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**Research Article**

# G Protein-Coupled Estrogen Receptor 1 Regulates Human Neutrophil Functions

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## What Is It about?

The role of estrogens in the immune system is relatively well known under both physiological and pathological conditions. Neutrophils are the most abundant circulating leukocytes in humans, and their abundance and function are regulated by estrogens. Although estrogens were thought to act via classical nuclear estrogen receptors, it was observed that some estrogens may induce rapid biological effects via a membrane-anchored receptor called G protein-coupled estrogen receptor 1 (GPER1). We report here that GPER1 regulates the activation and the life span of human neutrophils through several signaling pathways, pointing to GPER1 as a potential therapeutic target in immune diseases.

## Keywords

G protein-coupled estrogen receptor 1 · Neutrophils · Estrogens · Cell signaling · Human

## Abstract

***Background:*** The role of estrogens in immune functioning is relatively well known under both physiological and pathological conditions. Neutrophils are the most abundant circulating leukocytes in humans, and their abundance and function are regulated by estrogens, since they express estrogen receptors (ERs). Traditionally, estrogens were thought to act via classical nuclear ERs, namely ERα and ERβ. However, it was observed that some estrogens induced biological effects only minutes after their application. This rapid, "nongenomic" effect of estrogens is mediated by a membrane-anchored receptor called G protein-coupled estrogen receptor 1 (GPER1). Nevertheless, the expression and role of GPER1 in the immune system has not been exhaustively studied, and its relevance in neutrophil functions remains unknown. ***Methods:*** Human neutrophils were incubated in vitro with 10–100 μM of the GPER1-specific agonist G1 alone or in combination with lipopolysaccharide. GPER1 expression and subcel-

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lular localization, respiratory burst, life span, gene expression profile, and cell signaling pathways involved were then analyzed in stimulated neutrophils. ***Results:*** Human neutrophils express a functional GPER1 which regulates their functions through cAMP/protein kinase A/cAMP response element-binding protein, p38 mitogen-activated protein kinase, and extracellular regulated MAPK signaling pathways. Thus, GPER1 activation in vitro increases the respiratory burst of neutrophils, extends their life span, and drastically alters their gene expression profile. ***Conclusions:*** Our results demonstrate that GPER1 activation promotes the polarization of human neutrophils towards a proinflammatory phenotype and point to GPER1 as a potential therapeutic target in immune diseases where neutrophils play a key role.



## Introduction

Estrogens play an important role in many areas of human physiology, including reproduction, immunity, and vascular and nervous system biology, as well as in diseases, such as cancer, depression, and reproductive disorders [1]. Moreover, differences between the sexes have been demonstrated in the number, differentiation state, and function of immune cells [2, 3], as well as in the incidence of autoimmune and chronic inflammatory diseases, to which women are more susceptible [4, 5]. This sex difference has been partially attributed to sex steroids, and estrogens are considered key modulators of the immune system [6]. The biological effects of estrogens are classically mediated by the estrogen receptors (ERs) ERα and ERβ, which function as hormone-inducible transcription factors, binding to the estrogen-responsive element located within the promoter region of target genes [7]. Although estrogens mainly act by this classical genomic mechanism, it has relatively recently been confirmed that they are also able to rapidly activate transduction pathways via nongenomic mechanisms. The membrane-associated G protein-coupled estrogen receptor 1 (GPER1), previously known as GPR30, an orphan receptor designation, was identified in the early 2000s by independent laboratories [8–10]. Thus, GPER1 was identified as the receptor mediating the nongenomic effects of estrogens through intracellular calcium mobilization and the synthesis of phosphatidylinositol 3,4,5-trisphosphate [9]. Moreover, the GPER1-signaling mechanisms include the rapid activation of mitogen-activated protein kinases (MAPKs), extracellular regulated MAPK 1 (ERK-1), ERK-2, and phosphatidylinositol 3-kinase, as well as increased cytosolic cAMP and calcium [8, 11, 12].

As GPER1 is known to bind many of the same ligands as classical ERs, the identification of a nonsteroidal, high-affinity, highly selective agonist of GPER1, G1 [13], which can selectively bind GPER1 in the same cell where ERs are present, has enabled the role of GPER1 in human physiology to be defined and opened the door to the generation of diagnostic and therapeutic strategies directed at individual ERs [14]. In addition, GPER1 knockout mice models reveal no reproductive deficits but multiple physiological alterations and a lack of estrogen-mediated effects in numerous tissues, including those of the immune system [15]. In addition, GPER1 activation has been shown to mediate anti-inflammatory protective effects in rodent models of multiple sclerosis [16–18] and ischemia-reperfusion injury [19–21]. These findings suggest that GPER1 could be a potential target for new therapies against a range of inflammatory or autoimmune diseases which display gender dimorphism as a result of the regulation of physiological and immunological processes by sex steroids [3]. Despite all this evidence pointing to the crucial role of GPER signaling in the regulation of the inflammatory response, the impact of GPER1 signaling in innate immune cells is largely unknown. We have previously shown that estrogens are able to modulate granulocyte functions in teleost fish through a GPER1/cAMP/protein kinase A (PKA)/cAMP response element-binding

protein (CREB) signaling pathway [22]. Whether this signaling pathway is evolutionarily conserved is unknown. Although human neutrophils have been shown to express ERα and ERβ [23], the direct impact of estrogens on neutrophil functions is largely unknown. Earlier studies showed that neither physiological nor pharmacological estrogen concentrations of estradiol affect human neutrophil apoptosis [24]. However, there are some observations showing that estrogens modulate neutrophil function. Thus, it has been described that estrogens are able to reduce superoxide anion release by human neutrophils [25, 26]. More recently, it has been reported that terminally differentiated neutrophil-like HL-60 cells express functional ERα, ERβ, and GPER1 [27]. Therefore, in this study we sought to address the question of whether human neutrophils express a functional GPER1, using its specific agonist G1. It was found that GPER1 is constitutively expressed by human neutrophils and that its engagement by G1 activates a cAMP/PKA/CREB signaling pathway that results in increased respiratory burst, extended life span, and the induction of genes encoding pro-inflammatory mediators.

## Materials and Methods

*Ethics Statement*

Human samples were collected from healthy donors after having obtained informed written consent. All experimental protocols were approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy). The procedures were carried out in accordance with the approved guidelines.

*Cell Purification and Culture*

Neutrophils were isolated under endotoxin-free conditions from buffy coats of healthy donors to reach 99.7% [28, 29]. Eosinophils were isolated from the granulocyte fraction by the Eosinophil Isolation Kit (Miltenyi Biotec). Human monocytes were isolated from peripheral blood mononuclear cells, after Ficoll-Paque gradient centrifugation of buffy coats, by anti-CD14 microbeads (Miltenyi Biotec). Dendritic cells were obtained from monocytes by 5-day culture with 20 ng/mL interleukin-4 (IL-4) and 20 ng/mL granulocyte-macrophage colony-stimulating factor (G-CSF) (R&D Systems) [30]. Immediately after purification, cells were suspended in RPMI-1640 culture medium supplemented with 10% low-endotoxin fetal bovine serum (<0.5 EU/mL; BioWhittaker-Lonza), treated or not with several stimuli, including 10–100 μM G1 (Tocris, 50 mM stock in dimethyl sulfoxide), 1 μg/mL ultrapure *Escherichia coli* lipopolysaccharide (LPS) (0111:B4; Alexis), 10 ng/mL tumor necrosis factor α (TNFα) (Peprotech), 1,000 U/mL G-CSF (Myelostim, Italfarmaco SpA), 100 U/mL interferon γ (IFNγ) (R&D Systems), and 200 U/mL IL-10 (R&D Systems), and then plated on 12/24-well tissue culture plates (Greiner Bio-One) to be cultured at 37°C in a 5% $CO_2$ atmosphere. Control cells were incubated in the presence of 0.2% dimethyl sulfoxide to achieve the same concentration of dimethyl sulfoxide as 100 μM G1-treated cells. After the desired incubation period, cells were collected and centrifuged at 300 *g* for 5 min. The resulting supernatants were immediately frozen and stored at –20°C, while the corresponding pellets were extracted for total RNA or lysed for protein analysis, as described below.

*Reverse Transcription Quantitative Real-Time PCR*

Briefly, total RNA was extracted from $10^7$ neutrophils using the RNeasy Mini Kit, according to the manufacturer's protocol (Qiagen) and reverse transcribed using SuperScript III (Life Technologies). Reverse transcription quantitative real-time PCR (RT-qPCR) was performed in triplicate from 5 ng cDNA for each sample, using the Fast SYBR Green Master Mix (Life

**Table 1.** Primers used in this study

| Gene | Name | Sequence (5′ → 3′) |
|---|---|---|
| *IL1B* | F | ACGAATCTCCGACCACCACT |
| | R | CCATGGCCACAACAACTGAC |
| *IL1RA* | F | TTCCTGTTCCATTCAGAGACGAT |
| | R | AATTGACATTTGGTCCTTGCAA |
| *CXCL8* | F | CTGGCCGTGGCTCTCTTG |
| | R | CCTTGGCAAAACTGCACCTT |
| *CFOS* | F | ATGAGCCTTCCTCTGACTCG |
| | R | ACGCACAGATAAGGTCCTCC |
| *TNFA* | F | GAGCACTGAAAGCATGATCC |
| | R | CGAGAAGATGATCTGACTGCC |
| *PTGS2* | F | CTCAGCCATACAGCAAATCCT |
| | R | TTCTCCATAGAATCCTGTCCG |
| *SOCS3* | F | GGCCACTCTTCAGCATCTC |
| | R | ATCGTACTGGTCCAGGAACTC |
| *GCSF* | F | GTGAGTGAGTGTGCCACCT |
| | R | TTCCCAGTTCTTCCATCTGCT |
| *RPL32* | F | AGGGTTCGTAGAAGATTCAAGG |
| | R | GGAAACATTGTGAGCGATCTC |

The gene symbols followed the HUGO Gene Nomenclature Committee (http://www.genenames.org/about/guidelines) sequences are given.

Technologies) and the ViiA™ 7 real-time PCR system (Life Technologies). RT-qPCR analyses were performed using gene-specific primer pairs from Life Technologies (Table 1). The reaction conditions, identical for all primer sets, were as follows: 95°C for 20 s, followed by 40 cycles at 95°C for 1 s and at 60°C for 20 s. Data were calculated using the qGENE [31] and the linRegPCR 7.0 [32] software (http://www.gene-quantification.de/download.html) and then expressed as relative mRNA levels ± SEM, after normalization with the control ribosomal protein L32 mRNA expression levels.

*Flow Cytometry Analysis*

Neutrophils ($10^5$) were suspended in 50 mL PBS containing 10% complement-inactivated human serum (for Fc receptor blocking). Cells were then stained for 15 min at room temperature with 1:25 FITC antihuman CD66b (clone G10F5), 1:25 PE antihuman CD11b, 1:50 PerCP-Cy5.5 antihuman CD16 (clone 3G8), and 1:25 APC-Cy7 antihuman CD62L (clone 145/15) antibodies (all from Miltenyi Biotec). GPER1 expression was analyzed using 0, 0.2, 0.4, 0.8, and 2 μg/mL (0, 1:1,000, 1:500, 1:250, and 1:100, respectively) of an affinity-purified rabbit polyclonal anti-GPER1 (sc-48525-R, Santa Cruz Biotechnology) for 30 min at 4°C, followed by a 1:500 dilution of a PE-conjugated goat antirabbit IgG (H+L, Life Technologies) for 30 min at 4°C. For competition studies, a 10-fold molar excess of a commercial blocking peptide (sc-48515-P, Santa Cruz Biotechnology) was used. Data analysis was performed using FlowJo software version 8.8.6 from Tree Star (Ashland, OR, USA). Phenotypic cell analysis was performed in live cells, identified as singlet Vybrant DyeCycle Violet-negative cells (Life Technologies).

*Western Blotting*

Whole-cell [29] and cytosol and light membrane (secretory vesicles plus plasma membrane) fraction [33] proteins were obtained, solubilized in Laemmli sample buffer, and then subjected to immunoblot by standard procedures using the following antibodies: 1:1,000 anti-phospho-CREB (#9198), 1:1,000 anti-phospho-p38 MAPK (#9211), and 1:1,000 anti-phospho-ERK (#9106) from Cell Signaling; 1:200 anti-GPER1 (sc-48525-R), 1:1,000 anti-PKA (sc-903), and 1:1,000 anti-IκBα (sc-371) from Santa Cruz Biotechnology; 1:2,000 monoclonal antibody anti-β-tubulin (#T5293) from Sigma-Aldrich; and anti-GP91-phox from Abcam (#ab139371). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

*Detection of Cytokine Release*

Cytokine concentrations in cell-free supernatants were measured by specific human ELISA kits for C-X-C motif chemokine ligand 8 (CXCL8) (Immunotools) and IL-1 receptor antagonist (IL-1RA) (R&D Systems), according to the manufacturers' instructions. The detection limits of these ELISAs were 40 pg/mL for IL-1RA and 8 pg/mL for CXCL8.

*Respiratory Burst*

Superoxide anion ($O_2^-$) release was estimated by the cytochrome C reduction assay, as described previously [34].

*Statistical Analysis*

Data are expressed as means ± SEM, unless indicated otherwise. Statistical evaluation was performed using 1-way ANOVA followed by Tukey's post hoc test. $p$ values <0.05 were considered statistically significant.

## Results

*Human Neutrophils Express GPER1*

Using Western blot, it was found that several human immune cell types, including monocytes, neutrophils, eosinophils, and dendritic cells, all robustly expressed GPER1 protein (Fig. 1a). The expression of GPER1 by neutrophils was further confirmed by flow cytometry, by means of which neutrophils were found to be immunostained with GPER1 antibody in a dose-dependent manner, while staining was fully blocked by pre-adsorption of the GPER1 antibody with a specific blocking peptide (Fig. 1b–d). Notably, neutrophils expressed GPER1 in the plasma membrane, since the immunostaining was performed with live neutrophils. This result was further confirmed by subcellular fractionation studies, in which GPER1 was found to be present in the light membrane fraction (Fig. 1e). In addition, stimulation of neutrophils for 6 and 24 h with G-CSF, IFNγ, TNFα, IL-10, or LPS failed to alter GPER1 protein levels, as assayed by Western blot (Fig. 1f). We next investigated whether GPER1 was functional in human neutrophils using its specific agonist G1. Stimulation of neutrophils with 10 and 100 μM G1 resulted in a dose-dependently increased transcript level of *CFOS*, a marker of GPER1 activation [35], in these cells (Fig. 1g). These G1 concentrations were selected for further experiments.

*GPER1 Regulates Human Neutrophil Activation and Life Span*

G1 was able to significantly prime the production of $O_2^-$ triggered by *N*-formylmethionine-leucyl-phenylalanine (fMLP) or phorbol myristate acetate (PMA) in human neutrophils, and even more sharply than LPS (Fig. 2). In addition, the transcript levels of *IL1B*, *CXCL8*,

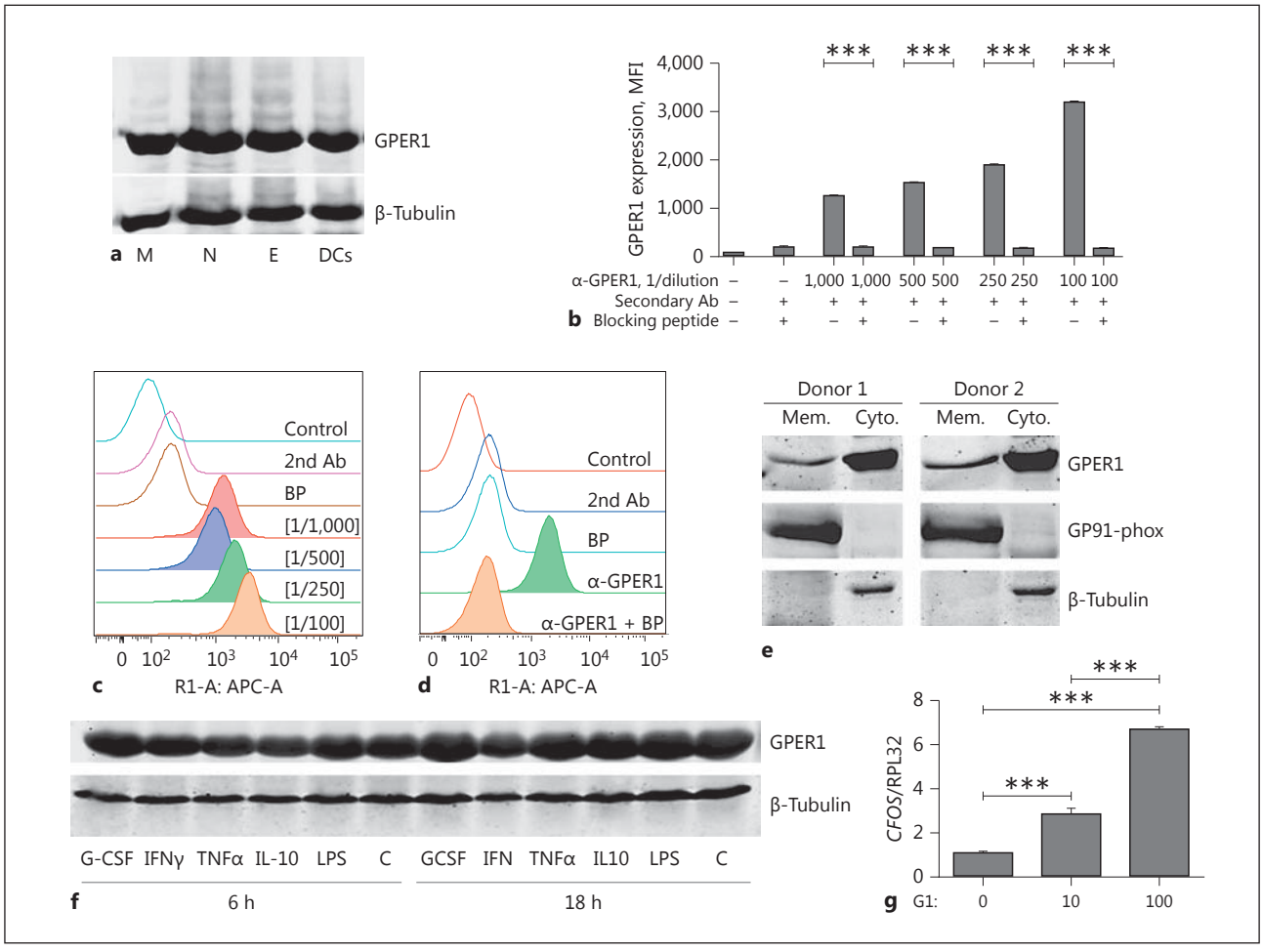


**Fig. 1.** GPER1 protein levels of human neutrophils and other human immune cells. **a** Western blot analysis of GPER1 in monocytes (M), neutrophils (N), eosinophils (E), and dendritic cells (DCs). **b** Mean fluorescence intensity (MFI) of the GPER1-positive cells measured by flow cytometry in human neutrophils (>99% purity): unstained (control), staining with secondary antibody (2nd Ab) and blocking peptide (BP) (as additional control), or with 1:1,000, 1:500, 1:250, and 1:100 dilution of α-GPER1 Ab, preincubated or not with the BP. Values represent the means ± SEM of 2 donors. Asterisks denote statistically significant differences between groups according to 1-way ANOVA and Tukey post hoc test. *** $p < 0.001$. **c**, **d** Representative fluorescence histograms of these same stainings. **e** Western blot analysis of GPER1 in cytosol and light membrane (secretory vesicles plus plasma membrane) fractions. Beta-tubulin and GP91-phox were used as markers of the cytosol and light membrane fractions, respectively. The results of neutrophil fractions obtained from 2 donors are shown. **f** Western blot analysis of neutrophils treated with G-CSF, IFNγ, TNFα, IL-10, or LPS for 6 and 18 h. Beta-tubulin was used as loading control (C). The results are representative of 3 different experiments. **g** Neutrophils were stimulated with 0 (control), 10, or 100 μM G1 for 1 h. Afterward, the mRNA levels of *CFOS* were determined by real-time RT-PCR. Gene expression is normalized against ribosomal protein L32 (RPL32) mRNA levels. Each bar represents the mean ± SEM of 3 donors. *** $p < 0.001$.

*PTGS2* (*COX2*), *SOCS3*, *GCSF*, and to some extent of *IL1RA* also increased upon 10 and 100 μM G1 stimulation, while those of *TNFA* were unaffected (Fig. 3a and data not shown). Consistent with these results, GPER1 engagement with 10 and 100 μM G1 increased in a dose-dependent manner the release of proinflammatory CXCL8 by human neutrophils (Fig. 3b), but had no effect on the release of anti-inflammatory IL-1RA (Fig. 3c).

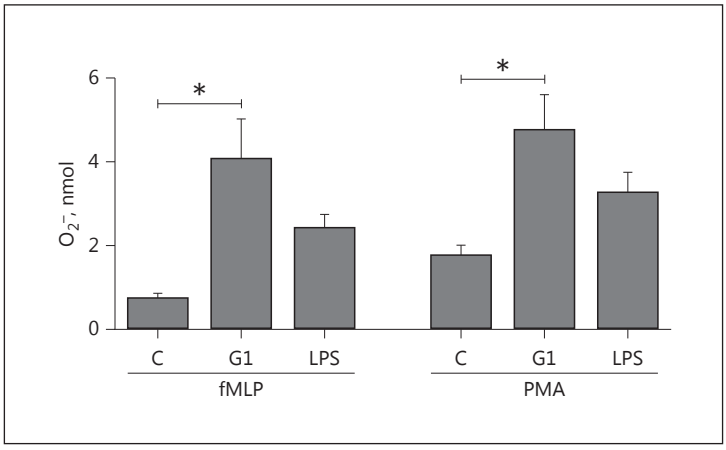


**Fig. 2.** GPER1 signaling modulates the respiratory burst of neutrophils. Neutrophils were incubated with 0 μM G1 (control, C), 100 μM G1 (G1), or lipopolysaccharide (LPS) for 2 h. Afterward, the superoxide anion ($O_2^-$) release (nmol) by these cells triggered by fMLP or by PMA was measured using a cytochrome C reduction assay. Values represent means ± SEM in triplicate and are representative of multiple independent experiments. Asterisks denote statistically significant differences between groups according to 1-way ANOVA and Tukey post hoc test. * $p < 0.05$. No symbol means not significant.

The above results prompted us to analyze the effect of GPER1 activation on neutrophil viability and the surface expression of CD11b and CD62L, 2 widely used activation markers [36]. Both LPS and G1 treatments were equally efficient in increasing neutrophil viability (Fig. 4a). In addition, a significant upregulation of total CD11b surface expression (Fig. 4b) and CD62L shedding (Fig. 4c) was observed in neutrophils stimulated with either LPS or G1. Collectively, these results suggest that GPER1 engagement promotes human neutrophil activation, which is characterized by the usual co-upregulation of CD11b and shedding of CD62L.

*GPER1 Signals via cAMP/PKA/CREB, ERK, and p38 MAPK Pathways in Human Neutrophils*

As GPER1 activation promotes adenylate cyclase, p38 MAPK, and ERK [8, 11, 12] activation in human cancer cells, we analyzed the activation of these signaling pathways in neutrophils stimulated with G1 for 30, 60, and 90 min, or with PMA as a positive control. CREB phosphorylation was seen to increase in neutrophils treated with G1 compared with the control at the 3 time points tested, although the increase was more pronounced after 60 and 90 min (Fig. 5a). After 30 min of exposure, G1 treatment weakly promoted p38 MAPK phosphorylation, but did not alter PKA levels, while modestly increasing ERK phosphorylation at the 3 time points tested (Fig. 5b), and failed to promote IκBα degradation (data not shown). As expected, PMA stimulation resulted in very strong phosphorylation of CREB, ERK, and p38 MAPK (Fig. 5a, b).

## Discussion

As estrogens have been shown to affect multiple cell components of the immune system, they have attracted significant interest as potential modulators of autoimmune diseases [1]. Although many important estrogenic responses are mediated by the 2 nuclear ERs ERα and ERβ [7], increasing evidence suggests that the nongenomic effects mediated by GPER1

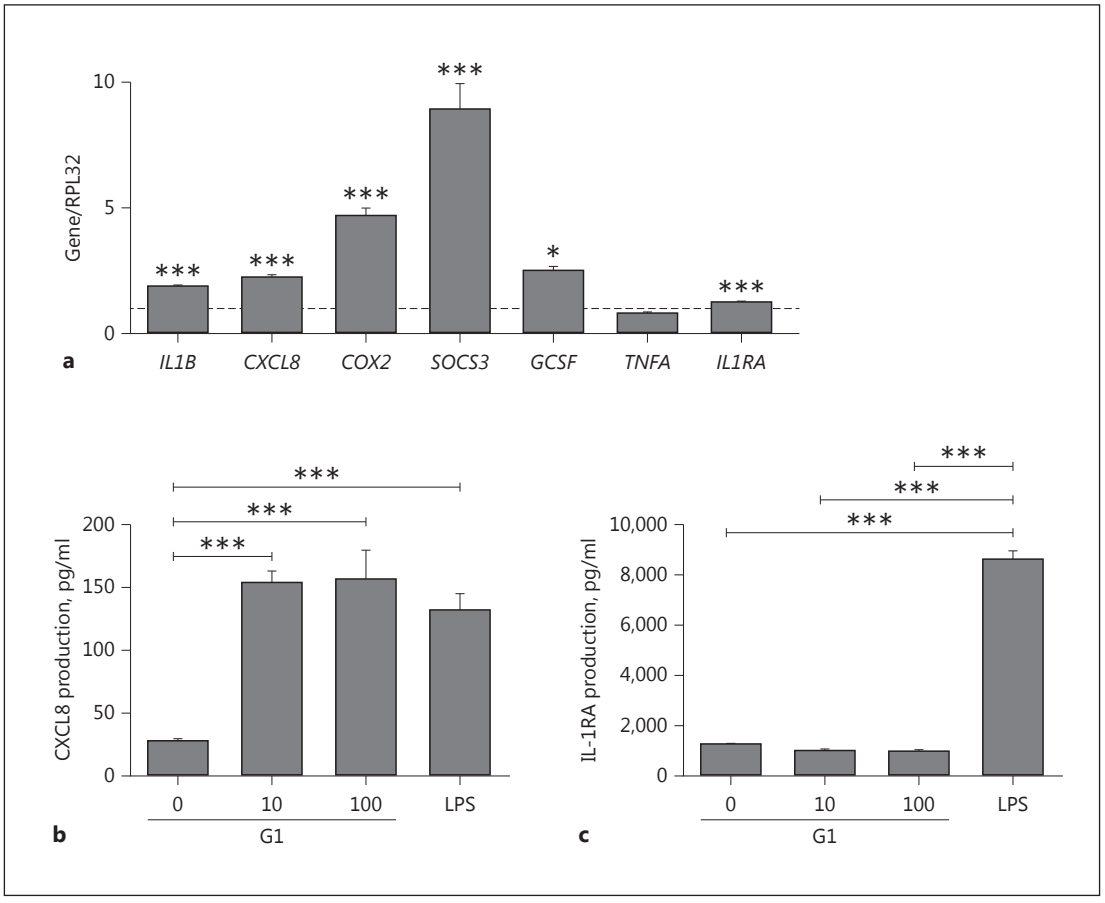


**Fig. 3.** GPER1 activation regulates the gene expression profile of human neutrophils. **a** Neutrophils were stimulated with 0 (control) and 100 μM G1 for 1 h. Afterward, the mRNA levels of *IL1B*, *CXCL8*, *COX2* (*PTGS2*), *SOCS3*, *GCSF*, *TNFA*, and *IL1RA* were determined by real-time RT-PCR. Gene expression is normalized against ribosomal protein L32 (RPL32) mRNA levels and then against unstimulated cells. Each bar represents the mean ± SEM of 3 donors. **b**, **c** Neutrophils were stimulated with 0 (control), 10, or 100 μM G1 or LPS for 16 h, and then CXCL8 (**b**) and IL-1RA (**c**) concentrations were measured by ELISA. The data represent the mean ± SEM of 3 donors. Asterisks denote statistically significant differences compared with the control group according to 1-way ANOVA and Tukey post hoc test. * $p < 0.05$, *** $p < 0.001$. No symbol means not significant.

signaling play crucial roles in mouse models of various human inflammatory disorders [17–21], where the receptor mainly shows an anti-inflammatory role. However, although there have been almost 900 publications on GPER1 since its discovery in the early 2000s and its expression in immune cells has been described [37], the in vivo role of GPER1 in the regulation of neutrophil function still remains enigmatic. The present study shows that GPER1 is functionally expressed in human neutrophils, extending a previous study reporting the functionality of this receptor in terminally differentiated neutrophil-like HL-60 cells [27]. Curiously, GPER1 expression was unaffected by neutrophil stimulation with different cytokines and LPS. Although GPER1 was originally reported to localize in the endoplasmic reticulum in different cancer cell lines [9], we found GPER1 in the plasma membrane of human neutrophils, as has been shown for human eosinophils [38]. Since its discovery, the localization of GPER1 has been a controversial aspect. Thus, although most studies reported an internal

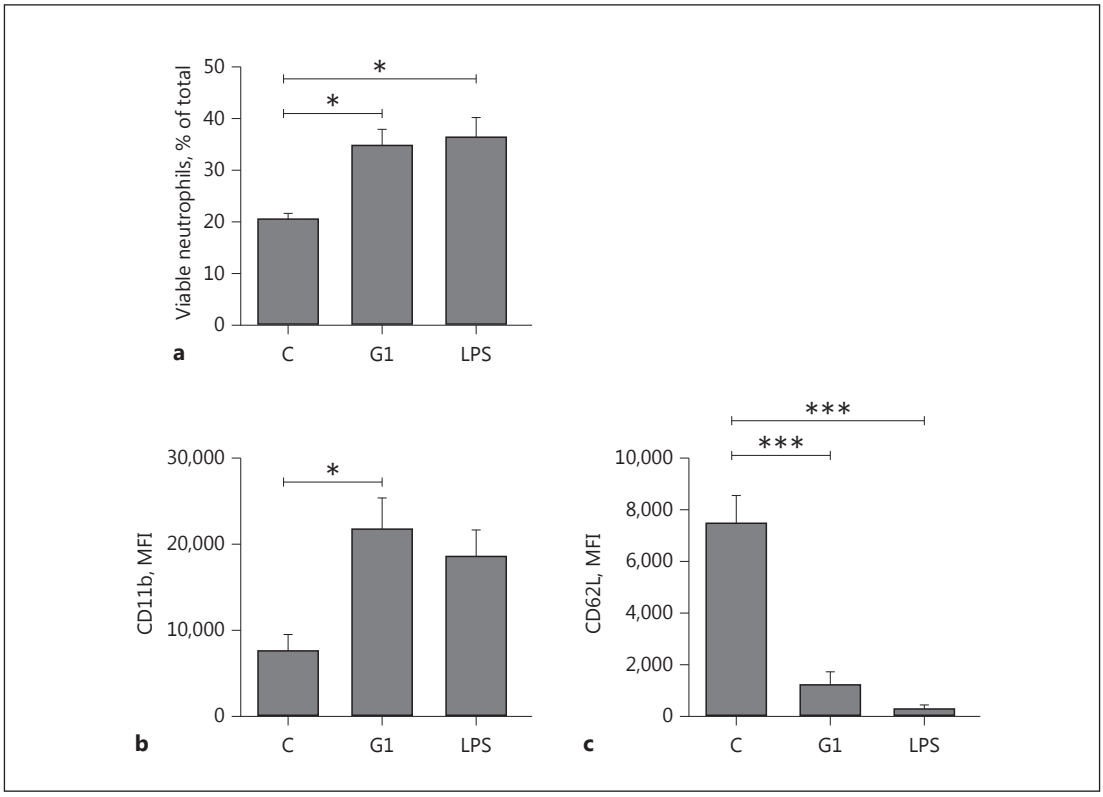


**Fig. 4.** GPER1 regulates human neutrophil activation and life span. Neutrophils were stimulated with 0 μM G1 (control, C), 100 μM G1, or LPS overnight. Afterward, the percentage of viable neutrophils (**a**) as well as the mean fluorescence intensity (MFI) of CD11b-positive cells (**b**) and CD62L-positive cells (**c**) were determined by flow cytometry. The data represent the mean ± SEM of 3 donors. Asterisks denote statistically significant differences compared with the control group according to 1-way ANOVA and Tukey post hoc test. * $p < 0.05$, *** $p < 0.001$. No symbol means not significant.

membrane localization, other studies showed it localized in plasma membrane [8] and even the nucleus [39]. Thus, the functional impact of this observation in human neutrophils is worthy of study.

GPER1 seems to be functional in human neutrophils, and its engagement with G1 promotes their activation, as evidenced by (1) the induction of *cFOS* expression, a known marker of the activation of the GPER signaling pathway [35], (2) the increased respiratory burst activity of the cells primed by different agents, e.g., LPS and fMLP, (3) the increase in CD11b expression and CD62L shedding, the 2 classical markers associated with neutrophil activation in vitro [36], and (4) the induction of several genes encoding key proinflammatory mediators, such as *IL1B*, *PTGS2*, *GCSF*, and *CXCL8*. However, the activation induced by GPER1 signaling is not identical to the classical activation observed with LPS, since while both G1 and LPS stimulate CXCL8 release, G1 fails to induce the release of *IL1RA*. These observations contrast with the inability of GPER1 to consistently enhance the respiratory burst of bony fish granulocytes and its anti-inflammatory effect on these cells in which the sustained induction of genes encoding Ptgs2, prostaglandin D2 synthase, and IL-10 was observed upon G1 stimulation [22]. Moreover, G1 promotes a differential effect on neutrophil-respiratory burst compared to estrogens, which have been found to reduce superoxide anion release by human neutrophils [25, 26]. However, it has an enhancing effect on reactive oxygen species production by human

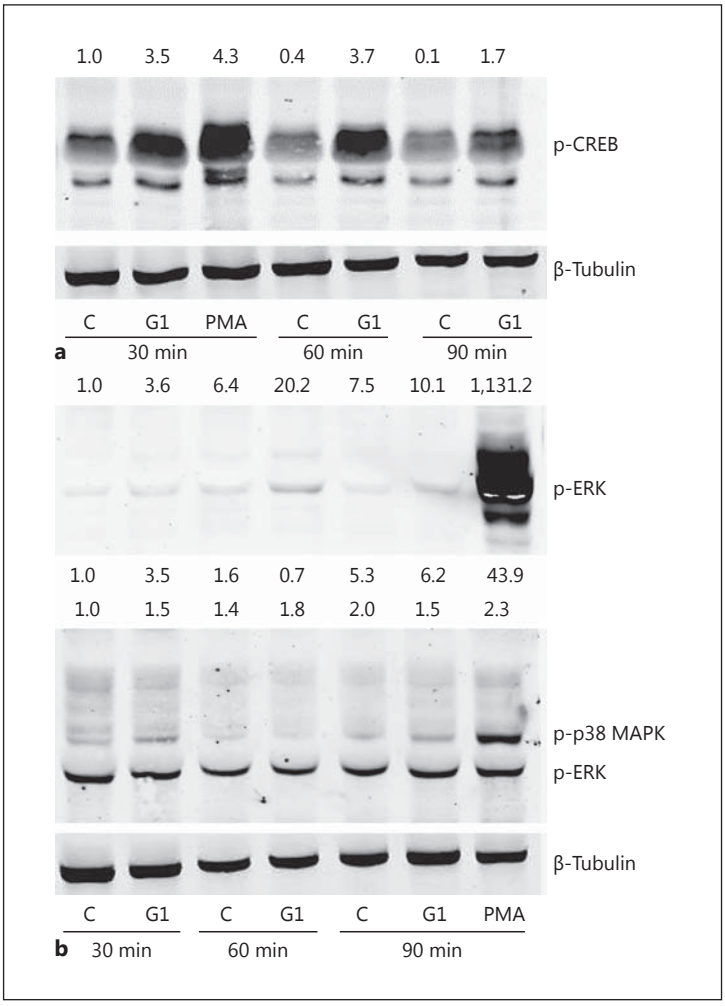


**Fig. 5.** GPER1 signals via the cAMP/PKA/CREB, p38 MAPK, and ERK pathways. Western blot analysis of phospho-CREB (p-CREB) (**a**) as well as phospho-p38 MAPK (p-p38 MAPK), PKA, and phospho-ERK (p-ERK) (**b**) in neutrophils stimulated with 0 (control, C), 100 μM G1 (G1) for 30, 60, or 90 min, or PMA was used as a positive control. The quantification of band intensity is shown over each lane. The blots are representative of 3 independent experiments.

neutrophils similar to that seen for para-nonylphenol, also described as a GPER1 agonist [14]. Taken together, these results suggest certain differences in the effect of GPER1 signaling in neutrophils across different vertebrate groups.

Another interesting observation made possible by the present study is that the stimulation of human neutrophils with the GPER1-specific agonist G1 resulted in an increased neutrophil life span, since estrogens delay human neutrophil apoptosis [40]. However, GPER stimulation has been found to inhibit spontaneous human eosinophil apoptosis through the inhibition of caspase-3 while promoting caspase-3-dependent apoptosis in IL-5-stimulated eosinophils [38]. Therefore, the regulation of human granulocyte life span by GPER1 seems to be complex and requires further research.

The signaling pathways downstream of GPER1 have been found to be very complex in human cancer cell lines, and although at least it is known that they involve adenylate cyclase/cAMP/PKA/CREB, p38 MAPK, and ERK [8, 11, 12, 35], no studies have so far examined GPER1 signaling in mammalian immune cells. We have previously reported that GPER1 signals via an adenylate cyclase/cAMP/PKA/CREB pathway in bony fish granulocytes [22]. It was found here that GPER1 not only uses a similar signaling pathway in human neutrophils, but also signals through p38 MAPK and ERK, as has been demonstrated in cancer cell lines [8, 11, 12, 35]. However, GPER1 failed to signal via NF-κB, in contrast to ER signaling which is known to

inhibit the NF-κB pathway by different mechanisms, including enhancement of IκBα levels [41].

Therefore, our results point to the relevance of the estrogen-mediated effects through GPER1 in human neutrophils in both physiological and pathological conditions. Furthermore, neutrophil regulation by GPER1 signaling must be considered in the context of endocrine disruption, since xenoestrogens, synthetic or natural substances of high stability that exert toxicity by mimicking the effects of estrogens, such as bisphenol A, some pesticides, and ethinylestradiol, are involved in autoimmunity [42] and have been found to activate GPER1 [15].

In summary, our results demonstrate that human neutrophils constitutively express a plasma membrane-anchored GPER1, which regulates their life span and promotes their activation. These results suggest a crucial role for GPER1 signaling in autoimmune and chronic inflammatory diseases in which estrogens and neutrophils are involved. They also point to GPER1 as a promising therapeutic target.

## Acknowledgments

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## Disclosure Statement

The authors declare no conflict of interest.

## Author Contributions

A. García-Ayala and V. Mulero conceived the study. N. Tamassia, M.A. Cassatella, A. García-Ayala, and V. Mulero designed the research. M.C. Rodenas, N. Tamassia, and F. Calzetti performed the research. M.C. Rodenas, N. Tamassia, I. Cabas, F. Calzetti, J. Meseguer, M.A. Cassatella, A. García-Ayala, and V. Mulero analyzed the data. M.C. Rodenas, I. Cabas, and V. Mulero wrote the manuscript, with minor contribution from other authors.

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