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.

Polymorphonuclear leukocytes (PMNs or neutrophils) are a first line of defense in the human innate immune response to bacterial pathogens. Most ingested bacteria are killed by the combined effects of PMN reactive oxygen species (ROS) and cytotoxic granule components (1). However, some pathogens have evolved means to circumvent killing by PMNs and cause disease. The mechanisms used by pathogens to evade destruction by the innate immune system are incompletely characterized.

*Anaplasma phagocytophilum*, the agent of human granulocytic anaplasmosis, is an obligate intracellular bacterium known to survive within PMNs (2, 3). *A. phagocytophilum* enters neutrophils primarily through a receptor-mediated endocytic pathway (4), and thus may ultimately reside in a modified endosomal compartment (5). Studies evaluating functional alterations in *A. phagocytophilum*-infected PMNs suggest that the pathogen delays PMN apoptosis (6, 7), minimizes proinflammatory cytokine release (8, 9), and inhibits and/or fails to activate ROS production (10–13). Mechanisms underlying the ability of *A. phagocytophilum* to inhibit production of PMN ROS are unclear (12–15).

Recent studies indicate bacterial phagocytosis induces an apoptosis differentiation program in human PMNs that most likely contributes to the resolution of infections (16, 17). The apoptosis differentiation program is characterized by changes in a common set of genes triggered by uptake of bacteria or other particles that accelerate normal PMN apoptosis (16, 17). In contrast, *A. phagocytophilum* delays normal PMN apoptosis to facilitate productive intracellular infection (6, 7). Inasmuch as induction of PMN apoptosis is regulated at the level of transcription (16–18), a comprehensive view of gene expression patterns in *A. phagocytophilum*-infected PMNs is critical to understanding processes that permit the pathogen to reside within PMNs. To that end, we studied global gene expression in human PMNs during infection with *A. phagocytophilum*. Our results suggest failure of *A. phagocytophilum* to trigger the PMN apoptosis differentiation program and ability of the organism to inhibit spontaneous neutrophil apoptosis are distinct processes that contribute to pathogen survival.

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. 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) for 3 min and centrifuged at 300 × g (T-500; Amersham) for 20 min at room temperature to sediment erythrocytes. The leukocyte-enriched supernatant was centrifuged at 670 × g for 15 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 preparations contained ~99% PMNs. Reagents used for neutrophil purification typically contained ≤250 µg/ml endotoxin (Limulus amoebocyte lysate assay; Fisher Scientific).

Growth of bacteria

A. phagocytophilum was isolated from a patient with human granulocytic anaplasmosis in Nantucket, Massachusetts (NCH-I isolate) (19). Patient blood (100 µl) was inoculated into SCID mice, as described (20). One drop of mouse blood, collected 3–8 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 reflected into diluted HL60 cells (10,000 × g for 10 min) and immediately inoculated into cultures of human PMNs. Alternatively, *A. phagocytophilum* (and control lysate) was heated for 10 min at 95°C and then inoculated into neutrophil cultures. Heat treatment of *A. phagocytophilum*, or PM (1–5 µg/ml) was combined in wells of a 24-well plate treated at 4°C to synchronize uptake (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 (OD600 = 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 10⁶ cells/ml. Glass coverslips (12 mm round; presoaked 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 at 37°C for 1 h and then washed twice with PBS. PMNs (3 × 10⁵ in 300 µl) were added to each well and allowed to settle at room temperature. 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. Uninfected 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 (Aquapor Mount; Polysciences). 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 uningested/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 (10⁶), bacteria (~1 × 10⁷ *S. aureus* or ~0.5–4 × 10⁶ live or heat-killed *A. phagocytophilum*), or PM (1–5 µg/ml) were combined in wells of a 96-well tissue culture plate treated at 4°C to synchronize uptake (except 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.

Neutrophil apoptosis

Neutrophil apoptosis was assessed by analysis of nuclear morphology (16) or with a modified TUNEL assay (Apop-BrdU; BD Biosciences), as described (15). Briefly, PMNs (2 × 10⁶) 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). In brief, PMNs (10⁶) were combined on ice with live *S. aureus* (10⁴) or with live or heat-killed *A. phagocytophilum* (bacteria isolated from 5 × 10⁶ 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 (15, 3, 6, 9, 12, 18) was in part 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) and was significantly repressed by 24 h, these time points were of interest. The 9-h time point was selected for analysis of *S. aureus*-induced changes in the gene expression set because the apoptosis differentiation program is well underway at that time and because it was the time at which the greatest number of genes was differentially expressed by *A. phagocytophilum* (Fig. 2) (16, 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* 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-time course after PMN uptake, plus supplemental material). Raw and normalized microarray data are also posted online in the Gene Expression Omnibus (http://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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4 The online version of this article contains supplemental material.
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 PMA or *S. aureus* (Fig. 1C). These findings are consistent with an earlier report that demonstrated that 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).4 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 3A). 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 following 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).4 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
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 p47phox (*NCF1*), p40phox (*NCF4*), and Rap1A (RAP1A), regulators of NADPH oxidase, were down-regulated
following phagocytosis of *S. aureus* (Fig. 2B). These genes, along
with p67phox (*NCF2*), Rac2, and p22phox (*CYBA*), remained unchanged or were up-regulated after infection of *A. phagocytophi-
lum* (Figs. 2B and 4, and Table I). Moreover, none of the genes
encoding NADPH oxidase components, including *CYBB* (gp91phox) and *CYBA* (p22phox), 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 pro-
teins, 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-1ε, CCL20, IL-12, 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 phago-
cytosis 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-
β, 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 pro-
tein 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 usu-
ally follows phagocytosis and ROS production (Fig. 1). As such,
the expression of genes involved in apoptosis was examined

categorization of genes, olive shading, superscript numbers, and parenthet-
ical annotation are described in the legend of Fig. 2. Results are presented
as the mean fold induction or repression of genes from three experiments
(three blood donors with assays done on separate days).

**FIGURE 4.** Global changes in PMN gene expression following inges-
tion of live (*A. phagocytophilum* or live *S. aureus*) or heat-killed
(*A. phagocytophilum* or heat-killed *S. aureus*) at the indicated times. Neutrophil gene expression data were
analyzed with GeneChip Operating Software version 1.2 (Affymetrix),
which replaced Microarray Suite version 5.0 and GeneSpring software ver-
sion 7.0 (Silicon Genetics, Redwood City, CA). Affymetrix microarrays
were scanned on a new GeneChip Scanner 3000 (Affymetrix). Functional
Importantly, the data provide an underlying molecular basis for the inability of \textit{A. phagocytophilum} to induce the apoptosis differentiation program in human PMNs. Taken together, the results indicate that these data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1). These data are consistent with the ability of live or heat-killed \textit{A. phagocytophilum} to delay normal neutrophil apoptosis (Fig. 1).

\textbf{Table I. Expression of NADPH oxidase components in human neutrophils following ingestion of \textit{A. phagocytophilum}}

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Live/HK</th>
<th>Unigenie</th>
<th>Encoded Protein</th>
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<td>Hs.88974</td>
<td>gp91$^{phox}$</td>
<td>nc nc nc nc 1.8</td>
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$^a$ Live or heat-killed (HK) \textit{A. phagocytophilum} was incubated with human PMNs for the indicated times, and expression of genes encoding NADPH oxidase proteins was measured with Affymetrix microarrays (HU133A), 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 \textit{A. phagocytophilum}. $^*, p < 0.05$ vs unstimulated (clarified HL60 lysate-treated) PMNs. Superscript numbers indicate paired probe sets.

\textit{A. phagocytophilum} inhibits Fas-induced PMN apoptosis

Although our results explain the inability of \textit{A. phagocytophilum} to induce PMN apoptosis, it is not known whether the pathogen actively blocks neutrophil death engaged by external stimuli. Therefore, we tested the ability of live and heat-killed \textit{A. phagocytophilum} to inhibit Fas-induced PMN apoptosis (Fig. 5). Consistent with results in Fig. 1D, viable and nonviable \textit{A. phagocytophilum} inhibited Fas-induced cell death to similar degrees (inhibition of Fas-induced PMN apoptosis was 69.3 ± 8.5% with live organisms ($p < 0.001$) and 68.3 ± 9.5% with heat-killed organisms ($p < 0.01$)) (Fig. 5C, left panel). These findings indicate that a pre-existing surface structure or protein promotes neutrophil survival during \textit{A. phagocytophilum} infection, presumably to facilitate growth and replication of the pathogen.

\textbf{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 humans, the ability of microorganisms to subvert PMN killing mechanisms is essential for pathogenesis. \textit{A. phagocytophilum} fails to trigger production of PMN ROS and delays neutrophil apoptosis, thereby promoting pathogen survival within granulocytes (6, 7, 10–15). Although progress has been made, the molecular basis for \textit{A. phagocytophilum} pathogenesis is not known.

To that end, we investigated global changes in human neutrophil gene expression following uptake of \textit{A. phagocytophilum} and gained new insight into pathogen survival mechanisms. First, we found that neutrophils infected with \textit{A. phagocytophilum} gradually up-regulated proinflammatory capacity, which peaked at 24 h postinfection, the latest time point studied. In contrast, there are immediate (within 30 min) and dramatic increases in neutrophil

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 orphan receptor TR3 (NR4A1), remained unchanged following uptake of \textit{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 \textit{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 enhanced gene transcript), B cell chronic lymphocytic leukemia/lymphoma 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 growth and replication of the pathogen.
proinflammatory capacity following receptor-mediated phagocytosis 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 or protein can modulate neutrophil function. In the end, the relevance of HL60 infection compared with PMNs exposed to nonviable organisms (29). Finally, heat killing or bacterial fixation, 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. phagocytophilum (~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.

FIGURE 5. Confirmation of microarray data and inhibition of Fas-induced 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. phagocytophilum (~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.

Second, our data clearly demonstrate that genes encoding components 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 infection 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 compared with human neutrophils. Thus, the relevance of HL60 infection models to human disease is unclear. The inability of A. phagocytophilum to trigger generation of ROS may be due in part to uptake by host cell endocytosis rather than phagocytosis (4, 5, 28). In addition, recent data support alterations in NADPH oxidase assembly and superoxide scavenging as the basis for failure of the pathogen to directly elicit significant ROS production, findings most compatible with our current data (13).

Third, our results demonstrate that failure of A. phagocytophilum to induce the neutrophil apoptosis differentiation program in part underlies intracellular survival and represents a novel mechanism for pathogen immune evasion. As pathogen uptake also fails to elicit PMN ROS production, it may be that inability to induce the apoptosis differentiation program is secondary to the absence of ROS production. This proposed mechanism is clearly distinct from that underlying the ability of A. phagocytophilum to delay spontaneous neutrophil apoptosis or block Fas-induced cell death (Figs. 1D and 5C). It is possible that changes in expression of a gene or group of genes, such as induction of those encoding antiapoptosis molecules, contribute to these latter phenomena (Fig. 4 and Table I).

Interestingly, live and heat-killed bacteria modulated early neutrophil 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 dissimilar 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. Conversely, 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 bacterial 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 structure 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.
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 human 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

NEUTROPHEL RESPONSE TO *A. phagocytophilum*