Role of proteinase-activated receptor-2 in anti-bacterial and immunomodulatory effects of interferon-γ on human neutrophils and monocytes

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Summary
Recent studies show that proteinase-activated receptor-2 (PAR₂) contributes to the development of inflammatory responses. However, investigations into the precise role of PAR₂ activation in the anti-microbial defence of human leucocytes are just beginning. We therefore evaluated the contribution of PAR₂ to the anti-microbial response of isolated human innate immune cells. We found that PAR₂ agonist, acting alone, enhances phagocytosis of *Staphylococcus aureus* and killing of *Escherichia coli* by human leucocytes, and that the magnitude of the effect is similar to that of interferon-γ (IFN-γ). However, co-application of PAR₂-cAP and IFN-γ did not enhance the phagocytic and bacteria-killing activity of leucocytes beyond that triggered by either agonist alone. On the other hand, IFN-γ enhances PAR₂ agonist-induced monocyte chemoattractant protein 1 (MCP-1) secretion by human neutrophils and monocytes. Furthermore, phosphoinositide-3 kinase and janus kinase molecules are involved in the synergistic effect of PAR₂ agonist and IFN-γ on MCP-1 secretion. Our findings suggest a potentially protective role of PAR₂ agonists in the anti-microbial defence established by human monocytes and neutrophils.

Keywords: innate immunity; monocyte chemoattractant protein-1; phagocytosis; proteinase-activated receptor; *Staphylococcus aureus*

Abbreviations: FCS, fetal calf serum; IFN-γ, interferon-γ; MFI, mean fluorescence intensity; MCP-1, monocyte chemoattractant protein-1 (other name is CCL2); PAR₂, proteinase-activated receptor-2; PAR₂-cAP, PAR₂-tc-activating peptide; PAR₂-cRP, PAR₂-tc-reverse peptide.
Introduction

Proteinase-activated receptor-2 (PAR2) plays a role in the development of allergic diseases of the skin\(^1\) and in certain inflammatory disorders.\(^2\) The impact of PAR2 activation on inflammation can be pro- or anti-inflammatory, depending on the stage of disease and the primary cell type involved in disease progression.\(^2\) During receptor activation, serine protease cleavage of PAR2 unmasks the N-terminal sequence of the ‘tethered ligand’. This unmasked sequence further serves as a receptor activator.\(^3\)

The PAR2 is activated by trypsin and tryptase, and also by proteases derived from immune cells and pathogens.\(^4\) However, serine proteases cause PAR-dependent as well as PAR-independent effects.\(^5,6\) As a result, specific synthetic activating peptides are important probes for investigating the role of PAR activation in different processes.

Interferon-\(\gamma\) (IFN-\(\gamma\)) is well-known as a mediator that has a wide range of anti-viral, anti-bacterial and anti-tumour or immunomodulatory activities.\(^7\) We recently demonstrated \textit{in vitro} that PAR\(_2\) activation on human monocytes enhances the suppressive effects of IFN-\(\gamma\) on influenza A virus replication.\(^8\) Moreover, \textit{in vivo} studies have shown that a protective role of PAR\(_2\) against influenza infection is also mediated by an IFN-\(\gamma\)-dependent mechanism.\(^9\) These studies revealed interplay between PAR\(_2\) activation and IFN-\(\gamma\) during the anti-viral response and raise the intriguing question of whether PAR\(_2\) activation also contributes to anti-bacterial and immunomodulatory effects triggered by IFN-\(\gamma\) in monocytes and neutrophils.

Human neutrophils and monocytes are not only ‘professional’ phagocytes, they are cells that, when activated, secrete different chemokines and cytokines. Stimulation of PAR\(_2\) agonist affects chemokine [IFN-inducible protein-10, interleukin-8 (IL-8) and cytokine (IL-1\(\beta\), IL-6) secretion by human neutrophils and monocytes.\(^8,10\) Among the chemokines secreted by neutrophils there is a molecule that appears to link neutrophils and monocytes during the time-delayed immune response to local infection. Monocyte chemoattractant protein-1 (MCP-1) is an essential mediator for monocyte and macrophage recruitment towards the site of infection.\(^11,12\) Neutrophils are a source of MCP-1 in time-delayed responses,\(^13\) and so may attract monocytes and macrophages. However, MCP-1 is not only a chemoattractant molecule for monocytes and macrophages, it also enhances the engulfment of apoptotic neutrophils (efferocytosis), thereby helping to resolve acute inflammation.\(^14\) In addition, MCP-1 is involved in fibroblast activation and influences collagen production, which makes MCP-1 an important participant in initial events during systemic scleroderma and skin fibrosis.\(^15\)

Interferon-\(\gamma\) is known to increase the secretion of MCP-1 by human neutrophils 48 hr after stimulation.\(^13\) However, whether PAR\(_2\) agonists enhance MCP-1 release or influence the IFN-\(\gamma\)-induced secretion of MCP-1 by human neutrophils has received little study.

We therefore evaluated the contribution of PAR\(_2\) to the anti-microbial response of isolated human innate immune cells. We investigated whether PAR\(_2\) agonist acting alone affects the phagocytic and bactericidal activity of human neutrophils and monocytes. We also investigated whether IFN-\(\gamma\) enhances the effect of PAR\(_2\) agonist on the MCP-1 release by human neutrophils and monocytes, and examined the intracellular signalling molecules involved in the effects of PAR\(_2\) agonist on MCP-1 secretion.

Materials and methods

Materials

Human recombinant IFN-\(\gamma\) was purchased from TebuBio (Offenbach, Germany). Lipopolysaccharide (LPS) from \textit{Escherichia coli} 055:B5 was purchased from Sigma (Munich, Germany; cat.#L2880). Human PAR\(_2\) activating peptide with the sequence \textit{trans}-cinnamoyl-LIGRLO-NH\(_2\) (cAP) and reverse peptide with sequence \textit{trans}-cinnamoyl-OLRGIL-NH\(_2\) (cRP) (Peptide Synthesis Facility, University of Calgary, Canada, Director: Dr Denis McMaster; the web page: http://www.ucalgary.ca/peptides).\(^8,10\) Fluorescein-conjugated killed \textit{Staphylococcus aureus} was purchased from Molecular Probes (Karlruhe, Germany). The \textit{E. coli} strain JM109 was obtained from Promega (Mannheim, Germany). Cell culture reagents were purchased from Bio-Whittaker (Aachen, Germany), PAA Laboratories (Coelbe, Germany) and Gibco-Life Technologies (Karlruhe, Germany). Cell-permeable inhibitors of intracellular signalling molecules [SB203580, rottlerin, LY 294002 and janus kinase (JAK) inhibitor I pyridone 6] were purchased from Calbiochem (Nottingham, UK).

Cell culture and isolation of human neutrophils and monocytes

Buffy-coats with blood cells for \textit{in vitro} experiments with human neutrophils and monocytes were obtained from healthy adult volunteers via the German Red Cross (Deutsches Rotes Kreuz, Münster, Germany). Neutrophils were isolated by Biocoll (Biochrom, Berlin, Germany) density gradient centrifugation followed by a hypotonic shock procedure.\(^10,16\) Peripheral blood monocytes were isolated by leukapheresis as previously described.\(^17\) Isolated human monocytes were cultivated in Teflon bags in McCoy’s medium (Biochrom) supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine and 1% non-essential amino acids. Monocytes were allowed to rest for 24 hr before stimulation. Isolated neutrophils were cultured in RPMI-1640 medium supplemented with 0.9% FCS, 2 mM L-glutamine and 1% non-essential amino acids.
and allowed to recover for 2 hr before stimulation. The following concentrations of reagents were used for stimulation during experiments: LPS 100 ng/ml; IFN-γ 10 or 100 ng/ml; PAR2-cAP 1 × 10^{-4} M. The corresponding reverse peptide with the reverse-sequence (PAR2-cRP) was used at a concentration of 1 × 10^{-4} M and served as a negative control.

**Bacterial killing assay**

Bacterial killing assay using *E. coli* (strain JM109) was performed as described previously^{18,19} with modifications. In brief, *E. coli* bacteria were cultured into Luria broth medium overnight at 37°C. Isolated uninfected human neutrophils were pre-stimulated with 10^{-4} M PAR2-cAP and/or IFN-γ (100 ng/ml) for 2 hr (37°C, 5% CO_2). Unstimulated neutrophils were used as control samples. After 2 hr incubation of neutrophils with stimuli, the cell culture medium (RPMI-1640 with 0.9% FCS, 2 mM l-glutamine and 1% non-essential amino acids) with stimuli was removed and cells were washed. Human neutrophils (2 × 10^8 cells) were resuspended in 200 μl RPMI-1640 containing 2 mM l-glutamine, 1% non-essential amino acids, 0.2%-BSA, 0.01% CaCl_2 and 0.01% MgCl_2 (this medium was designated the ‘assay medium’). Collected and washed bacteria (40 × 10^6 cells) were opsonized for 15 min at 37°C. For opsonization, bacteria were incubated in the assay medium containing 5% human serum from the same donor from whom the neutrophils were obtained. After opsonization, bacteria were washed. Neutrophils and opsonized bacteria were co-incubated in assay medium in the absence (for unstimulated control samples) or presence of stimuli (10^{-4} M PAR2-cAP and/or 100 ng/ml IFN-γ) for 1 hr at 37°C on a shaker. Neutrophils were removed from samples by hypotonic lysis (for this purpose sterile water was added to samples). A series of dilutions were prepared from the remaining bacteria. Bacteria were cultured on Luria broth agar plates without antibiotic at 37°C overnight. Colonies were counted the next day.

**Phagocytosis assay with killed S. aureus**

The phagocytosis assay was performed as described previously^{19,20}. In brief, FITC-conjugated killed *S. aureus* (Invitrogen, Darmstadt, Germany) was used for assay. The bacteria were opsonized before the assay. For this purpose, bacteria were incubated with 5% serum (from the same donor from whom neutrophils were isolated) for 25 min at 37°C. Non-infected neutrophils were pre-stimulated with PAR2-cAP 10^{-4} M, PAR2-cRP 10^{-4} M and/or IFN-γ 100 ng/ml for 2 hr at 37°C and 5% CO_2. Neutrophils and opsonized bacteria were co-incubated at 1 : 20 ratio (neutrophils : *S. aureus*). During co-incubation of bacteria and neutrophils, PAR2-cAP 10^{-4} M and/or IFN-γ 100 ng/ml were applied in the concentrations indicated above. Co-incubation took place in assay medium on a shaker for 30 min at 37°C. The phagocytosis assay was stopped by the addition of ice-cold PBS containing 0.5 mM EDTA (500 μl PBS to 1 ml of sample medium). Samples were then centrifuged at 169 g and neutrophil pellets were resuspended in ice-cold PBS containing 0.9% FCS and 2 mM EDTA. Trypan blue quench, which helps to discriminate adherent and ingested bacteria, was performed as described previously.^{21} The efficacy of phagocytosis was estimated using flow cytometry (FACS analysis). Measurements were performed for the next 15 min and all samples were kept on ice during measurements. At least 30 000 cells were analysed with the FACSCalibur and Cell Quest Pro Software (Becton Dickinson, Heidelberg, Germany).

**Phagocytosis assay with live S. aureus**

*Bacteria*. The *S. aureus* (SH1000) was kindly provided by Dr C. Eiff^{22} and *S. aureus* was grown for 18 hr in Mueller–Hinton bouillon at 37°C. Bacterial density was measured spectrophotometrically at 540 nm, after two PBS washings. The number of bacterial cells was calculated using a previously determined standard curve (based on the counts of colony-forming units). Finally, the concentration of bacteria in PBS was adjusted to 5 × 10^8 cells/ml. For the purpose of the quantitative analysis of phagocytosis by flow cytometry, *S. aureus* was incubated in PBS containing 0.1% FITC (Sigma Aldrich, Munich, Germany) for 1 hr at 37°C. After being labelled, bacteria were washed three times before incubation with pre-treated leucocytes.

**Assay.** During pre-treatment, human monocytes or neutrophils (1 × 10^6 cells) were cultured in medium either without stimuli (‘control’) or containing the following stimuli: 100 ng/ml LPS; 1 × 10^{-4} M PAR2-cAP, 10 ng/ml or 100 ng/ml of IFN-γ. Monocytes or neutrophils were pre-treated for 2 hr at 37°C and subsequently co-incubated with live FITC-labelled *S. aureus* at a ratio of 1 : 10 (cells : *S. aureus*) for 30 min at 37°C. During co-incubation of leucocytes and bacteria, PAR2-cAP and IFN-γ were applied in the same order and the same concentrations as during the pre-treatment of leucocytes by these stimuli. The efficacy of phagocytosis was determined by FACS analysis as described previously.^{23}

**Measurement of MCP-1 release. The inhibition of cell signalling molecules involved in the changes of MCP-1 release**

Non-infected human neutrophils (3.75 × 10^6 cells) and monocytes (3 × 10^6 cells) were treated with PAR2-cAP
and/or IFN-γ for 20 or 24 hr. Cell culture supernatants were collected and used for MCP-1 ELISA. Concentration of MCP-1 in the cell culture supernatants was measured with a human CCL2/MCP-1 (R&D Systems, Wiesbaden-Nordenstadt, Germany) ELISA kit according to the manufacturer’s instructions.

Specific inhibitors of intracellular signalling molecules were used to reveal which ones are involved in the effects of PAR2-cAP and/or IFN-γ at MCP-1 secretion by human neutrophils and monocytes. The inhibitors were used in the following concentrations: rottlerin [inhibits protein kinase Cδ (PKCδ)] 5 μM; LY294002 [inhibits phosphoinositide 3 (PI3) kinase] 50 μM; SB203580 (inhibits p38 kinase) 1 μM; and JAK inhibitor I pyridone 6 (pan-JAK inhibitor) 500 nM. All inhibitors were dissolved in DMSO, so the vehicle DMSO (1:1000) was used as an additional control. Human neutrophils and monocytes were pretreated with the inhibitors for 30 min and then PAR2-cAP (1×10^{-4} M) alone or in combination with IFN-γ (100 ng/ml) was applied for 24 hr (the maximum effect of the stimuli at MCP-1 release was noticed at this time-point). After treatment, cell culture supernatants were collected and used to measure MCP-1 concentration by human CCL2/MCP-1 (R&D Systems) ELISA kit.

**Statistical analysis**

Results are expressed as mean ± SEM. At least three independent experiments were performed. Statistical evaluation was performed by paired two-tailed Student’s t-tests. Significance was set at \( P < 0.05 \).

**Results**

PAR2 agonist alone enhances the phagocytic activity of human neutrophils and monocytes, but does not affect IFN-γ-stimulated phagocytosis

Neutrophils and macrophages from PAR2-deficient mice have been shown to display a significantly reduced phagocytic efficiency of *Pseudomonas aeruginosa* compared with cells from wild-type animals.\(^{24}\) However, the ability of PAR2 agonist to enhance the phagocytic activity of human neutrophils and monocytes and to affect IFN-γ-stimulated phagocytosis has yet to be evaluated. To investigate whether PAR2 agonist might potentially enhance the IFN-γ-induced phagocytosis we first carried out the phagocytosis assay with FITC-conjugated killed *S. aureus*. The treatment of human neutrophils with either PAR2-cAP (1×10^{-4} M) or IFN-γ (100 ng/ml) alone led to a similar enhancement of the mean fluorescence intensity (MFI) of human neutrophils (increased by around 40 ± 7% compared with untreated cells), indicating that the phagocytic activity of treated neutrophils increased (see supplementary material, Fig. S1). The combined action of PAR2-cAP and IFN-γ did not enhance the phagocytic activity of neutrophils above that triggered by either agonist acting alone (combined treatment increased phagocytic activity by around 51 ± 12% as compared with untreated cells) (Fig. S1). Treatment with the control peptide that cannot activate PAR2 (PAR2-cRP: 1×10^{-4} M) either alone or together with IFN-γ did not affect the phagocytic activity of human neutrophils (Fig. S1).

In our next experiments, we used live FITC-conjugated *S. aureus* (strain SH1000) to investigate the effect of PAR2-cAP alone or together with IFN-γ on the phagocytic activity of human monocytes and neutrophils against viable bacteria. We found that PAR2-cAP (1×10^{-4} M) or IFN-γ (100 ng/ml) alone enhanced phagocytic activity (Fig. 1a–d; a,b for neutrophils and c,d for monocytes). Although IFN-γ already appeared to stimulate phagocytic activity of monocytes at a concentration of 10 ng/ml, these effects were not statistically significant (Fig. 1c,d). Interferon-γ at a higher concentration (100 ng/ml) also enhanced phagocytic activity of human monocytes and neutrophils. The effects of IFN-γ at a concentration of 100 ng/ml reached statistical significance (Fig. 1a–d). Stimulation with IFN-γ increased the number of FITC-positive human monocytes (49 ± 13% of change compared with untreated cells) and FITC-positive human neutrophils (41 ± 7% of change compared with untreated cells). The MFI also increased in IFN-γ-treated human monocytes (increased by 53 ± 14%) and neutrophils (increased by 80 ± 18%) compared with untreated controls. PAR2-cAP led to an increase in the amount of FITC-positive monocytes (increased by 35 ± 7%) and FITC-positive neutrophils (increased by 24 ± 4%) compared with untreated samples. The MFI also increased in monocytes treated with PAR2-cAP (increased by 38 ± 8%) and in neutrophils (increased by 38 ± 4%) compared with untreated control samples. The combined action of PAR2-cAP and IFN-γ using the same concentrations did not enhance the phagocytic activity of neutrophils or monocytes beyond that triggered by either agonist acting alone (Fig. 1a–d).

Interferon-γ is a well-known endogenous modulator of phagocytic bacteria killing and secretory activity of human neutrophils and human monocytes.\(^{25,26}\) As an exogenous activator, LPS also affects phagocytic activity of both cell types. We wondered whether PAR2-cAP stimulation might interfere with LPS-modulated phagocytic activity of human neutrophils and monocytes. However, PAR2-cAP stimulation of human neutrophils as well as monocytes did not enhance the LPS-induced phagocytic activity against *S. aureus* (see supplementary material, Fig. S2). Hence, despite the fact that PAR2-cAP alone up-regulates the phagocytic activity of human neutrophils and monocytes against *S. aureus*, this agonist failed to enhance IFN-γ-induced and LPS-induced phagocytic activity.
PAR₂ agonist alone increases bacterial killing by human neutrophils, but does not enhance IFN-γ-stimulated bacterial killing

We next investigated whether treatment of isolated human neutrophils with PAR₂-cAP alone or in combination with IFN-γ affects the bactericidal activity of these phagocytes. In accordance with biosafety limitations, we used live E. coli bacteria in our experiments to estimate neutrophil killing activity. Human neutrophils were pre-treated for 2 hr with PAR₂-cAP or IFN-γ either alone or in combination. Either PAR₂-cAP (1 × 10⁻⁴ M) or IFN-γ (100 ng/ml) alone had a similar effect on bacteria killing by human neutrophils (killing efficacy increased by 62 ± 16% after PAR₂-cAP and by 72 ± 10% after IFN-γ) (Fig. 2). The PAR₂ agonist and IFN-γ in combination were not more effective in stimulating bacteria killing activity against E. coli than either was alone (Fig. 2).

Stimulation with IFN-γ enhances PAR₂-cAP-induced release of MCP-1 by human neutrophils and monocytes

It is known that MCP-1 facilitates monocyte recruitment to the site of bacterial infection and enhances the engulfment of apoptotic neutrophils (efferocytosis), thereby helping to resolve acute inflammation. Moreover, neutrophils may be a source of MCP-1 in time-delayed responses. We therefore studied the changes of MCP-1 secretion by human neutrophils and monocytes to reveal the effects of the PAR₂ agonist acting either alone or in combination with IFN-γ. For this experiment, neutrophils and monocytes were treated with PAR₂-cAP (1 × 10⁻³ M), PAR₂-cRP (1 × 10⁻⁴ M), or IFN-γ (100 ng/ml) either alone or in combination. We found that PAR₂-cAP alone did not lead to a notable change in MCP-1 secretion by human neutrophils after 20 hr of treatment; the level of secreted MCP-1 was still slightly below the threshold level.

**Figure 1.** Influence of proteinase-activated receptor-2 (PAR₂) agonist and interferon-γ (IFN-γ) stimulation on phagocytic activity of human neutrophils (a, b) and monocytes (c, d). Isolated leucocytes were pre-stimulated with 10⁻⁴ M activating protein (cAP), 10 or 100 ng/ml IFN-γ, or a combination of IFN-γ and cAP for 2 hr. Subsequently, leucocytes were co-incubated for 30 min with bacteria in the presence or absence of the stimuli mentioned. Results are presented as average values of stimuli effects on mean fluorescence intensity (MFI) of leucocytes, which engulfed live FITC-conjugated Staphylococcus aureus, compared with unstimulated leucocytes (mean ± SEM) (a, c). Results are presented as average values of stimuli effects on the amount of FITC-positive leucocytes (b, d) compared with unstimulated leucocytes (mean ± SEM; Student’s t-test versus controls: *P < 0.05; **P < 0.01). Three independent experiments were performed with human neutrophils and human monocytes.
of the ELISA (Fig. 3a). However, treatment of human neutrophils with PAR₂-cAP for 28 hr resulted in a significant increase of MCP-1 secretion by these cells (MCP-1 level in PAR₂-cAP stimulated samples was 36 ± 4 pg/ml, but was undetectable in unstimulated control samples) (Fig. 3b). Treatment of neutrophils with IFN-γ alone did not affect MCP-1 secretion at the 20 and 28 hr time-points. The level of secreted MCP-1 was below the threshold level of the ELISA at 20 hr and at 28 hr (Fig. 3a,b). Surprisingly, the co-application of IFN-γ with PAR₂-cAP enhanced the effect of the PAR₂ agonist on MCP-1 secretion 20 hr after stimulation (Fig. 3a). This effect was statistically significant even at 20 hr after stimulation (Fig. 3a). However, this effect was even more prominent at 28 hr (MCP-1 level was 284 ± 37 pg/ml versus 36 ± 4 pg/ml in samples treated by PAR₂-cAP alone) (Fig. 3b). Treatment with the PAR₂-inactive control peptide PAR₂-reverse peptide (cRP) and interferon-γ (IFN-γ) stimulation. Neutrophils were stimulated with 10⁻⁴ M cAP, 10⁻⁴ M cRP, 100 ng/ml IFN-γ alone or in combination for 20 hr (a) or 28 hr (b). Monocytes were stimulated with 10⁻⁴ M cAP, 100 ng/ml IFN-γ alone or in combination for 28 hr (c). The sensitivity threshold of the assay was 15-6 pg/ml. This level is marked on (a, b) by dotted lines. Results are presented as the average values of MCP-1 secretion by unstimulated (control) as well as by stimulated leucocytes in pg/ml ± SEM. Student’s t-test versus controls: **P < 0.01. Five independent experiments were performed for human neutrophils and four independent experiments for human monocytes.

We also investigated whether treatment of human monocytes with PAR₂-cAP alone or in combination with IFN-γ affects MCP-1 secretion. Here, we measured the level of secreted MCP-1 at 28 hr after stimulation of human monocytes with PAR₂-cAP or IFN-γ alone or in combination. We found that stimulation of human neutrophils for 28 hr with PAR₂-cAP alone, but especially in combination with IFN-γ, led to a statistically significant increase of MCP-1 secretion. We wondered whether monocytes would also be responsive to such stimulation at this time-point. Indeed, PAR₂-cAP enhanced MCP-1 secretion by human monocytes (Fig. 3c). The level of MCP-1 in untreated monocytes was 71 ± 10 pg/ml, but it was 271 ± 60 pg/ml in monocytes treated with PAR₂-cAP. When monocytes were stimulated with IFN-γ alone
MCP-1 secretion was not notably affected (Fig. 3c). However, when IFN-γ and PAR2-cAP were used together, MCP-1 secretion was enhanced significantly (1686 ± 335 pg/ml versus 271 ± 60 pg/ml in samples treated by PAR2-cAP alone) (Fig. 3c).

The inhibition of PI3 kinase and PKCδ abolishes the enhancement of MCP-1 secretion after co-stimulation of human neutrophils with IFN-γ and PAR2-cAP

We next investigated which intracellular signalling molecules were involved in the effects of PAR2 agonist on MCP-1 secretion by human neutrophils, when this agonist was applied alone or in combination with IFN-γ. In these experiments, we investigated the effects of the inhibitors of different intracellular signalling molecules: rottlerin (inhibits PKCδ), LY294002 (inhibits PI3 kinase), SB203580 (inhibits p38 kinase), and JAK inhibitor I pyridone 6 (inhibits JAKs). Experiments were performed with neutrophils treated for 28 hr with PAR2 agonist alone (PAR2-cAP 1 × 10⁻⁴ μl) or in combination with IFN-γ (100 ng/ml), because the maximum effect on the MCP-1 secretion was revealed at that time-point. We found that rottlerin and LY294002 each completely abolished the effect of co-application of PAR2-cAP and IFN-γ on MCP-1 release by human neutrophils (Fig. 4a). These results indicate the crucial role of PI3 kinase and PKCδ in enhancing MCP-1 secretion after co-stimulation of human neutrophils with PAR2-cAP and IFN-γ. In addition, treating neutrophils with either pyridine 6 or SB203580 only weakened the effect of PAR2-cAP and IFN-γ on MCP-1 secretion, which shows that p38 kinase and JAKs are involved in the combined action of both agonists (Fig. 4a).

We also examined which intracellular signalling molecules are involved in the enhanced secretion of MCP-1 by human neutrophils after treatment with PAR2-cAP alone (Fig. 4b). For these experiments, rottlerin, LY294002, SB203580 and pyridine 6 were used to check whether PI3 kinase, p38 kinase and PKCδ on the effect of PAR2 stimulation. However, pyridine 6 did not significantly affect the changes in MCP-1 release, indicating that JAKs do not participate in the effect induced by PAR2 agonist alone (Fig. 4b).

The inhibition of PI3 kinase and JAKs in human monocytes reduces the enhancement of MCP-1 secretion caused by co-stimulation with PAR2-cAP and IFN-γ

We also investigated whether rottlerin (inhibits PKCδ), LY294002 (inhibits PI3 kinase), SB203580 (inhibits p38 kinase), SB203580 (inhibits p38 kinase) and SB203580 (inhibits p38 kinase) abolished the combined action of both agonists (Fig. 4a). In these experiments, we investigated the effects of the inhibitors of different intracellular signalling molecules: rottlerin (inhibits PKCδ), LY294002 (inhibits PI3 kinase), SB203580 (inhibits p38 kinase), and JAK inhibitor I pyridone 6 (inhibits JAKs). Experiments were performed with neutrophils treated for 28 hr with PAR2 agonist alone (PAR2-cAP 1 × 10⁻⁴ μl) or in combination with IFN-γ (100 ng/ml), because the maximum effect on the MCP-1 secretion was revealed at that time-point. We found that rottlerin and LY294002 each completely abolished the effect of co-application of PAR2-cAP and IFN-γ on MCP-1 release by human neutrophils (Fig. 4a). These results indicate the crucial role of PI3 kinase and PKCδ in enhancing MCP-1 secretion after co-stimulation of human neutrophils with PAR2-cAP and IFN-γ. In addition, treating neutrophils with either pyridine 6 or SB203580 only weakened the effect of PAR2-cAP and IFN-γ on MCP-1 secretion, which shows that p38 kinase and JAKs are involved in the combined action of both agonists (Fig. 4a).

We also examined which intracellular signalling molecules are involved in the enhanced secretion of MCP-1 by human neutrophils after treatment with PAR2-cAP alone (Fig. 4b). For these experiments, rottlerin, LY294002, SB203580 and pyridine 6 were used to check whether PI3 kinase, p38 kinase and PKCδ on the effect of PAR2 stimulation. However, pyridine 6 did not significantly affect the changes in MCP-1 release, indicating that JAKs do not participate in the effect induced by PAR2 agonist alone (Fig. 4b).

The inhibition of PI3 kinase and JAKs in human monocytes reduces the enhancement of MCP-1 secretion caused by co-stimulation with PAR2-cAP and IFN-γ

We also investigated whether rottlerin (inhibits PKCδ), LY294002 (inhibits PI3 kinase), SB203580 (inhibits p38 kinase), and JAK inhibitor I pyridone 6 (inhibits JAKs) affected the induction of MCP-1 secretion after stimulation of human monocytes with PAR2-cAP and IFN-γ (Fig. 5a). Experiments were performed with monocytes treated with PAR2-cAP together with IFN-γ for 28 hr. We found that LY294002 and JAK inhibitor I pyridone 6 each reduced the effect of co-stimulation with PAR2-cAP and IFN-γ on the MCP-1 secretion by human monocytes (MCP-1 level was 1686 ± 335 pg/ml in samples treated with both PAR2-cAP and IFN-γ, 333 ± 140 pg/ml in samples treated with LY294002, and 352 ± 121 pg/ml in samples treated with JAK inhibitor) (Fig. 5a). SB203580 had...

![Figure 4](image-url)
no effect on MCP-1 secretion by human monocytes (Fig. 5a). Surprisingly, rottlerin enhanced the effect of co-stimulation with PAR$_2$-cAP and IFN-γ on MCP-1 secretion by monocytes (Fig. 5a) and also enhanced PAR$_2$-cAP-induced MCP-1 release when PAR$_2$ agonist was used alone (Fig. 5b). However, rottlerin did not affect MCP-1 levels in IFN-γ stimulated cells (data not shown).

We were also interested in whether rottlerin alone might affect MCP-1 secretion by human monocytes and found that it did increase secretion (Fig. 5c). SB203580 and JAK inhibitor each did not affect MCP-1 secretion triggered by PAR$_2$-cAP (Fig. 5b). LY294002 slightly reduced the effect of PAR$_2$-cAP stimulation on MCP-1 secretion by human monocytes (the level of MCP-1 after PAR$_2$-cAP application was 271 ± 60 pg/ml and if LY294002 was also added, the level of MCP-1 was 154 ± 72 pg/ml) (Fig. 5b). In all cases, treatment of monocytes with DMSO did not affect MCP-1 secretion (Fig. 5a–c).

**Discussion**

The most important finding of our study is that PAR$_2$ activation enhances phagocytic activity against Gram-positive (*S. aureus*) bacteria and the killing of Gram-negative (*E. coli*) bacteria by human leucocytes. The magnitude of the bactericidal effect induced by PAR$_2$ agonist was similar to that induced by IFN-γ (Figs 1 and 2; see supplementary material, Fig. S1). Since PAR$_2$ agonist can synergize with IFN-γ in enhancing anti-viral responses, we investigated whether co-application of PAR$_2$-cAP and IFN-γ led to stronger anti-bacterial responses of innate immune cells, but found that the response was no greater than when each compound was used alone (Figs 1 and 2; Fig. S1). In addition, PAR$_2$ agonist stimulation also failed to enhance LPS-stimulated phagocytic activity of neutrophils and monocytes (see supplementary material, Fig. S2). Hence, PAR$_2$ stimulation might trigger additional mechanisms that enhance the phagocytic activity of innate cells.
immune cells, and these mechanisms do not synergize with IFN-γ or LPS-triggered ones. Unfortunately, it remains problematic to investigate whether the classic PAR2 activators trypsin and tryptase can affect phagocytic and bacteria-killing activity of human innate immune cells. Trypsin and tryptase are known to induce PAR-independent effects.\(^3,6\) These effects could confound the data obtained using these enzymes as PAR2 agonists.

Cytokines and chemokines influence the recruitment of phagocytes to the site of pathogen infection. The PAR2 agonists reportedly affect the secretion of IFN-inducible protein-10, IL-8, IL-6 and IL-1β by human neutrophils, monocytes and endothelial cells.\(^8,10,27\) Among chemokines, MCP-1 appears to play a distinct role linking neutrophils and monocytes during time-delayed inflammatory response, and helping to resolve inflammation via activation of efferocytosis.\(^14\) In addition, IFN-γ reportedly enhances time-delayed MCP-1 secretion by human neutrophils.\(^13\) In our study, we investigated whether PAR2 activation interferes or synergizes with IFN-γ-enhanced MCP-1 secretion. We found that MCP-1 secretion by human neutrophils and monocytes was enhanced 28 hr after stimulation with PAR2-cAP (Fig. 3). Moreover, the treatment of human neutrophils and monocytes with IFN-γ together with PAR2-cAP resulted in a synergistic action of these agents, and so enhanced secretion of MCP-1 by innate immune cells (Fig. 3). These findings indicate that the combination of PAR2-cAP and IFN-γ is apparently effective at enhancing secretion of MCP-1 by human neutrophils and monocytes.

In our study, we were interested in which intracellular signalling molecules were involved in the synergetic action of PAR2-cAP and IFN-γ on MCP-1 secretion by human neutrophils and monocytes. Several signalling molecules are known to be involved in the regulation of MCP-1 secretion. For example, a serine protease plasmin induces MCP-1 expression in human monocytes via activation of p38 kinase and JAK/signal transducer and activator of transcription (STAT) pathways.\(^28\) Inhibitors of PI3 kinase attenuate IFN-γ-induced expression of MCP-1 in macrophages.\(^29\) Moreover, IFN-γ-induced activation of PI3 kinase results in downstream activation of PKCδ.\(^30\) Conversely, PAR2 induces some effects via signalling cascades involving PI3 kinase and PKCδ.\(^31\) Altogether, these facts led us to hypothesize that p38 kinase, PI3 kinase, PKCδ and JAKs were involved in the synergistic effect of PAR2 agonist and IFN-γ on MCP-1 secretion by human monocytes and neutrophils. Indeed, our experiments with inhibitors of these signalling molecules indicate that they all participate in synergistic effects of PAR2-cAP and IFN-γ on MCP-1 secretion by human neutrophils (Fig. 4a). Our results show that the enhanced effect of combined PAR2-cAP and IFN-γ treatment on MCP-1 secretion by human neutrophils appears to be associated with the signalling pathway JAK–PI3K–PKCδ (Fig. 6a). Possibly, STAT1 could be the next participant in this pathway in neutrophils. Interferon-γ is known to activate the PI3K–PKCδ axis, and activated PKCδ, in turn, affects STAT1 phosphorylation.\(^30\) The PKCδ is involved in a dual mechanism by which it participates in regulating IFN-dependent responses: (i) via STAT1 phosphorylation and (ii) via p38 mitogen-activated protein (MAP) kinase activation.\(^32\) The results of our study strongly suggest that PKCδ is the upstream activator of p38 MAP kinase during combined action of PAR2-cAP and IFN-γ on MCP-1 secretion by human neutrophils. We found that PKCδ inhibition abolished the effect of co-application of PAR2-cAP and IFN-γ on MCP-1 secretion, but that p38 MAP kinase inhibitor just weakened MCP-1 secretion by human neutrophils (Fig. 4a). In addition, we found that the PI3K–PKCδ axis plays a crucial role for MCP-1 secretion by human neutrophils stimulated with PAR2-cAP alone (Fig. 4b).

However, our study shows that human monocytes demonstrate not only similarities, but also differences, in the set of signalling molecules involved in the synergistic effect of PAR2-cAP and IFN-γ on MCP-1 secretion (Fig. 6b). The inhibition of PI3K and JAKs reduced, but did not abolish, the enhanced MCP-1 secretion, which was induced after monocytes were treated with PAR2-cAP together with IFN-γ (Fig. 5a). This reduced level of secreted MCP-1 was similar to the level reached after monocytes were stimulated with PAR2-cAP alone (Fig. 5a,b). These data indicate that PAR2-cAP effects on

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**Figure 6.** Suggested signalling events involved in the effects induced by combined treatment with proteinase-activated receptor-2 activating peptide (PAR2-cAP) and interferon-γ (IFN-γ) on monocyte chemoattractant protein 1 (MCP-1) secretion by human neutrophils (a) and monocytes (b). Experiments with selective inhibitors of signalling molecules led us to suggest that the phosphoinositide 3 kinase–protein kinase δ (PI3K–PKCδ) axis plays a key role in changes of MCP-1 secretion induced by co-application of PAR2-cAP and IFN-γ in human neutrophils (a). However, the inhibition of the PI3K molecule in human monocytes just weakened the effect of combined PAR2-cAP and IFN-γ application on MCP-1 secretion. This indicates the involvement of the PI3K-independent pathway in the effect of PAR2-cAP and IFN-γ co-application on MCP-1 secretion by monocytes (b).
MCP-1 secretion by human monocytes are mediated not only via a signalling pathway involving PI3K activation, but also via another pathway (Fig. 6b). Surprisingly, the PKCδ inhibitor rolletrin enhanced the effect of PAR2-cAP and IFN-γ on MCP-1 secretion by monocytes (Fig. 5a). Rolletrin also synergized with PAR2-cAP in its action on MCP-1 secretion (Fig. 5b). Moreover, rolletrin, when applied alone, enhanced MCP-1 secretion by human monocytes (Fig. 5c). Treatment with the p38 inhibitor SB203580 did not influence the increased MCP-1 secretion caused by either PAR2-cAP stimulation or combined application of PAR2-cAP and IFN-γ (Fig. 5a,b). The levels of secreted MCP-1 after IFN-γ stimulation were below the threshold in the neutrophil samples and could therefore not be determined (Fig. 3a,b). The treatment of human monocytes with IFN-γ yielded no significant changes in MCP-1 levels (Fig. 3c). Hence, the effects of the inhibitors of signalling molecules at MCP-1 release were not studied after IFN-γ stimulation of human monocytes and neutrophils. Altogether, the results of our experiments allowed us to suggest a possible scheme of signalling events involved in the enhancement of MCP-1 secretion triggered after combined stimulation of human neutrophils and monocytes with PAR2-cAP and IFN-γ (Fig. 6a,b).

In summary, our study demonstrates that PAR2 agonist acting alone can enhance a bactericidal response of human neutrophils and monocytes in vitro. However, PAR2 agonist is unable to synergize with IFN-γ in the enhancement of the bactericidal response. On the other hand, PAR2 agonist and IFN-γ do synergize to increase MCP-1 secretion by human neutrophils and monocytes during the late phase (after 24 h) of the inflammatory response. This synergistic action of PAR2 agonist and IFN-γ on MCP-1 release apparently involves the activation of PI3 kinase and JAKs in neutrophils and monocytes.

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Disclosures

The authors have no financial conflict of interest.

References

Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Influence of proteinase-activated receptor-2 (PAR2) agonist and interferon-γ (IFN-γ) stimulation on phagocytic activity of human neutrophils against killed FITC-conjugated *Staphylococcus aureus*. Isolated neutrophils were pre-stimulated with $10^{-4}$ M activating peptide (cAP), $10^{-4}$ M reverse peptide (cRP), or 100 ng/ml IFN-γ, or a combination of IFN-γ and cAP or cRP for 2 hr.

**Figure S2.** Influence of proteinase-activated receptor-2(PAR2) agonist and lipopolysaccharide (LPS) stimulation on phagocytic activity of human neutrophils (a, b) and monocytes (c, d). Isolated leucocytes were pre-stimulated with $10^{-4}$ M activating peptide (cAP), or 100 ng/ml LPS, or a combination of LPS and cAP for 2 hr. Subsequently, leucocytes were co-incubated for 30 min with bacteria in the presence or absence of the stimuli mentioned.

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