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# *Anaplasma phagocytophilum* Delay of Neutrophil Apoptosis through the p38 Mitogen-Activated Protein Kinase Signal Pathway

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**Human granulocytic anaplasmosis is caused by the obligate intracellular bacterium *Anaplasma phagocytophilum*. The bacterium avoids host innate defenses in part by infecting, surviving in, and propagating in neutrophils, as well as by inhibiting neutrophil apoptosis. However, the mechanisms of *A. phagocytophilum* survival in neutrophils and the inhibition of spontaneous apoptosis are not well understood. In this study, we demonstrated that antiapoptotic Mcl-1 protein (Bcl-2 family) expression is maintained and that inhibition of procaspase-3 processing occurs in *A. phagocytophilum*-infected human neutrophils. An evaluation of p38 mitogen-activated protein kinase (MAPK) showed evidence of increased phosphorylation with infection. Moreover, antagonism of p38 MAPK by the inhibitor SB203580 reversed apoptosis inhibition in live or heat-killed *A. phagocytophilum*-infected neutrophils. A role for the autocrine or paracrine production of antiapoptotic interleukin 8 (IL-8) expressed with *A. phagocytophilum* infection was excluded by the use of IL-8-, IL-8R1 (CXCR1)-, and IL-8R2 (CXCR2)-blocking antibodies. As previously demonstrated, the antiapoptotic effect was initially mediated by exposure to *A. phagocytophilum* components in heat-killed bacteria. However, an important role for active infection is demonstrated by the additional delay in apoptosis with intracellular growth and the refractory abrogation of this response by the p38 MAPK inhibitor 3 to 6 h after neutrophil infection. These results suggest that the initial activation of the p38 MAPK pathway leading to *A. phagocytophilum*-delayed neutrophil apoptosis is bypassed with active intracellular infection. Moreover, active intracellular infection contributes more to the overall delay in apoptosis than do components of heat-killed *A. phagocytophilum* alone.**

Human granulocytic anaplasmosis is an emerging tick-borne disease caused by *Anaplasma phagocytophilum* (7, 51). Neutrophils, the major target cell of this obligate intracellular bacterium, are activated for degranulation and a proinflammatory response by *A. phagocytophilum* (12, 28). Activated neutrophils are ordinarily regulated by an induction of apoptosis that precludes the sustained release of proinflammatory and toxic contents and precedes the phagocytosis of apoptotic neutrophils by professional phagocytes.

Mature neutrophils have the shortest life span of the various leukocytes and die rapidly via apoptosis in vivo and in vitro. A hallmark of neutrophil biology is spontaneous induction of apoptosis. Rapid expression of apoptosis in neutrophils and the subsequent engulfment of the apoptotic cells by phagocytes are important in the rapid resolution of inflammation. This is necessary to avoid unwanted tissue damage caused by activated neutrophils (2, 5, 14, 21). The signal transduction pathways mediating neutrophil apoptosis or delayed apoptosis remain unclear but may involve either p38 mitogen-activated protein kinase (MAPK) or phosphoinositol-3 kinase/Akt pathways (4, 10, 18, 53). Previous investigators have reported either induction or inhibition of apoptosis by *A. phagocytophilum* (22, 54).

p38 MAPK, a MAPK family member, is phosphorylated and activated by cellular stress and inflammatory stimuli, and its physiologic role seems to involve the regulation of important cellular responses, such as apoptosis and inflammation (5). p38 MAPK activation was previously shown in monocytes, but not neutrophils, exposed to *A. phagocytophilum* (27). However, given the known regulatory function of p38 MAPK in apoptosis, this pathway requires a more in-depth examination of *A. phagocytophilum*-infected neutrophils.

In this study, we confirm delayed neutrophil apoptosis and present evidence that p38 MAPK in fact plays an important role in the delayed apoptosis of *A. phagocytophilum*-infected neutrophils. We found that p38 MAPK is continuously phosphorylated and activated in live and heat-killed *A. phagocytophilum*-infected neutrophils. The inhibition of p38 MAPK by SB203580 abrogated delayed neutrophil apoptosis, but this pathway was bypassed after 3 to 6 h of active intracellular infection. These findings show that the p38 MAPK signal transduction pathway and active intracellular infection are required to prolong the survival of *A. phagocytophilum*-infected neutrophils.

## MATERIALS AND METHODS

**In vitro growth and preparation of cell-free *A. phagocytophilum*.** The *A. phagocytophilum* Webster strain was cultivated in HL-60 cells as described previously (12). Cell-free *A. phagocytophilum* organisms were prepared from approximately $10^7$ HL-60 cells when >90% were infected, as determined by Romanowsky staining (HEMA 3; Biochemical Science Inc., Swedesboro, NJ). Infected HL-60 cells were lysed in the presence of protease inhibitors (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL) by 5 to 10 passages through a 25-gauge needle, and cellular debris was removed by centrifugation at 750 × *g* for 10 min. The supernatant was centrifuged (2,500 × *g* for 15 min) to obtain pellets containing the cell-free *A. phagocytophilum* organisms, which were used immediately to infect $5 \times 10^6$ human peripheral blood neutrophils, estimated to provide a multiplicity of infection of 100:1. To use heat-killed bacteria, we heated cell-free *A. phagocytophilum* at 65°C for 10 min before use.

**Isolation of neutrophils and culture conditions.** Human peripheral blood neutrophils were isolated from EDTA-anticoagulated blood from healthy donors by

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dextran sedimentation and density gradient centrifugation (Ficoll-Paque; Amersham Pharmacia Biotech, Sweden) under a protocol approved by the Johns Hopkins Medicine Internal Review Board. Contaminating erythrocytes were lysed in hypotonic (0.2%) NaCl for 30 s and then neutralized with hypertonic (1.6%) NaCl. Neutrophil purity was always >95%, as determined microscopically after Romanowsky staining (Hema-3; Fisher Scientific Co., VA) of cytocentrifuged slides, and the viability of cells was >98% as assessed by trypan blue dye exclusion. Neutrophils were then suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine. When used, the inhibitor SB203580 was added to neutrophils at the same time as (0 h), 3 h after, or 6 h after *A. phagocytophilum* infection, and then the neutrophils were incubated overnight. SB203580 had no effect on uninfected neutrophil trypan blue viability or the rate of constitutive morphological apoptosis at the concentrations used.

**Apoptosis detection by morphological analysis, Annexin-V staining, and TUNEL assays.** Several methods for the identification of apoptosis exist, including morphological assessment of karyorrhexis, detection of annexin-V expression, and detection of DNA fragmentation by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Annexin-V exhibits calcium-dependent binding to phosphatidylserine (PS) expressed in the outer membrane leaflets of cells. Increased PS on cell surfaces is an early marker of neutrophil apoptosis mediated by the inhibition of membrane flippases, which maintain a normal distribution of PS between inner and outer leaflets (17). The TUNEL assay detects DNA fragmentation, a late event with apoptosis (20). Freshly isolated neutrophils were plated in 24-well tissue culture plates at $3 \times 10^6$ cells/ml in 1 ml per well as follows: (i) with medium only (no additional stimulation) to allow spontaneous apoptosis; (ii) with 30 μg/ml lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4; Sigma) known to delay apoptosis; (iii) with approximately $10^8$ viable *E. coli* organisms (ATCC 25922) propagated in LB medium to exponential phase; or (iv) with approximately $10^8$ cell-free *A. phagocytophilum* organisms. Apoptosis was always confirmed by morphological assessment (42, 46) of Romanowsky-stained cytocentrifuged preparations by counting the proportion of all cells with typical small, condensed, karyorrhectic nuclear bodies.

For flow cytometric detection of annexin-V staining, the cells were harvested at 3 h and 18 h, washed in binding buffer, and stained with annexin-V fluorescein isothiocyanate (FITC) alone according to the manufacturer's recommendations (Oncogene Research Products, Boston, MA). Cells that stained for annexin-V FITC were identified and quantitated by flow cytometry. Data acquisition and analysis were performed with CellQuest software (BD Pharmingen, San Diego, CA).

A flow cytometric TUNEL assay (Fluorescein-FragEL DNA fragmentation detection kit; Oncogene Research Products) was used in some cases to detect apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-terminal deoxytransferase (TdT) (20). Briefly, cells were fixed in 4% formaldehyde–phosphate-buffered saline for 10 min at room temperature, resuspended in 80% ethanol, and then stored at 4°C. After fixation, cells were rehydrated with Tris-buffered saline and suspended in permeabilization solution (proteinase K in 10 mM Tris) for 5 min at room temperature. Cells were washed, resuspended in 100 μl of TdT equilibration buffer, and then incubated in 60 μl of TdT labeling reaction mixture for 1 h in a dark chamber. Green fluorescence of apoptotic nuclei was detected and quantitated by flow cytometry as described above.

**Immunoblot analyses.** Purified neutrophils ($5 \times 10^6$ cells/ml) were incubated without any additional stimulation, with 30 μg/ml LPS, or with cell-free *A. phagocytophilum* for the time periods described above. Cells were pelleted at each time point (at 3 h and 18 h), resuspended in lysis buffer (Pierce, Rockford, IL), and assayed for protein content (bicinchoninic acid protein assay; Pierce). Protein samples (cell lysates) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under reducing conditions, transferred to nitrocellulose membranes, and then washed in a blocking solution containing 5% nonfat dry milk in Tris-buffered saline–Tween 20 (FisherBiothech, Fair Lawn, NJ). After being blocked, the membranes were incubated with anti-Mcl-1 (BD Pharmingen), anti-Bcl-2 (Zymed Laboratories Inc., South San Francisco, CA), anti-caspase-3/CPP32 (procaspase-3; BD Transduction Laboratory), anti-Akt, anti-phospho-Akt (Ser-473), anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK1/2 MEK, anti-phospho-ERK1/2 MEK (Cell Signaling Technology Inc., Beverly, MA), and anti-actin (Sigma, St. Louis, MO). The nitrocellulose blots were then washed and incubated with alkaline phosphatase-conjugated secondary antibody to mouse or rabbit immunoglobulin G (KPL, Gaithersburg, MD). Bands were detected using enhanced chemiluminescence (Bio-Rad, Hercules, CA) and exposure film (Pierce). Equal loadings of protein in lanes were confirmed in all experiments by stripping and reprobing the blots for actin. Quantitation of Mcl-1, Bcl-2, and procaspase-3 was performed by densitometry on immunoblots and normalized to actin expression. The quantified data were used for statistical comparison.

**RT-PCR.** Total cellular RNA was isolated from *A. phagocytophilum*-infected neutrophils using a QIAamp RNA blood mini kit (QIAGEN Inc., Valencia, CA). Reverse transcription (RT)-PCR was performed with SuperScript one-step RT-PCR with Platinum *Taq* (Invitrogen Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. The *bcl-2* family gene primers used were 5′-TGCACCTGACGCCCTTCAC-3′ and 5′-AGACAGCCAGGAGAAATC AAACAG-3′ (29). Reverse transcription was performed at 50°C for 30 min, and pre-PCR denaturation was performed at 94°C for 5 min, followed by 40 cycles of amplification (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min). The reactants were subjected to agarose (1%) gel electrophoresis and visualized by ethidium bromide staining. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers were run simultaneously as controls.

**Chemokine and cytokine assay.** Chemokine and cytokine detection was performed using the RayBio human cytokine antibody array (RayBiotech, Inc., Norcross, GA) according to the manufacturer's instructions. Briefly, purified neutrophils ($5 \times 10^6$ cells/ml) were incubated with or without cell-free *A. phagocytophilum*, and then supernatants were harvested at 3 h and 18 h. Each membrane supplied was placed into a tray and incubated with the appropriate cell culture supernatant. After incubation and thorough washing, diluted biotin-conjugated antibodies, followed by horseradish peroxidase-conjugated streptavidin, were added to each membrane. The signal was detected on the membrane using chemiluminescence and quantitated by image analysis, resulting in a ratio of IL-8 production compared to background.

**Chemokine receptor blocking experiments.** To examine whether the production of chemokines or IL-8 induced delayed neutrophil apoptosis of infected cells, *A. phagocytophilum*-infected neutrophils were cultured with 1 to 4 μg/ml CXCR1 (Biosource International Inc., Camarillo, CA)-, CXCR2 (Cell Sciences, Canton, MA)-, or IL-8 (Cell Sciences)-neutralizing monoclonal antibodies in 24-well plates for 18 h. Cells were harvested, and the expression of procaspase-3 and phosphorylated p38 MAPK was assessed by Western blot analysis.

**Statistical analysis.** Where appropriate, means from groups were compared by Student's *t* test. A value of less than 0.05 was considered significant.

## RESULTS

***Anaplasma phagocytophilum* delays neutrophil apoptosis.** We calculated the percentage of apoptotic cells by morphological examination in *A. phagocytophilum*-infected neutrophils in five replicated experiments. Typical neutrophil apoptotic morphology was observed in the majority (70 to 80%) of mock-stimulated (medium only) cultures (Fig. 1) and in all neutrophils stimulated by viable *E. coli* at 18 h (not shown), whereas incubation of neutrophils with cell-free *A. phagocytophilum* demonstrated more neutrophils with normal multilobular nuclei and only 15 to 20% apoptotic cells ($P < 0.01$). When cells were stimulated with LPS, neutrophils exhibited low proportions (15 to 27%) of apoptotic neutrophils ($P < 0.01$). These data show that *A. phagocytophilum* inhibited apoptosis to a degree similar to that observed for LPS.

Annexin-V FITC analysis (Fig. 2) showed an increased detection of PS, usually indicative of apoptosis, in *A. phagocytophilum*-infected neutrophils and in *E. coli*-stimulated neutrophil cultures as early as 30 min (not shown) to 3 h, whereas little staining was demonstrated before 6 h in mock- and LPS-stimulated neutrophils (not shown). After cultivation in vitro for 18 h, high-level annexin-V staining indicated significant cell surface PS expression on most mock (90%)- and LPS (83%, $P < 0.01$)-stimulated neutrophils, as anticipated, but levels of *A. phagocytophilum*-infected neutrophils were still discrepant compared with the 20% apoptosis evaluated by morphology (Fig. 1); at 18 h all *E. coli*-stimulated cultures contained dead cells that could not be analyzed. However, in contrast to the observations at early time intervals, incubation with *A. phagocytophilum* resulted in markedly fewer cells with annexin-V binding ($P < 0.01$) than with mock-stimulated cells.

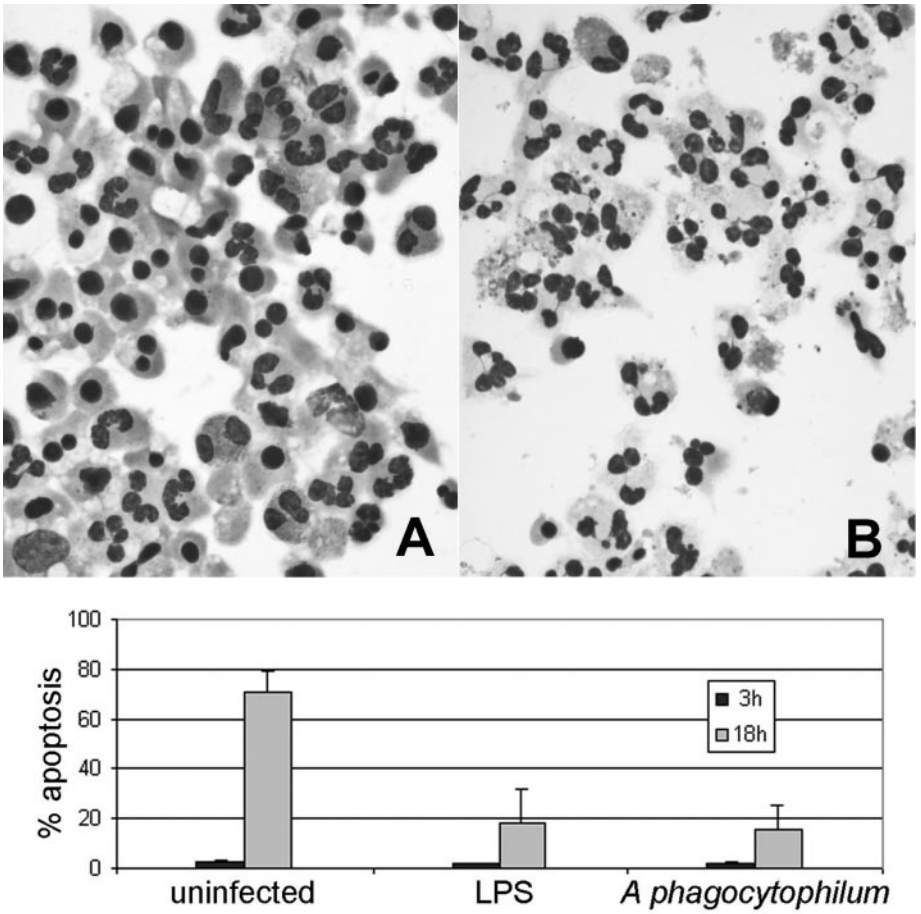


FIG. 1. Morphological appearance of human neutrophils incubated in the presence and absence of *A. phagocytophilum*. Infected and uninfected neutrophils were cultivated in vitro for 18 h, and cytocentrifuged preparations were Romanowsky stained. A, Uninfected neutrophils exhibit morphological features of apoptosis. B, *A. phagocytophilum*-infected neutrophils show only a small proportion of cells with an apoptotic morphology.

In contrast to the results of annexin-V staining at early time points, the inhibitory effect of *A. phagocytophilum* on neutrophil apoptosis at 1 h and 3 h was demonstrated by TUNEL assay. By 3 h, many mock (22%)-, LPS (92%)-, and *E. coli* (96%)-stimulated neutrophils became TUNEL positive, whereas *A. phagocytophilum*-infected neutrophils remained largely (12%) TUNEL negative (not shown). These findings demonstrate the differences in apoptosis kinetics and are in agreement with findings by previous investigators using other pathogens (1, 49) and our morphological analyses.

***A. phagocytophilum* prevents the cleavage of procaspase-3.** Caspase-3 is a key enzyme involved in spontaneous apoptosis that reflects the endpoint of activation via both mitochondrial and nonmitochondrial pathways. Activation of caspase-3 was analyzed indirectly by protein immunoblotting that detects proteolytic cleavage of procaspase-3 (26, 49). After 18 h of infection, expression of the proteolytically inactive 32-kDa procaspase-3 was increased in *A. phagocytophilum*-infected neutrophils compared to that in mock-infected cells (Fig. 3). This finding indicates that *A. phagocytophilum* exerts a protective effect by direct or indirect inhibition of proteolytic cleavage of procaspase-3 into its active form.

***A. phagocytophilum* inhibits the downregulation of neutrophil Mcl-1 and Bcl-2 expression and Bcl-2 family gene transcription.** The antiapoptotic protein Mcl-1 was recently found to be a key element in the regulation of human neutrophil apoptosis (9, 15, 38, 39), and the direct role of Bcl-2 in neutrophils is controversial (2). The expression level of both Mcl-1 and Bcl-2 decreased (32% and 35% of the 3 h values, respectively) as mock-infected neutrophils were cultured and normally entered apoptosis. However, compared with mock-infected neutrophils, the expression of both Mcl-1 and Bcl-2 (117% and 92% of mock-infected neutrophils, respectively) and also the transcription of *bcl-2* were maintained at 3 h and 18 h after *A. phagocytophilum* infection (Fig. 4).

**Phosphorylation of p38 MAPK is increased with *A. phagocytophilum* infection.** Multiple pathways (p38 MAPK, p42/44 extracellular signal-regulated kinases [ERKs], and Akt) may lead to or delay apoptosis (3, 4, 18, 30, 31), and the activation of these pathways was investigated in *A. phagocytophilum*-infected neutrophils. Western blots of Akt and ERK showed that both proteins were present in neutrophils, but with *A. phagocytophilum* infection, the phosphorylation of ERK and Akt was not detected (Fig. 5).

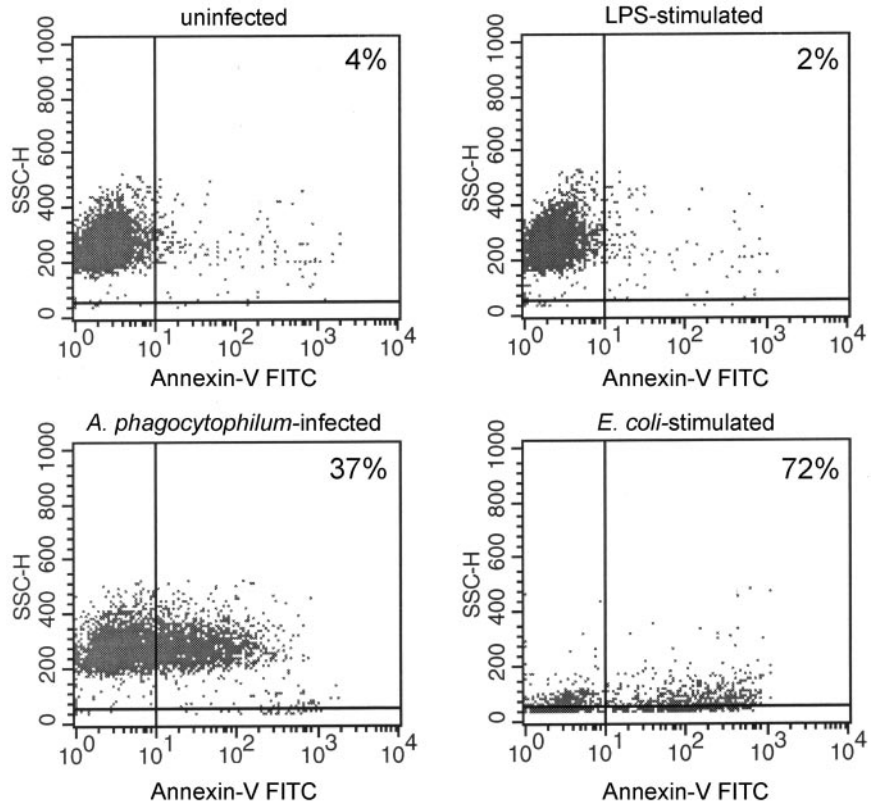


FIG. 2. Annexin-V apoptosis assays of *A. phagocytophilum*-infected neutrophils. Neutrophils were cultivated with or without LPS or cell-free *A. phagocytophilum*; cells were examined by flow cytometry. Annexin-V staining analyzed at 3 h shows early expression of phosphatidyl serine, a membrane marker correlated with apoptosis.

In contrast, significant p38 MAPK phosphorylation in *A. phagocytophilum*-infected neutrophils was observed after overnight (18 h) infection compared to what occurred with mock-infected cells (Fig. 5), an observation also made with other systems where apoptosis was inhibited by a variety of stimuli, such as LPS, tumor necrosis factor alpha, IL-8, and granulocyte-macrophage colony-stimulating factor (4, 40, 52). Additional supportive data to link this observation to the transient inhibition of neutrophil apoptosis in live or heat-killed *A. phagocytophilum*-infected neutrophils were sought by use of SB203580, a p38 MAPK inhibitor. Incubation of *A. phagocytophilum*-infected neutrophils with 10 μM p38 MAPK inhibitor at the time of infection reversed the delayed apoptosis of neutrophils when examined at 18 h ($P < 0.01$). This phenomenon was also observed in p38 MAPK inhibitor-treated neutrophils exposed to heat-killed *A. phagocytophilum* ($P < 0.01$), similar to that reported previously (54). We next examined the effect of p38 MAPK inhibitor when it was added at 3 to 6 h after exposure to heat-killed bacteria or active intracellular infection. Treatment with p38 MAPK at the same time of infection reversed the antiapoptotic phenotype (increased apoptosis; $P = 0.01$). However, when the p38 MAPK inhibitor was

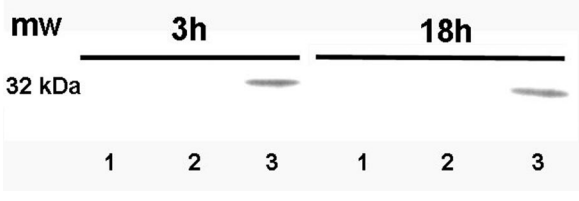


FIG. 3. Inhibition of procaspase-3 processing in *A. phagocytophilum*-infected neutrophils. Apoptosis results with procaspase-3 cleavage into active caspase-3 in neutrophils after 3 and 18 h of exposure to medium only (lane 1) or LPS (lane 2), compared with *A. phagocytophilum* infection (lane 3). A representative immunoblot from five replicated experiments is shown.

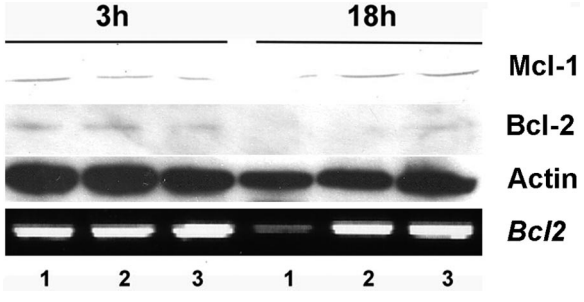


FIG. 4. Mcl-1 and Bcl-2 expression and *bcl-2* transcription are maintained in *A. phagocytophilum*-infected neutrophils. Neutrophils cultured for 3 and 18 h in medium only (lane 1) rapidly lose Mcl-1 and Bcl-2 expression and *bcl-2* family transcription (lane 1) compared to stimulation by LPS (lane 2) or infection with *A. phagocytophilum* (lane 3). Representative immunoblots and RT-PCRs from three experiments are shown.

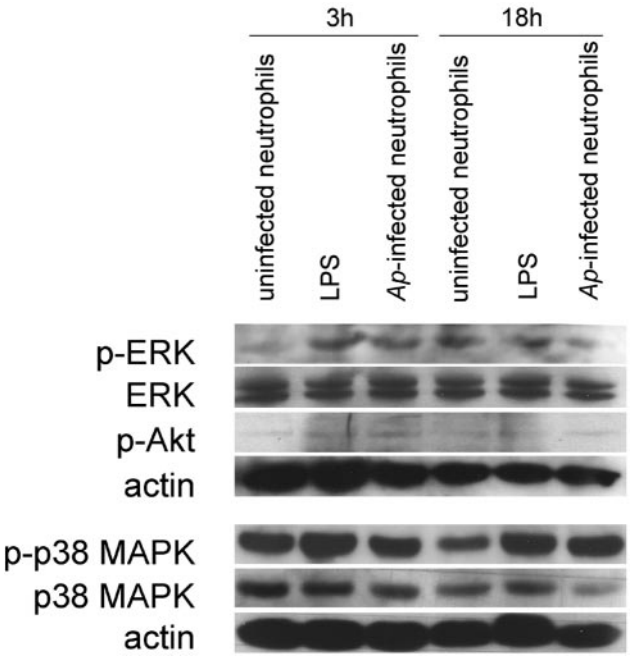


FIG. 5. p38 MAPK, but not ERK or Akt, are phosphorylated in *A. phagocytophilum* (*Ap*)-infected neutrophils. p38 MAPK was consistently found phosphorylated with *A. phagocytophilum* infection of neutrophils or LPS controls. Increased phosphorylation of neither ERK nor Akt was detected. The results are representative of three independent experiments. p-ERK, p-Akt, and P-p38, phosphorylated ERK, Akt, and p38, respectively.

introduced at 3 to 6 h later, significantly less apoptosis ($P < 0.02$) and increased quantities of procaspase-3 were detected with active infection. Both decreased apoptosis and procaspase-3 processing were also observed to a lower degree with exposure to heat-killed *A. phagocytophilum*, although the difference between treatment at 0 h and the 3-h time point was not significant ($P \geq 0.06$) (Fig. 6A and B). No additional reduction of apoptosis inhibition was observed in cells to heat-killed *A. phagocytophilum* at 3 or 6 h ($P \geq 0.09$). These data suggest that the inhibition of apoptosis with exposure to either viable or killed *A. phagocytophilum* organisms proceeds initially through the p38 MAPK pathway. However, the data also provide some evidence that with continued exposure to *A. phagocytophilum* or active intracellular infection, inhibition of the pathway at or proximal to p38 MAPK no longer reverses the antiapoptotic phenotype.

***A. phagocytophilum* infection induces IL-8 expression in neutrophils.** Various cytokines and chemokines synthesized by neutrophils, including IL-8, are reported to delay neutrophil apoptosis (25, 31, 40, 52, 55). Although *A. phagocytophilum*-infected HL-60 cells are known to increase the expression of many chemokines, direct study of this phenomenon in human neutrophils has not been conducted. Thus, to investigate whether *A. phagocytophilum* affects neutrophil apoptosis by inducing the secretion of chemokines linked to delayed apoptosis via the growth receptor family and extracellular signal pathways, IL-8 and other chemokines were measured in culture supernatants of *A. phagocytophilum*-infected neutrophils. The production of IL-8 was detected within the first 3 h of infection and maintained at a relatively high level at 18 h (Fig. 7); in addition, substantial increases in the expression of several other CXCL chemokines, including GROα (CXCL1), MCP-1 RANTES, and IL-6, were also detected at 18 h. Because such chemokines could provide an autocrine- or paracrine-like feedback loop that promotes the inhibition of apoptosis, we sought to block this process by using CXCR1-, CXCR2-, and IL-8-blocking monoclonal antibodies that would be predicted to "unmask" spontaneous apoptosis. In contrast to our expectations, all neutralizing antibodies did not reverse apoptosis inhibition but promoted an antiapoptotic phenotype (data not shown).

## DISCUSSION

In this study, we report that the inhibitory effect of *A. phagocytophilum* on spontaneous neutrophil apoptosis is a multistep process. This process appears to be mediated in part by the p38 MAPK signal transduction pathway that is later bypassed with active infection or prolonged exposure and potentially promoted by an autocrine/paracrine mechanism related to IL-8 or other CXCL chemokines induced with infection. The first observations of apoptosis with *A. phagocytophilum* infection were made with infected HL-60 cells, a promyelocytic cell line (8, 22). These cells become apoptotic more rapidly than uninfected HL-60 cells in a process relating to a dysfunctional transition from $G_1$ to S phase (8, 22) and with a reduction in the expression of the cell cycle proteins proliferating cell nuclear antigen, retinoblastoma protein (pRB), cyclins D3 and E, and the cyclin-dependent kinase inhibitor p21WAF1/CIP1, conclusions further supported by the suppression of Bcl-2 and high levels of Bax in infected cells. In contrast, studies of ex vivo human neutrophils reveal a rapid, protease-sensitive delaying effect of *A. phagocytophilum* on neutrophil apoptosis, especially at high multiplicities of infection (54). The effect is attributed to binding at the neutrophil cell surface and is not related to protein kinase A, NF-κB, or IL-1β; however, the effect is overcome by proteasome and eukaryotic protein synthesis inhibitors. These observations have recently been extended to implicate the maintenance of host *bfl-1* (Bcl-2 family) transcription, the stabilization of mitochondrial membranes, and decreased caspase-3 activation (19). These data seem to imply a greater role for bacterial components than active intracellular infection in the mechanism of apoptosis inhibition. Owing to the confusion inherent with such discrepancies, we sought to better define the signaling pathways that might be involved in this process.

Several intracellular microorganisms have been shown to delay the apoptosis of their host cells (1, 34, 49), but the molecular mechanisms underlying this process are not yet known. We initially observed that *A. phagocytophilum* interaction with neutrophils resulted in an early surface exposure of PS (Annexin-V binding) in the absence of significant morphological apoptosis or DNA fragmentation. This indicated that a defect in the apoptosis signaling pathways distal to aminophospholipid-translocase (flippase) activation could be induced by the pathogen. PS expression is routinely associated with $Ca^{2+}$ influx, and we suspected that it initially related to interactions of the bacterium or its products with cell surface ligands that would ordinarily trigger the apoptosis program. The lack of morphological or biochemical evidence for apoptosis progression fur-

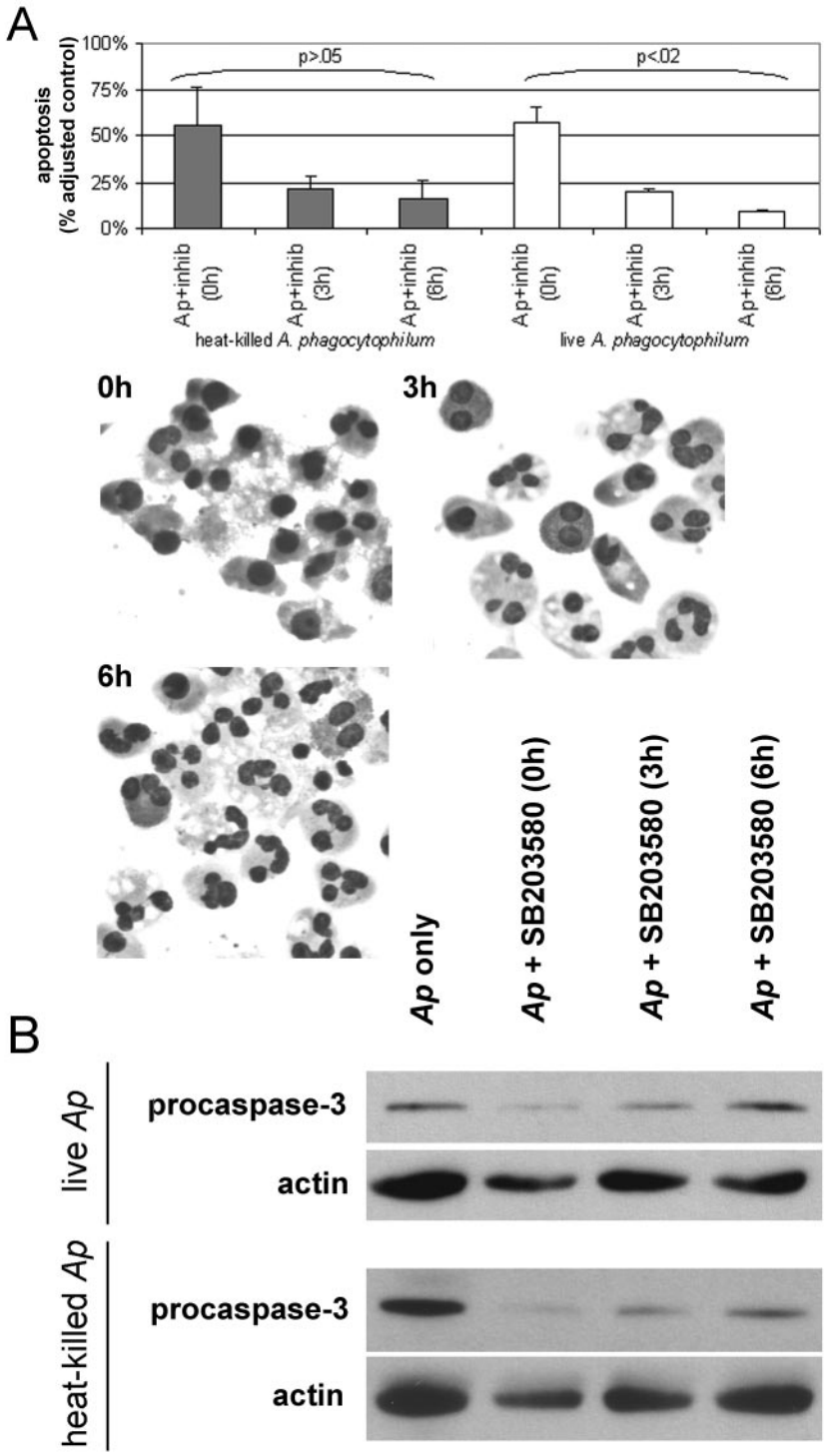


FIG. 6. p38 MAPK inhibitor SB203580 reverses the inhibition of neutrophil apoptosis by *A. phagocytophilum*. A, Apoptotic cells were identified by morphology upon light microscopy (Romanowsky stains; original magnification, ×260), and the percentages of apoptotic cells were calculated; 100% was set at the level of uninfected neutrophils, and 0% was set at the level of untreated *A. phagocytophilum* (*Ap*)-infected neutrophils. Note that the antiapoptotic effect was reversed when infected cells were treated with inhibitor at 0 h, regardless of infection with live bacteria or exposure to heat-killed bacteria. In contrast, apoptosis inhibition was reversed in neutrophils with live *A. phagocytophilum* to a significantly lower degree when p38 MAPK inhibitor was added at 3 or 6 h postinfection ($P < 0.02$), whereas neutrophils exposed to heat-killed bacteria showed no increased antiapoptotic effect with inhibitor added at these times ($P > 0.05$). B, As anticipated from changes in morphological apoptosis, p38 MAPK inhibitor promoted procaspase-3 cleavage at 0 h, but less so at 3 and 6 h.

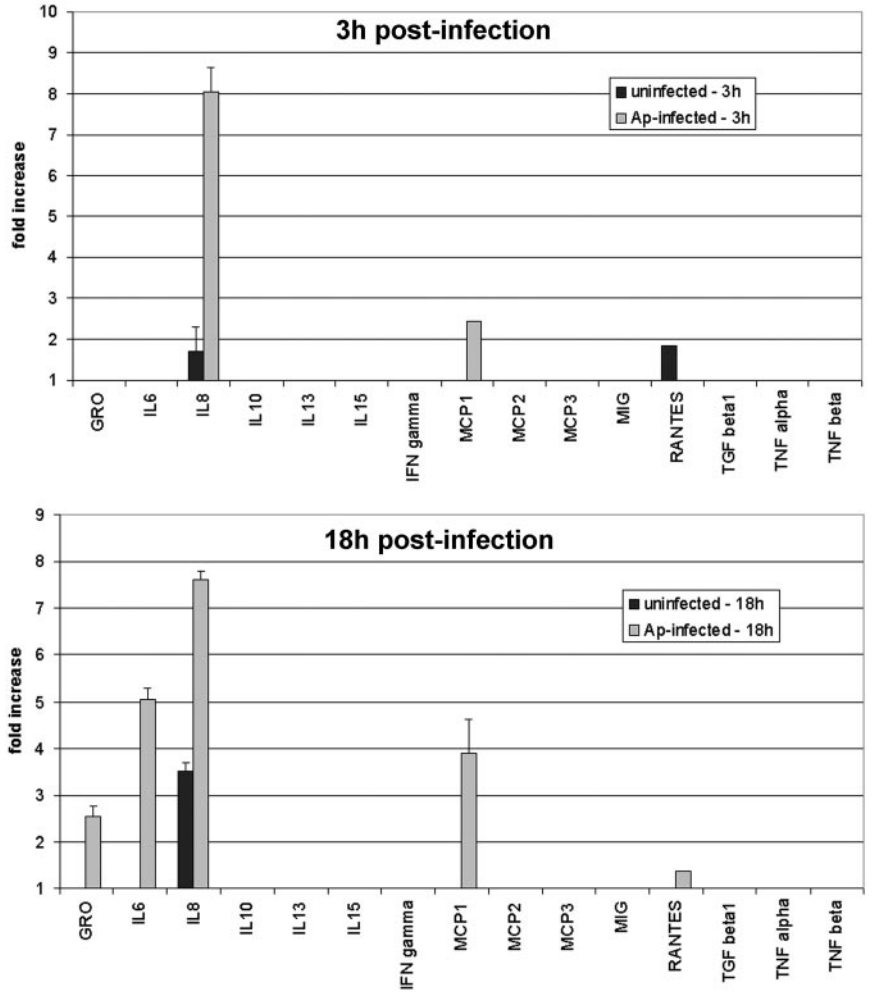


FIG. 7. *A. phagocytophilum*-infected neutrophils express IL-8, CCL, and CXCL chemokines. Neutrophils were incubated for 3 h and 18 h in medium alone or with *A. phagocytophilum*. IL-8, CCL, CXCL chemokine, and cytokine expression in culture supernatants was detected by solid-phase antibody capture and chemiluminescence; results are expressed as increases (*n*-fold) above background (medium alone). GRO, growth-related oncogene; MIG, monokine induced by gamma interferon; TGF, transforming growth factor.

ther suggested interference with the intracellular propagation of apoptosis program signaling.

Despite this link to cell surface events, *A. phagocytophilum* affects neutrophil survival via a mechanism that inhibits spontaneous neutrophil caspase-3 activation (procaspase-3 cleavage), the key enzyme involved in the proteolytic cleavage of many cellular targets, eventually leading to DNA fragmentation and cell death (19). Caspase activation can be related to either intrinsic (mitochondrial) or extrinsic (extracellular death ligands) pathways. Owing to its intracellular niche, we speculated that *A. phagocytophilum* might interfere with the intrinsic pathway and so evaluated the balance of pro- and antiapoptotic signals active at maintaining mitochondrial integrity, especially members of the Bcl-2 family.

Mcl-1, the predominant neutrophil antiapoptotic protein of the Bcl-2 family, is an induced survival protein subject to rapid turnover and is expressed in neutrophils, diminishing prior to the onset of apoptosis (35, 38, 39). The treatment of neutrophils with agents that delay apoptosis either increases or maintains antiapoptotic Mcl-1 levels, explaining cytokine-mediated neutrophil survival (9, 43, 44). Levels of Mcl-1 and Bcl-2 and the transcription of *bcl-2* family genes were found maintained in infected neutrophils, in part extending and contradicting observations in previous studies (19), despite their loss from spontaneous apoptosis in uninfected neutrophils by 18 h. Our observations that inhibited spontaneous neutrophil apoptosis by *A. phagocytophilum* is in part mediated by offsetting the normal loss of Bcl-2 antiapoptotic proteins was also reported but not further investigated by Ge et al. (19).

Although we focused on changes in turnover rates of Bcl-2 family proteins because of their key roles in the regulation of neutrophil cell death and survival, to further extend the understanding of the signaling involved in this process, a mechanism for altering Bcl-2 family expression by *A. phagocytophilum* was investigated. Because *bcl-2* family transcription is influenced by multiple pathways that result in transnuclear localization of activating transcription factors, such as CREB (23, 41), we analyzed three distinct signal transduction pathways previously characterized to impact *bcl-2* transcription, including the ERK, Akt, and p38 MAPK pathways (14, 33, 36, 37, 47, 48, 50).

Our results show that phosphorylation of p38 MAPK is always observed with apoptosis inhibition in *A. phagocytophilum*-infected neutrophils. A central role for this pathway is shown with the abrogation of apoptosis inhibition after the treatment of infected cells with SB203580, a highly specific inhibitor of the p38 MAPK α and β isoforms. Likewise, exposure of neutrophils to heat-killed *A. phagocytophilum* leads to the phosphorylation of p38 MAPK, to inhibition of caspase activation, and to apoptosis inhibition, and this phenotype is also reversed with the p38 MAPK inhibitor. Unexpectedly, we found that treatment of cells with the p38 MAPK inhibitor after 3 to 6 h of infection did not completely block the inhibition of spontaneous apoptosis with live or heat-killed *A. phagocytophilum*-infected neutrophils. This indicates that *A. phagocytophilum* delays neutrophil apoptosis by other signaling mechanisms besides p38 MAPK; alternately, p38 MAPK γ and δ isoform function could be preserved. That the change in apoptosis with p38 MAPK inhibitor used after established infection is significant for killed but not live *A. phagocytophilum* suggests that intracellular infection alters apoptosis by an alternative mechanism that does not directly involve p38 MAPK.

MAPKs are signal transduction proteins involved in numerous inflammatory events. In vitro, p38 MAPK is activated in neutrophils upon ligation of cell surface receptors with LPS, IL-8, and GROα (CXCL1) (5). Neutrophil effector functions, such as respiratory burst and chemotaxis, depend on functional p38 MAPK (5, 16, 24, 45). We demonstrated upregulation of CD11b, down-modulation of L-selectin (CD62L) (13), and prolonged degranulation in *A. phagocytophilum*-infected neutrophils (12), and these phenomena could be mediated in part by p38 MAPK as well. Kim and Rikihisa also examined p38 MAPK in human neutrophils infected by *A. phagocytophilum* and concluded that this pathway was not activated (27). The reasons for these discrepant results are unclear; however, the uniform production from *A. phagocytophilum*-infected neutrophils of chemokines known to stimulate p38 MAPK and the activation of this pathway in neutrophils by other bacterial components, such as LPS (4), argue against its absence. Moreover, p38 MAPK activation is a well-recognized signal for the delay of apoptosis in neutrophils (4) and is an obvious potential target for the control of neutrophil viability and enhanced *A. phagocytophilum* survival.

Several studies have demonstrated that the prototype chemokine IL-8 affects programmed cell death of neutrophils, resulting in delayed spontaneous apoptosis (25). As shown by Klein et al. for infected HL-60 cells (32), we show that a rapid and strong induction of IL-8 and other chemokines occurs during *A. phagocytophilum* infection of terminally differentiated peripheral blood neutrophils. Additionally, these cytokines that are antiapoptotic help to promote the survival of neighboring neutrophils through paracrine mechanisms (11, 25). Since the possibility of an autocrine mechanism of apoptosis inhibition could not be excluded, we examined the effects of blocking IL-8 and the chemokine receptors CXCR1 and -2 for the modulation of apoptosis. However, in contrast to what was anticipated, both treatments paradoxically enhanced the inhibition of apoptosis in infected and uninfected neutrophils. Although these data exclude the autocrine antiapoptotic hypothesis for *A. phagocytophilum*-infected neutrophils, the mechanisms involved with antibody inhibition of apoptosis are unclear.

How *A. phagocytophilum* initiates and sustains the effects of p38 MAPK activation is unclear. Most signaling through this pathway requires the interaction of pathogen-associated molecular pattern components, such as LPS, or growth factors and cytokines or chemokines with their cognate receptors on the surfaces of cells (4, 40, 52, 55). Therefore, it is likely that the initial triggering of the p38 MAPK pathway with *A. phagocytophilum* infection in neutrophils proceeds through a similar surface interaction and does not require live bacteria. Such interactions are often mediated through G protein-coupled responses that interdigitate with other signaling pathways, such as ERK and the c-Jun $NH_2$-terminal kinase pathway (6). However, neither the ERK nor the Akt pathway seems to mediate antiapoptosis with *A. phagocytophilum* infection, since highly specific inhibitors did not alter the delay in apoptosis. In contrast to the initial results with p38 MAPK activation, with bacterial internalization, and the time after infection, a decreasing effect on delayed apoptosis was observed, suggesting the additional requirement for live bacterial infection to sustain the antiapoptotic phenotype.

In summary, these data advance the understanding of how *A. phagocytophilum*-induced apoptosis inhibition is regulated in infected neutrophils by showing that (i) p38 MAPK plays an important role in the initial process driving the delayed apoptosis of *A. phagocytophilum*-infected neutrophils and that (ii) active intracellular infection, not simply exposure to *A. phagocytophilum* components, plays a partial role after infection is established. Clarification is needed of the overall role that p38 MAPK, its isoforms, its regulators, and its targets play in perturbing neutrophil function and permitting intracellular infection. In addition, more investigation of other signaling pathways that could facilitate ongoing apoptosis inhibition should be conducted. Further definition of how *A. phagocytophilum* triggers or influences the p38 MAPK pathway and how live infection bypasses this pathway will provide clues to the complex mechanisms underlying the pathogenesis of human granulocytic anaplasmosis.

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