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G protein–coupled receptor 21 in macrophages: An in vitro study

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G protein–coupled receptor 21 in macrophages: An in vitro study

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ABSTRACT

GPR21 is an orphan and constitutively active receptor belonging to the superfamily of G-Protein Coupled Receptors (GPCRs). GPR21 couples to the G_q family of G proteins and is markedly expressed in macrophages. Studies of GPR21 knock-out mice indicated that GPR21 may be involved in promoting macrophage migration. The aim of this study was to evaluate the role of GPR21 in human macrophages, analyzing (*i*) its involvement in cell migration and cytokine release and (*ii*) the consequence of its pharmacological inhibition by using the inverse agonist GRA2. THP-1 cells were activated and differentiated into either M_1 or M_2 macrophages. GPR21 expression was evaluated at gene and protein level, the signalling pathway was investigated by an IP_1 assay, and cytokine release by ELISA. Cell migration was detected by the Boyden chamber migration assay, performed on macrophages derived from both the THP-1 cell line and human peripheral blood monocytes. In addition, we compared the effect of the pharmacological inhibition of GPR21 with the effect of the treatment with a specific GPR21 siRNA to downregulate the receptor expression, thus confirming that GRA2 acts as an inverse agonist of GPR21. GRA2 does not affect cell viability at the tested concentrations, but significantly reduces the release of TNF-α and IL-1β from M_1 macrophages. The analysis of the migratory ability highlighted opposite effects of GRA2 on M_1 and M_2 macrophages since it decreased M_1, while it promoted M_2 cell migration. Therefore, the pharmacological inhibition of GPR21 could be of interest for pathological conditions characterized by low grade chronic inflammation.

1. Introduction

G-Protein-Coupled Receptors (GPCRs) are the largest protein superfamily in mammals (Katritch et al., 2013; Wang et al., 2021). Most respond to distinct and varied stimuli, including hormones, neurotransmitters, odorants, light, flavors and pheromones (Alexander et al., 2011; Calebiro, 2021; Weis and Kobilka, 2018). However, the endogenous ligand is still unknown for a substantial number of GPCRs. These orphan receptors have attracted particular interest in the field of drug discovery as they might be novel therapeutic targets for pharmacological intervention in a wide range of conditions (Civelli et al., 2013; Stockert and Devi, 2015; Tang et al., 2012). GPR21 is an orphan receptor whose gene is located on chromosome 9 in humans and chromosome 2 in mice (Gardner et al., 2012). It is broadly expressed, including in macrophages and some brain regions, especially the hypothalamus (Gardner et al., 2012; Osborn et al., 2012). Interestingly, it was demonstrated that GPR21 is constitutively active and couples to G_q type G proteins, in particular $G_{\alpha q}$ and $G_{\alpha 15/16}$ (Bresnick et al., 2003; Leonard et al., 2016; Xiao et al., 2008). Literature data reports that GPR21 is involved in the development of insulin resistance, since two different research

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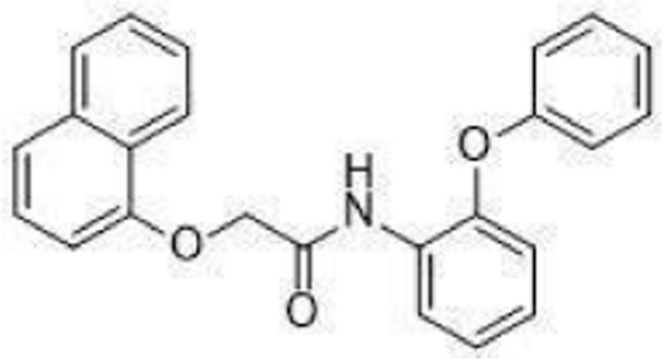

Fig. 1. Structure of GRA2, (2-(1-naphthyloxy)-N(2-phenoxyphenyl)acetamide).

groups found that GPR21 knockout (KO) mice are protected from inflammation and insulin resistance induced by a high fat diet (HFD), and tend to maintain a lean phenotype (Gardner et al., 2012; Osborn et al., 2012). Osborn et al. suggested that this protection may be ascribable to a decreased chemotaxis of GPR21 KO macrophages into the adipose tissue and liver, which would reduce both tissue inflammation and insulin resistance, thus suggesting that this receptor may be a positive regulator of macrophage migration (Osborn et al., 2012). Subsequently, our group showed that GPR21 impaired insulin signaling and induced MAPKs activation, an effect reported to promote macrophage accumulation in the tissue (Kinsella et al., 2021; Leonard et al., 2016). In addition, we showed that these events were antagonized by GRA2 (see Fig. 1), an inverse agonist of GPR21 capable of inhibiting receptor activation. Unfortunately, despite these promising findings there is scarce information available on the role of GPR21 in macrophage function, which involves many processes, such as tissue remodeling, wound healing, angiogenesis, metabolism, and especially inflammation (Chawla et al., 2011; Lavin et al., 2015; Wynn and Vannella, 2016). Since low grade chronic inflammation is an important contributor to many diseases (Iyengar et al., 2016; Liu and Nikolajczyk, 2019; Saltiel and Olefsky, 2017; Ying et al., 2020) including type 2 diabetes, understanding the role of GPR21 in macrophage activity is crucial to progress pharmacological targeting of this receptor. The aim of this study was to evaluate GPR21's function in human M_1 (pro-inflammatory phenotype) and M_2 (anti-inflammatory phenotype) macrophages, by analyzing (i) its involvement in cell migration and cytokine production, and (ii) its pharmacological significance by using the inverse agonist GRA2.

2. Materials and methods

2.1. Macrophage differentiation

THP-1 cells, were cultured in Roswell Park Memorial Institute (RPMI, Aurogene Srl, Rome, Italy) 1640 medium supplemented with 2 mM L-glutamine (Aurogene Srl, Rome, Italy), 100 μg/ml penicillin-streptomycin (Aurogene Srl, Rome, Italy) and 10% (v/v) foetal bovine serum (FBS, Aurogene Srl, Rome, Italy), at 37 °C in a humidified 5% CO2 atmosphere incubator. Cells were differentiated into macrophages by a 48 h culture with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, Sigma-Aldrich, St Louis, MO, USA) for 48 h, and subsequently in either pro-inflammatory (M_1) or anti-inflammatory (M_2) macrophages by a 24 h culture with either 100 ng/ml lipopolysaccharide (LPS, Sigma, Sigma-Aldrich, St Louis, MO, USA) plus 20 ng/ml interferon γ (IFNγ, R&D System, Minneapolis, MN, USA) or 20 ng/ml interleukin-4 (IL-4, R&D Systems, Minneapolis, MN, USA) plus 20 ng/ml interleukin-13 (IL-13, R&D Systems, Minneapolis, MN, USA), respectively (Freytes et al., 2013; Tjiu et al., 2009). The THP-1 cell line was obtained from LGC Standards S.r.l., Sesto San Giovanni-Milan, Italy.

Peripheral blood mononuclear cells (PBMC) were separated from buffy coats provided by the local Blood Transfusion Service (Novara, Italy) by density gradient centrifugation using the Ficoll-Hypaque reagent (Limpholyte-H, Cedarlane Laboratories, Burlington, ON, Canada). Monocytes derived macrophages (MDM) were prepared from $CD14^+$ monocytes isolated with the EasySepTM Human CD14 Negative Selection Kit (StemCells Techologies, Vancouver, BC, USA). The study was conducted according to the guidelines of the Declaration of Helsinki and the use of buffy coats was approved by the local ethical committee (n. CE 88/17). Informed consent was obtained from all subjects involved in the study.

Monocytes (0.5×10^6 cells/well) were plated in a 6-well plate and cultured for 6 days in a differentiation medium (DM) composed of RPMI-1640 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 2 mM L-glutamine, 10% FBS (Thermo Fisher Scientific), 25 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin, and recombinant human GM-CSF for M_1 polarization (100 ng/ml; R&D System, Minneapolis, MN, USA) or M-CSF for M_2 polarization (100 ng/ml; R&D Systems, Minneapolis, MN, USA). The DM was changed every 3 days. In the activation assays, MDM were cultured for 2 additional days in DM in the presence of LPS (1 μg/ml, *Escherichia coli*, serotype 055:B5, Sigma-Aldrich, St Louis, MO, USA) in the presence or absence of IFNγ (50 ng/ml, R&D System, Minneapolis, MN, USA) for M_1 or IL-4 (10 ng/ml, R&D System, Minneapolis, MN, USA) for M_2.

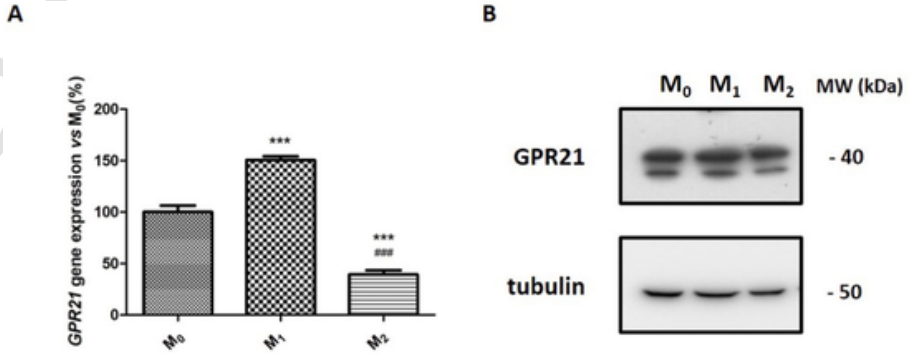

Fig. 2. Expression of GPR21 in macrophage-like cells. THP-1 cells were differentiated in M_0, M_1 or M_2 macrophages and expression of GPR21 was evaluated by Real-Time PCR **(A)** or Western-blot **(B)**. Gene expression was expressed as percentage of M_0 (100%). Data are expressed as mean ± SEM of three independent experiments run in triplicate. *$^{***}P < 0.001$ vs M_0; $^{\#\#\#}P < 0.001$ vs M_1.*

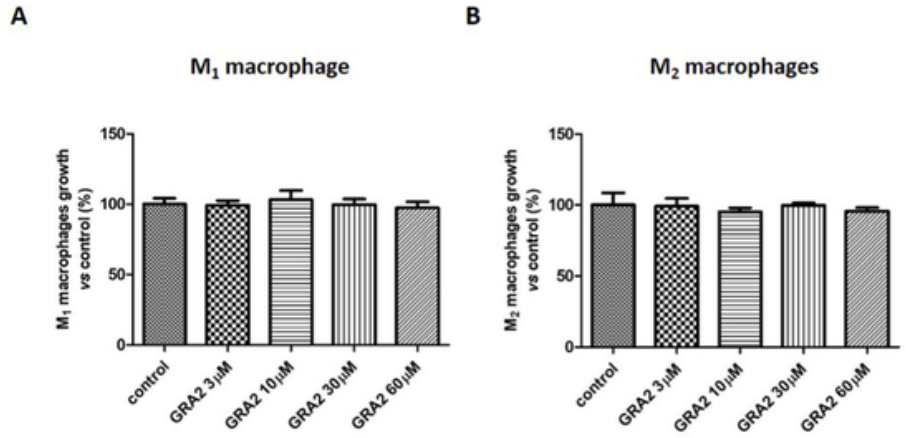

Fig. 3. Effects of GRA2 on cell viability of macrophage-like cells. THP-1 differentiated in either M_1 **(A)** or M_2 **(B)** macrophages were exposed to either vehicle alone (control) or increasing concentrations of GRA2 (3–60 μM). Cell growth was measured by MTT assay. Cell growth was expressed as percentage of control cultures (100%). Data are expressed as mean ± SEM of three independent experiments run in triplicate.

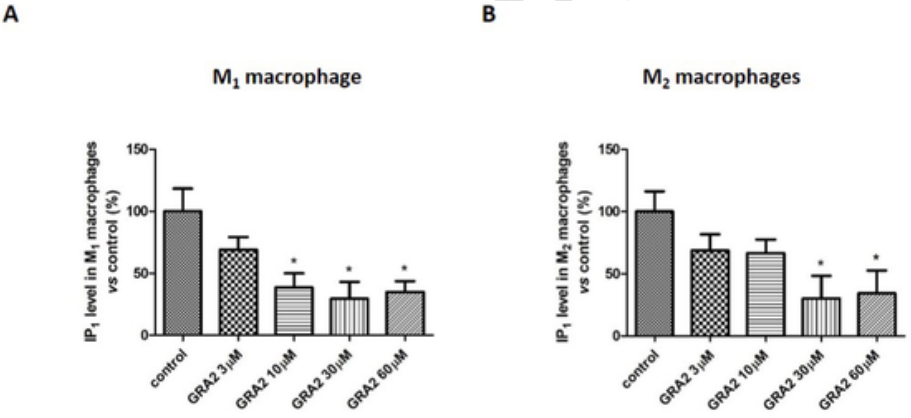

Fig. 4. Effect of GRA2 on basal IP_1 production in macrophage-like cells. THP-1 cells were differentiated in either M_1 **(A)** or M_2 **(B)** macrophages, activated and exposed to either vehicle alone (control) or increasing concentration of GRA2 (3–60 μM). GPR21 constitutive activation was quantified by measuring intracellular IP_1 level. Data are expressed as mean ± SEM of four independent experiments run in duplicate. *$P < 0.05$ vs control.*

2.2. Measurement of cell viability

Cells were plated (4×10^3 cells/well) in 24-well culture plates and exposed to vehicle alone (control, DMSO) or GRA2, the inverse agonist of GPR21 (the compound was kindly provided by Professor John Stephens of Maynooth University, Ireland). GRA2 has a molecular formula (C24 H19 N O3) and SMILES representation of O═C(COc1ccc2c-cccc2(c1))Nc4ccccc4(Oc3ccccc3). The compound was originally sourced from Specs (https://specs.net/).

Cell growth was evaluated in sub-confluent cultures by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Sigma-Aldrich, St Louis, MO, USA) colorimetric assay; results were confirmed by determining cell density, as previously described (Miglio et al. 2011, 2017).

2.3. Inositol phosphate (IP)-one (IP_1) homogenous time resolved fluorescence (HTRF) assay

Cellular IP-one levels were measured using an IP-one HTRF assay kit (Cisbio, PerkinElmer, MA, USA) (Trinquet et al., 2006) as previously described (Veglia et al., 2015). Briefly, subconfluent cells were detached from the cell culture dish and resuspended in the appropriate volume of the assay stimulation buffer. Cell suspension was added to a white half-volume 96 well plate (OptiPlate) along with different doses of the compound to be tested and incubated at 37 °C for 1 h. Then, IP-one lysis buffer containing IP-one-d2 conjugate was added to the appropriate wells, followed by the anti-IP-one cryptate Tb conjugate. Samples were incubated for 1 h at room temperature. The plate was read on a VICTOR X4 (PerkinElmer, MA, USA) plate reader with emission at 615 nm and 665 nm. The fluorescence resonance energy transfer (FRET) ratio (665 nm/615 nm) was converted to IP_1 concentrations by interpolating values from an IP_1 standard curve.

2.4. siRNA-mediated GPR21 knockdown

RNA interference experiments to knockdown GPR21 expression in macrophages derived from THP-1 cells were performed using Selected Negative Control siRNA (Silencer Select Negative control siRNA, Ambion, Thermo Fisher Scientific Inc., Rockford, IL, USA) or GPR21 siRNA (GPR21 Silencer Select Pre-designed siRNA cod. s6037, Ambion, Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's protocol.

THP-1 cells were seeded and differentiated into macrophages M_1 or M_2 as described above (see *Macrophage differentiation section)*, transfected with Transfection Reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions for 24 h and then harvested for sample preparation.

2.5. Western blot analyses

About 20 μg of total proteins was loaded for Western blot experiments, as previously described (Miglio et al., 2017; Benetti et al., 2016).

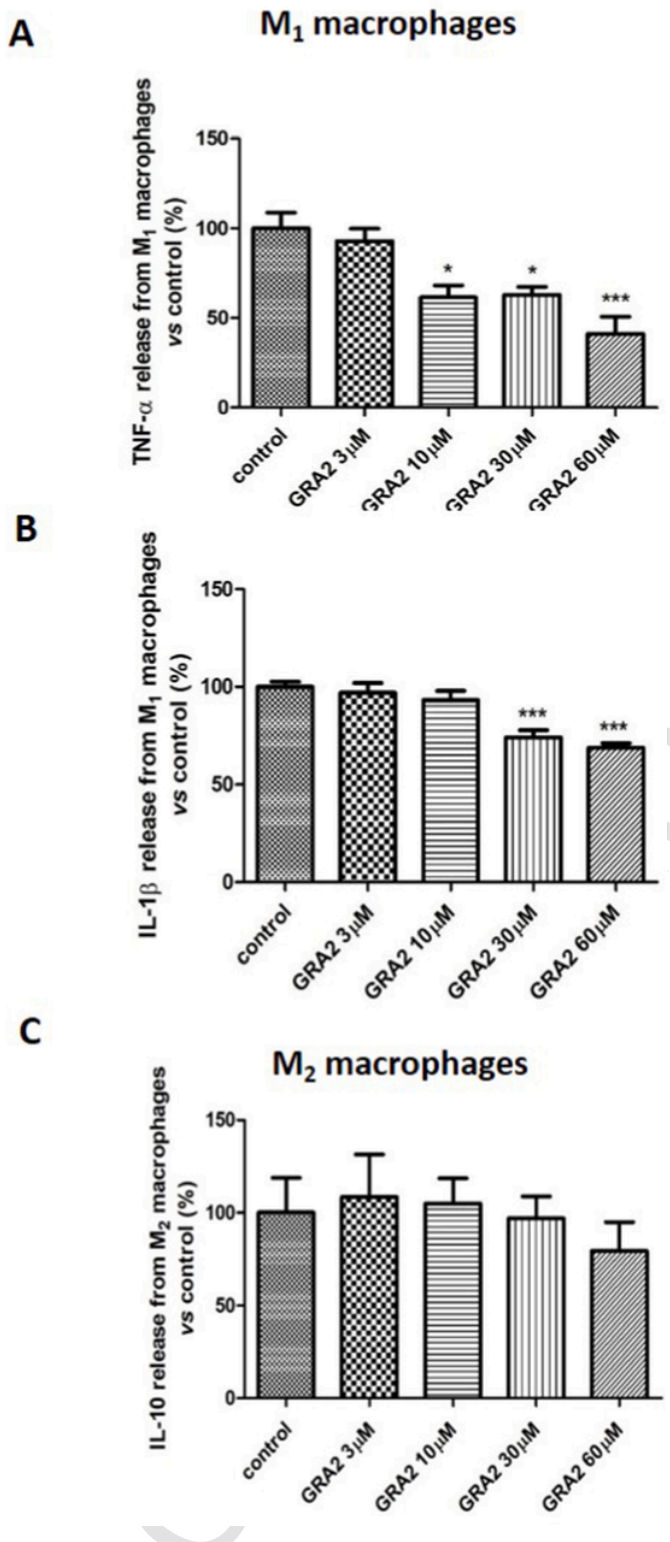

Fig. 5. Effect of GRA2 on cytokines release by macrophage-like cells. THP-1 cells were differentiated and activated in either M_1 **(A, B)** or M_2 **(C)**, and exposed to vehicle alone (control) or increasing concentration of GRA2 (3–60 μM). Cytokine release was quantified by ELISA. Data are expressed as mean ± SEM of four independent experiments run in duplicate. *$P < 0.05$, ***$P < 0.001$ vs control.*

After blocking, the PVDF membranes were incubated at 4 °C overnight with antibodies against GPR21 (1 μg/μl, Origene). To confirm equal protein loading, membranes were stripped and incubated with an anti-tubulin (1:5000, Abcam) or β-actin (1:5000, Sigma) monoclonal antibodies. Proteins of interest were detected with horseradish peroxidase-conjugated secondary antibody (1:5000, Cell Signaling Technology) for 1 h at room Temperature.

2.6. RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted using TRI Reagent® (Sigma, Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Complementary DNA synthesis and quantitative real-time PCR (q-PCR) reactions were performed on cells samples as previously described (Di Maira et al., 2022). mRNA levels were measured by q-PCR, using the SYBR® green method. The housekeeping human gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified in parallel in all amplification sets. Oligonucleotide sequence of primers used for qPCR are the following:

Primer	Sense	Reverse
Human ***TNF-α***	5′-AACCTCCTCTCTGCCATCAA -3′	5′-GGAAGACCCCTCCCAGATAC-3′
Human ***GPR21***	5′-TTTTCCACTGGGGCAAACCT-3′	5′-TTGGCAGATGCGGAAGATGT-3′
Human ***CCR1***	5′- ACCTGGTTAAATGGCTCCCC-3′	5′-AGAGTTCATGCTCCCCTGTG-3′
Human ***CCR3***	5′-CACAAGCCAGGGAGAAGTGAA-3′	5′-GCAGGCCCACGTCATCATAG-3′
Human ***GAPDH***	5′-TGGTATCGTGGAAGGACTCATGAC-3′	5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′
Human ***IL-1β***	5′-TGAAAGCTCTCCACCTCCAG-3′	5′-CACGCAGGACAGGTACAGAT-3′
Human ***IL-10***	5′-CCCCAACCACTTCATTCTTG-3′	5′-TCCCAAAGTGCTGGGATTAC-3′
Human **CD80**	5′-CCTACTGCTTTGCCCCAAGA-3′	5′-CAGGGCGTACACTTTCCCTT-3′
Human **CD86**	5′-TGGAAACTGACAAGACGCGG-3′	5′-CAAGGAATGTGGTCTGGGGG-3′
Human **CD206**	5′-GGCGGTGACCTCACAAGTAT -3′	5′-ACGAAGCCATTTGGTAAACG-3′
Human **CCL22**	5′-CCCCAACCACTTCATTCTTG -3′	5′-TCCCAAAGTGCTGGGATTAC -3′

mRNA amounts were calculated according to the threshold cycle of individual genes and their relative expression was quantified by serial dilutions of the amplified products compared with external standard curves of the reference genes containing known amounts of each gene product. The results were expressed as a relative ratio of the target to the housekeeping gene using the Light Cycler Relative Quantification software 4.05 (Roche Diagnostics, Monza, Italy). Samples were run in triplicate, and mRNA expression was generated for each sample. Specificity of the amplified PCR products was determined by melting curve analysis and confirmed by agarose gel electrophoresis and ethidium bromide staining.

2.7. Measurement of cytokines concentrations

Cell culture supernatants were collected and the levels of TNF-α, IL-1β and IL-10 were quantified with an enzyme-linked immunosorbent assay (ELISA) kit (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8. Cell motility assay

In the Boyden chamber (BD Biosciences, San Jose, CA, USA) migration assay, cells (5×10^3 cells/well) differentiated and activated into macrophages M_1 and M_2 were plated onto the apical side of 50 μg/ml Matrigel-coated filters (8.2 mm diameter and 5 μm pore size, Neuro Probe inc, BIOMAP) in serum-free medium with or without increasing

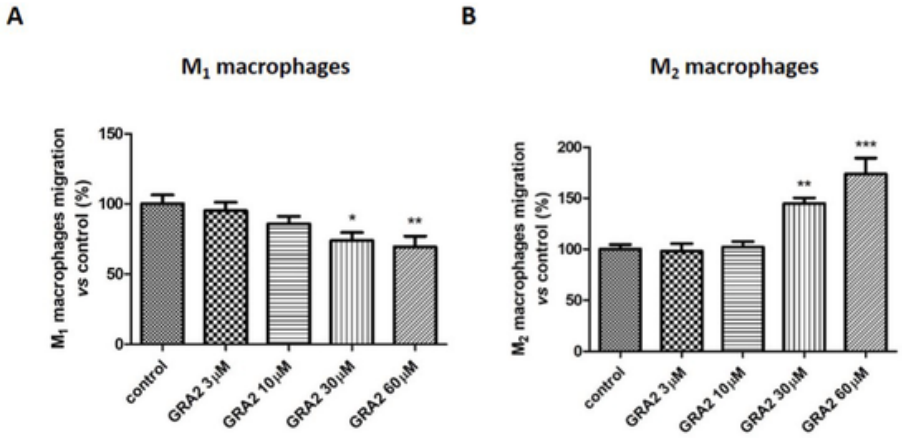

Fig. 6. Effect of GRA2 on migration of macrophage-like cells. THP-1 cells were differentiated and activated in either M_1 **(A)** or M_2 **(B)** macrophage-like cells, and exposed to vehicle alone (control) or increasing concentrations of GRA2 (3–60 μM). Cell migration was quantified by using the Boyden chamber migration assay. Data are expressed as mean ± SEM of six independent experiments run in triplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control.

concentrations of GRA2. Nontoxic drug concentrations were used for this assay. Medium containing C–C Motif Chemokine Ligand 7 (CCL7, 30 nM; ImmunoTools GmbH, Germany) was placed in the basolateral chamber as a chemo attractant for macrophages. After 6 h, cells on the apical side were wiped off with Q-tips. Cells on the bottom of the filter were stained with crystal violet, and all the fields were counted with an inverted microscope.

2.9. Data analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance was evaluated by one-way analysis of variance (ANOVA) with Bonferroni post-hoc test or by *t*-test (Prism 5, GraphPad Software, La Jolla, CA, USA). Differences were judged statistically significant when $P < 0.05$. Significance was denoted as $P < 0.05$*, $P < 0.01$**, $P < 0.001$***.

3. Results

3.1. Expression of GPR21, by human THP-1 cells differentiated into M_1 or M_2 macrophages

The expression of GPR21 in human THP-1 cells differentiated in M_0 macrophages by culture with PMA, and then in M_1 or M_2 macrophages by culture with either IFNγ + LPS or IL-4 + IL-13 (see Fig. 1S for the evaluation of the expression of specific markers of both macrophages M_1 and M_2), respectively, was analyzed at the mRNA level by Real-Time PCR and at protein level by Western-blot. As shown in Fig. 2 panel A, compared to M_0 cells, GPR21 mRNA levels were significantly increased in M_1 cells and decreased in M_2 cells, indicating that the GPR21 expression level correlates with the pro-inflammatory activity of macrophages, which is maximal in M_1 cells. Consistently, by analyzing the levels of GPR21 protein, we observed a trend in line with the gene expression (Fig. 2, panel B).

3.2. Effect of GRA2 on cell viability

To assess whether GRA2 exerts cytotoxic effects, THP-1 cells differentiated in M_1 or M_2 macrophages were exposed to either vehicle alone or increasing concentration of GRA2 (3–60 μM); cell growth was measured 24 h later by MTT assay. As shown in Fig. 3, in comparison to control cells no effect was exerted by GRA2 both on M_1 **(A)** and M_2 **(B)** macrophages. As indicated by these results, GRA2 did not affect the rate of growth of our cells.

3.3. Effect of GPR21 inhibition on IP_1 production

As GPR21 is a constitutively active receptor coupled to $G_{\alpha q}$ proteins, inositol-1-phosphate (IP_1) level was measured in M_1 and M_2 cells derived from THP-1 cells to quantify the activation state of this pathway and the effects of GRA2. The basal values were respectively $4{,}6 * 10^{-8}$ M $\pm 1{,}23 * 10^{-8}$ for M_1 and $2{,}3 * 10^{-8}$ M $\pm 3{,}87 * 10^{-9}$ for M_2 macrophages. Our results confirm that GRA2 acts as an inverse agonist of GPR21 in macrophages, with a concentration-dependent effect that became statistically significant at the concentration of 10 μM and 30 μM in M_1 and M_2 macrophages, respectively (Fig. 4). This is consistent with the different expression levels of GPR21 in these cells.

3.4. Effect of GRA2 on cytokine release

To investigate the role of GPR21 and the effect of GRA2 in macrophages, we evaluated the release of the characterizing cytokines in M_1 and M_2 cells derived from THP-1 cells, i.e. TNF-α and IL-1β in M_1 and IL-10 in M_2 cells. Results showed that GRA2 significantly decreased the secretion of the pro-inflammatory cytokines TNF-α and IL-1β in M_1 cells but had no significant effect on IL-10 secretion in M_2 cells (Fig. 5).

3.5. Effect of GRA2 on macrophage migration

As previous animal studies suggested an important role for GPR21 in promoting macrophage migration, we used a chemotactic assay to evaluate the effect of GRA2 in migration of M_1 and M_2 cells derived from THP-1 cells, using CCL7, a suitable chemokine to induce chemotaxis of both macrophage phenotypes, as a chemoattractant (Xuan et al., 2015). Results showed that GRA2 exerted an opposite effect in these cells, since it inhibited the migration of M_1 cells (Fig. 6, panel A), but it increased migration of M_2 cells (Fig. 6, panel B). The effect was statistically significant from the concentration of 30 μM for both the macrophage phenotypes.

To confirm this intriguing finding, we performed the same analysis on primary cells, obtained by differentiating human peripheral blood monocytes in M_1 cells by culture with GM-CSF and subsequent treatment with IFNγ + LPS or in M_2 cells by culture with M-CSF and subsequent treatment with IL-4. Analysis of cell migration induced by CCL7 showed that GRA2 significantly inhibited migration of M_1 cells treated with IFNγ + LPS, with a statistically significant effect since the dose of 30 μM (Fig. 7, panel A). By contrast, no significant effect was detected on M_1 cells treated with either IFNγ or LPS (Fig. 7, panel B and C). Moreover, GRA2 increased the migratory ability of M_2 cells, in line with what was observed in macrophages derived from THP-1 cells, with a

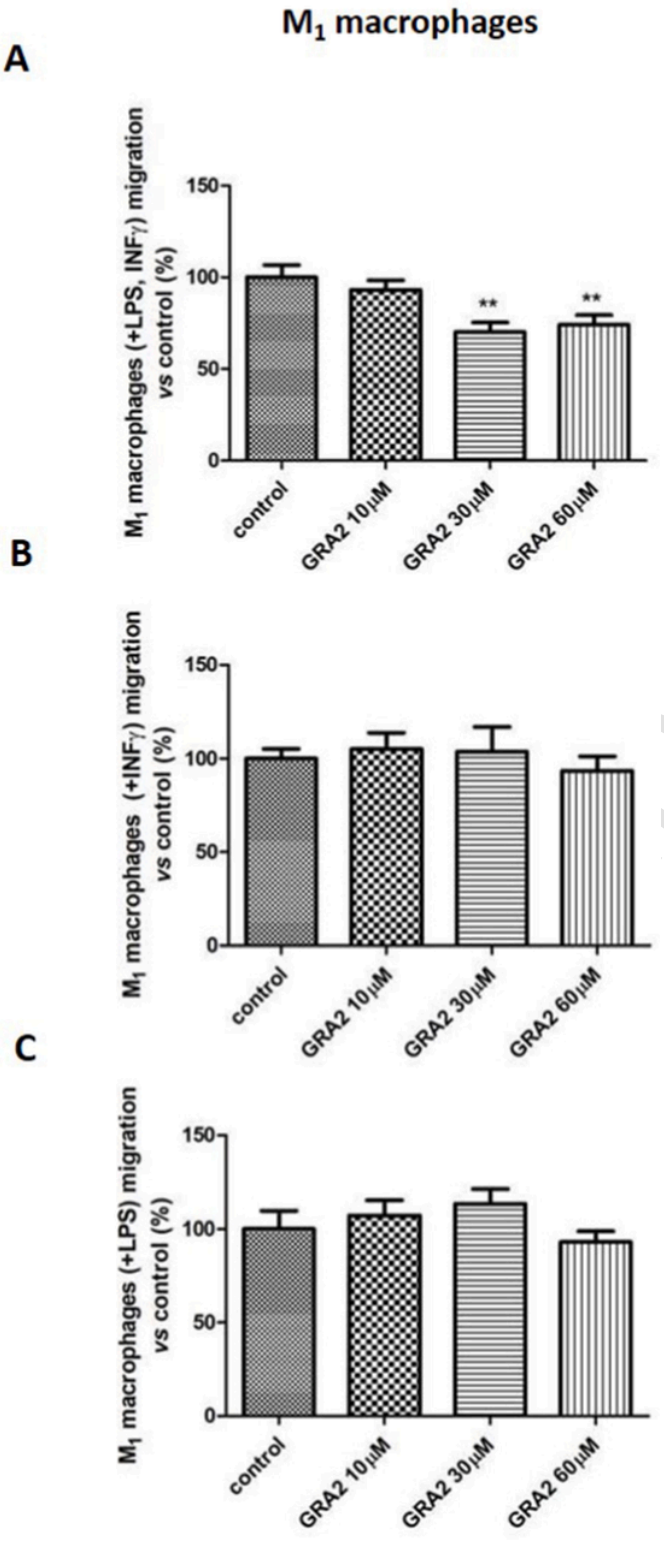

Fig. 7. Effect of GRA2 migration of M_1 macrophage-like cells. Human peripheral blood monocytes were differentiated in M_1 macrophages with GM-CSF, activated with IFNγ + LPS **(A)** and exposed to increasing concentrations of GRA2 (10–60 μM). Macrophages migration was quantified by using the Boyden chamber invasion assay. Panel **(B)** and **(C)** show the results obtained on INFγ-treated and LPS-treated M_1 macrophages, respectively. Data are expressed as mean ± SEM of eight independent experiments run in duplicate. *$P < 0.05$ vs control.*

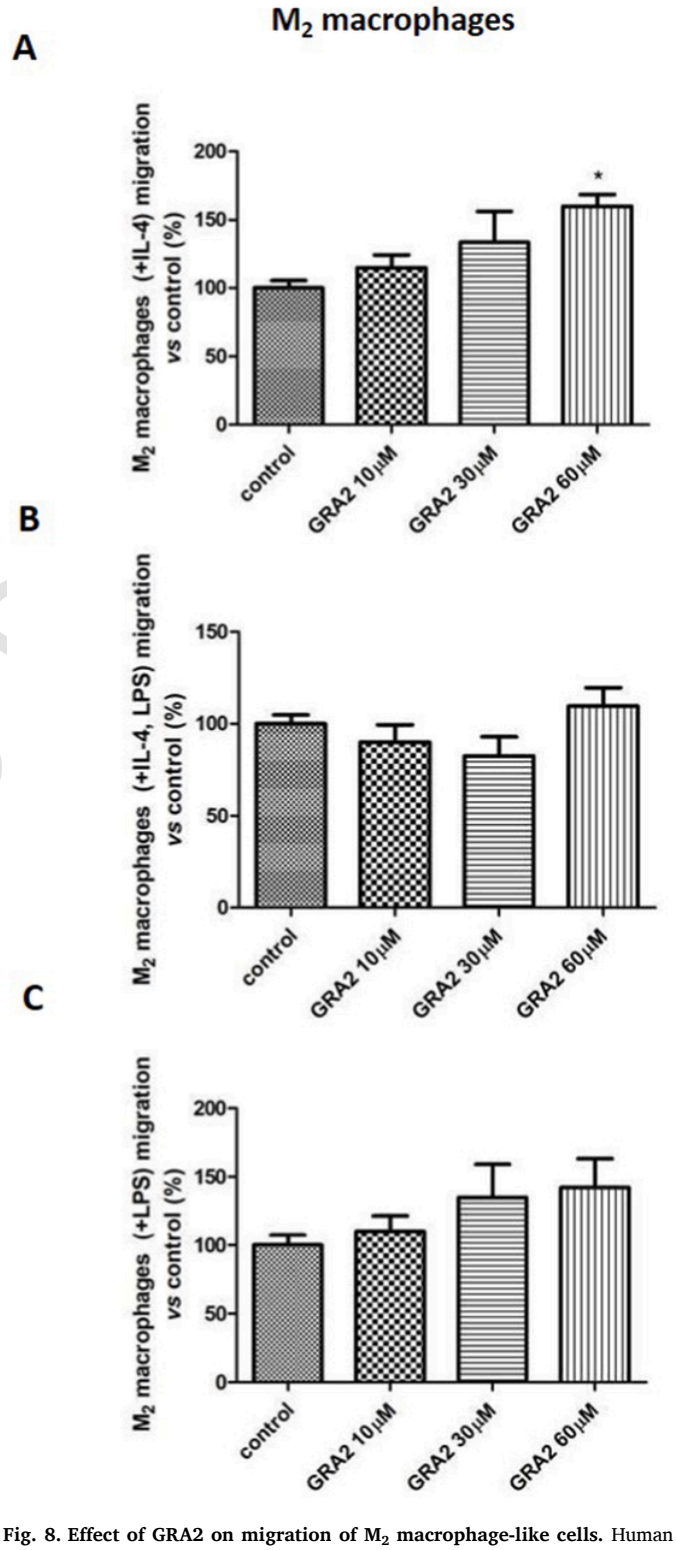

Fig. 8. Effect of GRA2 on migration of M_2 macrophage-like cells. Human peripheral blood monocytes were differentiated in M_2 macrophages with M-CSF, activated with IL-4 **(A)** and exposed to increasing concentrations of GRA2 (10–60 μM). Macrophages migration was quantified by using Boyden chamber migration assay. Panel **(B)** and **(C)** show the results obtained on IL-4, LPS-treated and LPS-treated M_2 macrophages, respectively. Data are expressed as mean ± SEM of seven independent experiments run in duplicate. *$P < 0.05$ vs control.*

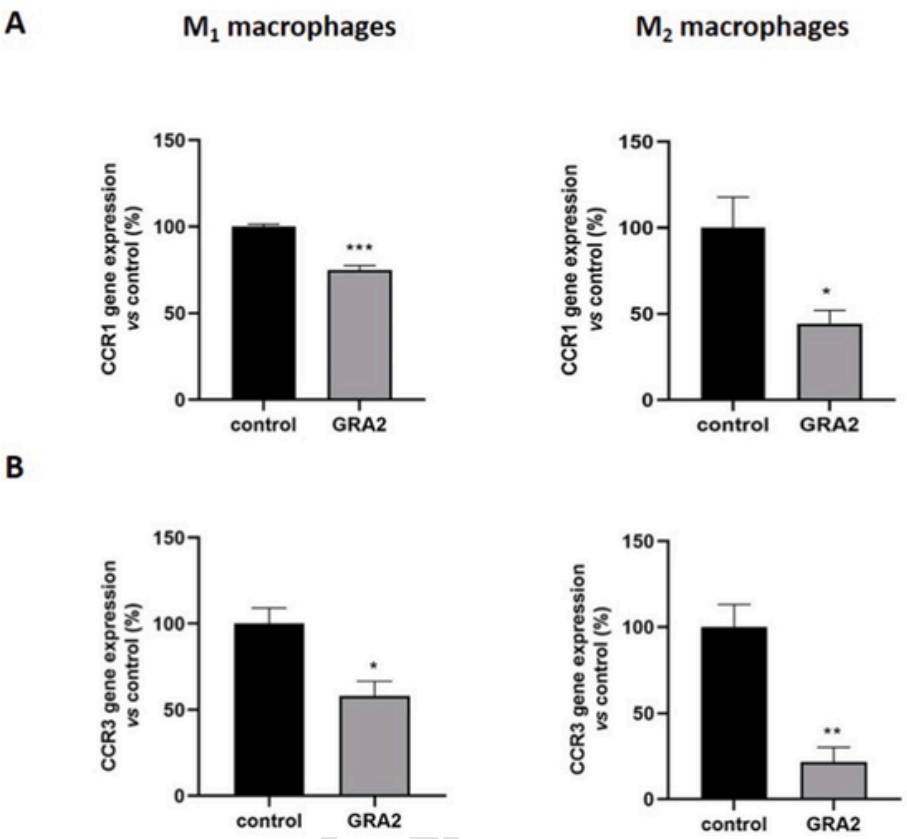

Fig. 9. Effect of GRA2 on CCL7 receptors expression in macrophage-like cells. THP-1 cells were differentiated and activated in either M_1 or M_2 macrophage-like cells and exposed to vehicle alone (control) or the highest concentration of GRA2 (60 μM). The expression of the gene encoding for CCR1 **(A)** and CCR3 **(B)** was evaluated by Real-Time PCR and expressed as percentage of M_1 or M_2 (100%). Data are expressed as mean ± SEM of three independent experiments run in triplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* M_1 or M_2.

significant effect at the highest concentration (Fig. 8, panel A). By contrast, no significant effect was detected on M_2 cells treated with LPS in the presence or absence of IL-4 (Fig. 8, panel B and C).

To investigate if the effect observed in presence of GRA2 could be due to CCL7 receptor modulation induced by the inverse agonist, we evaluated the gene expression of CCR1, CCR2 and CCR3 in the presence or absence of GRA2 in M_1 and M_2 macrophages derived from THP-1 cells. The expression of CCR2 in our cells was very low, at the minimum detectable level, therefore the results about this receptor are not shown.

Our data show that GRA2 induced a significant reduction of CCR1 (Fig. 9 panel A) and CCR3 (Fig. 9 panel B) transcript levels in both M_1 and M_2 macrophages, thus indicating that a modulation of CCL7 receptors could in part explain the observed effects on migration. However, by performing the experiments with other chemotactic factors (Fig. 3S), i.e. Fetal Bovine Serum (FBS) and osteopontin, we achieved the same results, thus showing that the effects of GPR21 inhibition on macrophages migration are not exclusively dependent on the chemokine CCL7 and its receptors.

3.6. Effect of GPR21 gene knockdown on M_1 and M_2 macrophages derived from THP-1 cells

To confirm that GRA2 effects are mediated via the GPR21 receptor, we evaluated cytokines release and migration ability of M_1 and M_2 macrophages (derived from THP-1 cells) treated, or not, with a specific siRNA against GPR21.

As show in Fig. 10, the use of specific siRNA against GPR21 was able to efficiently reduced GPR21 transcript (Fig. 10 panel A) and protein levels (Fig. 10, panel B) in both M_1 and M_2 cells. Consistently, the knockdown of the receptor resulted in a significant decrease expression and release of TNF-α (Fig. 10, panel C) and IL1-β (Fig. 10, panel D) in silenced M_1 macrophages. By contrast, no significant changes were observed in gene expression and release of IL-10 in GPR21-knockdown M_2 macrophages (Fig. 10, panel E). A similar effect on cytokines gene expression was obtain with GRA2 (Fig. 2S).

Moreover, by using the Boyden chamber migration assay, we observed (Fig. 10, panel F) an opposite effect in relation to cell phenotype, with inhibition of migration of M_1 cells and increase in migratory effect of M_2 polarized macrophages. These effects are fully consistent with results observed in cells treated with GRA2.

Finally, we assessed the effect of GPR21 inhibition, by either gene knockdown or GRA2 treatment, affects the mRNA expression of phenotypic markers of M_1 (CD80, CD86) and M_2 (CD2206, CCL22) macrophages (Fig. 11). Data shows that both inhibition strategies induced a significant decrease of the expression of CD80 and CD86 in M_1 macrophages. In contrast for M_2 macrophages, GRA2 treatment decreased expression of both CD206 and CCL222, whereas GPR21 knockdown did not have these effects.

4. Discussion

GPCRs are recognized to be important targets for drug discovery, representing the target of approximately 35% of marketed drugs (Santos et al., 2017; Sriram and Insel, 2018). In this field, particular interest is focused on the orphan receptors as their study could facilitate novel target validation (Hauser et al., 2017). This study confirmed the presence of the orphan receptor GPR21 on human macrophages and the ability of this receptor to modulate their activity. Our results showed that GPR21 is constitutively active in our experimental model, since its

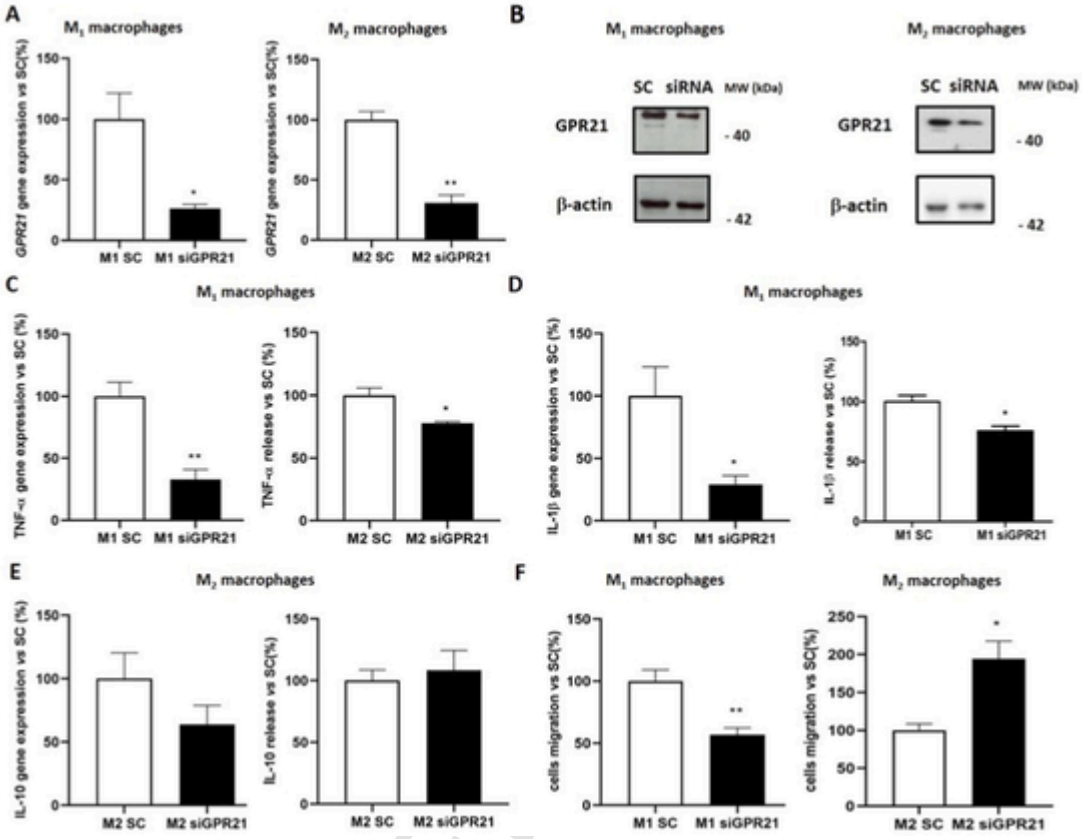

Fig. 10. Effect of GPR21 gene silencing on M_1 and M_2 macrophages derived from THP-1 cells. THP-1 cells were differentiated and activated in either M_1 or M_2 macrophage-like cells, and transfected with non-silencing siRNA (SC, Scramble) or silenced with siRNA against GPR21 for 24 h. The expression of GPR21 was evaluated by Real-Time PCR (panel A) and Western blot (panel B). TNF-α, IL-1β and IL-10 were evaluated at the gene level and by ELISA (panel C, D and E respectively). Macrophages migration was quantified by using Boyden chamber migration assay (panel F). Data are expressed as mean ± SEM of three independent experiments run in triplicate. $*P < 0.05$, $**P < 0.01$ *vs* M_1 or M_2 SC.

inverse agonist GRA2 inhibits its baseline signalling activity assessed as IP_1 production. Therefore, some functional aspects of this receptor can be studied even in the absence of a known endogenous ligand, by focusing on inhibition of its constitutive activity.

Macrophages can be found in tissues as either resident cells or cells deriving from blood monocytes leaving the circulation and migrating into tissues mainly in response to inflammatory stimuli. By a process named 'macrophage polarization', macrophages acquire different functional phenotypes in response to the micro-environmental encountered in different contexts (Murray et al., 2014; Sica and Mantovani, 2012). The resulting different macrophage populations are characterized by substantial heterogeneity, in morphology, function and cytokine production (Viola et al., 2019). The best characterized functional subsets are M_1 and M_2 macrophages, where M_1 cells play a key role in the effector phase of inflammation, whereas M_2 cells play a role in resolution of inflammation and starting of tissue repair (Das et al., 2015; Gordon, 2003; Mosser, 2003). These different activities are reflected by cytokine production, since M_1 macrophages produce high amounts of pro-inflammatory cytokines, such as TNF-α and IL-1β, and play a role also in activation of adaptive immunity (Dall'Asta et al., 2012; Mantovani et al., 2013), whereas M_2 macrophages preferentially produce anti-inflammatory cytokines, such as IL-10, and play a role also in switching down the adaptive immune response by supporting differentiation of regulatory T cells (Porta et al., 2015; Schultze and Schmidt, 2015; Schultze et al., 2015).

Key findings of our work were the distinct coordinated anti-inflammatory effects of GRA2 on M_1 and M_2 cells exerted on the one hand by inhibiting migration of M_1 macrophages and their expression of the pro-inflammatory cytokines TNF-α and IL-1β and the costimulatory molecules CD80 and CD86, involved in T cell activation; on the other hand, by increasing migration of M_2 macrophages, without inhibiting their expression of IL-10. These effects were ascribable to inhibition of the constitutive GRP21 function, since they were obtained also by knockdown of the GRP21 gene. Moreover, the effect on M_1 cells was detected only in M_1 cells fully activated with IFNγ + LPS, whereas the effect on M_2 cells was lost when GRA2 was used on M_2 cells activated with LPS, that attenuates the anti-inflammatory phenotype of these cells promoting their plasticity toward M_1 cells. It is possible that GRA2 may display different effects on different subsets of M_2 cells, which have been subdivided in M_{2a}, M_{2b}, M_{2c} and M_{2d} cells with distinct functions, being M_{2a} the classically anti-inflammatory ones, corresponding to those used in our experiments. The opposite migration response of M_1 and M_2 macrophages was only partly surprising since they have been reported to display different migration properties in both mice and humans (Cui et al., 2018; Vogel et al., 2014).

The function of GPR21 has been little investigated so far, but a role in inflammation has been previously suggested by Gardner et al., who observed a lower level of inflammatory markers in the plasma of GPR21 KO mice fed with a high fat diet to induce insulin resistance (Gardner et al., 2012). At the same time, another group studied the effect of GPR21 in a similar model of obesity-induced insulin resistance and suggested that GPR21 may play a role in the macrophage migration into tissue (Osborn et al., 2012). This hypothesis was questioned by Wang et al., (2016), but very recently further supported by Riddy et al., (2021), that by a specific KO-model confirmed that GPR21 affects both glucose homeostasis and macrophages migration. Consistently, Romero-Nava et al., (2021) suggested GPR21 as a potential therapeutic target for metabolic syndrome and very recently we demonstrated that GPR21 inhibi-

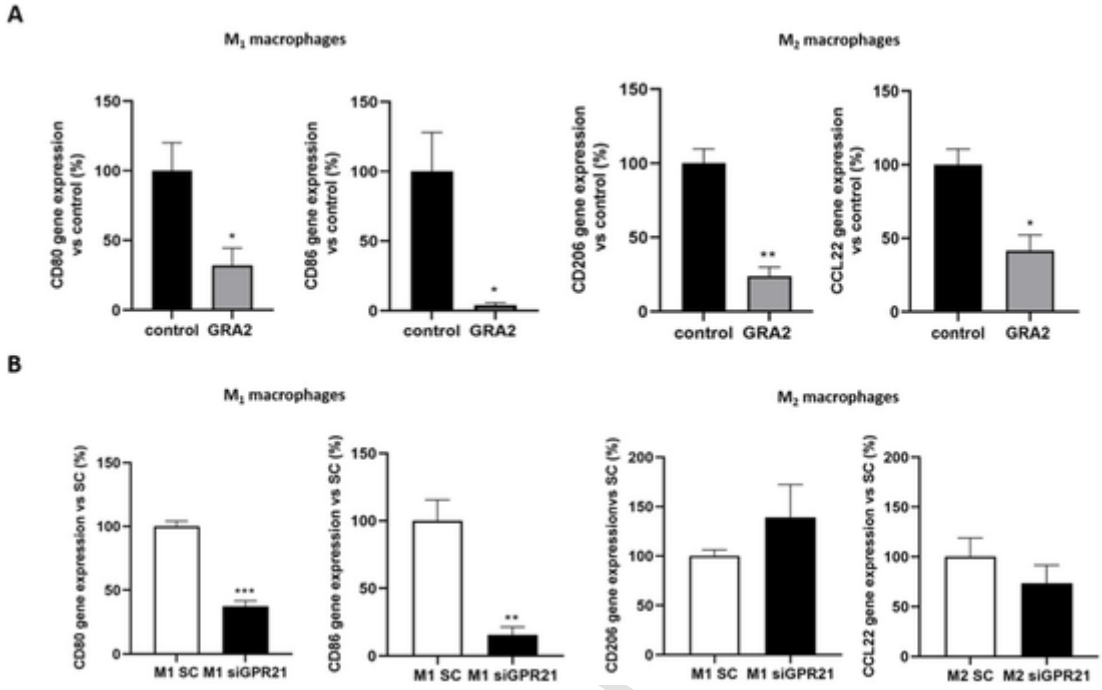

Fig. 11. Effect of GPR21 inhibition on phenotypic markers of M_1 and M_2 macrophages derived from THP-1 cells. THP-1 cells were differentiated and activated in either M_1 or M_2 macrophage-like cells, and treated with or without GRA2 (panel A) or transfected with non-silencing siRNA (SC, Scramble) or silenced with siRNA against GPR21 (panel B) for 24 h. The gene expression level of CD80, CD86, CD206 and CCL22 were evaluated by Real-Time PCR. Data are expressed as mean ± SEM of three independent experiments run in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.01$ *vs* relative control.

tion, by GRA2 or siRNA, improves glucose uptake in HepG2 cells (Kinsella et al., 2021).

Here, we confirm the presence of GPR21 in human macrophage, showing its expression is significantly different with respect to cell phenotype. More important, our results indicate that pharmacological inhibition or gene knockdown of GPR21 decreases M_1 pro inflammatory activity, known to be implicated in all the pathological conditions characterized by low grade of inflammation, such as insulin resistance. In addition, we demonstrated an improvement of the migration of M_2 macrophages, cells known to promote insulin sensitivity (Chawla et al., 2011).

Thus, our data supports previous observations (Osborn et al., 2012; Riddy et al., 2021) and helps to shed light on the mechanisms underlying the GPR21 involvement in inflammation and the potential of its targeting with pharmacological inhibitors, expanding the context from macrophage migration to cytokine production and selective activity on macrophage subsets.

A limit of this study is the high concentration of GRA2 necessary to achieve the observed effects, which decreases the pharmacological interest of this molecule. However, this study highlights the potential benefit induced by GPR21 inhibition and suggests that this strategy deserves further investigation, including a structure-activity relationship study of GRA2 aimed to construct molecules with optimized structure and increased potency.

In conclusion, our results suggest that GPR21 inhibition could inhibit inflammation by a double mechanism: through inhibition of the proinflammatory activity of M_1 macrophages and stimulation of the migration of the M_2 macrophages. Therefore, by modulating macrophage behaviour, inhibition of GPR21 activity might represent a novel and promising pharmacological strategy for pathological conditions involving low grade chronic inflammation.

Preliminary results of this study were presented as a poster at the 40° annual meeting of Italian Society of Pharmacology. The abstract was published in Pharmadvances, vol. 3, 2021.

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CRediT authorship contribution statement

Valentina Bordano: Investigation. **Gemma K. Kinsella:** Conceptualization, Writing – review & editing, Funding acquisition. **Stefania Cannito:** Methodology, Formal analysis, Investigation. **Chiara Dianzani:** Methodology, Formal analysis, Investigation. **Casimiro Luca Gigliotti:** Methodology, Resources. **John C. Stephens:** Resources. **Chiara Monge:** Investigation. **Claudia Bocca:** Methodology, Investigation, Resources. **Arianna C. Rosa:** Formal analysis. **Gianluca Miglio:** Writing – review & editing. **Umberto Dianzani:** Resources, Writing – review & editing. **John B.C. Findlay:** Conceptualization, Writing – review & editing. **Elisa Benetti:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Project administration, Funding acquisition.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2022.175018.

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