

## Research Article

# Proteinase-Activated Receptor-2 Agonist Activates Anti-Influenza Mechanisms and Modulates IFN $\gamma$ -Induced Antiviral Pathways in Human Neutrophils

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Received 29 April 2013; Accepted 12 August 2013

Academic Editor: Richard Tucker

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Proteinase-activated receptor-2 (PAR<sub>2</sub>) is expressed by human leukocytes and participates in the development of inflammatory diseases. Recent studies demonstrated an ability of PAR<sub>2</sub> agonist to enhance IFN $\gamma$ -induced antiviral responses of human leukocytes. However, the precise cellular antiviral defense mechanisms triggered in leukocytes after stimulation with IFN $\gamma$  and/or PAR<sub>2</sub> agonist remain elusive. Therefore, we aimed to identify neutrophil defense mechanisms involved in antiviral resistance. Here we demonstrated that PAR<sub>2</sub> agonist enhanced IFN $\gamma$ -related reduction of influenza A virus (IAV) replication in human neutrophils. PAR<sub>2</sub>-mediated decrease in IAV replication was associated with reduced NS-1 transcription. Moreover, PAR<sub>2</sub>-dependent neutrophil activation resulted in enhanced myeloperoxidase degranulation and extracellular myeloperoxidase disrupted IAV. The production of ROS was elevated in response to PAR<sub>2</sub> activation. Interestingly, IFN $\gamma$  did not influence both effects: PAR<sub>2</sub> agonist-triggered myeloperoxidase (MPO) release and reactive oxygen species (ROS) production, which are known to limit IAV infections. In contrast, orthomyxovirus resistance gene A (MxA) protein expression was synergistically elevated through PAR<sub>2</sub> agonist and IFN $\gamma$  in neutrophils. Altogether, these findings emphasize two PAR<sub>2</sub>-controlled antiviral mechanisms that are independent of or modulated by IFN $\gamma$ .

## 1. Introduction

The impact of proteinase-activated receptor-2 (PAR<sub>2</sub>) activation on inflammatory processes varies and depends on the stage of disease and the primary cell type(s) involved in disease progression [1, 2]. Trypsin, tryptase, and pathogen-derived proteases could trigger PAR<sub>2</sub> activation [3]. However, these enzymes cause PAR<sub>2</sub>-dependent as well as PAR<sub>2</sub>-independent effects [4, 5]. Moreover, trypsin-like serine proteases could assist influenza A replication via cleavage of viral hemagglutinin [6]. Together, these facts exclude the use of

trypsin and tryptase as appropriate PAR<sub>2</sub> activators in studies involving influenza A virus. Thus we used influenza A/FPV/Bratislava/79 (H7N7) containing a multibasic-cleavage site, which efficiently replicates without the necessity of trypsin. Moreover, specific synthetic PAR<sub>2</sub>-activating peptides, used in our study, do not affect hemagglutinin maturation but reportedly serve as important tools for investigating the role of PAR<sub>2</sub> activation in a wide range of anti-influenza responses.

Interferon- $\gamma$  (IFN $\gamma$ ) regulates the cellular antiviral state and shapes the antiviral and inflammatory response [7].

Recent *in vitro* and *in vivo* studies revealed a cooperation between IFN $\gamma$  and PAR<sub>2</sub> agonists in the induction of antiviral responses and in the regulation of the chemokine levels [8–10]. However, it remains unclear which cellular antiviral defence mechanism(s) in leukocytes are affected after concomitant IFN $\gamma$  and PAR<sub>2</sub> agonist application.

Neutrophils participate in the defence against influenza A virus (IAV) infection. Although it is well established that neutrophils contribute to lung injury during IAV infection, neutropenia is associated with enhanced virus replication in lungs and high mortality [11]. Moreover, neutrophils limit spreading in the organism of IAV strains with intermediate or high virulence [12]. Human neutrophils express functional PAR<sub>2</sub> [13, 14], which regulates motility and bactericidal activity of neutrophils [1, 10]. Although the PAR<sub>2</sub>-induced bactericidal activity is not enhanced in the presence of IFN $\gamma$  in neutrophils [10], PAR<sub>2</sub> agonist and IFN $\gamma$  synergize boosting anti-influenza effects in human monocytes [8]. Nonetheless, the role of PAR<sub>2</sub> and IFN $\gamma$  in neutrophils during IAV infection remains elusive.

Neutrophils possess a broad spectrum of weapons against viral and microbial pathogens including compounds of neutrophil granules (defensins, elastase, and some others), reactive oxygen species (ROS), and orthomyxovirus resistance gene (Mx) proteins [15, 16]. Thus, we investigated how PAR<sub>2</sub> activation affects IAV replication in neutrophils and which defence mechanism(s) are activated. We also evaluated whether PAR<sub>2</sub> agonist and IFN $\gamma$  synergize to strengthen the antiviral response.

## 2. Material and Methods

**2.1. Materials.** Human PAR<sub>2</sub>-activating peptide with the sequence *trans*-cinnamoyl-LIGRLO-NH<sub>2</sub> (tcAP) and the reverse peptide with the sequence *trans*-cinnamoyl-OLRGIL-NH<sub>2</sub> (tcRP) were synthesized at the University of Calgary (Peptide Synthesis Facility, Dr. D. McMaster, Calgary, Canada; <http://www.ucalgary.ca/peptides/>) and used at a concentration of 10<sup>-4</sup> M as described previously [8]. Human recombinant IFN $\gamma$  was received from Peprotech (Hamburg, Germany) and used at a concentration of 200 U/mL. The following antibodies were used: mouse anti-human  $\beta$ -actin (Sigma Aldrich); mouse monoclonal anti-MxA antibody (M143) which was a kind gift from the Department of Virology of the University of Freiburg and was used as described previously [17]. All cell culture reagents were obtained from PAA (Cölbe, Germany) or otherwise stated in the text.

**2.2. Isolation and Culture of Neutrophils.** Buffy coats from healthy adult human volunteers were obtained from the Deutsches Rotes Kreuz (Münster, Germany), and neutrophils were prepared as described previously [18]. Isolated neutrophils (1–1.5  $\times$  10<sup>6</sup> cells/mL) were allowed to recover in RPMI 1640 (Lonza) supplemented with 1% L-glutamine, 1% nonessential amino acids, 1% penicillin/streptomycin, and 0.9% fetal calf serum for at least 1 hr.

**2.3. Virus and Infections.** Avian influenza virus A/FPV/Bratislava/79 (H7N7; FPV) was originally obtained from the

virus strain collection of the Institute of Virology (Justus-Liebig-University, Gießen, Germany). For infection, human neutrophils were washed with PBSi (PBS supplemented with 0.01% CaCl<sub>2</sub>, 0.01% MgCl<sub>2</sub>, and 0.2% bovine serum albumin (BSA)) and infected with a multiplicity of infection of 0.75. Therefore, the virus was diluted accordingly in PBSi and applied to the cells for 30 min at 37°C and 5% CO<sub>2</sub>. Then, the inoculum was aspirated and replaced by RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% penicillin and streptomycin, 0.2% BSA, 0.01% CaCl<sub>2</sub>, and 0.01% MgCl<sub>2</sub>. For inhibitor studies, 1 mM myeloperoxidase (MPO) inhibitor (Calbiochem) or vehicle was added to the medium. Subsequently, cells were stimulated with agonists or left untreated. Cells were incubated for 0–20 hrs (as indicated in the text) at 37°C and 5% CO<sub>2</sub> depending on the readout system. In a second experimental approach, neutrophils were primed with agonists for 2 hrs and, subsequently, infected with IAV for 30 min as described above. Following infection primed cells were rechallenged with agonists (b/a stimulation protocol) for 20 hrs. Only if stated in the text, the b/a stimulation protocol was applied.

**2.4. Quantification of Neutrophil Degranulation.** After recovery, neutrophils were treated for 2 hrs with the indicated agonists or used immediately without prestimulation. Then, cells were spun down and resuspended at a ratio of 1  $\times$  10<sup>6</sup> cells per 100  $\mu$ L in PBS. Neutrophils were pretreated with 5  $\mu$ g/mL of the degranulation-promoting agent Cytochalasin B (Sigma Aldrich) (for 5 min at 37°C) and, subsequently, rechallenged with appropriate agonists for 30 min at 37°C. Cells were removed by centrifugation, and the supernatant was analysed for elastase and MPO activity. To measure the elastase release, the supernatant was prediluted 1/100 and incubated with 100  $\mu$ g/mL alpha-1-antitrypsin ( $\alpha$ 1AT) for 30 min at 37°C. Then, elastase/ $\alpha$ 1AT mixture was applied to PMN elastase ELISA (Abnova, Heidelberg, Germany). The assay was performed according to the manufacturer's instructions. To quantify the MPO levels, 100  $\mu$ L of degranulated supernatant was mixed with 100  $\mu$ L 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma Aldrich). Changes in the optical density at 630 nm were monitored for 20 min.

**2.5. IAV Disruption by Neutrophil Supernatant.** Supernatant from degranulated neutrophils was prepared as described above. The virus was diluted to 1  $\times$  10<sup>6</sup> PFU/mL. Then, neutrophil supernatant and virus dilution were mixed in a ratio of 1:1 and supplemented with 1 mM H<sub>2</sub>O<sub>2</sub> (Merck) or vehicle as indicated. After incubation for 1 hr at 37°C and 5% CO<sub>2</sub>, samples were collected and analysed in a standard plaque assay.

**2.6. Measurement of Intracellular Reactive Oxygen Species (ROS).** Intracellular generation of ROS was detected using the fluorescent dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Invitrogen). To induce ROS production, neutrophils (1.5  $\times$  10<sup>6</sup> cells/mL) were stimulated with the indicated agonists in the absence of cytochalasin B. Thirty minutes before the stimulation was stopped, 5  $\mu$ M CM-H2DCFDA was added. Then, cells were

put on ice, spun down at 4°C, and washed with PBS. Finally, neutrophils were resuspended in PBS supplemented with 1% FCS, 2 mM EDTA, and 2% paraformaldehyde and analysed with the FACScalibur and Cell Quest Pro software (BD Biosciences).

**2.7. Calcium Mobilization Studies.** Changes in intracellular calcium levels were measured as described previously [8, 14, 15]. Briefly, isolated neutrophils were washed, resuspended in HEPES-buffered salt solution (140 mM NaCl, 3 mM KCl, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM HEPES, 5 mM glucose, and 1 mM MgCl<sub>2</sub> (pH 7.4)) with or without 0.8 mM CaCl<sub>2</sub>, and incubated with 3.5 μM Fura-2 acetoxymethyl for 30 min at 37°C. Cells were washed twice, resuspended in HEPES-buffered salt solution with or without 0.8 mM CaCl<sub>2</sub>, and PAR<sub>2</sub>-triggered elevation in intracellular calcium levels was measured in a FluoroMaxx spectrophotometer (Yobin Yvon). For inhibitor studies, cells were pretreated with 100 μM 2-aminoethoxydiphenyl borate (2-APB) for 3 min before the PAR<sub>2</sub> agonist was applied.

**2.8. Real-Time RT-PCR.** Steady-state levels of MxA, oligoadenylate synthetase (OAS), and the viral nonstructural protein (NS-1/2) were evaluated by real-time fluorescence detection using Absolute SYBR Green ROX mix (Applied Biosystems, Foster City, CA, USA). Reactions in duplicate were analysed in an ABI Prism 7300 sequence detector supplied with SDS 2.1 software (Applied Biosystems). Specific primer pairs were used: MxA forward, 5'-AGAGAAGGTGAGAAGCTGATC-C-3', and reverse, 5'-TTCTTCCAGCTCCTTCTCTCTG-3'; oligoadenylate synthetase (OAS) forward, 5'-GCTCCTACCCTGTGTGTGTGT-3', and reverse, 5'-TGGTGAGAGGAC-TGAGGAAGA-3'; NS-1/2 forward, 5'-GAGGACTTGAAT-GGAATGATAACA-3', and reverse, 5'-GTCTCACTTCTT-CAATCAACCATC-3'.

**2.9. Immunoblot Analysis.** Stimulated neutrophils were collected, disrupted in preheated (100°C) lysing buffer (4 M urea, 0.5 M Tris pH 6.8, 25% glycerine, 10% SDS, and 0.005% bromophenol blue) supplemented with freshly prepared 1x protease inhibitor cocktail (Roche Diagnostics) and 200 mM dithiothreitol, and boiled for 5 min. Whole cell lysate preparations of stimulated neutrophils were separated by SDS-PAGE and transferred onto nitrocellulose membrane. To assess MxA expression 35 μg of protein lysate was applied per lane. Densitometric analysis was performed using ImageJ software.

**2.10. Statistical Analysis.** Results are expressed as mean ± SEM. At least three independent experiments were performed ( $n \geq 3$ ). Statistical evaluation was done by an analysis of variance and Student's *t*-test or Wilcoxon matched-pairs signed rank test. Significance was set at  $P < 0.05$ .

### 3. Results

**3.1. IAV Replication in Neutrophils Is Reduced by PAR<sub>2</sub> Agonist and IFN $\gamma$ .** Previously, we revealed that PAR<sub>2</sub> and IFN $\gamma$  cooperate to interfere with IAV replication in human monocytes [8]. Here, we investigated whether such a cooperation also exists in neutrophils, as they appear to play an important

role during IAV infections. Therefore, we aimed to confirm the replication of the avian IAV strain H7N7 in human neutrophils. Indeed, infection of neutrophils led to a time-dependent upregulation of viral NS-1 mRNA after 2 and 4 hrs. In noninfected neutrophils, viral NS-1 mRNA was not detectable (Figure 1(a)). Next, we treated IAV-infected neutrophils with PAR<sub>2</sub>-tcAP, IFN $\gamma$ , or a combination thereof and measured viral titers after 20 hrs. PAR<sub>2</sub> agonist stimulation decreased IAV titers by  $80 \pm 2\%$ , whereas IFN $\gamma$  treatment had no significant effect (Figure 1(b)). Concomitant stimulation with PAR<sub>2</sub> agonist and IFN $\gamma$  reduced IAV progeny by 3-4-fold (Figure 1(b)). To evaluate whether primed neutrophils are more resistant to IAV replication, we primed neutrophils with PAR<sub>2</sub> agonist, IFN $\gamma$ , or their combination for 2 hrs before cells were infected with IAV and rechallenged cells after infection (b/a-stimulation). In this stimulation protocol, PAR<sub>2</sub> and IFN $\gamma$  reduced viral titers by  $68 \pm 4\%$  and by  $57 \pm 5\%$ , respectively (Figure 1(c)). Combining PAR<sub>2</sub> agonist and IFN $\gamma$  additively decreased IAV titers by approximately  $86 \pm 2\%$  (Figure 1(c)). Scrambled PAR<sub>2</sub> peptide (tcRP) was used as control and did not affect viral titers (Figure 1(c)). Together, our data revealed that IAV replicates in neutrophils and that PAR<sub>2</sub> agonist and IFN $\gamma$  reduce IAV titers.

**3.2. PAR<sub>2</sub> Activation Triggers Degranulation and Production of Reactive Oxygen Species (ROS) in Neutrophils.** Myeloperoxidase (MPO) as well as other compounds of azurophil granules were demonstrated to have anti-influenza activity [19, 20] and, thus, may contribute to host protective rather than harmful functions. PAR<sub>2</sub>-AP was shown to increase plasma MPO activity indicating enhanced neutrophil degranulation in mice [21]. Therefore, we analysed whether stimulation with PAR<sub>2</sub>-tcAP or IFN $\gamma$  triggers human neutrophil degranulation of azurophil granules *in vitro*. In our preliminary experiments, where neutrophils (app.  $4 \times 10^6$  cells/100 μL) were primed with PAR<sub>2</sub> agonist for 2 hrs, a second dose of PAR<sub>2</sub> agonist elicited the release of elastase. However, variations in the magnitudes of the effect did not allow this effect of PAR<sub>2</sub> agonist to reach statistical significance (unpublished observations).

In contrast, preactivation of neutrophils with cytochalasin B led to a robust elevation of elastase and MPO release after PAR<sub>2</sub> activation. Basal release of MPO and elastase in cytochalasin B primed neutrophils was determined as  $26.9 \pm 4.6$  mU and  $113.6 \pm 21.0$  ng/mL, respectively (Figure 2(a)). Further addition of PAR<sub>2</sub>-tcAP enhanced extracellular MPO ( $86.5 \pm 19.3$  mU) and elastase ( $265.8 \pm 76.4$  ng/mL) levels significantly, but degranulation was unaffected by IFN $\gamma$ . Concomitant stimulation with PAR<sub>2</sub> agonist and IFN $\gamma$  failed to overcome the effect induced by PAR<sub>2</sub>-tcAP alone.

PAR<sub>2</sub>-tcAP primed, then cytochalasin B treated and rechallenged neutrophils (see "Quantification of Neutrophil Degranulation" in Material and Methods Section for details) behaved in different way. Applying the b/a stimulation, the second PAR<sub>2</sub> activation resulted in significantly less elevated MPO levels ( $87.9 \pm 20.4$  mU) as compared to  $128.6 \pm 24.0$  mU in nonpreactivated cells (Figure 2(b)). However, this reduction was not detected in neutrophils activated with both PAR<sub>2</sub> agonist and IFN $\gamma$  (Figure 2(b)).

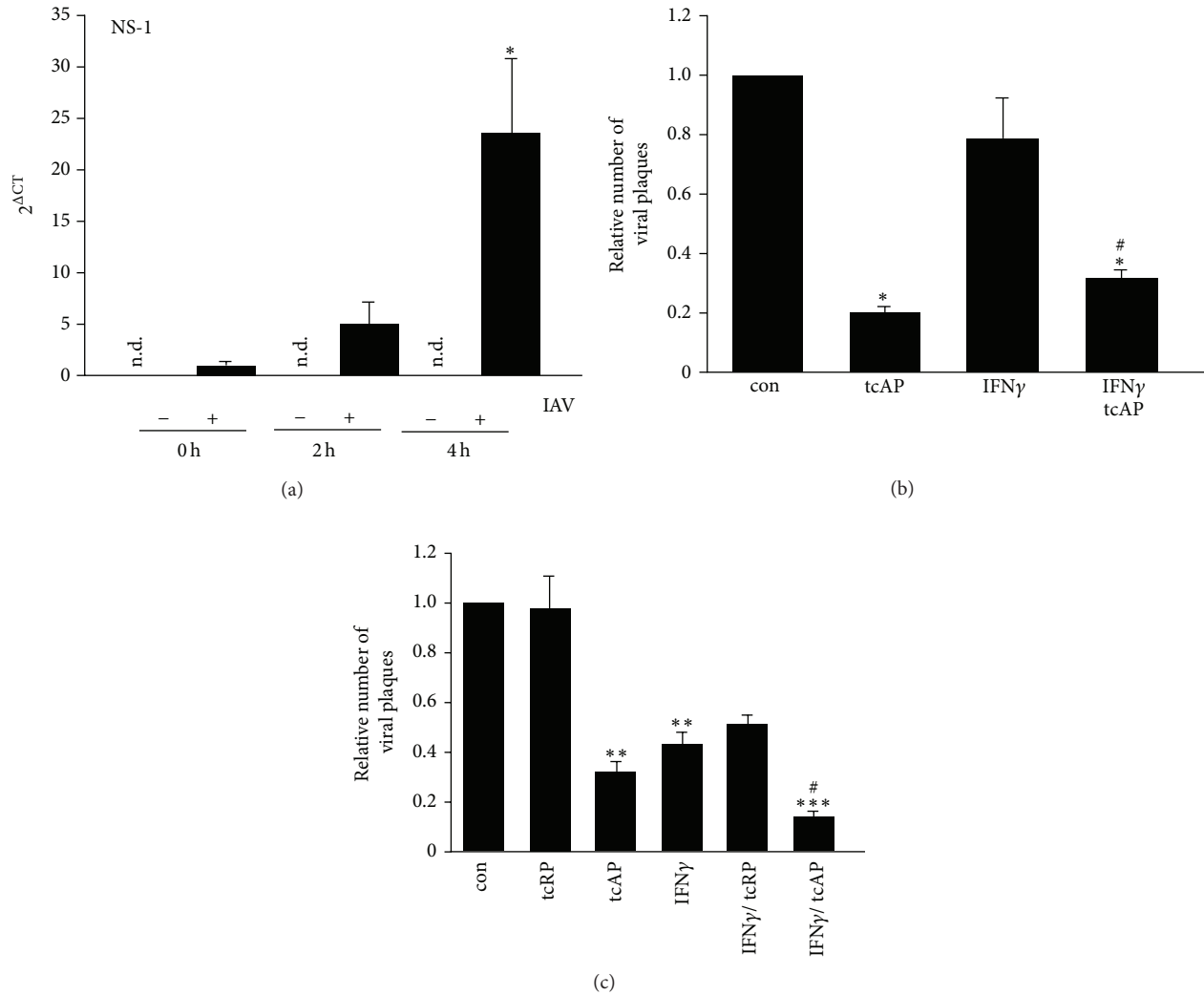


FIGURE 1: IAV replication in neutrophils was restricted by PAR<sub>2</sub> activation and IFN $\gamma$ . (a) Replication of IAV in neutrophils was determined by detection of NS-1 mRNA levels at different time points after infection. At 4 hrs, a significant induction of NS-1 mRNA expression was revealed. In noninfected neutrophils NS-1 mRNA was not detectable. (b) IAV-infected neutrophils were treated with agonists as indicated for 20 hrs. Analysis of IAV titers showed a significant reduction in PAR<sub>2</sub> agonist and PAR<sub>2</sub> agonist/IFN $\gamma$  treated neutrophils. (c) In cells that were primed with agonists for 2 hrs, infected with IAV for 30 min, and rechallenged with agonists for 20 hrs, both PAR<sub>2</sub> agonist and IFN $\gamma$  decreased viral replication. Moreover, combining PAR<sub>2</sub> agonist and IFN $\gamma$  further reduced IAV titers as compared to both agonists alone. For student's *t*-test: #\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.005. The symbol \* marks the significance as compared to control and the symbol # as compared to IFN $\gamma$  sample.

Because degranulation is often triggered by Ca<sup>2+</sup> signaling, we also investigated the contribution of Ca<sup>2+</sup> fluxes to PAR<sub>2</sub>-induced degranulation. PAR<sub>2</sub> agonist induced a rapid increase in intracellular Ca<sup>2+</sup> signaling in both Ca<sup>2+</sup>-free or Ca<sup>2+</sup>-supplemented buffer. However, extracellular Ca<sup>2+</sup> boosted PAR<sub>2</sub> agonist-induced intracellular calcium signals by 3-fold as compared to extracellular Ca<sup>2+</sup> starvation (Figure 2(c), green columns). However, PAR<sub>2</sub>-induced release of azurophil granules was independent of additional extracellular Ca<sup>2+</sup> (data not shown). 2-APB is known as an inhibitor of InsP3-induced Ca<sup>2+</sup> release and, probably, concomitant Ca<sup>2+</sup> entry [22]. 2-APB inhibited PAR<sub>2</sub>-induced Ca<sup>2+</sup> release

(Figures 2(c) and 2(d)) and, subsequently, reduced degranulation of azurophil granules as measured by elastase release (Figure 2(e)).

Reactive oxygen species (ROS) shape the inflammatory response during IAV infections [23]. In neutrophils, PAR<sub>2</sub>-tcAP, without any priming with cytochalasin B, induced ROS production that peaked at 2 hrs and then declined to baseline levels within 20 hrs. At 2 hrs, PAR<sub>2</sub> significantly upregulated ROS levels by 1.6 ± 0.2-fold as compared to controls. However, combination of PAR<sub>2</sub> agonist and IFN $\gamma$  was not more potent in induction of ROS than PAR<sub>2</sub>-tcAP alone. IFN $\gamma$  alone did not affect ROS production in neutrophils (Figure 2(f)). Together, our data indicated a regulatory role

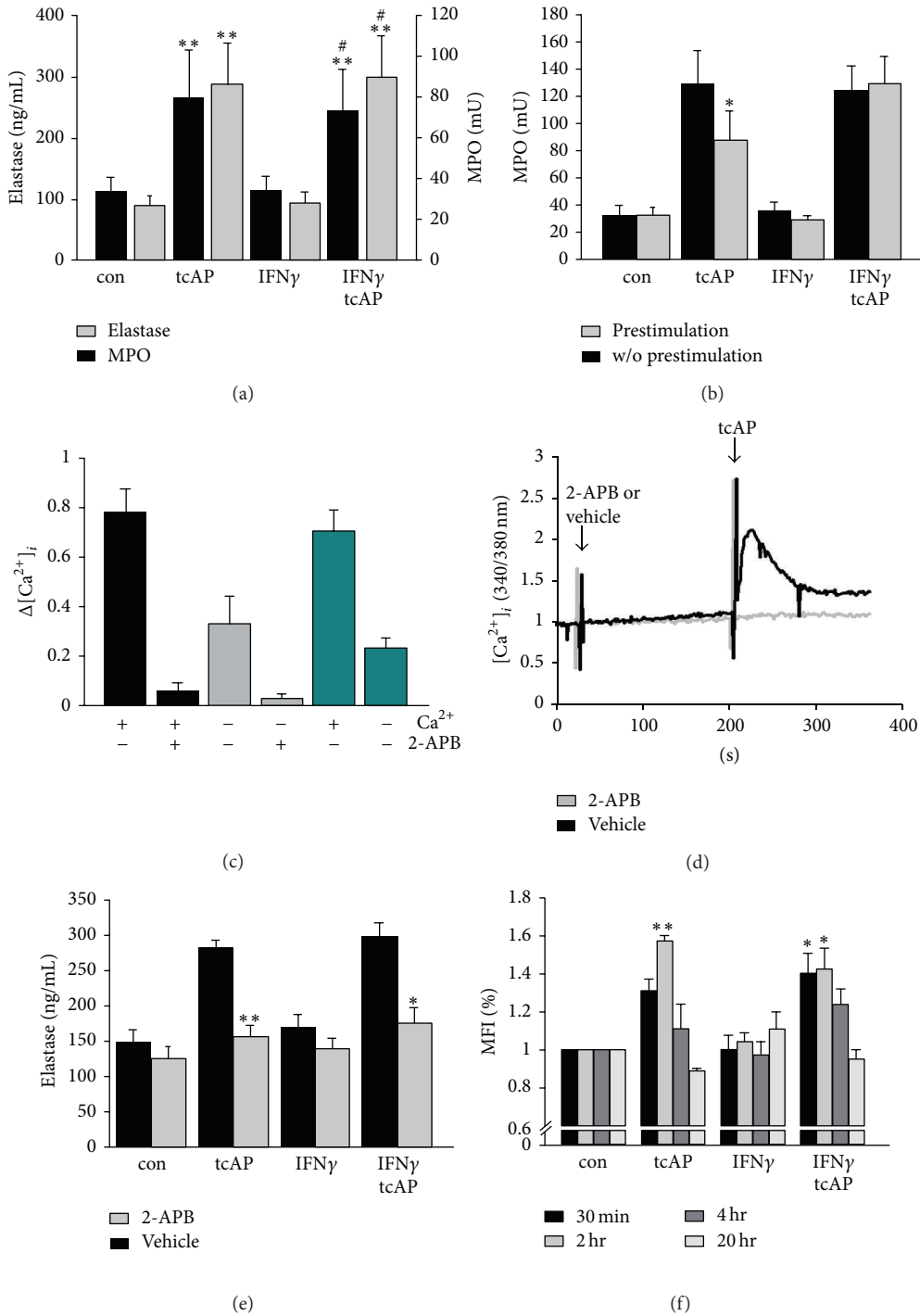


FIGURE 2: PAR<sub>2</sub> stimulation induced neutrophil degranulation in a Ca<sup>2+</sup>-dependent manner and upregulated ROS production. Neutrophils were treated as described in Material and Methods Section. (a) After stimulation with PAR<sub>2</sub> agonist and IFN $\gamma$ , the concentration of MPO and elastase was quantified in cytochalasin B primed neutrophils. (b) Comparing MPO levels in cytochalasin B primed neutrophils that were either pretreated with agonists (b/a-stimulation) or not showed a reduction in PAR<sub>2</sub> agonist stimulated neutrophils. Concomitant stimulation with PAR<sub>2</sub>-agonist and IFN $\gamma$  induced similar MPO levels in both pretreated and nonpretreated cells. (c, d) Neutrophils were loaded with Fura-2 AM (30 min), washed, and then PAR<sub>2</sub> agonist was added, and calcium mobilization was investigated. The availability of extracellular Ca<sup>2+</sup> led to increased intracellular calcium levels after PAR<sub>2</sub> agonist application. 2-APB almost completely blocked intracellular Ca<sup>2+</sup> fluxes, independent of extracellular Ca<sup>2+</sup>. (e) Pretreatment of neutrophils with 2-APB prevented PAR<sub>2</sub> agonist induced elastase release. (f) Changes in ROS levels were measured using a fluorescent substance (CM-H2DCFDA) that was added 30 min before the stimulation was stopped (see Material and Methods Section). Only at early time points, PAR<sub>2</sub> agonist elevated ROS level as measured by changes of the MFI. IFN $\gamma$  did not induce ROS upregulation. For student's *t*-test: \*\**P* < 0.05; \*\*\**P* < 0.01. The symbol \* marks the significance as compared to control and the symbol # as compared to IFN $\gamma$  sample.

for PAR<sub>2</sub>, but not for IFN $\gamma$ , in neutrophil degranulation of azurophil granules and ROS production.

**3.3. MPO Activity Disrupts IAV, but MPO Inhibition Is not Sufficient to Reverse PAR<sub>2</sub> Agonist-Induced Reduction of IAV Replication.** MPO and ROS are required for extracellular disruption of IAV [20]. Therefore, we hypothesized that degranulation fluid (DF) from PAR<sub>2</sub>-activated neutrophils may disrupt IAV. Neutrophils were treated with PAR<sub>2</sub> agonist, IFN $\gamma$ , or their combination, and the DF was collected. In the presence of H<sub>2</sub>O<sub>2</sub>, DF from PAR<sub>2</sub> agonist-treated neutrophils decreased IAV titers by 20-fold ( $95 \pm 5\%$ ) as compared to controls, whereas DF from IFN $\gamma$ -stimulated neutrophils only marginally decreased viral titers by  $14 \pm 1.5\%$  (Figures 3(a) and 3(b)). DF from PAR<sub>2</sub> agonist and IFN $\gamma$  costimulated neutrophils. In the presence of H<sub>2</sub>O<sub>2</sub>, the DF from PAR<sub>2</sub> agonist and IFN $\gamma$  co-stimulated neutrophils reduced viral titers by 20-fold as compared to controls. In the absence of H<sub>2</sub>O<sub>2</sub>, DF did not reduce viral titers (data not shown). Of note, purified elastase failed to disrupt IAV (data not shown).

To further specify the role of MPO and H<sub>2</sub>O<sub>2</sub> in neutrophil response against IAV, we treated IAV-infected neutrophils with a specific MPO inhibitor prior to stimulation with PAR<sub>2</sub> agonist, IFN $\gamma$ , or their combination. In IAV-infected untreated neutrophils, MPO inhibition increased viral titers by approximately 4-fold (Figure 3(c)). It is worth to notice that PAR<sub>2</sub> activation significantly decreased viral titers 2-fold ( $50 \pm 10\%$ ) even in the presence of the MPO inhibitor (Figure 3(c)). In contrast, IFN $\gamma$  did not reduce viral titers in neutrophils treated with MPO inhibitor. The combination of PAR<sub>2</sub>-tcAP and IFN $\gamma$  showed a trend to decrease viral progeny even in the absence of functional MPO.

We next analysed whether reduction of viral progeny originated from intracellular events. Therefore, neutrophils were infected with IAV. Further, viral NS-1 mRNA synthesis was measured as a marker for virus replication. In the case of PAR<sub>2</sub> agonist as well as combined PAR<sub>2</sub> agonist and/IFN $\gamma$  costimulation viral NS-1 mRNA levels were decreased by  $70 \pm 10\%$  and  $50 \pm 18\%$ , respectively (Figure 3(d)). Again, IFN $\gamma$  alone had no effect on reduction of viral NS-1 mRNA synthesis (Figure 3(d)).

Thus, PAR<sub>2</sub> agonist-induced disruption of IAV is associated with the MPO-H<sub>2</sub>O<sub>2</sub> axis and intracellular antiviral mechanisms interfering with IAV gene transcription, indicating at least two PAR<sub>2</sub>-regulated antiviral mechanisms.

**3.4. PAR<sub>2</sub> Agonist Stimulation Affects IFN $\gamma$ -Induced MxA Expression in Human Neutrophils.** We investigated the regulation of OAS and MxA levels. IFN $\gamma$  triggered OAS mRNA expression at 4 hrs and 16 hrs by  $61 \pm 18$ -fold and  $197 \pm 88$ -fold, respectively, as compared to controls (Figures 4(a) and 4(b)). When applied together, PAR<sub>2</sub> agonist and IFN $\gamma$  induced OAS mRNA expression at 4 hrs and 16 hrs by  $56 \pm 20$ -fold and  $210 \pm 96$ -fold, respectively (Figures 4(a) and 4(b)). PAR<sub>2</sub> agonist alone did not induce either OAS or MxA expression (Figures 4(a) and 4(b)). IFN $\gamma$  induced MxA mRNA levels by  $48 \pm 13$ -fold (4 hrs) and  $20 \pm 7$ -fold (16 hrs) as compared to controls. Concomitant stimulation with PAR<sub>2</sub> agonist and IFN $\gamma$  enhanced MxA expression by

$25 \pm 6$ -fold at 4 hrs and  $46 \pm 11$ -fold at 16 hrs (Figures 4(a) and 4(b)) as compared to controls. Since mRNA upregulation not necessarily leads to protein upregulation, the mRNA data were further verified by analysis of MxA on protein levels. As shown in Figures 4(c) and 4(d), the analysis of MxA protein expression after agonist stimulation resembled the expression profile observed on mRNA level. However, only the concomitant stimulation with PAR<sub>2</sub> agonist and IFN $\gamma$  upregulated the MxA protein expression significantly (Figures 4(c) and 4(d)). Although MxA was also slightly increased after IFN $\gamma$  treatment alone, this effect never reached statistical significance. In two samples out of six, MxA was just barely detectable after IFN $\gamma$  stimulation (data not shown). However, in other samples MxA expression was detectable and just slightly enhanced after IFN $\gamma$  stimulation (Figures 4(c) and 4(d)).

Thus, PAR<sub>2</sub> agonist stimulation appears to be an important factor enhancing IFN $\gamma$ -induced expression of MxA.

## 4. Discussion

The central hypothesis of our current work focuses on the role of PAR<sub>2</sub>-mediated degranulation-dependent antiviral responses and PAR<sub>2</sub>-induced intracellular defence mechanisms. Therefore, we investigated whether PAR<sub>2</sub> activates MPO release or triggers intracellular events that interfere with transcription of viral genes. We also explored whether antiviral defence mechanisms (e.g., MxA) might be regulated by PAR<sub>2</sub> agonist and IFN $\gamma$ .

First of all, we proved the ability of PAR<sub>2</sub> and IFN $\gamma$  to synergize reducing IAV replication in human neutrophils (Figure 1). Indeed, simultaneous pretreatment with both agonists followed by their coapplication after infection was more effective in the reduction of IAV replication than any of agonists alone (Figure 1(c)). Moreover, PAR<sub>2</sub> agonist application, but not IFN $\gamma$ , reduced IAV amplification in infected human neutrophils even without pretreatment (Figure 1(b)), suggesting different antiviral activities of IFN $\gamma$  and PAR<sub>2</sub> agonist. We hypothesized that PAR<sub>2</sub> elicits immediate effects based on neutrophil degranulation, whereas the antiviral action of IFN $\gamma$  is time-delayed. Thus, further, we investigated cellular anti-influenza defence mechanisms triggered by both substances.

Neutrophilic MPO was shown to possess anti-pathogenic activity in the presence of H<sub>2</sub>O<sub>2</sub> [20]. Moreover, PAR<sub>2</sub>-AP application was demonstrated to enhance MPO release in mice [21]. However, it remained unclear whether PAR<sub>2</sub> agonists directly induce neutrophil degranulation and whether released MPO inactivates or disrupts the IAV strain H7N7. We revealed that PAR<sub>2</sub> agonist application triggers Ca<sup>2+</sup>-dependent degranulation of human neutrophils and, thus, enhances MPO and elastase release (Figure 2). To measure degranulation, we pretreated neutrophils with cytochalasin B. Cytochalasin B is an artificial substance, which mimics neutrophil priming potentially via induction of a state of GPCRs reactivation [24]. However, in preliminary studies, rechallenge of PAR<sub>2</sub> agonist-primed neutrophils also showed a trend of elevated elastase levels indicating that degranulation may partially occur without cytochalasin B pretreatment

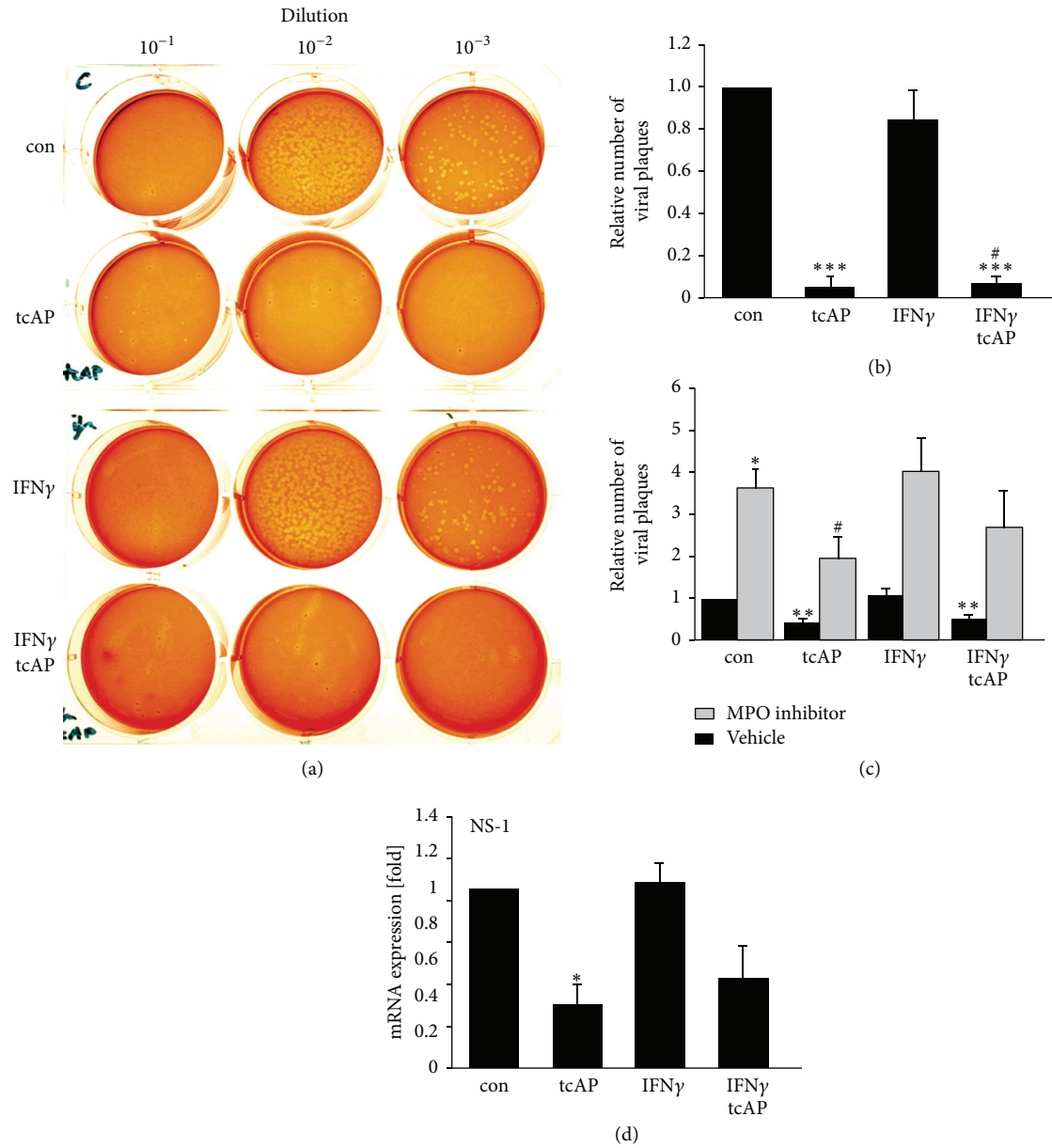


FIGURE 3: Influenza titers were controlled through extracellular MPO and on transcriptional level through PAR<sub>2</sub>. (a, b) DF from stimulated neutrophils was supplemented with H<sub>2</sub>O<sub>2</sub>, and the virucidal activity was determined. DF from PAR<sub>2</sub> agonist treated neutrophils disrupted IAV. (c) Application of a MPO inhibitor enhanced viral titers. Interestingly, despite MPO inhibition, PAR<sub>2</sub> activation reduced viral replication in neutrophils. (d) Analysis of viral gene replication displayed reduced NS-1 mRNA expression in PAR<sub>2</sub> agonist stimulated neutrophils. IFN $\gamma$  had no effect on viral replication. For student's *t*-test: #,\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.005. The symbol \* marks the significance as compared to control and the symbol # as compared to IFN $\gamma$  sample.

(unpublished data). Interestingly, PAR<sub>2</sub> agonist stimulation, without cytochalasin B pretreatment, was capable of enhancing ROS production by human neutrophils (Figure 2(f)), amongst which H<sub>2</sub>O<sub>2</sub> is the substrate for MPO. Moreover, we demonstrated that DF derived from PAR<sub>2</sub> agonist-activated neutrophils contains MPO and disrupts extracellular IAV (Figure 3(a)), indicating a MPO-dependent anti-influenza action. In contrast, IFN $\gamma$  failed to enhance PAR<sub>2</sub>-triggered MPO release, and ROS production (Figure 2). Thus, PAR<sub>2</sub> appears to induce an anti-influenza defence mechanism in

human neutrophils based on degranulation, MPO release and ROS production. However, these mechanisms are clearly independent of and not regulated by IFN $\gamma$  and, thus, represent no cross-point regarding simultaneous PAR<sub>2</sub> and IFN $\gamma$  antiviral action.

Although we demonstrated a substantial role for MPO in influenza disruption (Figure 3(a)), application of a MPO inhibitor did not completely reverse the downregulation of intracellular IAV replication in PAR<sub>2</sub> agonist-activated neutrophils (Figures 3(b) and 3(c)), suggesting the existence of

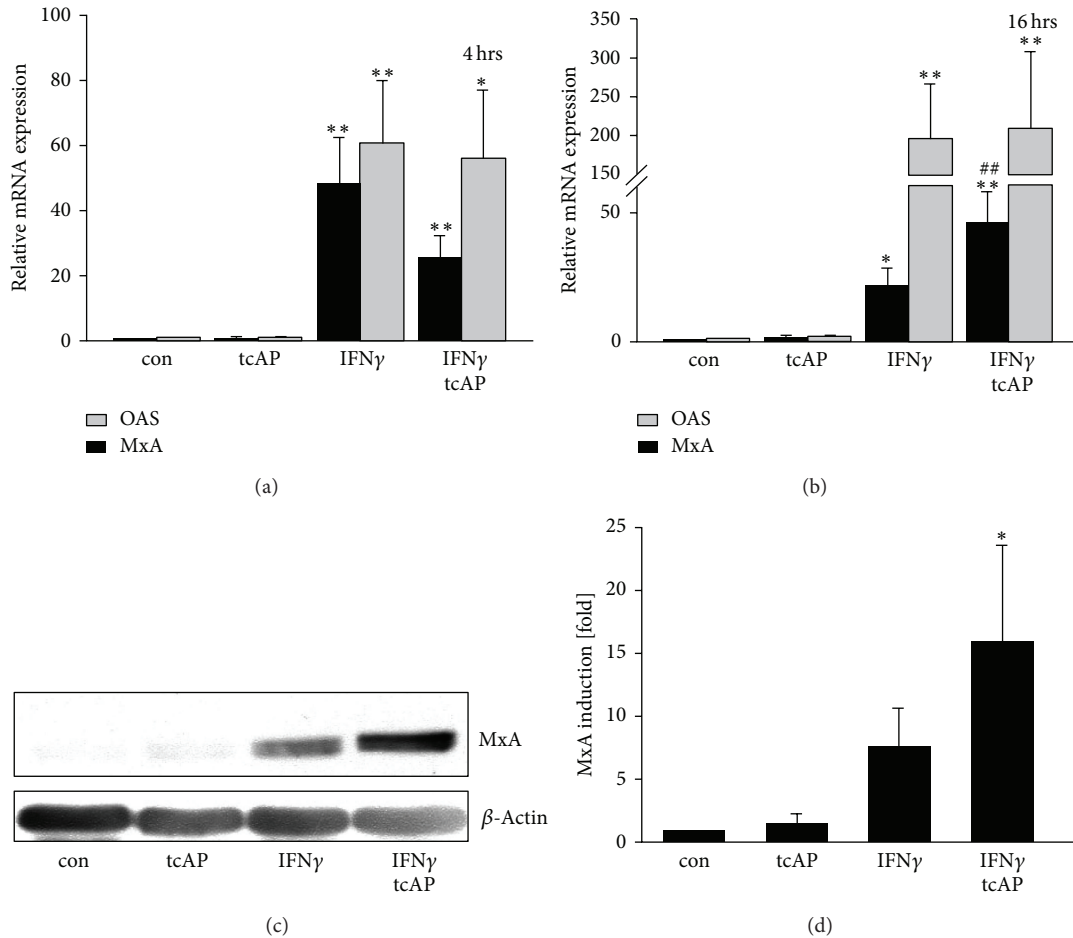


FIGURE 4: Regulation of MxA and OAS expression. (a, b) IFN $\gamma$ -induced expression of OAS remained unaffected after application of PAR $_2$  agonist. But PAR $_2$  agonist synergizes with IFN $\gamma$  to elevate MxA expression at 16 hrs, although this effect was not evident at early time points (4 hrs). (c, d) MxA expression was further analysed on protein level. Similar to mRNA results, concomitant stimulation with PAR $_2$  agonists and IFN $\gamma$  induced MxA protein (at 20 hrs time point). In contrast, IFN $\gamma$  upregulated MxA only slightly and nonsignificantly. For students *t*-test: \*\**P* < 0.05; \*\*\**P* < 0.01. The symbol \* marks the significance as compared to control and the symbol # as compared to IFN $\gamma$  sample. (d) Densitometric results were received for Western blot samples. Wilcoxon matched-pair signed rank test was applied for analysis: \**P* < 0.05 as compared to control.

a redundant mechanism(s) that are controlled by PAR $_2$ . For example, the defensin, cathelicidin LL37, which is stored in neutrophil secondary granules, has been shown to exert anti-influenza activity [25]. Moreover, PAR $_2$  agonist application also reduced NS-1 production in IAV infected neutrophils (Figure 3(d)), further pointing to PAR $_2$ -mediated transcriptional regulation during virus replication.

IFN $\gamma$  application as a pretreatment and during infection (b/a stimulation) was able to reduce IAV replication in human neutrophils (Figure 1(c)). Moreover, in the b/a stimulation model, concomitant IFN $\gamma$  and PAR $_2$  stimulation reduced IAV amplification in human neutrophils as compared to other stimulations (Figure 1(c)). Thus, antiviral mechanisms might require the presence of both PAR $_2$  agonist and IFN $\gamma$ . Indeed, application of PAR $_2$  agonist together with IFN $\gamma$  resulted in stronger induction of MxA mRNA expression as compared to the stimulation with IFN $\gamma$  alone (Figure 4(b)). Antiviral MxA, classically inducible by type I interferons [26], was demonstrated to be elevated by IFN $\gamma$  on transcriptional level

[27]. To our knowledge, the detection of MxA protein upon IFN $\gamma$  stimulation remains elusive. Although we confirmed the induction of MxA mRNA upon IFN $\gamma$  treatment, we found variations in the MxA protein expression amongst the investigated donors. These variations could not be explained by the Western blot artefacts since the experimental protocol was kept constant during all the time. Only combined PAR $_2$  agonist/IFN $\gamma$  stimulation significantly raised MxA protein levels in all investigated samples revealing a potential backup system for type I interferons for efficient fight against IAV infections intracellularly. 2'-5' oligoadenylate synthetase (OAS) also participates in cellular defence against RNA viruses and could be induced by IFN $\gamma$  [26, 28]. But OAS expression was not affected by PAR $_2$  agonist application even in combination with IFN $\gamma$  (Figures 4(a) and 4(b)). Our data suggests that PAR $_2$  shapes the antiviral response through activation of a defined set of defence mechanisms.

In summary, our data demonstrate that PAR $_2$  agonist and IFN $\gamma$  synergize to reduce IAV progeny in human neutrophils.



Enhanced MxA production is revealed as a cellular antiviral mechanism, which is synergistically activated by PAR<sub>2</sub> agonist and IFN $\gamma$  in human neutrophils. However, in neutrophils PAR<sub>2</sub> agonist controls IFN $\gamma$ -independent antiviral mechanism(s) such as enhanced MPO release, ROS production, and reduction of viral gene transcription.

## Abbreviations

2-APB: 2-Aminoethoxydiphenyl borate  
 $\alpha$ 1AT: Alpha-1-antitrypsin  
 AP: Activating peptide  
 b/a: Before/after  
 DF: Degranulated fluid  
 IAV: Influenza A virus  
 IFN $\gamma$ : Interferon gamma  
 MPO: Myeloperoxidase  
 mU: Milli units  
 MxA: Orthomyxovirus resistance protein A  
 OAS: Oligoadenylate synthetase  
 PAR<sub>2</sub>: Proteinase-activated receptor-2  
 ROS: Reactive oxygen species  
 RP: Reverse peptide  
 tc: *Trans-cinnamoyl*.

## Conflict of Interests

The authors of this paper declare no conflict of interests associated with data presented in the manuscript.

## Authors' Contribution

Micha Feld designed the study, performed experiments, analysed data, and wrote the manuscript. Victoria Shpacovitch performed experiments and wrote the draft of the manuscript. Christina Ehrhardt, Tobias Goerge, and Michaela Fastrich performed experiments. Stephan Ludwig and Martin Steinhoff designed the study and reviewed the paper.

## Acknowledgments

This work was supported by the IMF Muenster FE 1 1 09 05 (to Micha Feld) and SH 120709 (to Victoria Shpacovitch), Deutsche Forschungsgemeinschaft (STE 1014/3-1 to Martin Steinhoff), IZKF Muenster (Stei3/034/09 to Martin Steinhoff), and Weston-Havens Foundation San Francisco (to Martin Steinhoff). The research fellow position of Victoria Shpacovitch in Leibniz Institute for Analytical Sciences (ISAS) is supported by SFB 876 Project B2.

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