The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells

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Abstract

G protein-coupled receptor 55 (GPR55) is activated by endogenous, plant-derived and synthetic cannabinoids. Recent studies reported a broad tissue distribution for GPR55 and found prominent roles for this receptor in inflammatory pain, gut and bone physiology, as well as cancer. However, little is known about the expression and function of GPR55 in immune cells. To address this question, we performed a detailed characterization of GPR55 in different human innate and adaptive immune populations using polychromatic flow cytometry and we found that monocytes and NK cells expressed remarkable levels of this receptor compared to several cells of adaptive immunity. GPR55 activation by the specific agonist O-1602 boosted IL-12 and TNF-α production, and decreased endocytic activity, in LPS-activated monocytes. In addition, it increased CD69 activation marker expression, granzyme B and CD107a-dependent cytotoxicity and IFN-γ and TNF-α production in NK cells activated by both IL-2 and IL-12. These over-stimulatory effects of GPR55 were antagonized by its selective antagonist cannabidiol. Altogether, our data thus unveil a proinflammatory role for GPR55 in innate immunity that may be important for the design of new immune therapeutic strategies.

Keywords: cannabinoid receptors, immunoregulation, innate immunity

Introduction

To date, two G protein-coupled cannabinoid receptors have been identified: CB1, mostly located in neuronal cells, and CB2, predominantly expressed by immune cells as well as in the central nervous system under different stresses, including neuroinflammatory states (1–3). Recently, orphan G protein-coupled receptor 55 (GPR55) (4) has emerged as a candidate for mediating some of the non-CB1/non-CB2 responses induced by endogenous, plant-derived and synthetic cannabinoids, leading to the hypothesis that it might be a putative ‘type-3’ cannabinoid receptor (5, 6). However, the low aminoacidic sequence identity of GPR55 with CB1 and CB2, as well as controversy regarding its signaling, makes it disputable that GPR55 is indeed a true ‘CB1’ receptor (6, 7). GPR55 is widely expressed in the brain, especially in the cerebellum, and in many peripheral tissues, from the gastrointestinal tract, spleen and bone to vascular and endothelial cells.

Such a rather wide distribution suggests that GPR55 might be involved in many biological processes, in both central and peripheral tissues (8). Indeed, a major role for this receptor has been proposed in neuropathic or inflammatory pain (9), in the pathophysiology of the gut (10) and in bone remodeling (11). Additional roles for GPR55 have been suggested in the modulation of vascular function (12, 13) and in angiogenesis (14), in fetoplacental development during pregnancy (15), as well as in cancer progression where it modulates cancer cell proliferation and migration (16, 17). Interestingly, in contrast to CB1 and CB2, that mostly trigger inhibitory actions, GPR55 mainly exerts excitatory and stimulatory effects. As yet, the only evidence for a role of GPR55 in peripheral blood immune cells has been documented in human neutrophils and mast cells, where it appears to regulate neutrophil migration, degranulation and production of reactive oxygen species.
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(18, 19) and also to modulate mast cell-mediated anti-inflammatory actions (20). However, expression, cellular distribution and function of GPR55 in human peripheral blood cells have not been investigated yet. Here, we sought to characterize for the first time GPR55 in distinct populations of human mononuclear cells of both innate and adaptive immunity. Our data point to novel proinflammatory functions of GPR55 in monocytes and NK cells, where this receptor was particularly expressed.

Methods

Cell preparation and reagents

PBMCs were isolated after venous puncture from healthy donors and were separated by density gradient over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) according to standard procedures (21). Isolated PBMCs were cultured in RPMI-1640 complete medium supplemented with 10% (v/v) heat-inactivated human serum. K562 cells, a human myeloid leukemia cell line, were sub-cultured in RPMI-1640 complete medium supplemented with 10% FCS without phenol red and were used as target cells. WIN55,212-2 [(R)-(+)2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoazin-6-yl]-1-naphthalenylmethanone mesylate] and O-1602 [5-Methyl-4-[(1R,6R)-3-methyl-6-(1-cyclohexen-1-yl)-1,3-benzenediol] were purchased from Tocris Bioscience (Bristol, UK), and 3-α-lysocephatidylinositol (LPI) was purchased from Sigma-Aldrich (St Louis, MO, USA).

GPR55 expression by flow cytometry

Freshly isolated PBMCs (2 × 10⁶ cells) were washed twice with PBS and stained with a rabbit IgG anti-GPR55 antibody (Cayman Chemical Co., Ann Arbor, MI, USA) in stain buffer (PBS supplemented with 0.5% FCS and 0.02% NaN₃) for 10 min at 4°C. Cells were then washed with PBS and stained with anti-rabbit Alexa-633 secondary antibody (1:100) and with two different combinations of fluorescence-conjugated antibodies (Mix 1 and Mix 2) against markers of specific immune cell populations for 15 min in staining buffer at 4°C, as reported in Table 1. Cells were also stained with IgG isotype and Alexa-633 secondary antibody alone in order to assess background staining and specificity. Surface expression of GPR55 in different immune cells was analyzed by FACS (FACSCyan, Coulter).

Cell sorting

For the identification and subsequent purification of monocytes and NK cells, PBMCs underwent a three-color staining (anti-CD14 APC-750, anti-CD3 and anti-CD19 PE-Cy7 and anti-CD56 PE), and then were sorted using a MoFlo Cell Sorter (Beckman Coulter, Fullerton, CA, USA). The correct identification of the different cell populations required multiparametric flow cytometry, as previously reported (22). Thus, monocytes were gated as CD14⁺ cells and NK cells were identified as CD56⁺ cells by excluding CD3⁻ T lymphocytes and CD19⁺ B lymphocytes. This gating strategy allowed us to identify and purify with confidence two subsets of immune cells.

Immunoblotting

Purified human CD3⁺ T cells, CD14⁺ monocytes and CD56⁻ NK cells were lysed with lysis buffer and cell homogenates were subjected to 10% SDS–PAGE (50 μg/lane) under reducing conditions. Gels were then electrophoresed onto 0.45-μm nitrocellulose filters (Bio-Rad, Hercules, CA, USA) and were immunoreacted with anti-GPR55 polyclonal antibody (1:200, Cayman Chemical Co.), or with anti-β-actin monoclonal antibody (1:5000, Bio-Rad), and goat-anti-rabbit polyclonal antibody (1:2000, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) as a secondary antibody. The specificity of anti-GPR55 antibody was ascertained by pre-incubating 1 μg of this antibody with 10mg of its specific blocking peptide (Cayman Chemical Co.).

Confocal microscopy

Highly purified CD3 T cells, CD14⁺ monocytes and CD56⁻ NK cells were sorted with a MoFlo Cell Sorter and seeded on Chamber Slides (Lab-Tek; Electron Microscopy Sciences, Hatfield, PA, USA) at 5 × 10⁵ cells/well, fixed with 3% formaldehyde for 10 min and then stained with anti-GPR55 antibody (1:100) (Cayman Chemical Co.). The expression and localization of GPR55 receptor was visualized by confocal microscopy in a TCS SP Leica microscope (Wetzlar, Germany), acquiring images through the LAS AF program (Leica) as previously reported (23).

Intracellular staining by flow cytometry

Highly purified human monocytes and NK cells were left untreated or were pre-treated with GPR55 agonist O-1602, alone or in presence of GPR55 antagonist cannabidiol (CBD). To allow cytokine synthesis by monocytes and NK cells, the former were stimulated with 100ng/ml LPS for 6h, and the latter were cultured with 100U/ml IL-2 and 1ng/ml IL-12 for 24h. Release of cytokines was inhibited by adding 1 μg/ml Brefeldin A (Sigma-Aldrich), 5 h before the end of stimulation. At the end of the incubation, cells were stained at the cell surface with specific antibodies, fixed with 4% formaldehyde for 10min and then stained intracellularly with anti-TNF-α-PercP5.5 (1:100) and anti-IFN-γ-v450 (1:100) antibodies, as well as with anti-granzyme FITC (1:50) and anti-perforin-PE...
(1.50) in 0.5% saponin, at room temperature. Intracellular production of cytokines and/or granzyme/perforin by monocytes and NK cells was analyzed by flow cytometry in a FACSCyan ADP (Beckman Coulter), as previously reported (22). For each analysis, at least 300,000 events were acquired and viable cells were analyzed by using the FlowJo software (TreeStar, Ashland, OR, USA).

**Endocytosis assay**
Highly purified human monocytes were left untreated or were pre-treated with GPR55 agonist O-1602, alone or in the presence of GPR55 antagonist CBD. Cells were then incubated with 1 mg/ml FITC–dextran (molecular weight: 70 kDa; Sigma-Aldrich) at 37°C for 30 min in the dark to allow particle uptake. Endocytosis was stopped by adding ice-cold PBS and cells were extensively washed with cold PBS. Cells were collected and analyzed for FITC–dextran uptake by Flow Cytometry (FACSCanto, Becton Dickinson).

**Cytotoxicity assays**
Sorted and highly pure effector NK cells were incubated with or without target K562 cells at 37°C, 5% CO₂, for 4 h. Non-specific lysis was determined by incubation of NK cells, alone or in the presence of 1% saponin, at room temperature. Intracellular production of cytokines and/or granzyme/perforin by monocytes and NK cells was analyzed by flow cytometry in a FACSCyan ADP (Beckman Coulter), as previously reported (22). For each analysis, at least 300,000 events were acquired and viable cells were analyzed by using the FlowJo software (TreeStar, Ashland, OR, USA).

**Statistical analysis**
Statistical analysis (mean ± SD) was performed using Prism 5.0 (GraphPad Software, San Diego, CA, USA). Significant differences were calculated using one-way ANOVA followed by the non-parametrical Mann–Whitney U-test. Differences were considered significant at P values <0.05.

**Results**

**GPR55 expression in peripheral blood cells**
Initial studies were performed to assess the differential expression of GPR55 in the main cell subsets of human peripheral blood, i.e. CD³⁺ T lymphocytes (CD⁴⁺ and CD⁸⁺), CD₁⁹⁺ B lymphocytes, CD₁₅⁶⁺ NK cells, CD₁₄⁺ monocytes, CD₁₁c⁺ CD₁₂₃⁺ myeloid dendritic cells (mDCs), CD₁₁c⁻ CD₁₂₃⁺ plasmacytoid dendritic cells (pDCs), CD⁴⁺ CD₂₅⁻ naïve and CD⁴⁺ CD₂₅⁺⁰ Treg cells. CD⁴⁺ CD₂₅⁻ naïve and CD⁴⁺ CD₂₅⁺⁰ Treg cells were constantly analyzed for surface CD₁₂₇ and intracellular Foxp3 expression as *bona fide* markers to distinguish them, as previously reported (data not shown) (24). Such immunophenotypic characterization was performed by means of two different polychromatic staining mixes (Table 1) that allowed to correct identification of each cell population by means of the gating strategy shown in Fig. 1(A and B). As reported in Fig. 1, surface staining revealed low mean fluorescence intensity (MFI) levels of GPR55 expression in both subtypes of CD³⁺ T lymphocytes (MFI = 64.6 ± 9.3), namely CD⁴⁺ (MFI = 74.4 ± 5.2) and CD⁸⁺ T cells (MFI = 51.2 ± 9.7), as well as in naïve T cells, either CD₂₅low (MFI = 64.6 ± 6.2) or CD₂₅⁺ (MFI = 57.9 ± 5.9). Even lower levels of GPR55 expression were observed in pDCs (MFI = 36.0 ± 1.5) compared to CD⁴⁺ CD₂₅⁻ (MFI = 16.9 ± 3.6) and higher levels in mDCs (MFI = 235.0 ± 49.1). Interestingly, intense fluorescence was apparent in CD3⁺ T cells (MFI = 1114.0 ± 222.3) and monocytes (MFI = 1198.0 ± 118.5). Furthermore, Treg showed the largest variation (~30%) in GPR55 expression levels, whereas for each of the other cell populations, GPR55 levels were similar among different subjects (variation of ~10–20%). As reported in Fig. 1C, the signal intensity could be ranked as follows: monocytes/NK cells > mDCs > B cells > Treg > T cells > pDCs. Since monocytes and NK cells are both subdivided into different subsets, according to the expression of CD₁₆, we also investigated whether GPR55 expression differs in their major circulating subsets. Interestingly, we found that, among monocyte subsets, GPR55 was particularly expressed in intermediate (CD₁⁴⁺⁺ CD₁⁶⁻) and in non-classical (CD₁⁴⁺ CD₁⁶⁺⁺) monocytes, although the expression of this receptor on classical monocytes (CD₁⁴⁺⁺ CD₁⁶⁻) was also very high (Fig. 2A). As for NK cells subsets, GPR55 was almost equally expressed in CD₅₆⁺⁰⁺ CD₁⁶⁻ and in CD₅₆⁻ CD₁⁶⁺ NK cells, whereas the least represented population of CD₅₆⁺ CD₁⁶⁺ NK cells expressed low levels of GPR55 (Fig. 2B). In order to confirm such a marked expression of GPR55 in monocytes and NK cells, these cell populations were sorted through MoFlo, and the expression of this receptor was validated on highly purified cells through flow cytometry (Fig. 2C), immunoblotting (Fig. 2D) and confocal microscopy (Fig. 2E). All three methods showed that GPR55 was indeed more expressed in NK cells and monocytes compared to CD³⁺ T cells as well as to non-expressing HEK293 cells, which were used as negative controls (Supplementary Figure 1A, available at International Immunology Online).

**Role of GPR55 activation in monocytes and NK cells**
Since monocytes and NK cells expressed remarkable levels of GPR55, we next sought to investigate the possible functional activity of this receptor in these innate immune cells. Initially, we checked whether LPS-activated monocytes and IL-2 + IL-12-activated NK cells underwent any variation of GPR55 expression at their cell surface, and we found a respective 1.2- and 1.3-fold up-regulation of the receptor in activated cells (Supplementary Figure 1B, available at International Immunology Online). Then, its potential functional role was investigated upon activation with the GPR55 synthetic agonist O-1602, alone or in combination with the GPR55 antagonist CBD (6, 7). Compared to resting monocytes, LPS-activated monocytes produced high levels of pro-inflammatory IL-12 and TNF-α and ingested greater amounts of dextran particles. However, when challenged with O-1602, the production of both cytokines increased even further, whereas endocytic activity was inhibited (Fig. 3). In particular, GPR55 activation induced a significant ~50% and ~30% increase in IL-12 (Fig. 3A) and TNF-α (Fig. 3B) production,
respectively, from LPS-activated monocytes. Additionally, LPS-activated monocytes reduced by more than half their ability to ingest dextran particles upon GPR55 activation (Fig. 3C). These over-stimulatory and proinflammatory effects of GPR55 were also corroborated not only by the evidence that cytokine production and endocytic activity were counteracted when GPR55 was blocked by CBD (Fig. 3), but also by showing a significant increase in IL-12 and TNF-α upon treatment with the GPR55 endogenous ligand LPI but not with its non-selective agonist WIN55,212-2 (Supplementary Figure 1C, available at International Immunology Online). Interestingly, we also observed that GPR55 activation up-regulated cytokine production and suppressed endocytic activity even in resting monocytes, suggesting that this receptor might also exert a stimulatory effect under physiological conditions. Functional activity of GPR55 was further assessed in NK cells, where expression of activation and inhibitory markers, cytokine production and cytolytic activity were assessed. To investigate the influence of GPR55 on activated NK cells, a combination of IL-2 and IL-12 cytokines was used for 48h (25). Figure 4A shows that cytokine-activated NK cells had a relevant (~83%) surface expression of the activation marker CD69 compared to unstimulated control cells (~10%). When GPR55 was activated with O-1602, an up-regulation of CD69 was observed in both unstimulated and cytokine-activated NK cells (up to ~28% and ~90%, respectively), with a subsequent decline following GPR55 antagonism by CBD (down to ~17% and ~84%, respectively). Then, we investigated cytolytic activity of NK cells evaluated as granzyme B and perforin production, as well as by CD107a-mediated killing. When cytolytic activity was analyzed in unstimulated NK cells, no granzyme B- nor perforin-mediated killing activity was found. Instead, in cytokine-activated NK cells, a specific ~25% granzyme B-mediated and ~15% perforin-mediated lysis was observed. Interestingly, granzyme B-mediated (Fig. 4B) rather than perforin-mediated (Fig. 4C) cytolytic activity was enhanced by GPR55 agonism, with a decline similar to the levels observed in cytokine-activated NK cells in the presence of CBD. To clearly demonstrate that GPR55 enhances the cytolytic activity of NK cells, we also evaluated the ability

![Fig. 1. GPR55 surface expression in several innate and adaptive immune cells from human PBMCs through polychromatic flow cytometry. (A) According to physical parameters and using Mix 1, total lymphocytes were gated on CD3− and further gated as CD8+ or CD4+ cells. Subsequently, CD4+ cells were gated against CD25, and three cell populations were identified: CD4+ CD25high (Treg), CD4+ CD25mid and CD4+ CD25− cells. CD3+ cells were then gated against CD19 (B cells) and CD56− CD16− (NK cells). (B) According to physical parameters and using Mix 2, all PBMCs were gated and monocytes were identified as CD14+ HLA-DR+. DCs were then gated on HLA-DR+, excluding cells that expressed the lineage antibodies (CD3, CD19, CD56). Within HLA-DR− Lineage− cells, mDCs were characterized as CD11c+ CD123− cells, whereas pDCs are CD123+ CD11c−. In (A) and (B), FACS blots are representative of a single experiment, whereas histograms of their corresponding blots represent the MFI. (C) Summary of GPR55 expression in all cell subsets. Data represent the MFI ± SD of 12 independent experiments.](https://academic.oup.com/intimm/article/27/3/153/659079)
Fig. 2. GPR55 expression in NK cells and monocytes subsets and in highly purified cells. Major circulating subsets of NK cells and monocytes were gated according to CD16 expression and GPR55 levels were evaluated (A). FACS blots are representative of a single experiment, whereas histograms of their corresponding blots represent the MFI of four independent experiments. Highly purified CD3+ T cells, CD56+ CD16+ NK cells or CD14+ monocytes were analyzed for the expression of GPR55 by means of flow cytometry (C), western blotting (D) or confocal microscopy (E), as detailed in Methods. Densitometry data are shown as mean ± SD of three independent experiments. *P < 0.05 versus CD3+ T cells.
of NK cells to kill target K562 cells by a CD107a degranulation assay and by apoptosis assessment by Annexin-V/PI dot plots of control target K562 cells and K562 cells co-cultured with O-1602- or CBD-treated NK cells. We found a significant increase in CD107a expression on NK cells after K562 stimulation (Fig. 4F) and in both early and late apoptosis on K562 target cells in the presence of effector NK cells (Fig. 4G), and such effects were enhanced when NK cells were pre-treated with O-1602 but not with CBD. In parallel, we also evaluated the ability of GPR55 to affect intracellular cytokine production by NK cells. A relevant increase in IFN-γ (Fig. 4D) and TNF-α (Fig. 4E) was observed in cytokine-activated NK cells compared to unstimulated cells, an effect that was significantly increased upon GPR55 activation, and that was blunted by blocking the receptor with CBD (Fig. 4D and E). The significant increases in both IFN-γ and TNF-α with LPI, but not with WIN55,212-2 (Supplementary Figure 1D, available at International Immunology Online), confirmed the specificity of GPR55 in mediating such proinflammatory effects.

Discussion

This is the first study reporting a detailed characterization of GPR55 expression in the main cell populations of human PBMCs, along with the functional activity of this receptor in these cells. Although the expression and function of ‘classical’ CB1 and CB2 receptors has been widely studied in several immune cells, as yet no studies have addressed the expression of GPR55 within the immune system. In the attempt to shed some light on the mononuclear cells of innate and adaptive immunity that could express GPR55, we performed polychromatic flow cytometry that allowed simultaneous interrogation of receptor expression in distinct cell populations. Such a multiparametric approach revealed remarkable levels of GPR55 in monocytes and NK cells and modest levels in mDCs. Interestingly, the evidence that GPR55 is by far more abundant in innate rather than adaptive immune cells suggests that its function might be associated with the regulation of several innate immune responses. This hypothesis supports recent reports showing that GPR55 plays a critical stimulatory effect in neutrophils and mast cells (18–20). The latter are indeed essential parts of the innate immune system, because they are one of the first responders that migrate towards the site of inflammation, herein secreting their inflammatory granules (26, 27). Of note, monocytes and their macrophage and DC progeny serve three main functions in the immune system, i.e. phagocytosis, antigen presentation and cytokine production,
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Fig. 4. GPR55 potentiates NK cell inflammatory responses. Highly purified CD56+ CD16+ NK cells were cultured in the absence or presence of GPR55 agonist O-1602 or antagonist CBD and then were challenged or not with IL-2 and IL-12 for 24 h. By means of flow cytometry, the expression of CD69 activation marker (A), the production of cytolytic granules containing granzyme (B) and perforin (C), as well as the production of IFN-γ (D) and TNF-α (E) were investigated. Data are shown as mean ± SD of six independent experiments. *P < 0.05 versus control (CTRL); **P < 0.05 versus O-1602. For the cytotoxic assay, resting or treated NK cells were co-cultured with target K562 cells for a further 4 h at a ratio of 5:1 and the cell mixture was stained with Annexin-V/PI and with anti-CD56/16-PE-Cy7 and CD107a-FITC (F and G). Data are shown as mean of fluorescence intensity ± SD or as % mean of positive cells ± SD of four independent experiments. *P < 0.05 versus K562; **P < 0.05 versus O-1602.

thus orchestrating immune defense (28). The observation that GPR55 negatively affects the ability of monocytes to phagocytose, concomitantly enhancing IL-12 and TNF-α production, suggests that this receptor might be involved in controlling the actual production of proinflammatory mediators, rather than in potentiating antigen capture, overall reinforcing immune responses. In addition, NK cells are among the most important players in innate immunity, being unique in recognizing and lysing stressed, infected or cancer cells in the absence of antibodies, thus allowing a much faster immune reaction (29). Interestingly, also in NK cells, GPR55 exerted a stimulatory effect, enhancing signature cytokines and cytolytic activity. In order to sense the environment and respond to alterations caused by infections, cellular stress or transformation, NK cells take advantage of synergistic signals from combinations of receptors, which are integrated to activate natural cytotoxicity and cytokine production (30). In this scenario, our data suggest that GPR55 might represent a novel stimulatory receptor for NK cells, and that natural or synthetic antagonists of this receptor could participate in the reversible tuning of NK cell responsiveness. Notably, the GPR55-mediated NK cell over-stimulation is particularly important in tumor immunosurveillance, where these cells are considered the first and most relevant executioners that recognize and eliminate malignant cells (31). Although several endogenous or synthetic GPR55 ligands can also bind to CB1 and CB2 receptors (6), albeit weakly, it is interesting to point out that CB2 is barely represented in immune cells and, when up-regulated, it either exerts proinflammatory effects or is involved in immune cell apoptosis or in proliferation suppression (32). Instead, the remarkably expressed CB1 is primarily anti-inflammatory (33). In conclusion, our unprecedented results demonstrate that GPR55 is mainly expressed within cells of the innate immune compartment, thus giving solid ground to previous evidence that receptor activation triggers proinflammatory events. Although further research needs to be pursued in order to investigate GPR55 signaling, and thus the possibility to exploit its activity for the modulation of innate immune cells, the finding that GPR55 may have a central role in innate immune responses represents a
promising clue for targeting this receptor for the treatment of chronic inflammatory disorders or cancer.

**Supplementary data**

Supplementary data are available at International Immunology Online.

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