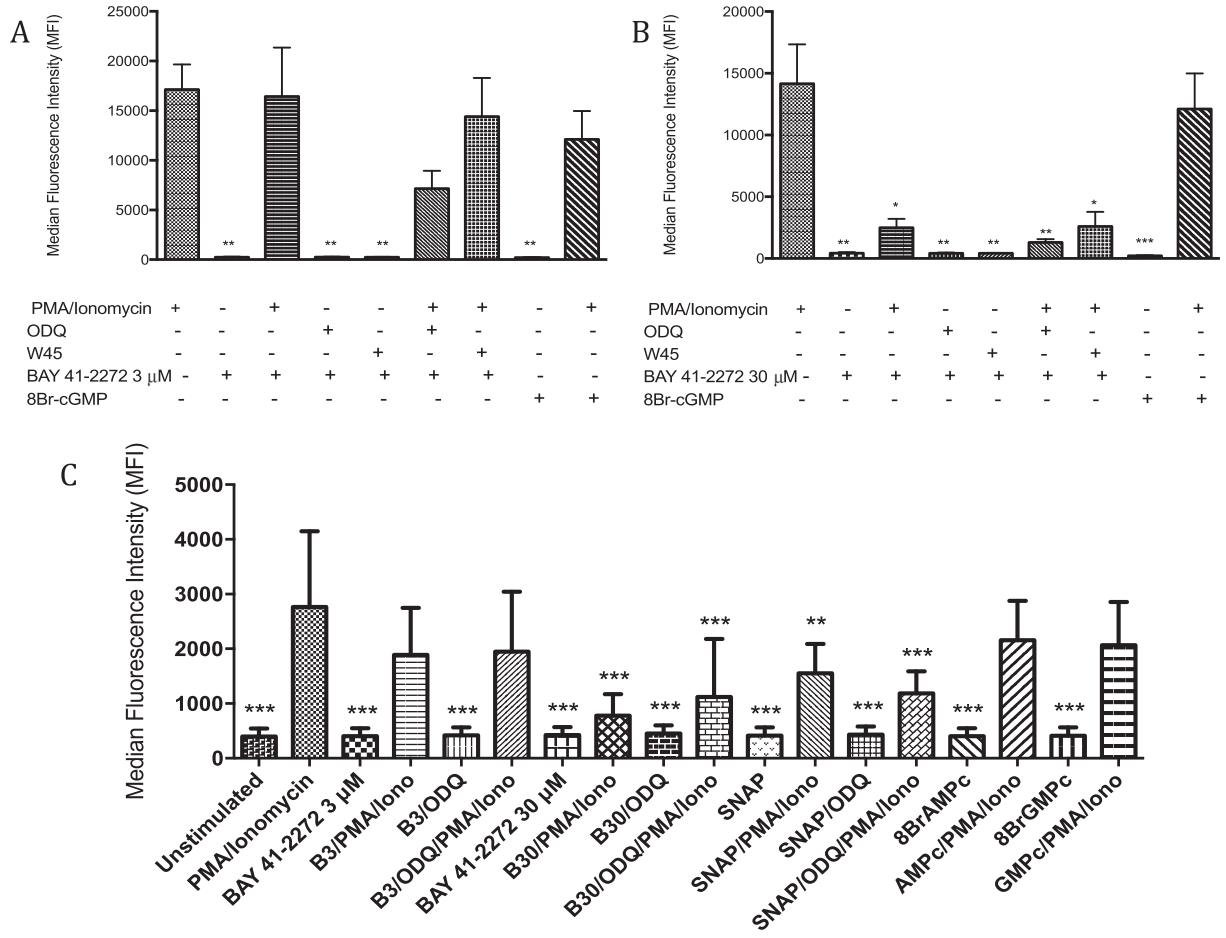

Fig. 2. CD69 expression among T lymphocytes after pretreatment with BAY 41-2272 for 24 h. Populations of $CD3^+CD8^+CD69^+$ (A) T lymphocytes, $CD3^+CD4^+CD69^+$ (B) T lymphocytes, and (C) T lymphocytes. Lymphocytes were divided into two groups, which were each pretreated with ODQ (100 μM) or W45 (1 μM) for 30 min. The cells were then incubated with BAY 41-2271 (3 μM and 30 μM, A and B), 8Br-cGMP (100 μM), 8Br-cAMP (100 μM), or SNAP (1 μM) for 24 h, and then the cells were stimulated with PMA and ionomycin (90 nM and 500 ng/mL, respectively) for 3 h and labeled with anti-CD3, anti-CD4, anti-CD8 and anti-CD69 antibodies for flow cytometry analysis. N = 6 (A and B), N-9 (C), median and maximum, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the untreated control group stimulated with PMA/ionomycin, ANOVA followed by Tukey's test.

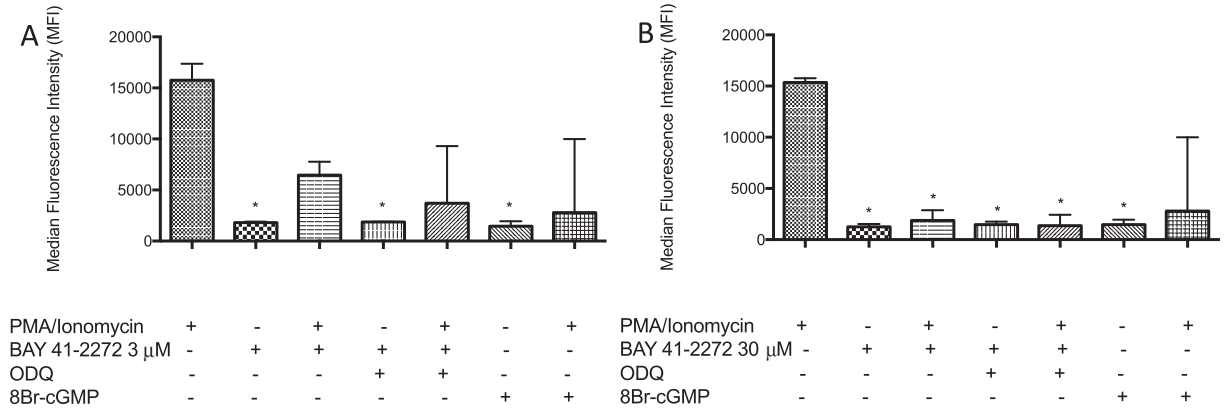

Fig. 3. CD25 expression on $CD4^+$ T lymphocytes after pretreatment with BAY 41-2272 for 24 h. $CD3^+CD4^+$ T lymphocytes were divided into two groups, which were each pretreated with ODQ (100 μM) or W45 (1 μM) for 30 min. The cells were then incubated with BAY 41-2271 3 μM (A) and 30 μM (B) or 8Br-cGMP (100 μM), 8Br-cAMP (100 μM), or SNAP (1 μM) for 24 h and stimulated with PMA and ionomycin (90 nM and 500 ng/mL, respectively). After 3 h, the cells were labeled with anti-CD3, anti-CD4, and anti-CD25 antibodies for flow cytometry analysis. N = 3, median and maximum, * $p < 0.05$ compared to the untreated control group stimulated with PMA/ionomycin, ANOVA followed by Tukey's test.

on lymphocytes. Within this context, we evaluated the expression of CD25, which is one of many Treg cell markers. We observed that treatment with BAY 41-2272 alone did not induce CD25 expression on $CD3^+CD4^+$ T lymphocytes (Supplementary Fig. 6). Although pretreatment with 3 μM BAY 41-2272 and subsequent PMA/ionomycin-induced activation did not alter the CD25 expression profile, pretreatment with 30 μM BAY 41-2272 significantly inhibited CD25 expression compared to that observed after PMA/ionomycin stimulation in the

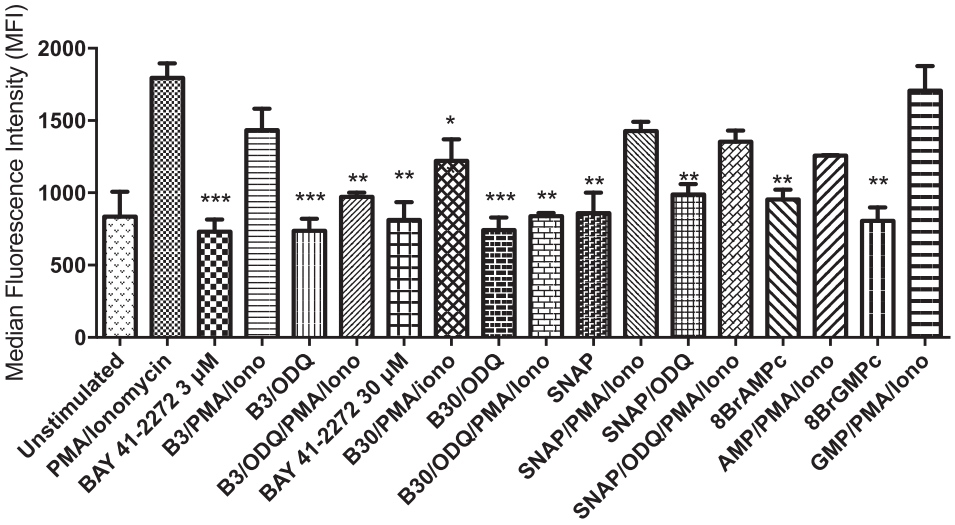

Fig. 4. T-bet expression in T lymphocytes after treatment with BAY 41-2272. Peripheral blood lymphocytes were stimulated with PMA and ionomycin (90 nM and 500 ng/mL, respectively), BAY 41-2271 (3 µM and 30 µM), 8Br-cGMP (100 µM), 8Br-cAMP (100 µM), or SNAP (1 µM) for 24 h and labeled with anti-CD3 antibodies. After extracellular labeling, the samples were fixed and permeabilized and then labeled with anti-T-bet antibodies for flow cytometry analysis. (N = 3, median and maximum, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the control group treated with PMA/ionomycin, one-way ANOVA followed by Tukey's test.

absence of pretreatment with BAY 41-2272 (Fig. 3).

3.4. BAY 41-2272 inhibits T-bet expression

We next investigated the expression of FOXP3, RORγT, GATA3, and T-bet after treatment with BAY 41-2272 and found that compared to the nonstimulated control group, treatment with BAY 41-2272 alone did not induce the expression of these transcription factors in T lymphocyte populations (Supplementary Fig. 7). Moreover, the levels of FOXP3, RORγT, and GATA3 in the T lymphocyte population pretreated with BAY 41-2272, 8-Br-GMP, 8-Br-cAMP or SNAP and stimulated with PMA/ionomycin were not altered compared to those in the untreated control group stimulated with PMA/ionomycin (Supplementary Figure 8). Nonetheless, pretreatment with 30 µM BAY 41-2272 for 24 h resulted in a statistically significant decrease in T-bet expression (Fig. 4).

3.5. BAY 41-2272 inhibits lymphoproliferation

Stimulation with BAY 41-2272 alone did not induce lymphoproliferation (Supplementary Fig. 9). In contrast, pretreatment with BAY 41-2272 significantly decreased lymphoproliferation in cells stimulated with phytohemagglutinin (Fig. 5).

3.6. BAY 41-2272 induces cGMP production

As cGMP is the product of sGC activation, we evaluated cGMP production after stimulation with BAY 41-2272. Treatment with BAY 41-2272 for 10 min induced cGMP production in lymphocytes compared to SNAP-stimulated control cells and cells treated with a nitric oxide donor and GC activator (Fig. 6).

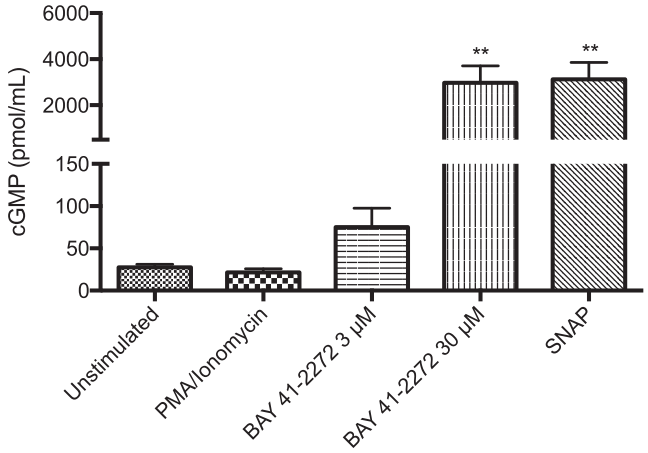

Fig. 6. cGMP production by lymphocytes after treatment with BAY 41-2272. Peripheral blood lymphocytes were stimulated with 90 nM PMA, 500 ng/mL ionomycin, BAY 41-2271 (3 µM and 30 µM) or 10 µM SNAP for 10 min. After treatment, the cells were lysed for cGMP dosing. N = 5, median and maximum, ** $p < 0.01$ compared to the nonstimulated control group, one-way ANOVA followed by Tukey's test.

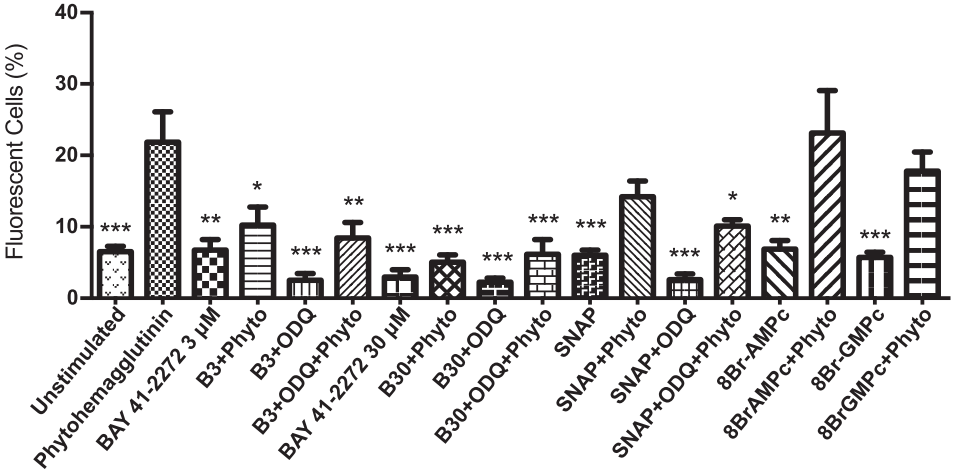

Fig. 5. Lymphoproliferation assay. Peripheral blood lymphocytes were pretreated with BAY 41-2271 (3 µM and 30 µM), 8Br-cGMP (100 µM), 8Br-cAMP (100 µM), or SNAP (1 µM) for 24 h and stimulated with phytohemagglutinin (1%) for 5 days. The samples were then analyzed by flow cytometry. N = 5, median and maximum, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the group treated with phytohemagglutinin, one-way ANOVA followed by Tukey's test.

4. Discussion

BAY 41-2272 increases guanosine 3′,5′-cyclic monophosphate (cGMP) levels by stimulating soluble guanylate cyclase (sGC). The potential of this drug in modulating the immune response has been explored in recent years. For example, our group demonstrated that BAY 41-2272 stimulated gp91phox expression and the release of superoxide by human THP-1 cells [3]. We have also demonstrated that BAY 41-2272 induced the production of superoxide, increased phagocytosis, and promoted microbicidal activity by human monocytes [4]. On the other hand, we have also demonstrated that BAY 41-2272 inhibits human neutrophils [8]. In this study, we investigated the effect of BAY 41-2272 on human T cells.

$CD4^+$ T lymphocytes are functionally classified by the pattern of cytokines that they produce and their transcription factors. Four major subtypes of lymphocytes are described in the literature: Th (T helper) 1, Th2, Th17, and Treg cells. Th1 cells mainly produce IFN-γ and are crucial for the elimination of intracellular pathogens [15,16]. Th2 cells mainly produce IL-4 and are involved in the production of antibodies, immune responses to extracellular parasites and hypersensitivity, including eosinophilia [1].

In contrast, Tregs are involved in maintaining peripheral tolerance by suppressing the activation and expansion of autoreactive T cells [17]. These cells act via several mechanisms, including induction of death by granzyme A-dependent cytolysis, granzyme B and perforin; cAMP deprivation; suppression of dendritic cell maturation; and production of inhibitory cytokines such as interleukin-10 (IL-10) [15,18]. Previous studies have demonstrated that the sGC pathway can modulate the lymphocyte response [9,19,20]. In the present study, we demonstrated that BAY 41-2272 inhibits the activation of human T lymphocytes and lymphoproliferation as well as cytokine production and transcription factor expression by these cells.

Several studies have sought to provide a better understanding of sGC activation in lymphocytes, showing that this process induced by low NO levels selectively increases Th1 cell differentiation and expansion in a murine model [9,19]. Another group demonstrated that NO modulates cytokine expression in human T lymphocytes [20]. In addition, NO has been reported to induce the differentiation of a population of Treg cells that are similar to natural Tregs in mice [19]. In the present work, we observed that pretreatment with BAY 41-2272 significantly inhibited the release of IFN-γ, IL-4, and IL-10 by lymphocytes. Conversely, treatment with ODQ, an sGC inhibitor, did not block the action of BAY 41-2272, and the drug maintained its inhibitory activity. Thus, we assume that BAY 41-2272 functions via other pathways in addition to the sGC pathway.

Regardless, low doses of NO may influence the activation, proliferation and differentiation of lymphocytes [21]; low doses of NO may also have a cytotoxic effect. Studies have shown that increased levels of chemokines and iNOS lead to attenuation of the T cell response [22], corroborating the results of this work in which a high dose of BAY 41-2272 inhibited activation of human $CD4^+$ T lymphocytes and lymphoproliferation. Apparently, this cell subpopulation is more sensitive to NO than murine $CD4^+$ T cells [21]. In addition, we observed that SNAP treatment inhibited CD69 expression, although 8BR-cGMP did not inhibit CD69 expression, suggesting that BAY 41-2272 acts in a GC-dependent but cGMP-independent manner.

NO has a paradoxical role in the immune response, where it sometimes exerts pro-inflammatory effects and other times leads to suppression of cellular activation. Several studies have demonstrated the role of NO in T cell differentiation. Intermediate SNAP (NO donor) concentrations stimulate the development of IFN-γ (Th1)-producing $CD4^+$ T cells in the presence of IL-12 and anti-IL-4 antibodies, with no alteration in the Th2 population [12,23]. $CD4^+$ T cells are thought to be delimited to two major subpopulations: Th1 and Th2 cells [24]. In contrast to reports in the literature, we observed inhibition of T-bet expression after pretreatment with BAY 41-2272, suggesting that this drug does not induce lymphocyte differentiation but can inhibit the expression of transcription factors related to cell differentiation.

Recently, NO has been shown to induce a population of Treg cells with characteristic marker profiles ($CD25^+$, $CD27^+$, $FOXP3^-$, $GITR^+$, T-bet^{low}, $GATA3^{high}$) that are similar to those of natural Treg cells [12]. However, we did not observe increased CD25 expression or altered FOXP3 expression in the $CD4^+$ T lymphocyte population after pretreatment with BAY 41-2272, again suggesting that this drug does not induce lymphocyte differentiation but can inhibit the expression of related factors via the pathway that it activates.

Some studies have shown that NO is required for the induction and stability of the Th17 response [25]. In contrast, NO has been shown to negatively regulate Th17 cell differentiation of murine and human T cells [26,27]. Furthermore, CD69-deficient mice exhibit a greater capacity for Th17 cell differentiation [28]. Nonetheless, the present work revealed that CD69 expression was inhibited after treatment with BAY 41-2272, although RORγT expression was not altered.

Activation of T lymphocytes begins with peptide-MHC recognition by a T cell response (TCR) associated with the signaling of costimulatory molecules as well as the cytokines and chemokines present in the microenvironment. The antigen recognition response by T cells is characterized by a series of divisions, and this process is termed clonal expansion or lymphoproliferation [29]. Thus, we evaluated the effect of BAY 41-2272 on cell proliferation and found that pretreatment with this drug inhibited cell growth, once again demonstrating the inhibitory capacity of BAY 41-2272 in lymphocytes.

Activation of the sGC pathway leads to cGMP production, an important mediator that regulates various cellular functions. Once produced, cGMP, which can be rapidly hydrolyzed by PDEs, exerts its function through protein kinases; the main kinases are PKG-I and PKG-II (PKG, protein kinase G), which share several targets with protein kinase A (PKA, protein kinase A) [30]. In our work, we observed cGMP production after stimulation with BAY 41-2272, demonstrating that the observed effects are dependent on activation of the sGC pathway.

$CD4^+$ T lymphocytes are important for homeostasis and defense but are also the main contributors to the pathology of autoimmune, inflammatory and allergic diseases. When dysregulated, each subpopulation of $CD4^+$ T lymphocytes may promote an immunopathology; for example, an excessive Th1 response may result in tissue damage, whereas an exaggerated Th2 response may result in atopy or hypersensitivity [31].

In addition, defects in T cells lead to the development of various diseases and predispose an individual to a broad spectrum of infections. Among the primary immunodeficiencies (PIDs) of T lymphocytes, some specific diseases result in increased susceptibility to a given pathogen, particularly in X-linked lymphoproliferative syndrome (XLP), which leads to failure to control T cell proliferation or the natural killer (NK) cells that arise after infection with Epstein-Barr virus (EBV) [32]. Another PID, IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy and X-linked), results from a deficiency of the FOXP3 transcription factor, which is involved in regulating the immune response to self-antigens, thus generating serious autoimmune manifestations [33].

Regulating and controlling responsiveness is an intrinsic property of the immune system. Excessive $CD4^+$ T cell responses are usually controlled in most individuals. Although several mechanisms control this response, failure may eventually occur, leading to the onset of certain diseases. Thus, therapies to regulate this response must be developed.

Our results suggest that BAY 41-2272 has an inhibitory effect on the production of cytokines by lymphocytes. This drug has also been shown to act in a GC-dependent and cGMP-independent manner and to inhibit cell activation without altering the expression of transcription factors such as FOXP3, RORγT, and GATA3 to inhibit T-bet expression and lymphoproliferation. These data suggest that BAY 41-2272 may be explored as an immunomodulator in patients with impaired immune response control, such as those with autoimmune diseases and

inflammatory diseases, as well as patients with compromised immune systems and lymphoproliferative syndromes. Taken together, our experience showing the stimulatory effects of BAY 41-2272 on THP-1 cells and human monocytes and the inhibitory effects of this drug on human neutrophils and T cells suggest that this drug should be explored as a potential modulator to help the host control and conclude the immune response against pathological stimuli.

5. Fundingxxx

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

6. Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105976.

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