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Distinct Cell Death Programs in Monocytes Regulate Innate Responses following Challenge with Common Causes of Invasive Bacterial Disease

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Peripheral blood monocytes represent the rapid response component of mononuclear phagocyte host defense, generating vigorous but finite antibacterial responses. We investigated the fate of highly purified primary human monocytes following phagocytosis of different bacteria. Exposure to high bacterial loads resulted in rapid loss of cell viability and decreased functional competence. Cell death typically involved classical apoptosis. Exposure to high numbers of *Escherichia coli* and *Klebsiella pneumoniae* induced nonapoptotic death with loss of cell membrane integrity, marked disruption of phagolysosomes, and caspase-1 activation, while a subset of cells also released caspase-1-regulated extracellular traps. Classical apoptosis increased if extracellular bacterial replication was reduced and decreased if intracellular ATP levels were reduced during these infections. Both classical apoptosis and the alternative forms of cell death allowed monocytes, whose functional competence was exhausted, to down-regulate reactive oxygen species and proinflammatory cytokine responses. In contrast, sustained stimulation of glycolytic metabolism and mitochondrial oxidative phosphorylation, with associated hypoxia inducible factor-1α upregulation, maintained intracellular ATP levels and prolonged monocyte functional longevity, as assessed by maintenance of phagocytosis, reactive oxygen species production, and proinflammatory cytokine generation. Monocyte innate responses to bacteria are short-lived and are limited by an intrinsic program of apoptosis, a response that is subverted by overwhelming infection with *E. coli* and *K. pneumoniae* or bacterial stimulation of cell metabolism. In this regard, the fate of monocytes following bacterial challenge more closely resembles neutrophils than macrophages. *The Journal of Immunology*, 2010, 185: 000–000.

Circulating peripheral blood monocytes represent a pleiotropic population of cells that are essential for innate immune responses to pathogenic microorganisms in the blood, mucosal surfaces, and tissues (1). Peripheral blood monocytes, derived from bone marrow precursors, are recruited to sites of inflammation during bacterial infection (2). In tissues they function as recruited phagocytes but, being less numerous than polymorphonuclear phagocytes, their principal roles are in tissue homeostasis and in maintenance of the pool of resident tissue macrophages (3). For certain infections, monocytes may be the key mononuclear phagocyte mediating cell-mediated immunity (4). Monocytes demonstrate marked phenotypic differences from differentiated macrophages but their roles in the context of high bacterial loads, as occurs in sepsis, are incompletely characterized (2).

During bacterial infection monocytes perform distinct roles in the innate host response. Monocytes actively phagocytose bacteria and promptly kill ingested organisms as part of the rapid response component of the mononuclear phagocyte limb of innate immunity (5). They demonstrate high-output secretion of proinflammatory cytokines, such as IL-1β and TNF-α, in response to engagement of TLR4 or other pattern recognition receptors. Ingestion of particles by phagocytic cells is ATP-dependent and is associated with a fall in intracellular ATP (6, 7). Lysosomal-mediated intracellular degradation of particles is also ATP-dependent (8). In comparison with tissue macrophages, monocytes have a lower concentration of mitochondria, which places them at risk for depleting intracellular ATP levels rapidly during antibacterial innate responses (9). Monocytes thus have a greater reliance on constitutive glycolytic metabolism for maintenance of intracellular ATP, in comparison with tissue macrophages, such as alveolar macrophages, which rely to a greater extent on oxidative phosphorylation by mitochondria (10).

During the interaction of myeloid cells with bacterial pathogens a variety of cell death processes have been described, which frequently benefit host defense. Apoptosis is characterized by nuclear condensation and fragmentation, DNA cleavage, cell shrinkage, and preservation of membrane integrity, and it is frequently, although not always, viewed as an anti-inflammatory death process (11, 12). In contrast, pyroptosis, which also involves nuclear condensation and DNA cleavage, is associated with caspase-1 activation and membrane rupture and may therefore be viewed as a more inflammatory death process (11). More recently, neutrophils have

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Abbreviations used in this paper: DCF, 2',7'-dichloro-dihydrofluorescein diacetate; 2-DG, 2-deoxyglucose; Eco, *E. coli*; ETosis, extracellular traposis; HIF, hypoxia-inducible factor; Kpn, *K. pneumoniae*; LDH, lactate dehydrogenase; ΔΨm, inner mitochondrial transmembrane potential; Mi, mock infection; MOI, multiplicity of infection; Nme, *N. meningitidis*; ROS, reactive oxygen species; Spn, *S. pneumoniae*; -ve, negative; +ve, positive.
been shown to use a death program termed extracellular traposis (ETosis) in which nuclear DNA, histones, and serine proteases are released as an antimicrobial strategy (13). Factors that may influence the death process include the bacterial strain, bacterial load, and the activation state of the cell, with apoptotic and non-apoptotic death processes occurring after the same infection depending on the prevailing conditions (11, 14).

The paradigm of phagocyte cell death during antibacterial host defense is provided by neutrophils, which undergo apoptosis after a period of sustained phagocytosis and killing (15). In contrast to tissue macrophages, monocytes have enhanced susceptibility to apoptosis (16–19) and a relatively short lifespan of 1–2 d in the circulation (20, 21). Proinflammatory stimuli and growth factors, however, are potent prosurvival stimuli for monocytes and reverse the intrinsic susceptibility to constitutive apoptosis (22, 23). Phagocytosis and killing of bacteria can induce a delayed program of apoptosis in macrophages as a component of innate host defense (24). Alternatively, early cell death of macrophages may be a pathogen-driven mechanism by which the immune system is evaded and can take various forms ranging between apoptosis, pyroptosis, or necroptosis, with varying consequences for the overall inflammatory response (11). In some forms of severe sepsis, for example, meningococcal disease, extremely high counts of bacteria can be present within the bloodstream, and the highest counts are associated with the most severe manifestations of disease (25). Comparatively little is known concerning the fate of highly purified primary human monocytes following high-dose bacterial challenge, and their altered susceptibility to apoptosis suggests that responses may differ from differentiated macrophages.

In view of their central role in the inflammatory response to overwhelming bacterial infection, we have investigated the cell fate of monocytes following exposure to a range of bacteria and demonstrated rapid cell death either by classical apoptosis or by an alternative death processes, concomitant with exhaustion of functional competence and downregulation of proinflammatory responses. In contrast, bacterial stimulation of monocyte metabolism prevents apoptosis, enabling cell survival and prolonging proinflammatory responses.

Materials and Methods
Monocyte isolation and culture
Human PBMCs were isolated by Ficoll Paque (GE Healthcare, Buckinghamshire, U.K.) density centrifugation of whole blood, donated by healthy volunteers. The South Sheffield Research Ethics Committee approved the studies, and subjects gave written, informed consent. Monocyte cultures were enriched from freshly isolated PBMCs using a MACS monocyte isolation kit II and MACS LS columns (Miltenyi Biotech, Auburn, CA), yielding an average 98% purity. Purified monocytes were cultured for 1 h in RPMI 1640 culture medium (Lonza, Basel, Switzerland) with 2 mM l-glutamine (Life Technologies, Rockville, MD) containing 10% human AB serum (First Link, Brierley Hill, U.K.) in 24-well plates (Costar, Sigma-Aldrich, St. Louis, MO) with or without coverslips at 5 × 10^5 cells/well or 25-cm² flasks (Costar, Sigma-Aldrich) at 2 × 10^6 cells per flask to allow monocyte adherence. The media was then replaced with RPMI 1640 with 2 mM l-glutamine containing 10% heat-inactivated FCS (Bioclear), maintained at 5% CO₂ at 37°C and cells used within 12 h.

Bacterial infection
Neisseria meningitidis (MC58, B:15:P7.16 serogroup B), Klebsiella pneumoniae subsp. pneumoniae (ATCC 43816), Escherichia coli (C29 group 2 capsular serotype K54) (26), Neisseria lactamica (Y92-1009, sequence type 3493 complex 613), and serotype 2 Streptococcus pneumoniae (D39 strain, NCTC 7466) were the strains studied. In specific experiments, the exception of N. lactamica, which was grown on chocolate blood agar. Individual colonies were selected and grown in liquid media to midlog phase (D39 strain, NCTC 7466) were the strains studied. In specific experiments, extracellular DNA structures were stained with 5 μM Hoechst 33342 (Invitrogen, Paisley, U.K.) and fixed in 2% paraformaldehyde, then incubated with a 1/200 dilution of rabbit anti-human histone H2A.Z Ab (no. 2718; Cell Signaling Technology, Beverly, MA) at 4°C overnight before staining with a 1/100 dilution of a goat anti-rabbit IgG FITC-conjugated secondary Ab (Sigma-Aldrich) for 1 h at room temperature. Images were processed by LSM software and AxioVision 4.7.2 software (Zeiss). Quantification was provided by analyzing 300 cells per field. In certain experiments, 10 μM of the caspase-1 inhibitor z-YVAD-fmk (Calbiochem, San Diego, CA) or DMSO vehicle control was added 30 min prior to infection. In select experiments, fluorescence microscopy was also performed with a Leica DMRB microscope with a 40×/0.7 objective lens (Leica Microsystems, Wetzlar, Germany). Image processing involved use of LAS AF software (Leica Microsystems).

Transmission electron microscopy
Monocytes (2 × 10^5) were mock infected or challenged with bacteria for 12 h. Cells were centrifuged at 1000 × g for 5 min, washed three times in PBS, and fixed in ice-cold 3% glutaraldehyde, 0.1 M phosphate buffer over night at 4°C. The cell pellets were then washed in 0.1 M phosphate buffer twice. Secondary fixation was carried out in 2% aqueous osmium tetroxide for 2 h at room temperature and washed twice in 0.1 M phosphate buffer. Specimens were then dehydrated through a graded series of ethanol: 75% ethanol for 15 min, 95% ethanol for 15 min, 100% ethanol for 15 min twice followed by 100% ethanol dried over anhydrous copper sulfate for 15 min. The specimens were then placed in propylene oxide for 15 min twice. Infiltration was accomplished by placing the pellets in a 0.5:0.5 mixture of propylene oxide and Araldite resin overnight at room temperature. The pellets were then left in Araldite resin for 6 h at room temperature and finally embedded in fresh Araldite resin for 48 h at 60°C. Sections (85 nm) were cut on a Reichert Ultracut E ultramicrotome and stained with 1% toluidine blue in 1% borax. Sections were examined using an FEI Tecnai transmission electron microscope at an accelerating voltage of 80 kV and micrographs were taken using a Gatan digital camera.

Determination of monocyte viability
The cell culture media from mockinfected or bacteria-exposed monocytes were analyzed for lactate dehydrogenase (LDH) release using a commercially available kit (Cytotoxic 96; Promega, Madison, WI) following the manufacturer’s instructions. For trypan blue dye exclusion, mock and infected monocytes were harvested by cell scraping and stained with 0.2% (w/v) trypan blue dye (Sigma-Aldrich). Cell counts were made using a hemocytometer by brightfield microscopy.

Morphological analysis of cell death
Monocytes on coverslips were fixed in 2% (w/v) paraformaldehyde (Sigma-Aldrich). Monocytes were stained by TUNEL using an ApopTag fluorescein direct in situ apoptosis detection kit (Chemicon International, Temecula, CA) following the manufacturer’s instructions and counterstained with DAPI containing mounting medium (Vector Laboratories, Burlingame, CA) (28). Nuclear morphology was analyzed by fluorescence microscopy, assessing 300 cells per field.

Measurement of loss of inner mitochondrial transmembrane potential
Monocytes were stained with 10 μM 5,5’6,6’-tetraethylrhodamine-1,1’-3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR) for 30 min at 37°C, 4 h and 12 h postinfection to measure loss of inner mitochondrial transmembrane potential (ΔΨm) by flow cytometry (29). Loss of ΔΨm was determined by the loss of red fluorescence.

Flow cytometry
All flow cytometric measurements were made using a four-color FACS-Calibur (BD Biosciences, Mountain View, CA). Forward and side scatter light was used to identify “viable” monocyte populations based on size and granularity. Ten thousand events were recorded and all data were analyzed using FlowJo software, version 8.8.4 (Tree Star, Ashland, OR).

Brightfield and fluorescence microscopy
Brightfield and fluorescence images of whole cell bacterial morphology, nuclear morphology, and extracellular traps were captured using a Zeiss LSM 510 confocal microscope with a Zeiss ×63/1.4 oil objective (Zeiss, Oberkochen, Germany). Extracellular DNA structures were stained with 5 μM Hoechst 33342 (Invitrogen, Paisley, U.K.) and fixed in 2% paraformaldehyde, then incubated with a 1/200 dilution of rabbit anti-human histone H2A.Z Ab (no. 2718; Cell Signaling Technology, Beverly, MA) at 4°C overnight before staining with a 1/100 dilution of a goat anti-rabbit IgG FITC-conjugated secondary Ab (Sigma-Aldrich) for 1 h at room temperature. Images were processed by LSM software and AxioVision 4.7.2 software (Zeiss). Quantification was provided by analyzing 300 cells per field. In certain experiments, 10 μM of the caspase-1 inhibitor z-YVAD-fmk (Calbiochem, San Diego, CA) or DMSO vehicle control was added 30 min prior to infection. In select experiments, fluorescence microscopy was also performed with a Leica DMRB microscope with a 40×/0.7 objective lens (Leica Microsystems, Wetzlar, Germany). Image processing involved use of LAS AF software (Leica Microsystems).

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obtained using an FEI Tecnai transmission electron microscope.

Nuclear morphology by transmission electron microscopy in monocytes exposed to bacteria. Representative transmission electron microscopy images of monocytes, stained with toluidine blue, following mock infection (original magnification ×11,500), K. pneumoniae (original magnification ×14,000), N. meningitidis (original magnification ×11,500), or S. pneumoniae (original magnification ×11,500) at an MOI of 10 for 12 h obtained using an FEI Tecnai transmission electron microscope.

Latex bead phagocytosis

Green fluorescent carboxylate-modified latex beads with a mean diameter of 1 μm (L3030; Sigma-Aldrich) were opsonized in 10% human AB serum with RPMI 1640 for 30 min at 37°C and incubated with monocytes for 1 h to allow phagocytosis. To account for extracellular binding, F-actin–dependent phagocytosis was inhibited by adding 2 μM cytochalasin D (Sigma-Aldrich) to control samples for 30 min (37°C, 5% CO₂) prior to infection (30). The extent of phagocytosis was determined by flow cytometry with an increase in fluorescence associated with internalized latex beads.

Determination of intracellular ATP concentration

Infected monocytes were treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. The media was then replaced with serum-free RPMI 1640 for 5, 10, 15, and 30 min and the cells were lysed in 100 μl of 1% (v/v) NP-40. Cell lysates were washed for 10 min to inactivate ATPase activity. Intracellular ATP was determined using a commercially available bioluminescent assay kit following the manufacturer’s instructions (ATP bioluminescent assay kit; Sigma-Aldrich) with results normalized to numbers of viable cells for each culture, as assessed by the numbers of adherent cells without altered cell morphology (31). Luminescence was measured using a Fusion universal microplate analyzer (Packard Instrument, Meriden, CT) and analyzed by Fusion instrument control application software, version 4.00. To determine the effect of oxidative phosphorylation or glycolysis on ATP generation, cultures were treated with either 50 μM oligomycin (Sigma-Aldrich), an inhibitor of mitochondrial respiration, 10 mM 2-deoxy-D-glucose (Sigma-Aldrich), an inhibitor of glycolysis, or a combination of both inhibitors 30 min prior to cell lysis (or at the indicated time if different).

Detection of reactive oxygen species

Production of intracellular reactive oxygen species (ROS) was measured using the cell-permeable molecule 2’,7’-dichloro-dihydrofluorescein diacetate (DCF; Sigma-Aldrich) (32). Monocytes were preincubated with DCF for 30 min before infection for the indicated time periods. Cells were washed in PBS and then analyzed by flow cytometry. To determine extracellular ROS production, monocytes preincubated with DCF for 30 min were infected for 12 h, supernatants centrifuged at 8000 × g for 10 min, then transferred to a white-walled, 96-well plate (Costar; Sigma-Aldrich), and fluorescence was detected using a Fusion universal microplate analyzer (Packard Instrument) and analyzed by Fusion instrument control application software, version 4.00. As a positive control for ROS, monocytes were treated with 1 μM PMA (Sigma-Aldrich) for 1 h before analysis and to measure the production of ROS by S. pneumoniae, bacteria were cultured in the absence of monocytes using the same dose of bacteria as used to infect the monocytes.

Cytokine bead array

Monocyte culture supernatants were collected 12 h postinfection and then centrifuged at 8000 × g for 10 min to remove monocyte and bacterial debris. Cytokines were analyzed using a BD CBA Flex set (IFN-γ, TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70) and measured using a BD FACSArray bioanalyzer (BD Biosciences, Mountain View, CA). The limits of detection were 1.8 (IFN-γ), 1.2 (TNF-α), 2.3 (IL-1β), 1.6 (IL-6), 1.2 (IL-8), 0.13 (IL-10), and 0.6 pg/ml (IL-12p70).

SDS-PAGE and Western blotting

Infected monocytes grown in T25 flasks were lysed for protein as previously described (29). Protein concentration was determined using a modified

FIGURE 1. Bacterial challenge results in loss of monocyte viability. Monocytes were mock infected or exposed to the indicated bacteria at an MOI of 10 for 4, 12, or 20 h and (A) extracellular CFU were estimated, (B) intracellular colony counts were estimated at 4 h by gentamicin killing assay, and (C) monocyte trypan blue exclusion was assessed by brightfield microscopy. The percentage of cells staining positive with trypan blue are indicated (n = 4/group). *p < 0.05; **p < 0.01; ***p < 0.001, all comparisons versus mock infections by ANOVA with Dunnett’s posttest comparison. Mi, mock infection.

Analysis of loss of lysosomal acidification

Monocyte cultures, in the presence or absence of gentamicin, were washed three times with PBS at 4 h postinfection, incubated at 37°C in RPMI 1640 containing 5 μM acridine orange (Sigma-Aldrich) for 15 min, washed, and resuspended in ice-cold PBS for analysis by flow cytometry with loss of orange fluorescence recorded as a marker of loss of lysosomal acidification.

FIGURE 2. Nuclear morphology by transmission electron microscopy in monocytes exposed to bacteria. Representative transmission electron microscopy images of monocytes, stained with toluidine blue, following mock infection (original magnification ×11,500) or infection with N. meningitidis (original magnification ×14,000), K. pneumoniae (original magnification ×11,500), or S. pneumoniae (original magnification ×11,500) at an MOI of 10 for 12 h obtained using an FEI Tecnai transmission electron microscope.
MONOCYTE DEATH IN BACTERIAL INFECTION

A

Mi

B

S. pneumoniae

C

K. pneumoniae

D

E

F

G

% TUNEL positive

% TUNEL-ve and fragmented nuclei

% TUNEL-ve without fragmented nuclei

% LDH release of control
Lowry assay (DC protein assay; Bio-Rad, Hercules, CA) and equal protein was added to all lanes. Protein samples were separated by SDS-PAGE (12%) and blotted onto nitrocellulose membrane (Bio-Rad). Blots were incubated with anti–capsase-1 (Abcam, Cambridge, U.K.), anti–hypoxia-inducible factor (HIF)-1α (Abcam), or anti–actin (Sigma-Aldrich) Abs for 12 h at 4°C. Protein was detected using HRP-conjugated goat anti–rabbit IgG (Dako, Glostrup, Denmark) and ECL (EZ-ECL; Geneflow, Staffordshire, U.K.).

Cell lysates from MCF-7 cells cultured for 24 h in normoxia (normal incubator 5% CO2) or hypoxia (3 kPa O2), maintained using an Invivo 400 hypoxic work station (Ruskinn, Pencoed, U.K.) with a 5% CO2/balance N2 gas mix, were used as negative and positive controls for HIF-1α.

### Statistics

All data were recorded as mean ± SEM unless otherwise stated. Statistical testing was performed using GraphPad Prism 5.02 software (GraphPad Software, San Diego, CA) with relevant statistical tests described in the figure legends. Significance was defined as $p < 0.05$.

### Results

#### Bacterial infection is associated with loss of micromolecule transport by monocyte cell membranes

We first examined whether the initial interaction with bacteria altered permeability of monocytes to trypan blue, an assay often regarded as a marker of loss of cell viability but that strictly measures altered cell membrane transport of micromolecules (33). The bacteria studied included pathogens, which are leading causes of invasive disease and sepsis, as well as a commensal organism, *N. lactamica*, which is shielded from the adaptive immune system by a polyclonal IgM response and rarely causes invasive disease. This organism was used for comparison with *N. meningitidis* (34–36). All of these bacteria are phagocytosed and killed by mononuclear phagocytes without intracellular persistence. We found increasing numbers of extracellular bacteria with time in each culture, from 4 to 20 h postinfection (Fig. 1A). We demonstrated that monocytes could internalize each bacterium, as shown by intracellular bacterial colony counts 4 h postinfection (Fig. 1B), although this required opsonization of *S. pneumoniae* to stimulate phagocytosis, as has been previously shown (28). Internalization was lowest for *N. meningitidis*. We found that all microorganisms tested, with the exception of *N. meningitidis*, significantly reduced monocyte cell membrane transport of micromolecules 4–12 h postinfection, as shown by trypan blue staining (Fig. 1C). The levels and timing of this effect varied between bacteria, but these findings suggested altered cellular homeostasis after sustained bacterial challenge in most infections.

#### Bacterial infection can induce apoptotic and nonapoptotic cell death

We hypothesized that altered levels and kinetics of trypan blue staining might reflect the existence of distinct monocyte cell death mechanisms after bacterial challenge and that responses to specific bacteria might favor particular forms of cell death. To address this, we focused on morphological and biochemical changes, recognizing that while both apoptosis and pyroptosis induce nuclear condensation and DNA fragmentation, apoptosis frequently involves nuclear fragmentation, whereas pyroptosis induces prominent disruption of the cell membrane (11, 12). Examination of nuclear morphology by electron microscopy revealed that some bacteria (e.g., *S. pneumoniae*) induce features of apoptosis such as nuclear fragmentation, but that other infections, such as *K. pneumoniae*, do not (Fig. 2). In keeping with the cell viability data, we observed that monocytes exposed to *N. meningitidis* had normal cell morphology. Further assessment with fluorescence microscopy enabled the combined assessment of nuclear morphology and DNA strand breaks, as detected by TUNEL staining. At 12 h postinfection with *S. pneumoniae*, we observed nuclear fragmentation (Fig. 3A, 3B). In contrast, *K. pneumoniae* infection produced TUNEL-positive cells but not nuclear fragmentation (Fig. 3C). The percentage of monocytes with DNA strand breaks and fragmented (apoptotic) nuclei and with DNA strand breaks but without nuclear fragmentation were quantified after each infection (Fig. 3D–F). As shown, most of the TUNEL-positive monocytes following *S. pneumoniae* or *N. lactamica* exposure had nuclear fragmentation (Fig. 3D–F). The combination of nuclear fragmentation with DNA strand breaks in these infections was consistent with apoptotic cell death. In contrast, after exposure to *E. coli* or *K. pneumoniae*, most monocytes were TUNEL-positive but lacked fragmented apoptotic nuclear morphology (Fig. 3F).

We next determined the integrity of the cell membrane after exposure to each bacterium. As shown in Fig. 3C, some bacteria were associated with permeabilization of the cell membrane. For example, *K. pneumoniae* infection resulted in marked disruption to the cell membrane, as assessed by light microscopy. To formally evaluate the effect of each bacterial species on the integrity of the cell membrane, we measured LDH release (Fig. 3G), a marker of significant membrane disruption (37). Only *E. coli* and *K. pneumoniae* exposure resulted in significant LDH release from monocytes. This suggested that these infections were associated with a nonapoptotic cell death mechanism, with absence of nuclear fragmentation and membrane permeabilization being features of pyroptosis (11).

The monocyte death processes following exposure to *E. coli* and *K. pneumoniae* are influenced by bacterial numbers

Different forms of programmed cell death can occur following identical stimuli (38), and we next investigated whether the nonapoptotic death process seen with *E. coli* and *K. pneumoniae* exposure was related to bacterial numbers. We repeated experiments in which bacterial numbers were reduced by early addition of antimicrobials. As shown for *E. coli*, this approach reduced both extracellular and intracellular bacterial burden (Fig. 4A). Addition of antimicrobials resulted in similar levels of nuclear fragmentation following *S. pneumoniae*, *E. coli*, or *K. pneumoniae* challenge (Fig. 4B).

Following *E. coli* and *K. pneumoniae* exposure in the absence of antimicrobials, the degree of membrane disruption (37) and the lack of apoptotic nuclear morphology suggested a death mechanism distinct from classical apoptosis (39). Loss of lysosomal acidification can be detected by a reduction in orange fluorescence of acridine orange, and this may be more extensive after some nonapoptotic death process

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**FIGURE 3.** Varying forms of cell death are observed in monocytes exposed to bacteria. A and B, Representative fluorescent images of DAPI (blue images)- and TUNEL (green images)-stained monocytes following mock infection (A) or exposure to *S. pneumoniae* (B) at an MOI of 10 for 12 h. The arrow illustrates a fragmented nucleus C, Representative brightfield (grayscale image) or fluorescent images of DAPI (blue image)- and TUNEL (green image)-stained monocytes following *K. pneumoniae* infection at an MOI of 10 for 12 h. Arrows indicate cells showing membrane rupture and TUNEL-positive nuclei without fragmentation. All images were obtained with a Zeiss LSM 510 confocal microscope with a Zeiss ×63/1.4 oil objective. D–F, Following mock infection or exposure to *N. meningitidis*, *K. pneumoniae*, *E. coli*, *N. lactamica*, or *S. pneumoniae* at an MOI of 10 for 4 and 12 h, monocytes were stained with TUNEL and DAPI and observed using fluorescence microscopy. The total percentage of TUNEL-positive monocytes (D), the percentage of TUNEL-positive monocytes with fragmented nuclei (E), and the percentage of TUNEL-positive monocytes without fragmented nuclei (F) were recorded (n = 3). G, Monocytes were mock infected or exposed to bacteria, as above, and the percentage LDH release was compared with positive controls at each time point (n = 4/group). *p* < 0.05; **p** < 0.01; ***p*** < 0.001, all comparisons versus mock infection by ANOVA with Dunnnett’s posttest comparison. Bacterial CFUs in these experiments are shown in Supplemental Table 1A. Mi, mock infection; +ve, positive.
Levels of bacterial internalization were lower for N. meningitidis than for non-Neisseria species (Fig. 1B). To exclude the possibility that maintenance of intracellular ATP merely reflected differing rates of delivery of bacteria to phagolysosomes, and hence reduced ATP requirements for phagocytosis and killing, we used an unencapsulated mutant, since N. meningitidis capsule has been shown to cause cell death processes following bacterial challenge, we next investigated if changes in cell homeostasis involved cell metabolism, initially measuring $\Delta\varphi_{\text{mi}}$ as a marker of mitochondrial homeostasis (43) and measuring intracellular ATP. One hour after exposure, some bacterial infections induced loss of $\Delta\varphi_{\text{mi}}$ in some cells, but no infection was associated with a marked reduction in the concentration of intracellular ATP in the population of cells (Supplemental Fig. 1). After 4–12 h, all of the bacteria studied induced significant loss of $\Delta\varphi_{\text{mi}}$ (Fig. 6A). Despite this, N. meningitidis infection differed from other infections in that intracellular ATP concentration in the population of viable cells 12 h postinfection was maintained at levels similar to those observed in mock-infected cells, after normalization of the number of viable cells to the level of those detected in the mock-infected culture (Fig. 6B). Viable monocytes after N. lactamica exposure also maintained intracellular ATP levels, but at lower levels than after N. meningitidis exposure. We addressed whether ATP preservation was dependent on glycolytic metabolism or oxidative phosphorylation. Mock infected monocytes required the simultaneous inhibition of both oxidative phosphorylation and glycolysis to deplete intracellular ATP and to inhibit ATP-dependent functions such as phagocytosis (Fig. 6C, 6D). Intracellular ATP was maintained at levels close to normal following inhibition of either oxidative phosphorylation or glycolytic metabolism in monocytes exposed to N. meningitidis. Maintenance of intracellular ATP following exposure to N. meningitidis occurred in association with upregulation of HIF-1$\alpha$ (Fig. 6E), an important factor upregulating a range of metabolic pathways in mononuclear phagocytes (44).

Levels of bacterial internalization were lower for N. meningitidis than for non-Neisseria species (Fig. 1B). To exclude the possibility that maintenance of intracellular ATP merely reflected differing rates of delivery of bacteria to phagolysosomes, and hence reduced ATP requirements for phagocytosis and killing, we used an unencapsulated mutant, since N. meningitidis capsule has been shown to decrease adherence and phagolysosomal compartmentalization, and increased the infecting dose (45). The unencapsulated mutant re-
sulted in comparable intracellular ATP concentrations, despite increased internalization, and also comparable low levels of apoptosis to the parental strain, suggesting capsular inhibition of internalization did not explain the monocyte survival (Supplemental Fig. 2). To determine whether there was any relationship between ATP levels and the mechanism of cell death, we studied monocytes exposed to *K. pneumoniae* incubated in the presence or absence of gentamicin. To some of these cultures we added inhibitors of glycolytic metabolism and oxidative phosphorylation. As shown in Supplemental Fig. 3, addition of gentamicin increased the apoptotic morphology (as in Fig. 4B), in association with a less marked drop in intracellular ATP than seen in the absence of gentamicin. Inhibitors of ATP generation increased the percentage of cells with the pyroptotic appearance, but they reduced the apoptotic appearance of cells with fragmented nuclei, despite the presence of antibiotics.

**Prolonged monocyte survival allows sustained innate immune responses**

In view of the sustained metabolic competence of monocytes after exposure to *N. meningitidis*, we next addressed whether these cells...
exposed to the mock infected cultures at 1 h (Supplemental Fig. 4) were associated with comparable phagocytosis of latex beads to cytokines, as determined by cytometric bead array. All infections also showed sustained evidence of innate immune function, measured by similar, levels of IFN-α, TNF-α, IL-1β, IL-6, and IL-12 being lower and those of IL-8 being similar, levels of IFN-γ, IL-1β, IL-6, and IL-12 were lower and those of IL-10 were greater. This demonstrated that prolonged survival of monocytes following 12 h of mock infection or exposure to N. meningitidis or S. pneumoniae at an MOI of 10. In the case of K. pneumoniae, ROS production remained noteworthy in the monocyte cultures early after bacterial challenge (Fig. 7B). In the case of S. pneumoniae, there was also evidence of extracellular ROS expression, in keeping with the known capacity of this organism to generate ROS (46) (Supplemental Figs. 4B, 5). By 12 h, ROS production remained noteworthy in the N. meningitidis–exposed cultures (Fig. 7C), in keeping with the ongoing capacity of these cultures to phagocytose bacteria. Other bacterial infections differed in their capacity to stimulate extracellular ROS production at this time. Cultures associated with monocyte apoptosis (e.g., S. pneumoniae and N. lactamica) also stimulated ROS production in the cells retaining viability. E. coli or K. pneumoniae exposure resulted in no detectable extracellular ROS in cultures 12 h after challenge even though intracellular ROS was detectable at earlier time points following exposure to these bacteria.

In keeping with the sustained viability of monocytes and the continued capacity to phagocytose opsonized particles, we observed that monocytes were still producing significant quantities of proinflammatory cytokines 12 h after N. meningitidis exposure (Fig. 8). This was unlike the situation with most of the other infections investigated, which demonstrated minimal cytokine production, or, in the case of E. coli exposure levels below the limit of detection (data not shown), by 12 h postinfection. N. lactamica–exposed monocytes retained functional capacity despite significant levels of apoptosis. These cells produced a different pattern of cytokines to N. meningitidis–challenged monocytes. While TNF-α and IL-8 levels were similar, levels of IFN-γ, IL-1β, IL-6, and IL-12 were lower and those of IL-10 were greater. This demonstrated that prolonged survival of monocytes following bacterial challenge has a significant impact on the resulting pattern of cytokine release.

Discussion

Investigation of cell death mechanisms in highly purified primary human monocytes challenged with bacteria has been a comparatively neglected area in comparison with other myeloid cells,
including macrophages (11). We demonstrate a rapid loss of cell viability during the first 12 h of exposure to bacteria. Some infections (E. coli and K. pneumoniae) induce nonapoptotic death. Only monocytes exposed to N. meningitidis maintained significant viability by 12 h postinfection. Loss of cell viability terminated antibacterial innate responses. The prolonged immune competence of monocytes exposed to N. meningitidis, however, comes at the cost of an extended proinflammatory immune response.

Monocytes are short-lived cells with a high level of susceptibility to constitutive caspase-dependent apoptosis (19, 20, 22). During interaction with bacteria, monocytes phagocytose bacteria briskly and kill these in their phagolysosomes, and thus phagolysosomal escape is an important bacterial survival strategy (47–49). Monocytes phagocytose bacteria with slightly less efficiency than neutrophils, but microbicidal killing appears comparable (5). The lysosomal system of monocytes is less developed than in tissue macrophages, lacking the capacity for more prolonged microbial killing (9, 50). Monocytes are therefore more reliant on acute microbicidal strategies, such as ROS-dependent killing, than are macrophages, and they are better equipped to control extracellular bacteria that can be rapidly killed in phagolysosomes than those that rely on prolonged intracellular killing in phagolysosomes (7). The bioenergetic demands of rapid bacterial internalization and intracellular killing stress phagocytes (6, 51), and monocytes have a lower density of mitochondria than differentiated macrophages (9). This places monocytes under considerable bioenergetic stress, and we show herein that the fate of monocytes is more similar to that of neutrophils than the differentiated macrophage.

Our findings emphasize the variety of cell death processes that occur following the interaction of monocytes with bacteria, reinforcing the need for careful characterization (52). Trypan blue positivity did not correlate closely with specific cell death features, suggesting that subtle disruption of micromolecular transport may be observed without other features of loss of viability (33). By analysis of nuclear morphology and membrane integrity we identified infections predominantly associated with apoptotic cell death, as evidenced by specific features of apoptosis, such as cell shrinkage, nuclear fragmentation, and preservation of cell surface membrane integrity (24, 39), whereas for E. coli or K. pneumoniae challenge DNA fragmentation was associated with cytolysis but not nuclear fragmentation. Caspase-1 activation, a feature of pyroptosis but not usually of macrophage apoptosis, was also observed (11), and loss of lysosomal acidification occurred in a greater percentage of cells in these infections. Loss of lysosomal acidification can result from impaired fusion of phagosomes with lysosomes, impairment of hydrogen ion pumps, or lysosomal membrane permeabilization (53–55), although we did not distinguish which of these mechanisms contributed in this case. More marked disruption of the lysosomal membrane, however, favors nonapoptotic death processes (56, 57) and caspase-1 activation by “inflammasomes,” containing nucleotide binding and oligomerization domain-like receptor family members, activated by the release of microbial components into the cytosol (58).

There was also evidence of extracellular DNA and histone release from monocytes and of association of bacteria with these extracellular structures following E. coli and K. pneumoniae infection. The release of DNA, to form extracellular traps containing histones and proteases, is observed from neutrophils and has also been described for basophils (13, 41, 42). To our knowledge, a similar process has not been described for monocytes. The large disruption of the cellular membranes, we observed with these infections, would facilitate the release of intracellular contents, including DNA, from these cells. This process increased in proportion to the infectious inoculum and was reduced by caspase-1 inhibition. Extracellular traps were not identified after S. pneumoniae exposure in keeping with the known ability of this organism to produce an endonuclease, which degrades extracellular DNA (59).

Individual microorganisms can mediate more than one death process (11, 52). Bacterial numbers, cell activation state, and, as we suggest, energy state may be other interrelated factors. We have not addressed activation state in this study, but Bergsbaken and Cookson made the important observation that activation of macrophages can convert apoptosis to caspase-1-dependent pyroptosis (14). Macrophage activation by LPS reduces apoptosis (60) and could have reduced apoptosis following E. coli and K. pneumoniae infection. The influence of bacterial numbers on the death process was complex. We have previously shown that apoptosis in differentiated macrophages is related to the intracellular burden of S. pneumoniae (61), but we did not specifically examine the influence

FIGURE 7. N. meningitidis–exposed monocytes demonstrate sustained phagocytosis and production of ROS. A, The percentage of monocytes phagocytosing opsonized fluorescent latex beads following 12 h mock infection or exposure to N. meningitidis, K. pneumoniae, E. coli, N. lactamica, or S. pneumoniae at an MOI of 10 (n = 6). **p < 0.01; ***p < 0.001, versus mock infection, ANOVA with Dunnett’s posttest. The percentage of monocytes with detectable intracellular ROS (B) 1–4 h and (C) 12 h after mock infection or exposure to N. meningitidis, K. pneumoniae, E. coli, N. lactamica, or S. pneumoniae (MOI of 10). ROS was measured by flow cytometry following incubation with DCF (n = 3), *p < 0.05, ANOVA with Dunnett’s posttest. Bacterial CFUs in these experiments are shown in Supplemental Table 1B. Mi, mock infection.
of intracellular, as opposed to extracellular, bacterial numbers in this study. The nonapoptotic death processes, observed after *E. coli* and *K. pneumoniae* exposure, were associated with higher numbers of extracellular bacteria but not higher numbers of intracellular bacteria 4 h postinfection. Nevertheless, reducing extracellular and intracellular bacterial numbers by antimicrobial treatment increased rates of apoptosis, relative to pyroptosis, while increasing the infectious dose increased rates of extracellular trap formation for these infections. Survival of monocytes following *N. meningitidis* exposure was associated with lower intracellular bacterial numbers but was not altered by increasing intracellular bacterial numbers. These conclusions emphasize that unique microbial factors interact with bacterial burden and cellular factors to determine the fate of cells.

The concentration of intracellular ATP influences the mechanism of cell death. Apoptosis requires intracellular ATP, but processes with a high affinity for ATP, such as many of the executors of apoptosis, are only compromised when cellular levels are depleted 100-fold (62). If there is not a source for this low level of ATP, then necroptosis, a necrosis-like cell death, results in membrane disruption with the potential release of phagocytosed bacteria and inflammatory mediators (12, 39). Little is known of the exact energy requirements of pyroptosis, but caspase-1 activation also requires ATP (63). Since the levels of ATP required for these death processes are so low, estimation of these differential energy requirements will require ultrasensitive assays. Nevertheless, our preliminary analysis suggests that by further depleting cellular ATP the death program shifts from apoptosis to pyroptosis. Monocytes must balance the large energy requirements of their rapid antibacterial response and their relatively limited capacity to generate ATP with the need to execute the most appropriate cell death program while there are still sufficient ATP reserves for its completion.

Execution of a program of apoptosis allows a monocyte, which has exhausted its functional capacity to kill ingested bacteria, to enhance intracellular bacterial killing (64, 65) and terminate proinflammatory responses (66). Increased cell activation, a fall in intracellular ATP below the levels required for apoptosis, and cytosolic sensing of microbial products would favor pyroptosis (11, 14, 63). Pyroptosis is known to facilitate control of intracellular bacteria, which escape into the cytoplasm (67). Extracellular ATP release could activate the same process in bystander cells (68). This phlogistic death process could be viewed as a compromise in overwhelming infection in which the monocyte although terminating its own inflammatory response by cell death does not terminate the overall tissue inflammatory response, as would happen with apoptosis, but instead ensures its perpetuation with a further round of inflammatory cell recruitment. In contrast, extracellular traps would ensure that unphagocytosed extracellular bacteria could be contained (13, 41, 42). Linking extracellular bacterial numbers to intracellular bacterial numbers and sensing this through recognition of levels of cytosolic microbial products or extracellular ATP would provide the monocyte with an effective mechanism by which the apoptotic program could be overridden when the antibacterial capacity of the phagolysosomal compartment is overrun.

Infection with *N. meningitidis*, the meningococcus, was distinct since it resulted in sustained monocyte viability in association with maintenance of intracellular ATP. Preservation of intracellular ATP levels was the result of activation of both glycolytic metabolism and oxidative phosphorylation and was associated with upregulation of HIF-1α (44). This response appeared to be intrinsic to *N. meningitidis* since intraphagolysosomal loading with an uncapsulated mutant (45) failed to reduce ATP or enhance apoptosis. Exposure to *N. meningitidis* resulted in sustained innate responses. This is noteworthy since marked proinflammatory cytokine release is central to the pathogenesis of meningococcal disease (69). A subpopulation of viable monocytes after *N. lactamica* infection maintained intracellular ATP, continued to phagocytose latex beads, and showed distinct cytokine expression with reduced proinflammatory cytokine expression but enhanced IL-10 responses. When the cytokine expression was normalized to numbers of adherent viable cells, the levels of IL-1β, IL-6, and IL-12 became comparable between *N. lactamica* and *N. meningitidis* and the levels of TNF-α and IL-8 became greater for *N. lactamica* infection, but the increase in IL-10 expression for *N. lactamica* infection was

**FIGURE 8.** Sustained proinflammatory cytokine production by monocytes exposed to *N. meningitidis*. Cytokine production by monocytes 12 h after mock infection or exposure to *N. meningitidis*, *K. pneumoniae*, *N. lactamica*, or *S. pneumoniae* at an MOI of 10 is shown. Cytokine levels in the culture media were determined by cytokine bead array: IFN-γ (A), TNF-α (B), IL-1β (C), IL-6 (D), IL-8 (E), IL-10 (F), and IL-12p70 (G) (*n* = 8). *p < 0.05; **p < 0.01; ***p < 0.001, ANOVA with Tukey’s posttest. Mi, mock infection.
even more accentuated (data not shown). In addition to showing increased levels of trypan blue positivity, N. lactamica infection resulted in a loss of cell adherence as well as decreased cell numbers, with evidence that apoptosis was the predominant mechanism of cell death. N. lactamica is more readily internalized than N. meningitidis, but the simultaneous ingestion of apoptotic bodies would be predicted to deactivate proinflammatory cytokine responses and enhance IL-10 release (66). In contrast, maintenance of intracellular ATP and failure to induce apoptosis in N. meningitidis–exposed cultures would be anticipated to have both direct effects by sustaining cytokine production in the monocytes ingest- ing meningococci as well as indirect effects by limiting the capacity of apoptotic bodies to downregulate proinflammatory cytokine responses. Additionally, the persistent generation of factors such as ROS would contribute to tissue injury (70). Failure to engage one of the regular death processes that result when monocytes ingest bacteria may underpin the proinflammatory features of meningococcal infection, providing an important illustration of the im- portance of cell death in limiting persistent high-level innate responses in monocytes.

In summary, we provide evidence that highly purified human monocytes engage an apoptotic program to downregulate innate responses to bacteria. The host response to some infections may prevent this process and substitute an alternative death process, which aims to contain extracellular bacteria by ensuring recrui- tement of additional inflammatory cells. Failure to engage either of these programs is likely to result in persistent innate responses, which place the host at risk for sepsis and multorgan failure as observed in meningococcal sepsis.

Note added in proof. During revision of this paper, we became aware of the publication of a paper by Bartneck et al. (71) de- scribing the production of extracellular traps by monocytes in response to nanoparticles.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Loss of inner-mitochondrial membrane potential and ATP levels at 1h post infection.

(A) Monocytes were mock-infected (Mi) or exposed to *N. meningitidis, K. pneumoniae, E. coli, N. lactamica, or S. pneumoniae* at an MOI of 10 for 1h, and the percentage of cells with loss of inner-mitochondrial transmembrane potential ($\Delta\psi_m$) were estimated by flow cytometry n=4, *p<0.05, ***p<0.001 vs. Mi*, ANOVA with Dunnett’s post-test. (B) Monocytes were mock-infected or exposed to bacteria as in (A) and 1h post infection intracellular ATP levels were estimated by bioluminescence.
Supplementary Figure 2. Enhanced bacterial internalization does not reduce intracellular ATP or increase apoptosis following *N. meningitidis* exposure.

(A) Monocytes were mock-infected (Mi) or exposed to *N. meningitidis* or the unencapsulated strain (ø13) and 12h post infection were washed three times and lysed before intracellular ATP levels were estimated by bioluminescence. There was no significant difference compared to Mi for either strain, n=4, ANOVA with Bonferroni post test. (B) The intracellular colony forming units (CFU) per ml of monocyte culture lysate 4h post infection with *N. meningitidis* or ø13 were determined by gentamicin assay. n=4, * p<0.05, unpaired t test. (C) Monocytes were mock-infected (Mi) or exposed to *N. meningitidis* or ø13 and 12h post infection the percentage of monocytes with fragmented nuclei were recorded. No significant difference was detected, n=4, ANOVA with Bonferroni post test.
Supplementary Figure 3. ATP levels determine the monocyte cell death program.
Monocytes were mock-infected (M) or exposed to K.pneumoniae (Kpn) at an MOI of 10 for 12h in the presence (+) or absence (-) of gentamicin, oligomycin or 2-deoxyglucose (2-DG).
At 12h cells were stained with TUNEL and DAPI and the percentage of TUNEL positive (+ve) cells with or without fragmented nuclei were recorded. Mean ATP levels for M without inhibitors =1.2µM, Kpn without gentamicin =0.1µM, Kpn with gentamicin =0.5µM, Kpn with gentamicin and 2-DG plus oligomycin = < 0.1µM, n=4, 2-way ANOVA with Bonferroni post test.
Supplementary Figure 4. Phagocytosis and extracellular levels of reactive oxygen species (ROS) in monocytes following bacterial challenge.

(A) The percent monocytes phagocytosing opsonised fluorescent latex beads 1h following mock-infection or exposure to *N. meningitidis*, *N. lactamica*, *K. pneumoniae*, *E. coli*, or *S. pneumoniae*, n=4, ANOVA with Dunnell's post-test. (B) Extracellular reactive oxygen species (ROS) generation by monocytes after mock-infection or exposure to *N. meningitidis*, *N. lactamica*, *K. pneumoniae*, *E. coli*, or *S. pneumoniae*. Phorbol 12-myristate 13 acetate (PMA) was used as a positive control. ROS were measured in the culture supernatants 1h and 4h post infection following pre-incubation with 2′,7′- dichloro-dihydrofluorescein diacetate (DCF), n=4, * p<0.05, *** p<0.001 vs. Mi, ANOVA with Dunnell's post test.
Supplementary Figure 5. Extracellular ROS production by *S. pneumoniae*.
Extracellular reactive oxygen species (ROS) generation by monocytes, *S. pneumoniae* or the combination of both. ROS were measured in the culture supernatants 1h and 4h post infection following pre incubation with 2',7' dichloro-dihydrofluorescein diacetate (DCF), n=4, * p<0.05, ** p<0.01 vs. Mi, ANOVA with Dunnett's post test. n=4.
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Supplemental Table 1. Colony forming units.

Monocytes were exposed to *N. meningitidis, K. pneumoniae, E. coli, N. lactamica,* or *S. pneumoniae* at an MOI of 10. Extracellular and intracellular colony forming units (CFU) were estimated 4, 12, and 20 hours after exposure. Results represent CFU corresponding to experiments in Figure 3 (A) and Figure 6 (B). Results are presented as Mean ± SEM. n=4-8.