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Macrophages Acquire Neutrophil Granules for Antimicrobial Activity against Intracellular Pathogens

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A primary goal of the innate immune response is to recognize and destroy microbial pathogens. One of the cell types that is endowed with prodigious antimicrobial activity is the neutrophil, containing granules with high concentrations of antimicrobial factors in the range of milligrams per milliliter (1). Of the four subsets of neutrophil granules, two are significant reservoirs for antimicrobial substances: azurophilic (primary) granules that contain α-defensins, bacterial/permeability-increasing protein, myeloperoxidase, and serprocidins, and specific (secondary) granules that contain lactoferrin, cathelicidin, and neutrophil gelatinase-associated lipocalin. These two subsets and gelatinase (tertiary) granules carry lysozyme while secretory granules are important for exocytosis of membrane-associated proteins (2). However, many intracellular pathogens, including Mycobacterium tuberculosis, target macrophages and reside in their vacuoles.

In human macrophages, the antimicrobial peptide granulysin can be delivered to macrophages by cytotoxic granules of Ag-specific T lymphocytes (3, 4). In addition, the expression of cathelicidin in macrophages is triggered by TLR2 in a vitamin D-dependent manner (5). However, macrophages substantially lack preformed granules with large quantities of antimicrobial mediators. This raised the question of whether infected macrophages could acquire neutrophil granules and use their antimicrobial cargo to combat an intracellular pathogen.

The initial discovery that eukaryotic cells ingest phagocytes was made by Metchnikoff in 1891 (6). In recent years, numerous studies showed that the major function of this process is the removal of apoptotic neutrophils without inducing inflammation or tissue damage (7, 8). However, the uptake of apoptotic cells by macrophages does not solely serve as a waste disposal of the immune system. It was suggested that macrophages acquire functionally active myeloperoxidase by the phagocytosis of apoptotic neutrophils (9) and increase antimicrobial activity of infected cells (10). The transfer of microbial Ag from apoptotic bodies to dendritic cells was shown to initiate adaptive immune responses directed against viral (11) and bacterial (12) pathogens.

We reasoned that the ability of macrophages to uptake apoptotic neutrophils, and their granules, could provide a pathway for transferring the antimicrobial activity between the two cell types. We chose to study tuberculosis as a model for the interaction between neutrophils and macrophages. A key role for neutrophils is demonstrated in host defense against tuberculosis infection. In particular, circulating neutrophils become activated and are recruited to lungs early in infection. Their role in host defense against M. tuberculosis is supported by studies showing that depletion of neutrophils before i.v. challenge with M. tuberculosis compromises the immune response against mycobacterial infection (13–19). Because the tuberculosis bacilli primarily reside in macrophages, we studied whether macrophages could acquire antimicrobial granules from apoptotic neutrophils and whether the uptake of neutrophil...
granules increased the antimicrobial activity of macrophages against intracellular mycobacteria.

Materials and Methods

Abs and reagents

mAbs used for immunofluorescence and FACS included the following: CD14 (Zymed Laboratories), CD15 (Caltag Laboratories), human neutrophil peptide 1 (HNP-1; although this Ab does not distinguish between HNP-1-4, the nomenclature of HNP-1 alone will be used; BD Pharmingen), CD68 (R&D Systems), CD71 (BD Pharmingen), IgG controls (Sigma-Aldrich; Caltag Laboratories), Alexa 488- and 568-conjugated anti-mouse IgG (Molecular Probes), Cy5-conjugated anti-mouse IgG (Caltag Laboratories). Cell stains used included: Cell Tracker Green (5-chloromethylfluorescein diacetate (CMFDA); Molecular Probes), Alexa 568-dextran (Molecular Probes).

Mycobacteria

*M. tuberculosis* virulent strain H37Rv was grown in suspension with constant, gentle rotation in roller bottles containing Middlebrook 7H9 broth (BD Biosciences) supplemented with 1% glycerol (Roth), 0.05% Tween 80 (Sigma-Aldrich), and 10% Middlebrook oleic acid, albumin, dextrose, and catalase enrichment (BD Biosciences). Aliquots from logarithmically growing cultures were frozen in PBS containing 10% glycerol, and representative vials were thawed and enumerated for viable CFU on Middlebrook 7H11 plates. Staining of bacterial suspensions with fluorochromic substrates differentiating between live and dead bacteria (BacLight; Molecular Probes) revealed a viability of the bacteria above 90%.

GFP-BCG were passed through PMA treated (10 ng/ml) THP-1 (American Type Culture Collection (ATCC)) for 1 wk and frozen in PBS containing 10% glycerol. Representative vials were thawed revealing 90% viability; the determined by CFU on Middlebrook 7H11 plates. Bacteria for infections were prepared by streaking frozen stocks on Middlebrook 7H11 plates; colonies were removed from plates, placed in PBS 0.05% Tween 80 (Sigma-Aldrich), and H2O bath-sonicated (Branson Ultrasonics) before enumeration (OD660).

Preparation of alveolar macrophages (AM), monocyte-derived macrophages, and neutrophils

AM were obtained from the bronchoalveolar lavage fluid of patients, who underwent bronchoscopy for diagnostic purposes. AM from patients given a diagnosis of an infectious lung disease or a disease affecting the alveolar space were excluded. Lavage fluid was filtered through a cell strainer (40-μm; BD Biosciences) and centrifuged (1200 rpm, 10 min). at 4°C. The pellet was resuspended in RPMI 1640 supplemented with amphotericin B (5.6 μg/ml; Sigma-Aldrich), penicillin (60 μg/ml), and 10% human serum. Cells were plated in 6-well plates (Costar) at a density of 1 × 10^6/ml and after 1 h nonadherent cells were removed by vigorous washing with PBS.

Flow cytometry confirmed the purity of the population (CD3<1%, CD19, CD56, CD66, CD1 negative).

To prepare monocyte-derived macrophages, PBMC were obtained following informed consent from healthy human donors by Ficoll (Amersham Biosciences) density centrifugation. Monocytes were enriched using Percoll density centrifugation (Amersham Biosciences). Briefly, PBMC at 30–50 × 10^6 were overlaid to a 50% solution of isosmotic Percoll (12.6:1 of Percoll:10× PBS, and medium), washed with cold monocyte wash buffer (2 mM EDTA, 2% FCS in PBS (pH 7.2–7.4)) and further purified by adherence using 6-well plates (Corning) and Iscove’s DMEM (Invitrogen Life Technologies) supplemented with 2 mM penicillin, 50 μg/ml streptomycin, and 10% autologous serum. Cells were incubated in Iscove’s 10% autologous serum for 5–7 days at 37°C in 5% CO2 to allow macrophage differentiation. To prepare autologous serum, nonheparinized blood was clotted, pelletted at 3000 rpm for 15 min, and uncultured plasma was passed through a 0.2-μm filter. To prepare neutrophils, RBC pellets remaining from Ficoll PBMC were further partitioned with 3% dextran plus 0.9% NaCl. Washes were performed with cold PBS and contaminating RBCs were lysed with cold NH4Cl lysis buffer.

Preparation of apoptotic neutrophils and B cells

Neutrophils were incubated at 37°C with 5% CO2 in Iscove’s medium plus 10% FCS (HyClone) for 20 h to allow apoptosis. Annexin V-PE FACS analysis indicated ~70% apoptotic cells. CMFDA labeling was performed as described (20). B cells were purified from the peripheral blood of healthy donors by positive selection using magnetic anti-CD19 beads following the protocol supplied by the manufacturer (Miltenyi Biotec). Briefly, 50 × 10^6 PBMC were incubated with 100 μl of anti-CD19 beads and separated magnetically. The yield was typically 1–3 × 10^6. Purity was above 90% in all experiments as determined by CD20-PE staining. The majority of contaminating cells were T cells and the absence of granulocytes was confirmed by CD66b staining. B cells were UV irradiated (1 min, wavelength 312 nm, 6 W) which induced apoptosis during the following 12 h of incubation. Labeling of B cells with Annexin V-PE and propidium iodine revealed that this treatment induced apoptosis in >80% of the B cells.

Immunofluorescence confocal microscopy

Adherent macrophages were removed from culture plates after 5–7 days in culture with trypsin-EDTA and plated in LabTek II 8-well chamber slides (Nalge Nunc International) for infection and/or treatments (see below). Nonadherent neutrophils and PBMC were plated on poly-l-lysine coated printed slides. For immunolabeling, cells were fixed with 4% paraformaldehyde and blocked for 1 h in 5% human serum, 10% goat serum, and 2% nonfat milk. For labeling intracellular proteins, cells were permeabilized using 0.01% saponin and 0.1% Triton X-100 during blocking. Immunofluorescence was examined using a Leica TCS SP inverted confocal laser scanning microscope (Leica Microsystems) fitted with krypton, argon, helium-neon, and two-photon lasers at the Carol Moss Spivak Cell Imaging Facility in the Brain Research Institute (University of California, Los Angeles, CA).

Flow cytometry analysis

Expression of cell surface antigenic determinants and intracellular HNP-1 were detected by flow cytometry. For detecting intracellular proteins, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin before labeling. Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility. Data were analyzed using WinMDI 2.8 (J. Trotter, The Scripps Research Institute, San Diego, CA).

Preparation of granules

A total of 1 × 10^6 neutrophils was placed in 1 ml of sucrose (0.34 M (pH 7.4)) and lysed through 10-s bursts of a probe sonicator while incubated on ice. Lysates were centrifuged at 200 × g for 10 min (4°C) to separate granules from nuclei and cell debris. Supernatants were then centrifuged for 30 min (4°C) at 1800 rpm and pellets containing granules were resuspended in 0.34 M sucrose and stored at −20°C. For bacterial assays, granules were thawed, centrifuged for 10 min at 1800 rpm, and resuspended in cell culture medium. Small aggregates were disrupted by sonication in a preheated water bath (37°C). The number of granules was calculated as equivalent to the number of granulocytes used for the preparation of the granules.

Preparation of macrophages for confocal microscopy

For confocal experiments using monocyte-derived macrophages, infections and treatments were performed in chamber slides. Uninfected macrophages were treated with CMFDA-stained apoptotic neutrophils at a ratio of 10 neutrophils per macrophage for 30 min before washing and immunostaining. For infections, macrophages were infected with GFP-BCG (multiplicity of infection 3) for 16 h, washed with PBS to remove remaining extracellular bacteria, and treated with unstained apoptotic neutrophils at a ratio of 10 neutrophils per macrophage for 1–12 h before immunostaining.

Quantification of mycobacterial growth

Incorporation of [3H]uracil (GE Healthcare Europe) into mycobacterial RNA was used to determine extracellular growth of *M. tuberculosis* as described previously (21). Briefly, 2 × 10^6 mycobacteria resuspended in RPMI 1640 (Biochrom) supplemented with glutamine (2 mM; Sigma-Aldrich), 10 mM HEPES and 13 mM NaHCO3 and 5% human AB serum (Cambrex) were cultured in 96-well round-bottom plates. Granules were isolated as described above and cultured overnight to induce apoptosis. Apoptotic granulocytes were then coincubated with mycobacteria for a total of 96 h. In selected experiments, freshly thawed, purified granules were added instead of the granulocytes. [3H]uracil (1 μCi) was added for the final 12–18 h of incubation. Before harvesting onto glass fiber filters, mycobacteria were killed by treatment with 4% paraformaldehyde for 30 min. [3H]uracil incorporation was measured in a beta counter. Background radioactivity in wells containing only culture medium was <300 cpm in all experiments.

4 Abbreviations used in this paper: HNP-1, human neutrophil peptide 1; CMFDA, 5-chloromethylfluorescein diacetate; BCG, bacillus Calmette-Guérin; AM, alveolar macrophage.
For determining the effect of apoptotic neutrophils and purified granules on the growth of intracellular mycobacteria, AM were infected with single-cell suspensions of *M. tuberculosis* (multiplicity of infection 5) in 6-well culture plates. After 4 h, extracellular bacteria were removed by rinsing with PBS. Adherent cells were detached by treatment with EDTA (1 mM, 10 min) and replated at a concentration of 5 × 10⁴ cells in 500 µl in a 24-well plate. The efficiency of infection, as quantified by staining of control cultures on Permanox chamber slides (Nunc) in every experiment varied between 18 and 38%. In selected experiments, viability of the cells was measured by trypan blue exclusion and was generally higher than 90%. Granulocytes that had been cultured overnight or thawed purified granules were added to the AM and incubated for 5 days. Alternatively, freshly thawed purified granules were given to the infected AM. The number of viable bacilli was determined by lysing the cells with 0.3% saponin (Sigma-Aldrich) to release intracellular bacteria. Cell lysates were resuspended vigorously, transferred into screw caps, and sonicated in a preheated water bath for 5 min. Aliquots of the sonicate were diluted 10-fold in 7H9 medium. Four dilutions of each sample were plated in duplicate on 7H11 agar plates and incubated at 37°C in 5% CO₂ for 21 days before determining the number of CFU.

**Results**

**Macrophage phagocytosis of apoptotic neutrophils inhibits intracellular mycobacterial growth**

A necessary event during resolution of inflammation is the clearance of apoptotic neutrophils by local macrophages. To investigate whether this could result in the transfer of antimicrobial activity, we first established an in vitro model to follow the trafficking of apoptotic neutrophils. Neutrophils were first labeled with a stable cytoplasmic dye (CMFDA) that retains fluorescence during apoptosis and allows detection of large and small apoptotic material (20). To track macrophage uptake of neutrophil apoptotic bodies, CMFDA-labeled apoptotic neutrophils were incubated with macrophages for 1 h before labeling with the macrophage marker CD68. By confocal laser microscopy, CMFDA-labeled apoptotic neutrophils were readily detected within CD68⁺ macrophages (Fig. 1a). To determine whether apoptotic neutrophils could mediate an antimicrobial activity against intracellular *M. tuberculosis*, apoptotic neutrophils were added to AMs infected with virulent *M. tuberculosis* for 5 days and the number of CFU was determined. Apoptotic B cells served as a control because they do not contain antimicrobial effector molecules and to exclude that host cell antimicrobial mechanisms are induced by the interaction with apoptotic debris. Addition of apoptotic neutrophils reduced the viability of intracellular *M. tuberculosis* in a dose-dependent manner while in contrast, apoptotic B cells had no effect on the viability of *M. tuberculosis* (Fig. 1b). The antimicrobial effect was bacteriostatic, because the bacterial load after 5 days was moderately higher than the initial inoculum (1.9 ± 0.4 × 10⁶ CFU at day 0 vs 2.2 ± 0.9 × 10⁶ after 5 days). These data show that apoptotic neutrophils provide a vehicle for the delivery of antimicrobial activity to macrophages infected with intracellular mycobacteria.

**HNP-1 is a marker for neutrophil granules**

The antimicrobial activity of neutrophils is known to be concentrated in the granules, which contain several antimicrobial peptides and enzymes. Among granule constituents, human neutrophil α-defensins (HNP, isoforms 1–4 differ in a single N-terminal residue but have similar antimicrobial properties) and cathelicidins have been well-described and are found within azurophilic and specific granules, respectively. In selecting an appropriate granule marker for our studies, we chose HNP-1 because this peptide and its isoforms constitute ~30% of the protein in azurophilic granules (22) and possesses a broad spectrum of antimicrobial activity against a variety of pathogens including mycobacteria (23, 24).

Because neutrophil antimicrobial peptides have been reported in several subsets of leukocytes (25, 26), we sought to determine whether HNP-1 was expressed in cells of the monocyte/macrophage lineage. HNP-1 was visualized in PMN using confocal laser microscopy. HNP-1 was readily detected within CD15⁺ neutrophils, but not within CD68⁺ monocytes or CD68⁺ macrophages at a ratio of 10:1. Cells were cultured for 1 h, washed, and plated onto poly-l-lysine-coated glass slides for labeling with anti-CD68. Scale bar, 10 µm. b, AM were infected with *M. tuberculosis* at a multiplicity of infection of 5 and seeded at 5 × 10⁴/well. The average number of viable intracellular mycobacteria at day 0 was 2.3 ± 1 × 10⁶. Apoptotic PMN or B cells were added in different amounts as indicated. Samples were incubated for 5 days before cells were lysed and CFUs were determined. The data represent the average number of CFU ± SEM from experiments performed with 12 different donors. *, Statistically significant differences (p < 0.05).

**Macrophages phagocytose apoptotic neutrophils including granule contents**

Neutrophils enter the site of infection as part of the acute inflammatory response and undergo apoptosis hours after arrival. Apoptosis results in a silent death that prevents release of proinflammatory toxic cell contents (27). During this terminal stage, neutrophils decrease surface adhesion molecules and are less responsive to activating or degranulating stimuli (28–30). To investigate whether apoptotic neutrophils retain the contents of their antimicrobial granules, we used two-color immunofluorescence microscopy to examine HNP-1 in viable and apoptotic neutrophils. CMFDA-labeled neutrophils were cultured for 20 h to spontaneously trigger apoptosis. Cells at time 0 and at 20 h were then labeled with anti-HNP-1 mAb and visualized by confocal laser...
microscopy. Both viable neutrophils at time 0, and apoptotic cells and bodies from 20 h were found to contain HNP-1 (Fig. 3a). Therefore, apoptosis of neutrophils does not result in loss of granule contents that can presumably remain available for antimicrobial activity.

We next asked whether granule contents could be detected in macrophages after these cells phagocytosed neutrophil apoptotic bodies. CMFDA-labeled apoptotic neutrophils were incubated with macrophages and then labeled with anti-CD68 and anti-HNP-1 mAbs. Using three-color confocal laser microscopy, 90% of CD68+ macrophages were found to contain CMFDA+ apoptotic neutrophils including HNP-1 enclosed within apoptotic bodies, as a marker of granule contents (Fig. 3b). These data demonstrate that the contents of neutrophil granules are delivered into macrophages through phagocytosis of apoptotic neutrophils.

The uptake of apoptotic neutrophils by macrophages leads to the delivery of granule contents to early endosomes and mycobacterial phagosomes

Intracellular localization of the internalized granule contents is important in the context of mycobacterial infection because the bacteria reside in specialized host cell compartments (31). Mycobacteria block phagolysosomal fusion and reside for extended periods within phagosomes expressing early endosome markers (32). Soluble factors such as transferrin, an iron-carrying serum protein, have been shown to enter mycobacterial phagosomes (33). To determine whether the contents of neutrophil granules traffic to macrophage endosomes, macrophages were incubated with CMFDA-labeled apoptotic neutrophils for 1 h before labeling with CD71, the transferrin receptor expressed on early endosomes. Cells were colabeled with anti-HNP-1 Ab as a marker for neutrophil granule contents and visualized by two-color confocal laser microscopy. These experiments revealed that HNP-1-labeled neutrophil bodies enclosed within CD71+ compartments (Fig. 4a), indicating that granule contents internalized through apoptotic vesicles can be delivered to early endosomes of macrophages.

Because mycobacterial phagosomes continue to exchange material with early endosomes, the localization of neutrophil granule contents within these CD71+ compartments suggests that antimicrobial contents may enter mycobacterial compartments. We chose GFP-labeled BCG as a model organism to investigate the biological consequence of granule internalization by macrophages. We first verified that the GFP-BCG demonstrated the hallmark macrophage pathology found in mycobacterial infection, inhibition of phagolysosomal fusion. When macrophages were first infected with GFP-BCG and then pulsed for 12 h with the lysosomal marker Alexa 568-labeled dextran, no colocalization was observed indicating that phagosomes had not fused with lysosomes (data not shown).

We next tested whether the uptake of apoptotic neutrophils could deliver granule contents to GFP-BCG phagosomes. Infected macrophages were incubated with apoptotic neutrophils for 1–12 h. After 1 h, HNP-1 was detectable within macrophages but not in mycobacterial phagosomes. However, at 12 h, we detected colocalization in ~70% (data not shown) of GFP-BCG with HNP-1 including evidence of alteration in bacterial morphology at higher magnification (Fig. 4b). These data indicated that the contents of neutrophil granules, acquired by macrophages by uptake of apoptotic neutrophils, could traffic to compartments containing mycobacteria and further suggested the possibility that neutrophil-derived materials could arm macrophages to decrease the viability of intracellular mycobacteria.

Purified neutrophil granules inhibit the growth of extracellular and intracellular mycobacteria

Although the data clearly indicate that uptake of apoptotic neutrophils could result in the delivery of granule contents to mycobacterial phagosomes and inhibit intracellular bacterial growth, they do not provide evidence that the granules themselves mediate the antimicrobial activity. Initially, we investigated whether purified granules modify mycobacterial growth in axenic culture. Extracellular mycobacteria were seeded in a 96-well plate and purified granules were added in increasing numbers. As a correlate of mycobacterial viability, the incorporation of [3H]uracil was measured during the final 24 h of the 4-day incubation period. Granules prepared from 1 × 10^5 or 1 × 10^6 neutrophils limited the metabolic activity of virulent mycobacteria by 80 and 88%, respectively (Fig. 5a). To demonstrate that preformed products found within neutrophil granules can confer antimicrobial activity against mycobacteria residing within human host cells, macrophages were treated with purified neutrophil granules prepared by sucrose density centrifugation. Macrophages were able to take up these granules, and like apoptotic neutrophils, HNP-1 as a marker for granule contents colocalized with early endosome marker CD71 as shown by two-color confocal laser microscopy (Fig. 5b). Uptake of granules by M. tuberculosis-infected AM limited bacterial growth in a dose-dependent manner and reached a maximum if AM were cultured with granules purified from 1 × 10^5 neutrophils (Fig. 5c). Inhibition of mycobacterial proliferation became apparent after 3 days.
days of culture and was maintained throughout the 7-day observation period (Fig. 5d). Similar to the effect of apoptotic neutrophils, the antimicrobial activity was bacteriostatic because growth of *M. tuberculosis* was delayed, but not abolished when compared with the initial inoculum. These data provide evidence that the uptake of neutrophil granules by macrophages confers an antimicrobial activity against intracellular *M. tuberculosis*.

**Discussion**

A key function of the innate immune system is the detection and destruction of microbial pathogens. The activation of antimicrobial pathways has been studied in specific cell types, yet one enigma is the mechanism by which neutrophils, with their preformed granules containing antimicrobial effector molecules, contribute to host defense against intracellular pathogens. In this study, we provide evidence for the cell-cell cooperation of two distinct cell types of the innate immune system, neutrophils, and macrophages, in combating an intracellular pathogen that resides within macrophages, *M. tuberculosis*. Specifically, our data demonstrate that macrophages take up apoptotic neutrophil granules, including granule contents, which traffic to endosomes and colocalize with the intracellular mycobacteria. The uptake of neutrophils, and importantly purified neutrophil granules results in a direct antimicrobial activity against the intracellular pathogen, and is mediated by transfer of the granules alone. These data provide evidence that macrophages acquire antimicrobial activity against intracellular pathogens through the uptake of neutrophil granules and extend earlier findings on the cooperation between murine neutrophils and macrophages infected with avirulent mycobacteria (10).

Although macrophages are the key targets of mycobacterial infection, there is evidence that neutrophils are required for an effective host response (14, 16). First, neutrophils are found at the site of disease both at the onset of mycobacterial infection and several days after the initial response (16, 19). Depletion of neutrophils before infection enhanced mycobacterial growth in the lungs of infected animals, whereas treatment locally with MIP-2 enhanced neutrophil recruitment and decreased mycobacterial growth (14, 16). Multiple functional roles for neutrophils in the host response to mycobacteria have been suggested, including the release of chemokines (17, 18), the induction of granuloma formation (18), and macrophage uptake of neutrophil-specific factors such as myeloperoxidase (34) and lactoferrin (10). Bacilli can be found in human lung neutrophils (35), but whether the neutrophils contribute directly to killing remains unclear (36, 37). Our data provide a mechanism whereby neutrophils can indirectly contribute to killing of mycobacteria, by the ability of infected macrophages to acquire the contents of neutrophil granules and their antimicrobial molecules. These findings are consistent with the observation that neutrophils shed “pseudoplatelets” or “cell-like” particles, containing myeloperoxidase and nuclear debris, and that these particles can be ingested by macrophages (34). It is likely that these cell-like particles are apoptotic bodies that in addition to myeloperoxidase (38), contain lactoferrin (10) and other antimicrobial peptides. The demonstration here that macrophage uptake

**FIGURE 3.** Neutrophil (PMN) granules are delivered to macrophages through phagocytosis of apoptotic PMN. *a*, PMN isolated from a single donor were stained with fluorescent CMFDA dye (green) for 20 min and washed. Cells were incubated for 20 h to allow apoptosis. Stained cells from an aliquot designated as time 0 were plated onto poly-l-lysine-coated glass slides, fixed, and stained with anti-HNP-1. After 20 h, apoptotic cells were washed and similarly plated onto a glass slide for staining. Scale bar, 25 μm. *b*, A diagram is shown to illustrate the experimental design. Autologous apoptotic PMN were stained with CM-FDA dye and prepared as described and added to macrophages at a ratio of 10:1, respectively. After incubating for 30 min, wells were gently washed to remove excess free PMN and debris. Remaining adherent macrophages were fixed, permeabilized, and double stained with anti-CD68 and anti-HNP-1. Samples were visualized using confocal microscopy in addition to differential interference contrast. Scale bar, 10 μm.
of apoptotic neutrophils results in growth inhibition of intracellular mycobacteria indicates the biological significance of this uptake pathway.

A key finding of the present study is the elucidation of a mechanism by infected macrophages that can acquire neutrophil granule contents. We provide evidence that apoptotic neutrophils are readily phagocytosed by macrophages infected with mycobacteria. This mechanism is consistent with knowledge of neutrophil function in inflammatory reactions. Upon entrance into tissues, neutrophils readily undergo apoptosis, an important component of host defense (39, 40) accelerated by *M. tuberculosis* ligands triggering TLR2 and/or other pathogen recognition receptors (41, 42). Phagocytic cells, including macrophages, normally clear the apoptotic neutrophils to avoid tissue injury from the toxic debris. The data presented here also show that the ability of macrophages to phagocytose apoptotic neutrophils allows macrophages to acquire antimicrobial activity against *M. tuberculosis*.

Many microbes, including *M. tuberculosis*, attempt to evade host defense mechanisms by infecting macrophages, residing in intracellular compartments and inhibiting fusion with lysosomes that contain antimicrobial mediators. Extensive characterization of mycobacterial phagosomes reveals that the transferrin receptor (CD71), rab5, and early endosome Ag 1 (EEA1), all markers of early endosomes, decorate these phagosomes while late endosomal proton-ATPases are blocked from recruitment to phagosomal compartments (31, 32, 43). Using HNP-1 as a marker of azurophilic granules, our data provide evidence that macrophage uptake of apoptotic neutrophils as well as purified neutrophil granules resulted in the trafficking of granule contents to CD71+ early endosomes, and to the phagosomes in which mycobacteria resides. The

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**FIGURE 4.** Granules within apoptotic neutrophils are delivered to early endosomes and colocalize with intracellular GFP-BCG. *a*, Macrophages were incubated with unstained apoptotic neutrophils for 1 h, gently washed to remove excess neutrophils, fixed, permeabilized, and double stained with anti-HNP-1 and anti-CD71 (mAb to transferrin receptor). Scale bar, 25 μm. *b*, Macrophages were infected for 16 h with GFP-BCG at a multiplicity of infection of 3. Cells were washed to remove any extracellular bacteria before incubation with unstained apoptotic neutrophils. After 1 or 12 h, cells were fixed and permeabilized before labeling with anti-HNP-1. Higher power magnification at 12 h is shown at the bottom. Scale bars, 10 μm.

**FIGURE 5.** Purified neutrophil granules colocalize to early endosomes and inhibit growth of extracellular and intracellular *M. tuberculosis*. *a*, A total of 2 × 10^6 *M. tuberculosis* were cultured with increasing numbers of granules (numbers on the x-axis give the number of neutrophils from which granules were prepared) for 96 h. [3H]Uracil (1 μCi) was present during the final 24 h of incubation. The figure shows the average uracil-uptake (cpm) ± SEM calculated from four independent experiments. All wells were set up in triplicates (*, p < 0.01). *b*, Macrophages were incubated with purified neutrophil granules for 1 h, gently washed to remove excess granules, fixed, permeabilized, and double stained with monoclonal anti-HNP-1. Scale bar, 20 μm. *c*, Human AM were infected with *M. tuberculosis* at a multiplicity of infection of 5. After overnight infection, extracellular bacteria were washed off and purified granules were added in different concentrations (given as the amount of neutrophils used for the purification of granules). After 3 days of culture, CFU were determined by plating cell lysates. The data represent average CFU ± SEM from independent experiments performed with seven different donors (*, p < 0.05; **, p < 0.01). *d*, A total of 5 × 10^5 infected AM were incubated with granules purified from 1 × 10^5 neutrophils. At different time points, the number of viable intracellular mycobacteria was determined by plating cell lysates. The bars depict the number of CFU in cultures incubated in the absence (□) or presence (■) of granules. The data represent averages ± SEM from independent experiments performed with five different donors (*, p < 0.01).
fusion of phagosomes with endosomes containing apoptotic neutrophils provides a potential and critically important microbicidal pathway, because mycobacterial phagosomes do not fuse with late endosomes/lysosomes. Therefore, these data provide a mechanism by which neutrophil granule contents can be acquired by macrophages and directed to the intracellular compartments containing a microbial pathogen, providing a novel mechanism for cell-cell cooperation in host defense against intracellular pathogens.

Importantly, the ability of infected macrophages to acquire pu- rified neutrophil granules was sufficient to mediate an antimycobacterial activity. Although we use HNP-1 as a marker for granules contained within apoptotic bodies, other neutrophil antimicrobial mediators are likely acquired. For example, these granules contain myeloperoxidase, cathelicidin, lactoferrin, and other factors, many of which have also been shown to mediate antimicrobial activity. The ability of macrophages to acquire granules carrying antimicrobial contents from apoptotic neutrophils provides a mechanism of cell-cell cooperation in establishing an effective innate immune response. This kind of intercellular cooperation also occurs to activate and regulate the adaptive immune response. Dendritic cells can acquire pathogen-derived Ag during the uptake of infec- ted cells that have undergone apoptosis, with the Ag deliv- ered into classical and nonclassical Ag presentation pathways (12). The phagocytosis of apoptotic cells inhibits transcription of the IL-12 p40 gene (44), and hence provides an anti-inflammatory signal. Thus, the cooperative interaction between apoptotic cells and nonapoptotic cells can serve to regulate in- flammation and to mount effective host defense strategies against intracellular pathogens.

We conclude that the sophisticated host evasion strategies of intracellular pathogens are effectively counterbalanced by the ability of macrophages to acquire neutrophil-derived antimicrobial agents contained within neutrophil granules. Moreover, the delivery of antimicrobial substances to macrophages is not restricted to neutrophils, but maintained during the adaptive immune response whereby T cells secrete granulysin into infected targets (3). The transfer of antimicrobial granules between immune effector cells constitutes an important host defense strategy of the innate immune system.

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10. Wagner and his bronchoscopy team for supplying bronchoalveolar lavage.