Neutrophil Adhesion Is a Prerequisite for Antibody-Mediated Proteolytic Tissue Damage in Experimental Models of Epidermolysis Bullosa Acquisita

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Although uncontrolled proteolytic activity mediated by activated neutrophils is a major reason for tissue damage, therapeutic approaches using protease inhibitors are inefficient. Here, we investigated the role of the immune complex-induced neutrophil adhesion and protease release in tissue damage. We show both in vitro and in vivo that immune complex-mediated neutrophil adhesion to the target tissue depends on β2 integrins. Without affecting elastase or reactive oxygen species release, blocking of adhesion drastically inhibited tissue damage in an experimental model of autoantibody-mediated skin blistering disease. By using a cell-bound fluorescent resonance energy transfer-based elastase sensor, we detected elastase enzyme activity on the surface of adherent cells resistant to protease inhibitors. Inhibitor resistance was lost by CD18 blockade or deficiency in vitro and in vivo. Immune complex-induced neutrophil adhesion created an enclosed protected space between the cell and its target structure where proteinases and reactive oxygen species can execute their tissue-damaging effect. Because immune complex-induced neutrophil adhesion represents an indispensable step for tissue damage of many diseases, our findings may facilitate the development of strategies for the treatment of such disorders.


INTRODUCTION

Equipped with a plethora of toxic chemical weapons, neutrophils play an essential role as first-line effector cells in host defense against microbial invaders. However, because they lack specificity, these defense mechanisms always bear the risk for the host to be damaged by its own immune system. As a consequence, the range of action of mediators like reactive oxygen species (ROS) or proteolytic enzymes is limited and tightly controlled by a variety of scavenger and inhibitor molecules abundantly present in blood and tissues (Roche et al., 2008; Stoller and Aboussouan, 2005).

The fine balance in neutrophil activation between host defense and tissue protection can be disturbed under some pathological conditions. In many chronic disorders like pemphigoid autoimmune blistering diseases, chronic obstructive pulmonary disease, or cystic fibrosis, the release of ROS and aggressive proteinases, such as elastase, cathepsin G, or proteinase 3 by activated neutrophils represents the pathomechanism responsible for at least partly irreversible tissue damage and destruction (Hoenderdos and Condille, 2013; Liu et al., 2000; Voynow et al., 2008). Consequently, inhibition of proteinases as a therapeutic concept has been under broad investigation for more than 30 years. Despite this enormous scientific effort, only the elastase inhibitor Sivelestat has been approved for clinical use in Japan (Lucas et al., 2013). This limited success indicates that mechanisms are present that prevent the effectiveness of the antiproteinase approach. The release of enzymatically active proteinases appears to be in coincidence with neutrophil adhesion. This can be observed not only under pathological conditions but also during transmigration of cells through endothelial and epithelial barriers, which depends on the interaction between integrins and cellular adhesion molecules (ICAMs and VCAM) (Colom et al., 2015; Zen et al., 2011). Already more than three decades ago, in vitro experiments have shown that a closed compartment between the neutrophils and the target are essential for proteolysis (Campbell et al., 1982; Weiss and Regiani, 1984). In the so-called “closed compartment” hypothesis, phagocyte adhesion creates a secluded microenvironment that excludes proteinase inhibitors and allows a high local concentration of...
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CD18 is required for the IC-induced neutrophil adhesion in vivo

Although it has been shown by several lines of evidence that CD18 is required for IC-induced neutrophil adhesion in vivo in the process of tissue damage (Soriano et al., 1999; Tang et al., 1997; van Spriel et al., 2001), this has not been proven by direct in vivo imaging so far. To visualize and confirm the role of CD18 for the adhesion of IC-activated neutrophils in vivo, cells were labeled with cell tracker and injected intradermally into mouse ear skin. Two hours later, laser-induced focal tissue damage was set on the ear, and the movement of the injected neutrophils toward the laser damage was monitored by two-photon microscopy. As expected, neutrophils derived from wild-type mice were recruited toward the focal damage (see Supplementary Movie S3a online). To quantify the behavior of neutrophils, the distance to the damage of all labeled neutrophils in the recorded three-dimensional stack was determined at different time points. This mean distance is influenced by cells that move toward the damage, thereby reducing the mean distance over time and other cells entering the three-dimensional stack. Depending on their entry point, cells initially can have a large distance to the focal damage and thereby initially increase the mean distance of all cells before migrating to the damage.

As expected, in the presence of irrelevant antibody of the same isotype, the mean distance of all cells was reduced over time, despite new cells entering the imaged volume (Figure 2a). In the presence of anti-mCOL7 IgG bound to the dermal-epidermal junction, neutrophils were inhibited in their migration to the focal damage (Figure 2a, and see Supplementary Movie S4a online). Because neutrophils entering the examined volume were also arrested once in contact with the anti-mCOL7 IgG-antigen complex, an increase of the mean distance of all neutrophils present in the three-dimensional stack was observed. This shows that the decrease by approximately 50% compared with controls of IC-induced neutrophil adherence was observed in the presence of anti-CD18 (Figure 1b and c). A comparable effect was seen when murine neutrophils were used and β₂-integrins were blocked with an anti-murine CD18 antibody, indicating no principal difference between both species with regard to this function (see Supplementary Figure S2 online). In the next step, we investigated the effect of the anti-CD18–blocking antibody on IC-induced neutrophil ROS production and elastase release, which represent essential pathomechanisms in experimental models of EBA (Shimanovich et al., 2004) and bullous pemphigoid (Liu et al., 2000). After exposure to IC, neutrophils immediately started to generate ROS, which reached a peak after 10 minutes and returned to background levels after 60 minutes (see Supplementary Figure S3 online). This process was not affected by the blockage of CD18 (Figure 1d, and see Supplementary Figure S3). Furthermore, determination of the elastase enzyme activity in the same supernatants showed that blocking CD18 resulted not in a reduced but in a slightly enhanced IC-induced release of elastase (Figure 1e). Taken together, these results suggest that blocking CD18 specifically inhibits the IC-induced neutrophil adhesion but does not affect ROS production or elastase release in these cells.

RESULTS

Blocking CD18 specifically inhibits IC-induced neutrophil adhesion in vitro

Previously, we and others observed that neutrophils adhere to surfaces coated with immobilized ICs (Tang et al., 1997; Yu et al., 2010). Time kinetics of this process, analyzed by microscope, showed that neutrophils adhered to the surface immediately after contact to the immobilized IC. Adhesion reached a peak at approximately 30 minutes, followed by a process of de-adhesion that lasted for more than 2 hours (see Supplementary Figure S1a and Supplementary Movie S1 online). Neutrophils exposed to uncoated surfaces did not show any significant morphological changes (see Supplementary Figure S1a and Supplementary Movie S2 online). These findings were confirmed by real-time impedance measurement, whereby increased cell adhesion directly correlates with an increase in the electrical impedance (expressed as cell index) of the cell layer (Scrace et al., 2013) (see Supplementary Figure S1b and c).

Given the importance of β₂-integrins in neutrophil adhesion (Arnaout, 2016), we first addressed the question of whether blocking CD18, the β subunit of β₂-integrins, could affect IC-mediated neutrophil adhesion. As expected, inhibition of CD18 by blocking antibodies reduced the IC-induced adhesion-related morphological changes of the cells dramatically (Figure 1a). This effect could be confirmed by real-time impedance measurement, whereby a significant effect of neutrophil activation are ill defined, in epidermolysis bullosa acquisita (EBA), a well-investigated, rare autoantibody-mediated skin blistering disease, neutrophil activation is directly associated with the presence of autoantibodies against type VII collagen (COL7) (Schmidt and Zillikens, 2013; Woodley et al., 1988). Furthermore, binding to Fcγ-receptors and releasing granule constituents and ROS have been identified here as essential components in neutrophil-mediated skin blistering in patients and disease models, making EBA a prototype of neutrophil-driven diseases (Chiriac et al., 2007; Mihai et al., 2007; Sitaru et al., 2005). In this study, we aimed to investigate whether neutrophil adhesion to the basement membrane zone is an essential step for tissue damage in experimental models of EBA. Using a previously established assay system in which neutrophils are activated by immobilized immune complexes (ICs) in vitro (Yu et al., 2010) and an ex vivo experimental model of inflammatory EBA, we investigated the functional role of IC-induced neutrophil adhesion in the attack and destruction of host tissue. We show that IC-induced neutrophil adhesion is an indispensable prerequisite in the process of tissue damage. Furthermore, we provide a mechanism for how neutrophil proteases escape from regulation by protease inhibitors and offer a therapeutic approach to how control of protease activity can be accomplished under pathophysiological conditions.

In contrast to many other chronic disorders where effector molecules (Campbell and Campbell, 1988; Wright and Silverstein, 1984). This closed compartment hypothesis has been used to explain cytotoxic and anti-tumor functions of neutrophils (Ottonello et al., 1999; Soriano et al., 1999; Tang et al., 1997; van Spriel et al., 2001).

Supplementary Figure S1a and Supplementary Movie S2 online). Because neutrophils entering the examined volume were also arrested once in contact with the anti-mCOL7 IgG-antigen complex, an increase of the mean distance of all neutrophils present in the three-dimensional stack was observed. This shows that the
in vivo anti-mCOL7—mCOL7 IC can induce neutrophil adhesion to the dermal-epidermal junction. In the next step, this experimental setting was used to investigate the role of CD18 in IC-induced neutrophil adhesion. Comparable to cells derived from wild-type mice, $Cd18^{-/-}$ neutrophils were recruited toward the site of focal damage (Figure 2b, and see Supplementary Movie S3b), indicating no general impairment of migration in these cells. However, in contrast to wild-type neutrophils, $Cd18^{-/-}$ cells were recruited to the site of the tissue lesion also when anti-mCOL7—antigen complexes were present (Figure 2b, and see Supplementary Movie S4b), showing that CD18 deficiency compromises the IC-induced neutrophil adhesion. In summary, these results show that CD18 is required for the IC-induced neutrophil adhesion in vivo.

Inhibition of $\beta_2$-integrins prevents anti-COL7 IgG-mediated tissue damage ex vivo

The facts that CD18 is required for the IC-induced neutrophil adhesion in vitro and in vivo and that CD18 blockade can specifically inhibit the IC-induced neutrophil adhesion provided a tool to investigate the pathophysiological role of this function in an experimental model of EBA. Hence, we used a modified ex vivo model of the disease where mouse skin section were sensitized with anti-mCOL7 IgG and subsequently incubated with human neutrophils (Yu et al., 2014). In this model, neutrophils activated by the mCOL7—anti-mCOL7 IC caused dermal-epidermal separation of $29.2 \pm 11.6\%$, and no separation was observed in the control (Figure 3a, b, and d). In the presence of anti-CD18 blocking antibody, skin separation was dramatically reduced to $0.8 \pm 2.0\%$ (Figure 3c and d). This result suggests that IC-induced neutrophil adhesion is indispensable for tissue damage in this ex vivo model.

IC-induced neutrophil adhesion prevents elastase inhibition in vitro

Our result gave rise to the question of how reduced adhesion could result in attenuated tissue damage although release of elastase and ROS were unaltered. To prevent the host from proteolytic tissue damage, potentially harmful proteinases such as neutrophil elastase are under tight control.

![Figure 1. Effect of anti-human CD18 blocking antibody on IC-induced neutrophil activation in vitro.](image-url)
control of ubiquitously present proteinase inhibitors such as A1AT. We hypothesized that the IC-induced neutrophil adhesion creates a closed space between cell and surface where macromolecular inhibitors have no or limited access. To verify this, we determined elastase activity on single cell level by using the cell surface-bound fluorescent resonance energy transfer (FRET)-based elastase sensor NEmo-2 (Cobos-Correa et al., 2009; Gehrig et al., 2012). Proteinase activity is determined by the loss of FRET caused by cleavage of the peptide substrate connecting donor and acceptor fluorophore and is expressed as the ratio of donor to acceptor fluorescence intensities (D/A).

Figure 2. Effect of CD18 on the neutrophil adhesion and movement in vivo. Bone marrow-derived neutrophils from wild-type and CD18-deficient neutrophils were labeled with cell tracker or NEmo-2 sensor and injected intradermally in presence of amCOLVII or isotype antibodies into the ventral ear skin 2 hours before laser-induced focal tissue damage was set. Neutrophil recruitment toward local damage was recorded by 2P-IVM (TriM Scope II [LaVision BioTec, Bielefeld, Germany] equipped with an XL Plan N25 X 1.05 W MP objective [Olympus, Hamburg, Germany]) over a 20-minute time lapse. (a) Distance of wild-type neutrophils and (b) Cd18−/− neutrophils to the injured site were normalized to the starting distance value and plotted over time. Data of three independent experiments per group with at least three ear samples were quantified and compared among these groups. Data are presented as mean ± standard error of the mean. Statistically significant differences were compared between groups using unpaired t test analysis. COLVII, collagen VII; min, minute.

Figure 3. Anti-CD18 blocking antibody reduces tissue damage in an ex vivo model of epidermolysis bullosa acquisita. Mouse skin cryosections were incubated with 0.2 mg/ml (a) rabbit control IgG or (b, c) rabbit-anti-mCOL7 IgG for 1 hour at 37°C. Subsequently, specimens were exposed to (a) freshly isolated human neutrophils or (b) neutrophils in the presence of 10 μg/ml anti-CD18 IgG or (c) 10 μg/ml isotype IgG. Images of the skin section were acquired using NIS-Element D 3.0 software (Nikon, Tokyo, Japan) and an Olympus (Tokyo, Japan) Bx41 microscope with a 20×/0.50 objective (corresponding to ×200 magnification). Sections of a representative experiment are shown. Scale bar = 100 μm. Arrows indicate the dermal-epidermal separation. (d) Furthermore, skin separation was quantified as percentage of the length of epidermis detachment in relation to the length of the total dermal-epidermal zone. Data are presented as mean ± standard deviation of six independent experiments. Statistically significant differences were indicated. IC, immune complex.
As shown in Figure 5a and b, 2 hours after the injection, the isotype control, were injected intradermally into the ear skin. Neutrophils, together with A1AT and anti-mCOL7 IgG or its eactivity. By contrast, no such increase could be induced IC-mediated activation induces extracellular elastase enzyme activity. Furthermore, CD18-mediated adhesion is essential to enable extracellular protease activity in vivo.

**DISCUSSION**

Neutrophil-mediated tissue damage as an adverse effect of an acute or chronic inflammatory process represents one of the most dangerous threats of the host by its own immune system. Based on the theory of a dysregulated balance between proteases and anti-proteases, which was originally described in lung emphysema (reviewed in Janoff, 1985, and Suki et al., 2003), the therapeutic application of inhibitory molecules capable of reducing protease activity in vivo has been under broad investigation. However, small and often membrane permeable inhibitors exerted frequently substantial toxic effects, and more physiological macromolecular inhibitors were characterized by a low efficiency (Lucas et al., 2013). Enhancement of the efficiency of nontoxic biological inhibitors could be a promising future strategy in the treatment of protease-mediated tissue damage.

A fundamental role for neutrophil elastase in the pathogenesis of autoantibody-mediated skin tissue damage has been described by Liu et al. (2000) using elastase-deficient mice in a local antibody-transfer model of bullous pemphigoid. In our study, we have characterized and investigated the pathophysiological role of IC-induced neutrophil adhesion using experimental models of EBA. We showed in vitro and in vivo that IC-induced neutrophil adhesion is dependent on CD18. Moreover, we showed that inhibition of adhesion prevents tissue damage. These data clearly show that IC-induced neutrophil adhesion represents an essential step for the tissue damage in experimental models of EBA.

In the closed compartment hypothesis, neutrophil adhesion to the target creates a closed space that excludes...
proteinase inhibitors and allows proteinase (e.g., neutrophil elastase) to cause tissue damage. Previously, Campbell and Campbell (1988) showed that the closed compartment is able to exclude A1AT. By the use of a membrane-bound protease sensor, we here provide direct evidence in vitro and in vivo that IC-induced neutrophil adhesion is required for elastase to escape from the access of proteinase inhibitors, supporting this hypothesis.

After recruitment into the skin, neutrophils are activated by immobilized anti-COL7/COL7 IC via FcγR, which initiates a tight adherence to the dermal-epidermal junction and forms a closed space preventing a molecular exchange with the environment (Figure 6). Neutrophil products such as ROS and proteases accumulate to high concentrations in this space and overrun the control of residual inhibitors (Naskalski et al., 2002). As a consequence, uncontrolled protease activity inaccessible to exogenous inhibitors will attack and destroy the structurally important proteins of the dermal-epidermal junction zone.

CD18 is the common β-2 subunit of four different β2 integrins, including lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18), macrophage-1 (Mac-1; CD11b/CD18), integrin αXβ2 (CD11c/CD18), and integrin DJβ2 (CD11d/CD18) (Amaout, 2016). Under physiological conditions, circulating neutrophils express LFA-1, Mac-1, and CD11d/CD18 (Amaout, 2016; Miyazaki et al., 2014). Given the essential role of CD18 in IC-mediated neutrophil adhesion and consequent tissue damage, it is interesting to know

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**Figure 5. Effect of CD18 on elastase enzyme activity on IC-activated neutrophils in the presence of A1AT in vivo.** NEmo-2–labeled WT and CD18-deficient neutrophils were combined with anti-mCOLVII antibody or an irrelevant antibody of the same isotype and injected in presence of A1AT into the intact ear dermis of anesthetized mice. Neutrophil elastase activity on the surface of injected neutrophils in mice ears was quantified 2 hours after cell injection by 2P-IVM (TriM Scope II [LaVision BioTec, Thebarton, Australia] equipped with an XPLanN 25×1.05 WMP objective), and fluorescence intensities of the donor and acceptor as well as the ratio of the intensities of D/A are shown. (a) Images of donor and acceptor fluorescence were processed to calculate D/A ratio values. Look-up table: black indicates low D/A ratio, intact NEmo-2; white indicates high D/A ratio, indicative of cleaved NEmo-2. Scale bar = 50 μm. (b, c) The average NE activity of each experiment in the presence of amCOLVII was quantified by normalization of D/A ratio value of WT and Cd18−/− to the respective isotype controls. Statistically significant differences were compared between groups using unpaired t test analysis. Observations indicated a significantly increased number of activated neutrophils compared with unstimulated cells. Results of one representative experiment out of three to eight independent experiments (ear samples) are shown. COLVII, collagen VII; D/A, donor to acceptor fluorescence intensities; IC, immune complex; Norm, normalized; ns, not significant; WT, wild type.
which β2 integrins are involved in this process. Because CD11b-deficient mice are susceptible to an antibody transfer-induced experimental model of EBA (Deng et al., 2017), Mac-1 appears to be dispensable for IC-induced neutrophil adhesion, but the roles of LFA-1 and CD11d/CD18 need to be elucidated.

With regard to potential counter-ligands for β2 integrins involved, integrins can be activated in both a ligand-dependent and -independent manner (Petridou and Skourides, 2016). Because our in vitro and ex vivo models were carried out under serum-free conditions and, therefore, in the absence of complement components, a role of the complement system can be excluded here. However, although IC-induced adhesion to plastic surfaces is clearly ligand independent in our in vitro model, this remains unclear in our ex vivo and in vivo approaches. It is likely that β2 integrin is activated via an inside-out signaling of integrins (Das et al., 2014) initiated by interaction of IC with FcγRs. Treatment with anti-human CD18 blocking antibody reduced only partially IC-induced neutrophil adhesion in vitro but mediated a complete abrogation of the tissue damage in the EBA ex vivo model. This may indicate that already a limited disturbance of the closed space could be sufficient to allow the entry of inhibitory molecules.

In addition to their role in IC-induced neutrophil adhesion, β2 integrins are also essentially involved in the adhesion processes, which enables the emigration of these cells from the vasculature into the inflamed skin. However, although both processes are dependent on CD18, they differ from each other significantly. During emigration from blood vessels, neutrophils interact with endothelial cells through binding of LFA-1 and Mac-1, enabling stable adherence, crawling, and transmigration (Henderson et al., 2001; Lu et al., 1997; Phillipson et al., 2006). However, in contrast to IC-induced neutrophil activation, β2-integrin-mediated processes depend here strictly on the binding to the corresponding counter-ligands ICAM-1 and ICAM-2 (Kolaczkowska and Kubes, 2013). Targeting specific unwanted immune functions to avoid systemic immunosuppression is a major goal in the development of treatment strategies. Therefore, our finding that β2-integrins represent central players in the IC-induced disruption of the target tissue might open a therapeutic avenue for EBA and related diseases. Specific inhibition of IC-induced neutrophil adhesion will not affect the recruitment of these cells from circulation to tissues but will be able to block antibody-mediated tissue damage. Furthermore, blocking IC-induced neutrophil adhesion will not affect neutrophil ROS generation and elastase release involved in host defense against microbial invaders. In addition to autoimmune bullous disorders, interference in neutrophil adhesion could also have a beneficial effect on other diseases, such as cystic fibrosis or chronic obstructive pulmonary disease, in which uncontrolled elastase activity is a major pathomechanism (Hoenderdos and Condliffe, 2013; Voynow et al., 2008). Treatment of these lung diseases with macromolecular proteinase inhibitors like A1AT are hampered by a rather low efficiency of these agents (Griese et al., 2008). Breaking the closed space of adherent neutrophils could facilitate the access of intrinsic and therapeutic inhibitors at the site of tissue damage.

In conclusion, our study shows an essential role of IC-induced neutrophil adhesion in autoantibody-mediated tissue damage. These results help explain the pathophysiological functions of neutrophils in the effector phase of neutrophil-mediated disorders and enable us to open a previous unseen window for their treatment.

**MATERIALS AND METHODS**

**Mice**

Female Cd18−/− mice (B6.129S7-Itgb2tm2Bay/J) were kindly provided by Karin Scharffetter-Kochanek, University of Ulm, Germany. Female C57BL/6J mice were bred at the animal facilities of the Research Center Borstel under specific pathogen-free conditions. Eight- to 14-week-old mice were used for the experiments. All
animal studies comply with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and have been carried out according to European Union Directive 2010/63/EU. All studies have been reviewed and approved by the Animal Research Ethics Board of the Ministry of Environment, Kiel, Germany.

**Antibodies**
Pathogenic rabbit anti-mCOL7 IgG was generated as previously described (Sitaru et al., 2005). Azide-free anti-human CD18 IgG (clone: TS/18) and the corresponding mouse IgG1 isotype control (clone: MOPC-21) were obtained from BioLegend (San Diego, CA). Azide-free anti-murine CD18 IgG (clone: GAME-46) and the corresponding rat isotype control (clone: R3-34) were obtained from BD Bioscience (Heidelberg, Germany).

**Neutrophil isolation and differentiation**
Human neutrophils were isolated from citrated blood of healthy donors as described (Kasper et al., 2004), whereas murine neutrophils were differentiated in vitro from bone marrow cells (described in detail in the Supplementary Materials and Methods online).

**Activation of neutrophils in vitro**
Activation of neutrophils in vitro by immobilized IC was performed as described previously, with modification (Yu et al., 2010). Generation of ROS and release of neutrophil elastase was determined by measurement of chemiluminescence and elastase enzyme activity in cell-free supernatants, respectively. Neutrophil adhesion was monitored by phase contrast microscopy quantified by real-time impedance measurement by using the xCELLigence system (Roche, Penzberg, Germany) (Scrace et al., 2013). The relative electrical impedance is expressed as cell index in arbitrary units.

**Dermal-epidermal separation ex vivo**
Skin dermal-epidermal separation was evaluated using an ex vivo model performed with mouse skin cryosections, rabbit anti-mCOL7 IgG, and human neutrophils as described in a previous study (Yu et al., 2014). Sections were washed in phosphate buffered saline, fixed with acetone acid, and subsequently stained with hematoxylin and eosin. Skin dermal-epidermal separation was evaluated by light microscopy, and the extent of dermal-epidermal separation was analyzed in a blinded fashion.

**Histology**
Hematoxylin and eosin staining was performed using paraffin sections. Deposition of rabbit IgG and C3 were detected on frozen sections of skin specimens by immunofluorescence staining using polyclonal DyLight649-conjugated donkey anti-rabbit IgG (Dianova, Hamburg, Germany) for detection of rabbit IgG and a rat anti-murine C3 antibody IgG2a; clone RMC11H9; Biozol, Eching, Germany), followed by staining with an Alexa Fluor 488-conjugated polyclonal goat anti-rat IgG antiseraum (Invitrogen, Darmstadt, Germany) for detection of complement component C3.

**Determination of elastase enzyme activity by FRET sensors**
Synthesis and purification of the FRET reporter NEmo-2, specific for neutrophil elastase activity and determination of elastase activity by cleavage of the cell-bound proteinase substrate, was performed as described previously (Gehrig et al., 2012). Briefly, 4 x 10^5 murine neutrophils were suspended in 100 µl chemiluminescence (CL) medium supplemented with 100 µmol/L A1AT (Sigma Aldrich, Hamburg, Germany), followed by incubation with rat anti-mouse CD18 or rat IgG1 isotype control (10 µg/ml) for 5 minutes at room temperature. After addition of NEmo-2 (2 µmol/L), 100 µl CL-medium was added to each aliquot, and the cell solution was spread on ibiTreat microscopy chamber slides (Ibidi Bio-Diagnostics, Munich, Germany). Cells were imaged by confocal microscopy as described in the Supplementary Materials and Methods.

**In vivo imaging of neutrophils and elastase activity**
Migration and elastase activity were determined in neutrophils adoptively transferred into mouse ears by two-photon microscopy. A detailed description of labeling, cell transfer, and microscopic techniques is presented in the Supplementary Materials and Methods.

**Data analysis**
Confocal microscopy images were further processed by ImageJ 1.38r software (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, MD) using background subtraction, smoothing with a median filter, and thresholding, and Coumarin 343/S-Carbocyanetetramethylrhodamine (TAMRA) (DA) ratio images were calculated.

Image processing of intravital imaging was performed using Imaris Software (Bitplane, Zurich, Switzerland) using background subtraction, smoothing with a median filter, thresholding, and calculation of DA ratio images. Cell movements over 20 minutes were compared using image volumes of 200 x 200 x 20 µm. Neutrophil migration was tracked by providing three-dimensional cell spatial coordinates (x, y, z) over time. Data were further analyzed using open source programming language R, as previously described (Lämmermann et al., 2013), to determine dynamic parameters for individual cells (distance-time plot). Plots including regression lines were generated using the ggplot2 in RStudio, version 0.99.902, for R, version 3.3.0. Source codes were kindly provided by Tim Lämmermann and Bastian R. Angermann.

Data are presented as mean ± standard deviation or mean ± standard error of the mean for the number of samples indicated in the figure or figure legends. Sample sizes in animal experiments were estimated on an empirical basis obtained from data of comparable studies. Statistically significant (P < 0.05) differences among the groups were calculated using one-way analysis of variance test or t test using GraphPad (La Jolla, CA) Prism software.

**CONFLICT OF INTEREST**
The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**
XY, RA, MP, SG, and CS performed experiments; XY, RA, MP, PK, TS, and FP analyzed results, made the figures, and designed the research; and XY, DZ, and FP wrote the manuscript.

**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.03.1499.

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