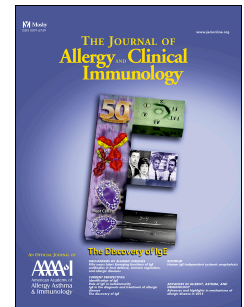


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Adhesion-induced eosinophil cytolysis requires the RIPK3-MLKL signaling pathway which is counter-regulated by autophagy

Running title: Mechanism of adhesion-induced eosinophil cytolysis

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Abbreviations used

cfegs: clusters of free eosinophil granules

CR3: complement receptor 3

DPI: diphenyleneiodonium-chloride

ECP: eosinophil cationic protein

EPX: eosinophil peroxidase

HES: hypereosinophilic syndromes

iC3b: inactive complement component 3b

IVIG: intravenous immunoglobulins

mAb: monoclonal antibody

MAPK: mitogen-activated protein kinase

MLKL: mixed lineage kinase-like

mTOR: mechanistic target of rapamycin

Nec-1: necrostatin-1

RIPK: receptor-interacting protein kinase

ROS: reactive oxygen species

TEM: transmission electron microscopy

Abstract

Background: Eosinophils are a subset of granulocytes which can be involved in the pathogenesis of different diseases, including allergy. Their effector functions are closely linked to their cytotoxic granule proteins. The release takes place by several different mechanisms, one of which is cytolysis, which is associated with the release of intact granules, so-called clusters of free eosinophil granules. The mechanism underlying this activation-induced form of cell death in eosinophils has remained unclear.

Objective: We aimed to elucidate the molecular mechanism of eosinophil cytolysis.

Methods: Isolated blood eosinophils were incubated on glass cover slips coated with intravenous immunoglobulin (IVIg) and inactive complement component 3b (iC3b). A morphological characterization of the distinct stages of the proposed cascade was addressed by means of time-lapse automated fluorescence microscopy, electron microscopy, and immunohistochemistry. Experiments with pharmacological inhibitors were performed to elucidate the sequence of events within the cascade. Tissue samples of patients suffering from eosinophilic skin diseases or eosinophilic esophagitis were used for *in vivo* analyses.

Results: Following eosinophil adhesion, we observed reactive oxygen species (ROS) production, early degranulation, and granule fusion processes leading to a distinct morphology exhibiting cytoplasmic vacuolization and, finally, to cytolysis. Using a pharmacological approach, we demonstrate the presence of a receptor-interacting protein kinase 3 (RIPK3) – mixed lineage kinase-like (MLKL) signaling pathway in eosinophils, which, following its activation, leads to the production of high levels of reactive oxygen species (ROS) in a p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K) - dependent manner. All these steps are required for cytoplasmic vacuolization and subsequent cytolysis to occur. Interestingly, triggering cytolysis is associated with an induction of autophagy in eosinophils and additional stimulation of autophagy by pharmacological inhibition of the mechanistic target of rapamycin (mTOR) counter-regulates cell death. Moreover, MLKL phosphorylation, cytoplasmic vacuolization, and cytolysis were observed in eosinophils under *in vivo* inflammatory conditions.

Conclusion: We report that adhesion-induced eosinophil cytolysis takes place by a RIPK3-MLKL-dependent necroptosis which can be counter-regulated by autophagy.

Key words: Autophagy, eosinophil cytolysis, eosinophilic disease, necrosis, necroptosis, non-apoptotic cell death, MLKL, RIP kinase, ROS.

Capsule Summary

The receptor-interacting protein kinase 3 (RIPK3) - mixed lineage kinase-like (MLKL) signaling pathway mediates eosinophil cytolysis, a phenomenon often observed in eosinophilic tissues of patients suffering from diseases with eosinophil-mediated immunopathology.

Eosinophils are a subset of granulocytes found in increased numbers in both inflamed tissues and peripheral blood under different pathological conditions, such as allergy, parasitic infections and autoimmune and neoplastic diseases.¹ Eosinophils contain granules, made up of highly cytotoxic granule proteins that have been linked to destruction of too-large-to-phagocytose pathogens such as helminthes. Early studies highlighted the potential of eosinophils to lyse IgG-and/or complement-coated *Schistosoma mansoni* *in vitro*,² whereby the presence of complement enhanced the eosinophil-mediated cytotoxicity.³ Addition of the calcium ionophore A23187 to eosinophils also induced stable adhesion and degranulation, resulting in damage to *Schistosomula*.² Transmission electron microscopy (TEM) revealed granule release through large cytoplasmic vacuoles upon treatment of eosinophils with A23187.⁴ Cytolysis, a non-apoptotic form of eosinophil death,⁵ was also described as the mechanism underlying A23187-induced degranulation.⁶ Whether eosinophil vacuolization and cytolysis are functionally linked or separate and independent degranulation processes has remained unknown.

Cytolysis is characterized by cytoplasmic membrane disintegration⁶ and the release of intact granules which accumulate as clusters of free eosinophil granules (cfegs) in the extracellular space.⁷ The release of free granules correlated with epithelial cell damage upon allergen challenge in guinea pig models of asthma.⁸ Moreover, the presence of cfegs, eosinophil vacuolization, and cytolysis has been linked to the severity of asthma exacerbation in children.⁹ These findings support the hypothesis of a crucial role of eosinophil vacuolization and cytolysis for eosinophil-driven diseases.

Although the presence of cytolytic eosinophils and cfegs in eosinophilic tissues has been described under various *in vivo* conditions,^{10,11} the mechanisms leading to cell death and granule release are still largely unknown. Recently, it has been reported that eosinophil cytolysis is mediated by an NADPH oxidase-dependent and caspase-independent cell death characterized by extracellular DNA trap formation (ETosis).¹² In the present study, we have investigated morphological changes and biochemical pathways leading to eosinophil cytolysis and propose that it is a form of regulated necrosis, most likely necroptosis,¹³ since the death process depends on receptor-interacting protein kinase 3 (RIPK3) and mixed lineage kinase-like (MLKL) protein. Interestingly, prior pharmacological activation of autophagy counter-regulates eosinophil cytolysis. Thus, inhibiting the RIPK3-MLKL pathway and stimulating autophagy in eosinophils represent two new ideas as therapeutic strategies for preventing eosinophil-mediated tissue damage.

METHODS

Cells

Human blood eosinophils from healthy individuals were isolated as described previously.¹⁴ Briefly, cell separation was performed by Ficoll-Hypaque centrifugation (Seromed-Fakola AG, Basel, Switzerland). The granulocyte fraction was depleted of erythrocytes by lysis with a buffer containing NH_4Cl , KHCO_3 and EDTA. Eosinophil separation from neutrophils was achieved by CD16-negative selection using the human eosinophil enrichment kit (Miltenyi Biotec (Bergisch-Gladbach, Germany)). The morphology of eosinophils was assessed by staining with Diff-Quik (Baxter, Düringen, Switzerland) and light microscopic analysis. The purity of the isolated human eosinophil populations was always >95%. Eosinophil morphology was also analyzed in Diff-Quik - stained blood smears, obtained from 20 healthy donors and 20 HES patients. Written, informed consent was obtained from all blood donors, and the Ethics Committee of the Canton Bern had previously approved the study.

Cell cultures

Eosinophils were cultured at 1×10^6 per ml in culture medium (RPMI 1640 plus GlutaMAX (Invitrogen) supplemented with penicillin/streptomycin and different concentrations of heat-inactivated FCS). Two systems were used to induce cytolysis: In initial experiments, we cultured eosinophils for indicated times on naked glass coverslips in culture medium containing 0.5% FCS. In most experiments, however, we specifically stimulated eosinophils with IVIG + iC3b in culture medium containing 5% FCS. In some experiments, eosinophils were additionally stimulated with 25 ng/ml IL-5. Briefly, glass coverslips were coated with 10 mg/ml IVIG overnight at 4°C. Following three washing steps, 10 µg/ml iC3b was added at 37°C for 1 h. Upon further washing steps, the reaction was stopped with addition of 1% BSA to each well. As control conditions, single-coated (10 mg/ml IVIG or 10 µg/ml iC3b) and 1% BSA-coated coverslips served. Freshly isolated blood eosinophils were hereupon cultured on coated coverslips in culture medium containing 5% FCS at 37°C for indicated times in a humidified atmosphere containing 5% CO_2 . Cells were then morphologically examined by staining with Diff-Quik and light microscopic analysis (Axiovert 35, Carl Zeiss Micro Imaging, Jena, Germany). Alternatively, we stained cells with 0.4 µM calcein-AM, 1 µg/ml Hoechst 33342, 1 µM DHR 123, and 0.1 µM ethidium homodimer-1 (EthD-1) (Invitrogen) and followed cells using time-lapse automated fluorescence microscopy (LSM 510 Exciter, Carl Zeiss Microimaging). For mechanistic studies, we cultured eosinophils in the presence of 5 µg/ml anti-CD18 F(ab')_2 fragments as well as 100 nM wortmannin, 10 µM PD169316, 1 µM DPI, 10 µM Nec-1, 20 µM z-VAD, 50 µM GSK'843, 10 µM GSK'872, 5 µM GW806742X, and 5 µM rapamycin. For Nec-1, GSK'843, GSK'872, and

GW806742X, we performed concentration-dependent experiments to determine the optimal inhibitory concentration. Pre-incubations with antibodies and pharmacological inhibitors, respectively, were performed for 30 min. To test for TNF- α -mediated effects, anti-TNFR1 (10 μ g/ml), anti-TNFR2 (10 μ g/ml), and anti-TNF- α (5 μ g/ml) mAbs were added 90 min before IVIG + iC3b activation.

Confocal laser scanning microscopy

Eosinophils were fixed with 4% paraformaldehyde for 5 min, washed with PBS pH 7.4, and subsequently permeabilized using 0.05% saponin in PBS pH 7.4 for 5 min at room temperature (RT) and acetone for 10 min at -20°C. For analysis of phospho-MLKL expression, eosinophils were fixed with acetone only and staining was performed in the presence of 0.01% saponin. Nonspecific binding was prevented by pre-incubation of the adherent cells with a blocking buffer (containing human immunoglobulins, normal goat serum, 7.5% BSA) at RT for 1 h. Subsequently, indirect immunofluorescence staining was performed as previously described,¹⁵ using primary antibodies diluted in blocking solution (4°C, overnight) as well as subsequent appropriate FITC- or Cy3-conjugated secondary antibodies (RT, 1 h). PI, Hoechst 33342 (both for nuclear staining) or Celltracker red (for cellular staining) were added for an additional 10 min. Staining with cell permeable fluorescent dyes, such as Autodot, Syto 13, Celltracker red and MitoSox or with the cell membrane dye PKH67 were performed on live cells prior to fixation according to the corresponding manufacturer's instructions. As primary antibodies, anti-MLKL (phospho S358; 1:250), anti-ECP (5 μ g/ml), and anti-EPX mAbs (1 μ g/ml) were employed. As staining controls, mouse or rabbit isotype controls served, employed at the corresponding concentrations, and cells were stained with secondary antibody only. Staining analysis was performed using the confocal laser scanning microscopes LSM 510 and LSM 5 Exciter (Carl Zeiss Microimaging). To produce a high resolution stack for subsequent 3D reconstruction, z-sections were performed at the resolution limit. To determine the corresponding morphology within the process of cytolysis, transmitted light was used. The percentage of vacuolated eosinophils in eosinophil associated skin diseases was determined in a total area of 0.087 mm².

Eosinophil vacuolization and MLKL phosphorylation in tissues was also analyzed by indirect immunofluorescence following staining of the indicated eosinophilic tissues with anti-ECP (5 μ g/ml), anti-EPX (1 μ g/ml), and anti-MLKL mAbs (phospho S358; 1:250) as previously described.¹⁶⁻¹⁸

Oxidative burst measurements

Eosinophils (1×10^6 per ml in RPMI-1640 plus 5% FCS) were pretreated with the indicated pharmacological inhibitors and antibodies, respectively, in Eppendorf tubes for 20 min in a CO_2 incubator at 37°C . DHR 123 ($1 \mu\text{M}$) was added to each Eppendorf tube for the last 10 min of incubation. 100 μl of cell suspension was added to each well using black, glass-bottom 96-well plates (Greiner Bio-One GmbH), previously coated with IVIG + iC3b, and kept at 37°C . Fluorescent activity of the DHR 123 dye was immediately measured at excitation 502 nm and fluorescence emission intensity 530 nm, using a spectrofluorimeter (SpectraMax M2, Molecular Devices, Biberach an der Riß, Germany). The kinetics of superoxide production was time-dependently analyzed every 5 min, at 37°C up to 1 h. Relative DHR 123 fluorescence was obtained upon subtraction of the corresponding DHR 123 fluorescence of medium without cells at the corresponding time point and subsequent norming of the values to initially measured fluorescence. Cells added to 1% BSA-coated wells served as negative and 25 nM PMA stimulated cells as positive controls.¹⁹ ROS production in single eosinophils was analyzed by time-lapse automated fluorescence microscopy (see the description of additional methods in this article's Online Repository at www.jacionline.org).

Statistical analysis

Data were analyzed using Graph Pad Prism 5 software (La Jolla, CA, USA). Figures depict mean levels $\pm$ standard errors of the mean (SEM) and the numbers of independent experiments performed are provided in the Figure Legends (n). The one-way ANOVA followed by Tukey's multiple comparisons test or the Kruskal-Wallis test were applied. P-values of < 0.05 were considered as statistically significant.

RESULTS

Adhesion to IVIG + iC3b results in eosinophil cytolysis

To address morphological and molecular events in the process of eosinophil cytolysis, we first applied a nonspecific adhesion system in which the reduction of the FCS content in the medium of cultured eosinophils from 5 to 0.5% evoked vacuolization and, in approximately 20% of the cells, lysis with the dispersion of nuclear material within 1 h (Fig 1, A, upper left panel). Pre-treatment of the glass surface with poly-2-hydroxyethyl methacrylate (Poly-Hema), an anti-adhesive substratum,²⁰ prevented this cytolysis (Fig 1, A, upper right panel), suggesting that adhesion, not starvation, is

critical in the initiation of the death process. Pharmacological inhibition of caspases did not prevent eosinophil cytolysis (Fig 1, A, lower left panel).

Since the eosinophil-mediated lysis of *Schistosoma mansoni* was reported to be more efficient in the presence of complement,³ we thought to develop a more specific system for triggering eosinophil adhesion and subsequent cytolysis. Stimulation of eosinophils with IVIG + iC3b, the latter known to be a ligand for complement receptor 3 (CR3), significantly induced cell death within 1 h of adhesion (Fig 1, A, lower right panel). The type of cell death corresponded morphologically to that induced by adhesion to glass coverslips owing to reduced exposure to FCS (Fig 1, A, upper left panel). We subsequently quantified cell death using live cell imaging (Fig 1, B). Interestingly, the eosinophil death was accelerated in the presence of IL-5 (Fig 1, B, left panel). In contrast, IVIG alone, iC3b alone, or 1% BSA failed to induce cell death. Transmission electron microscopy (TEM) showed the hallmarks of cytolysis: loss of plasma membrane integrity and nuclear condensation accompanied by expulsion of intact single granules, resulting in the presence of cfebs (Fig 1, B, right panels).¹⁰ As expected, blocking of CD18 with F(ab')₂ fragments of an anti-CD18 antibody prevented cell spreading (Fig 1, C), development of vacuolization (Fig 1, D), and cell death (Fig 1, E), confirming that this form of cytolysis can be specifically initiated through CR3. It should be noted that blocking of CD18 also prevented eosinophil death induced by adhesion as a result of culturing the cells in medium with low FCS content (0.5% FCS) (data not shown). To use a specific death trigger, however, in all subsequent experiments, we induced eosinophil cytolysis with IVIG + iC3b in medium supplemented with 5% FCS.

Eosinophil vacuolization precedes cytolysis and occurs also under in vivo conditions

To investigate the morphological changes associated with eosinophil cytolysis in single cells in a time-dependent manner, we followed a total of 100 eosinophils from three independent healthy volunteers by live cell imaging over a time period of 2 h of continuous stimulation with IVIG + iC3b (see Fig E1 and associated Video E1 in this article's Online Repository at www.jacionline.org). Eosinophils were stained with calcein-AM, Hoechst 33342, and ethidium bromide. Within 15 min of stimulation, we observed vacuolization in 56% (+/- 9%) of the spread eosinophils, without, however, cells having become ethidium bromide positive. The remaining cells detached from the surface and remained viable over the entire observation period. Moreover, 98.1% (+/- 3.3%) of the vacuolated eosinophils finally underwent cytolysis within a time period of 2 h (see Fig E1 and associated Video E1 in this article's Online Repository at www.jacionline.org). These experiments emphasize an absolute requirement for adhesion and subsequent cell spreading for the induction of vacuolization and

cytolysis in eosinophils. Taken together, while spreading following adhesion is a reversible process, vacuolization, however, is an early morphological sign for subsequent cytolysis, at least in the setting of continuous eosinophil activation as it occurred under these *in vitro* conditions.

To obtain evidence for possible *in vivo* relevance of the findings described above, we analyzed morphological features of eosinophils from healthy donors and patients suffering from hypereosinophilic syndromes (HES).^{21,22} The HES patients showed no evidence of eosinophilic leukemia and mostly suffered from skin symptoms. The numbers of vacuolated eosinophils were much higher in HES patients compared to healthy donors, both in blood (see Fig E2, A, upper panels, in this article's Online Repository at www.jacionline.org) and following eosinophil isolation (Fig E2, A, lower panels, in this article's Online Repository at www.jacionline.org). Furthermore, in eosinophilic tissues, we observed not only vacuolated eosinophils (arrows), but also nuclear disintegration (empty arrowhead) and cfebs (rhombus) (see Fig E2, B, in this article's Online Repository at www.jacionline.org), suggesting that vacuolated eosinophils could proceed to cytolysis also under *in vivo* conditions. We have quantified the numbers of vacuolated eosinophils in a relatively large cohort of patients with different eosinophilic skin diseases. Vacuolization ranged between 0 and 44%. In contrast, eosinophils physiologically infiltrating the duodenum were only rarely vacuolated (see Fig E3 in this article's Online Repository at www.jacionline.org).

Considering the role of adhesion in the process of cytolysis and the observation of vacuolated and cytolytic eosinophils in eosinophilic tissues, we addressed the question of whether cytolysis might develop already within the bloodstream at sites of potential extravasation. IL-5 primed eosinophils were added to monolayers of IL-1 β -primed human dermal endothelial cells.²³ Morphological changes were followed using live microscopy over a time period of 1 h. Similar to IVIG + iC3b - activated eosinophils, we observed rapid adhesion, spreading, and vacuolization in a subpopulation of IL-5 – primed eosinophils (see Fig E2, C, in this article's Online Repository at www.jacionline.org). The majority of eosinophils, however, detached and remained viable under these *in vitro* conditions.

Eosinophil vacuolization results from fusion of granules and depends on increased production of reactive oxygen species (ROS)

Using TEM, we addressed the question of the origin of cytoplasmic vacuolization observed with live-cell imaging. Granules of unstimulated eosinophils are characterized by an electron-dense crystalline core surrounded by a less electron-dense matrix (see Fig E4, A, upper panels, g, in this article's Online Repository at www.jacionline.org). Eosinophil granules exhibited a prominent loss of

both matrix and core density within 5 min following adhesion (see Fig E4, A, upper panels, hg, in this article's Online Repository at www.jacionline.org). Sombbrero vesicles were found adjacent to these hypodense granules (see Fig E4, A, upper panels, sVES, in this article's Online Repository at www.jacionline.org), implying ongoing piecemeal degranulation (PMD).²⁴ Furthermore, the fusion of hypodense granules to large convolutes with beginning vacuolization (see Fig E4, A, upper panels, arrowheads, in this article's Online Repository at www.jacionline.org) and subsequent dissolution of the vacuole content were observed (see Fig E4, A, middle panels, in this article's Online Repository at www.jacionline.org). Vacuoles were surrounded by a single membrane, which, however, lost its integrity before nuclear or plasma membrane changes were detected (see Fig E4, A, lower panels, in this article's Online Repository at www.jacionline.org).

Using immunofluorescence, we detected eosinophil peroxidase (EPX) in beginning vacuoles upon 5-min adhesion, supporting the assumption that they originate from granules (see Fig E4, B, in this article's Online Repository at www.jacionline.org). TEM images did not allow a conclusive answer to the question of whether degranulation occurred out of or into the vacuoles. We addressed this question using immunofluorescence and observed that the margins of vacuoles could be stained by a cell membrane dye (see Fig E4, C, in this article's Online Repository at www.jacionline.org), suggesting that the vacuoles might represent intracellular degranulation tunnels with access to the cell surface. We also noticed that eosinophils release DNA within 5 min after IVIG + iC3b - mediated adhesion. The released DNA could be stained with Mitosox (stains DNA in the presence of reactive oxygen species (ROS), e.g. mitochondrial DNA), suggesting that such stimulation results in the generation of extracellular DNA traps in which the DNA scaffold contains mitochondrial DNA (see Fig E4, D, in this article's Online Repository at www.jacionline.org).¹⁴

We next addressed molecular signaling events responsible for vacuolization and subsequent eosinophil cytolysis. We measured the production of ROS in IVIG + iC3b - adherent eosinophils over a time period of 1 h and observed a significant increase within 15 min after CR3 activation (Fig 2, A). PMA stimulation was used as a positive control in these experiments. In eosinophils, ROS are mainly produced by the inducible NADPH oxidase system, which can be blocked with diphenyleneiodonium-chloride (DPI).¹⁴ Interestingly, DPI (1 μ M) not only reduced ROS production, but also completely blocked cytoplasmic vacuolization (Fig 2, B) and cytolysis (Fig 2, C), suggesting that increased ROS production is required for these CR3-mediated cellular responses. Single-cell analysis using time-lapse automated fluorescence microscopy confirmed that only eosinophils generating high levels of ROS underwent subsequent vacuolization and cell death (see Fig. E5 and associated Video E2 in this article's Online Repository at www.jacionline.org). In contrast, both increased ROS production and

cytoplasmic vacuolization were unaffected by pharmacological inactivation of caspases using a pan-caspase inhibitor (z-VAD) (Fig 2, A and 2, B).

CR3 mediates the activation of the RIPK3-MLKL pathway which is required for increased ROS production and cytolysis

Since the IVIG + iC3b - mediated death was caspase-independent and exhibited a necrotic phenotype, we investigated the possibility that the eosinophil cytolysis we observed may represent necroptosis, a type of death characterized by activation of the RIPK3-MLKL pathway.¹³ Freshly isolated eosinophils exhibited no evidence of MLKL phosphorylation, suggesting that this essential effector protein in the cell death pathway downstream of RIPK3 was not activated at this time.^{17,18} In contrast, as early as 5 min following activation with IVIG + iC3b, we observed phospho-MLKL signals. Between 5 and 15 min after stimulation, the number of phospho-MLKL positive eosinophils increased up to 21% (Fig 3, A), indicating activation of the RIPK3-MLKL pathway. Moreover, we observed strong phospho-MLKL expression in 30-60% of infiltrating tissue eosinophils in biopsies from patients with eosinophilic diseases (Fig 3, B), suggesting that this pathway is also activated in inflammatory eosinophils under *in vivo* conditions.

To test whether the RIPK3-MLKL pathway is required for the IVIG + iC3b - mediated eosinophil cytolysis, we used a pharmacological approach. Inhibition of RIPK3 by GSK'872 or GSK'843²⁵ significantly reduced ROS production (Fig 4, A), prevented vacuolization (Fig 4, B) and cell death (Fig 4, C). Similarly, treatment of eosinophils with GW806742X²⁶ for pharmacological inactivation of MLKL also reduced ROS production and blocked cell death (Fig 4, A-C). The effects of GSK'843, GSK'872 and GW806742X were concentration-dependent and IC50s for these inhibitors were in the micromolar range as previously reported (see Fig E6 in this article's Online Repository at www.jacionline.org).²⁵ Inhibition of RIPK1 by necrostatin-1 (Nec-1)²⁷ had no effect on IVIG + iC3b - mediated ROS production and vacuolization (Fig 4, A and Fig 4, B). However, we noticed a minor, but significant inhibitory effect on eosinophil death (Fig 4, C and Fig E6, B, in this article's Online Repository at www.jacionline.org). Based on these data, we conclude that RIPK3 and MLKL are involved in eosinophil cytolysis and their activation occurs proximal to ROS production. In contrast, RIPK1 appears to play no role or only a minor role.

Since autocrine TNF- α is required for necroptosis induction in some cell lines²⁸ and owing to our observation of early degranulation events (presence of sombrero vesicles, see Fig E4, A, in this article's Online Repository at www.jacionline.org), we next investigated whether this requirement also applies for IVIG + iC3b - mediated eosinophil death. The addition of neutralizing anti-TNF- α mAb

to the culture medium had no effect in our system (see Fig E7 in this article's Online Repository at www.jacionline.org). Moreover, blocking TNFR1 or TNFR2 by anti-TNFR1 or anti-TNFR2 mAb²⁹ also failed to prevent the eosinophil death induced by IVIG + iC3b (see Fig E7 in this article's Online Repository at www.jacionline.org). We were also unable to detect TNF- α in supernatants of 2-h IVIG + iC3b - stimulated eosinophils (data not shown). Thus, we obtained no evidence for any role of autocrine TNF- α in the cell death pathway triggered by IVIG + iC3b in eosinophils.

p38 MAPK and PI3K activation depends on the activation of the RIPK3-MLKL pathway and is required for increased ROS production and cytolysis

It had previously been reported that increased ROS production by the NADPH oxidase requires p38 MAPK and PI3K activation in neutrophils.¹⁵ Therefore, we pharmacologically blocked p38 MAPK with PD169316 and PI3K with wortmannin, observing markedly reduced ROS production (Fig 5, A), vacuolization (Fig 5, B), and a corresponding reduction in cell death (Fig 5, C) by IVIG + iC3b - activated eosinophils, suggesting that both p38 MAPK and PI3K are critical for eosinophil cytolysis. In order to directly analyze p38 MAPK in IVIG + iC3b - activated eosinophils, we performed immunoblotting experiments, although this approach did not allow us to follow single eosinophils. Following 15-min activation, phosphorylation of p38 MAPK and MAPKAP kinase-2 (MK2; a direct target of p38)³⁰ was increased as compared to unstimulated cells (Fig 5, D). To examine the possibility that the RIPK3-MLKL pathway is required for activation of p38 MAPK following IVIG + iC3b - stimulation in eosinophils, we again blocked RIPK1, RIPK3, and MLKL using pharmacological inhibitors in these experiments. Both inactivation of RIPK3 and MLKL prevented or reduced phosphorylation of both p38 and MK2, suggesting that p38 activation had been blocked under these conditions (Fig 5, D). In contrast, Nec-1 treatment resulted in no p38 inhibition. Moreover, wortmannin blocked p38 activation, suggesting that PI3K activation is also proximal to p38 activation (Fig 5, D). To confirm that the RIPK3-MLKL pathway is indeed proximal to p38, PI3K and NADPH oxidase activation, we analyzed MLKL phosphorylation in the presence of PD169316, wortmannin and DPI. None of these inhibitors was able to prevent MLKL activation (see Fig E8 in this article's Online Repository at www.jacionline.org).

Since Nec-1 inhibition did not markedly block eosinophil cytolysis, we analyzed the expression of RIPK1, RIPK3 and MLKL by immunoblotting. While RIPK3 and MLKL were clearly detectable in eosinophils, the expression of RIPK1 was low or undetectable. Similarly, RIPK1 expression was also low in neutrophils as compared to Jurkat cells (Fig 5, E). Taken together, one

concludes that the RIPK3-MLKL pathway acts proximal to p38 MAPK and PI3K, both of which are required for increased ROS production and eosinophil cytolysis.

Induction of mTOR-dependent autophagy rescues eosinophils from IVIG + iC3b – induced cytolysis

Autophagosomes engulfing cytosolic material, the hallmark of autophagy, could be detected in eosinophils within the process of cytolysis (see Fig E9, arrowheads, in this article's Online Repository at www.jacionline.org). Autophagosome formation was also monitored by Autodot, a monodansylcadaverine-like, lysosomotropic agent, which detects autophagosome formation via an ion-trapping mechanism and interaction with lipids concentrated in the autophagosome membrane.³¹ We observed enhanced Autodot fluorescence in eosinophils undergoing cytolysis, suggesting increased autophagosome formation in these cells (Fig 6, A). To investigate whether activation of autophagy during cytolysis was part of the cell death pathway or rather a compensatory pro-survival strategy of the eosinophil, we used rapamycin, an mTOR inhibitor, to induce autophagy prior to adhesion. Pretreating eosinophils with rapamycin resulted in increased autophagic activity (Fig 6, A) together with a significant reduction of vacuolization (Fig 6, B) and cell death (Fig 6, C). These data suggest that autophagy plays a counter-regulatory role in the adhesion-induced cytolysis of eosinophils.

DISCUSSION

So far, evidence for necroptosis in human diseases has been obtained only for epithelial cells of the liver, intestine, and skin.¹³ In the present paper, we provide evidence that adhesion can activate the RIPK3-MLKL pathway in eosinophils under inflammatory conditions, both *in vitro* and *in vivo*. Moreover, pharmacological inhibition of RIPK3 and MLKL blocked the death pathway triggered by CR3, suggesting that the cytolysis of eosinophils observed under these conditions is in fact a necroptosis. We also report that cytoplasmic vacuolization is an irreversible death event for eosinophils undergoing cytolysis, at least under *in vitro* conditions of continuous stimulation with IVIG + iC3b. A recent report demonstrated a correlation between eosinophil vacuolization, the presence of cfecs and cytolysis with the severity of asthma exacerbations in children,⁹ suggesting that eosinophil cytolysis/necroptosis might additionally trigger the inflammatory responses. Therefore, it is important to better understand the mechanism for this still largely unknown cell death pathway in eosinophils.

Necroptosis can be induced by death receptors, TLRs, and intracellular RNA and DNA sensors.¹³ Here we provide evidence that CR3 activation on eosinophils can also result in necroptosis. Interestingly, increased activation of β 2-integrins on eosinophils has been observed in asthmatic patients after allergen challenge,³² pointing to the likelihood that the death mechanism described here also occurs under *in vivo* conditions. Pharmacological inactivation of RIPK3 with two different inhibitors completely prevented ROS production and eosinophil cytolysis triggered by CR3 *in vitro*. In contrast, inhibition of RIPK1 had a minor effect only, perhaps as a consequence of low expression levels. It should be noted that necroptosis has also been described in the absence of RIPK1.^{13,33-35} In TNFR1-induced necroptosis, the necrosome, defined as the complex containing RIPK1 and RIPK3, plays a key role in the initiation of necroptosis.^{13,36} Using neutralizing and blocking Abs, we obtained no evidence that TNF- α mediates CR3-triggered eosinophil cytolysis. We cannot exclude, however, that the activation of TNFR1 in the absence of its ligand is a consequence of adhesion. Moreover, a previous report suggested that activation of CR3 with soluble ICAM-1 prolonged eosinophil survival.³⁷ The IVIG + iC3b - mediated death shown here strictly required adhesion. Therefore, it is possible that the strength of CR3 activation determines the functional response of the cell. Clearly, the mechanism of RIPK3 activation by CR3 in eosinophils remains to be further investigated.

Pharmacological inactivation of MLKL, known as a pseudokinase involved in executing cell death in necroptosis downstream of RIPK3,²⁶ completely prevented CR3-mediated ROS production in eosinophils and their subsequent cytolysis. Recent studies have suggested that RIPK3-mediated activation of MLKL leads to its binding at cellular membranes, thereby triggering ion fluxes.^{17,26,38} However, in eosinophil cytolysis, MLKL, in collaboration with RIPK3, was required to activate p38 MAPK and NADPH oxidase activation. A connection between MLKL and downstream effectors had been suggested previously. For instance, it has been shown that, in the absence of MLKL, RIPK3 fails to trigger phosphorylation of the mitochondrial protein phosphatase PGMA5S.³⁹ Moreover, overexpression of MLKL has been reported to activate JNK.⁴⁰ MLKL has also been shown to contribute to inflammasome activation,⁴¹ further supporting the idea that it may be involved in signaling pathways. Therefore, MLKL should be seen not only as a necroptosis executor protein, but also as an adaptor protein for necrosis signaling.

Eosinophil cytolysis and necrotic-like eosinophil death, respectively, have also been reported following treatment with calcium ionophores^{12,42} or ligation of Siglec-8.^{43,44} Whether the RIPK3-MLKL signaling pathway mediates eosinophil death under these conditions as well remains to be determined. On the other hand, the same critical role of the NADPH oxidase for eosinophil death had been demonstrated in these previous reports,^{12,43,44} suggesting that the molecular mechanisms overlap, at least partially. It should be noted that ROS-dependent cytoplasmic vacuolization has also

been described in GM-CSF-primed and CD44-activated neutrophils as a morphological feature of the programmed necrotic cell death pathway.¹⁵ Therefore, it has been suggested that this previously described caspase-independent neutrophil death represents a necroptosis as well.⁴⁵

Increased ROS production can be expected to bring about the induction of autophagy,⁴⁶ as we have observed in this study. The removal of irreversibly damaged biomolecules is likely to be a process that can support eosinophil survival. Clearly, ROS are likely to be involved in the permeabilization of granules¹⁵ and, as shown in this study, of vacuoles (see Fig E4, in this article's Online Repository at www.jacionline.org), but the exact mechanism of how necrosis occurs under these circumstances, remains to be investigated.

We observed PMD in eosinophils which subsequently underwent vacuolization and cytolysis. Previous morphological studies addressing the origin of cytoplasmic vacuolization resulted in two opposing viewpoints: vacuoles may result from PMD⁴⁷ or they may represent 3-dimensional degranulation tunnels.⁴ Our findings suggest that these previous observations may be interconnected: the first observation would lead to hypodense granules and a larger fusion product, which finally connects to the plasma membrane. In consequence, an increased cell surface available for degranulation is achieved. Moreover, we hypothesize that the degranulation tunnels of several eosinophils adherent to a pathogen may lead to accumulation of cytotoxic agents at these loci, resulting in a more efficient destruction of the target as opposed to simple degranulation. Morphological observations of vacuolated eosinophils showed an initial loss of plasma membrane integrity at the site of the vacuole/tunnel membrane, suggesting high cytotoxicity at these loci. It is possible that the loss of plasma membrane integrity contributes to the death process: As analyzed by single cell live microscopy, we observed that essentially all vacuolated eosinophils underwent lysis within 2 h. Whether MLKL, in addition to its role as an adaptor protein for signal transduction, also participates in the process of plasma membrane permeabilization remains to be investigated.

As assessed by TEM and fluorescence analyses, we observed autophagosomes in eosinophils undergoing cytolysis. Interestingly, pharmacological inhibition of mTOR by rapamycin not only increased autophagy, it also, at least partially, prevented cell death in IVIG + iC3b - activated eosinophils. Therefore, eosinophil cytolysis does not appear to represent autophagic cell death, instead, the induction of autophagy seems rather an attempt to maintain viability. These data support previously published work suggesting that autophagy and/or autophagy-related proteins (ATGs) can modify necroptosis. For instance, autophagy has been shown to promote the degradation of RIPK1⁴⁸ and ATG5 is able to activate necrosome formation on the autophagosome.⁴⁹ Although the mechanism of how autophagy counter-regulates eosinophil cytolysis remains to be investigated, it seems reasonable that preventing a pro-inflammatory cell death in the eosinophilic inflammatory

response could represent a new therapeutic strategy in diseases associated with eosinophil-mediated immunopathology, such as bronchial asthma and atopic dermatitis.⁵⁰

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Key messages:

- **Eosinophil cytolysis mediated by complement receptor 3 is a necroptosis, mediated by RIPK3 and MLKL.**
- **Eosinophil cytolysis is preceded by vacuolization, which results from granule fusion events.**
- **Eosinophil cytolysis is counter-regulated by autophagy.**

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Figure legends

FIG 1. Eosinophil cytolysis is induced upon adhesion to naked glass as well as IVIG + iC3b coated glass. **A, Upper left:** Untreated, freshly isolated human eosinophils were added to glass coverslips in low FCS containing culture medium and stained with Diff-Quik. Cytoplasmic vacuolization and necrotic cell death, characterized by deposition of eosinophil granules (rhombus) and nuclear disintegration (arrowhead), were observed. **Upper right:** Preventing adhesion with Poly-Hema resulted in an unchanged eosinophil morphology during the entire culture period. **Lower left:** Enzymatic inactivation of caspases with z-VAD failed to prevent the necrotic morphology. **Lower right:** Eosinophil adhesion to IVIG + iC3b coated glass also provoked cytoplasmic vacuolization and a necrotic cell death. Bars, 10 μ m. Data are representative of at least 3 independent experiments. **B,** Eosinophil adhesion to IVIG + iC3b coated glass provoked cell death as assessed by live cell imaging. Interestingly, additional stimulation with IL-5 resulted in accelerated eosinophil death. Values are means $\pm$ SEM. **, $p < 0.01$; ****, $p < 0.0001$; $n=3$. See also Fig E1 and associated Video E1. **Right:** TEM images of the necrotic eosinophils showed plasma and nuclear membrane disruption (upper panel), release of entire granules, and the typical clusters of free eosinophil granules (*cfegs*) (lower panel). Bars, 1 μ m. **C,** Blocking of CD18 with F(ab)₂ fragments of anti-CD18 mAb largely prevented eosinophil spreading as quantified 5 min after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. **, $p < 0.01$; $n=5$. **D,** Blocking of CD18 with F(ab)₂ fragments of anti-CD18 mAb largely prevented cytoplasmic vacuolization as quantified 15 min after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. **, $p < 0.01$; $n=5$. **E,** Blocking of CD18 with F(ab)₂ fragments of anti-CD18 mAb largely prevented eosinophil death quantified 1 h after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ****, $p < 0.0001$; $n=5$.

FIG 2. Cytoplasmic vacuolization and cytolysis of eosinophils requires ROS production but is caspase-independent. **A,** ROS production by IVIG + iC3b - stimulated eosinophils was analyzed in a time-dependent manner using a DHR oxidation assay. PMA was used as a positive control. DPI significantly inhibited ROS production. Data are representative of 3 independent experiments. **B,** DPI, but not z-VAD, largely prevented cytoplasmic vacuolization as quantified 15 min after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ****, $p < 0.0001$; $n=4$. **C,** DPI, but not z-VAD, largely prevented eosinophil death as quantified 1 h after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ***, $p < 0.001$; $n=3$. For single-eosinophil analysis, see Fig E5 and associated Video E2.

FIG 3. MLKL is phosphorylated in IVIG + iC3b - stimulated blood eosinophils *in vitro* and in tissue eosinophils under *in vivo* inflammatory conditions. **A**, Eosinophils were stimulated with IVIG + iC3b for the indicated times. Immunofluorescence staining was performed using anti-phospho-MLKL mAb (1:250). Staining controls were performed without a primary antibody (left panels). Data are representative of 3 independent experiments. The relative numbers of phospho-MLKL positive eosinophils (means $\pm$ SEM) are indicated in the upper right corner of each image. Bars, 10 μ m. **B**, Paraffin-embedded tissue specimens from patients suffering from the indicated eosinophilic diseases were analyzed. *Left*: Hematoxylin- and eosin-stained tissue sections of the investigated biopsy specimens. Staining was performed using anti-phospho-MLKL (1:250) and anti-ECP (1:100) mAbs. Arrows indicate phospho-MLKL positive eosinophils and arrowheads phospho-MLKL negative eosinophils. The relative numbers of phospho-MLKL positive eosinophils for each condition are indicated in the upper right corner of each image. Bars, 10 μ m.

FIG 4. Cytoplasmic vacuolization and cytolysis of eosinophils require a functionally intact RIPK3 - MLKL pathway. **A**, ROS production by IVIG + iC3b - stimulated eosinophils was analyzed in a time-dependent manner using a DHR oxidation assay. PMA was used as a positive control. Several pharmacological blockers of the RIPK3-MLKL pathway were shown to inhibit ROS production. Data are representative of 3 independent experiments. **B**, GSK'872, GSK'843, and GW806742X, but not Nec-1, prevented cytoplasmic vacuolization as quantified 15 min after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ***, $p < 0.001$; $n=4$. **C**, GSK'872, GSK'843, and GW806742X prevented eosinophil death as quantified 1 h after the initiation of IVIG + iC3b stimulation. In contrast, Nec-1 had much less inhibitory activity on eosinophil death. Values are means $\pm$ SEM. **, $p < 0.01$; ***, $p < 0.001$; $n=3$.

FIG 5. Cytoplasmic vacuolization and cytolysis of eosinophils requires an active p38 MAPK pathway distal from MLKL. **A**, ROS production by IVIG + iC3b - stimulated eosinophils was analyzed in a time-dependent manner using a DHR oxidation assay. PMA was used as a positive control and wortmannin (100 nm) and PD169316 (10 μ M) were used to block PI3K and p38 MAPK, respectively. Both inhibitors inhibited ROS production. Data are representative of 3 independent experiments. **B**, Wortmannin and PD169316 prevented cytoplasmic vacuolization as quantified 15 min after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ***, $p < 0.001$; $n=3$. **C**, Wortmannin and PD169316 prevented eosinophil death as quantified 1 h after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ***, $p < 0.001$; ****, $p < 0.0001$; $n=3$. **D**, Eosinophils were

cultured in the presence and absence of PD169316 (10 μ M), wortmannin (100 nM), Nec-1 (25 μ M), GSK'843 (50 μ M), GSK'872 (10 μ M) or GW806742X (5 μ M), and subsequently stimulated with IVIG + iC3b for 15 min. Cell lysates were analyzed by immunoblotting for phosphorylated Thr180/Tyr182 p38 and phosphorylated Thr334 MK2. p38 and GAPDH protein levels were analyzed as loading controls. Representative immunoblots are shown ($n \geq 3$). **E**, eosinophil lysates were analyzed by immunoblotting for RIPK3, RIPK1, and MLKL expression. Levels were compared with neutrophils and Jurkat T cells. GAPDH protein levels were analyzed as loading controls. Representative immunoblots are shown ($n \geq 3$).

FIG 6. Activation of eosinophil cytolysis is associated with the activation of autophagy and pharmacological hyperactivation of autophagy by rapamycin treatment largely prevents eosinophil vacuolization and cell death. **A**, Autophagosome formation is shown by Autodot staining (arrows). Eosinophils were cultured in the presence and absence of rapamycin (5 μ M) and either received no other stimulation (1% BSA) or were treated with IVIG + iC3b for 30 min. Bars, 10 μ m. Statistics were performed by analyzing 50 cells per experiment in each of three different experiments. Values are means $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; $n=3$. **B**, Rapamycin significantly prevented cytoplasmic vacuolization quantified 15 min after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ***, $p < 0.001$; $n=4$. **C**, Rapamycin significantly prevented eosinophil death quantified 1 h after the initiation of IVIG + iC3b stimulation. Values are means $\pm$ SEM. ***, $p < 0.001$; $n=3$.

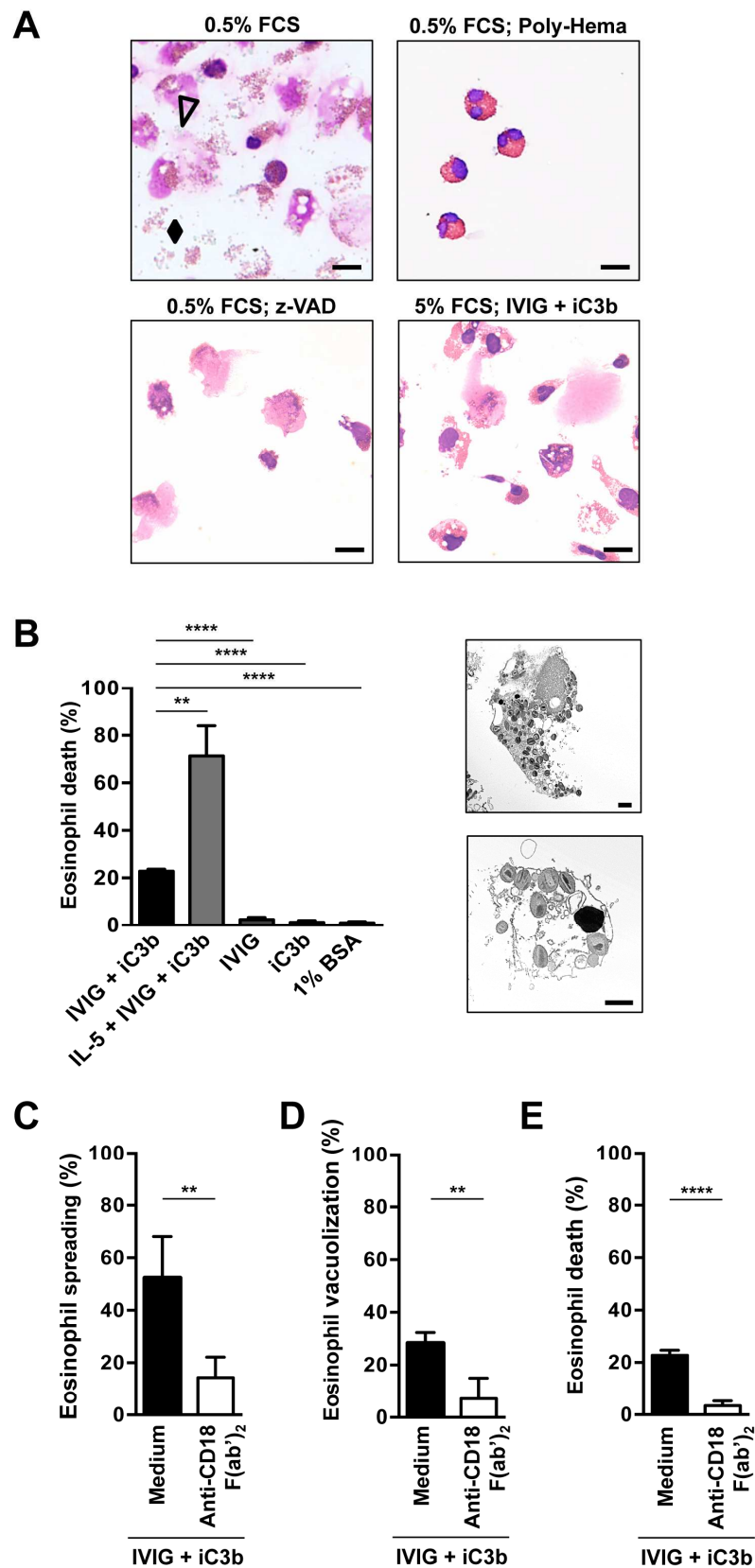


Figure 1

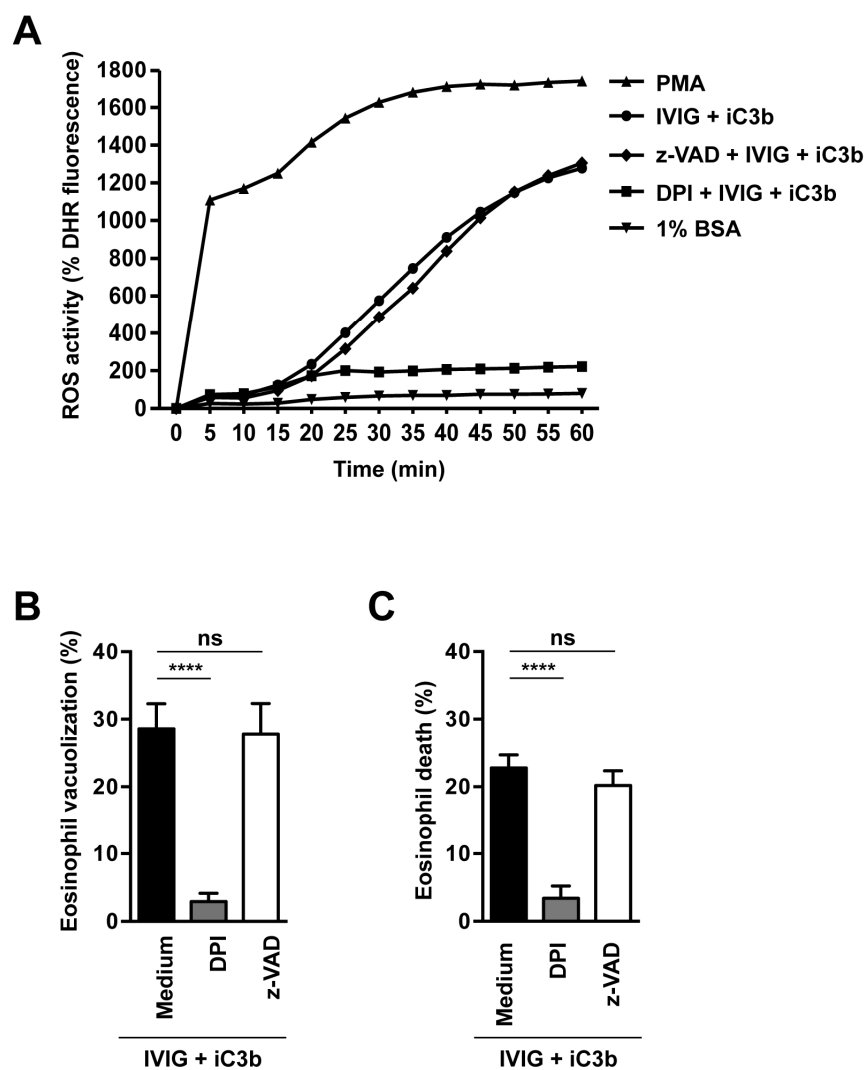


Figure 2

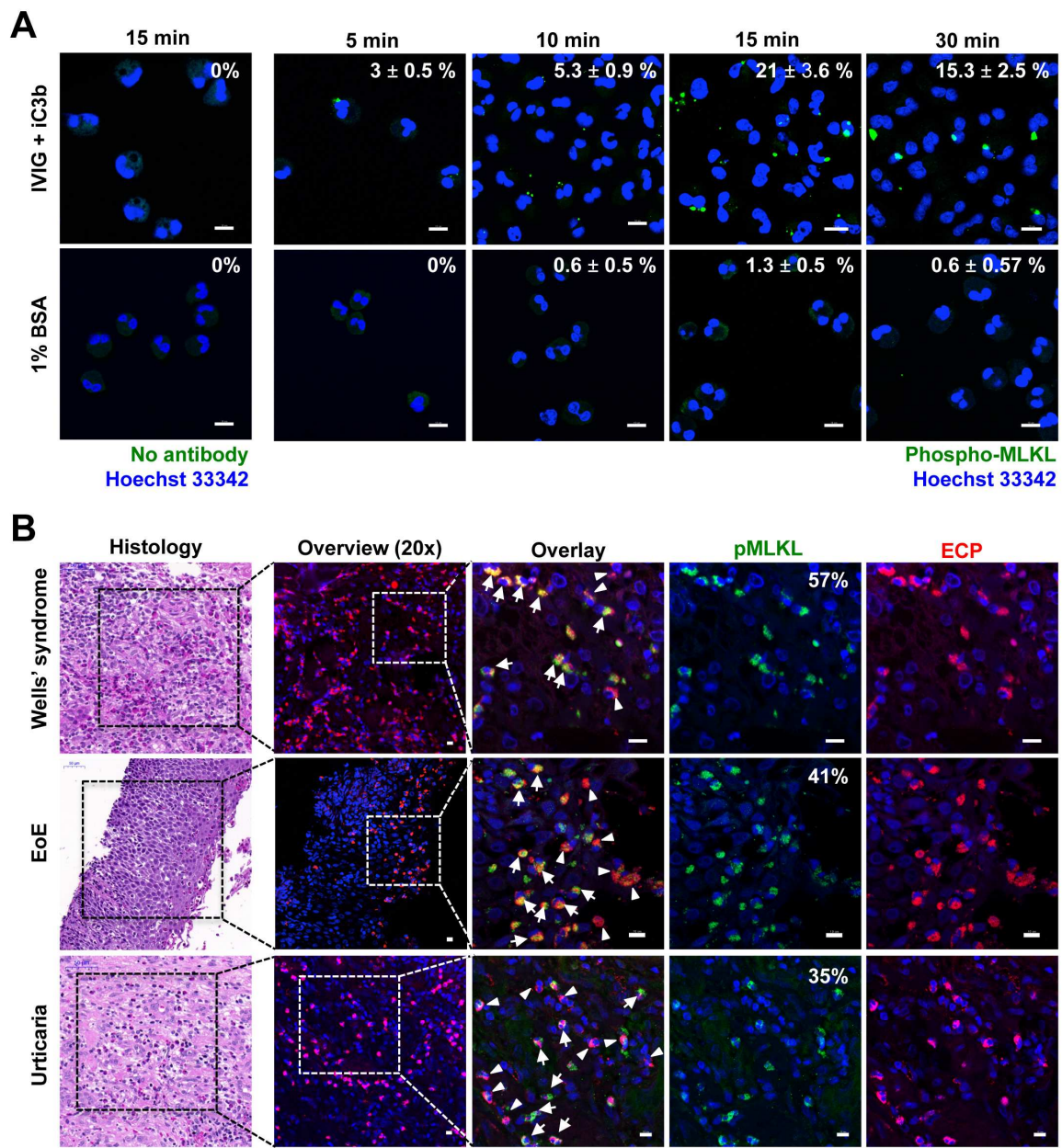


Figure 3

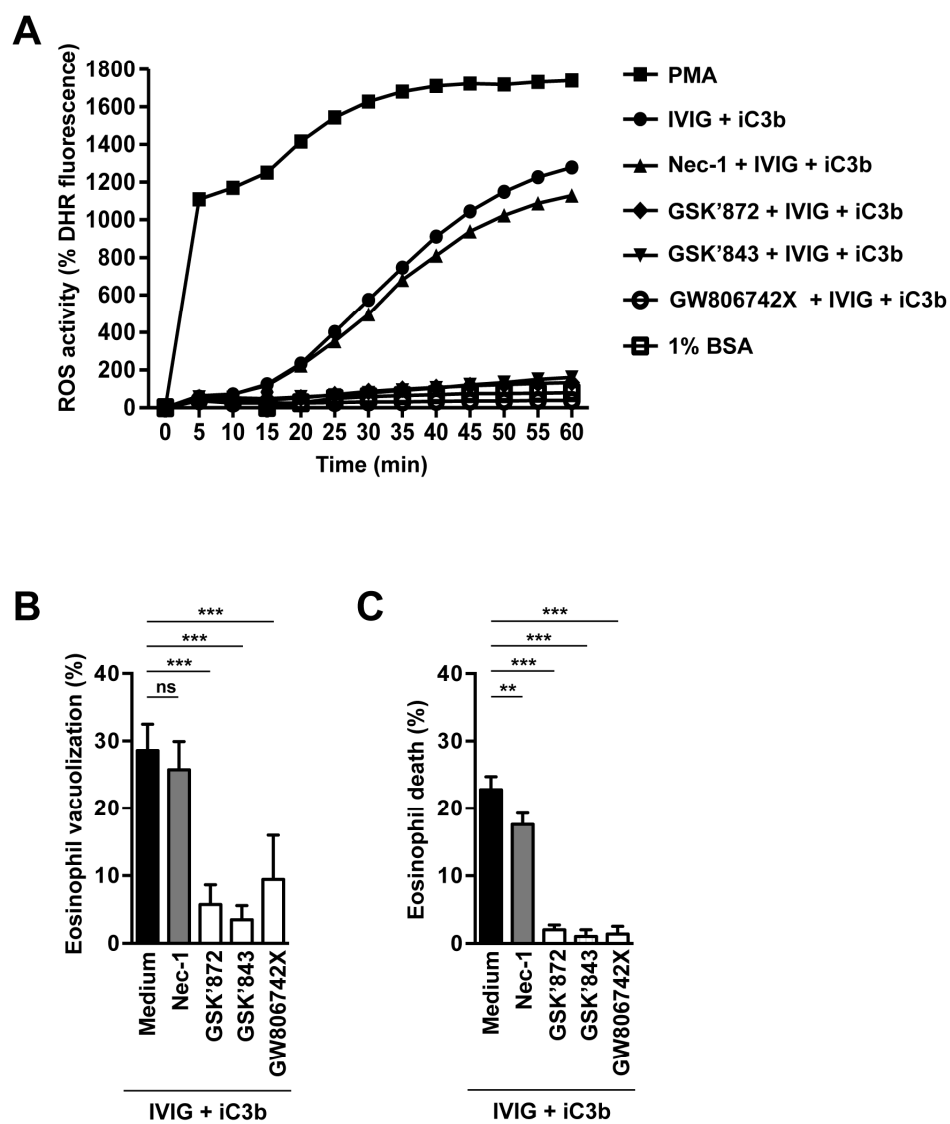


Figure 4

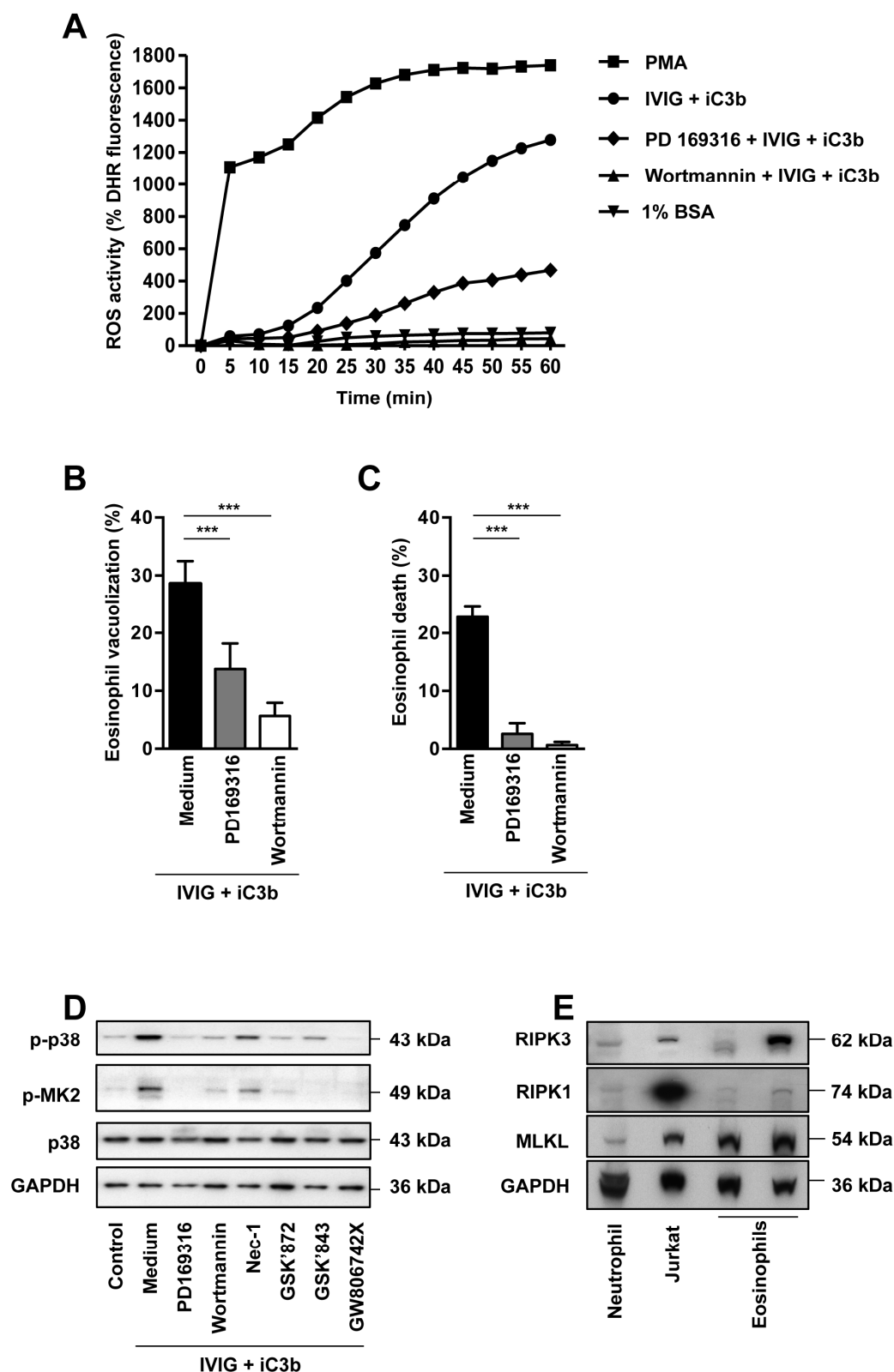


Figure 5

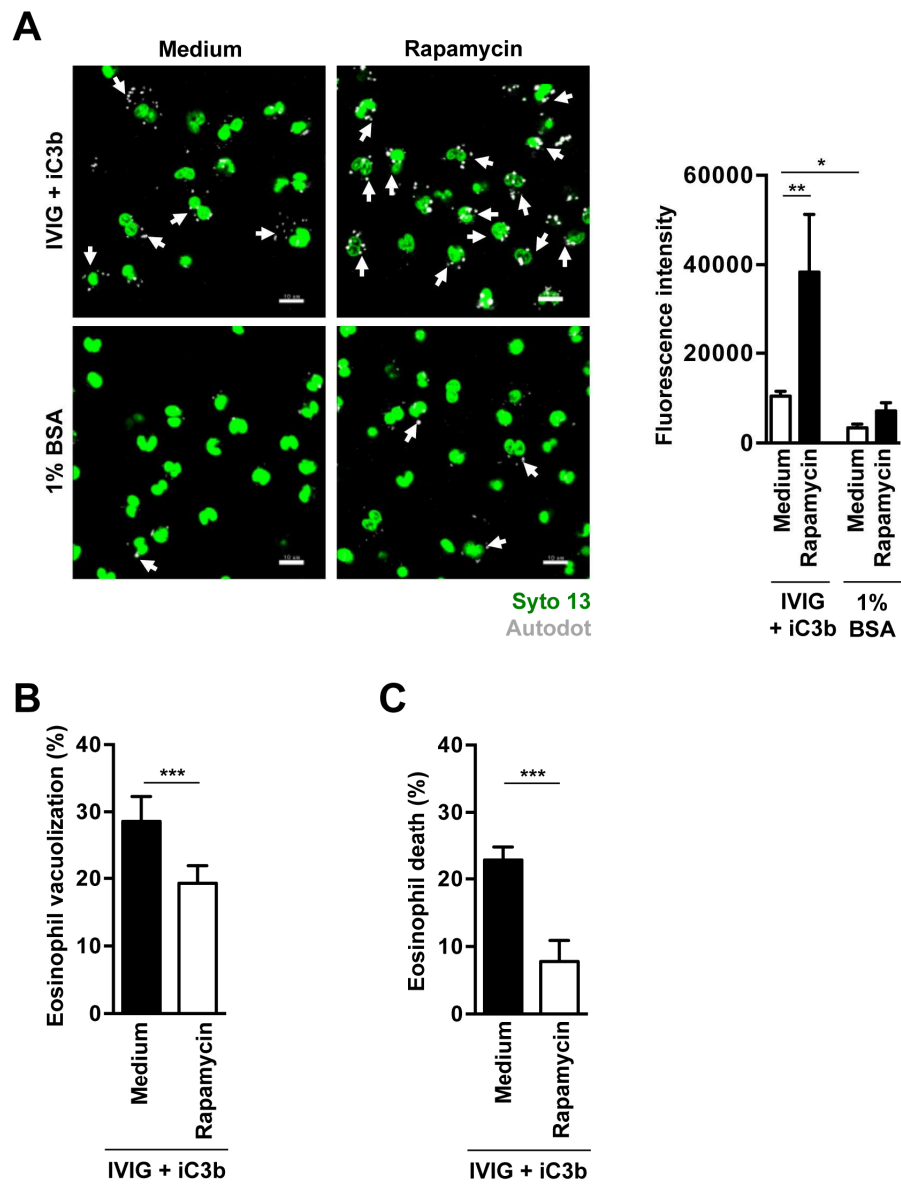


Figure 6

Online Repository

Adhesion-induced eosinophil cytolysis requires the RIPK3-MLKL signaling pathway which is counter-regulated by autophagy

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SUPPLEMENTARY METHODS

Reagents

Easy Sep Human Eosinophil Enrichment Kit was obtained from Stem Cell Technologies (Grenoble, France). Poly 2-hydroxyethyl methacrylate (Poly-Hema) was purchased from Sigma-Aldrich (Buchs, Switzerland). Intravenous immunoglobulins were kindly provided by CSL Behring AG (Bern, Switzerland). Human inactive complement factor 3b (iC3b) was obtained from Calbiochem (La Jolla, CA, USA). Human recombinant IL-5 was from R & D Systems Europe (Abingdon, UK). Anti-CD18 F(ab')₂ fragments (clone IB4) were from Ancell Corporation (Stillwater, MN, USA). Anti-TNFR1 (mAb225), anti-TNFR2 (mAb226), anti-TNF- α (AF-210-NA), and mouse IgG2a isotype control mAbs were obtained from R & D Systems (Abingdon, UK). For immunofluorescence and immunohistochemical staining, anti-EPX (Lee Laboratories, Mayo Clinic) and anti-ECP (EG1; Pharmacia Diagnostics AB, Uppsala, Sweden) mAbs, as well as rabbit anti-phospho-MLKL (phospho Ser358) mAb (Abcam, Cambridge, UK) were used. Control Abs were from DAKO (rabbit immunoglobulin and mouse IgG1). Goat-anti-mouse/-rabbit Alexa Fluor 488 (Invitrogen Molecular Probes, Paisley, UK) and donkey-anti-rabbit/-mouse Cy3 (Jackson ImmunoResearch) served as secondary Abs, respectively. LIVE/DEAD Cell Viability Assays, MitoSOX™ Red, and SYTO® 13 Green, calcein-AM, Hoechst 33342, ethidium homodimer-1 (EthD-1), propidium iodide (PI), CellTracker™ Red CMTPX dye were purchased from Molecular Probes (ThermoFisher Scientific). PKH67 membrane tracker and dihydrorhodamine (DHR) 123 were from Sigma-Aldrich, and AutoDot from Abgent (San Diego, CA, USA). For immunoblotting, we additionally used rabbit anti-p38, rabbit anti-phospho-Thr180/Tyr182-p38, rabbit anti-phospho-Thr334-MK2 (pMK2), and rabbit anti-RIPK3 Abs (all from Cell Signaling Technology; Danvers, MA, USA). Anti-MLKL mAb was from Abcam, anti-RIPK1 mAb from BD Biosciences, and anti-GAPDH mAb from Chemicon International (Chandlers Ford, U.K.). For inhibitor studies, z-VAD-FMK (z-VAD) was obtained from BD Biosciences, PD169316 from Merck Millipore, necrostatin-1 (Nec-1) and rapamycin from Enzo Life Sciences (Lausen, Switzerland), GSK'843 and GSK'872 from Glix Laboratories (Southborough, MA, USA), GW806742X from Adipogen AG (Liestal, Switzerland), and wortmannin and diphenyleneiodonium chloride (DPI) from Calbiochem. Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem.

Time-lapse automated fluorescence microscopy

IVIG + iC3b – activated freshly isolated eosinophils in RPMI 1640 plus 5% FCS (2.5×10^6 / ml) were monitored by live cell imaging for five parameters: morphology using differential interface

contrast (DIC), cell viability using 0.4 μ M calcein-AM (green), cell death using 0.1 μ M EthD-1 (red), according to LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells, ROS production using 1 μ M DHR 123 (green), and nuclear morphology using Hoechst 33342 (1 μ g/ml, blue). Cells were seeded on glass coverslips that had been pre-coated as described above. The live cell analysis was performed in a humidified atmosphere containing 5% CO₂ at 37 °C and observed by inverted confocal microscope LSM 510 Exciter (Carl Zeiss Micro Imaging, Jena, Germany) using 63x /1.40 Oil DIC objective and followed by analysis with IMARIS software. Images were obtained every minute for up to 300 min. Minimum laser, and pinhole values, and high speed scanning were applied to avoid phototoxicity. The multi-location and automated focusing option was used to obtain several live captures simultaneously. Important time points are shown in min. Vacuoles are indicated by arrows, and scale bars represent 10 μ m. Results are representative of at least 10 independent experiments.

Determination of cell death

Eosinophils (1×10^6 per ml in RPMI-1640 plus 5% FCS) were pretreated with the indicated pharmacological inhibitors and antibodies, respectively, in Eppendorf tubes for 20 min in a CO₂ incubator at 37°C. Eosinophils were then seeded on pretreated black, glass-bottom 96-well plates (Greiner Bio-One GmbH), previously coated with IVIG + iC3b or 1% BSA, and kept at 37°C as described above for 1 h at 37°C in a humidified atmosphere containing 5% CO₂. Calcein-AM (0.4 μ M) and EthD-1 (0.1 μ M) were added to each well upon 50 min incubation. Cell viability was determined by randomly acquiring images of each well with confocal imaging at 37°C. Images were analyzed by Imaris software Bitplane AG, Zurich, Switzerland), using Isosurface Rendering and Spots Detection to analyze 1000 cells for viability (viable cells maintain calcein-AM, in contrast dead cells take EthD-1 up).

Co-culture live cell imaging

Calcein-AM (0.4 μ M) - stained IL-5 primed eosinophils were added to a monolayer of IL-1 - stimulated human dermal vascular endothelial cells (HDEC), cultured on uncoated μ -Slide Angiogenesis (Ibidi GmbH, Martinsried, Germany), and stained with FITC- or rhodamine-conjugated lectin for cell membrane visualization and Hoechst H33342 (1 μ M) for visualization of the nuclei. Time course imaging as well as intermittent z-section imaging at resolution limits were performed within 1-h co-culture using Live 510 Duo confocal microscope with incubation chamber (Carl Zeiss Microimaging).

Transmission electron microscopy

Eosinophils were cultured in 0.5% FCS containing RPMI (1×10^6 per 100 μ l) and allowed to adhere to untreated glass coverslips for the indicated times. Upon supernatant withdrawal, eosinophils were fixed in 2.5% (v/v) glutaraldehyde solution buffered with sodium cacodylate (pH 7.4, 540 mOsm), postfixed in 1% osmium tetroxide [buffered with 0.1 M sodium cacodylate buffer (pH 7.4, 340 mOsm)], dehydrated through ascending concentrations of ethanol, and embedded in epoxy resin. Ultrathin sections were obtained at a nominal thickness of 80 nm and mounted on copper grids coated with Formvar (polyvinyl formal; Fluka, Buchs, Switzerland). Sections were then stained with lead citrate and uranyl acetate prior to viewing in a Philips EM-400 transmission electron microscope.

Immunoblotting

Eosinophils (1×10^6 per 100 μ l) were pretreated with the indicated pharmacological inhibitors and allowed to adhere to IVIG + iC3b-coated glass coverslips for 15 min. A proteinase inhibitor cocktail was added directly to the coverslip prior to centrifugation at 4° C. Upon supernatant withdrawal, cells were lysed in Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl [pH 7.4], and 1 mM EDTA) containing both protease (protease inhibitor cocktail) and phosphatase inhibitors (1 μ M okadaic acid, 1 mM Na_3VO_4 , and 5 mM NaF). In case of RIPK1 and RIPK3 detection, eosinophils were lysed with RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 1% SDS, and 1 mM EDTA; supplemented with protease inhibitor mixture). Protein extracts from Jurkat T cells and neutrophils served as controls. The lysates were heated at 95°C for 5 min. Proteins were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Immobilion-P; Millipore, Bedford, MA). The membranes were routinely blocked in TBS containing 0.1% Tween-20 and 5% nonfat dry milk, followed by incubation overnight with the indicated Abs (all antibodies were diluted at 1:1000) at 4°C. The next morning, samples were further incubated with the appropriate HRP-conjugated secondary Ab for 1 h at room temperature. Filters were developed using an ECL technique (ECL-Kit; GE Healthcare) according to the manufacturer's instructions.

SUPPLEMENTARY FIGURES AND VIDEOS

ACCEPTED MANUSCRIPT

Video E1. Cytoplasmic eosinophil vacuolization precedes eosinophil death. See Fig E1.

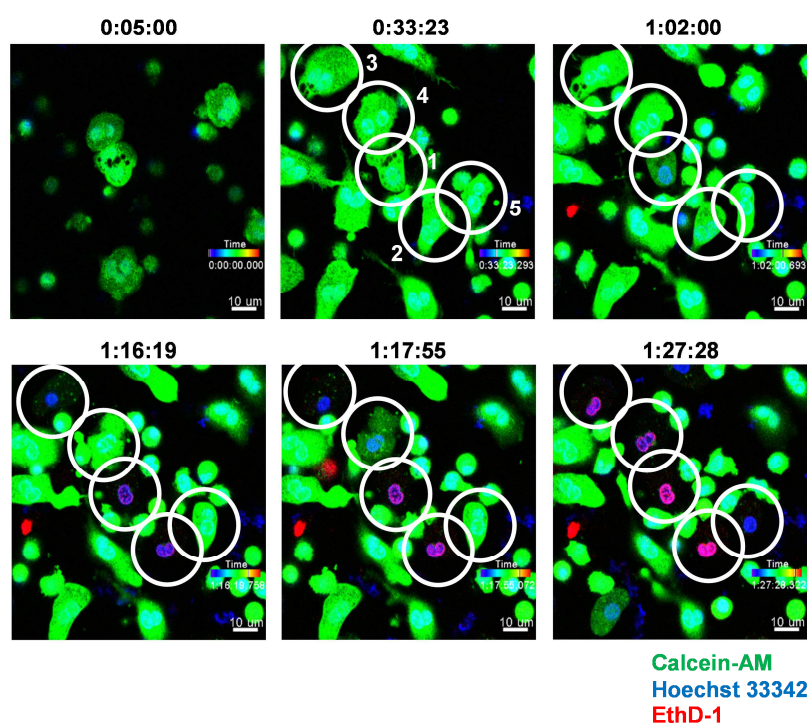


FIG E1. Cytoplasmic eosinophil vacuolization precedes eosinophil death. Eosinophil adhesion to IVIG + iC3b - coated glass provoked cell death as assessed by live cell imaging within a time period of 2 h. Eosinophils were stained with calcein-AM (green), Hoechst 33342 (blue), and EthD-1 (red). Single eosinophils were followed over time. Following adhesion to IVIG + iC3b - coated glass, eosinophils stop moving and develop vacuoles. These cells gradually lose the calcein-AM fluorescence and the blue nucleus becomes visible. Subsequently, the nucleus turns red, since the cells are no longer able to exclude EthD-1, indicating loss of plasma membrane integrity. Five cells are circled and can be followed over time in Video E1. Analyzing a total of 200 eosinophils, we observed that approximately 98% of the vacuolated eosinophils subsequently lost plasma membrane integrity within 2 h.

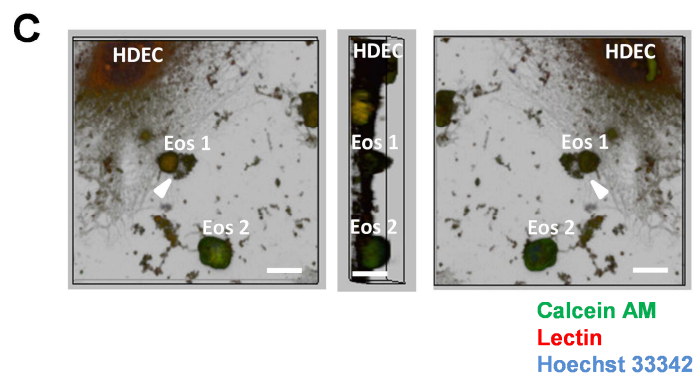
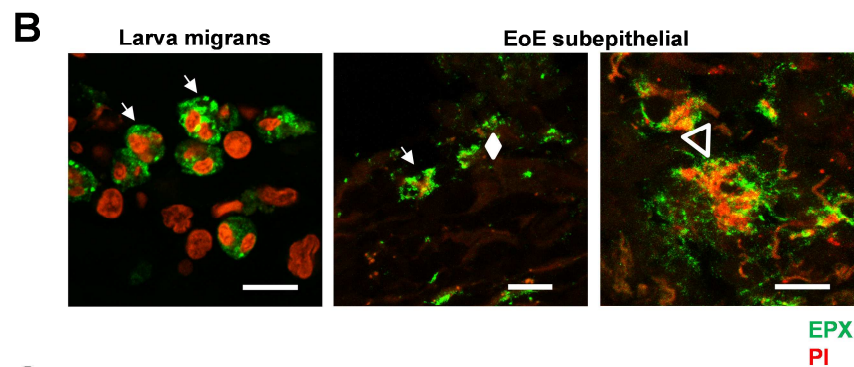
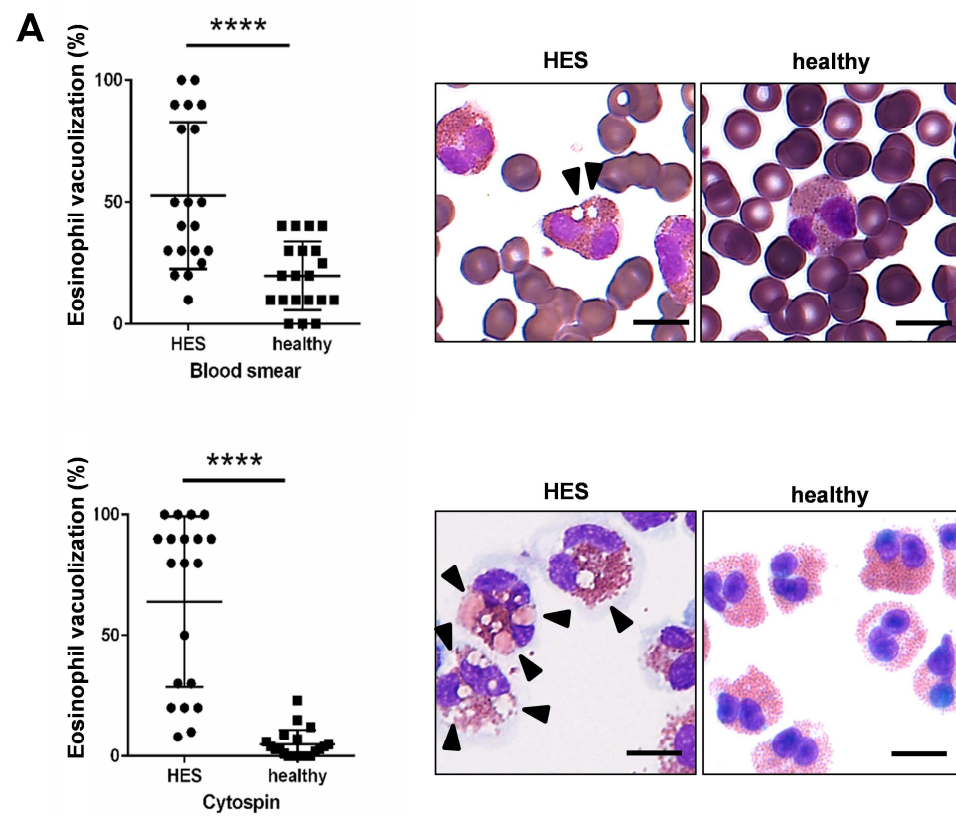
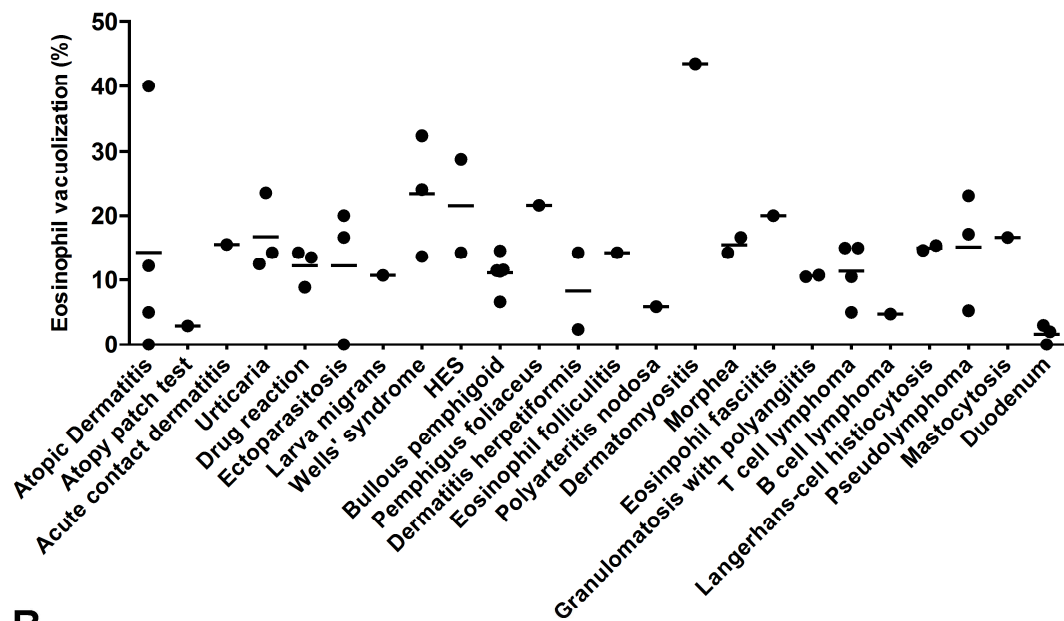


FIG E2. Cytoplasmic vacuolization is observed in both blood and tissue eosinophils under *in vivo* conditions as well as following *in vitro* co-culture with IL-1 β – stimulated dermal endothelial cells. **A**, Morphological analysis of blood eosinophils (blood smears and also after isolation) demonstrated a relatively higher frequency of cytoplasmic vacuolization in eosinophils from HES patients as compared to healthy donors. Values are means $\pm$ SEM. ****, $p < 0.0001$. *Right*: Representative original data (arrowheads point to vacuoles). Bars, 10 μ m. **B**, Prominent vacuolization of tissue eosinophils (arrows) was found in biopsies obtained from patients suffering from Larva migrans and eosinophilic esophagitis (EoE). Clusters of free eosinophil granules (*cfegs*) (rhombus) and nuclear disintegration (empty arrowhead) were also observed. Additional examples and a quantitative analysis of eosinophil vacuolization in different eosinophilic skin diseases is shown in Fig E3. Bars, 10 μ m. **C**, IL-5-primed calcein-AM-stained eosinophils were added to a monolayer of IL-1 β –stimulated human dermal vascular endothelial cells (HDEC) stained with rhodamine-conjugated lectin. Nuclear staining was performed using Hoechst 33342. Morphological changes in eosinophils adherent to HDEC were analyzed by z-stack images taken at different time points within a time frame of 1-h co-culture. A representative example of a 3D reconstruction shows both vacuolization (Eos1, arrowhead) and adhesion with spreading, but without vacuolization (Eos2), in eosinophils adherent to HDEC (label in the nucleus). Depicted are both top (right panel), bottom (left panel) and side views (middle panel). One can appreciate that the vacuole is seen both from top and bottom views, but not from side view, underlining the possibility that the vacuole is a 3D degranulation tunnel directed towards the adherent surface, e.g. the HDEC monolayer (see Fig E4, C). Bars, 10 μ m.

A



B

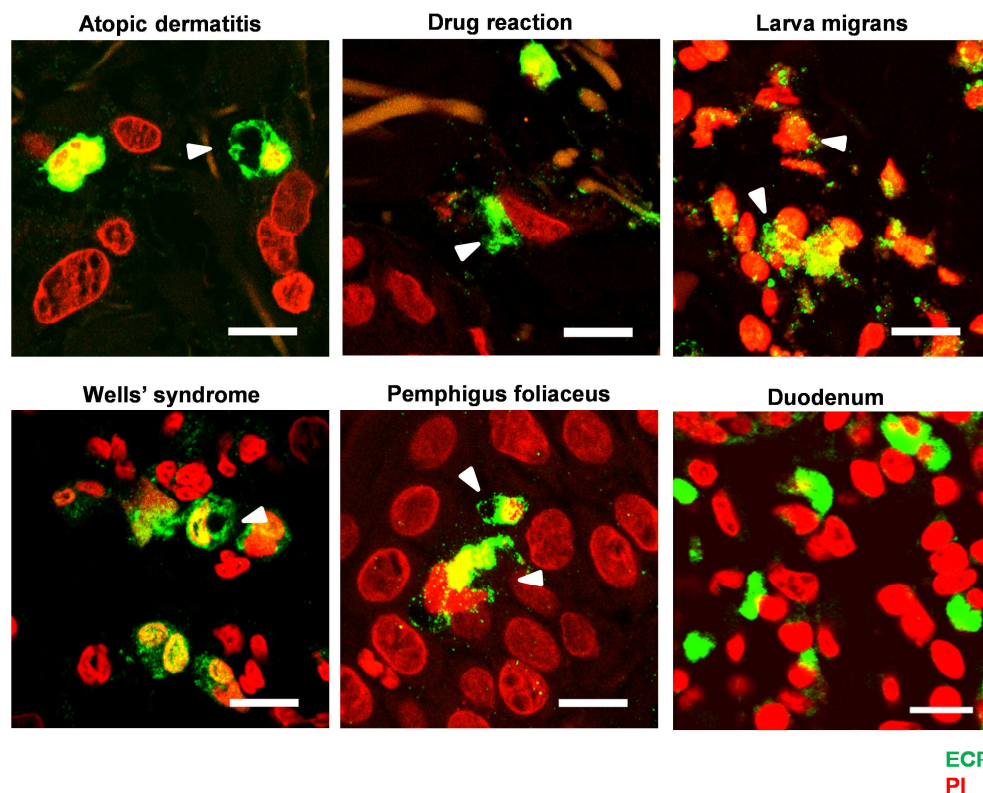
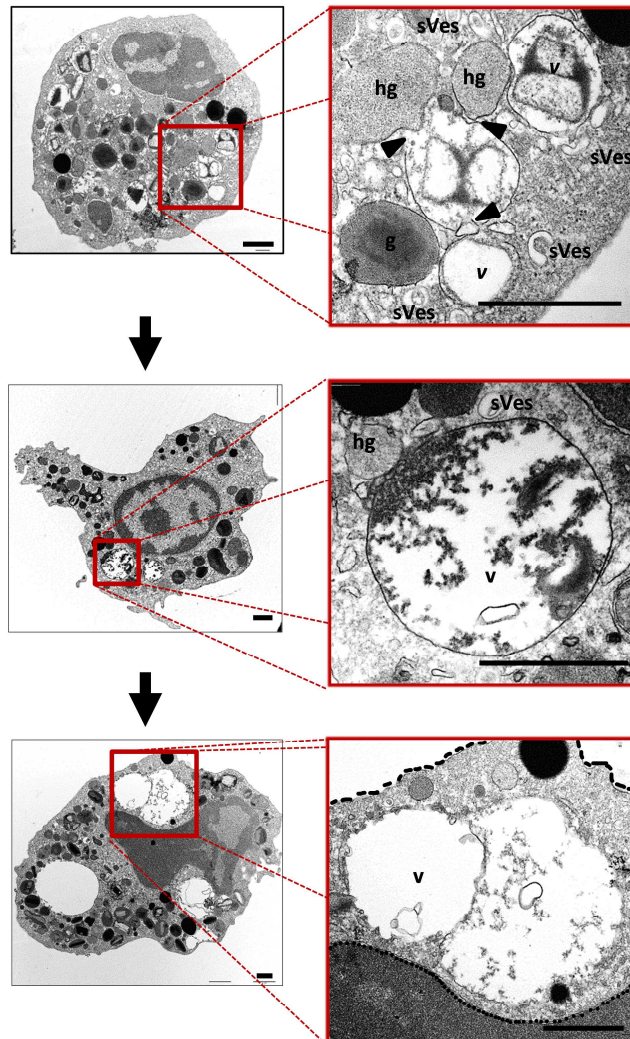
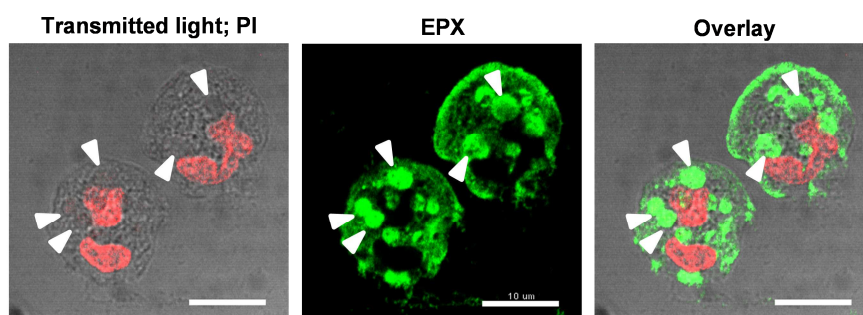


FIG E3. Cytoplasmic eosinophil vacuolization in eosinophilic skin diseases. **A,** Vacuolization of tissue eosinophils was quantified in skin biopsies obtained from patients suffering from eosinophilic skin diseases. **B,** Representative original data (arrowheads point to vacuolated eosinophils) is shown as 3D reconstruction of z-stack images (see Material and Methods). Biopsies from patients with the indicated diseases were stained with anti-ECP mAb and PI. As a control, we used normal duodenum tissues, in which the physiologically infiltrating eosinophils showed no or only little evidence for vacuolization (n=3). Bars, 10 μ m.

A**B**

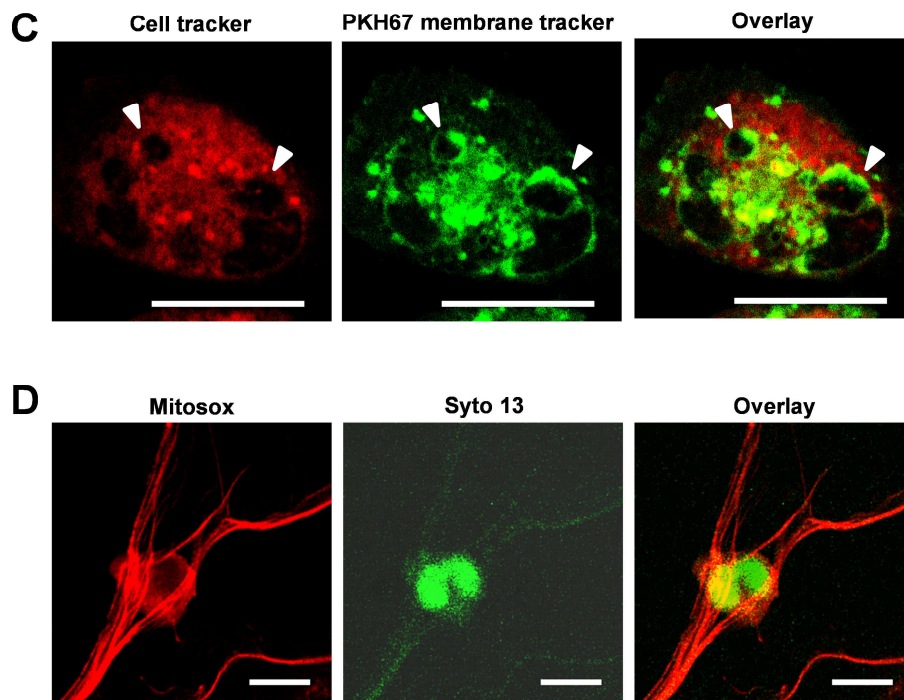


FIG E4. Cytoplasmic vacuoles evolve from granule-granule fusions and seem to represent large degranulation tunnels. **A**, Adherent eosinophils show an altered granule morphology as assessed by TEM (upper panel). Hypodense granules (h.g.) with loss of core and matrix density (in comparison to normal; g) as well as sombrero vesicles (sVes) could be documented in close proximity. Furthermore, prominent fusion constructs of altered granules were found (closed arrowheads indicating fusion sites). Additional diminution of granule content (v) resulted in beginning vacuolization. Vacuolated cells showed large, perinuclear spaces (v; middle and lower panels). Loss of membrane integrity seems to occur first at the level of the vacuole membrane, while the nuclear and plasma membrane are still intact (lower panel). Bars, 1 μ m. **B**, Vacuoles (red arrowheads) demonstrate strong EPX positivity within 5 min of adhesion as assessed by immunofluorescence. Bars, 10 μ m. **C**, Cell membrane staining with PKH67 membrane tracker showed positivity of the vacuole membrane, suggesting a connection to the cell surface. 3D reconstruction of z-stack images (see Material and Methods) supported this notion by revealing vacuoles as 3D tunnels directed towards the underlying surface. Bars, 10 μ m. **D**, IVIG + iC3b - stimulated eosinophils release mitochondrial DNA within 5 min. Eosinophils were stained with Syto 13 and MitoSOX Red before stimulation. Bars, 10 μ m. All data are representative of at least 5 independent experiments.

Video E2. Increases in ROS production precede eosinophil death. See Fig E5 and related Fig 2.

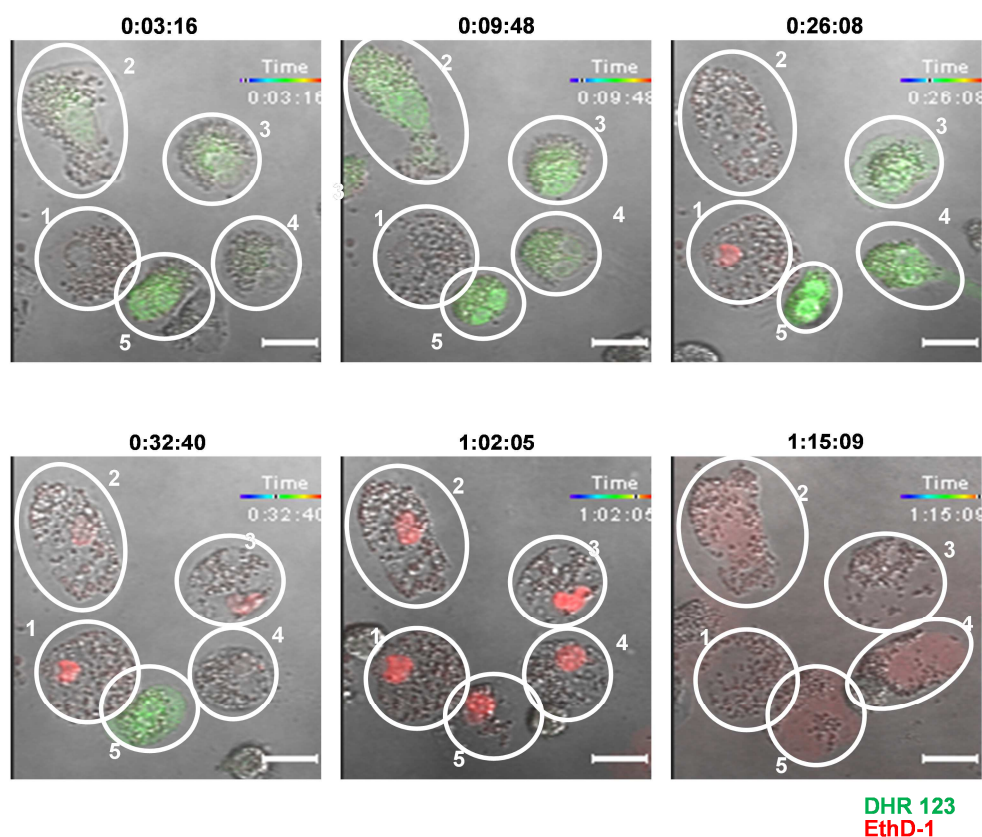


FIG E5. Increases in ROS production precede eosinophil death. Eosinophil adhesion to IVIG + iC3b - coated glass provoked cell death as assessed by live cell imaging within a time period of 2 h. Eosinophils were stained with DHR 123 (green) and EthD-1 (red). Single eosinophils were followed over time. As illustrated in Fig E1, adherent eosinophils rapidly spread on IVIG + iC3b - coated glass and subsequently generate increased levels of ROS (green). Later, the intensity of the green staining declines and the nucleus appears red, since the cells are no longer able to exclude EthD-1. This indicates loss of plasma membrane integrity. Subsequently, DNA clouds appear owing to loss of nuclear membrane integrity in the dying eosinophils. Five cells are circled and can be followed over time in Video E2. Analyzing a total of 200 eosinophils, we observed that all eosinophils demonstrating increased ROS levels subsequently lost plasma membrane integrity within 2 h.

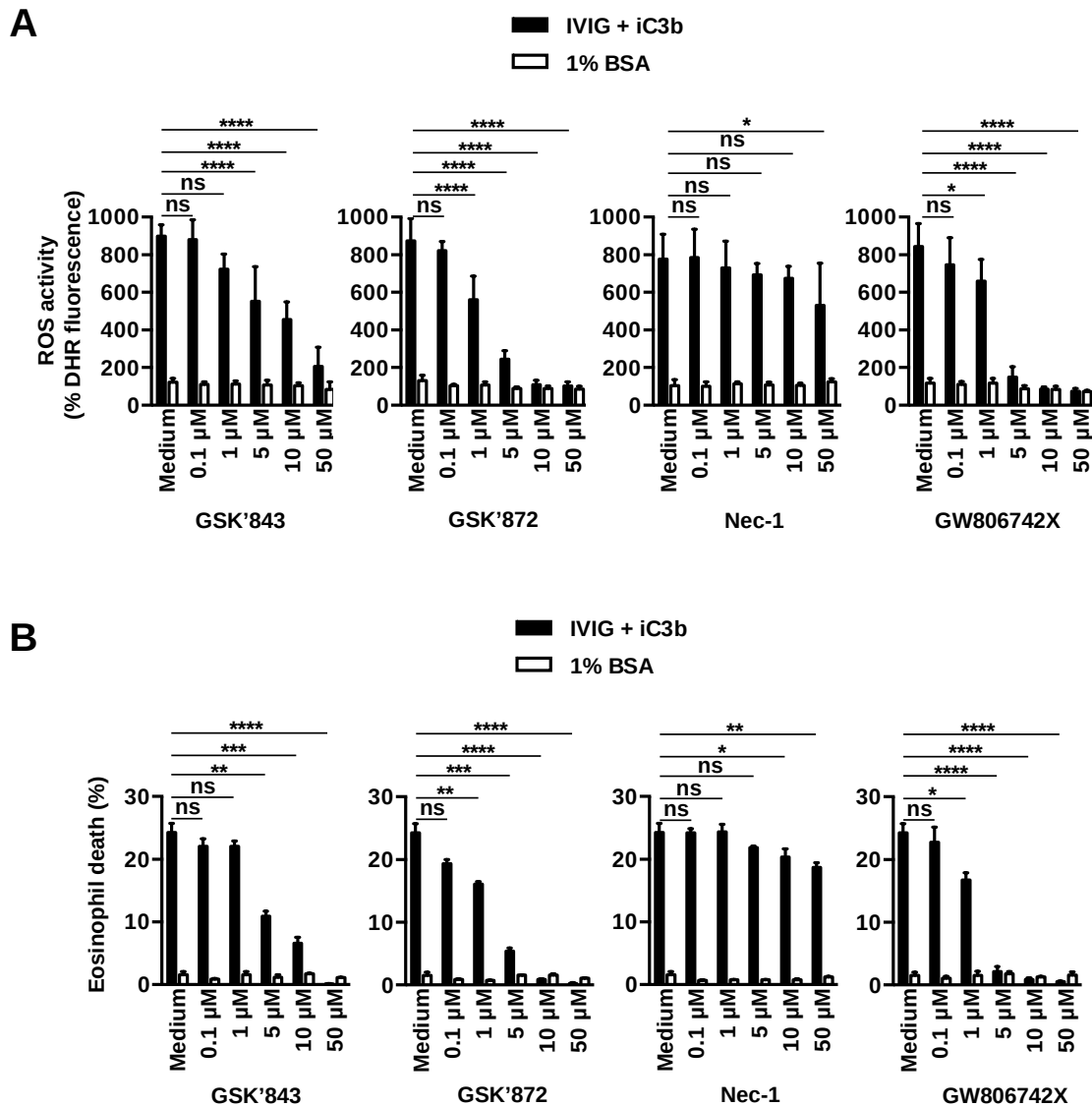


FIG E6. The inhibition of eosinophil ROS production and cell death by RIPK3 and MLKL inhibitors.

A, ROS production after 60 min by IVIG + iC3b - stimulated eosinophils was analyzed in a concentration-dependent manner. The IC₅₀s were as follows: 17.90 mM for GSK'843, 1.47 mM for GSK'872 and 1.93 mM for GW806742X. No obvious inhibition was observed using Nec-1. Values are means $\pm$ SEM. *, $p < 0.05$; ****, $p < 0.0001$; $n=3$. **B**, Cell death in IVIG + iC3b - stimulated eosinophils was analyzed 60 min after treatment in a concentration-dependent manner. The IC₅₀s were as follows: 5.42 mM for GSK'843, 2.10 mM for GSK'872 and 1.53 mM for GW806742X. Only little inhibition of death was observed using Nec-1. Values are means $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; $n=3$.

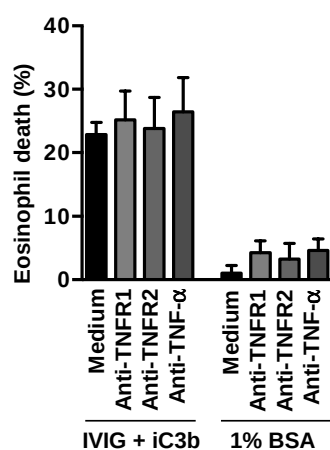


FIG E7. No evidence for a role of TNF- α – mediated eosinophil death following CR3 activation. Eosinophils were cultured in the presence and absence of the indicated blocking antibodies and either received no stimulation or were treated with IVIG + iC3b. Viability was assessed after a 1-h culture period. All values are means $\pm$ SEM; n=3.

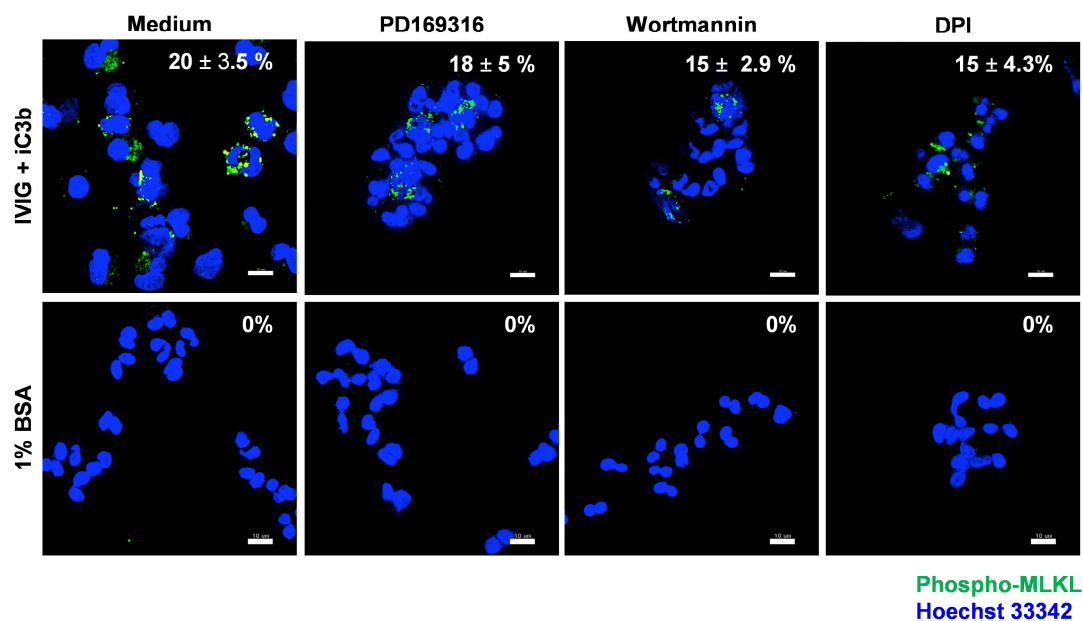


FIG E8. MLKL phosphorylation is proximal to activation of p38 MAPK, PI3K and NADPH oxidase. Eosinophils were stimulated with IVIG + iC3b for 15 min in the presence or absence of the indicated pharmacological inhibitors. Immunofluorescence staining was performed using anti-phospho-MLKL mAb (1:250). Data are representative of 3 independent experiments. The relative numbers of phospho-MLKL positive eosinophils (means ± SEM) are indicated in the upper right corner of each image. Bars, 10 μm.

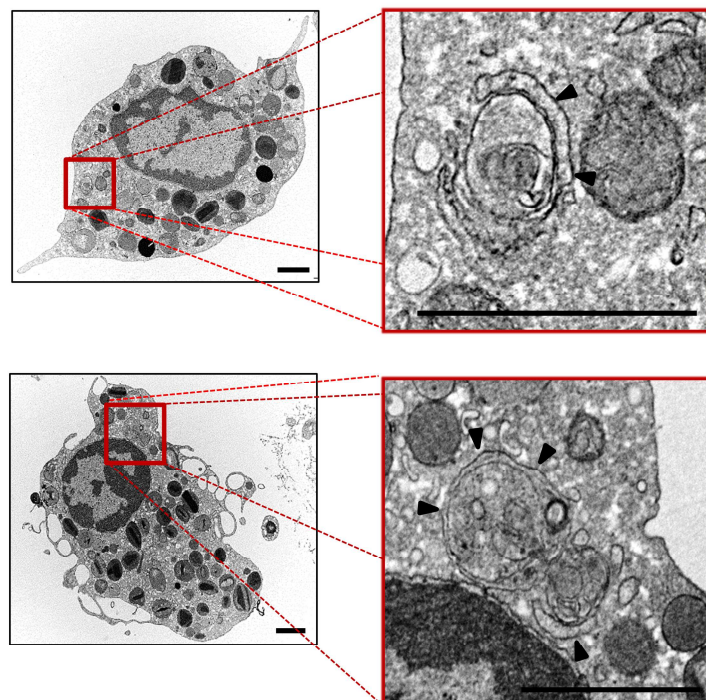


FIG E9. Autophagosome formation in eosinophils. Following 30 min of adhesion, we observed engulfment of cytosolic materials by double membrane vacuoles using TEM, suggesting autophagosome formation (upper and lower panels, closed arrowheads). Bars, 1 μm .