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Induction of eosinophil apoptosis by hydrogen peroxide promotes the resolution of allergic inflammation

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Eosinophils are effector cells that have an important role in the pathogenesis of allergic disease. Defective removal of these cells likely leads to chronic inflammatory diseases such as asthma. Thus, there is great interest in understanding the mechanisms responsible for the elimination of eosinophils from inflammatory sites. Previous studies have demonstrated a role for certain mediators and molecular pathways responsible for the survival and death of leukocytes at sites of inflammation. Reactive oxygen species have been described as proinflammatory mediators but their role in the resolution phase of inflammation is poorly understood. The aim of this study was to investigate the effect of reactive oxygen species in the resolution of allergic inflammatory responses. An eosinophilic cell line (Eo1-1) was treated with hydrogen peroxide and apoptosis was measured. Allergic inflammation was induced in ovalbumin sensitized and challenged mouse models and reactive oxygen species were administered at the peak of inflammatory cell infiltrate. Inflammatory cell numbers, cytokine and chemokine levels, mucus production, inflammatory cell apoptosis and peribronchiolar matrix deposition was quantified in the lungs. Resistance and elastance were measured at baseline and after aerosolized methacholine. Hydrogen peroxide accelerates resolution of airway inflammation by induction of caspase-dependent apoptosis of eosinophils and decrease remodeling, mucus deposition, inflammatory cytokine production and airway hyperreactivity. Moreover, the inhibition of reactive oxygen species production by apocynin or in $gp91^{phox-/-}$ mice prolonged the inflammatory response. Hydrogen peroxide induces Eo1-1 apoptosis *in vitro* and enhances the resolution of inflammation and improves lung function *in vivo* by inducing caspase-dependent apoptosis of eosinophils.

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Eosinophils express numerous receptors and secrete a wide variety of inflammatory mediators that influence many innate and adaptive immune responses. These multifunctional cells are important in the defense against helminth infection and are involved in the pathogenesis of many eosinophilic dominant allergic diseases.¹ High levels of eosinophil granule proteins (such as major basic protein (MBP)) have been found in bronchoalveolar lavage fluid from patients with asthma and evidence indicates that high-concentration granule products have contributed to the development of airway hyperreactivity (AHR), a cardinal feature of asthma.² Asthma is an inflammatory disease of the airways with participation of many cell types including leukocytes especially eosinophils and lymphocytes.^{3,4} Activation of these cells (mainly lymphocytes) leads to the release of proinflammatory mediators and cytokines such as leukotriene B₄, interleukin-4 (IL-4),

interleukin-5 (IL-5), interleukin-9 (IL-9), interleukin-13 (IL-13) and colony-stimulating factor granulocyte-macrophage (GM-CSF).^{3,5-7} Investigations using preclinical animal models of asthma and clinical studies in patients with asthma have demonstrated that the presence of eosinophils in the lungs are associated with epithelial damage, goblet cell hyperplasia, smooth muscle hypertrophy and airway hyperresponsiveness resulting in airflow limitation which can be fatal.^{3,8-10} Recently, anti-IL-5 treatment has been shown to ameliorate lung function in patients with eosinophilic asthma.¹¹

Apoptosis of leukocytes is regarded as an important process for the successful resolution of inflammatory responses. Reduced eosinophil apoptosis in bronchoalveolar lavage (BAL) fluid has been shown to correlate positively with severity of asthma.^{3,12-14} Indeed, defective leukocyte apoptosis and subsequent removal of apoptotic cells by

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Abbreviations: CAT, catalase; SOD, superoxide dismutase; MBP, eosinophil granule proteins; AHR, airway hyperreactivity; ROS, reactive oxygen species; RNS, Reactive nitrogen species; OVA, ovalbumin; EPO, eosinophil peroxidase

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phagocytes is thought to be important for the initiation and propagation of chronic inflammatory diseases such as asthma.¹⁵ Therefore, a balance in the tissue microenvironment between pro- and antiapoptotic signals is likely to greatly influence the load of eosinophils in the asthmatic lung.¹⁶ Thus, there is a great interest in understanding the mechanisms responsible for the elimination of eosinophils and other leukocytes and inactivation of proinflammatory mediators in inflammatory sites.¹⁷

Several molecular pathways have been shown to modulate the survival and death of leukocytes at sites of inflammation, including reactive oxygen species (ROS).¹⁸ ROS are a family of molecules containing oxygen and includes hydrogen peroxide (H₂O₂), superoxide O₂⁻, hydroxyl radical (OH) and nitric oxide (NO).¹⁹ In inflammatory conditions, ROS are increased as they help in neutralizing invading organisms during infection either directly or indirectly by formation of extracellular traps (ETs).²⁰ ROS have traditionally been regarded as quintessentially proinflammatory. However, evidence for ROS-mediated anti-inflammatory actions has been described.²¹ The importance for ROS production in the context of infection can be exemplified in patients with chronic granulomatous disease (CGD) where defective production in ROS results in multiple infections and often early death.^{22,23} Furthermore, studies in mouse models have shown that NADPH oxidase is key for regulating lung inflammation and injury as well as NF- κ B activation and downstream cytokine production in response to LPS.²⁴ More recently, our group has demonstrated that NADPH oxidase-derived H₂O₂ is directly linked to induction of apoptosis of neutrophils and resolution of inflammation in a model of antigen-induced arthritis.¹⁸ However, the role of ROS in the context of the resolution of allergic inflammation is still unknown.

Here, we evaluated whether H₂O₂ drives apoptosis of eosinophils and thereby influences the resolution of established eosinophilic inflammation and reduction of airflow obstruction. Our study provides evidence that H₂O₂ is released during allergic inflammation in a gp91^{phox}^{-/-}-dependent manner and induces a caspase-dependent proapoptotic effect in eosinophils, thus having a crucial role in the resolution of allergic inflammation.

Results

Kinetics of inflammation response after asthma induction in mice. We used a well-established murine model of asthma previously described by Stock *et al.*²⁵ In this model, the inflammatory response was characterized by accumulation of leukocytes detectable at 12 h and that reached maximal at 24–48 h after challenge with OVA (Supplementary Figure 1A). There was predominant accumulation of neutrophils at 12 h after antigenic challenge (Supplementary Figure 1B). Twenty-four hours after challenge, the number of neutrophils dropped and there was increased accumulation of eosinophils. The natural resolution of eosinophilic inflammation was initiated at 48 h and virtually completed at 72 h after antigen challenge (Supplementary Figure 1C). Likewise, similar results were obtained in an allergic pleurisy model in which there is also acute

self-resolving eosinophilic inflammation (Supplementary Figure 2A). Importantly, the increased number of mononuclear cells at 12 h and 48 h after antigen challenge coincided with the decrease of neutrophils and eosinophils, respectively (Supplementary Figure 1D).

Treatment with H₂O₂ or SOD decreases eosinophil accumulation in asthma models. Next experiments were designed to investigate the ability of H₂O₂ to resolve eosinophilic inflammation *in vivo*. We treated mice with SOD or H₂O₂ 24 h after OVA challenge, a time point at which the number of neutrophils is close to basal levels and eosinophil numbers are maximal. Treatment with SOD, which increases production of endogenous H₂O₂, or exogenous H₂O₂ treatment decreased the number of eosinophils in the BAL (Figure 1a) and reduced the levels of eosinophilic peroxidase (EPO) in the lung (Figure 1b). Treatment with catalase, which degrades H₂O₂, prevented the proresolving effects of SOD (Figures 1a and b). The lung parenchyma of challenged mice exhibited intense infiltration of inflammatory cells and loss of pulmonary architecture 48 h after antigen challenge when compared with the control group (PBS) (Figures 1c and d). Mice that received H₂O₂ at 24 h showed reduced parenchymal inflammation (Figure 1e). Likewise, treatment with SOD was associated with less parenchymal inflammation and administration of catalase prevented the anti-inflammatory actions of SOD (data not shown). Likewise, similar results were obtained in an allergic pleurisy model (Supplementary Figures 2B and C).

gp91^{phox} deficiency extends the duration of allergic inflammation. Because treatment with H₂O₂ resolved eosinophilic inflammation, a series of experiments were then performed in gp91^{phox}^{-/-} mice, which lack the ability to assemble the NADPH oxidase and have decreased capacity to generate ROS.¹⁸ ROS production in gp91^{phox}^{-/-} mice was significantly lower when compared to wild-type mice (Figure 2a). In these mice, natural resolution of eosinophilic inflammation, which usually is complete within 72 h in WT mice, was not observed even at 96 h after antigen challenge (Figure 2b). Likewise, intranasal treatment with apocynin (an inhibitor of NADPH oxidase) at a dose of 10 mg/kg prevented natural resolution of eosinophilic inflammation at 72 and 96 h after challenge (Figure 2b).

The histopathological analysis of lung sections showed that within 72 h the inflammation was present only in focal segments in wild-type mice (WT) and lung tissue was mostly preserved (Figure 2d). In contrast, there was much infiltration of inflammatory cells and derangement of tissue architecture at 72 h in gp91^{phox}^{-/-} mice exposed to antigen when compared with the control group (PBS, Figures 2d and e).

H₂O₂ treatment induced caspase-dependent apoptosis of eosinophils. Considering that the generation of endogenous H₂O₂ (via treatment with SOD) and administration of H₂O₂ reduced the number of eosinophils, we investigated whether death of eosinophils via apoptosis could underlie the resolution of eosinophilic inflammation. Indeed, there was significant increase in the number of apoptotic eosinophils as observed morphologically in mice treated with either SOD

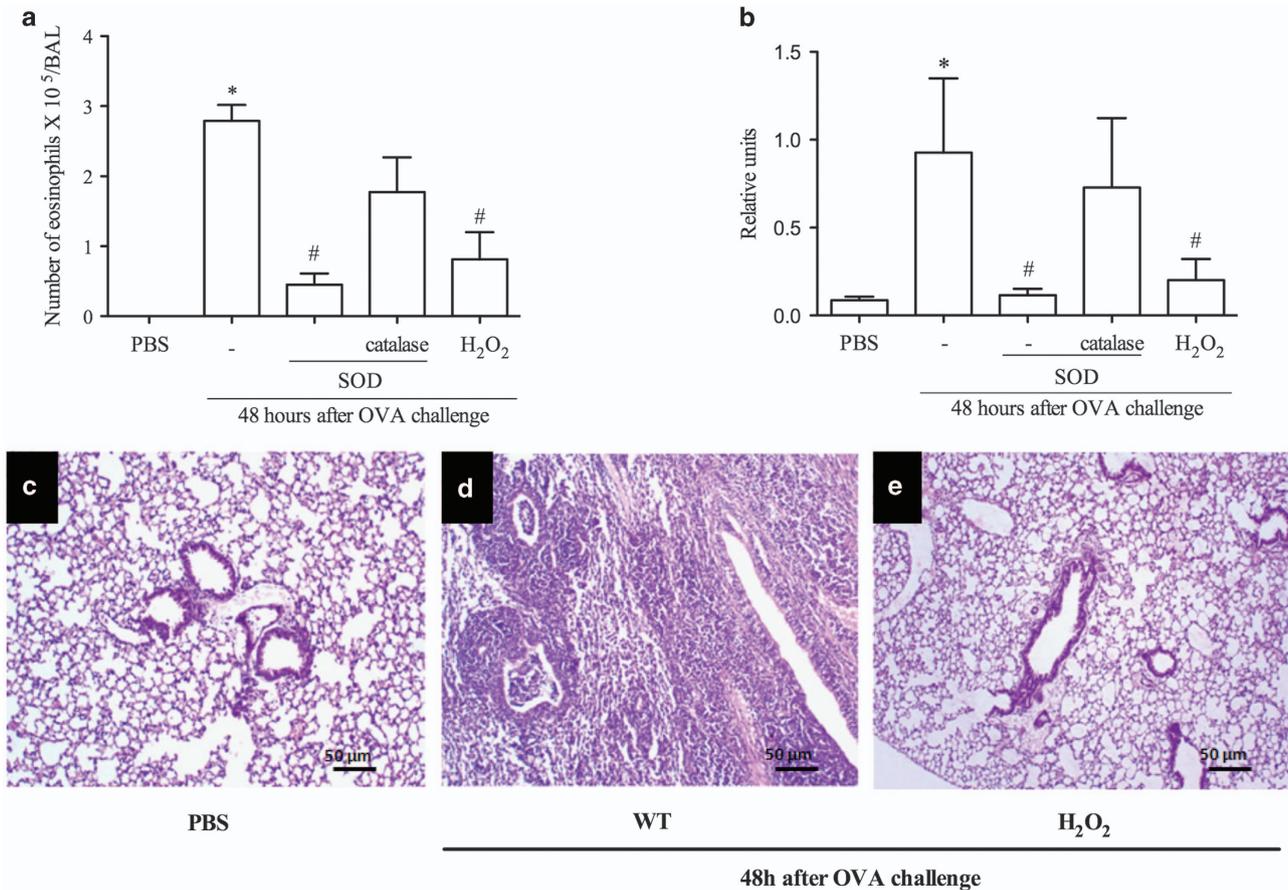


Figure 1 H₂O₂ promotes resolution of inflammation in a mouse model of asthma. Number of eosinophils after administration of SOD (0.3 mg/kg), SOD+catalase (1.2 mg/kg), H₂O₂ (0.5 M) or vehicle (30 μl PBS) (a). Eosinophil peroxidase (b). Photomicrographs of lung sections - PBS (c), asthma (d) H₂O₂ (e) H&E. 100 ×. Scale 50 μm. Data represent mean ± S.E.M. (n = 5). *P < 0.05 versus control mice. #P < 0.05 versus OVA mice

or H₂O₂ (Figures 3a and d–g and Supplementary Figure 2D) and demonstrated by flow cytometric analysis (Figure 3b). In mice treated with H₂O₂, there was an increase in expression of annexin V-FITC+ in CCR3+ cells at 2 h after treatment (Figure 3b). These data were confirmed by increased staining for cleaved caspase-3 in eosinophils, but not in mononuclear cells, in BAL from ova-challenged mice treated with H₂O₂ (Supplementary Figure 3). In contrast, T cells were not affected by H₂O₂ (% of cells CD3+/AnnX-V+: PBS: 0.69 ± 0.24; OVA: 0.74 ± 0.18, OVA+H₂O₂: 1.04 ± 0.22 P > 0.05, n = 6), suggesting that H₂O₂ treatment affected eosinophils more specifically. Treatment of animals with zVAD-fmk, a pan-caspase inhibitor, reversed the proresolution actions of H₂O₂, demonstrating the caspase dependency of the resolution of eosinophilic inflammation (Figure 3c).

H₂O₂ induces concentration-dependent apoptosis of an eosinophilic cell line *in vitro*. Next, we evaluated whether H₂O₂ could induce directly the apoptosis of a human eosinophil cell line, Eo1-1. This cell line has been extensively used as a model of eosinophil function, including chemotaxis, mediator release and apoptosis induction.^{26,27} Eo1-1 cells were incubated for a 4-h period with increasing concentrations of 0.3, 1.0 and 3.0 mM H₂O₂. Annexin-V-positive

cells were considered apoptotic cells and annexin-V/PI dual-positive cells were considered late apoptotic/secondarily necrotic cells. H₂O₂ markedly increased apoptosis of Eo1-1 cells in a concentration-dependent manner (Figure 4a). In order to confirm that Eo1-1 cells undergo apoptosis following incubation with H₂O₂, Eo1-1 was coincubated with H₂O₂ and Q-VD-OPH, a highly effective pan-caspase inhibitor. Coincubation of Eo1-1 with Q-VD-OPH abrogated the ability of H₂O₂ to induce apoptosis (Figure 4b). To demonstrate further the role of caspase-3 in Eo1-1 apoptosis induced by H₂O₂, we performed western blot analysis to investigate expression of cleaved caspase-3 on lysates from Eo1-1 cells 4 h after stimulation with different concentrations of H₂O₂. H₂O₂ caused caspase-3 cleavage when compared with cells treated with media alone (Figure 4c). Apoptosis was also assessed morphologically using light microscopy after cyto centrifugation and staining with Diff-Quick. We observed that apoptotic Eo1-1 cells exhibited nuclear condensation and cellular shrinkage, confirming flow cytometric data (data not shown).

H₂O₂ reduces airway hyperreactivity to methacholine. Airway hyperreactivity (AHR) has been studied in a well-established murine model of asthma.²⁸ In this model, the time course of the eosinophilic infiltrate was similar to that

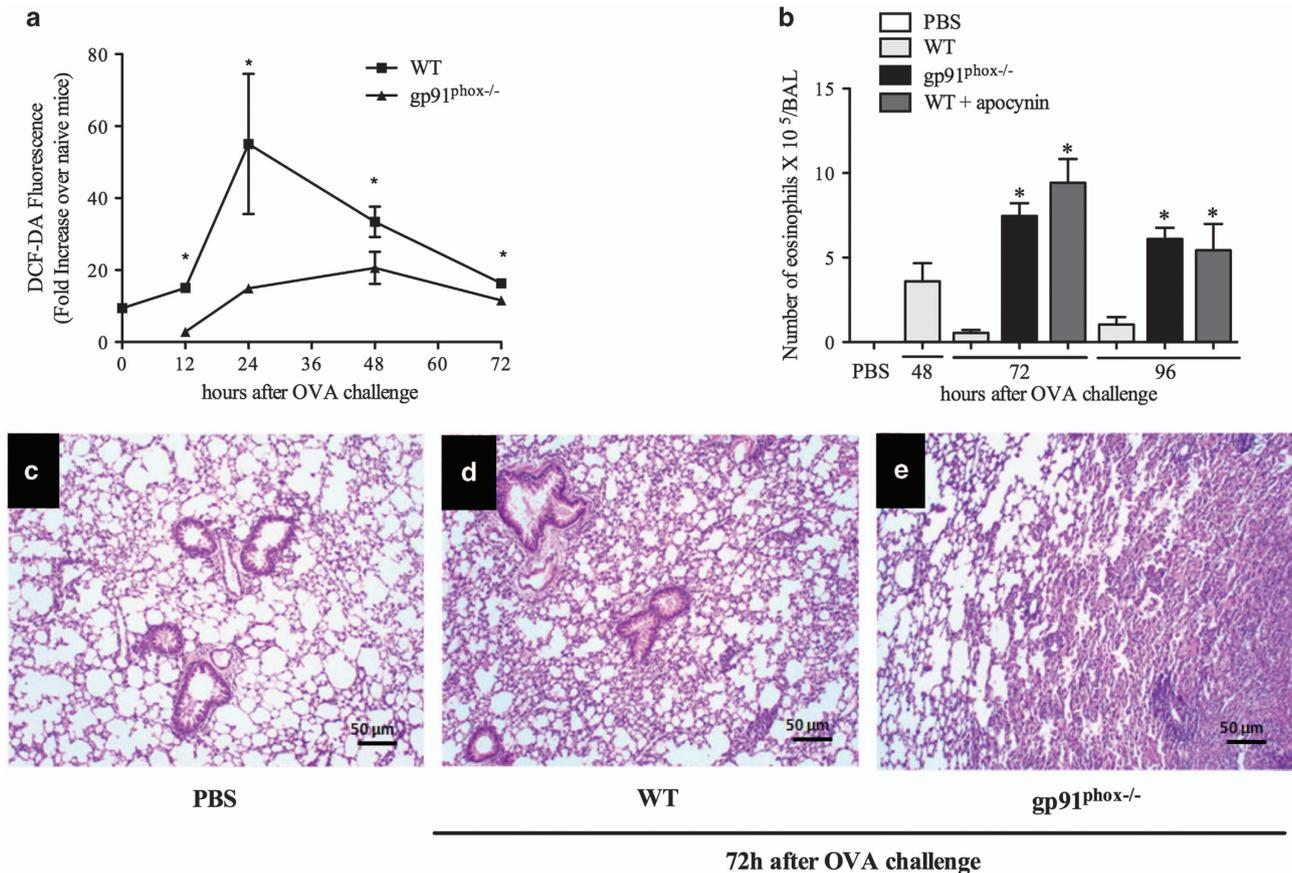


Figure 2 ROS derived from NADPH oxidase is necessary for inflammation resolution. ROS production in WT or gp91^{phox-/-} mice (a). Eosinophils 72 and 96 h after OVA in gp91^{phox-/-} mice and after apocynin treatment (b). Eosinophil peroxidase (c). Lung sections of PBS (d), WT (e) gp91^{phox-/-} (f). H&E. 100 \times . Scale 50 μ m. Data represent mean \pm S.E.M. ($n=5$). * $P<0.05$ versus control group

observed in our previous experiments (see Supplementary Figure 4 and compare with Supplementary Figure 1). Akin to experiments described previously, delayed treatment with H₂O₂ resolved the eosinophilic infiltrate in the BAL at 48 h after antigen challenge (Figures 5a and d) when compared with the control group (PBS, Figures 5a and b). In these mice, AHR was assessed by measuring airway resistance (RI) and lung elastance parameters in mechanically ventilated animals. As shown in Figure 6, antigen challenge with OVA exacerbated airway resistance and elastance of the lungs in response to inhaled methacholine (3–81 mg/ml), as compared with the control group challenged with PBS. Treatment with H₂O₂ reduced AHR, as seen by decreased airway resistance and elastance (Figure 6).

Effect of H₂O₂ administration on mucus deposition and lung remodeling. To evaluate mucus production, sections of lung tissue were stained with periodic acid-Schiff. The analysis of airway mucins demonstrated significant metaplasia of goblet cells and mucus accumulation at 48 h after the last OVA challenge in actively sensitized mice (Figures 5e and g) in comparison with the control group (PBS) (Figure 5f). Treatment with H₂O₂ reduced OVA-induced mucus accumulation and the percentage of mucus producing cells to values similar to those observed in nonallergic controls (Figures 5e, f and h). The lung sections stained with Gomori

trichrome demonstrated that OVA mice had increased peribronchiolar matrix deposition as compared with the PBS mice (Figures 5i–l). Quantitative analyses demonstrated that delayed treatment with H₂O₂ prevented extracellular matrix deposition in challenged mice (Figures 5i and l). Furthermore, H₂O₂ treatment decreased concentrations of CCL11, CCL24, IL-4, IL-5 and TNF- α that were measured at 48 h after antigen challenge (Table 1).

Discussion

There is strong evidence indicating an important role for eosinophils in the pathogenesis of allergic diseases through the release of a variety of inflammatory mediators, including MBP, EPO and cytokines.¹ Here, we evaluated the role of endogenous and effects of exogenous administration of H₂O₂ in the context of resolution of allergic inflammation. The results presented here can be summarized as follows: (i) treatment with H₂O₂ or strategies that enhanced H₂O₂ reduced eosinophil accumulation in the BAL and lung tissue; (ii) H₂O₂ promoted resolution of inflammation by inducing caspase-dependent apoptosis of eosinophils *in vivo*; (iii) H₂O₂ also induced caspase-dependent apoptosis of a human eosinophilic cell line *in vitro*. (iv) H₂O₂ derived from NADPH oxidase was necessary for natural resolution of allergic inflammation; (v) finally, treatment with H₂O₂ decreased secretion of mucus,

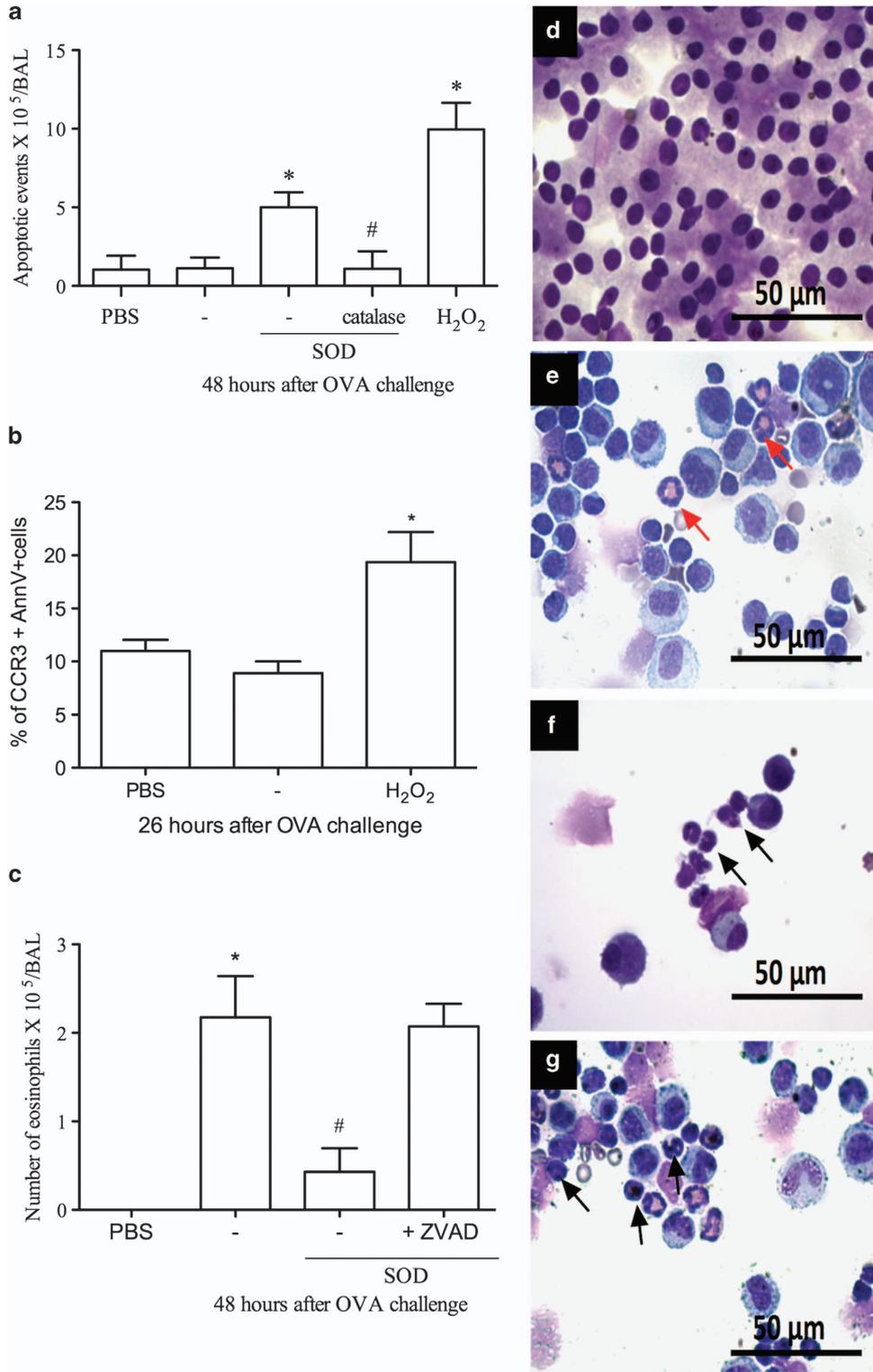


Figure 3 H₂O₂ induces apoptosis of eosinophils. SOD (0.3 mg/kg), SOD+catalase (1.2 mg/kg), H₂O₂ (0.5 M) or vehicle (30 μl PBS) i.n. Apoptotic morphology (a) Cells expressing Annexin V-FITC+CCR3+ (b). Eosinophils after zVAD-fmk administration (1 mg/kg, i.p) (c). Cell types. Red arrow normal eosinophil. Black arrow: eosinophil with apoptotic morphology. PBS (d) asthma (e) SOD (f) H₂O₂ (g). 100 × Scale: 50 μm. Data represent mean ± S.E.M. (n = 5). *P < 0.05 versus control mice. #P < 0.05 versus OVA mice

extracellular matrix deposition, inflammatory cytokine production and decreased AHR induced by antigen. Altogether, these results demonstrate a clear proresolving effect of H₂O₂ in allergic inflammation *in vivo*.

In our experiments, we have demonstrated that endogenous or exogenous H₂O₂ resolved eosinophilic inflammation in two different models of the allergic response. In the asthma model, this event correlated with increased number of

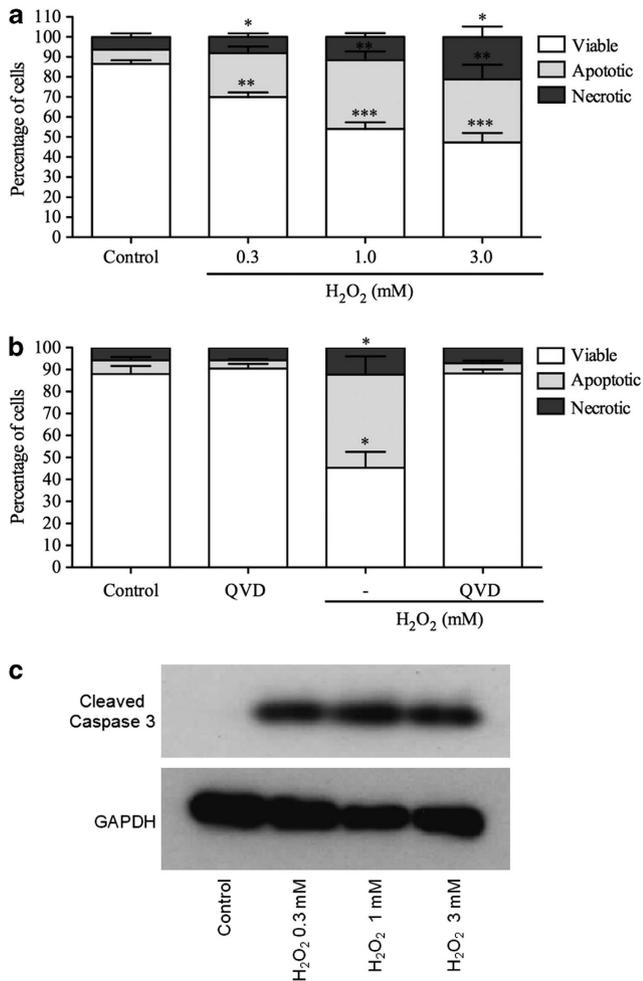


Figure 4 H₂O₂ induces concentration and caspase-dependent apoptosis in Eo1 cells. Eo1 cells with H₂O₂ (0.3 mM–3.0 mM) (a) or H₂O₂ (1 mM) and Q-VD-OPh (10 μM) (b). Western blotting for cleaved caspase-3 (17/19 kDa) and GAPDH (37 kDa) (c). Data represent mean ± S.E.M. *n* = 3. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Western blots representative on three experiments

apoptotic eosinophils. This is similar to the role of H₂O₂ in a model of arthritis, in which H₂O₂ limits inflammation associated with induction of caspase-dependent apoptosis of neutrophils.¹⁸ In fact, blockade of caspases with a pan-caspase inhibitor, zVAD-fmk, prevented apoptosis and the resolution of eosinophilic inflammation induced by H₂O₂ consistent with our previous studies on neutrophils. H₂O₂ has been shown to induce apoptosis in culture of different cell types including human hepatocyte cells,²⁹ epithelial cells,³⁰ endothelial cells³¹ and myocytes.³² Here, we demonstrate that H₂O₂ also induced apoptosis of a human eosinophilic cell line, as by assessed by flow cytometry, light microscopy and cleavage of caspase-3 assessed by western blotting. The experiments using Eo1 cells suggest that this pathway may be relevant in humans and clearly deserve further investigation in human disease. Indeed, ROS have been reported to increase apoptosis of human eosinophils.^{33,34} Therefore, our evidence suggests that resolution of eosinophilic inflammation by endogenous or exogenous H₂O₂ is due to the capacity of

this molecule to induce caspase-dependent apoptosis of eosinophils.

Results in gp91phox-deficient mice showed that influx was not altered but persistency of eosinophils in tissues was greatly prolonged. Moreover, the intranasal treatment with an inhibitor of NADPH oxidase delayed the resolution of inflammatory response. It has been reported that in conditions of oxidative stress, the NADPH oxidase complex catalyzes electron transfer from NADPH to molecular oxygen and generates superoxide anions (O₂⁻). The superoxide dismutase (SOD) degrades O₂⁻ in H₂O and produces H₂O₂.²³ Our work is the first to describe the importance of the oxidative pathway involving NADPH oxidase to resolve eosinophilic response. This is consistent with other studies in mice, which showed that gp91^{phox}-/- mice had delayed resolution of neutrophilic inflammation and this process was reversed by administration of exogenous H₂O₂ in a model of antigen-induced arthritis.¹⁸ These data support the concept that ROS, which are generated by the phagocytic NADPH oxidase and commonly considered harmful mediators of acute inflammation, have a role in limiting inflammation and may be pivotal in resolving acute inflammation.³⁵ Indeed, our results show that H₂O₂ not only induces resolution of inflammation, but it is also relevant in the context of the natural resolution of eosinophilic inflammation. Recent data show that administration of SOD accelerates resolution of inflammation associated to antigen-induced arthritis resulting in increased number of apoptotic neutrophils. This coincided with activation of caspase-3 and increased Bax expression in neutrophils recovered from the articular cavity.¹⁸ We also investigated whether reactive nitrogen species (RNS) participate in the resolution of the inflammatory response in the model of allergic asthma. It was observed that in the absence of NO production by iNOS there was no change in the resolution of allergic inflammation (Supplementary Figure 5). RNS such as NO are involved in the inflammatory process in the airways of asthmatic patients. NO has also been shown to be involved in the regulation of apoptosis: it can prevent or induce apoptosis depending upon the cell type and the concentration in which it is produced.^{36–40} The data suggest that RNS are not essential for resolution of allergic inflammation.

Our results showed that in addition to resolving eosinophilic inflammation, treatment with H₂O₂ had major physiological consequences in a model of allergic asthma. Indeed, treatment with H₂O₂ greatly reduced eosinophil accumulation, changes in airway reactivity, remodeling and mucus deposition induced by antigen challenge of immunized mice. Cytokines, including IL-4, IL-5 and TNF-α, and chemokines (CCL11 and CCL24) are thought to be involved in eosinophil accumulation and linked to pathophysiology of allergic disease.^{41–44} There is also evidence that IL-5 and TNF-α promote eosinophil survival *in vitro*.^{45–47} In addition, CCL11 and CCL24 are important for eosinophil recruitment from the blood to the parenchyma and then into the airway.⁴⁸ However, the evidence that antibody or drugs targeting these cytokines or chemokine induce resolution of eosinophilic inflammation *in vivo* is scanty. In addition, IL-4, IL-5 and TNF-α are essential for the development of AHR, mucus production and fibrosis in asthma.^{49–51} Similarly, chemokines, such as CCL11 and CCL24, may also contribute to AHR and fibrogenesis in

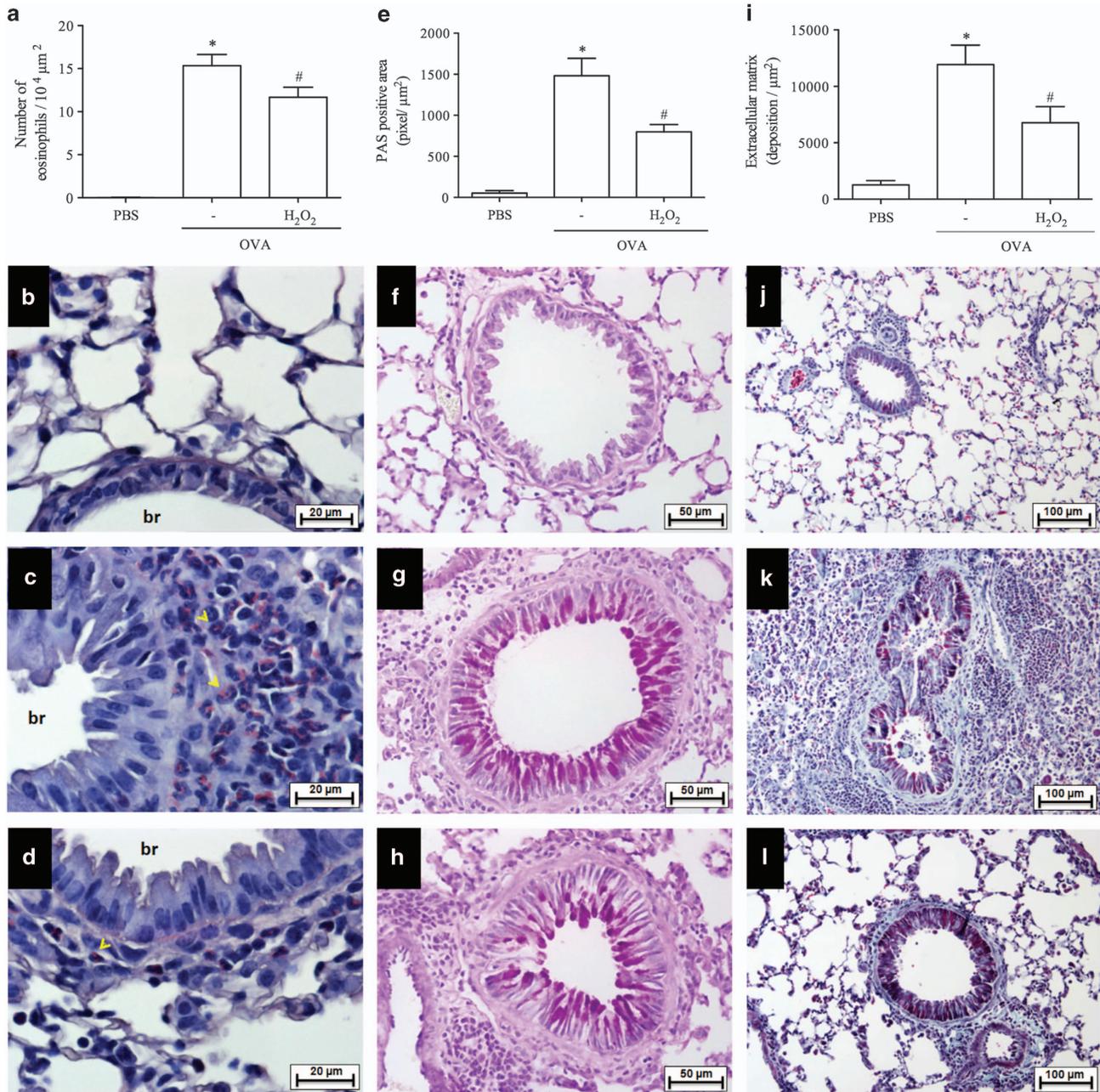


Figure 5 Effect of H₂O₂ on lung remodeling and mucus deposition. Leukocyte infiltrate around the bronchial region of the lung sections stained with hematoxylin and eosin (H&E) or Sirius Red (pH 10.2) (a–d). Mucus production from airway stained with hematoxylin and Periodic acid-Schiff (PAS) (e–h). 400 ×. Peribronchial fibrosis stained with hematoxylin and eosin and a combination of Gomori trichrome (i–l). 200 ×. **P* < 0.05 versus control mice. #*P* < 0.05 versus OVA mice

animal models of asthma.^{42,48} In our studies, there was a marked decrease in levels of these cytokines, which accompanied the resolution of the eosinophil numbers. Resolution of inflammation is known to exert potent anti-inflammatory effects and decrease of production of proinflammatory cytokines,^{52,53} suggesting that decreased levels of TNF, IL-5, IL-4 and chemokines are likely a consequence of the anti-inflammatory action of the resolution process. Alternatively, it is also possible that ROS induced a primary decrease of cytokines that could have then accounted for eosinophil apoptosis and in the resolution of eosinophilic inflammation.

The latter possibility is more difficult to ascertain in the *in vivo* situation and clearly more studies are needed to convincingly show that blockade of cytokines alter survival of eosinophils *in vivo*, as demonstrated *in vitro*. Whatever the mechanism, direct effect on eosinophils (shown here) or an indirect action via decrease on survival factors, our data clearly demonstrate the proresolving effects of H₂O₂ in the context of eosinophilic inflammation. Taken together our findings show that exogenous or endogenous generation of H₂O₂ resolve allergic inflammation by inducing eosinophil apoptosis in a caspase-dependent manner. Induction of eosinophil apoptosis by H₂O₂

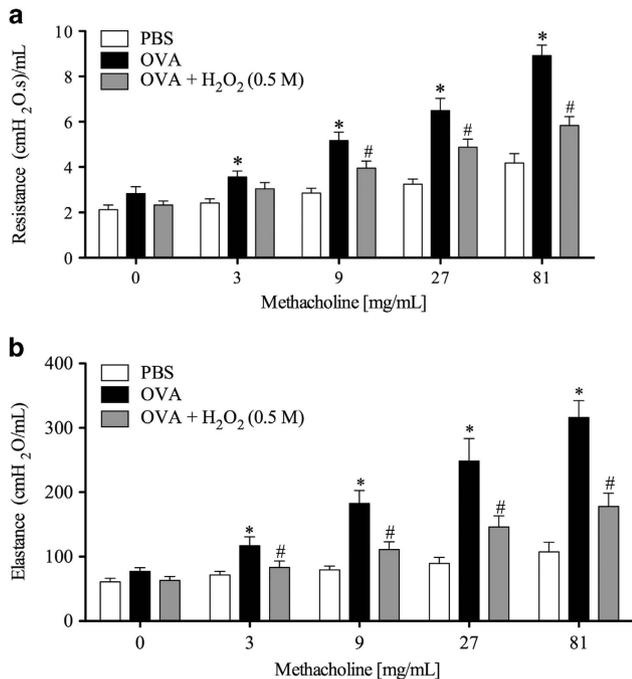


Figure 6 Airway resistance and dynamic elastance after increasing methacholine doses. H₂O₂ (0.5 M) or vehicle (30 μl PBS) was administered i.n. instillation 24 h after asthma induction. Airway resistance (a) and elastance (b) was measured in response to increasing doses of methacholine (3, 9, 27, 81). Data represent mean ± S.E.M. (n = 10). *P < 0.05 versus vehicle group. #P < 0.05 versus OVA mice

Table 1 Levels of cytokines in lung (pg/ml)

Cytokines	PBS	OVA	H ₂ O ₂
CCL11	743 ± 152	9109 ± 5063 ^a	5845 ± 2048 ^b
CCL24	903 ± 245	11956 ± 6304 ^a	6896 ± 1143 ^b
IL-4	110 ± 32	238 ± 149 ^a	127 ± 34 ^b
IL-5	847 ± 84	1226 ± 445 ^a	829 ± 140 ^b
TNF-α	182 ± 41	310 ± 135 ^a	157 ± 45 ^b

Abbreviations: TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5

^aSignificantly different from the control group (P < 0.05)

^bSignificantly different from the vehicle group (P < 0.05)

decreases airway remodeling and dysfunction. These results have fundamental implications to the basic concept of inflammation resolution and may have therapeutic implications. Indeed, our results demonstrate that resolution of eosinophilic inflammation by H₂O₂ maintains tissue integrity and function. Restoration of tissue inflammation to homeostasis may reverse airway function and remodeling in patients, a tenet that may be exploited for the development of novel therapies for the treatment of asthma.

Materials and Methods

Ethics statement. Male C57/BL6 and gp91^{phox} -/- mice (Gene *Cybb*; ES Cell Line name CCE/EK.CE)⁵⁴ (8–10 weeks) were bred and housed in a temperature-controlled room with free access to water and food. Animal Care and Use Committee and the study received prior approval from the local animal ethics committee (Animal Ethics Review Board – Comitê de Ética em Experimentação Animal-CETEA/Universidade Federal de Minas Gerais-UFMG (protocol number: 218/11).

Reagents and drugs. Superoxide dismutase (SOD) from bovine erythrocytes, catalase, H₂O₂, ovalbumin, Z-VAD-fmk, propidium iodide (PI) and 4,5-diaminofluoresceína-diacetato (DAF-2DA) were purchase from Sigma (St. Louis, MO, USA). Apo DETECT ANNEXIN-V-FITC KIT 2,7-diclorodihidrofluoresceína-diacetato (DCF-DA) and dihidrodamine-123 (DHR-123) were purchase from Invitrogen (Life Technologies, São Paulo, Brazil). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Q-VD-OPh (R&D Systems, Minneapolis, MN, USA), annexin-V-FLUOS (Roche, Mannheim, Germany).

Eol-1 cell culture and apoptosis induction. Human eosinophilic cell line (Eol-1 cells) was maintained in RPMI-1640 medium (PAA) with 2% FBS (Biosera), penicillin (100 U/ml) and streptomycin (100 U/ml) (PAA). Cells were aliquoted (2 × 10⁶ cells/ml) and incubated with H₂O₂ (Thermo Fisher Scientific, Waltham, MA, USA), Q-VD-OPh (R&D Systems) or combinations of these either in 96-well-flat bottomed-plates (final volume of 150 μl) or in 2 ml Eppendorf tubes (final volume of 500 μl) in a humidified, 37 °C incubator at 5% CO₂ atmosphere or on a shaking, temperature-controlled heat block. Q-VD-OPh stock was initially dissolved in dimethyl sulphoxide (Sigma) then diluted in buffer yielding a final concentration of 0.2%; a corresponding DMSO control of 0.2% was assessed as an appropriate vehicle control. Apoptosis was assessed by flow cytometry with a BD-LSR Fortessa (Becton Dickinson Biosciences, San Jose, CA, USA) using annexin-V-FLUOS (Roche) in combination with propidium iodide (PI) (Sigma) as described.⁵⁵ Data were analyzed using Flowjo software (TreeStar, Ashland, OR, USA). Morphological apoptotic changes were assessed by light microscopy of DiffQuick stained cytocentrifuged cells.⁵⁵

Western blotting. Cells at a concentration of 2 × 10⁶ cells/ml per condition were incubated with H₂O₂ (Thermo Fisher Scientific), Q-VD-OPh (R&D Systems) or combinations of these at 37 °C on a shaking heat block for 4 h. Eol-1 cells were pelleted by centrifugation at 3000 × g for 60 s and resuspended with whole-cell lysis buffer. Sample was incubated on ice for 10 min then NP-40 was added, briefly vortexed and centrifuged for 20 min at 3000 × g. Supernatant was removed and the remaining cell pellet was resuspended in sample buffer before boiling at 95 °C for 5 min. Lysate were run on 12% precast gels (Thermo Fisher Scientific, Rockford, IL, USA) and transferred onto PVDF (Immobilon-P; Millipore, Herts, UK). Membranes were blocked for 1 h in 5% (wt/vol) dried milk/TBS/0.1% Tween-20 before probing with antibodies to cleaved caspase-3 diluted 1 : 500 (Cell Signaling Technologies) at 4 °C overnight or GAPDH diluted 1 : 20 000 (Sigma-Aldrich, St. Louis, MO, USA) 1 h at room temperature. Following 3 × 5 min washes in TBS/0.1% Tween-20, the blots were incubated with HRP-conjugated secondary antibody (Dako, Glostrup, Denmark) diluted 1 : 2500 for 1 h at room temperature before incubation with ECL (GE Healthcare, Bucks, UK) exposure to BioMax MS-1 X-ray-sensitive film, and processing (X-Ograph Imaging Systems, Wilts, UK).

Induction of asthma. All mice were sensitized intraperitoneally (i.p.) with 100 μg of OVA (albumin from chicken egg white - A5503, Sigma-Aldrich) in 2% alum (aluminum hydroxide gel adjuvant; Brenntag) on day 0, then challenged intranasally (i.n.) on days 8–10 with 10 μg of OVA or PBS.⁵⁶ The treatments with apocynin, SOD, H₂O₂ and catalase were performed i.n. and the zVAD-fmk (Tocris Bioscience) was administered i.p.

Induction of pleurisy. Mice were immunized with OVA adsorbed to aluminum hydroxide gel as described.⁵² Briefly, mice were injected subcutaneously (s.c.) on days 1 and 7 with 0.2 ml of a solution containing 100 mg of OVA and 70 mg of aluminum hydroxide. Sensitized mice were then challenged with OVA (1 mg/cavity, in a total volume of 100 μl intrapleurally, i.pl) or PBS. Cells present in the pleural cavity were collected at different times by washing the cavity with 2 ml PBS and total cell counts performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytocentrifuge preparations (Shandon Cytospin III), stained with May-Grünwald-Giemsa using standard morphological criteria to identify cell types. The results are presented as the number of cells/cavity.

BAL analysis. BAL was performed to obtain leukocytes present in the alveolar space. Mice were killed by anesthetic overdose and the trachea of each animal was exposed and cannulated with a polypropylene catheter of 1.7 mm. Airways were washed with 2 ml of ice-cold PBS. Total cell counts were performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytocentrifuge preparations (Shandon Cytospin III), stained with May-Grünwald-Giemsa using standard morphological criteria to identify cell types. The results are

presented as the number of cells/BAL. In a separated set of experiments, apoptotic cells were morphologically identified in cytocentrifuged slides, which were also positively stained for cleaved caspase-3 (Alexa Fluor 488 rabbit anti-mouse cleaved caspase-3; Cell Signaling; 1 : 50). Fluorescence intensity was measured offline using Volocity software 6.3 (Perkin–Elmer, Waltham, MA, USA) and fluorescence profile was assessed using Image J (NIH).⁵⁷

Histological analysis. The lung was prepared as described.⁵⁸ Briefly, lungs were removed 24 h after the last challenge and fixed in Millonig buffer solution (pH 7.4) with 4% paraformaldehyde. For analysis of leukocyte infiltrate around the bronchial region, the lung sections were stained with hematoxylin and eosin (H&E) or were subjected to Sirius Red (pH 10.2) staining (Llewellyn's Sirius Red Direct Red 80, CI 35780; Aldrich, Milwaukee, WI, USA). Results were expressed as leukocytes/ $10^4 \mu\text{m}^2$. Mucus production was analyzed from tissue sections stained with Harris hematoxylin stain and a combination of Periodic acid-Schiff (PAS) stain (Schiff's reagent, Merck, Rio de Janeiro, Brazil). Photomicrographs of airways obtained at 400 \times magnification were analyzed using the software Image-Pro Plus (Image-Pro Plus, 4.1; Media Cybernetics, Houston, TX, USA). Nine to twelve bronchial areas per lung were outlined and quantified. Results were expressed as PAS positive area (pixels/ μm^2). Peribronchial fibrosis was analyzed from tissue sections stained with hematoxylin and eosin stain and a combination of Gömötrichrome stain (Trichrome Stain LG Solution; Sigma-Aldrich). Photomicrographs of airways obtained at 200 \times magnification were analyzed using the software Image-Pro Plus. Eight to twelve peribronchial area per lung were outlined and quantified.⁵⁹ Results were expressed as extracellular matrix deposition area (μm^2).

Assessment of leukocyte apoptosis. Apoptosis was assessed as described.^{52,60} Briefly, cells (5×10^4) collected 48 h after antigen challenge were cytocentrifuged, fixed and stained with May–Grunwald–Giemsa and counted using oil immersion microscopy ($\times 100$ objective) to determine the proportion of cells with distinctive apoptotic morphology (cell shrinkage, chromatin condensation, nuclear fragmentation and maintenance of membrane integrity). Twenty-five fields were counted per slide and the results expressed as the mean \pm S.E.M. of number of apoptotic cells in 25 fields. Assessment of apoptosis was also performed by flow cytometry using commercial kit annexin-V-FITC (Invitrogen) following the instructions of the manufacturer in cells previously stained with the following Abs to extracellular markers for 30 min on ice: anti-mouse CCR3 mAb (BD Biosciences, San Jose, CA, USA) or anti-mouse mAb CD3 (BD Biosciences). Flow cytometry was performed using a FACS Canto flow cytometer (BD). At least 10^4 events were recorded and analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA). Results are expressed as cells undergoing early stage apoptosis quantified by staining with annexin-V but not PI.

ELISA analysis. Murine IL-4, IL-5, TNF- α , CCL11 and CCL24 levels were measured in right lung tissue samples by means of ELISA technique using commercial DuoSet kits R&D Systems following the instructions of the manufacturer. Results were expressed in levels of cytokines per lung (pg/ml).

Quantification of eosinophil accumulation in lung. Pulmonary EPO activity was determined to estimate eosinophil recruitment into the lung parenchyma as described.⁶¹ Absorbance was read in an ELISA reader (Expert Plus ASYS Hitech GmbH, Eugendorf, Austria) at 492 nm. Values are expressed in O.D.

Determination of the production of reactive oxygen species and nitrogen by fluorimetry. Leukocytes obtained from the BAL were incubated separately with the probes '7'-dicloro-dihydrofluorescein diacetate (DCF-DA 20 μM), dihidrorodamin diacetate 123 (DHR-123 5 μM) of 4,5-diaminofluorescein (DAF-2DA 10 μM) for 30 min in an oven at 37 °C. This stage of the experiment was performed in the dark, because the markers are photosensitive. The reading of fluorescence was performed in a fluorescence spectrophotometer (Synergy 2, Biotek, Winooski, VT, USA) with wavelengths of excitation and emission of 488 and 515 nm, respectively.

Invasive assessment of respiratory mechanics. Airway reactivity was assessed as a change in airway function after challenge with aerosolized methacholine in a FinePoint R/C Buxco Platform. The parameters were measured as previously described.⁶² The analyses were performed 48 h after the last OVA challenge.

Statistical analysis. ANOVA followed by Student Newman–Keuls was applied to comparison of multiple groups. In order to test statistical significance between two

groups we used the unpaired Student's *t*-test (GraphPad Software, San Diego, CA, USA). All *in vitro* experiments were performed at least three times with each experiment carried out in triplicate. All *in vivo* experiments included six mice per group. Data were expressed as the mean \pm S.E.M. Differences were considered significant at $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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