Hydrogen sulfide limits neutrophil transmigration, inflammation, and oxidative burst in lipopolysaccharide-induced acute lung injury

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Transmigration and activation of neutrophils in the lung reflect key steps in the progression of acute lung injury (ALI). It is known that hydrogen sulfide (H₂S) can limit neutrophil activation, but the respective mechanisms remain elusive. Here, we aimed to examine the underlying pathways in pulmonary inflammation. In vivo, C57BL/6N mice received the H₂S slow releasing compound GYY4137 prior to lipopolysaccharide (LPS) inhalation. LPS challenge led to pulmonary injury, inflammation, and neutrophil transmigration that were inhibited in response to H₂S pretreatment. Moreover, H₂S reduced mRNA expression of macrophage inflammatory protein-2 (MIP-2) and its receptor in lung tissue, as well as the accumulation of MIP-2 and interleukin-1β in the alveolar space. In vitro, GYY4137 did not exert toxic effects on Hoxb8 neutrophils, but prevented their transmigration through an endothelial barrier in the presence and absence of MIP-2. In addition, the release of MIP-2 and reactive oxygen species from LPS-stimulated Hoxb8 neutrophils were directly inhibited by H₂S. Taken together, we provide first evidence that H₂S limits lung neutrophil sequestration upon LPS challenge. As proposed underlying mechanisms, H₂S prevents neutrophil transmigration through the inflamed endothelium and directly inhibits pro-inflammatory as well as oxidative signalling in neutrophils. Subsequently, H₂S pretreatment ameliorates LPS-induced ALI.

Acute lung injury (ALI) due to pulmonary inflammation still represents a major problem in critical care medicine and is associated with high rates of morbidity and mortality. In this regard, postoperative pulmonary complications as underlying cause are of great importance. By now, treatment or preventive options are limited, and new therapeutic strategies are needed.

ALI is characterised by alveolar barrier dysfunction, oedema formation, and accumulation of immune competent cells in the lungs. Especially the transmigration of neutrophils through endothelial cells promote the acute phase of pulmonary inflammation. Activated neutrophils are attracted by pro-inflammatory cytokines, e.g., macrophage inhibitory protein-2 (MIP-2), to the side of the injury. Subsequently, neutrophils react with excessive pro-inflammatory cytokine release and oxidative burst, which in turn further aggravate the overall cellular inflammatory response and lung tissue injury. Conversely, a reduction of neutrophil transmigration has been described to limit lung injury.

In order to evaluate possible (pre)treatment options, we and others have previously shown that inhalation of hydrogen sulfide (H₂S) prevents neutrophil accumulation in models of ventilator and lipopolysaccharide (LPS)-induced lung injury. Although recent data suggest that H₂S may limit neutrophil activation and transmigration by downregulating pulmonary expression of chemoattractant molecules or by reducing leukocyte rolling and adhesion, it remains completely unknown how H₂S interacts with the neutrophilic inflammatory response.
The current study was designed to thoroughly investigate the impact of H₂S on neutrophil vitality, transmigration, pro-inflammatory response, and oxidative burst in vivo and in vitro. We provide first evidence that H₂S prevents neutrophil activation, migration, cytokine release, and oxidative burst upon LPS challenge, subsequently ameliorating lung tissue inflammation and injury.

Results
Effects of LPS and GYY4137 on acute lung injury and neutrophil transmigration in vivo. First, we sought to induce acute lung injury in mice by nebulisation of LPS, either in the absence (LPS; study design Fig. 1a) or in the presence of the H₂S releasing compound GYY4137 (LPS + GYY). Compared to controls (control) or controls receiving GYY4137 (control + GYY), treatment with LPS alone resulted in alveolar wall thickening after 6 h. In contrast, additional GYY4137 significantly decreased alveolar wall thickness (P = 0.0413; Fig. 1b,c). Similar results were obtained by determining an overall ALI score (Fig. 1d). The relative amount of neutrophils in the BAL fluid was determined by cytospin analysis (e). Data represent means ± SEM for n = 8/group. ANOVA (Tukey’s post hoc test), *LPS vs. control (d: P = 0.0076; e: P < 0.0001); *LPS + GYY vs. control (e: P < 0.0001); *LPS vs. control + GYY (d: P = 0.0044; e: P < 0.0001); *LPS + GYY vs. control + GYY (e: P < 0.0001); *LPS + GYY vs. LPS (c: P = 0.0413; e: P < 0.0001).
Next, we analysed the expression of the chemotactic cytokine MIP-2 and its receptor C-X-C motif-chemokine receptor 2 (CXCR2) in lung tissue. Both are known mediators of neutrophil transmigration. In contrast to control and control + GYY groups, LPS inhalation induced MIP-2 mRNA expression in lung tissue homogenates (LPS, Fig. 2a), an effect that was prevented in the presence of GYY4137 (LPS + GYY, Fig. 2a). Analysis of CXCR2 mRNA expression yielded similar results, but failed statistical significance (Fig. 2b). In BAL fluid, the amount of MIP-2 (Fig. 2c) and IL-1β (Fig. 2d) was determined by ELISA. Graphs represent means ± SEM, n = 8/group. ANOVA (Tukey’s post hoc test), *LPS vs. control (a,c,d: P < 0.0001); *LPS + GYY vs. control (c: P = 0.0002; d: P = 0.0086); ^LPS vs. control + GYY (a,c,d: P < 0.0001); ^LPS + GYY vs. control + GYY (a,c,d: P < 0.0001) §LPS vs. LPS + GYY (a: P = 0.0007; c: P = 0.0413; d: P = 0.0192).

**Effects of LPS and GYY4137 on inflammatory response in the lung in vivo.** Next, we analysed the expression of the chemotactic cytokine MIP-2 and its receptor C-X-C motif-chemokine receptor 2 (CXCR2) in lung tissue. Both are known mediators of neutrophil transmigration. In contrast to control and control + GYY groups, LPS inhalation induced MIP-2 mRNA expression in lung tissue homogenates (LPS, Fig. 2a), an effect that was prevented by supplementary GYY4137 treatment (LPS + GYY, Fig. 2a). Analysis of CXCR2 mRNA expression yielded similar results, but failed statistical significance (Fig. 2b). In BAL fluid, the MIP-2 protein content remained minimal in both control groups (control, control + GYY, Fig. 2c). While LPS inhalation clearly increased MIP-2 protein (Fig. 2c), the additional application of GYY4137 significantly reduced the amount of MIP-2 as compared to LPS alone (P = 0.0413, Fig. 2c). Likewise, LPS inhalation increased the amount of interleukin-1β protein (IL-1β) in BAL fluid that was partially prevented in the presence of GYY4137 (P = 0.0192; Fig. 2d). Interesting to note, GYY4137 application per se tended to decrease IL-1β readings as compared to controls (Fig. 2d). According to these findings, the H2S releasing compound GYY4137 reduces neutrophil transmigration, most likely by limiting the accumulation of chemoattractant and pro-inflammatory cytokines in the lung.

**Effects of GYY4137 on Hoxb8 neutrophil vitality and migration through an endothelial monolayer in vitro.** Different pulmonary cell type, e.g., endothelial or neutrophil cells, can produce MIP-2 and IL-1β upon LPS stimulation. Because the results from our in vivo experiments would not allow to identify the specific cell type on which GYY4137 exerts its anti-inflammatory effects, we aimed to define the impacts of GYY4137 on differentiated Hoxb8 neutrophils in vitro.
We first investigated whether the inhibiting effect of GYY4137 on neutrophil accumulation might be a result of potential toxicity. To address this issue, Hoxb8 neutrophils were subjected to increasing concentrations of GYY4137. After 24 h of incubation, the cell vitality assays showed no differences between treated and untreated neutrophils (Fig. 3a).

After excluding potential toxicity, we further analysed whether GYY4137 directly affects neutrophil transmigration through an endothelial monolayer. As our results from co-culture experiments demonstrate (experimental design, Fig. 3b), MIP-2 led to an enhanced transmigration of neutrophils from the upper to the lower compartment (Fig. 3c). In contrast, incubation of neutrophils with GYY4137 in the upper compartment significantly reduced neutrophil transmigration through the endothelial monolayer (Fig. 3c). These effects have been observed in the absence of the stimulating cytokine (control vs control \( P = 0.0039 \)); MIP-2 vs. control + GYY (c: \( P < 0.0001 \)); MIP-2 + GYY vs. MIP-2 (c: \( P = 0.0002 \)).

Effects of LPS and GYY4137 on Hoxb8 neutrophil cytokine release and oxidative burst \textit{in vitro}. Finally, we tested whether GYY4137 exerts direct anti-inflammatory and/or anti-oxidative effects on Hoxb8 neutrophils by analysing cytokine accumulation and reactive oxygen species (ROS) formation. LPS incubation profoundly induced MIP-2 accumulation in Hoxb8 neutrophils. In contrast, supplemental GYY4137 significantly reduced MIP-2 liberation despite LPS treatment (\( P = 0.0010 \); Fig. 4a). The formation of ROS was clearly
induced by LPS compared to controls. Likewise, GYY4137 treatment in the presence of LPS completely prevented ROS production (P = 0.0130; Fig. 4b). The results from our in vitro experiments indicate that GYY4137 exerts direct inhibitory effects in neutrophil cells, thus preventing neutrophil migration, cytokine release, and ROS formation.

**Discussion**

We and others have previously shown that hydrogen sulfide prevents lung injury in models of LPS-induced ALI\(^{10,11,14}\). In these studies, protection was clearly associated with the reduction of neutrophil accumulation in the lungs. Because in a series of injury models\(^{7,8,10,13,20}\), H\(_2\)S-mediated inhibition of neutrophil influx appeared to be a major factor of its preventive effects, the rationale of the present study was to examine how H\(_2\)S interferes with the neutrophil inflammatory response. In order to specifically address the above mentioned problem of postoperative pulmonary complications as a source of ALI\(^{14}\), and to rule out potential preventive effects of H\(_2\)S, we chose to apply the H\(_2\)S releasing compound GYY4137 prior to the inflammatory insult.

We first induced ALI in vivo by nebulisation of LPS\(^{21}\). Lung injury was characterised by enlarged alveolar walls and an elevated ALI score. These findings are in line with the results of other rodent models, i.e., after intraperitoneal, intranasal, or intratracheal LPS application\(^{10,12,13,22,23}\). By contrast, prophylactic application of GYY4137, a water-soluble compound that slowly releases H\(_2\)S\(^{24}\), prevented all signs of lung injury despite LPS treatment. Our data support the findings of two related studies. Here, GYY4137 exerted lung protection in a mouse endotoxemia model\(^{11–13}\). With regard to LPS-induced ALI, the observed effects of GYY4137 are comparable to those of H\(_2\)S fast-releasing salts, i.e., sodium hydrosulfide\(^{25}\) or inhaled H\(_2\)S gas\(^{10,14}\), underlining the lung protective properties of H\(_2\)S irrespective of the form of application. Although we chose a ‘prophylactic’ time point of GYY4137 application, it seems likely that a more ‘therapeutic’ approach, i.e., administration after the injurious insult, can reduce lung inflammation and injury. First, GYY4137 showed anti-inflammatory effects in several experimental models using a post-injury time points of application\(^{26,28}\). Second, we recently demonstrated the time dependency of application\(^{27}\). After setting the insult, an earlier H\(_2\)S inhalation resulted in a more protective effect. However, postponed application still reduced lung inflammation\(^{27}\). Mechanistically, it has been demonstrated in LPS and other models that H\(_2\)S-mediated lung protection is associated with the reduction of inflammatory processes. Amongst them, H\(_2\)S significantly decreases the activity of NF-κB\(^{28,29}\), cystathionine-β-synthetase and cystathionine-γ-lyase\(^{14,35}\), and particularly pro-inflammatory cytokine and neutrophil accumulation in the lungs\(^{5,10,12,13,20,34}\).

Pulmonary neutrophil activation and transmigration display key events in the development of ALI after bacterial challenge\(^{5}\). Here, we show that upon LPS inhalation a substantial fraction of neutrophils was recruited into the alveolar space that was inhibited in the presence of GYY4137. These results are in line with previous work on endotoxemia in rats and mice demonstrating a decrease in lung myeloperoxidase activity and neutrophil influx in response to GYY4137 application\(^{11–17}\). While the limitation of neutrophil function appears to be a general mechanism of H\(_2\)S-mediated protection in various lung injury models\(^{10,12,13}\), the exact mechanism remains elusive. We therefore investigated the effect of the H\(_2\)S releasing compound GYY4137 on neutrophil reaction upon LPS stimulation in vivo and in vitro.

MIP-2 represents a central chemotactic mediator that is released following LPS challenge in various pulmonary cell types, e.g., endothelial cells\(^{35,36}\), epithelial cells\(^{37,38}\), or neutrophils\(^ {39,40}\), and subsequently activates the...
neutrophil inflammatory response. In the current study, we found that LPS increased MIP-2 mRNA expression in lung tissue homogenates and MIP-2 protein in the alveolar space. In contrast, GYY4137 application inhibited both MIP-2 mRNA expression and protein release even in the presence of LPS. Furthermore, GYY4137 tended to reduce MIP-2 mRNA expression of the corresponding receptor CXCR2 as compared to LPS alone in the same samples. Similar observations have been made by others in a model of ventilator-induced ALI [5], indicating that the chemotactic response of pulmonary cells may be suppressed in response to H$_2$S administration. These results allow us to hypothesise that decreased pulmonary chemoattractant signalling due to H$_2$S treatment may lead to reduced neutrophilic transmigration as we observed in vivo. Because our results derived from lung homogenates, we cannot differentiate between pulmonary cell types as effect sites of H$_2$S. We therefore tested the cell specific effects of H$_2$S on differentiated Hoxb8 neutrophils [19] as well as in co-culture with endothelial HUVEC cells in vitro.

In these experiments, we aimed to assess three potential mechanisms:

1. H$_2$S may induce toxic effects in neutrophils, thus preventing neutrophil transmigration.
After 24 h of incubation with the slow releasing H$_2$S donor GYY4137 in concentrations ranging from 0.1 µM to 1 mM, we were not able to detect any impact on cellular vitality in Hoxb8 neutrophils. To the best of our knowledge, no other study has yet tested the effect of GYY4137 on neutrophil vitality. The concentrations used in the current study follow and even exceed GYY4137 concentrations used in comparable vitality studies in other cell lines [39,40]. It is interesting to note that GYY4137 seems to affect cell viability after 3 to 5 days at the earliest but not up to 24 h [39,40]. In this context, incubation with H$_2$S enhanced short-term survival of cultured human neutrophils after 24 h in a recent study [41]. Taken together, we suggest that the observed reduction in neutrophil transmigration does not result from toxic side-effects of H$_2$S.

2. H$_2$S may prevent transmigration of neutrophils into the lungs.
Upon chemotactic stimulation in the lung, neutrophils first pass the endothelial barrier and migrate to the site of inflammation [32]. As it has been demonstrated in a model of small intestine ischemia-reperfusion injury, H$_2$S can prevent leukocyte adhesion and rolling [46-48].

To address our above mentioned hypothesis, we established an in vitro neutrophil transmigration model using an endothelial monolayer (HUVEC cells) on a perforated insert (Fig. 3b) that was co-cultured with differentiated Hoxb8 neutrophils [42]. Adding the chemoattractant cytokine MIP-2 to the bottom medium led to a substantially increased transmigration of Hoxb8 cells through the endothelial cell layer into the bottom medium as compared to the control group. Adding GYY4137 to the top medium completely inhibited this process. Our observations can be explained in several ways: First, the permeability of the endothelial barrier increased upon MIP-2 stimulation and decreased in response to H$_2$S supplementation [36,45,44], consequently reducing the capability of neutrophils to cross intercellular gaps. Second, H$_2$S limits the pro-inflammatory signalling in stimulated HUVEC cells [54,55], thus attracting fewer neutrophils. This interpretation is strengthened by our in vivo results in which GYY4137 inhibited MIP-2 mRNA and protein accumulation in lung tissue and BAL. While the latter two points might explain how H$_2$S impacts neutrophil transmigration through the endothelium during inflammation, the present migration experiment also suggests a direct effect of GYY4137 on neutrophils. As we observed, GYY4137 significantly reduced the number of spontaneously transmigrating neutrophils even in the absence of MIP-2 stimulation.

3. H$_2$S may directly inhibit neutrophil pro-inflammatory signalling and oxidative burst.
At the site of inflammation, activated and migrated neutrophils liberate pro-inflammatory cytokines and ROS in order to enhance the inflammatory process [5]. Upon LPS stimulation, we found a substantial increase of MIP-2 protein in the medium of Hoxb8 cell cultures, a response that is comparable to other neutrophil cell lines [46,47]. In contrast, we provide first evidence that additional application of GYY4137 prevented MIP-2 release, indicating that H$_2$S can suppress the inflammatory response in neutrophils.

In the current study, we show that LPS stimulation directly induced ROS formation in Hoxb8 neutrophils as previously reported in isolated human neutrophils [56,48]. In contrast, H$_2$S releasing GYY4137 prevented ROS production even in the presence of LPS, strongly suggesting that H$_2$S profoundly inhibits the oxidative burst in neutrophils.

In conclusion, the slow-releasing H$_2$S compound GYY4137 prevents lung injury and neutrophil transmigration in a mouse model of LPS-induced ALI. The inhibition of neutrophil transmigration by H$_2$S appears to be a critical step for preventing lung injury. We show in vivo and in vitro that GYY4137 limits neutrophil migration by reducing chemoattractant signalling in lung tissue and endothelial cells. Moreover, we demonstrate that H$_2$S directly suppresses the pro-inflammatory response and the production of ROS in neutrophils. These findings allow first insights into the cell specific effects of H$_2$S and underline the beneficial potential of H$_2$S releasing compounds in the prophylaxis of acute lung injury.

**Methods**

**Animals.** Animal experiments were performed in accordance with the guidelines of the local animal care commission (University of Freiburg, Freiburg, Germany) and in conformance with the journals’ requirements for human and animal trials (ARRIVE Animals in Research: Reporting In Vivo Experiments). The study was approved by the local government in consultation with an ethics committee (Regierungspräsidium Freiburg, Referat 35, Fachgebiet Tierschutz und Tierhaltung, abteilung3@rpf.bwl.de, Freiburg, Germany, permission No. G-12/73). C57BL/6N mice (n = 32, weighing 24.6 ± 0.2 g) were obtained from Charles River Laboratories (Sulzfeld, Germany).

**Experimental setting.** Preliminary experiments were conducted prior to the study in order to establish the appropriate dose for LPS-induced moderate lung injury and to determine the effective dose of intraperitoneally
breathe synthetic air for another 6 h. Group 3 (LPS): mice received 25 µl/g body weight PBS i.p. and were exposed to synthetic air for 1 h. Afterwards, mice were treated with 5 ml of aerosolised PBS and subjected to synthetic air for another 6 h. Group 2 (control + GYY): mice received 250 mg/kg body weight GYY4137 i.p. (freshly dissolved in PBS, 10 mg/ml Dichloromethane Complex, Sigma, Taufkirchen, Germany) and were exposed to synthetic air for 1 h. Afterwards, mice were treated with 5 ml of aerosolised PBS and subjected to breathe synthetic air for another 6 h. Group 4 (LPS + GYY): mice received 250 mg/kg body weight GYY4137 i.p. (freshly dissolved in PBS) and were exposed to breathe synthetic air for 1 h. Afterwards, mice were treated with 0.05 mg of aerosolised LPS (E.coli 055:B5, Sigma; dissolved in 5 ml PBS) and subjected to breathe synthetic air for another 6 h. Experiments were performed in a sealed plexiglas chamber with a constant air flow of 1.5 l/min. Mice had free access to food and water. Nebulisation was performed in a custom-built cylindrical chamber (20 cm in length, 9 cm in diameter) connected to an air nebuliser (MicroAir; Omron Healthcare, Vernon Hills, IL, USA), producing particles from 1–5 µm, as previously described. Nebulisation of the 5 ml solutions was ceased after 14–16 min in all experiments.

At the end of the experiment (6 h after PBS or LPS nebulisation), mice were euthanised by an overdose injection of ketamine (180 mg/kg, i.p.) and acepromazine (1.8 mg/kg, i.p.). Bronchoalveolar lavage fluid and lung tissue for histological examination and semi-quantitative polymerase chain reaction (sqRT-PCR) analysis were gained as described previously.

Histological examination and ALI Score. Cryosections of the left lung lobes and haematoxylin and eosin staining were performed and analysed in a blinded fashion as described previously. Alveolar wall thickness, cellular infiltration and haemorrhage were each rated from 0 (no injury) to 4 (maximal injury) for all individuals. Counts of each score were summed up, and the result was depicted as ALI score as described previously.

BAL cytokine measurements. BAL aliquots were analysed using interleukin-1β and macrophage inflammatory protein-2 ELISA kit (R&D Systems GmbH, Wiesbaden, Germany) according to the manufacturers’ instructions.

RNA preparation and semi-quantitative polymerase chain reaction (sqRT-PCR). RNA from lung tissue samples was extracted and purified as previously described. cDNA samples were synthesised from equal amounts of RNA using random hexamer reverse primers and a TaqMan Reverse Transcription kit (Applied Biosystems Inc., Foster City, USA). TaqMan PCR reactions were performed according to the manufacturers’ instructions. TaqMan Gene Expression Assays for CXCL2 (MIP-2; Mm00436450_m1), CXCR2 (Mm00438258_m1), and GAPDH (TaqMan Rodent GAPDH Control Reagent) were purchased from Applied Biosystems. The comparative CT (ÅΔCT) method to evaluate the expression profiles of the analysed samples was used.

Cell culture. Human umbilical vein endothelial cells (HUVEC, Pelobiotech, Planegg, Germany) were cultured in complete endothelial cell growth medium (Promocell GmbH, Heidelberg, Germany) supplemented with 10% fetal calf serum (FBS, Gibco, Life Technologies GmbH, Darmstadt, Germany) and 1% penicillin-streptomycin (Promocell). Hematopoietic progenitor Hoxb8 neutrophils were a generous gift from Prof. Häcker (Institute of Medical Microbiology and Hygiene, UMC, Freiburg, Germany). Cells were cultured in Opti-modified Eagle medium (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 30 µM 2-mercaptoethanol (Thermo Fischer, Paisley, UK), 1 µM estradiol (Sigma) and 1% stem cell factor. Stem cell factor was harvested from chinese hamster ovary cells (a generous gift from Prof. Häcker) as described earlier. Prior to the experiments, progenitor Hoxb8 neutrophils were cultured for four days in differentiating medium (estradiol free medium) All cells were grown and experiments were performed at standard growing conditions (37 °C, 5% CO₂, sufficient humidity). All neutrophil assays were performed in the presence of BFs.
Neutrophil cytokine measurement. Differentiated neutrophils were seeded in 24-well plates. As controls, cells were incubated in the absence (control) or presence of 1 μM GYY4137 (control + GYY) for 4h. In addition, cells were incubated with 100 ng/ml LPS in the absence (LPS) or presence of 1 μM GYY4137 (LPS + GYY) for 4h. Neutrophil cell culture supernatants were analysed using MIP-2 ELISA kit (R&D Systems GmbH) according to the manufacturers’ instructions.

Neutrophil detection of reactive oxygen species. Differentiated neutrophils were seeded in 24-well plates. As controls, cells were incubated in the absence (control) or presence of 1 μM GYY4137 (control + GYY) for 4h. In addition, cells were incubated with 100 ng/ml LPS in the absence (LPS) or presence of 1 μM GYY4137 (LPS + GYY) for 4h. Subsequently, neutrophils were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DHDHF-DA, Sigma) in order to detect reactive oxygen species as previously described54. Fluorescence was measured with Tecan infinite 2000 (Thermo Fisher).

Statistical analysis. In vivo experiments were performed with n = 8 mice per group. Power calculations were performed prior to the study in order to define group sizes. Cell culture experiments were performed from at least three subsequent cell passages with n = 3 per group. Graphs represent means ± standard error of means (SEM) and were created with SigmaPlot 11.0 software (Systat Software Inc., Erkrath, Germany). In Figs 2a,b and 4b, data were depicted as fold induction compared to untreated controls. Data were further analysed for normal variation prior to one way analysis of variance (ANOVA) followed by the Tukey’s post hoc test. P < 0.05 was considered significant. All calculations were performed with GraphPad Prism 7.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Data Availability
All data generated or analysed during this study are included in this published article (and its Supplementary Information file).

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Acknowledgements
The authors thank Prof. Häcker (Institute of Medical Microbiology and Hygiene, UMC, Freiburg, Germany) for sharing hematopoietic progenitor Hoxb8 neutrophils, Chinese hamster ovary cells, and expertise on these cells. The article processing charge was funded by the German Research Foundation (DFG) and the University of Freiburg in the funding programme Open Access Publishing.

Author Contributions
S.F., A.H. and S.G.S. developed the concept and designed the study; F.H., A.G., M.-N.A.I., V.G. and S.G.S. performed the experiments; S.F., F.H. and S.G.S. analysed the data; S.F., F.H., A.G., M.-N.A.I., V.G., A.H. and S.G.S. interpreted the results of the experiments. S.F. and S.G.S. prepared the figures; S.F., A.H. and S.G.S. drafted, edited, and revised the manuscript. All authors approved the final version of the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-33101-x.

Competing Interests: The authors declare no competing interests.

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