Monensin induces secretory granule-mediated cell death in eosinophils

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Background: Eosinophils contribute to the pathology of several types of disorders, in particular of allergic nature, and strategies to limit their actions are therefore warranted.
Objective: We sought to evaluate the possibility of targeting the acidic, lysosome-like eosinophil granules as a potential means of inducing eosinophil cell death.
Methods: To this end, we used monensin, an ionophoric drug that has previously been shown to permeabilize the secretory granules of mast cells, thereby inducing cell death.
Results: Our findings reveal that monensin induces cell death in human eosinophils, whereas neutrophils were less affected. Blockade of granule acidification reduced the effect of monensin on the eosinophils, demonstrating that granule acidity is an important factor in the mechanism of cell death. Furthermore, monensin caused an elevation of the granule pH, which was accompanied by a decrease of the cytosolic pH, hence indicating that monensin caused leakage of acidic contents from the granules into the cytosol. In agreement with a granule-targeting mechanism, transmission electron microscopy analysis revealed that monensin caused extensive morphological alterations of the eosinophil granules, as manifested by a marked loss of electron density. Eosinophil cell death in response to monensin was caspase-independent, but dependent on granzyme B, a pro-apoptotic serine protease known to be expressed by eosinophils.
Conclusions: We conclude that monensin causes cell death of human eosinophils through a granule-mediated mechanism dependent on granzyme B. (J Allergy Clin Immunol 2023;152:1312-20.)

Key words: *Eosinophils, monensin, apoptosis, granules, granzyme B*

Abbreviations used
AnnV: Annexin V
GzmB: Granzyme B
LSG: LysoSensor Green
TEM: Transmission electron microscopy

Eosinophil granulocytes are multifunctional leukocytes involved in the onset and propagation of diverse inflammatory responses. Apart from their role in host defense against pathogens, immune modulation, and tissue repair, they are potent proinflammatory cells involved in a range of human pathologies in many organ systems.[1]

Degranulation is central to eosinophil functions; the release of toxic granule proteins and production of oxygen free radicals is their main mechanism of killing invading pathogens. This is also a mechanism by which eosinophils damage cells and tissues of the host in eosinophilic inflammation.[2]

There are 4 major eosinophil granule proteins, namely, eosinophil cationic protein, major basic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase. The Charcot-Leyden crystal protein/galectin-10 is found mainly in the cytoplasm of the cells. On activation, eosinophils can also produce a range of cytokines and mediators such as leukotriene C_4 and platelet-activating factor.[3,4]

Several factors can activate and promote survival and effector functions of human eosinophils, including IL-3, IL-5, GM-CSF, immunoglobulins, and complement factors. Of these, IL-5 has been established as an essential cytokine in eosinophil biology.[5]

A number of inflammatory diseases are associated with eosinophilia, for example, asthma, allergic rhinitis, atopic skin diseases, idiopathic hypereosinophilic syndrome, eosinophil esophagitis, and inflammatory bowel disease. Thus, strategies that inhibit the activation and survival of eosinophils at the site of inflammation would be beneficial in the treatment of eosinophil-associated disorders.

Previous studies have shown that mast cells are highly sensitive to cell death induced by lysosomotropic agents (reviewed in Paivandy and Pejler[6]). Such agents diffuse across the membrane of acidic mast cell granules, and are trapped in the granules because of protonation. When the concentration of the lysosomotropic agents reaches a critical threshold, they acquire detergent-like properties, causing granule membrane permeabilization, which in turn induces cell death.[7,8]

Mast cells and eosinophils have many features in common, and are often found in proximity to each other in the tissue, particularly in allergic disorders but also in apparently cell-specific diseases such as mastocytosis and eosinophilic esophagitis, indicating a functional unity between the 2 cell types.[9] Similar to mast cells, eosinophils are rich in lysosome-like acidic granules; the pH of specific granules in resting eosinophils is approximately 5.2.[10] On the basis of this, we reasoned that lysosomotropic agents may induce eosinophil cell death through similar mechanisms as in mast cells.

In a recent study we showed that monensin, an ionophoric compound used in poultry for its antibacterial and antiparasitic

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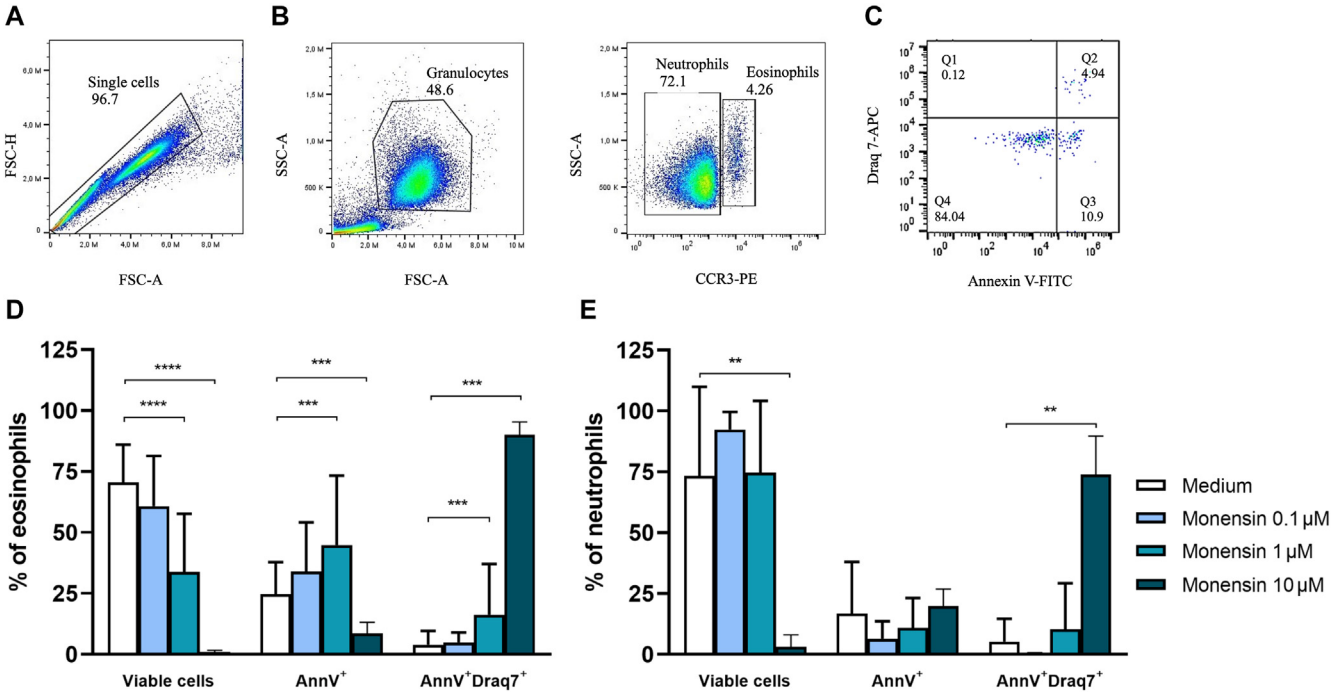

FIG 1. Monensin induces cell death in human peripheral blood eosinophils but has less effects on neutrophils. General gating strategies consisting of exclusion of doublets and debris (**A**), identification of human peripheral blood eosinophils and neutrophils (**B**), and differentiation of cell populations regarding viability (**C**). Eosinophils (**D**) and neutrophils (**E**) were treated for 2 hours with monensin at 0.1, 1, or 10 μM. Effect on cell viability was assessed by staining with AnnV and Draq7. Viable cells: $AnnV^{-}Draq7^{-}$; apoptotic cells: $AnnV^{+}$ $Draq7^{-}$; necrotic/late apoptotic cells: $AnnV^{+}Draq7^{+}$ (n = 15). *APC*, Antigen-presenting cell; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *FSC-A*, forward scatter-area; *FSC-H*, forward scatter-height; *SSC-A*, side scatter-area. The bar charts show mean values + SD. Significance was tested with 1-way ANOVA with *post hoc* Tukey test. $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

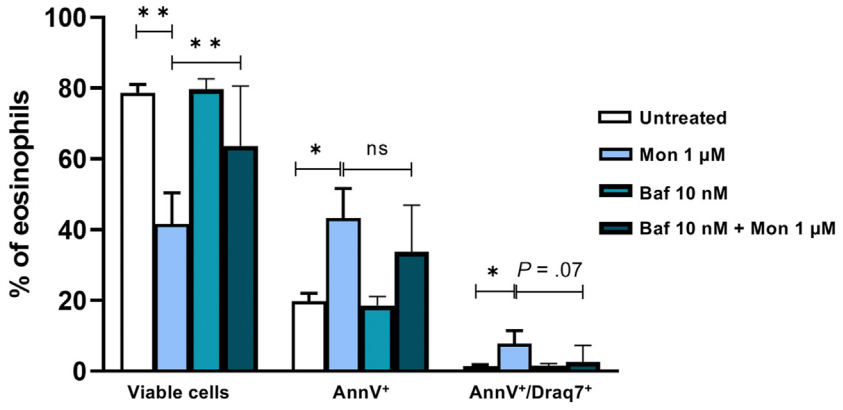

FIG 2. The V-ATPase-inhibitor Bafilomycin A1 reduces the cytotoxic effects of monensin on eosinophils. Human peripheral blood eosinophils were preincubated with 10 nM Bafilomycin A1 (Baf) for 2 hours followed by 2-hour treatment with monensin (1 μM). Cell viability was assessed by staining with AnnV and Draq7. Viable cells: $AnnV^{-}Draq7^{-}$; apoptotic cells: $AnnV^{+}Draq7^{-}$; necrotic/late apoptotic cells: $AnnV^{+}Draq7^{+}$ (n = 7). *ns*, Not significant; *V-ATPase*, vacuolar H^{+}-ATPase. The bar charts show mean values + SD. Significance was tested with 1-way ANOVA with *post hoc* Tukey test. $^{*}P < .05$; $^{**}P < .01$.

activity, has lysosomotropic and potent cytotoxic effects on mast cells, and that it has potent cytotoxic effects on these cells.[11] Here, we investigated whether monensin can also cause cell death of eosinophils. Indeed, we show that monensin is cytotoxic for human peripheral blood eosinophils, and that this occurs through a granule-mediated mechanism. Potentially, monensin could hence be explored for therapeutic purposes in diseases characterized by excessive amounts of eosinophils and mast cells, such as allergic disorders.

METHODS

For further experimental details, see this article's Methods section in the Online Repository at www.jacionline.org.

Preparation of peripheral blood granulocytes and isolated eosinophils

Eosinophil and neutrophil granulocytes were isolated from buffy coats obtained from peripheral blood of healthy donors. Granulocytes were isolated by Percoll gradient centrifugation; eosinophils and neutrophils were separated from each other using anti–CD16-coated magnetic particles.

Incubation of granulocytes with monensin and inhibitors

Granulocytes or purified eosinophils were cultured in RPMI supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, and antibiotics. Monensin at different concentrations was added. The effect of monensin on granulocyte viability was evaluated by flow cytometry analysis. In some experiments, granulocytes were pretreated with the caspase-3/7 inhibitor Z-DEVD-FMK or the granzyme B (GzmB) inhibitor Z-AAD-CMK. Caspase activation was tested by using the caspase-3/7 Green Detection Reagent. To assess whether the granule acidity has an impact on the responsiveness of granulocytes to monensin-induced cell death, granulocytes were pretreated with the vacuolar H^+-ATPase inhibitor Bafilomycin A1.

Flow cytometric analysis of cell viability

Treated granulocytes were incubated with Annexin V (AnnV) and Draq7, and cell viability was evaluated by flow cytometry. Granulocytes were gated by their forward- and side scatter properties, and by specific surface markers. Eosinophils were gated as cells double positive for CD15 and CCR3, with lower fluorescence intensity of CD15 than in neutrophils. Viable cells were identified as $AnnV^-Draq7^-$ cells and nonviable cells as either $AnnV^+Draq7^-$ or $AnnV^+Draq7^+$.

Measurement of granule and cytosolic pH

Changes in granule acidity were evaluated using LysoSensor Green (LSG). Cytosolic pH was measured using the cell-permeable fluorescent indicator BCFL-AM.

Transmission electron microscopy and β-hexosaminidase release assay

Transmission electron microscopy (TEM) analysis[12] and the β-hexosaminidase release assay[13] were performed as described.

Activation of eosinophils

Isolated eosinophils were primed with 4 nM rhIL-5 for 20 minutes and subsequently stimulated with 10 nM rhC5a for 15 minutes. To monitor eosinophil degranulation, the plasma membrane expression of the surrogate marker CD63 (Invitrogen, Waltham, Mass) was analyzed using flow cytometry.

GzmB immunofluorescence

Purified human eosinophils were left untreated or treated with monensin alone or in the presence of the GzmB inhibitor Z-AAD-CMK. Samples of the cells were applied into a circle drawn using a water repellent pen and cells were allowed to settle. The liquid was aspirated from the top of the samples by capillarity using filter paper, followed by fixation with formaldehyde, followed by permeabilization and blocking with goat serum. Slides were then incubated with rabbit antihuman GzmB antibody, and then incubated with goat antirabbit Alexa-488 antibody. Slides were then incubated with ActinRed 555, and NucBlue Samples were mounted using SlowFade diamond antifade mountant and analyzed by confocal microscopy.

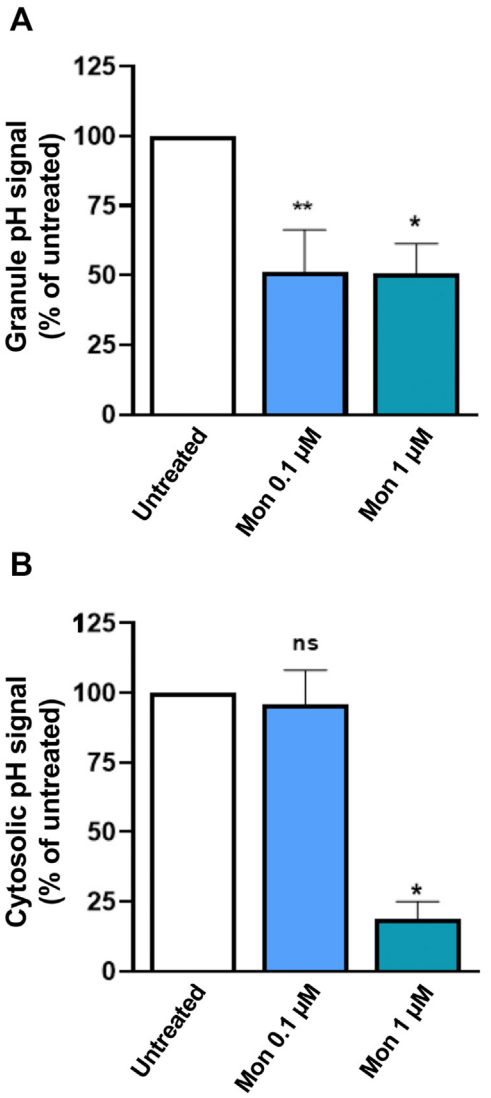

FIG 3. Monensin causes an increase in granule pH and acidification of the cytosol in eosinophils. Peripheral blood eosinophils were treated with monensin (0.1 μM or 1 μM) for 2 hours, followed by staining with LSG, which enters the secretory granules and displays higher fluorescence at low pH (**A**) (n = 7), or the cell-permeable fluorescent indicator BCFL-AM, which produces strong fluorescence at high pH (**B**) (n = 4). *ns,* Not significant. The bar charts show mean values + SD. Significance was tested with 1-way ANOVA with *post hoc* Tukey test. **P* < .05; ***P* < .01.

Statistical analysis and graphs

Data were analyzed using the GraphPad Prism 9.3 statistics and graphing software (GraphPad Software, San Diego, Calif). For comparison between treated and nontreated cells, Wilcoxon matched-pairs signed rank-test was used. Significance was tested with 1-way ANOVA with *post hoc* Tukey test. Statistical significance was set at *P* less than .05.

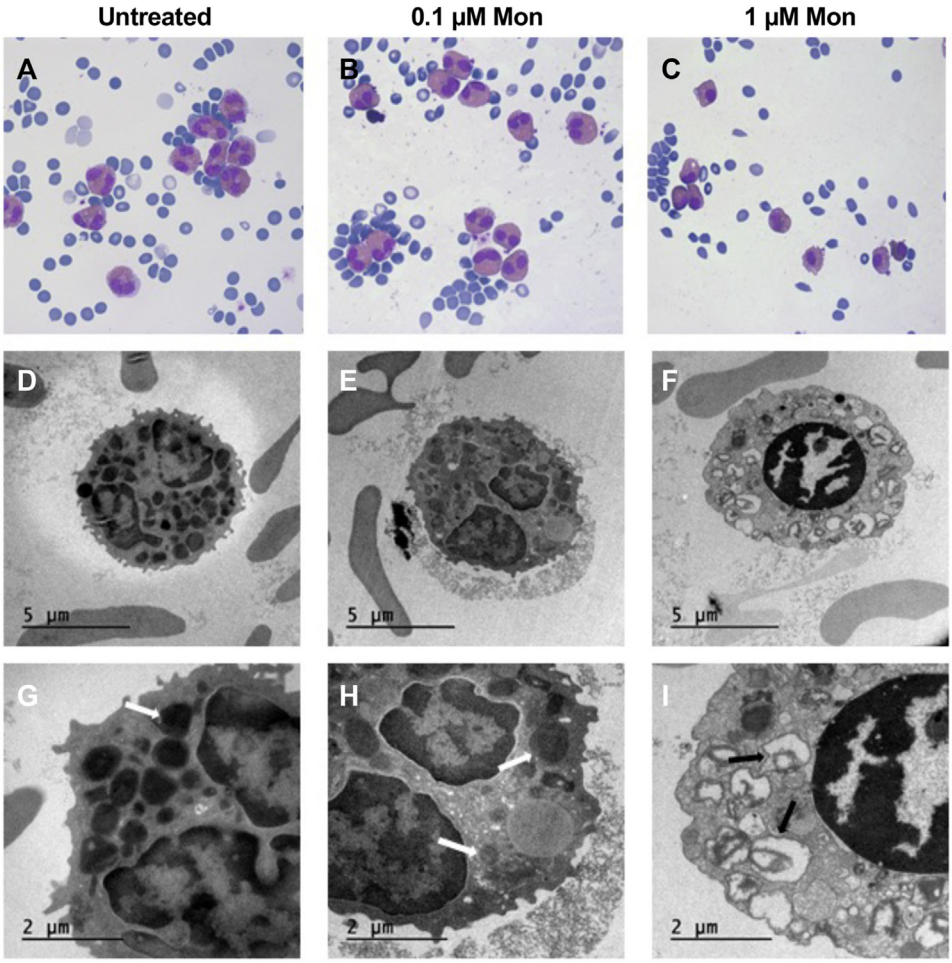

FIG 4. Monensin affects the morphology of eosinophils. **A-C,** Cytospin preparations (May Grünwald-Giemsa staining) of peripheral blood eosinophils, either untreated (Fig 4, *A*) or treated with 0.1 μM (Fig 4, *B*) or 1 μM (Fig 4, *C*) monensin for 2 hours. **D-I,** TEM images of peripheral blood eosinophils, either untreated (Fig 4, *D* and *G*) or treated with 0.1 μM (Fig 4, *E* and *H*) or 1 μM (Fig 4, *F* and *I*) monensin for 2 hours. White and black arrows in the enlarged images (Fig 4, *G-I*) highlight the marked alterations in granule morphology caused by monensin.

RESULTS

Monensin induces apoptotic cell death in eosinophils

Eosinophils and neutrophils were purified from peripheral blood of healthy donors (Fig 1, *A-C*) and incubated with monensin at 0 to 10 μM. The cells were then stained with AnnV and Draq7, and cell viability was assessed by flow cytometry. At the highest concentration (10 μM), monensin was cytotoxic to both eosinophils and neutrophils, causing cell death ($AnnV^+Draq7^+$) (Fig 1, *D* and *E*). In contrast, monensin at 1 μM caused mainly apoptosis-like cell death of eosinophils ($AnnV^+Draq7^-$), but did not affect neutrophil survival. At lower concentrations, monensin did not have any significant effect on either eosinophil or neutrophil survival (Fig 1, *D* and *E*).

Loss of granule acidity prevents the effect of monensin on eosinophil cell death

In resting cells, eosinophil-specific granules have an acidic pH (≈5.2), which is maintained by a vacuolar H^+-ATPase that serves as a proton pump across the granule membrane.[10] Studies on mouse and human mast cells have indicated that granule acidity is important for their responsiveness to monensin.[11] To assess the importance of granule acidity for the effect of monensin on eosinophils, we used Bafilomycin A1, a vacuolar H^+-ATPase inhibitor. Bafilomycin A1 alone did not have any effect on eosinophil survival (Fig 2). However, preincubation of eosinophils with 10 nM Bafilomycin A1 could partially protect the eosinophils against monensin-induced cell death, indicating that granule acidity is a prerequisite for monensin to exert its effect on eosinophils eosinophils (Fig 2).

Monensin causes an increase in granule pH and acidification of the cytosol

Monensin has previously been shown to target the lysosome-like granules of mast cells, causing increased pH and permeabilization of the granule membrane. To test the possibility of a similar mechanism in eosinophils, we stained untreated and monensin-treated eosinophils with LSG and BCFL-AM. LSG enters lysosome-like organelles and produces high fluorescence

under acidic conditions. Cytosolic pH was measured using BCFL-AM, which, in contrast to LSG, produces strong fluorescence at high pH. Untreated eosinophils stained strongly with LSG, in agreement with the high content of acidic granules in resting eosinophils (Fig 3, *A*). Treatment with monensin at 0.1 μM or 1 μM resulted in a significant decrease in the fluorescence intensity, indicating that monensin caused increased granule pH (Fig 3, *A*). Notably, only the higher concentration of monensin (1 μM) caused acidification of the cytosol, assessed as decreased BCFL-AM fluorescence (Fig 3, *B*). Thus, monensin at 0.1 μM affects the granule pH, but higher monensin concentrations are needed to permeabilize the granule membrane, leading to efflux of the acidic granule content into the cytosol (Fig 3, *B*).

Monensin causes morphological alterations in eosinophils

To provide further insight into how monensin affects eosinophils, the impact of monensin on eosinophil morphology was evaluated. For this purpose, purified peripheral blood eosinophils were incubated with monensin at 0, 0.1, or 1 μM, and were then prepared for TEM analysis or used for cytospin preparations. Light microscopy analysis (May Grünwald-Giemsa staining) of eosinophils treated with 1 μM of monensin revealed marked morphological alterations, as manifested by a condensed nucleus and decreased cell size. Such effects were not observed at the lower monensin concentration (Fig 4, *A-C*). More detailed insight into how monensin affects eosinophils was obtained by the TEM analysis. Unstimulated eosinophils showed the characteristic features of eosinophils: a bilobed nucleus and an abundance of specific granules with an electron-dense crystalline core and an outer electron-lucent matrix surrounded by a delimiting membrane (Fig 4, *D* and *G*). Eosinophils incubated with 0.1 μM of monensin showed no signs of cell death; the nucleus was intact, and the cell size was not significantly affected. However, the granules were less electron dense, indicating an effect of 0.1 μM monensin on the granule structure (Fig 4, *E* and *H*). In agreement with our flow cytometry data (see Fig 1), 1 μM of monensin caused morphological effects indicative of apoptosis-like cell death, including a profound nuclear condensation. Notably, and in agreement with a mechanism in which monensin acts through a granule-dependent pathway, extensive damage to the granules was also seen. Specifically, the dense core of the specific granules was lost, and the granules were partly emptied, with some remaining granule content accumulated at the border of the granules (Fig 4, *F* and *I*).

GzmB contributes to monensin-induced eosinophil cell death

To further address the mechanism of cell death in response to monensin, we investigated the importance of caspase activation. We found that monensin at 1 μM induced caspase-3/7 activation, and that this was inhibited by the caspase-inhibitor Z-DEVD-FMK (Fig 5, *A*). However, monensin-induced eosinophil cell death was not inhibited by Z-DEVD-FMK, suggesting that caspase-independent mechanisms of cell death are predominant (Fig 5, *B*).

To search for such caspase-independent mechanisms of cell death, we assessed the importance of GzmB. GzmB is a pro-apoptotic serine protease contained in cytotoxic granules of innate and adaptive immune killer cells. Notably, GzmB is also

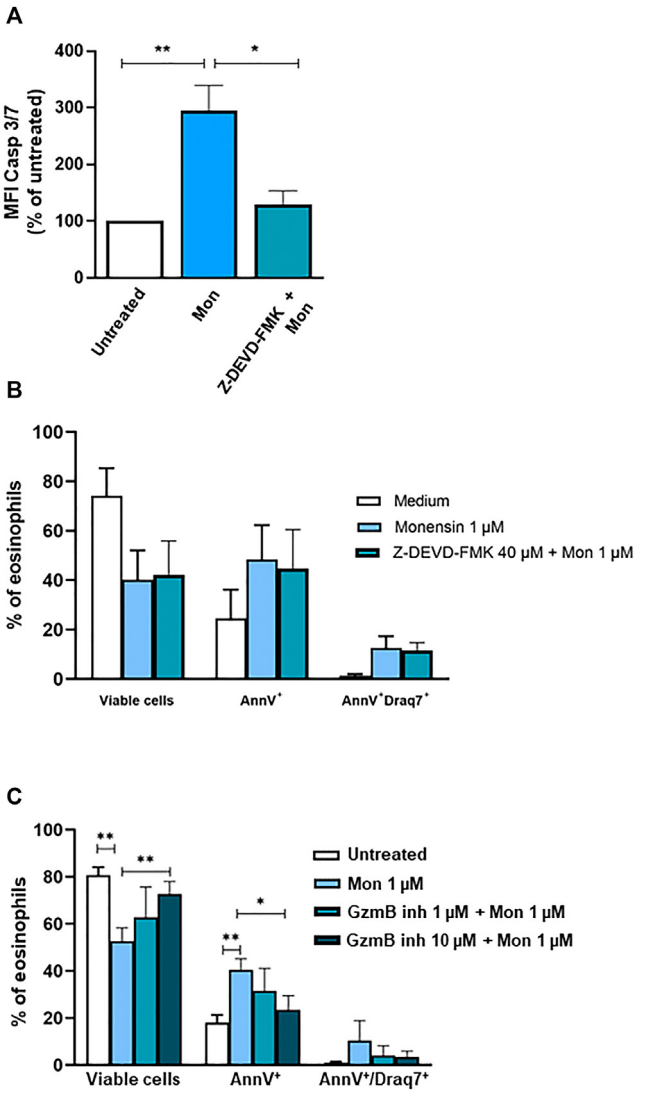

FIG 5. GzmB contributes to monensin-induced eosinophil cell death. **A,** Peripheral blood eosinophils were treated with monensin (1 μM) for 2 hours, and caspase-3/7 activation was assessed by flow cytometry (n = 7). **B** and **C,** Eosinophils were pretreated with either the caspase-3/7 inhibitor Z-DEVD-FMK (Fig 5, *B*) or the GzmB inhibitor Z-AAD-CMK (Fig 5, *C*) before stimulation with monensin (1 μM) for 2 hours (n = 4). Effects on cell viability were assessed by staining with AnnV and Draq7. Viable cells: $AnnV^-Draq7^-$; apoptotic cells: $AnnV^+Draq7^-$; necrotic/late apoptotic cells: $AnnV^+Draq7^+$ (n = 5). *MFI,* Mean fluorescence intensity. The bar charts show mean values + SD. Significance was tested with 1-way ANOVA with *post hoc* Tukey test. $*P < .05$; $**P < .01$.

expressed by mast cells and has been shown to induce mast cell apoptosis upon granule leakage caused by lysosomotropic agents such as L-leucyl-L-leucine methyl ester.[14] Because human eosinophils have been shown to express both mRNA and protein for GzmB,[15] we assessed the involvement of this serine protease in monensin-induced eosinophil cell death. Indeed, the GzmB inhibitor Z-AAD-CMK inhibited monensin-induced cell death in a dose-dependent manner (Fig 5, *C*), indicating that GzmB contributes to the execution of eosinophil cell death in response to monensin.

The findings above suggest that GzmB might be present in the eosinophil granules and is translocated into the cytosol on

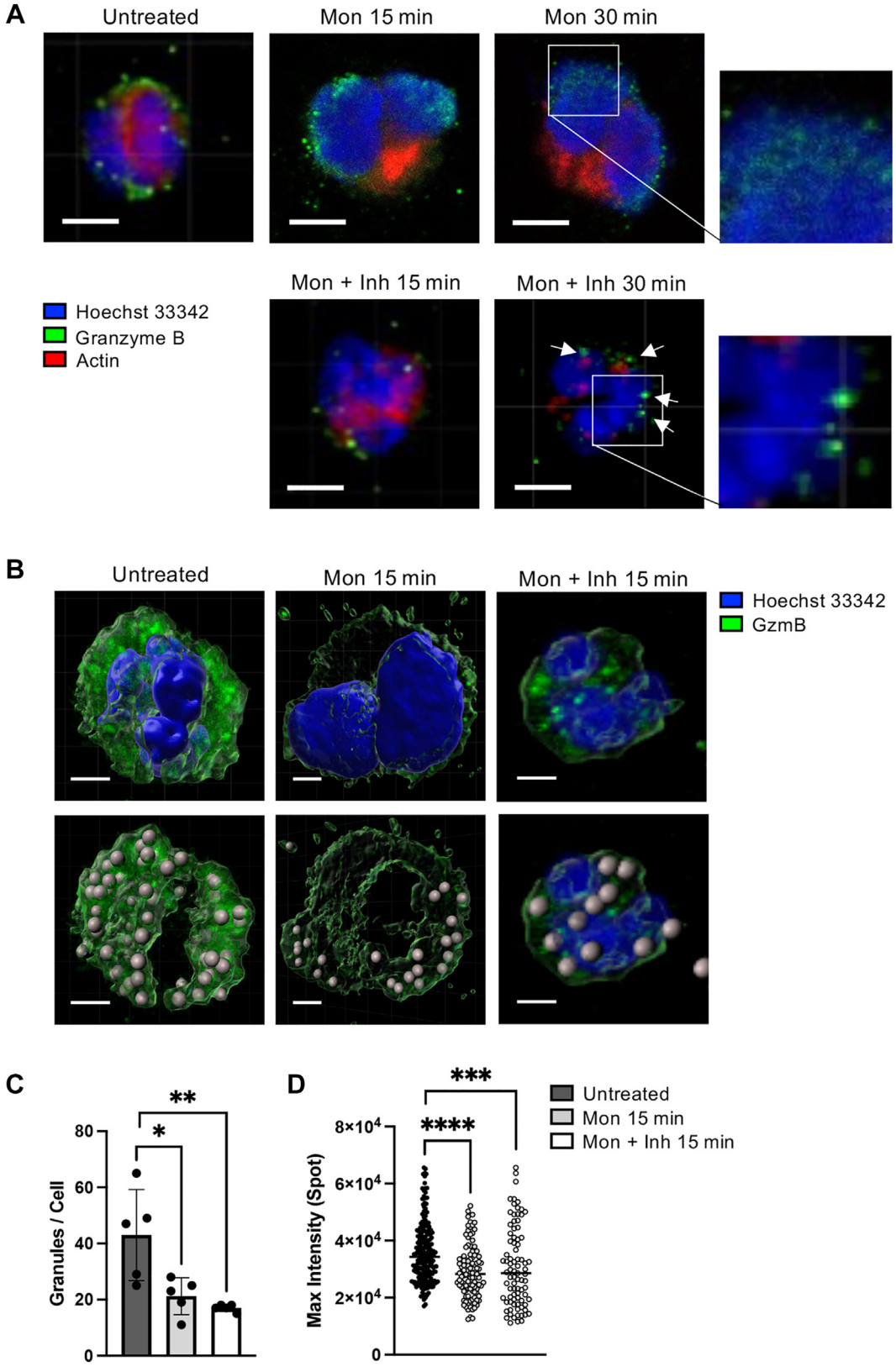

FIG 6. GzmB is present in eosinophil granules and translocates to the cytosol after treatment with monensin. Eosinophils were treated with monensin (Mon) (1 μM) alone or in the presence of the GzmB inhibitor Z-AAD-CMK (10 μM) for 15 or 30 minutes. **A,** Immunofluorescence staining for GzmB (green), actin (red), and nucleus (blue). Note the granule-like appearance of GzmB staining in controls and a more diffuse GzmB staining on monensin treatment. Note also that GzmB inhibition partly preserved the integrity of

exposure to monensin. To address this, confocal analysis was performed. Indeed, strong GzmB positivity was found in granule-like organelles within the nontreated eosinophils (Fig 6; see Fig E1 in this article's Online Repository at www.jacionline.org). After exposure to monensin, the number of GzmB-positive granules was clearly reduced, and the granule size was decreased. This was accompanied by an increased GzmB positivity in the cytoplasmic region. GzmB inhibition partly preserved granule size after exposure to monensin. Altogether, these findings suggest that GzmB is present in the eosinophil granules and translocates to the cytosol on exposure to monensin.

Monensin counteracts acidification of eosinophil granules on IL-5 priming but does not affect degranulation

IL-5 has previously been shown to cause acidification of eosinophil granules, which may be one of the prerequisites for degranulation.[16,17] Given that monensin increased the intragranular pH of eosinophils, we speculated that monensin could prevent this effect of IL-5. To test this, eosinophils were first treated with monensin at concentrations that did not cause cell death according to our previous experiments (0.1 μM and 0.05 μM), followed by stimulation with IL-5 (4 nM) for 20 minutes. The intragranular pH was then assessed by using LSG. These analyses revealed that monensin, at both 0.1 and 0.05 μM, prevented the granule acidification caused by IL-5 (Fig 7, *A*).

Next, we assessed whether monensin can prevent the priming effect of IL-5 on degranulation, by inhibiting IL-5–induced granule acidification. Previous investigations have established that C5a is highly efficient in stimulating degranulation in IL-5–primed human eosinophils.[18] We confirmed this by showing increased CD63 expression after incubation of purified eosinophils with 10 nM C5a following IL-5 priming (Fig 7, *B*). However, preincubation of the cells with 0.1 μM monensin did not significantly inhibit CD63 expression (Fig 7, *B*). In line with this, preincubation with monensin did not prevent the morphological changes caused by IL-5, as shown by TEM analysis. Notably, the IL-5–induced effects included emissions of multiple pseudopods and disarrangement of the crystalline cores of the secretory granules (Fig 7, *C-E*). As expected, IL-5 caused a robust release of β-hexosaminidase (a lysosome/granule marker) from the eosinophils. In contrast, no significant release of β-hexosaminidase was seen in response to monensin (Fig 7, *G*), indicating that monensin causes minimal eosinophil degranulation.

DISCUSSION

Eosinophils and mast cells play pivotal roles in allergic responses and often localize in close proximity in inflammatory disorders.[19] The interaction between the 2 cell types results in mutual activation, which can contribute to the perpetuation of inflammation.[20] Strategies to target mast cell-eosinophil interactions could therefore be of great interest, and one such strategy may be to induce selective eosinophil/mast cell death.[1]

In previous studies we have established a strategy for inducing selective mast cell death, through targeting their acidic secretory granules by using monensin and other types of lysosomotropic agents.[11,12,21-23] However, before this study, the impact of granule-targeting strategies on eosinophils has not been studied. Considering that eosinophils, similar to mast cells, are characterized by an exceptionally high content of acidic secretory granules, we reasoned that eosinophils also may be sensitive to granule-targeting drugs. Indeed, we show here that monensin has a potent cytotoxic effect on human eosinophils. Thus, monensin may emerge as a novel tool for the selective and simultaneous depletion of both eosinophils and mast cells at sites of inflammation.

An important finding was that monensin causes eosinophil cell death predominantly by an apoptosis-like mechanism, as judged by AnnV/Draq7 staining. This was also confirmed by TEM analysis, where we observed typical signs of apoptosis after monensin treatment, such as nuclear condensation and damaged granules, while the outer cell membrane was intact. In a clinical context, apoptosis is preferable over necrosis, because it causes minimal damage to the surrounding tissues. However, the cytotoxic effect of monensin was strictly dose-dependent: 1 μM of monensin induced eosinophil, but not neutrophil, cell death, whereas 10 μM of monensin was cytotoxic to both cell types, causing necrotic death. Monensin doses below 1 μM, however, had no effect on eosinophil survival.

Mechanistically, we could show that the acidity of eosinophil granules was essential for the ability of monensin to cause eosinophil cell death. Lysosomotropic agents are weak bases that accumulate in acidic granules by ion trapping, and this is a prerequisite for the membrane damage and subsequent leakage of granule contents into the cytosol. Notably, neutrophil granules are more heterogeneous than in eosinophils, and the intragranular pH in resting neutrophils is higher than in the eosinophils, ranging from 5.5 to 7.5.[24] Thus, the lower amounts of acidic granules in neutrophils than in eosinophils may explain the relative lack of effect of monensin on neutrophils.

In agreement with a mechanism in which monensin acts through a granule-mediated pathway, monensin caused an increase in the pH of the eosinophil granules. Interestingly, monensin concentrations as low as 0.1 μM caused a raised intragranular pH. At this drug concentration, however, there was apparently no leakage from the granules to the cytosol, as indicated by an absence of altered cytosolic pH. In concordance with this observation, TEM analysis revealed that 0.1 μM of monensin caused morphological changes in the granules with loss of the dense core, whereas the nucleus was not affected, and no other signs of cell death were seen. However, at higher monensin concentrations (1 μM), we observed that the loss of granule acidity was accompanied by a corresponding decrease in cytosolic pH, indicating leakage of acidic granule content into the cytosol. Notably, cytosolic acidification is a common feature of apoptotic

GzmB-positive granule-like structures (white arrows); bar scale = 5 μm. **B,** 3-D view generated from Z-stack sections; staining for GzmB (green) and nucleus (blue). Lower panels are showing areas (gray spheres) where GzmB staining is more intense (granules); bar scale = 2 μm. **C,** Number of intensely stained GzmB-positive spots (granules) per cell. **D,** Max fluorescence intensity per spot (granule); untreated (black circles), monensin-treated cells (gray circles), and monensin-treated cells in the presence of Z-AAD-CMK. Data are represented as mean values ± SD and analyzed with unpaired *t* test using PrismaPad 9 software. $*P \leq .05$; $**P \leq .01$; $***P \leq .001$; and $****P \leq .0001$.

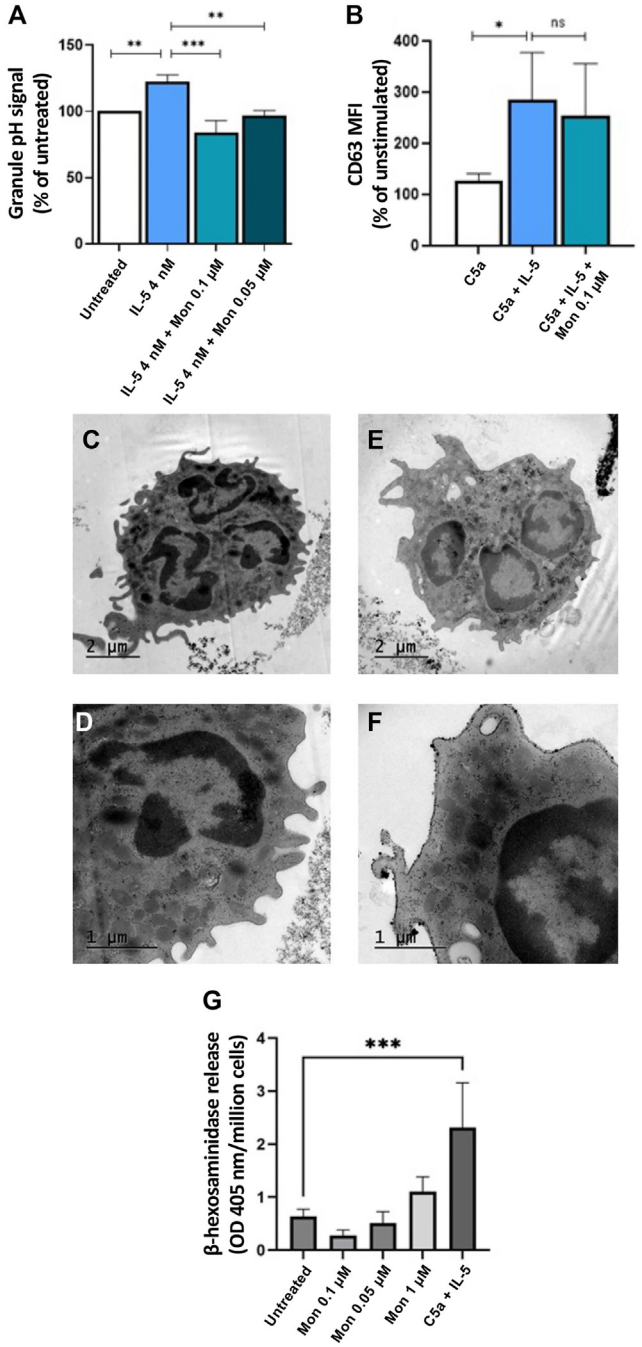

FIG 7. Monensin prevents IL-5–induced acidification of eosinophil granules but has no effect on degranulation. Purified eosinophils were primed with 8 nM rhIL-5 for 20 minutes followed by stimulation with 10 nM rhC5a for 15 minutes, with or without preincubation with monensin (Mon; 0.1 μM or 0.05 μM). Intragranular pH was evaluated by LSG (**A**); eosinophil degranulation was assessed by CD63 staining (**B**) analyzed by flow cytometry and by the β-hexosaminidase release assay (**G**) (untreated, n = 11; Mon 0.1 μM, n = 5; Mon 0.05 μM, n = 5; Mon 1 μM, n = 6; C5a + IL-5, n = 11). **C-F,** TEM images of eosinophils treated as above with IL-5 and C5a without Mon (Fig 7, *C* and *D*), or preincubated with 0.1 μM Mon for 2 hours (Fig 7, *E* and *F*). *MFI,* Mean fluorescence intensity. The bar charts show mean values + SD in *A* and *B* and mean values + SEM in *G*. Significance was tested with 1-way ANOVA with *post hoc* Tukey test (Fig 7, *A* and *B*), paired *t* test, or Friedman test (Fig 7, *G*). **P* < .05; ***P* < .01; ****P* < .001.

cells irrespective of pathway.[25] On the basis of our observations, we may thus propose that granule deacidification, as a result of granule permeabilization, plays a key role in the mechanism of cell death in eosinophils exposed to monensin. According to a recent classification of cell death mechanisms,[26] we may hence propose that monensin induces lysosome-dependent cell death in eosinophils.

Apoptotic cell death is typically dependent on effector caspases (caspase-3/7), but caspase-independent apoptosis can also occur.[27] To provide further mechanistic insight into how monensin causes eosinophil cell death, we thus investigated the involvement of caspases. Indeed, flow cytometric analysis revealed that monensin caused caspase-3/7 activation, and that this activation could be counteracted by the selective caspase-3 inhibitor Z-DEVD-FMK. However, the inhibition of caspase-3 did not prevent cell death, indicating that more complex, caspase-independent mechanisms are involved in the execution of cell death.

GzmB is a serine protease, mainly produced by natural killer cells and cytotoxic T cells, whose main function is to induce apoptotic cell death to eliminate virus-infected cells and tumor cells. A number of myeloid cells, including mast cells, basophils, and eosinophils, have also been demonstrated to express GzmB, and GzmB may thus have a role in regulating cell death in such cellular contexts.[15,27] For example, in a recent study on mouse mast cells, GzmB was shown to have a critical role in cell death induced by the lysosomotropic agent L-leucyl-L-leucine methyl ester.[14] In the cytosol, GzmB can induce cell death by activating caspases such as caspase-3, but GzmB can also activate the mitochondrial and DNA damage pathways independently of caspases. Therefore, caspase inhibitors have little effect on human GzmB-mediated cell death and DNA fragmentation.[27] Thus, one possibility would be that GzmB released to the cytosol from leaking granules may be responsible for the execution of cell death in monensin-treated eosinophils. Indeed, our findings support a major role for GzmB in the execution of eosinophil cell death in response to monensin.

Degranulation is one of the key effector functions of eosinophils in response to diverse stimuli, and IL-5 priming has been reported to significantly increase eosinophil granule protein release.[28] As we and others have shown (this study and Kurashima et al[10]), the eosinophil-specific granules are acidic, and the acidity increases on cellular activation. Potentially, this may be an important step in the degranulation process: major basic protein crystals stored in the granule core can be solubilized before release as a consequence of granule acidification, and both eosinophil peroxidase and major basic protein show increased cytotoxic activity at acidic pH.[16,17] On the basis of this, we here assessed whether IL-5 causes granule acidification in the eosinophils and whether this could be affected by monensin. Indeed, our finding confirms that IL-5 causes a significant granule acidification, and we also show that such granule acidification is inhibited by monensin. This prompted us to investigate whether monensin can inhibit the priming effect of IL-5 on eosinophil degranulation. Supporting this notion, a previous study demonstrated that the lysosomotropic agent NH_4Cl increased eosinophil granule pH and inhibited eosinophil peroxidase exocytosis induced by platelet-activating factor.[10] As expected, IL-5 priming caused prominent eosinophil

degranulation, as manifested by an increase in C5a-induced CD63 expression.[29] However, monensin did not have a significant effect on either CD63 expression or on the morphology changes caused by IL-5 activation.

Conclusions

Our study indicates that monensin causes eosinophil cell death through a granule-mediated pathway involving GzmB. Together with our previous findings on mast cells, this introduces the possibility of using monensin or other lysosomotropic agents as a therapeutic option for the selective reduction of eosinophil and mast cell numbers at sites of inflammation.

DISCLOSURE STATEMENT

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Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Clinical implications: Our results suggest that granule-targeted drug therapy by monensin or other lysosomotropic agents may have potential in the management of eosinophil-associated diseases.

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METHODS

Blood donors

Peripheral blood from healthy donors was obtained from the blood donor venue at Uppsala University hospital. In experiments with mixed granulocytes (neutrophils and eosinophils), 15 mL of heparinized blood from each donor was used. Freshly obtained buffy coats were used when eosinophils were further isolated from the granulocyte mixture.

Preparation of peripheral blood granulocytes and isolated eosinophils

Eosinophil and neutrophil granulocytes were isolated from peripheral blood from healthy donors: granulocytes were isolated by Percoll (1.082 g/mL) gradient centrifugation. Red blood cells were removed by hypotonic lysis. In some of the experiments, eosinophils and neutrophils were separated from each other using anti–CD16-coated magnetic particles (Miltenyi Biotec; Bergisch Gladbach, Germany) in a magnetic field. In these experiments, fresh buffy coats from healthy donors were used, to obtain sufficient cell numbers. Differential counts were obtained using cytospin slides (Cytospin, Shandon Southern Instruments, Sewickley, Pa) stained with May Grünwald-Giemsa (Sigma-Aldrich/Merck, Darmstadt, Germany), and were examined under a light microscope.

Incubation of granulocytes with monensin and inhibitors

Granulocytes or purified eosinophils were seeded in 96-well plates in RPMI (Gibco/Thermo Fisher Scientific, Waltham, Mass) supplemented with 10% heat-inactivated FBS (Thermo Fischer, Waltham, Mass), 4 mM L-glutamine, and antibiotics (penicillin/streptomycin; Gibco). Monensin (Sigma-Aldrich) at different concentrations was added to selected wells, and the plates were incubated for 2 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. The effect of monensin on granulocyte viability was evaluated by flow cytometry analysis (see below). To delineate the mechanisms behind monensin-induced cell death, granulocytes were pretreated with the caspase-3/7 inhibitor Z-DEVD-FMK (AH Diagnostics, Solna, Sweden) or the GzmB inhibitor Z-AAD-CMK (Sigma-Aldrich) before stimulation with monensin. The ability of monensin to cause caspase activation was tested by staining of monensin-treated granulocytes with caspase-3/7 Green Detection Reagent (Invitrogen) and evaluation of the induced fluorescein isothiocyanate fluorescence by flow cytometry. To assess whether the granule acidity has an impact on the responsiveness of granulocytes to monensin-induced cell death, granulocytes were pretreated with the vacuolar H^+-ATPase inhibitor Bafilomycin A1 (Sigma-Aldrich) at 10 nM before stimulation with monensin.

Flow cytometric analysis of cell viability

Treated granulocytes were incubated with AnnV and Draq7, and cell viability was evaluated by flow cytometry. Granulocytes were gated by their forward- and side scatter properties, and by specific surface markers. Eosinophils were gated as cells double positive for CD15 and CCR3 (Invitrogen), with lower fluorescence intensity of CD15 than neutrophils. Viable cells were identified as $AnnV^-Draq7^-$ cells, apoptotic cells as $AnnV^+Draq7^-$, and necrotic/late apoptotic cells as $AnnV^+Draq7^+$. Cells were analyzed with a 2 laser Accuri instrument (BD Biosciences, Franklin Lakes, NJ). Data analysis was performed using the Kaluza 2.1 software (Beckman Coulter, Indianapolis, Ind).

Measurement of granule and cytosolic pH

Changes in granule acidity were evaluated using LSG (Invitrogen), a probe that accumulates in acidic organelles, and whose fluorescence is dependent on protonation. Cytosolic pH was measured using the cell-permeable fluorescent indicator BCFL-AM (Sigma-Aldrich). This probe produces strong fluorescence at high pH. Granulocytes were treated with monensin (0.1 or 1 μM) before incubation with either probe, and the fluorescence intensity in the fluorescein isothiocyanate channel was measured by flow cytometry.

TEM and β-hexosaminidase release assay

TEM analysis[E1] and the β-hexosaminidase release assay[E2] were performed as described.

Activation of eosinophils

Isolated eosinophils were primed with 4 nM rhIL-5 (Peprotech, Waltham, Mass) for 20 minutes and subsequently stimulated with 10 nM rhC5a (R&D Systems, Minneapolis, Minn) for 15 minutes at 37°C in a humidified atmosphere of 5% CO_2 in air. To monitor eosinophil degranulation, the plasma membrane expression of the surrogate marker CD63 was measured by using an anti-CD63 antibody (Invitrogen), followed by flow cytometry analysis. The effect of monensin on eosinophil degranulation was evaluated by preincubating cells with monensin at 0.05 μM or 0.1 μM, which did not induce significant eosinophil cell death. This effect was also evaluated by TEM to visualize any morphological alterations of eosinophil secretory granules. In addition, the effect of IL-5 on granule acidification, with or without monensin, was assessed using LSG (as above).

GzmB immunohistochemistry

Purified human eosinophils ($\sim 0.2 \times 10^6$ cells/mL) were left untreated or treated with monensin (1 μM) alone or in the presence of GzmB inhibitor Z-AAD-CMK (10 μM) for 15 or 30 minutes. A 100-μL sample of about 0.2×10^5 cells/slide was applied into a circle drawn using a water repellent pen and cells were allowed to settle for 30 minutes. The liquid was gently aspirated from the top of the samples by capillarity using filter paper. Samples were left to dry at room temperature for 15 to 30 minutes and fixed with 4% formaldehyde in PBS for 10 minutes. Slides were washed 3 times with PBS and cells were permeabilized with 0.1% Triton X-100 diluted in PBS for 10 minutes, followed by blocking with 5% goat serum diluted in PBS for 15 minutes. Slides were washed 3 times with PBS and incubated with rabbit antihuman GzmB antibody (rabbit polyclonal anti-GzmB; Abcam, Cambridge, United Kingdom; ab53097) diluted in PBS 1% BSA (1:500) overnight at 4°C. Slides were washed 3 times with PBS and incubated with goat antirabbit Alexa-488 antibody (Thermo Fisher) diluted in PBS 1% BSA (1:1000) for 1 hour at room temperature. Slides were then washed 3 times with PBS, followed by incubation with ActinRed 555 (Invitrogen) and NucBlue (Invitrogen) probes for 30 minutes at room temperature according to the manufacturer's instructions. After 3 times washing with PBS, samples were mounted using SlowFade

diamond antifade mountant (Invitrogen) and cover glass. Images were acquired using a 63x NA 1.40 oil objective on a LSM700 confocal microscope (Zeiss, Oberkochen, Germany) and analyzed using Imaris software (Bitplane, Belfast, United Kingdom).

Statistical analysis and graphs

Data were analyzed using the GraphPad Prism 9.3 statistics and graphing software (GraphPad Software). For comparison between treated and nontreated cells, Wilcoxon matched-pairs signed rank-test was used. Significance was tested with 1-way ANOVA with *post hoc* Tukey test. Statistical significance was set at P less than .05.

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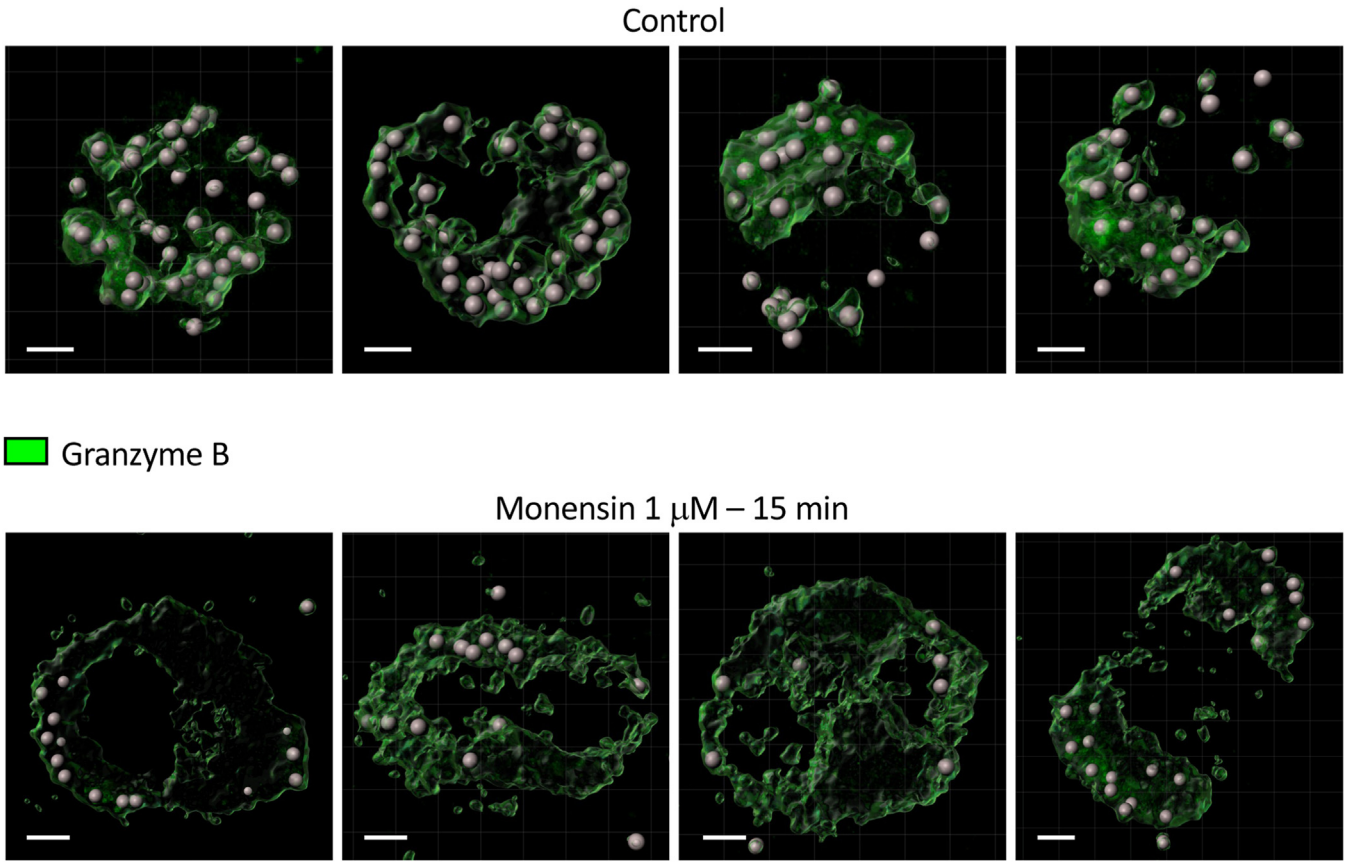

FIG E1. Monensin causes a reduction the number of GzmB intense stained areas (granules) in eosinophils. 3-D view generated from Z stacks sections of GzmB-stained cells (green). The spheres indicate the areas with more intense staining for GzmB; the upper panels represent 4 representative control cells; the lower panels represent 4 representative monensin-treated cells. Bar scale = 2 μm.