

Research Article

Proteinase-Activated Receptor-2 Agonist Activates Anti-Influenza Mechanisms and Modulates IFN γ -Induced Antiviral Pathways in Human Neutrophils

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Proteinase-activated receptor-2 (PAR₂) is expressed by human leukocytes and participates in the development of inflammatory diseases. Recent studies demonstrated an ability of PAR₂ agonist to enhance IFN γ -induced antiviral responses of human leukocytes. However, the precise cellular antiviral defense mechanisms triggered in leukocytes after stimulation with IFN γ and/or PAR₂ agonist remain elusive. Therefore, we aimed to identify neutrophil defense mechanisms involved in antiviral resistance. Here we demonstrated that PAR₂ agonist enhanced IFN γ -related reduction of influenza A virus (IAV) replication in human neutrophils. PAR₂-mediated decrease in IAV replication was associated with reduced NS-1 transcription. Moreover, PAR₂-dependent neutrophil activation resulted in enhanced myeloperoxidase degranulation and extracellular myeloperoxidase disrupted IAV. The production of ROS was elevated in response to PAR₂ activation. Interestingly, IFN γ did not influence both effects: PAR₂ 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 PAR₂ agonist and IFN γ in neutrophils. Altogether, these findings emphasize two PAR₂-controlled antiviral mechanisms that are independent of or modulated by IFN γ .

1. Introduction

The impact of proteinase-activated receptor-2 (PAR₂) 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 PAR₂ activation [3]. However, these enzymes cause PAR₂-dependent as well as PAR₂-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 PAR₂ 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 PAR₂-activating peptides, used in our study, do not affect hemagglutinin maturation but reportedly serve as important tools for investigating the role of PAR₂ activation in a wide range of anti-influenza responses.

Interferon- γ (IFN γ) 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 γ and PAR $_2$ 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 γ and PAR $_2$ 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 $_2$ [13, 14], which regulates motility and bactericidal activity of neutrophils [1, 10]. Although the PAR $_2$ -induced bactericidal activity is not enhanced in the presence of IFN γ in neutrophils [10], PAR $_2$ agonist and IFN γ synergize boosting anti-influenza effects in human monocytes [8]. Nonetheless, the role of PAR $_2$ and IFN γ 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 $_2$ activation affects IAV replication in neutrophils and which defence mechanism(s) are activated. We also evaluated whether PAR $_2$ agonist and IFN γ synergize to strengthen the antiviral response.

2. Material and Methods

2.1. Materials. Human PAR $_2$ -activating peptide with the sequence *trans-cinnamoyl*-LIGRLO-NH $_2$ (tcAP) and the reverse peptide with the sequence *trans-cinnamoyl*-OLRGIL-NH $_2$ (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 $^{-4}$ M as described previously [8]. Human recombinant IFN γ was received from Peprotech (Hamburg, Germany) and used at a concentration of 200 U/mL. The following antibodies were used: mouse anti-human β -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 6 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 $_2$, 0.01% MgCl $_2$, 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 $_2$. 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 $_2$, and 0.01% MgCl $_2$. 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 $_2$ 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 6 cells per 100 μ L in PBS. Neutrophils were pretreated with 5 μ 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 μ g/mL alpha-1-antitrypsin (α 1AT) for 30 min at 37°C. Then, elastase/ α 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 μ L of degranulated supernatant was mixed with 100 μ 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 6 PFU/mL. Then, neutrophil supernatant and virus dilution were mixed in a ratio of 1 : 1 and supplemented with 1 mM H $_2$ O $_2$ (Merck) or vehicle as indicated. After incubation for 1 hr at 37°C and 5% CO $_2$, 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-H $_2$ DCEFDA) (Invitrogen). To induce ROS production, neutrophils (1.5 \times 10 6 cells/mL) were stimulated with the indicated agonists in the absence of cytochalasin B. Thirty minutes before the stimulation was stopped, 5 μ M CM-H $_2$ DCEFDA 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 Na₂HPO₄, 10 mM HEPES, 5 mM glucose, and 1 mM MgCl₂ (pH 7.4)) with or without 0.8 mM CaCl₂, 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 CaCl₂, and PAR₂-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 PAR₂ 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'-GCTCCTACCTGTGTGTGTGT-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 PAR₂ Agonist and IFNγ. Previously, we revealed that PAR₂ and IFNγ 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 PAR₂-tcAP, IFNγ, or a combination thereof and measured viral titers after 20 hrs. PAR₂ agonist stimulation decreased IAV titers by 80 ± 2%, whereas IFNγ treatment had no significant effect (Figure 1(b)). Concomitant stimulation with PAR₂ agonist and IFNγ 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 PAR₂ agonist, IFNγ, or their combination for 2 hrs before cells were infected with IAV and rechallenged cells after infection (b/a-stimulation). In this stimulation protocol, PAR₂ and IFNγ reduced viral titers by 68 ± 4% and by 57 ± 5%, respectively (Figure 1(c)). Combining PAR₂ agonist and IFNγ additively decreased IAV titers by approximately 86 ± 2% (Figure 1(c)). Scrambled PAR₂ 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 PAR₂ agonist and IFNγ reduce IAV titers.

3.2. PAR₂ 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. PAR₂-AP was shown to increase plasma MPO activity indicating enhanced neutrophil degranulation in mice [21]. Therefore, we analysed whether stimulation with PAR₂-tcAP or IFNγ triggers human neutrophil degranulation of azurophil granules *in vitro*. In our preliminary experiments, where neutrophils (app. 4×10^6 cells/100 μL) were primed with PAR₂ agonist for 2 hrs, a second dose of PAR₂ agonist elicited the release of elastase. However, variations in the magnitudes of the effect did not allow this effect of PAR₂ 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 PAR₂ activation. Basal release of MPO and elastase in cytochalasin B primed neutrophils was determined as 26.9 ± 4.6 mU and 113.6 ± 21.0 ng/mL, respectively (Figure 2(a)). Further addition of PAR₂-tcAP enhanced extracellular MPO (86.5 ± 19.3 mU) and elastase (265.8 ± 76.4 ng/mL) levels significantly, but degranulation was unaffected by IFNγ. Concomitant stimulation with PAR₂ agonist and IFNγ failed to overcome the effect induced by PAR₂-tcAP alone.

PAR₂-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 PAR₂ activation resulted in significantly less elevated MPO levels (87.9 ± 20.4 mU) as compared to 128.6 ± 24.0 mU in nonpreactivated cells (Figure 2(b)). However, this reduction was not detected in neutrophils activated with both PAR₂ agonist and IFNγ (Figure 2(b)).

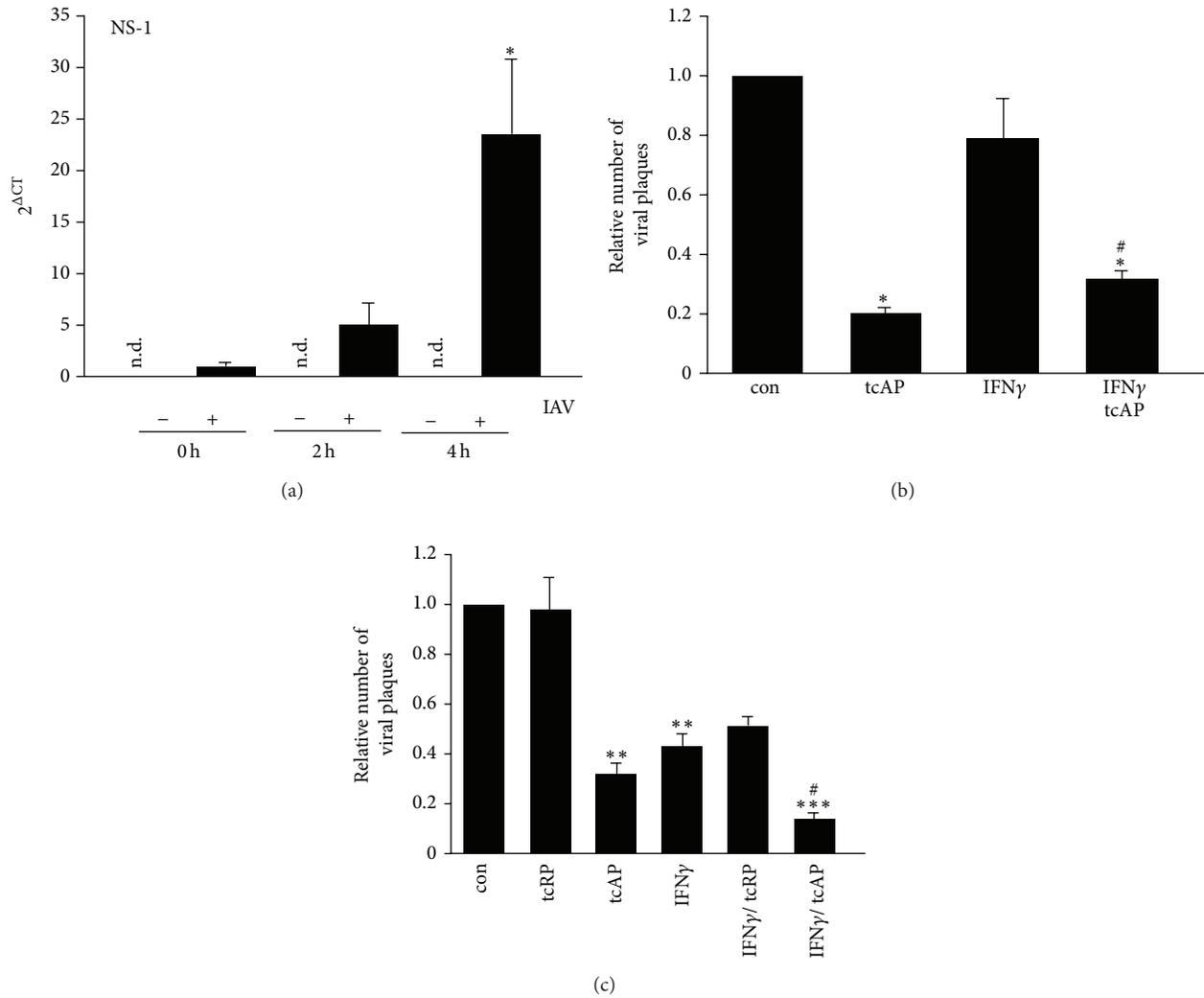


FIGURE 1: IAV replication in neutrophils was restricted by PAR₂ activation and IFN γ . (a) Replication of IAV in neutrophils was determined by detection of NS-1 mRNA levels at different time points after infection. At 4 hrs, a significant induction of NS-1 mRNA expression was revealed. In noninfected 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 PAR₂ agonist and PAR₂ agonist/IFN γ 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 PAR₂ agonist and IFN γ decreased viral replication. Moreover, combining PAR₂ agonist and IFN γ 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 γ sample.

Because degranulation is often triggered by Ca²⁺ signaling, we also investigated the contribution of Ca²⁺ fluxes to PAR₂-induced degranulation. PAR₂ agonist induced a rapid increase in intracellular Ca²⁺ signaling in both Ca²⁺-free or Ca²⁺-supplemented buffer. However, extracellular Ca²⁺ boosted PAR₂ agonist-induced intracellular calcium signals by 3-fold as compared to extracellular Ca²⁺ starvation (Figure 2(c), green columns). However, PAR₂-induced release of azurophil granules was independent of additional extracellular Ca²⁺ (data not shown). 2-APB is known as an inhibitor of InsP3-induced Ca²⁺ release and, probably, concomitant Ca²⁺ entry [22]. 2-APB inhibited PAR₂-induced Ca²⁺ 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, PAR₂-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, PAR₂ significantly upregulated ROS levels by 1.6 ± 0.2-fold as compared to controls. However, combination of PAR₂ agonist and IFN γ was not more potent in induction of ROS than PAR₂-tcAP alone. IFN γ alone did not affect ROS production in neutrophils (Figure 2(f)). Together, our data indicated a regulatory role

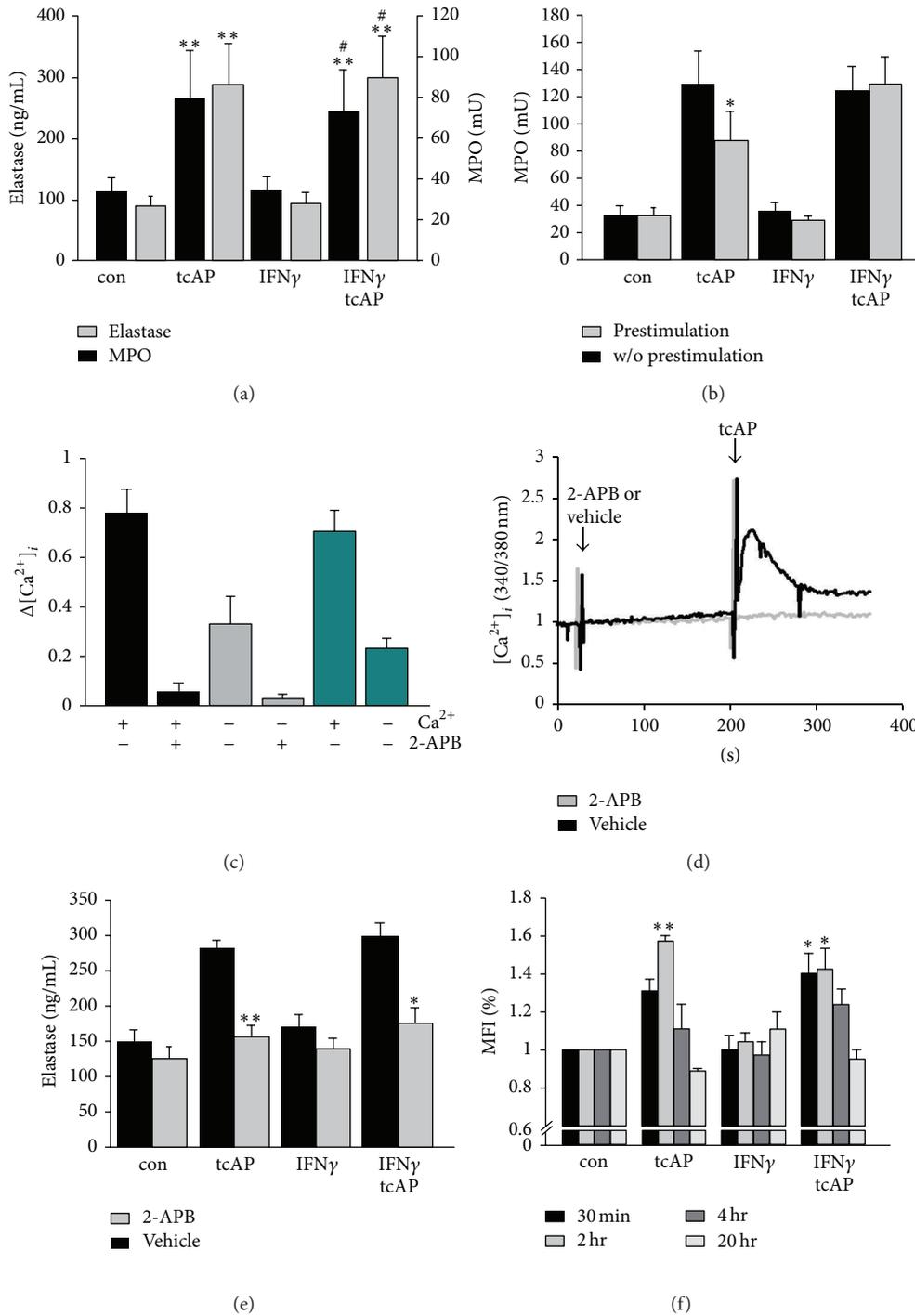


FIGURE 2: PAR₂ stimulation induced neutrophil degranulation in a Ca²⁺-dependent manner and upregulated ROS production. Neutrophils were treated as described in Material and Methods Section. (a) After stimulation with PAR₂ agonist and IFN γ , the concentration of MPO and elastase was quantified in cytochalasin 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 PAR₂ agonist stimulated neutrophils. Concomitant stimulation with PAR₂-agonist and IFN γ induced similar MPO levels in both pretreated and nonpretreated cells. (c, d) Neutrophils were loaded with Fura-2 AM (30 min), washed, and then PAR₂ agonist was added, and calcium mobilization was investigated. The availability of extracellular Ca²⁺ led to increased intracellular calcium levels after PAR₂ agonist application. 2-APB almost completely blocked intracellular Ca²⁺ fluxes, independent of extracellular Ca²⁺. (e) Pretreatment of neutrophils with 2-APB prevented PAR₂ 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, PAR₂ agonist elevated ROS level as measured by changes of the MFI. IFN γ 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 γ sample.

for PAR₂, but not for IFN γ , in neutrophil degranulation of azurophil granules and ROS production.

3.3. MPO Activity Disrupts IAV, but MPO Inhibition Is not Sufficient to Reverse PAR₂ 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 PAR₂-activated neutrophils may disrupt IAV. Neutrophils were treated with PAR₂ agonist, IFN γ , or their combination, and the DF was collected. In the presence of H₂O₂, DF from PAR₂ agonist-treated neutrophils decreased IAV titers by 20-fold ($95 \pm 5\%$) as compared to controls, whereas DF from IFN γ -stimulated neutrophils only marginally decreased viral titers by $14 \pm 1.5\%$ (Figures 3(a) and 3(b)). DF from PAR₂ agonist and IFN γ costimulated neutrophils in the presence of H₂O₂, the DF from PAR₂ agonist and IFN γ co-stimulated neutrophils reduced viral titers by 20-fold as compared to controls. In the absence of H₂O₂, 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 H₂O₂ in neutrophil response against IAV, we treated IAV-infected neutrophils with a specific MPO inhibitor prior to stimulation with PAR₂ agonist, IFN γ , 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 PAR₂ activation significantly decreased viral titers 2-fold ($50 \pm 10\%$) even in the presence of the MPO inhibitor (Figure 3(c)). In contrast, IFN γ did not reduce viral titers in neutrophils treated with MPO inhibitor. The combination of PAR₂-tcAP and IFN γ 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 PAR₂ agonist as well as combined PAR₂ agonist and/IFN γ costimulation viral NS-1 mRNA levels were decreased by $70 \pm 10\%$ and $50 \pm 18\%$, respectively (Figure 3(d)). Again, IFN γ alone had no effect on reduction of viral NS-1 mRNA synthesis (Figure 3(d)).

Thus, PAR₂ agonist-induced disruption of IAV is associated with the MPO-H₂O₂ axis and intracellular antiviral mechanisms interfering with IAV gene transcription, indicating at least two PAR₂-regulated antiviral mechanisms.

3.4. PAR₂ Agonist Stimulation Affects IFN γ -Induced MxA Expression in Human Neutrophils. We investigated the regulation of OAS and MxA levels. IFN γ triggered OAS mRNA expression at 4 hrs and 16 hrs by 61 ± 18 -fold and 197 ± 88 -fold, respectively, as compared to controls (Figures 4(a) and 4(b)). When applied together, PAR₂ agonist and IFN γ induced OAS mRNA expression at 4 hrs and 16 hrs by 56 ± 20 -fold and 210 ± 96 -fold, respectively (Figures 4(a) and 4(b)). PAR₂ agonist alone did not induce either OAS or MxA expression (Figures 4(a) and 4(b)). IFN γ induced MxA mRNA levels by 48 ± 13 -fold (4 hrs) and 20 ± 7 -fold (16 hrs) as compared to controls. Concomitant stimulation with PAR₂ agonist and IFN γ enhanced MxA expression by

25 ± 6 -fold at 4 hrs and 46 ± 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 PAR₂ agonist and IFN γ upregulated the MxA protein expression significantly (Figures 4(c) and 4(d)). Although MxA was also slightly increased after IFN γ treatment alone, this effect never reached statistical significance. In two samples out of six, MxA was just barely detectable after IFN γ stimulation (data not shown). However, in other samples MxA expression was detectable and just slightly enhanced after IFN γ stimulation (Figures 4(c) and 4(d)).

Thus, PAR₂ agonist stimulation appears to be an important factor enhancing IFN γ -induced expression of MxA.

4. Discussion

The central hypothesis of our current work focuses on the role of PAR₂-mediated degranulation-dependent antiviral responses and PAR₂-induced intracellular defence mechanisms. Therefore, we investigated whether PAR₂ 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 PAR₂ agonist and IFN γ .

First of all, we proved the ability of PAR₂ and IFN γ 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, PAR₂ agonist application, but not IFN γ , reduced IAV amplification in infected human neutrophils even without pretreatment (Figure 1(b)), suggesting different antiviral activities of IFN γ and PAR₂ agonist. We hypothesized that PAR₂ elicits immediate effects based on neutrophil degranulation, whereas the antiviral action of IFN γ 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 H₂O₂ [20]. Moreover, PAR₂-AP application was demonstrated to enhance MPO release in mice [21]. However, it remained unclear whether PAR₂ agonists directly induce neutrophil degranulation and whether released MPO inactivates or disrupts the IAV strain H7N7. We revealed that PAR₂ agonist application triggers Ca²⁺-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 PAR₂ agonist-primed neutrophils also showed a trend of elevated elastase levels indicating that degranulation may partially occur without cytochalasin B pretreatment

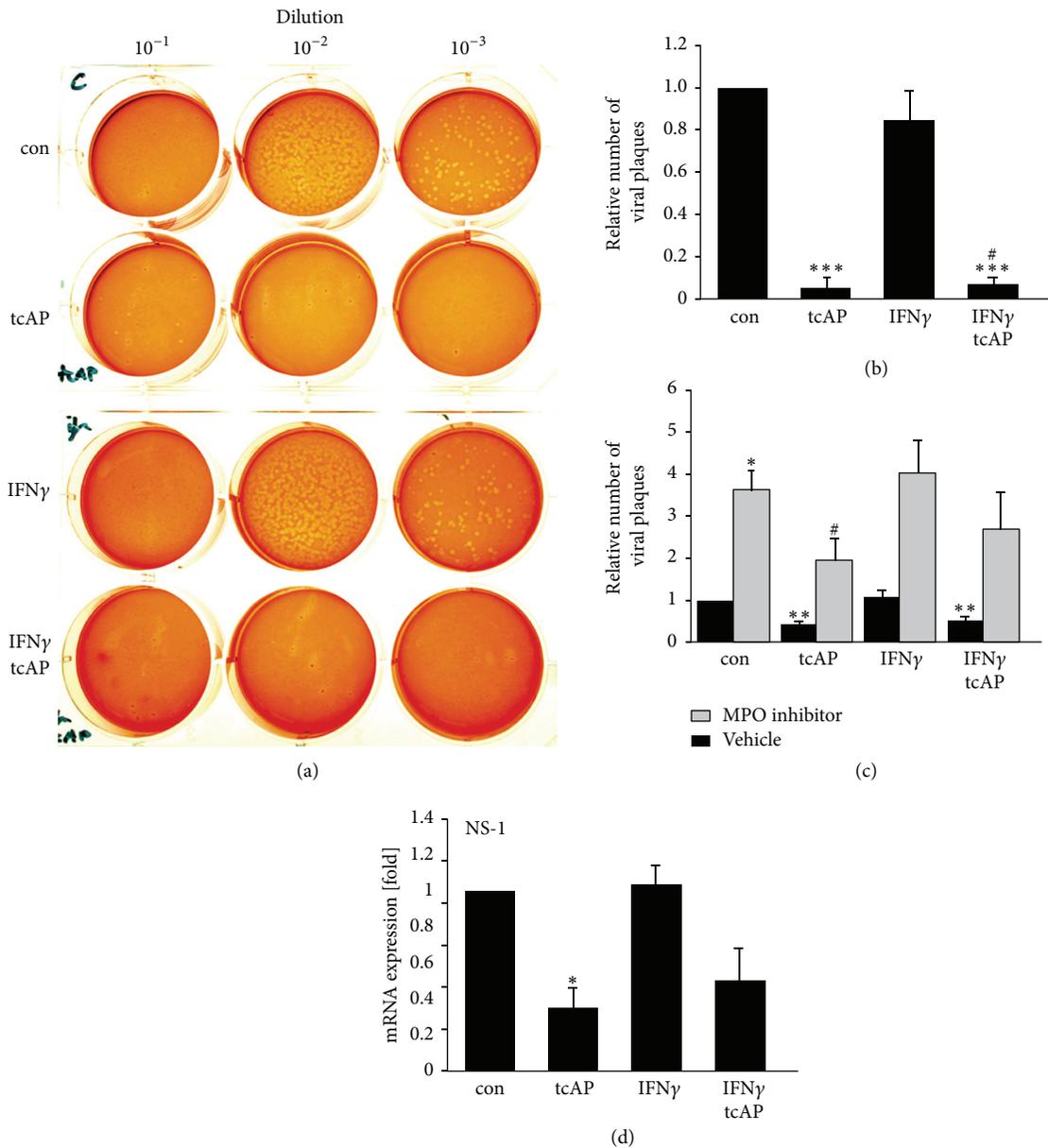


FIGURE 3: Influenza titers were controlled through extracellular MPO and on transcriptional level through PAR₂. (a, b) DF from stimulated neutrophils was supplemented with H₂O₂, and the virucidal activity was determined. DF from PAR₂ agonist treated neutrophils disrupted IAV. (c) Application of a MPO inhibitor enhanced viral titers. Interestingly, despite MPO inhibition, PAR₂ activation reduced viral replication in neutrophils. (d) Analysis of viral gene replication displayed reduced NS-1 mRNA expression in PAR₂ agonist stimulated neutrophils. IFN γ 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 γ sample.

(unpublished data). Interestingly, PAR₂ agonist stimulation, without cytochalasin B pretreatment, was capable of enhancing ROS production by human neutrophils (Figure 2(f)), amongst which H₂O₂ is the substrate for MPO. Moreover, we demonstrated that DF derived from PAR₂ agonist-activated neutrophils contains MPO and disrupts extracellular IAV (Figure 3(a)), indicating a MPO-dependent anti-influenza action. In contrast, IFN γ failed to enhance PAR₂-triggered MPO release, and ROS production (Figure 2). Thus, PAR₂ 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 γ and, thus, represent no cross-point regarding simultaneous PAR₂ and IFN γ 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 PAR₂ agonist-activated neutrophils (Figures 3(b) and 3(c)), suggesting the existence of

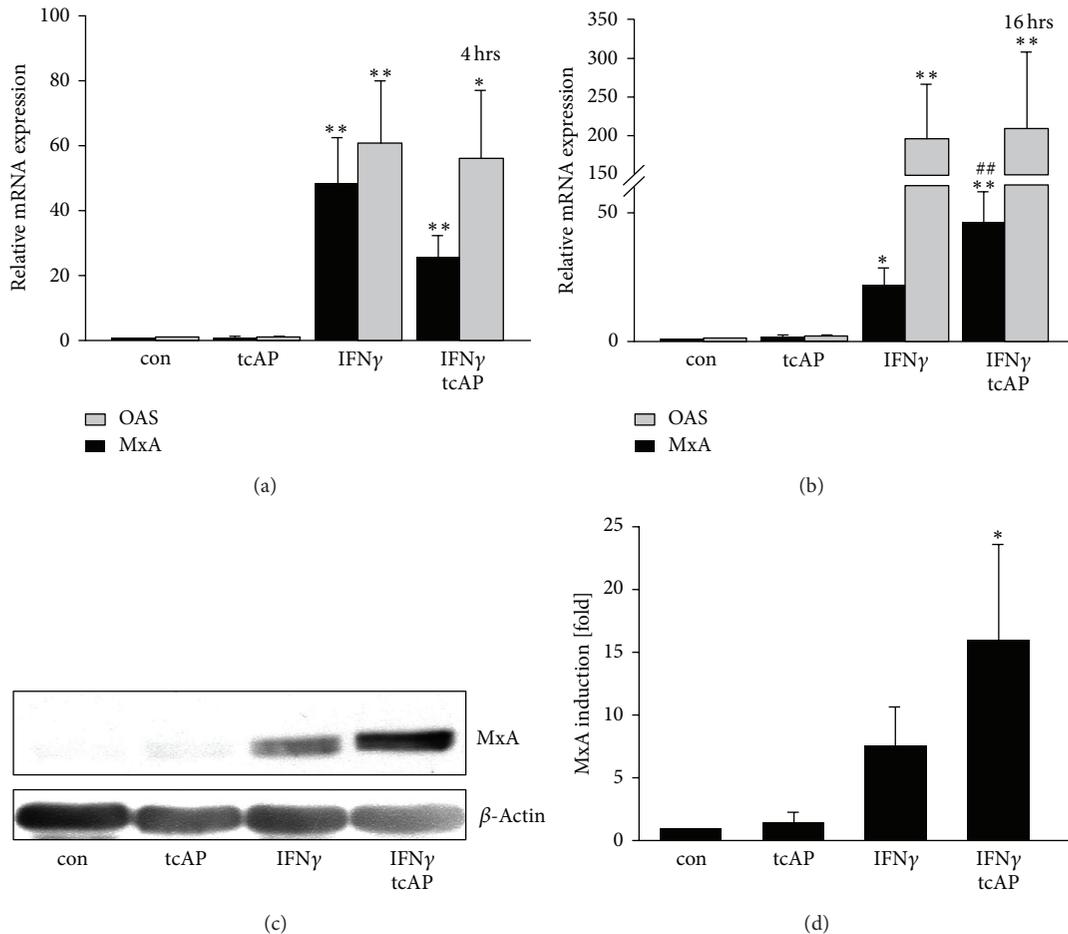


FIGURE 4: Regulation of MxA and OAS expression. (a, b) IFN γ -induced expression of OAS remained unaffected after application of PAR $_2$ agonist. But PAR $_2$ agonist synergizes with IFN γ to elevate MxA expression at 16 hrs, although this effect was not evident at early time points (4 hrs). (c, d) MxA expression was further analysed on protein level. Similar to mRNA results, concomitant stimulation with PAR $_2$ agonists and IFN γ induced MxA protein (at 20 hrs time point). In contrast, IFN γ 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 γ 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 γ 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 γ 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 γ . Indeed, application of PAR $_2$ agonist together with IFN γ resulted in stronger induction of MxA mRNA expression as compared to the stimulation with IFN γ alone (Figure 4(b)). Antiviral MxA, classically inducible by type I interferons [26], was demonstrated to be elevated by IFN γ on transcriptional level

[27]. To our knowledge, the detection of MxA protein upon IFN γ stimulation remains elusive. Although we confirmed the induction of MxA mRNA upon IFN γ 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 γ 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 γ [26, 28]. But OAS expression was not affected by PAR $_2$ agonist application even in combination with IFN γ (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 γ synergize to reduce IAV progeny in human neutrophils.

Enhanced MxA production is revealed as a cellular antiviral mechanism, which is synergistically activated by PAR₂ agonist and IFN γ in human neutrophils. However, in neutrophils PAR₂ agonist controls IFN γ -independent antiviral mechanism(s) such as enhanced MPO release, ROS production, and reduction of viral gene transcription.

Abbreviations

2-APB: 2-Aminoethoxydiphenyl borate
 α 1AT: Alpha-1-antitrypsin
 AP: Activating peptide
 b/a: Before/after
 DF: Degranulated fluid
 IAV: Influenza A virus
 IFN γ : Interferon gamma
 MPO: Myeloperoxidase
 mU: Milli units
 MxA: Orthomyxovirus resistance protein A
 OAS: Oligoadenylate synthetase
 PAR₂: 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.

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