Insights into Pathogen Immune Evasion Mechanisms: *Anaplasma phagocytophilum* Fails to Induce an Apoptosis Differentiation Program in Human Neutrophils

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Insights into Pathogen Immune Evasion Mechanisms: Anaplasma phagocytophilum Fails to Induce an Apoptosis Differentiation Program in Human Neutrophils

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Polymorphonuclear leukocytes (PMNs or neutrophils) are essential to human innate host defense. However, some bacterial pathogens circumvent destruction by PMNs and thereby cause disease. Anaplasma phagocytophilum, the agent of human granulocytic anaplasmosis, survives within PMNs in part by altering normal host cell processes, such as production of reactive oxygen species (ROS) and apoptosis. To investigate the molecular basis of A. phagocytophilum survival within neutrophils, we used Affymetrix microarrays to measure global changes in human PMN gene expression following infection with A. phagocytophilum. Notably, A. phagocytophilum uptake induced fewer perturbations in host cell gene regulation compared with phagocytosis of Staphylococcus aureus. Although ingestion of A. phagocytophilum did not elicit significant PMN ROS, proinflammatory genes were gradually up-regulated, indicating delayed PMN activation rather than loss of proinflammatory capacity normally observed during phagocytosis-induced apoptosis. Importantly, ingestion of A. phagocytophilum failed to trigger the neutrophil apoptosis differentiation program that typically follows phagocytosis and ROS production. Heat-killed A. phagocytophilum caused some similar initial alterations in neutrophil gene expression and function, which included delaying normal PMN apoptosis and blocking Fas-induced programmed cell death. However, at 24 h, down-regulation of PMN gene transcription may be more reliant on active infection. Taken together, these findings suggest two separate antiapoptotic processes may work concomitantly to promote bacterial survival: 1) uptake of A. phagocytophilum fails to trigger the apoptosis differentiation program usually induced by bacteria, and 2) a protein or molecule on the pathogen surface can mediate an early delay in spontaneous neutrophil apoptosis. The Journal of Immunology, 2005, 174: 6364–6372.

Materials and Methods

Materials

Dextran T-500 and Ficoll-Paque PLUS (1,077 g/L) were purchased from Amersham Biosciences. Sterile water (Irrigation, United States Pharmacopeia (USP)) and 0.9% sodium chloride (Irrigation, USP) were obtained from Baxter Healthcare. PE-conjugated mAb specific for IL-1R antagonist (IL-1Rn) (clone AS17) and isotype control mAb were purchased from BD Biosciences. RPMI 1640 medium was purchased from Invitrogen Life Sciences.
Technologies. Unless indicated, all other reagents were obtained from Sigma-Aldrich.

**Neutrophil isolation**

Human PMNs were isolated from heparinized venous blood of healthy individuals with a widely used method (18). All studies with human blood were performed in accordance with protocols approved by the Institutional Review Board for Human Subjects at the University of Minnesota and the National Institute of Allergy and Infectious Diseases. Blood was mixed 1:1 with 0.9% sodium chloride (Baxter Healthcare) and collected 3.0% Dextran T-500 (Amersham) for 20 min at room temperature to sediment erythrocytes. The leukocyte-enriched supernatant was centrifuged at 670 × g for 10 min and resuspended in 35 ml of 0.9% sodium chloride. Ficoll-Paque™ PLUS (10 ml) was pipetted carefully beneath the cell suspension and then centrifuged at 380 × g for 25 min to separate PMNs from PBMCs. PBMCs were removed by aspiration, sides of the tubes were swabbed, and erythrocytes were lysed with water (Irrigation USP; Baxter Healthcare) for 15–30 s, followed by immediate mixing with 1.7% sodium chloride. Purified PMNs were centrifuged at 380 × g, resuspended in RPMI 1640 medium buffered with 10 mM HEPES, pH 7.4 (RPMI/H), and enumerated by microscopy. Purity of PMN preparations and cell viability were routinely assessed by flow cytometry (FACS Calibur; BD Biosciences) or microscopy. The entire procedure was performed at room temperature, and cell suspensions contained ~99% PMNs. Reagents used for neutrophil purification typically contained <25.0 pg/ml endotoxin (Limulus amebocyte lysate assay; Fisher Scientific).

**Growth of bacteria**

_A. phagocytophilum_ was isolated from a patient with human granulocytic anaplasmosis in Nantucket, Massachusetts (NCH-1 isolate) (19). Patient blood (100 µl) was inoculated into SCID mice, as described (20). One drop of mouse blood, collected ~ 3 wk after infection, was inoculated into HL60 cells (5 × 10^5 cells/ml), which were cultured in IMDM containing 20% heat-inactivated FBS at 37°C with 5% CO₂, as described (20). Infected and uninfected HL60 cells were counted and lysed using gentle sonication (3 quick pulses at power 6, Model 100 Ultrasonic Dismembrator; Branson Ultrasonics). Host cell debris from each was removed by centrifugation (2200 rpm for 5 min). The supernatant containing cell-free _A. phagocytophilum_ was inoculated into HL60 cells that were centrifuged at 10,000 × g for 10 min to pellet bacteria. Pellet material containing _A. phagocytophilum_ or that from uninfected HL60 lysate (negative control) was resuspended in RPMI/H and immediately inoculated into cultures of human PMNs. Alternatively, _A. phagocytophilum_ (and control lystate) was heated for 10 min at 95°C and then inoculated into neutrophil cultures. Heat treatment of Gram-positive bacteria at 95°C for 5 min has been shown to effectively kill all _A. phagocytophilum_. These conditions (except that we heated for 10 min) also effectively killed _A. phagocytophilum_, as evidenced by their inability to infect or propagate within HL60 cells compared with freshly isolated, unheated _A. phagocytophilum_. All negative control samples (unstimulated PMNs) were inoculated with clarified HL60 lysate from uninfected cells that was prepared as described above. Thus, identical methods were used to prepare _A. phagocytophilum_ and negative control HL60 lysates.

_Staphylococcus aureus_ strain COL was cultured at 37°C to mid-exponential phase of growth (ODmax = 0.75) in tryptic soy broth, as described (16). Bacteria were washed once with 1 ml of PBS and opsonized with fresh 50% human serum for 30 min at 37°C, where indicated. Oposnized _S. aureus_ were washed twice with PBS and chilled on ice before use.

**Neutrophil uptake of _A. phagocytophilum_**

Uptake of bacteria by human PMNs was measured with a previously described method (16, 18), but with modifications. Following isolation, PMNs were resuspended in RPMI/H at a concentration of 10^6 cells/ml. Glass coverslips (12 mm round; pre-soaked in nitric acid and flamed with ethanol) were placed into the wells of a 24-well tissue culture plate. Coverslips were coated with 100% autologous human serum for 1 h and then washed twice with PBS. PMNs (3 × 10^5 in 300 µl) were added to each well and allowed to settle at room temperature for 15 min. Plates were then chilled on ice. Bacteria isolated from infected HL60 cells (ratio of 1 infected HL60 cell:2 PMNs, ~5–20 _A. phagocytophilum_ per PMN) were resuspended in 1 ml of PBS containing 7.5 µg/ml Alexa Fluor 488 (Molecular Probes), and incubated for 15 min at room temperature. Stained bacteria were washed twice with 1 ml of PBS to remove unbound Alexa Fluor 488 and resuspended in RPMI/H. A total of 100 µl of Alexa Fluor 488-labeled _A. phagocytophilum_ was added to each assay well, and plates were centrifuged at 380 × g for 8 min at 4°C to synchronize ingestion of bacteria by PMNs. Culture plates were incubated at 37°C in a humidified incubator with 5% CO₂ for up to 24 h. At the indicated times, medium was aspirated and cells were washed with 500 µl of cold PBS and fixed with 4% paraformaldehyde on ice for 30 min. Fixative was aspirated, and cells were washed with 500 µl of PBS. Unengested bacteria were then counterstained with anti-Alexa Fluor 488 Ab conjugated to Alexa Fluor 594 in 500 µl of PBS (7.5 µg/ml final Ab concentration) (Molecular Probes) for 15 min. Coverslips/Cells were washed twice in 500 µl of PBS and mounted onto microscope slides with mounting medium (Aquapolymount; Poly- sciences). One hundred PMNs from random fields of view were evaluated at each time point, and ingested bacteria were scored using a fluorescence microscope (Nikon E800 microscope; Nikon). Bacteria stained with Alexa Fluor 488 (green only) were scored as ingested, and those stained with Alexa Fluor 488 and Alexa Fluor 594 were counted as unengested/surface bound. The experiment was repeated using PMNs from three separate donors.

**Neutrophil ROS production**

PMN ROS production was measured using a published fluorometric method (16, 21), but with modifications. Neutrophils were incubated with 25 µM 2′,7′-dihydrodichlorofluorescein diacetate (Molecular Probes) for 30–45 min at room temperature in RPMI/H. Subsequently, PMNs (10^6), bacteria (~1 × 10^7 _S. aureus_ or ~0.5–4 × 10^5 live or heat-killed _A. phagocytophilum_), or PMN (1–5 µg/ml) were combined in wells of 24-well plates treated with 4% DMSO (and control lysate). 0.5–1 g/ml of 30–45 min at 37°C with excitation and emission wavelengths of 485 and 538 nm, respectively. _Vmax_ was calculated as the highest rate of ROS production within a 10-min time period using Softmax Pro version 3.1.2 ( Molecular Devices) (18).

**Neutrophil apoptosis**

Neutrophil apoptosis was assessed by analysis of nuclear morphology (16) or with a modified TUNEL assay (Apo-BrdU; BD Biosciences), as described (15). Briefly, PMNs (2 × 10^6) were plated directly into 24-well plates precoated with normal human serum and removed by aspiration at the desired time point. Following interaction with live or heat-killed _A. phagocytophilum_, determination of PMN apoptosis by light microscopy was performed on cells stained with a modified Wright-Giemsa. Neutrophil apoptosis was scored by morphological assessment of nuclei in ~250 PMNs per condition for each experiment. Alternatively, apoptosis was determined by DNA fragmentation in neutrophils following exposure to bacteria (described above) with a modified TUNEL assay, as described by the manufacturer (BD Biosciences). Samples (10,000 events each) were analyzed using a FACS Calibur flow cytometer (BD Biosciences). In some experiments, 500 ng/ml anti-Fas mAb (clone CH-11; Upstate Biotechnologies) was added to PMN cultures 30 min after _A. phagocytophilum_.

**PMN gene expression and microarray analysis**

PMN phagocytosis experiments were performed, as described previously (18). Briefly, PMNs (10^6) were combined on ice with live _S. aureus_ (10^6) or with live or heat-killed _A. phagocytophilum_ (bacteria isolated from 5 × 10^6 infected HL60 cells for a ratio of 1 infected HL60 cell:2 PMNs, ~5–20 _A. phagocytophilum_)/PMN in wells of a 12-well tissue culture plate (precoated with 20% autologous normal human serum). Unstimulated control assays received either buffer (for _S. aureus_ comparisons) or clarified HL60 lysate prepared as described above (for _A. phagocytophilum_ comparisons). Plates were centrifuged at 350 × g for 8 min at 4°C to synchronize uptake of bacteria and then incubated at 37°C with 5% CO₂ for up to 24 h. Selection of the times at which gene expression was measured in _A. phagocytophilum/-infected neutrophils was based on previous experience with PMN gene expression and timing for triggering of the apoptosis differentiation program (typically between 3 and 6 h after PMN-pathogen interaction) (16, 18, 22-25). Inasmuch as apoptosis was not triggered by _A. phagocytophilum_ at early time points (1.5–6 h), we selected experiments that showed distinct _A. phagocytophilum_/PMN interactions at later time points (15–30 min). The 9-h time point was selected for analysis of _A. phagocytophilum_-induced changes in gene expression, as this time point was selected for apoptosis induction. The expression profile at 9 h was used as a control for the 9-h experiments. PMNs incubated with _A. phagocytophilum_ were lysed, and _A. phagocytophilum_-infected HL60 cells were centrifuged at 10,000 × g and immediately transferred to a microplate fluorometer (Spectramax Gemini; Molecular Devices). ROS production was measured for up to 120 min at 37°C with excitation and emission wavelengths of 485 and 538 nm, respectively. _Vmax_ was calculated as the highest rate of ROS production within a 10-min time period using Softmax Pro version 3.1.2 ( Molecular Devices) (18).

At each time point, culture medium was aspirated from the plate and PMNs were lysed with RLT buffer (Qiagen) supplemented with 2-ME. Purification of PMN RNA and preparation of labeled cRNA target were performed, as described (16, 18, 22, 24, 25). Labeling of samples, hybridization of cRNA with Hu133A oligonucleotide arrays (Affymetrix), and GeneChip scanning (GeneArray 2500; Affymetrix) were performed with...
standard Affymetrix protocols (see www.affymetrix.com). Experiments were performed with PMNs from three healthy donors using a separate HU133A GeneChip (Affymetrix) for each donor. For experiments in Fig. 2, data were analyzed with Microarray Suite version 5.0 (Affymetrix) and GeneSpring expression analysis software version 6.0 (Silicon Genetics), as described previously (16, 18, 22, 24, 25). For microarray experiments presented in Figs. 3 and 4, data were analyzed with GeneChip Operating Software version 1.2 (Affymetrix), which replaced Microarray Suite version 5.0, and GeneSpring software version 7.0 (Silicon Genetics). Furthermore, Affymetrix microarrays for the experiments presented in Fig. 2 were scanned on a GeneArray 2500 scanner (Affymetrix), whereas those in Figs. 3 and 4 were scanned on a new GeneChip Scanner 3000 (Affymetrix). Changes in gene expression were determined by comparing neutrophil transcript levels from samples stimulated with *A. phagocytophilum* or *S. aureus*-treated with those from control, unstimulated cells. Briefly, genes identified as differentially expressed were called “Present” by GeneChip Operating Software version 1.2 (Affymetrix) in two of three individuals tested. The average fold change in gene expression was at least 1.5-fold for *A. phagocytophilum*-stimulated PMNs and/or 2.0-fold for those stimulated with *S. aureus*. Changes in PMN gene expression were also statistically significant in at least one of the pathogen treatments (at the level of *p* ≤ 0.05, unpaired Student’s *t* test). A complete set of microarray results is published as supplemental Table I (first set of microarray experiments encompassing a 24-h time course after PMN uptake, plus *S. aureus* data at 9 h) and supplemental Table II (second set of microarray experiments that compare live and heat-killed *A. phagocytophilum* at 9 and 24 h), supplemental material. Raw and normalized microarray data are also posted online in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; accession no. GSE2405).

**TaqMan real-time RT-PCR confirmation of microarray data**

Phagocytosis experiments and RNA preparation for TaqMan analysis were performed with conditions identical with those used for microarray experiments (see above). DNA was removed from RNA samples by treatment with RNase-free DNase, as suggested by the manufacturer (Qiagen). RNA from three individuals was analyzed in duplicate wells with an ABI 7700 thermocycler (Applied Biosystems), as described (18). Primers and probe sets were designed with Primer Express version 1.5a and manufactured by Applied Biosystems. RNA was normalized using the Applied Biosystems predeveloped assay reagent for human ribosomal RNA.

**Flow cytometric evaluation of intracellular IL-1RN production**

Following uptake of *A. phagocytophilum* (described above), intracellular production of IL-1RN by human PMNs was measured with mAb AS17 conjugated to PE (BD Biosciences) using a previously described method (22). Briefly, staining for intracellular IL-1RN was performed 14 h following initial exposure to *A. phagocytophilum* in the presence of GolgiPlug (brefeldin A), added 6 h before measurements, as suggested by the manufacturer (BD Biosciences). The ability to detect intracellular IL-1RN is due to the capacity of brefeldin A (or GolgiPlug) to block endoplasmic reticulum-to-Golgi trafficking. The length of incubation with brefeldin A dictates in part the percentage of PMNs in which IL-1RN can be detected. The percentage of *A. phagocytophilum*-treated PMNs staining positive for intracellular IL-1RN was routinely 0–0.25% at 9 h, and this percentage increased significantly over time to 6–8% by 14 h. This significant increase is due to accumulating cytokine in the endoplasmic reticulum after treatment with GolgiPlug. Thus, it is likely that all PMNs constitutively produce IL-1RN, albeit not to detectable levels. Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences), and a single gate was used to exclude autofluorescent cells and debris.

**Statistical analysis**

Statistics were performed with Student’s *t* test (Microsoft Excel 2002; Microsoft) or one-way ANOVA with a Tukey’s or Bonferroni’s posttest for multiple comparisons (GraphPad Prism version 4.0 for Windows; GraphPad).

**Results**

**Ingestion kinetics, ROS production, and apoptosis of *A. phagocytophilum*-infected human PMNs**

To evaluate ingestion kinetics of *A. phagocytophilum* by human PMNs in our assay system, pathogen uptake was visualized and quantified by fluorescence microscopy (Fig. 1, A and B). Compared with phagocytosis typically observed for other bacteria (16), there was relatively slow neutrophil uptake of *A. phagocytophilum*. However, most of the neutrophils (*75.3 ± 7.8%*) contained ingested bacteria 6 h after initiation of neutrophil-*A. phagocytophilum* interaction (Fig. 1B). In addition, PMNs generally contained

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*The online version of this article contains supplemental material.*

**FIGURE 1.** Interaction of *A. phagocytophilum* with human PMNs. A. Ingestion of *A. phagocytophilum* by human PMNs. Neutrophils were incubated with 5–20 *A. phagocytophilum*/PMN for the indicated times, and percentage of ingestion (number of PMNs containing internalized *A. phagocytophilum*) was determined by fluorescence microscopy. B. Micrographs illustrating ingestion of *A. phagocytophilum* by human PMNs. Green, intracellular bacteria. Red or red-green (yellow), extracellular bacteria. Scale bar, 20 μm. C. **PMN ROS production**. Percent ROS production was determined by flow cytometry at the indicated times. Data are the mean ± SEM to indicate the percentage of PMNs in which IL-1RN can be detected. **D. PMN apoptosis**. PMN apoptosis was determined by fluorescence microscopy at the indicated times. Data are the mean ± SEM to indicate the percentage of PMNs in which IL-1RN can be detected.
high numbers of minimally degraded *A. phagocytophilum* (Fig. 1B). The relatively slow PMN uptake of *A. phagocytophilum* is consistent with a recent report by Carlyon et al. (13).

We next evaluated generation of intracellular ROS by human PMNs during ingestion of *A. phagocytophilum* to determine whether pathogen uptake activates neutrophils in our assay system (Fig. 1C). As with previous studies (10, 11, 13), neither live nor heat-killed pathogens elicited significant PMN ROS production compared with either PMA or *S. aureus* (Fig. 1C). These findings are consistent with an earlier report that demonstrated inhibition of neutrophil superoxide generation is not dependent on viable or structurally intact bacteria (10).

Bacterial phagocytosis normally induces neutrophil apoptosis (16, 23). In contrast, uptake of live or heat-killed *A. phagocytophilum* failed to induce neutrophil apoptosis (Fig. 1D). Spontaneous neutrophil apoptosis was also delayed significantly by live or heat-killed organisms 24 h after initial *A. phagocytophilum*-PMN interaction (PMN apoptosis was inhibited 51.3 ± 6.1% by live bacteria and 52.8 ± 9.7% by heat-killed organisms compared with spontaneous apoptosis noted in control PMNs) (Fig. 1D). The ability of live *A. phagocytophilum* to delay neutrophil apoptosis diminished by 48 h (57.7 ± 2.9% of the cells were apoptotic after infection with live organisms vs 70.1 ± 10.5% for uninfected cells; data not shown), indicating some of the processes underlying the delay in neutrophil apoptosis occur early during infection. These findings are consistent with reports by Yoshiie et al. and Scaife et al. (6, 7) that suggest pathogen protein synthesis and/or intracellular proliferation are not required for the observed anti-apoptotic effect. The apoptosis differentiation program is typically triggered within hours of bacterial uptake (16, 23). Inasmuch as there was a significant delay in neutrophil apoptosis 24 h after infection (Fig. 1), we examined transcript levels in neutrophils at time points up to 24 h.
A. phagocytophilum induces global changes in PMN gene expression

To gain insight into the molecular processes that permit survival of A. phagocytophilum within PMNs, we screened 14,500 human genes for changes in expression following ingestion of A. phagocytophilum (Fig. 2, and supplemental Table I). Compared with phagocytosis of S. aureus (Fig. 2) or other bacterial pathogens (16), ingestion of A. phagocytophilum induced far fewer changes in PMN gene expression (Figs. 2, A and B, and 3A). For example, 2,040 human genes were differentially regulated 9 h after phagocytosis of S. aureus by PMNs (Fig. 3A). By comparison, only 722 genes (35.4% of those altered by ingestion of S. aureus) were induced or repressed by A. phagocytophilum at the same time point (Fig. 3A).

Previous work demonstrated that marked changes in PMN gene expression occur by 3–6 h after phagocytosis, and accompany induction of apoptosis and down-regulation of proinflammatory gene expression (16, 22). The S. aureus strain COL used for comparison in this study has been shown to elicit this typical response (16). Thus, 24 h following interaction with S. aureus, neutrophils have undergone apoptosis or primary necrosis and are no longer viable (our unpublished observations). By comparison, neutrophils containing ingested A. phagocytophilum 24 h after uptake were viable and had the greatest number of differentially regulated genes (1245) compared with any of the other times measured (Figs. 2A and 3, A and B). This response included genes involved in the acute inflammatory response, such as those encoding TNF-α, IL-1β, IL-1e, IL-6, CXCL1, CXCL2, CXCL3, CCL3, CCL4, CCL20, CD54, IL-1RN, IL-1R1, and orosomucoid (Figs. 2A and 4). The patterns of gene regulation were also strikingly different between S aureus and A. phagocytophilum at 9 h postinfection (Fig. 2B). Notably, a significant number of genes that regulate apoptosis and signal transduction and encode structural proteins were down-regulated after phagocytosis of S. aureus, but remained unchanged following ingestion of A. phagocytophilum (Fig. 2B). Furthermore, many changes in PMN gene expression following ingestion of A. phagocytophilum were at variance with those in S. aureus-activated cells, or the time at which the change in expression occurred was delayed (Figs. 2A and 4).

Live and heat-killed pathogen-induced alterations in PMN gene regulation

Live and nonviable (heat-killed) A. phagocytophilum had similar capacities to delay neutrophil apoptosis, and each failed to elicit generation of PMN ROS (Fig. 1, C and D). To test the hypothesis that PMN gene regulation following uptake of live or heat-killed organisms is concomitantly similar, we performed a second set of microarray experiments to directly compare the ability of live and heat-killed A. phagocytophilum to modulate human neutrophil gene expression (Figs. 3 and 4, and supplemental Table II). Compared with live pathogens at 9 or 24 h after uptake, interaction with heat-killed A. phagocytophilum resulted in fewer alterations in PMN gene expression (Fig. 3). For example, 722 neutrophil genes were differentially regulated by live A. phagocytophilum 9 h after ingestion compared with 409 genes (56.7% of those altered by live pathogens) induced or repressed by heat-killed bacteria (Fig. 3A). At 9 h following ingestion of A. phagocytophilum, there was a proportional number of up- and down-regulated genes. At 24 h, although the number of genes up-regulated by live and heat-killed A. phagocytophilum was generally similar (shared 72% of up-regulated genes) (Fig. 3B), there were far fewer genes down-regulated by heat-killed bacteria (only 8.3% of those down-regulated by live bacteria) (Fig. 3C). These results suggest that down-regulation of PMN genes late during interaction with A. phagocytophilum may be reliant on active intracellular infection, a finding that merits further investigation (Fig. 3, B and C). In contrast, there was striking similarity in the overall patterns of gene regulation within functional categories between live and heat-killed A. phagocytophilum (Fig. 4). Notably, there were large tracts of genes in all functional categories remaining unchanged after ingestion of live or dead A. phagocytophilum that were differentially regulated following phagocytosis of S. aureus (Fig. 4). Thus, our data may

FIGURE 3. Changes in PMN gene expression following uptake of live and heat-killed A. phagocytophilum. A. Following bacteria-neutrophil interaction (∼10 S. aureus per PMN, or ∼5–20 live or heat-killed A. phagocytophilum per PMN), neutrophil gene expression was measured with oligonucleotide microarrays. Numbers of differentially expressed genes at 9 h after bacteria-neutrophil interaction are compared between S. aureus, live and heat-killed A. phagocytophilum (B). Numbers of differentially expressed genes 24 h after ingestion of live or heat-killed A. phagocytophilum (C). Differentially expressed genes were categorized based on known or putative function and enumerated, as described in the legend of Fig. 2. Experiments were performed with PMNs from three healthy donors using a separate HU133A GeneChip for each donor. Data were analyzed with GeneChip Operating Software version 1.2 (Affymetrix) and GeneSpring software version 7.0 (Silicon Genetics, Redwood City, CA), as described in Materials and Methods. Red bars, up-regulated genes; blue bars, down-regulated genes.
suggest that the majority of differences in PMN gene expression between live and heat-killed *A. phagocytophilum* are primarily in the time frame and magnitude of change.

Expression of genes encoding NADPH oxidase components

Previous studies in promyelocytic HL60 cells suggest that the ability of *A. phagocytophilum* to inhibit ROS generation is linked to down-regulation of NADPH oxidase components (12–15). However, this mechanism has not been demonstrated in human neutrophils. To that end, we analyzed all Affymetrix probe sets encoding NADPH oxidase proteins in our microarray data set (Table I). Genes encoding p47<sup>phox</sup> (*NCF1*), p40<sup>phox</sup> (*NCF4*), and Rap1A (RAP1A), regulators of NADPH oxidase, were down-regulated following phagocytosis of *S. aureus* (Fig. 2B). These genes, along with p67<sup>phox</sup> (*NCF2*), RAC2, and p22<sup>phox</sup> (*CYBA*), remained unchanged or were up-regulated after ingestion of *A. phagocytophilum* (Figs. 2B and 4, and Table I). Moreover, none of the genes encoding NADPH oxidase components, including *CYBB* (gp91<sup>phox</sup>) and *CYBA* (p22<sup>phox</sup>), were significantly down-regulated after *A. phagocytophilum* infection (Table I). These results clearly indicate that failure of *A. phagocytophilum* to elicit production of ROS by PMNs, or to block ROS production by a second stimulus, is not due to repression of genes encoding NADPH oxidase proteins, as previously suggested (12–15).

Regulation of inflammatory response mediators after *A. phagocytophilum* infection

There were at least two general patterns of proinflammatory gene expression apparent in *A. phagocytophilum*-infected PMNs (Fig. 2). First, a group of genes encoding proinflammatory molecules, such as IL-1RN, TNF-α, IL-6, IL-1β, IL-1α, CCL20, IL-12<sub>H9252</sub>, CCL3, CCL4, CXCL1, CXCL2, CD16, IL-13RA1, and CD18, was similarly up- or down-regulated by uptake of *S. aureus* and *A. phagocytophilum* (Figs. 2 and 4). However, induction or repression of many of these genes by *A. phagocytophilum* was blunted or delayed compared with that by *S. aureus* (Fig. 2). Second, there were genes for which expression was repressed following phagocytosis of *S. aureus*, but transcript levels were up-regulated or remained unchanged after ingestion of *A. phagocytophilum*. This category included genes encoding lymphotoxin B, TGF-β1, IL-16, IL-17R, CD32, CD47, CD114 (G-CSF receptor), IL-1R2, MyD88, TLR4, leukocyte Ig-like receptors, STAT1, STAT3, STAT5B, STAT6, and formyl peptide receptor 1 (Fig. 2). These findings were confirmed by TaqMan real-time RT-PCR analysis, which was performed for six genes representative of the entire microarray data set and included three proinflammatory molecules (Fig. 5A). In addition, we used flow cytometry to confirm that IL-1RN protein accumulated following ingestion of *A. phagocytophilum* (Fig. 5B). The finding that *A. phagocytophilum*-infected PMNs retained proinflammatory capacity is consistent with the observation that the pathogen failed to induce neutrophil apoptosis.

A. phagocytophilum fails to induce an apoptosis differentiation program in human neutrophils

*A. phagocytophilum* did not induce rapid PMN apoptosis that usually follows phagocytosis and ROS production (Fig. 1). As such, the expression of genes involved in apoptosis was examined...
Importantly, the data provide an underlying molecular basis for the Taken together, the results indicate that delay normal neutrophil apoptosis (Fig. 1) and are an important component of the PMN apoptosis differentiators, B cell line 2-associated X-protein (BAX) and nuclear or- 

Expression of NADPH oxidase components in human neutrophils following ingestion of A. phagocytophilum.

Table I. Expression of NADPH oxidase components in human neutrophils following ingestion of A. phagocytophilum

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Live/HK</th>
<th>Unigene</th>
<th>Encoded Protein</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYBB²</td>
<td>Live A.p.</td>
<td>Hs.88974</td>
<td>gp91phox</td>
<td>1.8</td>
</tr>
<tr>
<td>CYBB²</td>
<td>Live A.p.</td>
<td>Hs.88974</td>
<td>gp91phox</td>
<td>1.6</td>
</tr>
<tr>
<td>CYBB²</td>
<td>HK A.p.</td>
<td>Hs.88974</td>
<td>gp91phox</td>
<td>1.8</td>
</tr>
<tr>
<td>CYBA³</td>
<td>HK A.p.</td>
<td>Hs.68877</td>
<td>p22phox</td>
<td>2.5</td>
</tr>
<tr>
<td>CYB³</td>
<td>Live A.p.</td>
<td>Hs.68877</td>
<td>p22phox</td>
<td>1.8*</td>
</tr>
<tr>
<td>NCF1¹</td>
<td>Live A.p.</td>
<td>Hs.458275</td>
<td>p47phox</td>
<td>2.0</td>
</tr>
<tr>
<td>NCF1¹</td>
<td>Live A.p.</td>
<td>Hs.448231</td>
<td>p47phox</td>
<td>2.8</td>
</tr>
<tr>
<td>NCF1¹</td>
<td>HK A.p.</td>
<td>Hs.1583</td>
<td>p47phox</td>
<td>2.1*</td>
</tr>
<tr>
<td>NCF1¹</td>
<td>HK A.p.</td>
<td>Hs.1583</td>
<td>p47phox</td>
<td>2.0*</td>
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³ Live or heat-killed (HK) A. phagocytophilum was incubated with human PMNs for the indicated times, and expression of genes encoding NADPH oxidase proteins was measured with Affymetrix microarrays (HGU133A), as described in Materials and Methods. Data are the mean fold change in genes from two or three individuals. The gene data for infection with live organisms is derived from the first set of microarray experiments. nc (no change). Gene failed to meet criteria for being differentially expressed (see Materials and Methods). HK, heat-killed A. phagocytophilum. * p < 0.05 vs unstimulated (clarified HL60 lysate-treated) PMNs. Superscript numbers indicate paired probe sets.

closely. Previous work has shown that many genes encoding apoptosis regulators are typically induced or repressed within 3–6 h after PMN phagocytosis (18). Notably, two such apoptosis regulators, B cell line 2-associated X-protein (BAX) and nuclear or- phan receptor TR3 (NR4A1), remained unchanged following up-
take of A. phagocytophilum (18) (supplemental Tables I and II). Recently, we used PMNs from patients with chronic granulomatous disease to demonstrate that ROS modulate expression of BAX and are an important component of the PMN apoptosis differentiation program induced by phagocytosis (24). Thus, our finding that A. phagocytophilum-infected PMNs do not elicit significant ROS production (Fig. 1C) most likely explains why expression of BAX remained unchanged (data not shown). Genes encoding apoptotic protease-activating factor 1, BAX-inhibitor 1 (testis en-
tended gene transcript), B cell chronic lymphocytic leukemia/lum-
phoma 10, caspase 3, caspase 8, glucocorticoid receptor (NRC31), programmed cell death proteins 6 and 10, myeloid cell leukemia sequence 1, p21-activated kinase, JUN, tumor up-regulated caspase recruitment domain-containing antagonist of caspase 9, TNF superfamily member 10, TNF receptor superfamily, member 10B, and TNF receptor superfamily, member 1A-associated via death domain (TNFR-associated death domain protein), were in-
duced or repressed after phagocytosis of S. aureus (Figs. 2 and 4). By contrast, these genes remained unchanged following uptake of A. phagocytophilum or were at variance with changes in S. aureus (Figs. 2 and 4). Importantly, several antiapoptosis genes, including BIRC2, BIRC3, CFLAR, TNFAIP8, and TNIP2, were up-regulated in PMNs after uptake of live or dead A. phagocytophilum (Fig. 4). These data are consistent with the ability of live or heat-killed A. phagocytophilum to delay normal neutrophil apoptosis (Fig. 1D). Taken together, the results indicate that A. phagocytophilum failed to induce the apoptosis differentiation program in human PMNs. Importantly, the data provide an underlying molecular basis for the inability of A. phagocytophilum to trigger PMN apoptosis.

A. phagocytophilum inhibits Fas-induced PMN apoptosis

Although our results explain the inability of A. phagocytophilum to induce PMN apoptosis, it is not known whether the pathogen ac-
tively blocks neutrophil death engaged by external stimuli. There-
fore, we tested the ability of live and heat-killed A. phagocytophi-

Discussion

There is limited understanding of how bacterial pathogens evade human innate host defense to cause disease. Inasmuch as neutrophils are the most prominent innate immune effector cell in hu-

Materials and Methods. Data are the mean fold change in genes from two or three individuals. The gene data for infection with live organisms is derived from the first set of microarray experiments. nc (no change). Gene failed to meet criteria for being differentially expressed (see Materials and Methods). HK, heat-killed A. phagocytophilum. * p < 0.05 vs unstimulated (clarified HL60 lysate-treated) PMNs. Superscript numbers indicate paired probe sets.
responses following infection with A. phagocytophilum rent neutrophil activation and inflammatory disease (26, 27) during of PMN proinflammatory cytokine production (8, 9) with concur-

regulation may help bridge previous findings indicating an absence

Our observation that there is a delay in proinflammatory gene up-

expression induced by heat-killed bacteria were in general similar to

rather than human PMNs (12, 14). HL60 cells are an immortalized

cell line with significant differences in functional capacity com-

pared with human neutrophils. Thus, the relevance of HL60 infec-

tion models to human disease is unclear. The inability of A. phago-

cytophilum to trigger generation of ROS may be due in part to

uptake by host cell endocytosis rather than phagocytosis (4, 5, 28).

and Tables I). 

Second, our data clearly demonstrate that genes encoding com-

ponents of NADPH oxidase, including gp91phox and Rac2, remain

unchanged or are up-regulated in human neutrophils during A. phagocytophilum infection (Table I). These results are at variance with previous studies that suggest the inability of PMNs to produce ROS production following uptake of A. phagocytophilum is due to decreased gp91phox and/or Rac2 transcript levels (12, 14). However, those studies were limited in scope and/or used HL60 cells rather than human PMNs (12, 14). HL60 cells are an immortalized cell line with significant differences in functional capacity com-

pared with human neutrophils. Thus, the relevance of HL60 infec-

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and Table I).

neutrophil apoptosis. A. phagocytophilum inhibits neutrophil apoptosis triggered by anti-Fas mAb. Neutrophils were preincubated with live or heat-killed A. phagocytophilum or clarified lysate from uninfected HL60 cells that was heated at 95°C for 10 min. Apoptosis was measured with a modified TUNEL assay using a 

flow cytometer. Results are the mean ± SEM of three experiments.

proinflammatory capacity following receptor-mediated phagocyto-

sis or ingestion of other bacterial pathogens, which (with receptor-

mediated phagocytosis) diminish dramatically by 24 h (16, 18, 22).

Our observation that there is a delay in proinflammatory gene up-

regulation may help bridge previous findings indicating an absence

of PMN proinflammatory cytokine production (8, 9) with concur-

rent neutrophil activation and inflammatory disease (26, 27) during A. phagocytophilum infection. The delay in PMN proinflammatory responses following infection with A. phagocytophilum might pro-
mote intracellular survival by limiting early neutrophil signaling and recruitment/activation of other immune cells. Furthermore, re-
tention of proinflammatory capacity in A. phagocytophilum-in-
fected cells is consistent with the observation that the pathogen
fails to induce PMN apoptosis.

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FIGURE 5. Confirmation of microarray data and inhibition of Fas-in-
duced neutrophil apoptosis. A, TaqMan analysis of PMN gene expression. There was strong correlation (88.9%) between TaqMan and microarray data. Results are the mean ± SEM fold change in PMN transcript levels from three individuals. *, p ≤ 0.05 vs unstimulated cells. B, Left panel, Intracellular accumulation of neutrophil IL-1RN 14 h following ingestion of A. phagocytophilum. Right panel, Mean ± SEM of five experiments. C, A. phagocytophilum inhibits neutrophil apoptosis triggered by anti-Fas mAb. Neutrophils were preincubated with live or heat-killed A. phagocyto-

philum (−5−20 bacteria per PMN), and then cultured with 500 ng/ml anti-Fas mAb, clone CH-11 for 6 h. ΔA.p. or Δlysate, A. phagocytophilum or clarified lysate from uninfected HL60 cells that was heated at 95°C for

10 min. Apoptosis was measured with a modified TUNEL assay using a flow cytometer. Results are the mean ± SEM of three experiments.

Interestingly, live and heat-killed bacteria modulated early neutro-

phils processes (inhibition of apoptosis and failure to elicit ROS generation) to similar degrees. Moreover, patterns of PMN gene expression induced by heat-killed bacteria were in general similar to those induced by viable A. phagocytophilum, but generally dis-
similar to the patterns induced by S. aureus (Figs. 2 and 4). This observation suggests that there is an A. phagocytophilum-specific response, very different from that in bacteria studied to date (e.g., S. aureus), which most likely involves in part a bacterial surface structure or protein. The observation that a bacterial surface structure is sufficient to alter PMN function has been reported by others (6, 10). At 9 h, over one-half of the genes differentially regulated by live A. phagocytophilum were similarly altered by heat-killed pathogen. At 24 h, up-regulated genes were, again, similar. Con-

versely, interaction with heat-killed pathogen by 24 h resulted in far fewer down-regulated genes compared with uptake of viable organisms. Although the number of PMN genes down-regulated by live or dead A. phagocytophilum at 24 h represents a minor percentage of the overall gene expression data at that time point (28.5%), these differences may suggest that living organisms are necessary for ongoing functional alterations in PMNs infected with A. phagocytophilum. Alternatively, this phenomenon may simply be due to bacterial replication and the associated increase in bac-
terial burden in viable infection compared with PMNs exposed to nonviable organisms (29). Finally, heat killing or bacterial fixation, in and of itself, may alter the efficiency with which a surface struc-
ture or protein can modulate neutrophil function. In the end, the

observation that there are differences between live and heat-killed A. phagocytophilum in numbers of down-regulated genes at 24 h still fails to explain the similar capacity of each to block apoptosis within that same time frame (Fig. 1). Thus, the mechanisms by which PMN functions are altered by long-term (>24-h) infection with A. phagocytophilum warrant additional study.

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Based on our current studies and other recent reports, the ability of bacteria to alter normal PMN apoptosis is most likely a component of pathogenesis (23). Importantly, our study suggests that failure of A. phagocytophilum to induce the neutrophil apoptosis differentiation program and concomitant ability to delay/block PMN apoptosis represent two separate mechanisms used by A. phagocytophilum to prolong pathogen survival. Prolonged pathogen survival permits ongoing PMN exposure to a host of pathogenic mechanisms. The combined strategies implemented by A. phagocytophilum to prolong neutrophil survival may thus represent a general mechanism used by obligate intracellular pathogens to evade host innate host defense, thereby contributing to disease. Notably, our studies provide a global view of the host-pathogen interface important for our understanding of human diseases caused by intracellular bacteria.

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Disclosures
The authors have no financial conflict of interest.

References