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Efferocytosis of Apoptotic Neutrophils Enhances Control of *Mycobacterium tuberculosis* in HIV-Coinfected Macrophages in a Myeloperoxidase-Dependent Manner

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Keywords

Mycobacterium tuberculosis · HIV · Reactive oxygen species · Myeloperoxidase · Macrophages

Abstract

Tuberculosis remains a big threat, with 1.6 million deaths in 2017, including 0.3 million deaths among patients with HIV. The risk of developing active disease increases considerably during an HIV coinfection. Alveolar macrophages are the first immune cells to encounter the causative agent *Mycobacterium tuberculosis*, but during the granuloma formation other cells are recruited in order to combat the bacteria. Here, we have investigated the effect of efferocytosis of apoptotic neutrophils by *M. tuberculosis* and HIV-coinfected macrophages in a human in vitro system. We found that the apoptotic neutrophils enhanced the control of *M. tuberculosis* in single and HIV-coinfected macrophages, and that this was dependent on myeloperoxidase (MPO) and reactive oxygen species in an autophagy-independent manner. We show that MPO remains active in the apoptotic neutrophils and can be harnessed by infected macrophages. In addition, MPO inhibition removed the suppression in *M. tuberculosis* growth caused by the apoptotic neutrophils. Antimycobacterial components from apoptotic neutrophils could thus increase the microbicidal activity of macrophages during an *M. tuberculosis*/HIV coinfection. This cooperation between innate immune cells could thereby be a way to compensate for the impaired adaptive immunity against *M. tuberculosis* seen during a concurrent HIV infection.

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Introduction

Tuberculosis is the leading cause of death from a single infectious agent. One of the major risk factors for developing an active disease is coinfection with HIV, which affects the protective adaptive immune response. Another problem is the rise in drug-resistant tuberculosis, making the treatment more difficult and increasing mortality [1]. The causative agent *Mycobacterium tuberculosis* enters the lungs and predominantly infects alveolar macrophages [2]. In most instances, the microbes cause a latent infection but if the host fails to control the pathogen, an active tuberculosis infection is established. Following infection, different immune cells are recruited to the lung and a granuloma is formed to control the infection [3, 4]. The normally well-structured tuberculosis granulomas in patients are disrupted in HIV-coinfected individuals,

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with more necrosis and a heterogeneous cellular population dominated by neutrophils and eosinophils [5].

The role of neutrophils in the defense against mycobacterium is controversial and has been implicated as both beneficial, especially during early infection, and detrimental at later stages [6–9]. However, compared to viable and apoptotic neutrophils, the necrotic phenotype leads to more severe disease progression and mycobacterial growth, especially in HIV patients [5, 10–12]. Apoptotic cells are eliminated mainly by macrophages, in a process called efferocytosis, and this can induce macrophage activation, cytokine release, and a decrease in *M. tuberculosis* growth. Depending on the study design, the mechanisms behind the increased control of *M. tuberculosis* in macrophages may vary, from increased phagosome maturation, to harnessing the antimicrobial content of granules from neutrophils [13–16].

Upon phagocytosis, a strong respiratory burst is triggered in neutrophils, generating reactive oxygen species (ROS) through the NADPH oxidase inside the phagosome in order to combat the ingested bacteria. Azurophilic granules containing myeloperoxidase (MPO) fuse with the phagosome and contribute to the bactericidal environment by producing hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) [17]. Although macrophages are able to produce reactive oxygen intermediates, their main route of antimicrobial action is through autophagy and lysosomal-endosomal fusion with the phagosome [18]. These innate defense mechanisms exerted by neutrophils and macrophages are inhibited by *M. tuberculosis* as a means to persist inside host cells [19–24].

It is well established that HIV coinfection impairs the activation of macrophages through the adaptive immune system by decreased antigen presentation and impaired stimulation of *M. tuberculosis* Ag-specific CD4 T cells [25–27]. Therefore, an alternative way to activate coinfected macrophages is needed. Previously, we have shown that apoptotic neutrophils are able to activate *M. tuberculosis*-infected macrophages, leading to increased proinflammatory signals and *M. tuberculosis* growth inhibition [13]. From these and other studies showing a role for the cooperation between apoptotic and viable cells in the defense against *M. tuberculosis* [14–16], we hypothesized that efferocytosis can enhance immune protection also in HIV/*M. tuberculosis*-coinfected cells. In our in vitro study using human cells, we show that apoptotic neutrophils enhance the control of intracellular *M. tuberculosis* both in *M. tuberculosis*-single and HIV-coinfected macrophages, and that this process was dependent on MPO and ROS in an autophagy-independent way.

Materials and Methods

Generation of Macrophages from Monocytes

Monocytes were isolated from buffy coats and the PBMCs were isolated through gradient centrifugation before adhesion to tissue culture flasks. Non-adherent cells were removed by extensive washing and the monocytes were cultured in DMEM supplemented with 10% pooled normal human serum for 7 days to differentiate into macrophages.

Isolation of Neutrophils and Induction of Apoptosis

Polymorphonuclear cells were separated through gradient centrifugation from peripheral blood. The red blood cells were lysed by hypotonic shock followed by KRG washing of the neutrophils. The neutrophils were then resuspended in RPMI containing 2 mM of L-glutamine and 10% heat-inactivated FBS, and left for around 20 h at 37 °C for spontaneous apoptosis. Staining with Annexin V (AV) and propidium iodide (PI) showed that around 70% were apoptotic after this incubation; 64.8% (±3.2) were apoptotic (AV+) and 4.8% (±0.5) were late apoptotic (AV+PI+). Prior to experiment, the apoptotic neutrophils were washed once before being resuspended in suitable medium for coculture with macrophages. For some experiments the neutrophils were stained with PKH26 red fluorescent membrane labeling kit (MINI26 Sigma) prior to apoptosis, according to the manufacturer's instructions.

Preparation of M. tuberculosis

M. tuberculosis H37Rv was cultured for 2 weeks in *M. tuberculosis* medium (Middlebrook 7H9 with 0.05% Tween-80, 0.5% glycerol, and 10% ADC enrichment) and passaged 1 week before use. For GFP-expressing *M. tuberculosis* the medium was supplemented with 20 μg/mL of kanamycin, and for luciferase-expressing *M. tuberculosis* 100 μg/mL of hygromycin was used. For infection, the bacteria were prepared as previously described [28].

HIV and M. tuberculosis *Coinfection*

Macrophages were infected with 0.06 ng/mL HIV-1BaL (Lot p4238), produced as previously described [28], for 1 week prior to *M. tuberculosis* infection at MOI = 1–5. After *M. tuberculosis* infection the macrophages were incubated with apoptotic neutrophils (1:2) for different time points, depending on the experiment.

Flow Cytometry and Confocal Microscopy

GFP-expressing *M. tuberculosis*-infected macrophages, seeded in 96-well plates or on cover slips, and treated with labelled apoptotic neutrophils, were stained with 75 nM of LysoTracker Deep Red (cat. No. L12492; Life Technologies) to visualize acidic organelles for 2 h prior to fixation with 4% PFA. These cells were then analyzed either by flow cytometry or confocal microscopy. Some cells were treated with 100 nM of bafilomycin A1 (from *Streptomyces griseus*, Sigma Aldrich) 1 h before and during the infection as a negative control for acidification.

MPO staining was performed after fixation of macrophages that had been infected and exposed to apoptotic neutrophils for 30 min to 24 h. The cells were permeabilized with 0.1% saponin for 30 min followed by wash and staining with the primary antibody polyclonal rabbit anti-human MPO (A0398, DAKO) diluted 1:400 for 1 h at room temperature. After washing with PBS, the secondary antibody goat anti-rabbit AF647 (A21244, Life Technologies) diluted 1:400 was added for 30 min at 37 °C. Following washing,

DAPI diluted 1:100 was added for 15 min at room temperature before the cover slips were washed and mounted.

All cover slips were analyzed in a LSM 700 Zeiss upright confocal microscope with a plan apochromat 63x, NA 1.40 objective. Images were acquired with Zen software and all samples were observed in a blinded fashion where 50–300 phagosomes/sample were examined. Image brightness and contrast were adjusted equally with Photoshop for representative micrographs only, after the completion of the blinded analysis. Pseudo colors were chosen for optimal display of the results.

Cytokine Measurement

Supernatants from infected macrophages treated with apoptotic neutrophils were analyzed for cytokines through cytometric bead array analysis, performed according to the manufacturer's instructions (BD Biosciences). Data were analyzed using Kaluza software (Beckman Coulter, Fullerton, CA, USA).

M. tuberculosis *Growth Assay*

For the *M. tuberculosis* growth assay, macrophages were infected with luciferase-expressing *M. tuberculosis* for 1.5 h, extracellular bacteria washed off with media, followed by addition of apoptotic neutrophils that had been pretreated with 500 μM of the MPO inhibitor 4-aminobenzoic hydrazide (ABAH; A41909, Sigma) for 1 h. The growth of *M. tuberculosis* was measured as described previously [29] after 5 days and compared to the day 0 values (phagocytosis).

Western Blot

Macrophages were infected for 1.5 h prior to washing and the addition of apoptotic neutrophils for a total of 24 h when the cells were collected and Western blot was performed as previously described [28]. The antibodies were: rabbit monoclonal anti-LC3B (D11; cat. No. 3868, Cell Signaling), mouse monoclonal anti-SQSTM1 D-3 (cat. No. sc-28359, Santa Cruz Biotechnology), and mouse monoclonal anti-β-actin (clone AC-74; cat. No. A2228, Sigma-Aldrich). The dilutions of the antibodies were 1:5,000 for LC3, 1:2,000 for SQSTM1, and 1:10,000 for β-actin. The secondary antibodies polyclonal goat anti-rabbit or anti-mouse immunoglobulins/HRP (Dako Cytomation) were diluted 1:2,000 for LC3 and SQSTM1, and 1:10,000 for β-actin. Band intensities were quantified using ImageJ.

ROS Measurement

The probe CM-H2DCFDA (C6827, Invitrogen) was used for general oxidative stress detection in macrophages. 5 μM was added together with *M. tuberculosis* for 1 h at 37 °C with a wash before and after, prior to the addition of apoptotic neutrophils. Measurements were performed 1 h after uptake of apoptotic neutrophils that had been pretreated with 500 μM ABAH for 1 h and washed prior to addition to the macrophages. The ROS from macrophages or apoptotic or viable neutrophils were measured as chemiluminescence using the substrate luminol (20 μg/mL; Sigma-Aldrich) upon ABAH inhibition (0–1,000 μM) and 0.1 μM PMA (Sigma-Aldrich) or 0.01 μM fMLP (Sigma-Aldrich) stimulation.

Statistical Analysis

All statistical analyses were performed with GraphPad prism software. The data were analyzed using repeated-measures ANOVA, with the post hoc Bonferroni's multiple comparison test (unless otherwise indicated). p values <0.05 were considered significant.

Results

Apoptotic Neutrophils Inhibit Growth of M. tuberculosis *in Macrophages*

It is not known if efferocytosis of apoptotic neutrophils can enhance the capacity of HIV-coinfected macrophages to control *M. tuberculosis*. Therefore, apoptotic neutrophils were added to *M. tuberculosis*-single and HIV/*M. tuberculosis*-coinfected macrophages, and the bacterial load was assessed 5 days after infection. When investigating the growth of *M. tuberculosis*, we washed away the non-phagocytosed bacteria after infection before adding apoptotic neutrophils in order to start the experiment with the same bacterial load in the different cells. Consistent with our previous results [13], *M. tuberculosis* growth was decreased by apoptotic neutrophils after 5 days (Fig. 1a, b). This growth inhibition effect was significant both when analyzing the intracellular *M. tuberculosis* (macrophage lysate) and the total bacteria (combined intra- plus extracellular contribution; Fig. 1a, b). Furthermore, there was no difference between single and coinfected macrophages, indicating that apoptotic neutrophils have the capacity to inhibit *M. tuberculosis* growth also in HIV-infected macrophages.

To confirm that apoptotic neutrophils were taken up by the infected/coinfected macrophages to the same extent, the uptake of apoptotic neutrophils was tracked. Already after 1 h, 50% of the macrophages had taken up apoptotic neutrophils (ApoN+), which further increased, reaching around 80% at 24 h (Fig. 2a). Infected and coinfected macrophages both ingested apoptotic neutrophils at a similar rate. Furthermore, the colocalization between apoptotic neutrophils and *M. tuberculosis* also increased with time (Fig. 2b). We also assessed *M. tuberculosis* uptake in order to evaluate if apoptotic neutrophils affected the phagocytosis of *M. tuberculosis*. Apoptotic neutrophils were added to infected macrophages without removing non-phagocytosed bacteria, thereby allowing further phagocytosis of *M. tuberculosis*. After 4 h of stimulation with apoptotic neutrophils (i.e., when >60% of the macrophages contained apoptotic neutrophils), we observed a 20% increase in *M. tuberculosis*-GFP+ macrophages that also contained apoptotic neutrophils, compared to those that had been stimulated with but did not contain apoptotic neutrophils (Fig. 2c). This was further confirmed by an increase in the total *M. tuberculosis*-GFP signal (MFI) in macrophages with apoptotic neutrophils from 2.2 to 3.8 in single infected and 2.0 to 3.6 in HIV-coinfected macrophages (Fig. 2d).

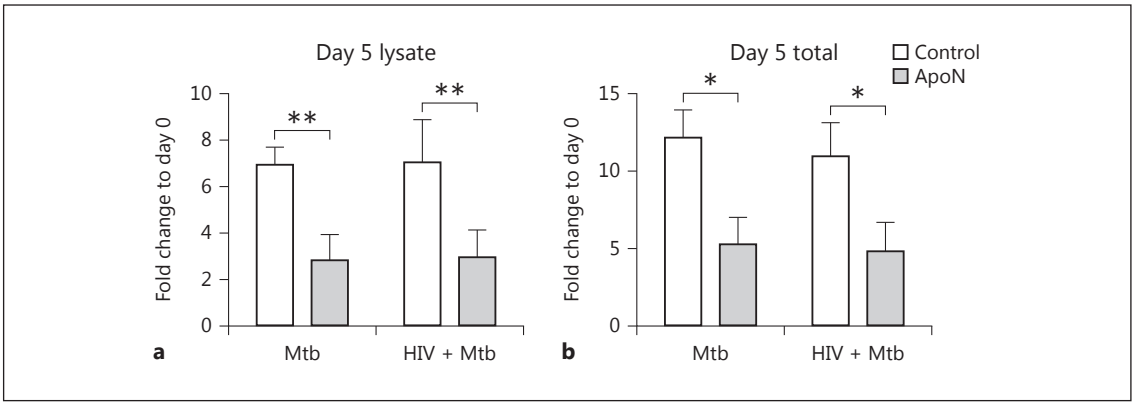

Fig. 1. Apoptotic neutrophils decreased *M. tuberculosis* growth both in single and HIV-coinfected macrophages. Human macrophages were preinfected with/without HIV for 7 days before infection with *M. tuberculosis* (Mtb, MOI = 1) for 1.5 h. After a wash, apoptotic neutrophils (ApoN) were added for 5 days and the signals from luciferase expressing *M. tuberculosis* in cell lysates (**a**) and total bacteria (supernatant + lysate; **b**) were measured. Data are the mean ± SEM from 4 independent experiments. * $p < 0.05$, ** $p < 0.01$, using repeated-measures ANOVA.

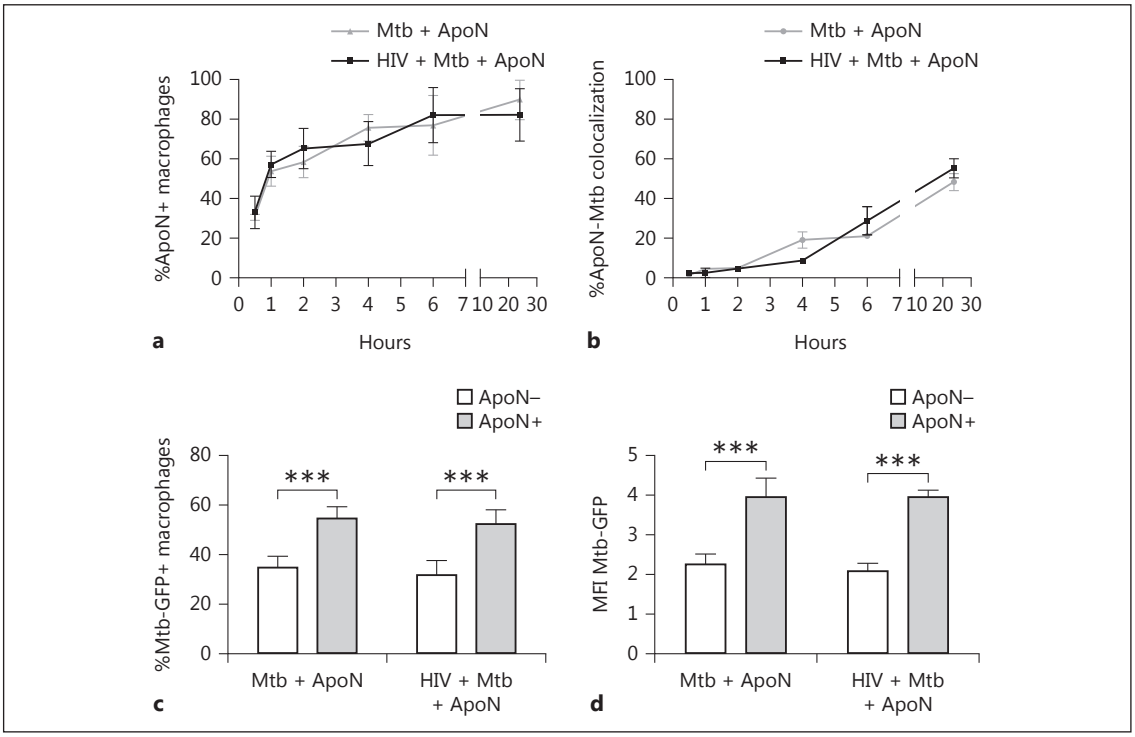

Fig. 2. Uptake of apoptotic neutrophils by macrophages is time dependent and causes an increase in bacterial phagocytosis. The percentage of macrophages containing apoptotic neutrophils (ApoN+; **a**) and percentage of colocalization of apoptotic neutrophils to *M. tuberculosis* phagosomes (**b**), at the indicated time points, as quantified by microscopy. The macrophages were first infected with HIV followed by *M. tuberculosis* (Mtb, MOI = 2) infection for 1.5 h, and extracellular bacteria were washed away before stimulation with apoptotic neutrophils for up to 24 h. The graph shows the mean ± SEM from 2 independent experiments. **c**, **d** Flow cytometry analysis revealed that phagocytosis of *M. tuberculosis* (% Mtb-GFP+ macrophages and MFI Mtb-GFP) was increased in infected macrophages containing apoptotic neutrophils (ApoN+) compared to those that were exposed but did not ingest apoptotic neutrophils (ApoN–). Data are the mean ± SEM from 6 independent experiments, where the macrophages were *M. tuberculosis* infected (MOI = 2) for 2 h followed by apoptotic neutrophil stimulation for 4 h without washing away the bacteria. *** $p < 0.001$, using repeated-measures ANOVA.

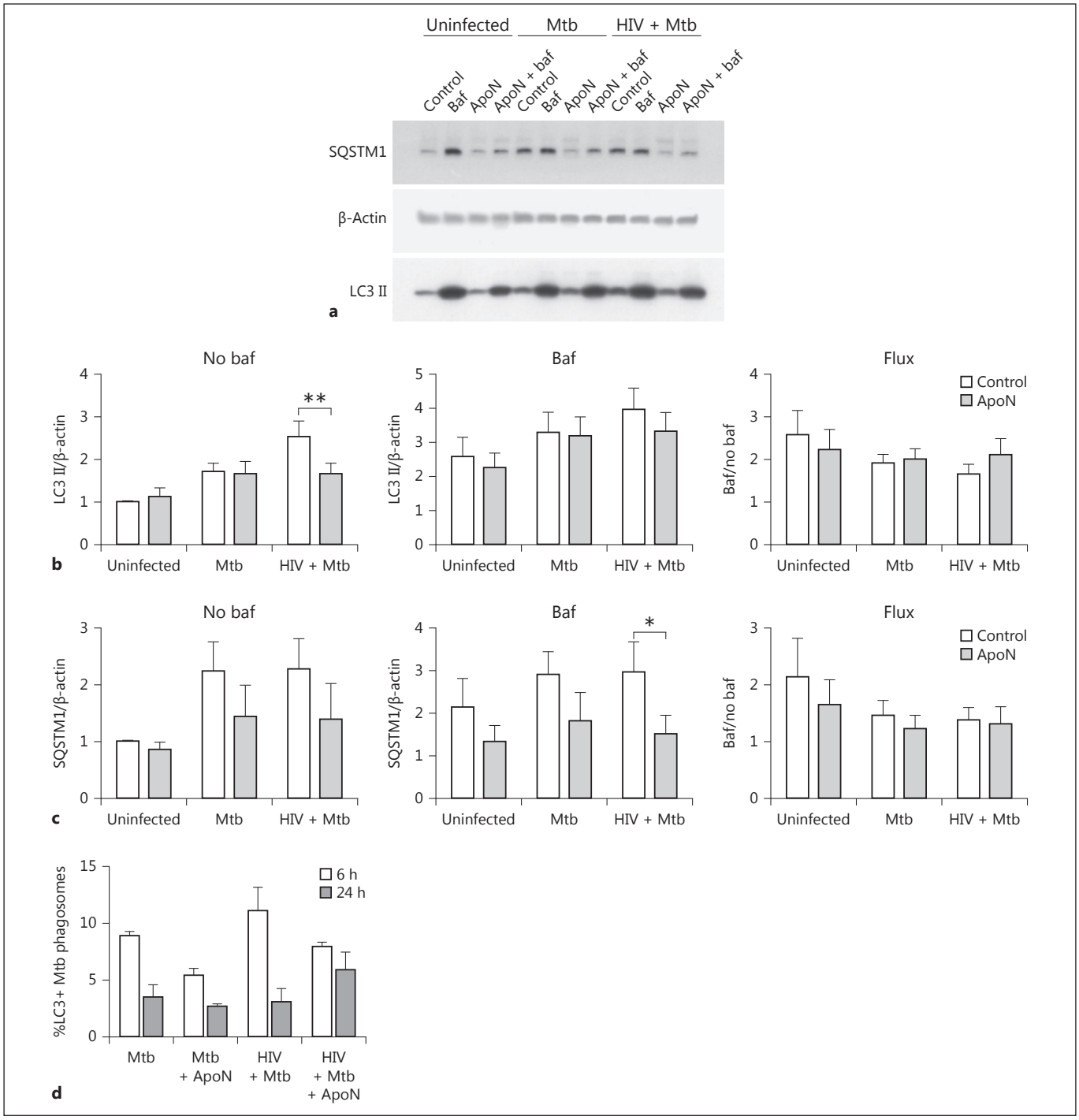

Fig. 3. Apoptotic neutrophils do not cause any changes in autophagic flux. **a** Representative immunoblots showing the autophagy markers LC3B and SQSTM1 (p62), with their β-actin loading controls. The autophagy markers LC3 II (**b**) and SQSTM1 (**c**) were quantified from Western blots and normalized to their respective β-actin control and presented as the ratio over uninfected macrophages without apoptotic neutrophils (ApoN). The macrophages were infected with *M. tuberculosis* (Mtb, MOI = 5) for 1.5 h, washed, and stimulated with apoptotic neutrophils for 22.5 h. "Baf" indicates that the macrophages were pretreated with bafilomycin for 1 h prior to infection, while the graphs named "Flux" show the autophagic flux (i.e., samples with baf/samples without baf). Data are shown as the mean ± SEM with * $p < 0.05$ and ** $p < 0.01$ using repeated-measures ANOVA ($n = 6$). **d** The percentage of LC3 colocalization to *M. tuberculosis* was quantified by microscopy after prior HIV infection and 1.5 h of *M. tuberculosis* infection (MOI = 2) followed by washing and stimulation with apoptotic neutrophils for 4.5 h (6 h in total) or 22.5 h (24 h in total). Data are the mean ± SEM from 4 independent experiments.

Apoptotic Neutrophils Do Not Cause Changes in Autophagic Flux

Since autophagy has been suggested to have a protective role during *M. tuberculosis* infection [30–32], we evaluated if efferocytosis of apoptotic neutrophils can stimulate autophagy or drive the autophagic flux. We studied the autophagy proteins LC3 II (Fig. 3a, b) and SQSTM1 (Fig. 3a, c) in whole cell lysates, and bafilomycin was used to inhibit the last steps of autophagy in order to evaluate the total accumulation of autophagosomes. The autophagic flux can be determined by the ratio of total autophagosomes formed (with bafilomycin) and those formed and being degraded (without bafilomycin). Values ≤1 indicated no flux, and values >1 indicated autophagic flux. Apoptotic neutrophils did not affect the autophagic flux (Fig. 3a–c), rather they decreased formation of autophagosomes, most noticeably for SQSTM1 with bafilomycin and with the same tendency without bafilomycin. Colocalization of LC3 with *M. tuberculosis* at 6 and 24 h postinfection further confirmed this. There was a tendency of decreased autophagosome formation at 6 h in single and coinfected macrophages when stimulated with apoptotic neutrophils (Fig. 3d). The LC3-*M. tuberculosis* colocalization levels decreased after 24 h, but to a lesser extent in the infected mac-

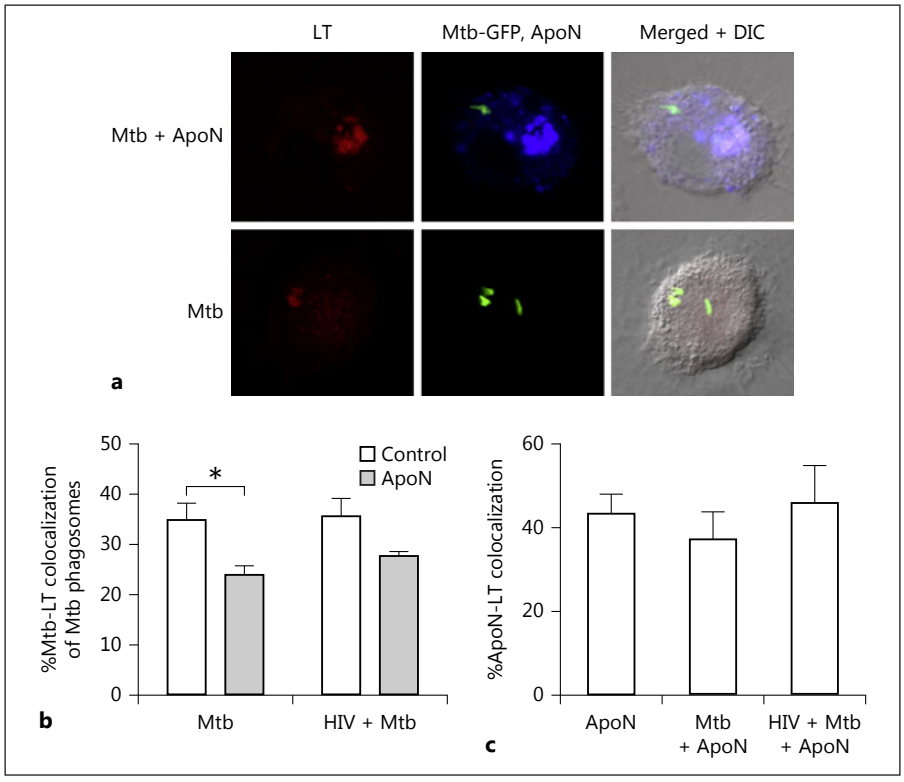

Fig. 4. Apoptotic neutrophils decrease acidification of *M. tuberculosis* phagosomes. **a** Representative micrographs of LysoTracker (LT) colocalization to apoptotic neutrophils (ApoN) or *M. tuberculosis* (Mtb) phagosomes in infected macrophages. Green, Mtb; red, LT; blue, ApoN. Macrophages were infected with *M. tuberculosis* for 2 h (MOI = 1), stimulated with apoptotic neutrophils for 4 h, with the addition of the probe LysoTracker (LT) for the last 2 h before fixation and colocalization studies by confocal microscopy. The percentage of colocalization of *M. tuberculosis* to LT (**b**) and apoptotic neutrophils to LT (**c**) for 6 donors, showing the mean ± SEM. * $p < 0.05$ using repeated-measures ANOVA.

Fig. 5. MPO, which is active even after apoptosis, is present in macrophages which have ingested apoptotic neutrophils (ApoN). ROS production by apoptotic neutrophils was measured through chemiluminescence upon stimulation with PMA (**a**) or fMLP (**b**), showing the mean from 3 independent experiments. **a** MPO was inhibited by 30 min of preincubation with increasing concentrations of ABAH (as indicated) before luminol and PMA was added to the apoptotic neutrophils. **b** Freshly isolated neutrophils (PMN) and apoptotic neutrophils were stimulated with fMLP and their ROS production measured in the presence of luminol. **c** Percentage of MPO+ macrophages at the indicated time points, as quantified by microscopy (**d**). **e** Percentage of MPO colocalization with *M. tuberculosis* (Mtb) phagosomes at the indicated time points. The macrophages were first infected with HIV followed by *M. tuberculosis* (MOI = 2) infection for 1.5 h, washed, and stimulated with apoptotic neutrophils for up to 24 h. The graph shows the mean ± SEM from 2 independent experiments. **d** The microscopy images visualize the macrophages by DIC, while apoptotic neutrophils are shown in yellow, *M. tuberculosis*-GFP in green, MPO in red, and DAPI in blue. The first row shows the cells 30 min after the addition of apoptotic neutrophils, the second after 4 h, and the last one after 24 h. The last column shows infected macrophages that have not been stimulated with ApoN but have been stained for MPO.

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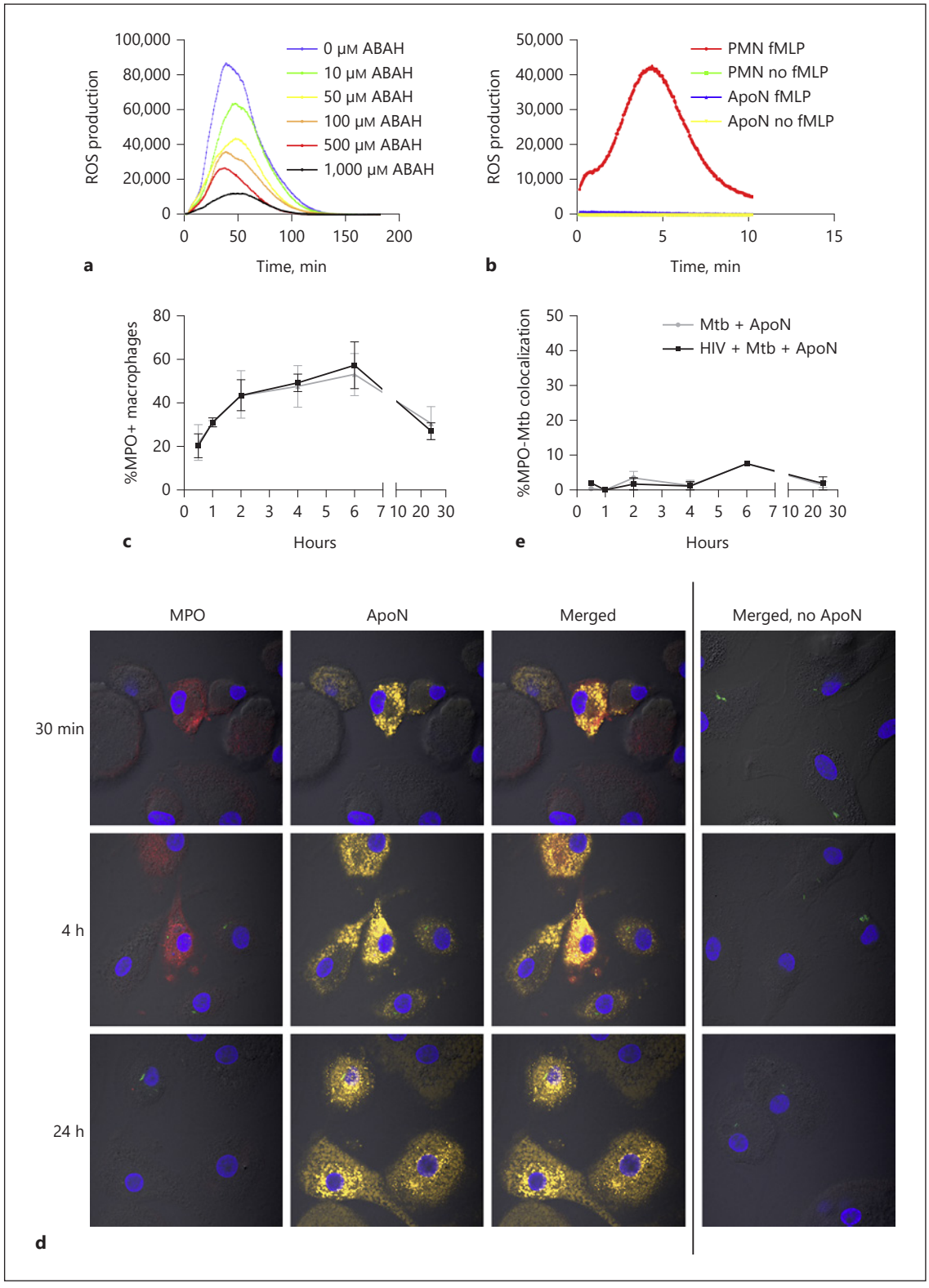
ROS production
100,000
80,000
60,000
40,000
20,000
0
0 μM ABAH
10 μM ABAH
50 μM ABAH
100 μM ABAH
500 μM ABAH
1,000 μM ABAH
0 50 100 150 200
Time, min
a
ROS production
50,000
40,000
30,000
20,000
10,000
0
PMN fMLP
PMN no fMLP
ApoN fMLP
ApoN no fMLP
0 5 10 15
Time, min
b
%MPO+ macrophages
80
60
40
20
0
0 1 2 3 4 5 6 7 10 20 30
Hours
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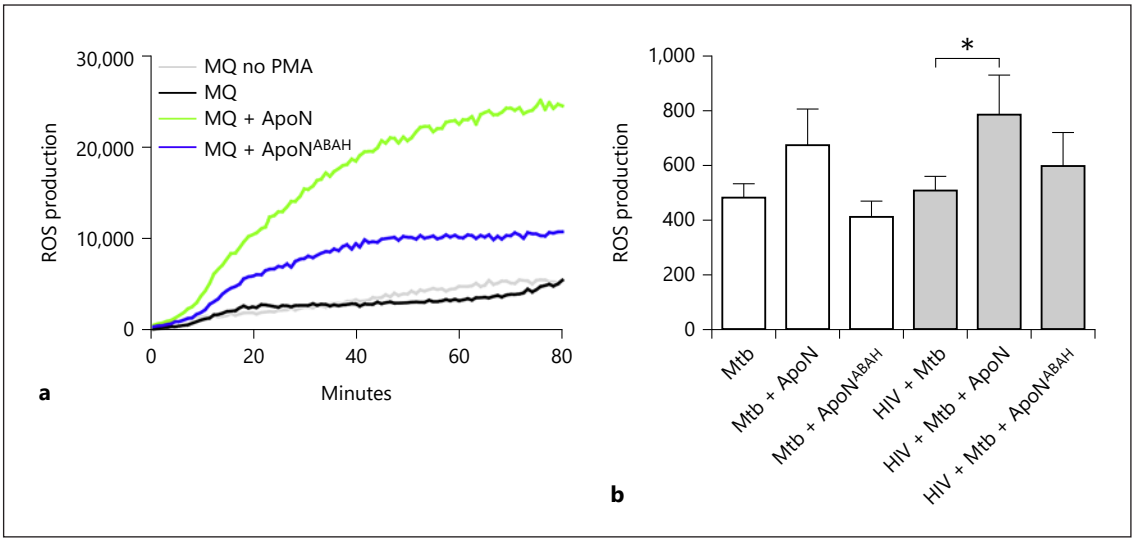

Fig. 6. Apoptotic neutrophils increase ROS production in macrophages in an MPO-dependent manner. **a** ROS production in macrophages was measured through chemiluminescence upon stimulation with PMA. Untreated apoptotic neutrophils (ApoN) and those treated with ABAH ($ApoN^{ABAH}$) were added to macrophages for 24 h, followed by washing and PMA stimulation in the presence of HRP. The graph shows the mean from 3 independent experiments. **b** ROS production measured with the probe CM-H2DCFDA in infected macrophages stimulated with apoptotic neutrophils for 1 h. Apoptotic neutrophils were untreated or treated with the MPO inhibitor ABAH ($ApoN^{ABAH}$) and washed, before being added to the macrophages. The graph shows the mean ± SEM from 5 independent experiments. * $p < 0.05$ using repeated-measures ANOVA.

rophages stimulated with apoptotic neutrophils, indicating less degradation of LC3 in those macrophages. Together, these results suggest that autophagosome formation is not directly involved when apoptotic neutrophils decrease the growth of *M. tuberculosis* in macrophages.

Lysosome Fusion with M. tuberculosis *Phagosomes Is Further Inhibited by Apoptotic Neutrophils*

To further analyze phagolysosome fusion during efferocytosis, we assessed the acidification of the *M. tuberculosis* phagosomes using the probe LysoTracker (Fig. 4a). There was a decreased colocalization between *M. tuberculosis* and LysoTracker in macrophages with apoptotic neutrophils (Fig. 4b). This indicated that acidification and maturation of the *M. tuberculosis* phagosomes were not involved in the decreased *M. tuberculosis* viability caused by the apoptotic neutrophils. Moreover, we observed a colocalization of around 40% between apoptotic neutrophils and LysoTracker in uninfected and infected macrophages (Fig. 4c). This was a higher colocalization than that seen between *M. tuberculosis* and LysoTracker, both without (approx. 35%) and with apoptotic neutrophils (approx. 25%; Fig. 4b). Flow cytometry data showed a significant ($p < 0.01$) overall increase in LysoTracker signal (MFI) in macrophages containing apoptotic neutrophils (30.1 ± 4.1 in single and 31.1 ± 5.5 in coinfected macrophages, respectively) compared to those that did not (17.1 ± 1.5 in single and 18.1 ± 3.0 in coinfected macrophages, respectively). However, from the microscopy data it is evident that this acidification was clearly concentrated to apoptotic neutrophils and did not localize to *M. tuberculosis*.

MPO, Which Is Active after Apoptosis, Is Present in Macrophages That Have Phagocytosed Apoptotic Neutrophils

Neutrophils can generate an oxidative burst owing to its high expression of the NADPH oxidase, generating superoxide anions (O_2^-), which dismutates to hydrogen peroxide (H_2O_2), which is further catalyzed into the more bactericidal HOCl by the azurophilic granular protein MPO [33, 34]. To investigate if this system is still intact in apoptotic neutrophils, we measured the ROS production using a luminol-enhanced chemiluminescence assay. Activation of apoptotic neutrophils with the phorbol ester PMA triggered a strong ROS response (Fig. 5a), whereas a receptor-dependent stimulus fMLP only induced an ROS response in live neutrophils, but not in apoptotic neutrophils (Fig. 5b). The PMA-induced ROS response by apoptotic neutrophils was additionally confirmed to be MPO dependent using the irreversible

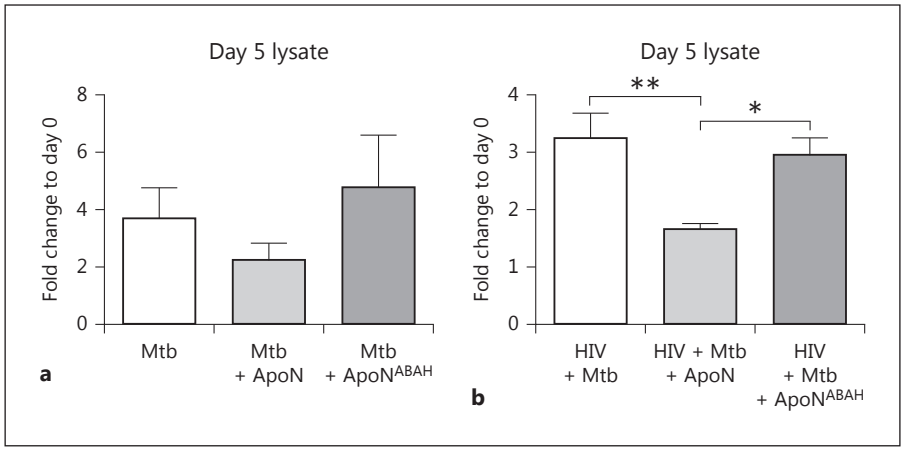

Fig. 7. The suppressed *M. tuberculosis* growth caused by apoptotic neutrophils is rescued through MPO inhibition. Apoptotic neutrophils (ApoN) were treated with 500 μM of ABAH ($ApoN^{ABAH}$) for 1 h prior to washing and added to *M. tuberculosis* (Mtb, MOI = 1) infected (**a**) and HIV-coinfected (**b**) macrophages for 5 days. The intracellular growth is shown as the mean ± SEM from 5 independent experiments. * $p < 0.05$, ** $p < 0.01$, using repeated-measures ANOVA.

MPO-inhibitor ABAH (Fig. 5a). There was a time-dependent increase of MPO inside the macrophages that had ingested apoptotic neutrophils, which was not seen in the absence of apoptotic neutrophils, confirming that the neutrophils were the source of MPO (Fig. 5c, d). The increase in MPO was correlated to the early kinetics observed for uptake of apoptotic neutrophils (Fig. 2a). However, in contrast to the uptake of apoptotic neutrophils, there was a decreased MPO inside the macrophages after 24 h (Fig. 5c, d). This indicated that the effect of MPO in efferocytosing macrophages could be transient. MPO seemed to be spread in the cytoplasm of macrophages upon uptake of apoptotic neutrophils. The colocalization of MPO to *M. tuberculosis* phagosomes remained below 10% after 24 h (Fig. 5e).

ROS Production in Macrophages Is Increased upon Phagocytosis of Apoptotic Neutrophils

We further observed an increased ROS production in uninfected (Fig. 6a) as well as HIV-coinfected macrophages after uptake of apoptotic neutrophils (Fig. 6b). Evidence for the involvement of MPO for the increased ROS production in macrophages was further supported by ABAH pretreatment of the apoptotic neutrophils, which decreased ROS production (Fig. 6a, b). Of interest is that the ROS experiments with uninfected macrophages (Fig. 6a) was performed 24 h after uptake of apoptotic neutrophils (at which time point there was less discernable MPO staining in macrophages), indicating that MPO could mediate a long-lasting functional response. These results showed that the complex for ROS generation in apoptotic neutrophils was sequestered by macrophages through efferocytosis and could enhance the antimicrobial capacity of infected macrophages.

M. tuberculosis *Growth Inhibition Caused by the Apoptotic Neutrophils Is MPO Dependent*

Since autophagy or phagosome maturation were not affected by uptake of apoptotic neutrophils, we next explored whether ROS and MPO from apoptotic neutrophils support *M. tuberculosis* killing. ABAH was therefore used to inhibit the MPO activity of the apoptotic neutrophils before they were added to the infected macrophages. When irreversibly inhibiting MPO with ABAH, the *M. tuberculosis* growth control caused by apoptotic neutrophils was reversed, although only significant in the HIV-coinfected macrophages (Fig. 7a, b). These results indicate that MPO and ROS are involved in the enhanced control of *M. tuberculosis* in macrophages after uptake of apoptotic neutrophils.

Apoptotic Neutrophils Do Not Increase the Proinflammatory Response in M. tuberculosis*-Infected Macrophages*

In order to determine whether the decrease in *M. tuberculosis* growth caused by the apoptotic neutrophils was due to an induced proinflammatory response by the macrophages, we measured the production of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 (Fig. 8a–c). We found that the apoptotic neutrophils caused a decrease in IL-1β production in both *M. tuberculosis*-single and HIV-coinfected macrophages. This was partly restored by the inhibition of MPO with ABAH. Furthermore, TNF-α was reduced in *M. tuberculosis*-infected macrophages upon stimulation with apoptotic neutrophils, with the same tendency for IL-6. These results suggest that the apoptotic neutrophils do not stimulate a proinflammatory cytokine response in macrophages infected with live virulent *M. tuberculosis*.

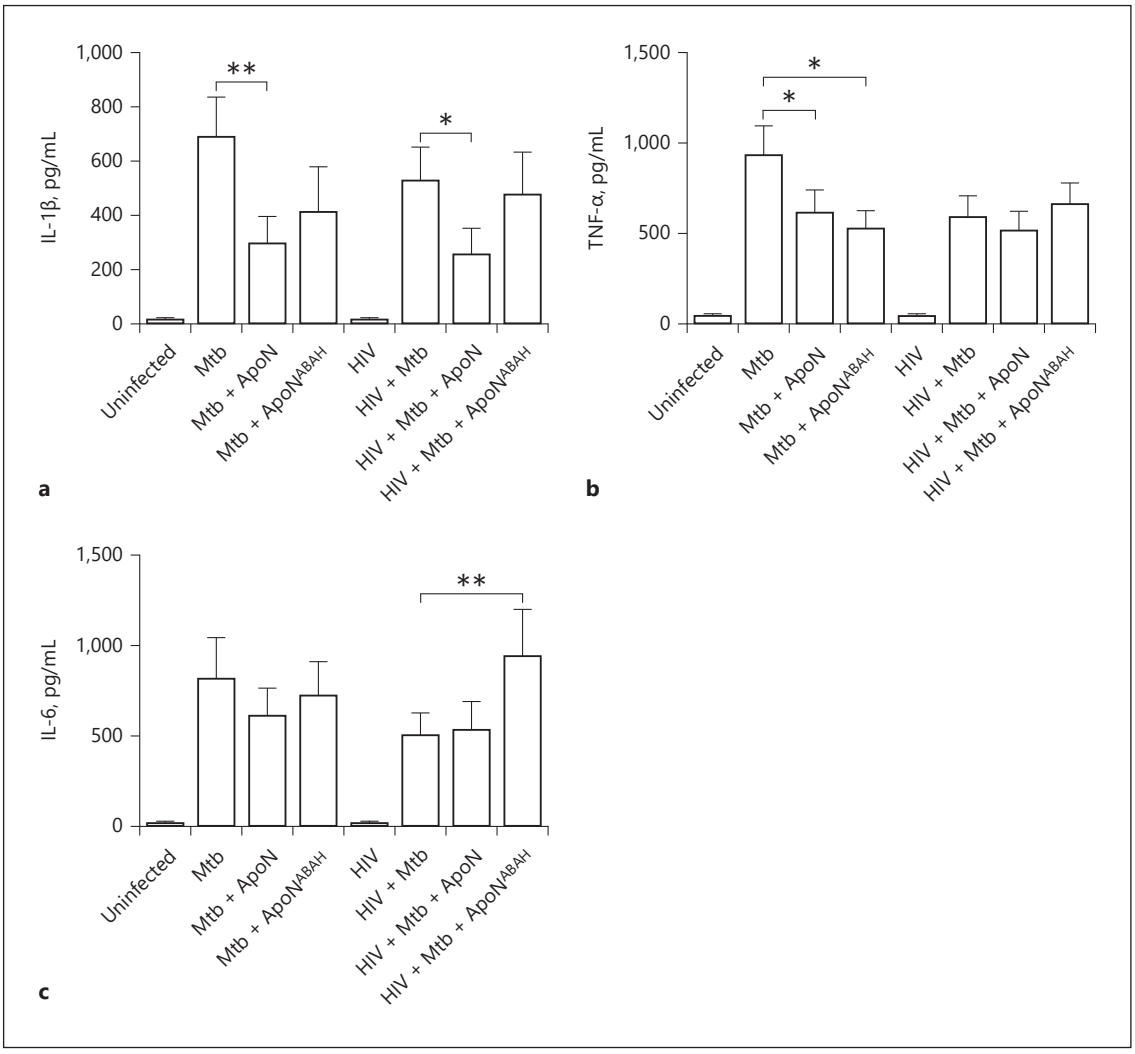

Fig. 8. Apoptotic neutrophils decrease the proinflammatory response in infected macrophages. IL-1β (**a**), TNF-α (**b**), and IL-6 (**c**) were measured in the supernatants of *M. tuberculosis* and HIV-coinfected macrophages. Apoptotic neutrophils (ApoN) were treated with 500 μM of ABAH ($ApoN^{ABAH}$) for 1 h prior to washing and added to the infected macrophages. The macrophages were infected with *M. tuberculosis* (Mtb, MOI = 2) for 1.5 h prior to stimulation with apoptotic neutrophils for 22.5 h. Data are the mean ± SEM from 11 independent experiments. * $p < 0.05$, ** $p < 0.01$, using repeated-measures ANOVA with Dunnett's multiple comparison test.

Discussion

M. tuberculosis Ag-specific CD4 T cells are depleted or are impaired in HIV/*M. tuberculosis*-coinfected individuals [35, 36], and we recently showed that HIV/*M. tuberculosis* coinfection skews these effector cells into a suppressive phenotype that fails to control *M. tuberculosis* in macrophages [25, 37]. As the adaptive immune response cannot support control of *M. tuberculosis* during HIV/*M. tuberculosis* coinfection, we here investigated if the function of coinfected macrophages can be boosted in other ways. Using an in vitro model, we showed a pronounced time-dependent uptake of the apoptotic neutrophils by coinfected macrophages. This uptake was associated with a reduction in *M. tuberculosis* growth, as only observed by others during *M. tuberculosis*-single infection [13–16]. We further established that the decreased *M. tuberculosis* growth was MPO dependent and that production of ROS was enhanced in efferocytosing macrophages, suggesting a role for ROS and MPO in the defense against *M. tuberculosis*. This cooperation between neutrophils and macrophages could thus be a way to compensate for the im-

pairment of the adaptive immunity against *M. tuberculosis* seen during HIV coinfection.

In contrast to other reports [14, 16], we did not observe any changes in autophagic flux or increased phagosomal maturation caused by the apoptotic cells. In contrast, we detected a higher percentage of LysoTracker colocalization to apoptotic neutrophils in macrophages than to the bacterial phagosomes. This is in concordance with other observations showing that apoptotic cells are swiftly cleared by macrophages in order to avoid secondary necrosis [38–41], whereas *M. tuberculosis* is able to inhibit phagosomal maturation [42, 43]. The additional decrease in maturation of *M. tuberculosis*-containing phagosomes in macrophages containing apoptotic neutrophils could be due to the increase in pH upon ROS production. A study supporting this theory showed that M1 macrophages exhibit higher phagosomal pH along with delayed phagosome-lysosome fusion compared to M2 macrophages, in part due to the increased proton consumption during intraphagosomal ROS production in M1 macrophages [44]. Neutrophil phagosomes normally have a higher pH than macrophage phagosomes, since neutrophils generate a strong oxidative burst, while the macrophages act mainly through lysosome fusion with the phagosomes [45–48]. However, since we observed very little colocalization between *M. tuberculosis* and apoptotic neutrophils or MPO, their effect was most likely not limited to the phagosome but could occur in the cytosol. There are scenarios when *M. tuberculosis* does not necessarily need to be in the cytosol for them to be affected by reactions occurring in the cytosol or reactions taking place in compartments with close proximity to the *M. tuberculosis*-containing phagosome. For instance, as part of their phagosomal escape strategy virulent *M. tuberculosis* express the ESX-1 type VII secretion system leading to pore formation and damage to the phagosomal membrane [49]. This inadvertently puts the bacterium also in contact with host defense mechanisms or components of the cytosol, as in this case the accumulation of MPO in macrophages that have ingested apoptotic neutrophils, leading to enhanced control of *M. tuberculosis*.

The antimicrobial activity of ROS and MPO against mycobacteria has been studied extensively, with earlier studies suggesting a role in bacterial killing [50–53], while more recent studies suggest that *M. tuberculosis* is only sensitive to endogenous ROS, generated within the bacteria [54, 55]. *M. tuberculosis* has several defense mechanisms against ROS, one being katG catalase-peroxidase which transforms H_2O_2 to oxygen and water. Isoniazid-resistant *M. tuberculosis*, lacking katG, is more susceptible to H_2O_2 [21, 22]. As our experiments were performed with virulent *M. tuberculosis* H37Rv with wild-type katG, this indicates that the efferocytosis-mediated ROS production is at a level beyond the threshold that *M. tuberculosis* can neutralize, and they therefore succumb to killing. Alternatively, the overall ROS production inside the macrophage triggers signaling cascades involved in killing of intracellular *M. tuberculosis*. Indeed, ROS have been implicated as signaling mediators, able to modulate phagocytosis, gene expression, and promoting a proinflammatory response [56, 57]. Although other studies have shown a role for MPO in stimulating TNF secretion by macrophages [58, 59], in this study using live virulent *M. tuberculosis* we did not find an induction of a proinflammatory response. In contrast to our earlier findings with γ-irradiated inactive *M. tuberculosis* H37Rv [13], we show that efferocytosis of apoptotic neutrophils by macrophages infected with live virulent *M. tuberculosis* H37Rv decrease the proinflammatory response. The reason for this could be that growth restriction of *M. tuberculosis* during efferocytosis reduce the stimulatory effect of *M. tuberculosis*. This would suggest that neutrophil granules are able to boost the immune response of macrophages coinfected with HIV and *M. tuberculosis*, without causing additional proinflammatory activation. In this study we have used HIV-1 BaL, which is a CCR5 tropic virus. HIV-1 BaL has the ability to infect T cells and dendritic cells in mucosa in a manner similar to the HIV isolates found to establish HIV infection in primary HIV-infected individuals [60], so the effects we found should be of relevance in vivo.

In conclusion we have found that efferocytosis of apoptotic neutrophils can inhibit *M. tuberculosis* growth in *M. tuberculosis*-single and HIV-coinfected macrophages. The decrease in *M. tuberculosis* growth in coinfected macrophages was MPO dependent, and we suggest that its effect is mediated by ROS and MPO rather than autophagy and lysosome fusion. Our study clearly shows the importance of cooperation between cells of the innate immune system and that apoptotic neutrophils can contribute to an enhanced killing of *M. tuberculosis* inside macrophages during HIV coinfection. Stimulating efferocytosis of apoptotic neutrophils or the uptake of MPO via neutrophil extracellular traps could therefore be a strategy to compensate for the impaired adaptive immune response during HIV infection.

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Statement of Ethics

The authors have no ethical conflicts to disclose. Blood, buffy coats, and normal human serum from heathy donors were obtained from the blood bank at Linköping University Hospital, who had given written consent for research use of the donated blood in accordance with the Declaration of Helsinki. Thus, this study did not require a specific ethical approval according to paragraph 4 of the Swedish law (2003:460) on Ethical Conduct in Human Research.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.-M.A., R.B., and O.S. conceived and designed the experiments; M.L. provided HIV; A.-M.A. performed the experiments and analyzed the data; A.-M.A. and R.B. wrote the paper, with inputs from all authors.

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