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Neutrophils exposed to *A. phagocytophilum* under shear stress fail to fully activate, polarize, and transmigrate across inflamed endothelium


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Schaff Y, Trott KA, Chase S, Tam K, Johns JL, Carlyon JA, Genetos DC, Walker NJ, Simon SI, Borjesson DL. Neutrophils exposed to *A. phagocytophilum* under shear stress fail to fully activate, polarize, and transmigrate across inflamed endothelium. *Am J Physiol Cell Physiol* 299: C87–C96, 2010. First published April 14, 2010; doi:10.1152/ajpcell.00165.2009.—Anaplasma phagocytophilum is an obligate intracellular bacterium that has evolved mechanisms to hijack polymorphonuclear neutrophil (PMN) receptors and signaling pathways to bind, infect, and multiply within the host cell. E-selectin is upregulated during inflammation and is a requisite endothelial receptor that supports PMN capture, rolling, and activation of integrin-mediated arrest. Ligands expressed by PMN that mediate binding to endothelium via E-selectin include sialyl Lewis x (sLe(x))-expressing ligands such as P-selectin glycoprotein ligand-1 (PSGL-1) and other glycolipids and glycoproteins. As *A. phagocytophilum* is capable of binding to sLe(x)-expressing ligands expressed on PMN, we hypothesized that acute bacterial adhesion to PMN would subsequently attenuate PMN recruitment during inflammation. We assessed the dynamics of PMN recruitment and migration under shear flow in the presence of a wild-type strain of *A. phagocytophilum* and compared it with a strain of bacteria that binds to PMN independent of PSGL-1. Acute bacterial engagement with PMN resulted in transient PMN arrest and minimal PMN polarization. Although the wild-type pathogen also signaled activation of β2 integrins and elicited a mild intracellular calcium flux, downstream signals including PMN transmigration and phosphorylation of p38 mitogen-activated protein kinase (MAPK) were inhibited. The mutant strain bound less well to PMN and failed to activate β2 integrins and induce a calcium flux but did result in decreased PMN arrest and polarization that may have been partially mediated by a suppression of p38 MAPK activation. This model suggests that *A. phagocytophilum* binding to PMN under shear flow during recruitment to inflamed endothelium interferes with normal tethering via E-selectin and navigational signaling of transendothelial migration.

bacterial pathogen; granulocytes; neutrophil trafficking

**ANAPLASMA PHAGOCYTOPHILUM** is a tick-borne pathogen and is the etiologic bacterial agent of granulocytic anaplasmosis (10, 16). Neutrophils (PMN), which normally function to phagocytose and destroy bacteria, are typically inhospitable hosts for bacterial propagation, yet *A. phagocytophilum* has evolved the capacity to subvert a variety of PMN innate immune functions. Pathogen binding and intracellular infection results in decreased reactive oxygen species production (9, 27, 33, 44), delayed proinflammatory response (6), and delayed apoptosis (6, 46). *A. phagocytophilum* also bind to selectin ligands that PMN employ to roll on inflamed endothelium including P-selectin glycoprotein ligand-1 (PSGL-1) (12), leading us to speculate that pathogen binding may substantially alter recruitment of PMN. Therefore, we developed a model to examine the effects of acute bacterial binding to PMN under shear flow in a custom microfluidic channel to directly image the ability of *A. phagocytophilum* to activate PMN and alter the dynamics of recruitment and migration on endothelium.

PMN trafficking on endothelial cells during an inflammatory response is initiated by selectin-mediated capture and rolling, followed by chemokine-induced β2 integrin-mediated firm adhesion. Binding of Gram-negative bacteria such as *Escherichia coli* to PMN in shear flow, rapidly induce rolling, firm adhesion, polarization, and transmigration in PMN interacting with vascular endothelium (25, 30). In contrast, there is evidence that *A. phagocytophilum*-infected PMN show diminished adhesion to endothelial cells in static and low-shear conditions (12). It has been proposed that this provides a means for propagation and maintenance of *A. phagocytophilum*-infected PMN within the vasculature that facilitates subsequent pathogen acquisition by the tick and completion of the pathogen life cycle (12). Defects in any one step of the PMN extravasation process during acute inflammation as a result of *A. phagocytophilum* uptake could maintain a greater portion of infected PMN in circulation thereby enhancing host-vector transmission. This hypothesis is supported by recent data showing that *A. phagocytophilum* is preferentially maintained in circulation with relatively low numbers in peripheral tissues, including PMN-rich hematopoietic tissue (4, 7, 24).

PSGL-1, specifically its sialyl Lewis x (sLe(x))-modified NH2-terminus, is one known human PMN receptor for *A. phagocytophilum* (17, 22). More generally, to productively infect PMN, *A. phagocytophilum* requires host cell fucosylation. Many E-selectin ligands have this fucosylation. E-selectin is unique among the selectins in that it binds receptors that transduce signals that activate PMN to arrest on inflamed endothelium in shear flow (19, 23). E-selectin engagement of PSGL-1 leads to slow rolling of PMN and activation of the spleen tyrosine kinase (Syk) signaling pathway, which in turn activates the CD11a subunit of β2 integrin (LFA-1) that supports cell deceleration to arrest (11, 20, 23, 47). *A. phagocytophilum* also activates PMN to upregulate β2 integrin expression and adhesive functions that support inflammatory recruitment (7), yet the mechanism by which PMN are retained in the circulation during infection have not been elucidated.

A naturally occurring subpopulation of *A. phagocytophilum* strain NCH-1 organisms, termed NCH-1A2, has been selected...
through cultivation in a sLeα-defective human promyelocytic leukemia cell line (HL60 A2 cells) (17, 35). NCH-1A2 demonstrated decreased dependence on sLeα and PSGL-1 for adherence to and infection of HL60 cells. Additionally, NCH-1A2 infects HL60 cells independent from Syk, which is required for NCH-1 infection through PSGL-1 (35), as well as PMN adhesion activated via PSGL-1 during rolling on E-selectin (47).

E-selectin ligands require a threshold shear to withstand repulsive forces in blood flow and initiate leukocyte rolling. However, assessment of A. phagocytophilum binding to PMN, identification of important host cell receptors for A. phagocytophilum, and evaluation of pathogen-induced PMN signaling have only been studied in static adhesion assays (6, 9, 17, 18, 22). The kinetics of interaction between the PSGL-1-independent bacterial strain (NCH-1A2) and human PMN have also not been reported. Although it is unknown how A. phagocytophilum passes between host cells, an extracellular stage is presumed given the robust, protective humoral immune response that develops after infection (28, 40). In this study we developed a model that allows direct visualization of the effect of acute bacterial binding to PMN interacting with inflamed endothelium under shear flow.

We hypothesized that A. phagocytophilum engagement of selectin ligands during PMN interaction with E-selectin alters the multistep sequence of PMN rolling, signaling, firm adhesion, and polarization that culminates in transmigration under shear flow conditions. We show that acute exposure and binding of A. phagocytophilum activates PMN integrins and can induce arrest, but subsequent migration and diapedesis on inflamed endothelium is diminished. The inhibition of migration corresponds to a reduction in intracellular calcium-mediated signaling and p38 mitogen-activated protein kinase (MAPK) activation. We propose that bacterial binding to PMN under shear flow may provide an adaptive mechanism that diminishes the capacity of PMN to arrest and migrate from the vasculature thus promoting a high blood concentration of this bacterial pathogen.

MATERIALS AND METHODS

Reagents. The anti-high affinity CD18 antibody 327C was a generous gift from ICOS (Eli Lilly, Indianapolis, IN). Anti-CD18 was purchased from Thermoscientific (formerly Pierce, clone TSI18), and anti-Mac-1 was purchased from Dako (Carpinteria, CA). In some experiments 327C was conjugated to fluorescent labels including Dylight 488 and Dylight 649 (Pierce, Rockford, IL) following manufacturer instructions. Anti-CD162 (PSGL-1, PL-1 clone) and anti-CD15s (CSLEX1 clone) antibodies were purchased from BD Biosciences (San Jose, CA). The anti-E-selectin and P-selectin mAb, and anti-Mac-1 was purchased from Dako (Carpinteria, CA). In some experiments, anti-CD162 was conjugated to fluorescent labels including Alexa Fluor 488 (Alexa Fluor 488 carboxylic acid, succinimidyl ester; Invitrogen) at 7.5 μg/ml for 15 min. Labeled organisms were then washed twice and resuspended in RPMI 1640 (Invitrogen) with 10 mM HEPES. PMN were suspended in RPMI 1640 with 10 mM HEPES at 2 x 10⁶ PMN/ml. PMN were placed in 1-ml cuvettes in warmed aggregometer chambers and stirred for 5 min. Fluorescent-labeled NCH-1 or NCH-1A2 A. phagocytophilum organisms were added to PMN suspensions, and aliquots were removed at 1, 5, 10, and 20 min postheparin. Aliquots were fixed in 4% paraformaldehyde, washed twice, and evaluated for binding of organisms via flow cytometry (Cytomics FC 500, Beckman Coulter). Stained PMN without bacteria served as control samples. Specific blockade of PMN surface ligands was performed by incubating aliquots of PMN suspended in RPMI 1640 with 10 mM HEPES as above with antibodies against PSGL-1 (CD162) and β2 integrin (CD18) at 10 μg/ml for 30 min. PMN were washed twice and then placed in aggregometry cuvettes and stirred. Labeled bacteria were added to stirred PMN as above, and aliquots were fixed in 4% paraformaldehyde for flow cytometric evaluation.

High affinity β2 integrin cytometry. PMN suspended in HEPES-buffered saline containing 1% human serum albumin were incubated with bacteria or chemokine for 5 min at 37°C in the presence of 10 μg/ml of the mAb 327C conjugated to Dylight 488 (Pierce). After stimulation, samples were cooled to 0°C for 30 min and then pelleted and resuspended in cold HEPES-buffered saline. Samples were read on a FACSscan cytometer (BD Biosciences) and quantified for median fluorescence intensity compared with baseline unstimulated samples. For analysis, the results of each experiment were normalized by comparing each response to the maximal response elicited by IL-8 (defined as 100%).}

PMN and bacterial infection over L-E cells. PMN were perfused over a monolayer of L-cells transfected with E-selectin (L-E cells) at 2 dyn/cm² and allowed to roll as previously described (39). A. phagocytophilum NCH-1, NCH-1A2, or uninfected HL-60 cell lysate were then infused into the flow stream to mimic bacterial interaction with PMN during early recruitment. After 3 min of exposure to bacteria, the instantaneous velocity of NCH-1, NCH-1A2, or lysate-exposed rolling PMN was calculated from image sequence acquired by video microscopy (Nikon TE200 microscope, Melville, NY). For some experiments, A. phagocytophilum bacteria were incubated in HEPES-buffered saline containing 1 μM CellTracker Green CMFDA (Invitrogen) for 40 min at 37°C (22). Labeled bacteria were centrifuged twice at 10,000 g for 10 min to remove excess fluorophore and resuspended each time in HEPES-buffered saline to a concentration of 5 x 10⁷/ml. PMN were infused over L-E cells, allowed to roll, and then perfused with 5 x 10⁷ bacteria/ml (as above). PMN were imaged under alternating brightfield and 488 nm excitation with a
×60, 1.45 numerical aperture (NA) oil objective (Nikon) to observe binding of labeled bacteria. The percentage of cells exhibiting at least one distinct fluorescent cluster (i.e., regions 1 standard deviation brighter than average cell intensity) were measured at 60 and 180 s following perfusion of bacteria, corresponding with maximal PMN arrest and polarization, respectively.

**Intracellular calcium concentration and velocimetry in microfluidic flow channel.** PMN were loaded with the cytosolic calcium indicator Fura-2 AM (1 μM, Invitrogen) for 30 min at 37°C and then washed with dye-free buffer. L-cells, a fibroblastic murine cell line previously transfected with E-selectin expression plasmids under stable conditions (39), were maintained in RPMI 1640 media with 10% FBS and grown to confluence on glass coverslips. Dye-loaded PMN were perfused over a monolayer of L-E cells in a microfluidic flow chamber and allowed to roll for 2 min as previously described (39). Buffer containing NCH-1, NCH-1A2, or *E. coli* at a concentration of 5 × 10^6 was injected into the inlet reservoir of the microfluidic flow chamber, and Fura-2 emission was measured by fluorescence video microscopy with alternating excitation at 340 and 380 nm at a frame rate of 1 per second over 5 min. Images were captured on a Nikon 1200 microscope equipped with ×40 NA = 1.3 S Fluor objective and an Orca-ER Hamamatsu camera (Leeds Precision Instruments, Minneapolis, MN). Images were acquired with SimplePCI 5.3 (Compix imaging).

Intracellular calcium concentration in PMN responding to bacterial influx was calculated from the Fura-2 emission ratio between the two excitation wavelengths as previously described (38).

In some experiments, PMN in the microfluidic flow chamber were imaged at a rate of 4 frames per second under phase-contrast bright-field illumination with a Nikon TE200 microscope equipped with ×20 NA = 0.45 phase-contrast objective and MTI CCD-300T camera. The shape and position of each PMN in each frame was determined from their bright appearance under phase-contrast illumination. Images were acquired with Image Pro Plus 5.1 (Mediacybernetics) and digitized by using a Meteor II framegrabber (Matrox). Adherent PMN were considered arrested if they did not move more than half a cell diameter within a span of 10 s. PMN with a length-to-width ratio greater than 1.5 were considered polarized. Instantaneous rolling velocity was determined by dividing the change in the position of the centroid of a PMN from frame to frame by the timestep between frames (0.25 s) as determined by using custom macros written for Image Pro Plus 5.1.

**PMN recruitment on inflamed endothelium.** Human umbilical vein endothelial cells (HUVEC) from pooled donors were purchased from Cascade Biologics (Invitrogen) and maintained in Media 200 with 2% FBS. HUVEC were passaged on glass coverslips coated with cross-linked gelatin and allowed to grow for 2–3 days until forming a confluent monolayer. Four hours before the experiment, the monolayers were treated with 5 ng/ml IL-1β and then coverslips were attached to microfluidic flow chambers by vacuum. PMN at a concentration of 1 × 10^6 were incubated with NCH-1 or NCH-1A2 at a concentration of 5 × 10^6 for 5 min, perfused over the inflamed HUVEC under a fluid shear stress of 2 dyn/cm², and imaged under phase-contrast illumination by videomicroscopy at 4 frames per second. PMN were allowed to accumulate on the HUVEC surface for 2 min and then the percentage of transmigrated cells in a series of image sequences were measured over 5 min. Transmigrated PMN were easily distinguished by their spread and dark under phase-contrast illumination. Under certain conditions HUVEC were incubated with 20 μg/ml of the mAb EP-5C7 for 10 min, which recognizes and blocks E-selectin and P-selectin. After antibody incubation, PMN in the presence of 10 mg/ml EP-5C7 were perfused over the HUVEC monolayers as in previous experiments.

**Western blots.** PMN (1 × 10^5) were shear mixed with L-E cells (2 × 10^5) causing them to phosphorylate p38 MAPK as previously described (20). In some experiments, PMN were sheared with untransfected L-cells (“L-P cells”) in place of L-E cells to separate the effect of E-selectin interaction from the effect of shear stress alone (20).

In contrast to PMN-pathogen binding

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**RESULTS**

**Kinetics of *A. phagocytophilum* binding to human PMN under shear stress.** *A. phagocytophilum* binding and infection can induce several PMN signaling pathways (1, 8, 9, 14, 26, 35, 42), but the effect of acute pathogen binding to PMN under shear flow has not been studied. We first determined pathogen binding on rolling PMN. Within seconds of infusion of NCH-1 or NCH-1A2 into the flow chamber, bacteria bound to rolling PMN (Fig. 1A). NCH-1 bound significantly more PMN at both 60 and 180 s (P < 0.03 at both time points, Fig. 1B). Binding generally increased between 60 and 180 s with ~30–60% PMN having bound bacteria at 60 s to ~60–95% PMN having bound bacteria at 180 s, although the difference was not significant (Fig. 1B). We next examined bacterial binding at later time points to determine binding kinetics and specificity. After 5 min of shear, NCH-1 showed slightly increased binding compared with NCH-1A2 with ~41% of PMN bound by at least one bacterium compared with ~27% of PMN bound by NCH-1A2 (Fig. 1, C and D). Between 10 and 20 min of shear stress, bacterial binding plateaued with ~40% of PMN having bound bacteria (Fig. 1D).
under static conditions (22), under shear mixing, PMN binding to both strains of bacteria was reduced in the presence of a blocking antibody to the β2 integrins (Fig. 1E), whereas a blocking antibody to PSGL-1 (PL1) only moderately reduced PMN binding to NCH-1 but not to NCH-1A2 (Fig. 1E). These data demonstrate that 1) both NCH-1 and NCH-1A2 bind PMN in shear flow, although the degree of PMN binding differs between strains; 2) A. phagocytophilum NCH-1A2 successfully binds human PMN in a PSGL-1-independent manner under shear stress, albeit with lower avidity than wild-type NCH-1; and 3) blocking CD18 reduces NCH-1 and NCH-1A2 binding to human PMN under shear stress.

A. phagocytophilum binding supports transient PMN arrest but fails to induce polarization following rolling on E-selectin. We next determined the role of bacterial binding to PMN in an in vitro model of inflammatory recruitment. PMN were sheared over an E-selectin-expressing substrate as previously described (39) and subsequently infused with chemokine or suspended over a monolayer expressing E-selectin. The fraction of PMN that subsequently transitioned to a polarized shape defined by an aspect ratio of 1.5 were measured. Virtually all PMN arrest and undergo shape polarization upon infusion of IL-8 or binding of the LPS-positive bacterium E. coli (Fig. 2A). We postulated that, in contrast to E. coli, A. phagocytophilum binding would diminish PMN arrest due to obscuring the binding and early critical signaling events brought on by interaction between E-selectin and its glycosylated ligands (e.g., PSGL-1) on the rolling PMN. The fraction of total interacting PMN that arrested following influx of A. phagocytophilum NCH-1 was significantly elevated compared with infusion of control PMN or PMN incubated with HL60 cell lysate (P < 0.05) but comparable arrest to NCH-1A2 (P > 0.05, both comparisons) but significantly decreased compared with E. coli and IL-8 (P < 0.005, both comparisons, Fig. 2A). A. phagocytophilum NCH-1A2 also elicited significantly less PMN arrest than E. coli or IL-8 (P < 0.05) but comparable arrest to NCH-1 (P > 0.05). A closer analysis of adhesion kinetics revealed that PMN arrest activated by capture of A. phagocytophilum was delayed compared with E. coli. Moreover, PMN began to detach under shear stress within 70 s of bacteria addition to the flow stream (Fig. 2B). This observation correlated with NCH-1 and NCH-1A2 being a less potent stimuli of PMN arrest and polarization compared with E. coli or IL-8 (P < 0.001, both comparisons, Fig. 2A).

A. phagocytophilum NCH-1 inhibits PMN transmigration on inflamed endothelial cells under shear flow. To determine whether A. phagocytophilum actively inhibits subsequent stages of PMN recruitment, we tested the affect of pathogen binding in
shear flow on transendothelial migration in the vascular mimetic flow channels. As a model of recruitment to inflamed endothelium, PMN were perfused over monolayers of HUVEC stimulated with IL-1β (37). Based on the impaired arrest and polarization, we hypothesized that binding of *A. phagocytophilum* via E-selectin ligands would decrease the extent of PMN transmigration normally induced on inflamed HUVEC under shear flow conditions. Consistent with previous studies, approximately 50% of control PMN transmigrate on inflamed HUVEC and this is significantly blocked with anti-E-selectin (EP-5C7) (Fig. 3, A and B) (39). NCH-1 binding to PMN also significantly decreased PMN transmigration when compared with control PMN, lysate-treated PMN, or PMN exposed to NCH-1A2 (*P* < 0.005, all comparisons, Fig. 3, A and B). Interestingly, NCH-1A2 binding to PMN did not alter PMN transmigration (similar to control PMN and lysate-treated PMN, *P* > 0.05). We conclude that binding of wild-type bacteria halved the efficiency of PMN transmigration on inflamed endothelium in shear flow similar in magnitude to that noted when blocking E-selectin on the endothelial surface. This suggests that NCH-1 recognition of glycolipid ligands critical to efficient signaling of PMN transmigration during rolling and arrest remain available on cells bound to NCH-1A2.

*A. phagocytophilum* elicits PSGL-1-dependent calcium flux and interferes with E-selectin signaling. A hallmark of activation during PMN rolling on E-selectin is a rapid intracellular calcium flux (38). Since intracellular calcium concentration increases in response to ligation of adhesion molecules, chemokine receptors, and Fc receptors (15, 38), it is an excellent measure of the extent of secondary signaling activity driving PMN polarization (38). *A. phagocytophilum* NCH-1 interacting with PMN triggered an increase in the concentration of intracellular calcium in rolling and arrested PMN over the first 5 min following bacterial exposure (Fig. 4A). The calcium flux elicited by NCH-1 was significantly less rapid, smaller in extent, and more transient than that caused by *E. coli* (*P* < 0.001, Fig. 4B) but significantly higher than that triggered by control lysate (*P* < 0.001, Fig. 4, B and C). The PSGL-
NCH-1 binding to PMN under shear stress induced an attenuation of the mean.

Columns indicate the average of 4 separate experiments, bars represent the SE.
P
NCH-1 maintained significantly lower intracellular calcium than different letters indicate significant differences between the means (P < 0.05). These data suggest that pathogen binding interferes with E-selectin-mediated PMN calcium flux and subsequent activation.

A. phagocytophilum interaction with PMN under shear flow suppresses E-selectin-mediated phosphorylation of p38 MAPK. PMN rolling on an E-selectin-bearing substrate activates high-affinity β2 integrins and facilitates migration through a p38 MAPK-dependent pathway (20, 43). Since inhibition of p38 MAPK effectively blocks PMN arrest and directional migration in chemotactic gradients, we assessed whether a deficiency in this pathway could account in part for the observed migration defects. We determined whether PMN binding to A. phagocytophilum altered E-selectin-mediated activation of p38 MAPK. PMN were sheared with L-E cells for 1 min and then lysed and analyzed for phospho-p38 by Western blot. E-selectin and TNF-α stimulation induced phosphorylation of p38 MAPK in human PMN (Fig. 5). Phosphorylation was dependent on E-selectin as PMN sheared with L cells transfected with P-selectin did not induce significant phospho-p38, and activation was specifically inhibited in the presence of SB-202190 (Fig. 5). In the presence of NCH-1 and NCH-1A2, p38 MAPK phosphorylation was suppressed compared with that induced by shearing PMN with L-E cells (Fig. 5). These findings suggest that binding of A. phagocytophilum in shear inhibits activation of p38 MAPK, a necessary component of E-selectin-mediated signaling of PMN arrest and polarization.

A. phagocytophilum alters tethering and slow, steady PMN rolling on E-selectin. To better gauge the mechanism of inhibition of PMN activation on E-selectin, we next investigated whether binding of A. phagocytophilum NCH-1 and NCH-1A2 to their cognate receptors under shear flow altered the kinetics of PMN rolling on E-selectin. PMN were perfused under a shear stress over a monolayer of L-E cells. A. phagocytophilum were then infused into the flow stream to mimic bacterial interaction with PMN in the microcirculation. Control PMN rolled on E-selectin at a characteristic velocity of 6 µm/s with independent strain NCH-1A2 failed to elicit significant calcium flux compared with control (P = 0.96, Fig. 4, B and C) and maintained significantly lower intracellular calcium than NCH-1 (P < 0.001, Fig. 4, B and C). These data suggest that NCH-1 binding to PMN under shear stress induced an attenuated calcium flux, whereas binding of NCH-1A2 does not. The transience in the A. phagocytophilum-mediated calcium signal correlates with the observed decrease in PMN arrest and polarization, suggesting that pathogen binding interferes with E-selectin-mediated PMN calcium flux and subsequent activation.

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a 4 μm/s variance in instantaneous velocity (Fig. 6A). Infusion and binding of NCH-1 resulted in a significant increase in mean velocity of the rolling fraction of PMN (P < 0.001, Fig. 6, A and B) and marked variations and transient increases from the mean compared with control PMN, measured by the standard deviation (P < 0.002, Fig. 6C). In contrast, NCH-1A2 infusion did not perturb PMN rolling (P = 0.88). Thus the PSGL-1–dependent strain of A. phagocytophilum altered tethering and impaired slow, steady rolling normally induced within seconds of when PMN roll on E-selectin. This bacterial-induced alteration in E-selectin–dependent rolling velocity is consistent with rapid binding and steric blocking of selectin ligand recognition and suggests that NCH-1, but not PSGL-1-independent NCH-1A2, competes for E-selectin ligands on the PMN surface and may account in part for A. phagocytophilum–induced defects in PMN activation.

Kinetics of A. phagocytophilum activation of human PMN under shear stress. Because rolling on E-selectin rapidly induces high affinity β2 integrin expression, which correlates with PMN deceleration to arrest, we hypothesized that bacterial binding itself under shear may activate β2 integrin–mediated adhesion. We examined the capacity for NCH-1 and NCH-1A2 to directly activate PMN following binding. Incubation of PMN with IL-8 elicits a maximal CD18 upregulation (100%; Fig. 7). Coincubation of PMN with NCH-1 increased the expression of high affinity β2 integrin by fivefold above the lysate control (P = 0.005) to a level comparable to E. coli stimulation (P = 0.625; Fig. 7) but significantly less than IL-8 stimulation (P = 0.016). NCH-1 was unable to suppress IL-8–mediated CD18 activation (P < 0.05 compared with both E. coli and NCH-1; Fig. 7), consistent with its inability to bind PSGL-1 and activate calcium flux. These findings suggest that contact with NCH-1 shifts PMN β2 integrins to an active conformation, whereas binding of NCH-1A2 does not.

**DISCUSSION**

PMN are endowed with a highly efficient adhesion and migratory apparatus that supports their primary function of rapid antimicrobial response during host defense. They have evolved a capacity for rapid activation of integrins that support the transition from cell rolling to arrest and transmigration on inflamed endothelium under the hydrodynamic shear of blood flow. One such process involves recognition of sLex–decorated receptors including L-selectin and PSGL-1 that redistribute and cluster at the trailing edge of a rolling PMN (36). This in turn triggers a cascade of intracellular signaling events including calcium flux, phosphorylation of MAPK, an upshift in integrin affinity, and shear-resistant adhesion (2, 3, 19). In this study,
we demonstrated that rapid binding of *A. phagocytophilum* to PMN in a shear flow channel results in rapid PMN arrest that correlates with a small calcium flux and an upshift in CD18 affinity. Despite this response to binding *A. phagocytophilum*, PMN failed to fully activate as detected by impaired p38 MAPK phosphorylation and transition to a polarized and trans migratory phenotype. This defect in the presence of NCH-1, but not NCH-1A2, was attributed to bacterial inhibition of normal E-selectin ligand recognition and signaling during rolling on E-selectin. Our data suggest that when bacteria are extracellular they may rapidly adhere to PMN under fluid flow and antagonize the multistep process of recruitment and transmigration.

Selectin mimicry is a strategy used by a number of pathogens including *Staphylococcus aureus*, whose superantigen-like 5 binds PSGL-1 and inhibits PMN rolling under shear flow (5). *A. phagocytophilum* engagement of PSGL-1 results in multiple signaling events critical to pathogen invasion and survival in host PMN including activation of the host cell kinase Syk, phosphorylation of host ROCK1, and host cell-mediated tyrosine phosphorylation of the bacterial effector AnkA (26, 31, 35, 42). Indeed, antibodies targeting PSGL-1 NH2-terminal peptide (KPL1 or PL1) prevent *A. phagocytophilum* engagement of PSGL-1, which in turn inhibits Syk activation, ROCK1 phosphorylation, and AnkA delivery (22, 34, 35, 42). The NCH-1A2 strain of *A. phagocytophilum* has shed light on the specific PSGL-1 engagement-dependent signaling events. NCH-1A2 were captured by PMN under shear flow; however, that binding was slower and less efficient overall. Nonetheless, NCH-1A2 binding through its fucosylated receptors did reduce PMN arrest and polarization similar to the wild-type strain. However, this binding did not alter PMN rolling velocity, shift the β2 integrins to an active conformation, or initiate a measurable calcium flux. As such, NCH-1A2 binding was unable to inhibit normal PMN transmigration on inflamed endothelial cells. The fact that both pathogens could suppress E-selectin-mediated p38 MAPK phosphorylation suggests that this signaling is central to *A. phagocytophilum* pathogenesis but is not the sole determinant of PMN arrest, polarization, and transmigration in this model of host-pathogen interaction. These findings build on functional differences reported for PSGL-1-independent infection, including Syk-independent infection and less efficient AnkA delivery (35) and fit the PMN recruitment model that specifically implicates Syk as required for integrin activation after E-selectin binding to PSGL-1 (47). Other E-selectin ligands on PMN may be bound and inhibited by *A. phagocytophilum*, including E-selectin ligand-1, L-selectin, CD44, CD43, and other glycoproteins and glycoproteins (23). It is also possible that acute inhibition of PMN calcium signaling and migration proceeds through nonselectin-mediated pathways. For instance, many pathogens express chemokine receptor inhibitors such as pertussis toxin, which could rapidly down-regulate signaling pathways not associated with E-selectin rolling. Although *A. phagocytophilum* does not have LPS, it does have an outer calyx that contains glycolipids and glycoproteins that appear to partially activate PMN. The fact that *A. phagocytophilum* capture did not result in full PMN activation as evidenced by cell arrest, polarization, and transmigration (as seen with *E. coli*) suggests that binding and inhibition of E-selectin ligands is at least one of the strategies the pathogen uses to inhibit inflammatory signaling. Further study is required to determine whether *A. phagocytophilum* can occupy and prevent activation of a significant fraction of the total E-selectin binding sites on PMN, or whether the bacteria downregulates activation by other mechanisms.

Our findings are consistent with previous reports regarding PMN activation by extracellular pathogen studied under static conditions but also highlight striking differences between host-pathogen interactions observed under hydrodynamic shear that more closely mimics events in the circulation. In the absence of shear, extracellular pathogen binding and uptake by PMN is relatively slow, with peak ingestion occurring within 2 h (6, 9). Conversely, under shear flow, pathogen binding was detected within seconds, and pathogen-induced calcium flux peaked within a minute of capture. PSGL-1-dependent CD18 activation was maximum by 5 min, and capture of bacteria under shear was reduced with CD18 blockade for both the wild-type and the PSGL-1-independent strain. This suggests that specific CD18-dependent pathogen binding can occur without overt PMN activation and that *A. phagocytophilum* activation of CD18 may provide a positive feedback for additional pathogen capture. Although *A. phagocytophilum* induces both murine and human PMN to upregulate CD18 in vivo and in vitro under static conditions (7, 8, 13), blocking CD18 does not alter pathogen binding in the absence of shear (17). This is consistent with the concept that CD18 is required for shear resistant but not static adhesion.

The mechanisms by which *A. phagocytophilum* transfers between host cells are currently unknown. It has been postulated that bacterial-induced upregulation of IL-8 attracts naive PMN toward another infected PMN by chemotaxis (1). Alternatively, infected endothelial cells may be a reservoir of *A. phagocytophilum* infection and serve to directly infect marginated PMN in the circulation (21). Both of these propagation mechanisms would be promoted by initial adhesion of PMN to inflamed endothelium. Based on this study, we hypothesize that *A. phagocytophilum* has adapted a mechanism for trafficking that alters the normal multistep adhesion cascade in which PMN adhere to the surface of infected endothelium, do not transmigrate, but subsequently demarginate back into the circulation. The pathogen is likely extracellular at one or many points postinfection as there is a strong humoral immune response to infection. In addition, cell-free bacteria are capable of efficient in vitro and in vivo infection. Thus alterations in PMN behavior in the seconds following contact with extracellular *A. phagocytophilum* may be relevant to PMN trafficking. Pathogen-induced changes in PMN trafficking in vivo are currently being studied in mouse models of infection (8, 29, 32). We have observed that differential trafficking with prominent PMN migration to and accumulation within the spleen occurs during infection (Borjesson et. al., unpublished data). Our in vitro flow system has permitted dissection of PMN signaling events initiated by pathogen binding. These studies will provide a basis for further evaluation of in vivo PMN–pathogen trafficking based on tissue specific blood flow and regulation of adhesion molecules.

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REFERENCES


