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The Anaplasma phagocytophilum adhesin Asp14 directs PDI-mediated disulfide reduction to promote infection

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The *Anaplasma phagocytophilum* adhesin Asp14 directs PDI-mediated disulfide reduction to promote infection

A dissertation submitted in partial fulfillment of the requirements for the degree of doctor of philosophy at Virginia Commonwealth University

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˚C	Degrees Celsius
ADP	Adenosine diphosphate
AipA	*Anaplasma phagocytophilum* invasion protein A
AnkA	Ankyrin repeat-containing protein A
ANOVA	One-way analysis of variance
AptA	*Anaplasma phagocytophilum* toxin A
ApV	*Anaplasma phagocytophilum*-occupied vacuole
ARDS	Acute respiratory distress syndrome
Asp14	14-kilodalton *Anaplasma phagocytophilum* surface protein
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Ats-1	*Anaplasma* translocated substrate-1
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CKO	Conditional knockout
CO_2	Carbon dioxide
Cys	Cysteine
DAPI	4’,6-diamidino-2-phenylindole
DC	Dense-cored
DMEM	Dulbecco’s-Modified Eagle Medium

DNA	Deoxyribonucleic Acid
dpi	Days post infection
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
g	Relative centrifugal force (RCF), or g-force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GTP	Guanine triphosphate
h	Hour(s)
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGA	Human granulocytic anaplasmosis
HGE	Human granulocytic ehrlichiosis
HL-60	Human Leukemia cell line-60
hpi	Hours post infection
HRP	Horseradish peroxidase
IFA	Immunofluorescence assay
IFN	Interferon
IMDM	Iscove's-Modified Dulbecco's medium
IP	Intraperitoneal

IPTG	Isopropyl ß-D-1-thiogalactopyranoside
kDa	Kilodalton
KD	Knock-down
KO	Knockout
LPS	Lipopolysaccharide
mg	Milligram
min	Minute(s)
ml	Milliliter
mM	Millimolar
MPB	N-(3-maleimidopropionyl)
MS	Mass spectrometry
Msp	Major surface protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential amino acids
ng	Nanogram
NPC1	Niemann-Pick protein type C1
NPC2	Niemann-Pick protein type C2
OmpA	Outer membrane protein A
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase

PFA	Paraformaldehyde
qPCR	Quantitative polymerase chain reaction
RC	Reticulate cell
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
T4SS	Type IV secretion system
TBS-T	Tris-buffered saline-Tween-20
TLR	Toll-like receptor
Trx	Thioredoxin-1
µg	Microgram
µl	Microliter
µM	Micromolar

Abstract

THE ANAPLASMA PHAGOCTYOPHILUM ADHESIN ASP14 DIRECTS PDI-MEDIATED DISULFIDE REDUCTION TO PROMOTE INFECTION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Obligate intracellular pathogens are a significant cause of global morbidity and mortality. These pathogens must invade host cells in order to survive, replicate, and cause disease. As such pathogen internalization is one of the most critical life cycle phases and represents an excellent therapeutic target. Through understanding pathogen internalization, potential therapies can be identified. Oxidoreductase exploitation is an invasion strategy that is thematic among obligate intracellular pathogens. However, pharmacologic inhibition of oxidoreductases is cytotoxic. Delineating the mechanisms and microbial proteins responsible for these interactions could identify novel therapeutic targets for many important pathogens. *Anaplasma phagocytophilum* infects neutrophils by an incompletely defined mechanism, resulting in the emerging potentially fatal disease, human granulocytic anaplasmosis. The bacterial adhesin, Asp14, contributes to invasion by virtue of its C-terminus engaging an unknown receptor. Herein we identify that receptor and mechanistically dissect the benefit of that interaction to *A. phagocytophilum* and determine the Asp14 residues that mediate that interaction. Yeast two-hybrid analysis identified protein disulfide isomerase (PDI) as a putative Asp14 binding partner. This

interaction was confirmed via co-immunoprecipitation and determined to be dependent upon the Asp14 C-terminus. *PDI* knockdown (KD) impaired bacterial infection of, but not binding to, host cells. *A. phagocytophilum* failed to productively infect myeloid-specific PDI conditional knock-out mice. This is the first demonstration of microbial PDI exploitation *in vivo*. Chemical inhibition of surface oxidoreductases, and antibody-mediated inhibition of PDI reductase activity impaired *A. phagocytophilum* infection of, but not binding to host cells. Infection of PDI inhibited cell was rescued when host and bacterial cells were reduced with recombinant PDI or the chemical reducing agent tris (2-carboxyethyl) phosphine (TCEP). Together these data indicate that *A. phagocytophilum* specifically relies on PDI-mediated disulfide reduction to promote infection. Furthermore, reduction of only bacterial surfaces, and not host surfaces, rescued bacterial infection. TCEP also restored bacterial infectivity after direct inhibition of Asp14 using a C-terminal blocking antibody that inhibits Asp14 interaction with host cells and bacterial infection. Mutational analyses determined that Asp14 residues E123 and S124 are critical for the interaction between Asp14 and PDI. Residues N119, T120, and K122 are contributory. These data demonstrate that Asp14 binds and brings PDI to disulfide bonds within *A. phagocytophilum* surface protein(s) that it reduces, enabling infection on a cellular and organismal level. Targeting the Asp14 C-terminus could benefit approaches to prevent/treat granulocytic anaplasmosis. A thematically similar approach would identify essential proteins from other obligate intracellular pathogens that could prove to be protective targets.

Chapter 1: Introduction and background

1.1 *Anaplasma phagocytophilum* background

Classification

Anaplasma phagocytophilum is an obligate intracellular Gram-negative bacterium of the order *Rickettsiales*. The *Rickettsiales* are α-proteobacteria that are comprised of the three families *Rickettsiaceae*, *Midichloriaceae*, and *Anaplasmataceae*.[6,7] The *Anaplasmataceae* are obligate intracellular bacteria that reside and replicate within host cell-derived vacuolar compartments. This family includes the endosymbiotic *Wolbachia* genus and the pathogenic genera: *Ehrlichia*, *Neorickesttsia*, and *Anaplasma*.[7] The *Anaplasma* genus contains a number of species that represent significant threats to the health of livestock and humans. Of those bacteria, *Anaplasma phagocytophilum* is unique, as it has the widest host range, including mammals, birds, and reptiles. Furthermore it is the only *Anaplasma spp.* capable of infecting humans, resulting in the disease human granulocytic anaplasmosis (HGA).[8]

Discovery

Disease caused by *A. phagocytophilum* was first documented in 1932 Scotland as the veterinary disease tick-borne fever of sheep. This disease was reported to be caused by the organism *Ehrlichia phagocytophila.* In 1969 a similar disease, equine granulocytic ehrlichiosis, was observed in the United States. This disease was reportedly caused by the bacterium, *Ehrlichia equi*.[9] The first case of HGA was documented in 1994, where the disease was described as human granulocytic ehrlichiosis.[10] Shortly thereafter in 2001 the bacteria *E. phagocytophila, E. equi,* and the agent of human granulocytic ehrlichiosis were all recognized as the same organism and redefined as *A. phagocytophilum*.[11] This

reorganization was determined on the basis of DNA sequencing analysis of *16S rRNA* and *groESL* genes.[11]

Morphology

A. phagocytophilum are small (0.4-1.3 µm) Gram-negative non-motile pleomorphic cocci. These obligate intracellular bacteria exhibit a biphasic lifecycle characterized by two morphologically distinct forms. The two forms of *A. phagocytophilum* are referred to as the dense-cored (DC) form and the reticulate cell (RC) (Figure 1). The DC is a metabolically inert, environmentally resistant, infectious form that is characterized by its condensed, ruffled outer membrane and an electron dense nucleoid.[12] The RC is found exclusively within the vacuolar niche that *A. phagocytophilum* produces within a host cell, where it replicates by binary fission (Figure 1).[13,14] Both morphological forms contain a thin outer membrane and minimal periplasmic space.[12] *A. phagocytophilum* lacks any discernable cell wall and the genes necessary to synthesize peptidoglycan and the lipid A component of lipopolysaccharide (LPS). Membrane stability is maintained by the incorporation of host-derived cholesterol into the bacterial membranes.[15]

Environmental life cycle

In nature, *A. phagocytophilum* persists in a zoonotic cycle. Under normal circumstances this bacterium cycles between *Ixodes* species (spp.) ticks and mammalian hosts. In the United States *Ixodes scapularis* and *I. pacificus* serve as vectors for this bacterium in the North Eastern/upper Midwestern and Western states, respectively. In Europe, Asia, and

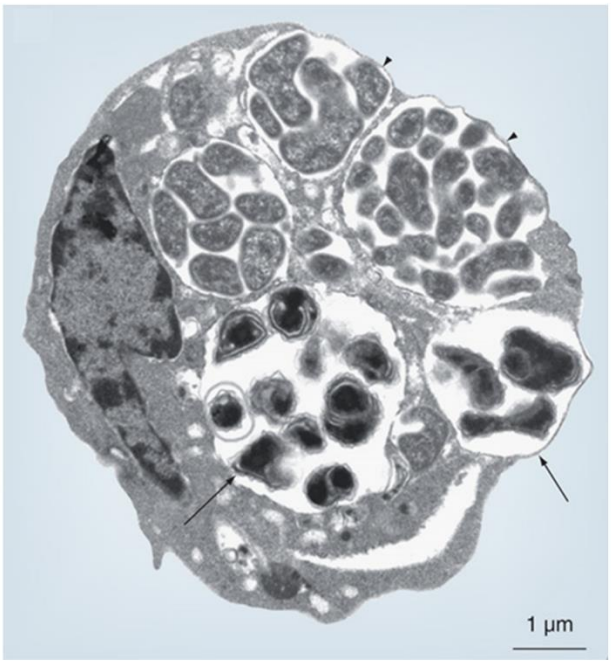


Figure 1: *A. phagocytophilum* is a vacuole-adapted obligate intracellular bacterium

An electron microscopy image of an *A. phagocytophilum*-infected HL-60 promyelocytic cell. Vacuoles containing infectious DC bacteria are indicated by arrows. Host-derived vacuoles containing replicative RC bacteria are indicated by arrowheads.

Adapted from: Thomas, Carlyon, and Dumler. (2009). Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and *Ehrlichia ewingii* ehrlichiosis. Expert Rev Anti Infect Ther. 7(6):709-22.[1]

Australia *A. phagocytophilum* is transmitted by *I. ricinus, I. persulcatus,* and *I. holcyclus,* respectively.[16-20] The tick lifecycle is defined into the stages: eggs, larvae, nymphs, and adult ticks.[20] As this bacterium is not transmitted transovarially from mother to egg, eggs develop free of infection.[21] Infection occurs after the eggs hatch into larvae that take a blood meal from an *A. phagocytophilum*-infected mammal. The initial site of infection is within cells of the tick midgut, later infecting hemocytes that carry transport bacteria to cells of the salivary glands, from where it is transmitted during feeding.[22] This stimulates the larva to molt into a nymph. The now *A. phagocytophilum*-infected nymphs will again take a blood meal to stimulate molting into an adult form, and in doing so transmit the bacteria to a new mammalian host. Female adult ticks also take blood meals, allowing additional opportunities to transmit harbored bacteria.[18,23] As *Ixodes* spp. ticks vector numerous pathogens, it is not uncommon for ticks to harbor and transmit multiple pathogens during transmission feeding, including *Babesia* spp. and *Borrelia burgdorferi.*[24-27]

As *A. phagocytophilum* has a broad host tropism, numerous spp. can serve as bacterial reservoirs from which ticks become infected. While the white-footed mouse (*Peromyscus leucopus)* has been described as the primary reservoir for *A. phagocytophilum,* other reservoirs are common, including deer, squirrels, voles, and rats.[18,28-31] In mammalian hosts, *A. phagocytophilum* bacteria primarily infect granulocytes. Endothelial cells have been implicated as becoming infected during murine infection.[10,32-34] However, these cells do not serve as a major site of bacterial replication during infection. When a human becomes infected, it is considered incidental.[35] While tick transmission is the primary

mechanism for the spread of HGA, cases of human-to-human spread through perinatal transmission and *A. phagocytophilum*-tainted blood transfusions have been reported.[36-40]

1.2 Human granulocytic anaplasmosis

Clinical manifestations and disease

A. phagocytophilum infection of humans results in the disease HGA. Following an incubation period of 5-14 days post infection, patients develop an acute febrile illness that is characterized by fever, myalgia, malaise and headaches.[41] Common laboratory findings include elevated hepatic transaminases and cytopenias, including anemia, leukopenia, and thrombocytopenia.[42-46] Although the majority of cases are self-limiting, this disease can be debilitating, with 36% of severe anaplasmosis cases requiring hospitalization.[46] Furthermore, this febrile presentation often results in misdiagnosis, as many other diseases present similarly. Misdiagnosis leading to delayed treatment can result in potentially lethal sequelae in certain populations, such as the elderly, the young, and the immunocompromised.[42,45] These complications include seizures, septic shock-like syndromes, acute respiratory distress syndrome, rhabdomyolysis, and immunosuppression resulting in opportunistic infections.[41,44,46]

Epidemiology and incidence

A. phagocytophilum is found worldwide with an endemic range including Europe, Asia, South America and North America.[8] In the United States the majority of cases are reported in New England, Wisconsin, and Northern California, mirroring the regions with

the highest density of the vectors *I. scapularis* and *I. pacificus,* respectively; however, this may not be truly representative.[47,48] Positive testing for anaplasmosis in canines, a population more regularly tested than humans, indicates the range of *A. phagocytophilum* is much greater (Figure 2A).[2,49] In the United States, HGA became reportable to the Centers for Disease Control and Prevention (CDC) in 1999. Since that time a 16.5-fold increase in the number of reported cases has been seen, with 5,762 reported in 2017, representing 17.9 cases per million people (Figure 2B). Furthermore, the disease incidence peaks seasonally with a major increase in June and July and a smaller increase in October and November, corresponding to seasonally increased prevalence of nymph and adult ticks, respectively.[50] Additionally, these periods coincide with increased human outdoor activity and subsequent exposure to bacteria carrying ticks. Within the population, it is seen that men are 1.4 fold more likely to contract disease than women.[20] Recent evidence has shown that female mice are innately more resistant to *A. phagocytophilum* infection than males, which may contribute to this observation.[51] Regardless of gender, the overall case fatality rate of HGA is low, at 0.3%.[3]

Diagnosis, treatment and prevention

Historically, HGA was diagnosed by the visibility of bacterial inclusions within granulocytes, as seen by microscopic evaluation. As *A. phagocytophilum* infects a low percentage of circulating neutrophils, this method is prone to false negative results.[48,50] Consequently, PCR-based approaches have become the most reliable method of diagnosis.[52] Due to its intracellular niche, *A. phagocytophilum* is resistant to antibiotics that cannot penetrate host cell membranes. Furthermore, this bacterium does not encode

peptidoglycan biosynthesis machinery, providing resistance to beta-lactams. Macrolide resistance likely arises from several mutations within the *23S rRNA* that confers macrolide resistance in other bacterial spp.[53] Despite these resistances, *A. phagocytophilum* infection is effectively treated with doxycycline.[53-55] Doxycycline is contraindicated in several populations including those with tetracycline allergies and children under the age of 18, for whom rifampin is effective. Both rifampin and doxycycline are contraindicated for pregnant women. Rifampin can be used to treat those with tetracycline allergies, but is also teratogenic.[38] No vaccine against *A. phagocytophilum* has been developed.[56]

1.3 *A. phagocytophilum* pathogenesis

Life cycle

A. phagocytophilum exhibits a biphasic intracellular life cycle, wherein it alternates between two morphologically distinct forms. While extracellular, *A. phagocytophilum* exists as a DC organism, which is infectious but metabolically inert.[13] Adhesin proteins on the DC surface allow binding to host cells. Following adherence, internalization occurs via receptor-mediated endocytosis at caveolin-1 enriched lipid rafts.[57] In this endosome, the bacterium converts from the DC form to the replicative RC between four and eight hours post infection (hpi). The RC produces and secretes proteins that begin remodeling the *A. phagocytophilum*-containing vacuole (ApV) into a distinct niche for bacterial growth. In this niche, the bacterium replicates by binary fission.[14] The vacuole-bound bacterial colony is referred to as a morula, Latin for mulberry, describing the appearance of the

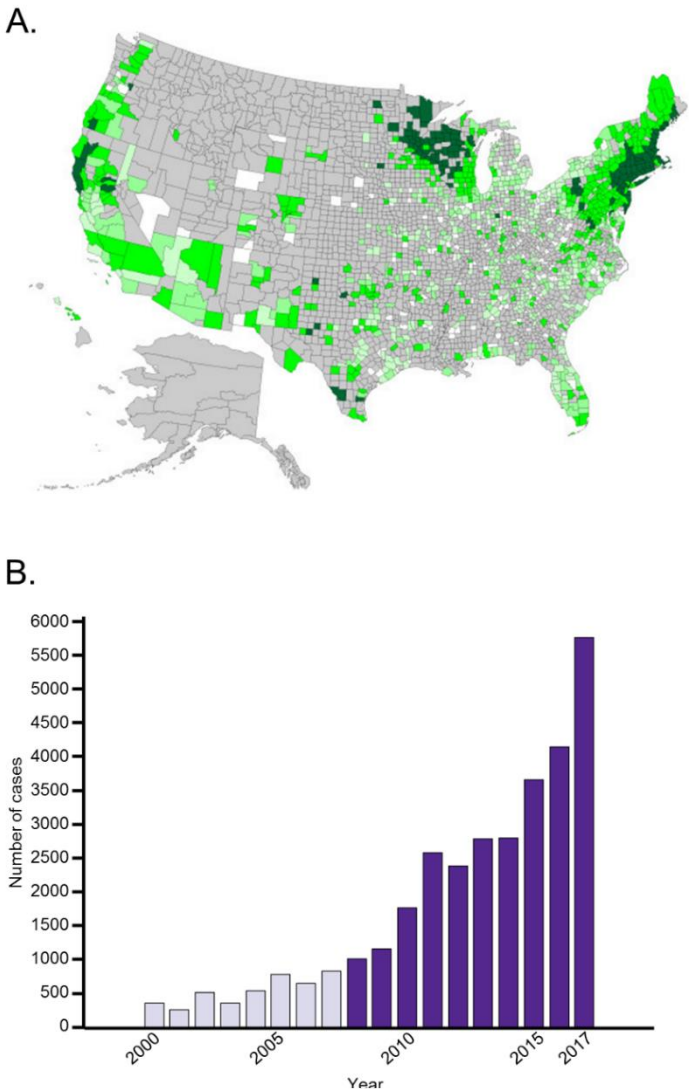


Figure 2: Regional prevalence and annual national incidence of granulocytic anaplasmosis

(A) Canine *A. phagocytophilum* exposure between 2010 and 2012. Counties in grey did not report to the study. Counties with darker shades of green correspond to greater percentages of samples that indicated exposure to *A. phagocytophilum*. White: 0%, Light green: 0.1-0.5%, green: 0.5-1.0%, dark green: 1.1-5.0%, very dark green: >5.0%. (B) The annual number of HGA cases reported to the CDC from 2000-2017. Light purple indicates cases of human granulocytic ehrlichiosis prior to its reclassification as HGA. Dark purple indicates reported HGA cases.

Adapted from: Little SE, Beall MJ, Bowman DD, Chandrashekar R, Stamaris J. Canine infection with *Dirofilaria immitis, Borrelia burgdorferi, Anaplasma spp*., and *Ehrlichia* spp in the United States, 2010-2012. (2014). Parasit Vectors. 7:257.[2]

Adapted from: Anaplasmosis, Epidemiology and Statistics. cdc.gov: Centers for Disease Control and Prevention; 2019.[3]

colony under microscopic evaluation.[58,59] Between 28 and 32 hpi, RCs begin converting back into DC organisms, which then exit the host cell via extrusion or host cell lysis.[14]

Nutritional parasitism

A. phagocytophilum has distinct nutritional requirements that are met by exploitation of host cell functions. *A. phagocytophilum,* like other obligate intracellular pathogens, lacks the ability to produce many necessary metabolites. Specifically, *A. phagocytophilum* is auxotrophic for sixteen amino acids that must be supplied by the host.[60] *A. phagocytophilum* also requires host-derived cholesterol and phospholipids that are incorporated into bacterial membranes.[15,61] Many of these nutrients are brought to the ApV via exploitation of host Rab GTPase-directed vacuole trafficking. Rab GTPases localize to specific organelles and dictate their identity. These proteins cycle from active to inactive states on endosomes to control endosomal maturation. The ApV becomes decorated with numerous Rab GTPases associated with recycling endosomes (Rab4A, Rab10, Rab11A, Rab14, Rab22A and Rab35), the endoplasmic reticulum (ER) (Rab 1 and Rab10) and the *trans*-Golgi network (TGN) (Rab10, Rab 11A, Rab14, and Rab22A). This labeling results in the trafficking of ER and TGN-derived vacuoles to the ApV, supplying the contained bacteria with necessary nutrients.[5,62] In the case of Rab10, the *A. phagocytophilum* surface protein uridine monophosphate kinase has been shown to directly interact with Rab10.[61] The host process of autophagy is also exploited. Autophagy is a host process through which damaged cellular components and intracellular pathogens can be targeted for destruction through encapsulation in an ER-derived membrane followed by lysosomal fusion.[63-65] *A. phagocytophilum* is able to exploit this

process while avoiding destruction. Furthermore, the ApV has been observed to resemble early autophagosomes, possessing a double-lipid bilayer membrane and the early autophagosome markers beclin-1 and LC3. This process of autophagosome formation is mediated by the type 4 secretion system (T4SS), a bacterial secretion system capable of translocating effector molecules directly across both the bacterial and host membranes into the host cytosol.[66-69] The T4SS effector *Anaplasma* translocated substrate-1 (Ats-1) has been shown to induce autophagosome formation during infection, which has been shown to promote bacterial growth by increasing amino acid availability.[70,71] Another required nutrient, cholesterol, is brought to the ApV through bacterial exploitation of the Niemann-Pick protein type C1 (NPC1) and Niemann-Pick protein type C2 (NPC2) to transport low-density lipoprotein-derived cholesterol from lysosomes to the ApV.[72-74] This cholesterol is then incorporated into bacterial membranes.[15]

Subversion of host defenses

A. phagocytophilum resides within host neutrophils, a cell with extreme bactericidal capabilities. Neutrophils are a class of phagocytic cells that serve as a first line of defense during the immune response.[75] As such, *A. phagocytophilum* has evolved numerous mechanisms to subvert bacterial killing.

Initially, toll-like receptors (TLR), lectins, and other neutrophil surface receptors identify pathogens by recognition of pathogen associated molecular patterns (PAMPs). Once identified, a pathogen will be phagocytosed.[75-77] During this process the pathogen will be internalized into a phagosome where the pathogen is exposed to a number of bactericidal

stresses including: lysosomal fusion, vacuolar acidification, reactive oxygen/nitrogen species, cationic peptides, and proteases.[75] *A. phagocytophilum* has not been shown to produce either peptidoglycan or LPS and lacks the biosynthetic genes necessary to produce these PAMPs in a complete form.[15,78] While these cell envelope components are important to cell stability, they are also PAMPs that initiate strong immune responses; therefore their loss is suspected to provide a unique form of molecular camouflage, allowing *A. phagocytophilum* to hide from this system. It is of note however, that this bacterium does stimulate TLR2 signaling though an unknown non-LPS ligand.[79]

Bacterial surface adhesins stimulate receptor-mediated endocytosis, avoiding phagocytosis.[80] In addition to innate avoidance of phagocytosis, *A. phagocytophilum* directly alters host gene expression to produce a more hospitable "host". *A. phagocytophilum* possesses a T4SS that secrets bacterial effectors from the bacterial cytosol through the bacterial and host membranes directly into the host cytosol.[67,81] One T4SS effector, ankyrin repeat-containing protein A (AnkA), becomes phosphorylated by host cytosolic machinery, inducing its translocation into the host cell nucleus.[82,83] In the nucleus, AnkA interacts with histone deacetylase 1, resulting in decreased expression of antimicrobial genes. Transcription of NADPH oxidase components, the enzyme complex responsible for reactive oxygen species production, is specifically altered. The genes encoding components of the NADPH oxidase complex including *CYBB* and *gp91^{phox}* are transcriptionally downregulated during infection.[84-87] Additionally, Rac2, a key regulator of NADPH activation, is transcriptionally downregulated. As such *A. phagocytophilum*

inhibits NADPH oxidase at the level of enzymatic components and regulation of activation.[88]

The bacteria also prevent recognition of the ApV as a pathogen-containing membrane compartment. *A. phagocytophilum* does this through camouflage of the ApV. As described above, the ApV becomes decorated with Rab GTPases associated with recycling endosomes, and autophagosome markers. These markers effectively disguise the ApV as a host compartment preventing its recognition for destruction.[5,61,62,70,71]

Assuming a pathogen cannot be destroyed, neutrophils have a secondary defense, a very brief lifespan. Even in the absence of pathogens, neutrophils have a half-life of 24 h in circulation, making the neutrophil one of the shortest lived cell types in mammals.[89] To prevent this, *A. phagocytophilum* utilizes multiple strategies to delay neutrophil apoptosis and to allow bacterial replication and development.[90-92] One mechanism is the effector Ats-1, which is trafficked to host mitochondria where it inhibits apoptosis by concurrently preventing cytochrome c release and translocation of the pro-apoptotic protein BAX into the mitochondria.[93] Expression of the anti-apoptotic BCL-2 family of mitochondrial proteins are increased.[94,95] This prevents activation of caspase-3 and caspase 9 while maintaining mitochondrial membrane potentials.[91] Additionally, *A. phagocytophilum* inhibits transduction of external pro-apoptotic signals through inhibition of CD95/Fas signal transduction and subsequent caspase-8 activation.[90,95]

In addition to its evasion of the innate host immune response, *A. phagocytophilum* also has the ability to evade host adaptive immune responses that rely on the production of B and T cells, which recognize antigens specific to the invading pathogen. B cells produce secreted antibodies, which, upon binding to a pathogen, label it for destruction by other immune cells. T cells recognize specific antigens in the context of MHC molecules via their surface T cell receptor. This interaction induces the T cell to secrete cytokines to engender a specific immune response or induce apoptosis of a pathogen-infected cell.[96] *A. phagocytophilum* is able to evade the adaptive response via antigenic variation. The 44-kDa *A. phagocytophilum* surface protein P44 is the most abundant protein on the bacterial surface and is highly immunogenic, yet also antigenically variable. The *A. phagocytophilum* genome encodes 113 *p44* paralogs. Of these, only one is expressed at a given time from the promoter-containing expression site. Most P44 genes possess conserved 5' and 3' regions and a central hypervariable region.[60] Through RecF-mediated gene conversion, the P44 isoform in the expression site can be converted to another isoform, altering the hypervariable region.[97,98] In this way the immunodominant *A. phagocytophilum* protein can change in response to humoral immune pressure, allowing the bacterium to evade a protective immune response from efficiently targeting it.[97-102]

1.4 Mechanisms of *A. phagocytophilum* binding and entry

Bacterial determinants of binding and entry

As an obligate intracellular bacterial pathogen, the survival of *A. phagocytophilum* is dependent upon its ability to bind to and invade host cells. Binding and invasion are known to occur at calveolin-1 rich lipid rafts and are mediated by adhesins, bacterial proteins

that promote binding to and invasion of host cells.[57,80] These proteins work cooperatively to facilitate entry and infection of host cells (Figure 3). The most well studied *A. phagocytophilum* adhesins and invasins are: outer membrane protein A (OmpA), *A. phagocytophilum* invasion protein A (AipA), and the 14-kDa *A. phagocytophilum* surface protein (Asp14)[103-107]. It has been shown that these three proteins interact through different mechanisms to cooperatively promote binding to and invasion of host cells.[103-107] As important adhesins, these proteins share some features: their expression is upregulated during tick feeding on mammalian hosts and during the conversion from the replicative RC form to the infectious DC form.[104,106,107] Antibody-mediated obstruction of these surface proteins inhibits binding to and/or invasion of host cells.[104-107] The regions of these proteins that mediate interactions with their cognate receptors, binding domains, has been published and allow refinement of antibody inhibition of these adhesins.[104-107] Antibody targeting of any combination of these proteins inhibits bacterial binding and invasion synergistically.[105] Recent unpublished data, which I contributed to, confirms this phenomenon in a murine model of granulocytic anaplasmosis. We determined that vaccination utilizing a carrier protein conjugated to peptides corresponding to the published binding domains of these three proteins inhibits the establishment of a productive *A. phagocytophilum* infection in mice.

OmpA is an *A. phagocytophilum* surface protein with homologs in many bacterial species across multiple orders. Some of these homologs are reported to promote adhesion; however, specific ligands are variable.[108,109] OmpA functions as an adhesin for *A. phagocytophilum,* wherein OmpA inhibition reduces binding of *A. phagocytophilum* to

host cells. OmpA interacts with α2,3-linked sialic acid and α1,3-linked fucose residues that comprise sialyl Lewis x (sLe^x) and other glycans that cap host selectin ligands.[107] The OmpA amino acid residue lysine 64 is critical to sLe^x binding. Glycine 61 is also important to sLe^x binding, likely through increasing the flexibility of the region.[105,107] In the case of neutrophils, OmpA binds to the sLe^x cap found on the surface protein P-type selectin glycoprotein ligand-1 (PSGL-1).[107] Infection of endothelial cells, which lack sLe^x and PSGL-1, still relies on OmpA in a manner dependent upon the same fucose and sialic acid residues.[107,110]

AipA is a bacterial surface protein that mediates invasion of host cells.[105,106] AipA is an integral outer membrane protein that, when inhibited, decreases the ability of *A. phagocytophilum* to infect host cells.[106] While the mechanism of AipA-mediated internalization is not yet known, it is known that the AipA residues 9-21 are critical for infection of host cells.[105] While the host receptor of AipA has not been identified, recent unpublished data has implicated the host protein CD13 as a putative AipA-interacting partner.

Asp14 is another *A. phagocytophilum* invasin that plays a dramatic role during host cell invasion.[104,105] Asp14 is a small outer membrane protein that is transcriptionally upregulated during host cell invasion and when the *A. phagocytophilum* bacterium engages with PSGL-1.[104] Antibody inhibition of Asp14 and competition assays indicate that Asp14 functions during the internalization process, but not during bacterial adhesion to host cells.[104] While the interacting partner of Asp14 was previously undetermined, it

was known that the interaction with host cells is mediated by the Asp14 amino acids 101-124, and that residues 113-124 are critical to bacterial invasion of host cells.[104,105] It is likely that, while the true interacting domain of Asp14 is found within residues 113-124, the residues 101-112, which have a net charge of +4.91, may play a role in overcoming the charge repulsion between *A. phagocytophilum* and its host, improving the efficiency of Asp14 interaction with its receptor.[104]

Additional *A. phagocytophilum* surface proteins have been implicated as potential adhesins and invasins. APH_1235 is an *A. phagocytophilum* surface protein that is transcriptionally upregulated in the DC organism, during the RC-to-DC conversion, and during tick transmission feeding. Antibody blocking of APH_1235 on bacterial surfaces has been shown to reduce infection.[111,112] Asp55 and Asp62 are both surface proteins that have been shown to similarly inhibit infection of host cells when obstructed by antibodies.[113]

Host determinants of binding and internalization

In addition to the previously mentioned proteins, there are host-cell-binding determinants to consider. The most important host cell determinant of *A. phagocytophilum* infection is PSGL-1.[14] Interaction between an *A. phagocytophilum* invasin and the N-terminal region of PSGL-1 results in the transduction of a signal into the host cell that then induces *A. phagocytophilum* internalization.[114] Furthermore, monoclonal antibody targeting of this N-terminal region nearly ablates *A. phagocytophilum* binding to host cells. This interaction is distinct from the interaction between OmpA and sLe^x and is thought to be mediated by

the *A. phagocytophilum* outer membrane protein major surface protein 2 (MSP2/P44).[115] This is because recombinant P44 binds to myeloid cells and competitively inhibits bacterial binding to host cells and monoclonal antibody binding to the N-terminus region of PSGL-1.[115] Antibody targeting of the N-terminal conserved region of P44 has been shown to block bacterial binding and infection of host cells, while targeting of the hypervariable region inhibited bacterial replication, but not binding.[116] These indicate that P44 may play a role in bacterial adherence and invasion and that it may engage PSGL-1. Bacterial engagement of PSGL-1 by *A. phagocytophilum* transduces a signal into the host cell resulting in the non-receptor tyrosine kinase (Syk)-mediated phosphorylation of effector kinase of RhoA GTPase (ROCK1).[114] ROCK1 phosphorylation plays a direct role in the internalization of *A. phagocytophilum.*[114] Under shear stress conditions, similar to that seen in circulating neutrophils, other host determinants of binding have been identified. Under these conditions glycophosphatidylinositol (GPI) anchored proteins has been shown to contribute to binding and internalization. CD18 have also been implicated as a receptor under shear stress conditions. How these proteins contribute to bacterial adherence and invasion is not known.[57,117] However, recent unpublished data from our lab indicate that CD18 may interact with APH_1235.

1.5 Models for studying *A. phagocytophilum*

A number of models are used to study *A. phagocytophilum* infection. Human neutrophils are primary cells that *A. phagocytophilum* infects *in vivo*. While these cells are used when possible, their short life span necessitates the use of immortalized cell lines for long term culture. The cell line used to study *A. phagocytophilum* binding and invasion is the HL-60

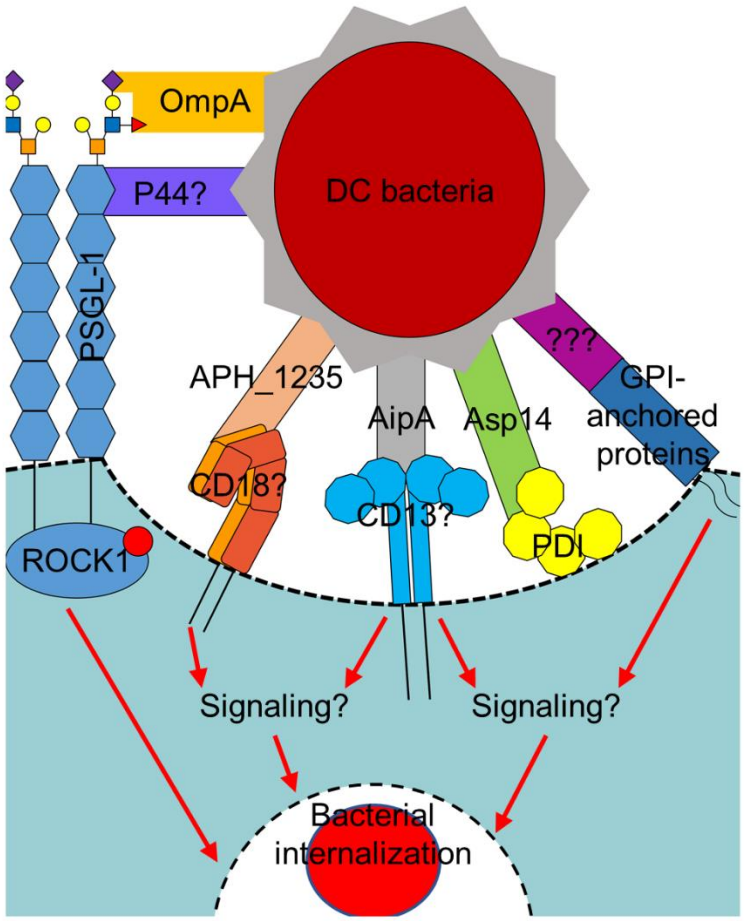


Figure 3: Mechanisms of *A. phagocytophilum* cellular invasion

An illustration of the known and implicated adhesins that mediate *A. phagocytophilum* binding to and internalization by host cells. Host PSGL-1 and its sLe^x cap are bound by bacterial P44 (potentially) and OmpA, respectively. This activates ROCK1 signaling, by way of Syk, to induce bacterial internalization. APH_1235 has been implicated as an adhesin. Recent unpublished work has identified CD18 as its potential receptor. GPI-anchored proteins are engaged by unknown bacterial adhesins (labeled as: ???). Engagement of Asp14 with PDI contributes to internalization through a mechanism described in this work. Recent unpublished work has implicated CD13 as a potential interacting partner of the adhesin AipA. Engagement of CD18, CD13 and GPI anchored proteins may transduce signals into host cells that mediate internalization.

Adapted from: Truchan HK, Seidman D, Carlyon JA. Breaking In and Grabbing A Meal: Anaplasma phagocytophilum Cellular Invasion, Nutrient Acquisition, and Promising Tools for Their Study. Microbes and infection / Institut Pasteur. 2013;15:1017-1025.[5]

cell line, an immortalized promyelocytic leukemia cell line that expresses a complement of known *A. phagocytophilum* receptors similar to that of human neutrophils.[32,118,119] HEK-293T cells are amenable to both *A. phagocytophilum* infection and transfection, unlike HL-60 cells. Thus HEK-293T cells are utilized during all experiments requiring plasmid or siRNA transfection.[120] For *in vivo* testing of *A. phagocytophilum* infection a murine model was established in 1996. Initially C3H/HeJ and C3H/HeN background mice were utilized for the study of *A. phagocytophilum* infection.[121] Since that time the C57Bl/6J inbred mouse strain has become the most common background for the study of *A. phagocytophilum*, as this strain is susceptible to bacterial infection and reproduces certain histopathologic lesions associated with HGA.[51,121-123] Furthermore, I was involved in recently published work further specifying the usefulness of this mouse strain. We found that female mice are less susceptible to *A. phagocytophilum* infection than male mice, indicating that murine sex is a biological variable that should be considered when investigating this bacterium.[51] Conveniently, this strain is also a common background used for the development of transgenic mouse lines. Numerous transgenic mouse lines have been used for the study of *A. phagocytophilum in vivo.*[74,122,124,125] I was involved in one such published work utilizing acid sphingomyelinase knockout (KO) mice to study the relevance of that enzyme during *A. phagocytophilum* infection.[74] Recently a line of transgenic mice has been developed wherein genes may be conditionally knocked out (CKO) only within myeloid cells, specifically lysozyme-expressing monocytes and neutrophils[126,127].[128] In these mice, the *Cre* recombinase gene has been put under the control of the *LysM* promoter, allowing expression of Cre recombinase only in myeloid

cells. With this background, *loxP* sites may flank any gene of interest. *loxP* sites are DNA motifs recognized by Cre recombinase, which will then excise the intervening genomic space, producing a CKO of that gene only in Cre-expressing cells.[126,129] In this way any gene of interest may now be studied during *in vivo A. phagocytophilum* infection, even genes once considered to be lethal KOs.

1.6 Protein disulfide isomerase background

Biological sulfur chemistry

Reduction and oxidation (redox) reactions describe the movement of electrons that occur in many biological reactions from energy production, anabolism, metal utilization, and oxidative stress among others. One of the most common redox reactions concerns the formation of disulfide bonds between cysteines (Cys). These bonds are formed when the thiol groups of two Cys residues become oxidized, forming a covalent sulfur-sulfur bond between the two residues. This process is common in secreted proteins where intra- and intermolecular disulfide bonds improve protein stability. Intermolecular disulfide bonds also maintain multimeric protein complexes.[130] Conversely, the reduction of disulfide bonds to individual thiol groups often serves a role in molecular signaling events.[130-132] The formation and dissolution of these bonds is catalyzed by disulfide oxidoreductase enzymes.[4,133-135]

There are numerous disulfide oxidoreductases that function to interconvert free thiol groups and disulfide bonds. These proteins catalyze this chemical reaction using a “CxxC” active site motif, wherein the two Cys residues may exist in reduced state or as

an oxidized disulfide bond.[4,135,136] The intervening "x" residues can represent any amino acid, and determine the active sites redox potential, a value that represents the tendency of the active site to reduce or oxidize a substrate. Depending upon the redox state of the active site Cys, when a substrate protein comes into contact with the free sulfhydryl groups of the active site, nucleophilic attack of substrate disulfide bonds by an active site Cys sulfhydryl group results in the formation of a mixed disulfide, a disulfide bond between an enzyme Cys and a substrate Cys. This mixed disulfide is resolved by nucleophilic attack by the other oxidoreductase active site Cys. Through this process, the substrate disulfide bond is reduced to free Cys and a disulfide bond is formed within the enzyme active site. This process is reversible allowing the enzyme to form disulfide bonds within a substrate (Figure 4A).[4] While there are many oxidoreductases, they differ in their redox potentials and their localization. The most thoroughly studied oxidoreductase is protein disulfide isomerase (PDI).[4]

PDI is a multifunctional protein common to all eukaryotic cells. Within cells it is found ubiquitously in the cytosol, ER lumen, and cell surface. Depending upon its environment, PDI catalyzes the oxidation, reduction and isomerization of disulfide bonds.[4] PDI is a member of the thioredoxin superfamily of redox proteins and contains 4 thioredoxin like domains, labeled αββ'α'. These domains serve unique functions. The α and α' domains each contain an active site: N-WCGHCK-C. The active site Cys residues catalyze disulfide oxidation and reduction, while the intervening amino acids determine the reduction potential of the reaction.[4,135,137] The reduction potential of PDI is -162.7 mV, which is less conducive to disulfide reduction than thioredoxin, which has a reduction

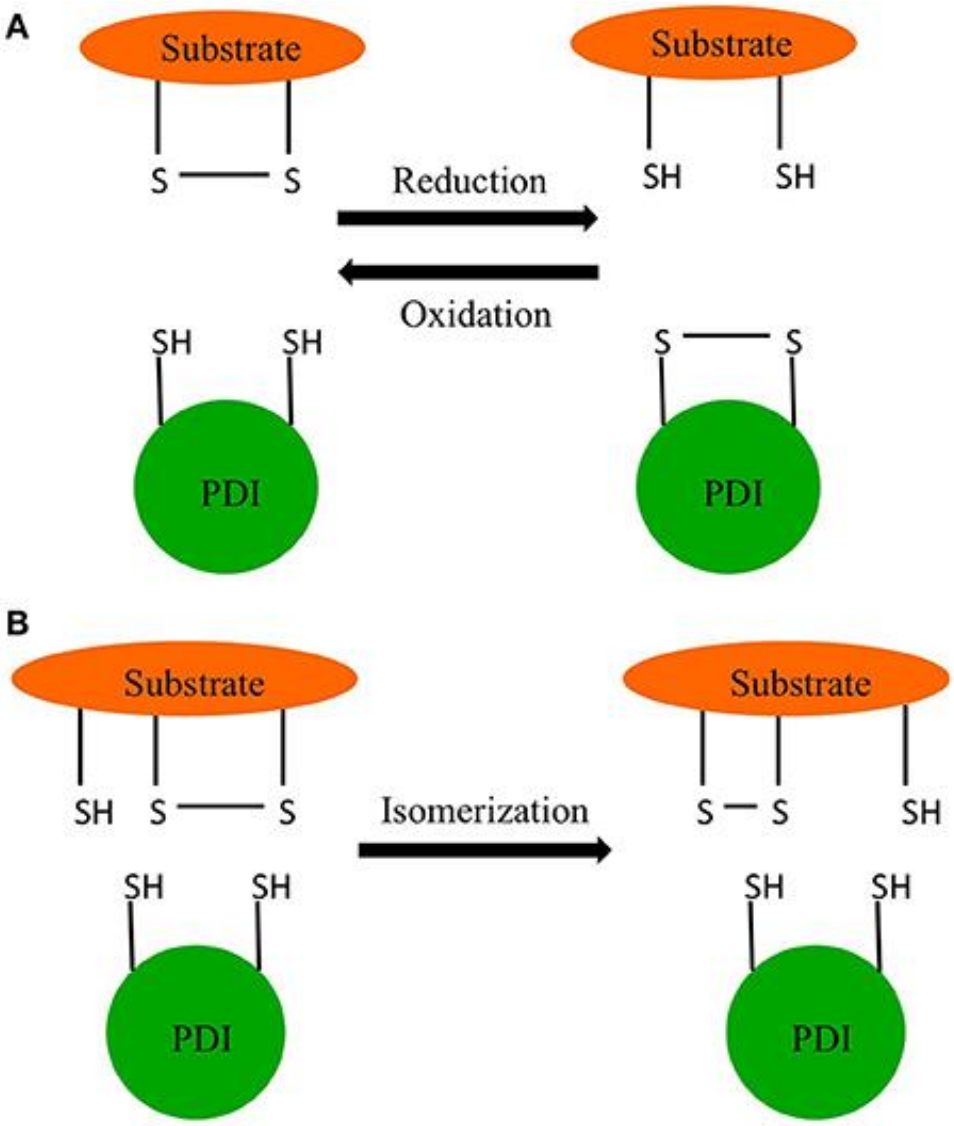


Figure 4: PDI reduces, oxidizes, isomerizes disulfide bonds

(A) PDI, by virtue of the Cys within its active sites catalyzes the reversible reduction and oxidation of disulfide bonds. (B) Through multiple rounds of oxidation and reduction PDI is able to isomerize disulfide bonds within a substrate.

Adapted from: Ali Khan H, Mutus B. Protein disulfide isomerase a multifunctional protein with multiple physiological roles. Front Chem. 2014;2:70[4]

potential of -270 mV.[138,139] The β' domain contains a large multivalent hydrophobic surface that functions as a binding site. An inter-region linker between the β' and α' domains provides flexibility to bring the α' active site into proximity of the β' ligand binding site.[135,136]

PDI function in the ER

The canonical subcellular localization of PDI is within the ER lumen. At this site PDI functions as a chaperone protein. Newly synthesized unfolded and misfolded proteins interact with the β' subunit binding site. This is mediated by the interaction between the β' subunit hydrophobic binding pocket and surface-exposed hydrophobic regions common to unfolded and misfolded proteins.[135,136] If PDI is in an oxidized state it will catalyze the oxidation of the substrate protein, forming disulfide bonds within the protein. If the PDI active site is reduced, it will reduce any disulfide bonds present on the substrate protein. It is through numerous cycles of oxidation and reduction of a substrate that PDI eventually isomerizes disulfide bonds between different substrate Cys until the substrate achieves the native conformation and no longer interacts with the PDI binding site (Figure 4B).[133,134] Once PDI has accomplished its task of producing the necessary disulfide bonds in proteins to be secreted, its active site will remain in a reduced conformation. In order to convert PDI back to an oxidized state, another oxidoreductase, Ero1, is required. Ero1 contains a "CxxC" active site, which when reduced, transfers electrons to O_2 producing H_2O_2 within the ER lumen. Once oxidized, Ero1 oxidizes the PDI α' active site. The oxidized α' active site then oxidizes the α active site, resetting PDI for another round of oxidation and isomerization.[133]

PDI function at the host cell surface

It has become increasingly apparent that PDI serves an important role outside of the ER, especially at eukaryotic surfaces. PDI possesses a canonical C-terminal KDEL motif, which serves to label the protein to be returned to the ER by KDEL receptors within Golgi and ER membranes. In the case of most proteins, a KDEL retention signal prevents secretion of proteins.[140] How PDI is translocated to the host cell surface is not well understood. It is known that on the surface of endothelial cells, PDI is secreted in part through classical Golgi body-mediated secretion. In fact recent studies have shown that surprisingly PDI cell surface association and possibly secretion is dependent upon interaction with its KDEL motif and KDEL receptor 1.[141,142] Regardless of the mechanisms of PDI secretion, extracellular PDI levels are highly regulated [141]. PDI at eukaryotic cell surfaces is known to exclusively function as a reductase, reducing disulfide bonds to free Cys. PDI has been found to be important in integrin function on leukocyte surfaces. On thrombocytes it is known that PDI-mediated reduction converts $\alpha IIb\beta_3$ and $\alpha_2\beta_1$ integrins into an active conformation that allows binding to fibrinogen and collagen-coated surfaces, promoting coagulation. β_3 integrins on the surface of endothelial cells are also regulated in this way.[143-145] Recently, the platelet adhesion mediating protein platelet glycoprotein Ibα has been identified as a substrate of surface PDI. PDI reduces a disulfide bond within this protein, promoting cellular adhesion to neutrophils.[146] On activated neutrophils it has been found that a greater proportion of surface PDI is in a reduced state and that αM integrin affinity for PDI is increased.[128] Furthermore, PDI-mediated disulfide reduction of the $\alpha_M\beta_2$ integrin promotes adhesion to ICAM-1 and fibrinogen, which promotes neutrophil rolling and eventual diapedesis during infection events.[128,145]

Disulfide bonds as a molecular switch

The importance of disulfide bonds to protein stability has long been known. Recently it has become apparent that disulfide bonds also have to potential to control the function of secreted and transmembrane proteins.[132] The secreted protein thrombospondin-1 is known to mediate communication between cells to alter adhesion. Differential arrangement of the disulfide bonds within thrombospondin-1 results in differences in cellular adhesion.[147] Furthermore, multiple conformations of thrombospondin-1 have been confirmed *in vivo.*[148] It has also been reported that activated thrombospondin-1 reduces disulfide bonds in von Willebrand factor, as a regulatory step of thrombus formation.[149,150] This process has also been shown to be important in angiogenesis. Plasmin is a serum proteinase that is important during the clearance of thrombi. Upon cleavage of its disulfide bonds, plasmin undergoes proteolysis producing angiostatin, an inhibitor of angiogenesis.[151,152] As referenced above, PDI is also known to reduce structural disulfide bonds within integrins, resulting in a conformational change from an inactive to active conformation.[143-146]

1.7 Exploitation of PDI by intracellular pathogens

Intracellular pathogens include a number of bacteria, protists, and all viruses. This group represents a significant source of global morbidity and mortality. All obligate intracellular pathogens share the feature that they must all be internalized by a host cell in order to survive and replicate. Many of these pathogens have exhibited convergent evolution, wherein they use similar internalization strategies. One such conserved strategy is the use of disulfide bonds and host oxidoreductases during the internalization process.[4,153]

Human immunodeficiency virus (HIV)

The most studied example of a pathogen that exploits PDI is human immunodeficiency virus (HIV).[153-155] HIV induces entry through interaction with CD4 followed by interaction with the co-receptor CXCR4/CCR5 on the surface of macrophages and T cells.[154] Additionally, PDI has been determined to play a critical role in this process. The HIV 120 kDa glycoprotein (gp120) envelope protein initially interacts with CD4 on the host cell surface. Thioredoxin-1 (Trx), another disulfide oxidoreductase, which normally interacts with CD4, is exploited to reduce CD4 inter- and intramolecular disulfide bonds. Reduction of intermolecular disulfide bonds within the CD4 dimer, converts CD4 from a dimer to an oxidized monomer. Further reduction of intramolecular disulfide bonds within CD4 monomers produces a reduced monomer, which has a higher affinity for gp120 than either the dimer or oxidized monomer.[156,157] After this point, disulfide bonds within gp120 are reduced by PDI, or another surface oxidoreductase, resulting in a conformational change that induces binding to the gp120 co-receptor (CXCR4/CCR5).[158] It is thought that reduction-driven conformational changes in gp120 and its interaction with the co-receptor (CXCR4/CCR5) induces conformational changes in gp41, the HIV fusion protein associated with gp120. This exposes a hydrophobic fusion peptide that inserts into the host membrane, driving fusion of the HIV envelope with the host plasma membrane.[159,160] More recently it has been noted that other surface oxidoreductases: Trx and glutaredoxin-1, can function in the place of PDI and induce fusion more efficiently than PDI, due to their stronger reduction potential.[161,162] To date no HIV protein has been shown to bind directly to PDI.

Newcastle disease virus

Newcastle disease virus enters host cells in a similar manner to HIV. Hemagglutinin-neuraminidase on the viral envelope brings the virus proximal to surface oxidoreductases, including PDI.[163] The viral fusion glycoprotein becomes reduced, inducing a conformational change that drives fusion of the viral envelope with the host plasma membrane.[164,165]

Dengue virus

Dengue virus has also been implicated in exploiting PDI during its internalization. While the process of Dengue virus internalization is not understood, it is known that lipid raft-localized PDI-mediated disulfide reduction of β1 and β3 integrins enhances viral entry.[166,167] It is suggested that PDI-mediated reduction of β1 and β3 integrins increases their affinity for their natural ligands, which is thought to increase the affinity of β1 and β3 integrins for a viral ligand. This interaction may promote viral internalization.[167]

Chlamydia spp.

Chlamydiaceae is a family of medically important intracellular bacteria. To date very little is known about how they promote their internalization. *C. trachomatis* is the most well-known of the pathogenic *Chlamydiaceae*. No host receptor has been identified across all strains, except for PDI.[168-171] It has been determined that the presence of PDI is important for Chlamydial binding to the host cell surface; however, the bacteria do not bind directly to PDI and disulfide reduction is not important for binding.[169] However, PDI-mediated internalization requires disulfide reductase activity.[169-171] A bacterial effector may bind to

a PDI-containing multiprotein complex, such as the estrogen receptor, collagen prolyl-4-hydroxylase, or the microsomal triglyceride transfer protein, amongst others [171]. While the target protein of PDI is unknown, it is thought that PDI may reduce a host protein, such as an integrin or L-selectin, to transduce a signal into the host cell and trigger internalization. Another hypothesis is that PDI may be acting upon a bacterial protein to induce internalization. *Chlamydia spp.* lack a classical peptidoglycan cell wall and instead possess a periplasmic P-layer composed of heavily disulfide bonded Cys rich proteins that may require reduction to induce internalization and infection.[169,172,173] Alternatively, *Chlamydia* possess a Type 3 secretion system (T3SS), its needle protein, CdsF, is one of the few such proteins to contain Cys residues, suggesting that T3SS activity may be activated by PDI via targeting of CdsF.[169]

Other Pathogens

Other pathogens have been reported to rely on PDI or disulfide reduction during their invasion processes. Surface expression of PDI improves internalization of the parasite *Leishmania chagasi*.[174] *Toxoplasma gondii* expresses a surface-exposed PDI homolog that plays a role during pathogen internalization.[175] Vaccines targeting this protein have been shown to be protective against *T. gondii* infection of mice.[176] Murine leukemia virus contains its own “CxxC” catalytic motif and is thought to isomerize its own surface disulfide bonds to mediate internalization.[177]

1.8 Research objective

Obligate intracellular pathogens rely on their ability to bind to and invade host cells in order to survive and replicate. Therapeutic targeting of shared internalization processes could eliminate infection not only by *A. phagocytophilum*, but also by numerous intracellular pathogens. In spite of the therapeutic promise of these determinants, there is currently no vaccine for HGA.[166,169,170] Herein I investigate the mechanism through which the *A. phagocytophilum* adhesin, Asp14, mediates bacterial internalization and infection. I show that Asp14 interacts with the host protein PDI. Furthermore, this interaction results in the reduction of disulfide bonds of an *A. phagocytophilum* surface protein, allowing efficient infection of host cells. Importantly the requirement for PDI is maintained *in vivo,* as confirmed through human neutrophil and murine infection experiments. This marks the first *in vivo* example of PDI exploitation by a pathogen. In addition to determining the relevance of PDI towards *A. phagocytophilum* infection, I have also determined the specific residues of Asp14 that mediate its interaction with PDI. This represents the first instance of a bacterial protein directing PDI-mediated reduction of another protein and the first identification of microbial amino acid residues that are essential to interacting with PDI. In this work I have established a research strategy wherein PDI utilization by pathogens can be dissected in detail. This could lead to the identification of PDI-interacting determinants that, unlike PDI itself, could prove suitable therapeutic targets for the treatment or prevention of diseases caused by the pathogens that exploit similar invasion strategies.

Chapter 2: Materials and methods

Cultivation of uninfected and *A. phagocytophilum*-infected cell lines

Uninfected and *A. phagocytophilum* (NCH-1 strain)-infected HL-60 human promyelocytic cells (CCL-240; American Type Culture Collection (ATCC, Manassas, VA)) were cultured in Iscove's Modified Dulbecco's Media (IMDM) as previously described.[178] Human embryonic kidney HEK-293T cells (CRL-3216, ATCC) were cultured as previously described.[179] Primary human *ex vivo* neutrophils were isolated and cultured as previously described.[180] All mammalian cells were cultured at 37°C and 5.0% CO_2. *Ixodes scapularis* embryonic ISE6 cells were cultured as described previously.[180] Cell health and survival was determined by trypan blue exclusion assay, which was performed as per manufacturer's instructions and as previously described.[88,181,182]

Reagents and antibodies

Antisera specific to the OmpA, Asp14, and AipA binding domains were generated as previously described.[105] The PSGL-1 N-terminus-specific antibody KPL-1 (BD Biosciences, San Jose, CA) and the PDI specific antibodies BD34 (BD Biosciences), RL90 (ThermoFisher scientific, Rockford, IL) and rabbit polyclonal PDI-targeting antisera (MilliporeSigma, Darmstadt, Germany) were obtained commercially. HRP and Alexa Fluor 488-conjugated secondary antibodies, GFP-targeting antibodies and Flag-targeting antibodies were purchased from Invitrogen (Carlsbad, CA). HRP-conjugated secondary antibodies were procured from Cell Signaling Technology (Danvers, MA). β-actin and GAPDH-targeting antibodies were procured from Santa Cruz biotechnology (Dallas, TX). The bacitracin and TCEP were procured from Alfa Aesar (Ward Hill, MA) and ThermoFisher scientific respectively. Lipofectamine 2000, N-(3-maleimidopropionyl)

biocytin (MPB), streptavidin-conjugated beads, and isopropyl ß-D-1-thiogalactopyranoside (IPTG) were also obtained from ThermoFisher scientific. DharmaFECT transfection reagent was procured from Dharmacon (Lafayette, CO). Protein A/G agarose resin and M2 FLAG affinity resin were procured from ThermoFisher scientific and MilliporeSigma, respectively. Fluorescent sulfate-modified 1.0 µm microfluorospheres were procured from ThermoFisher scientific. Iodoacetimide and glutathione were purchased from MilliporeSigma.

Plasmid Constructs

Mammalian codon-optimized *asp14* was synthesized by Biomatik (Wilmington, DE) and mammalian codon-optimized *ech_0377,* $asp14_{1\text{-}112}$*:ech_0377*$_{95\text{-}104}$*,* $ech\_0377_{1\text{-}94}$*:asp14*$_{113\text{-}124}$ by Genescript (Piscataway, NJ). Alanine substitution mutants of *asp14:* $asp14_{G117A}$*,* $asp14_{A118L}$*,* $asp14_{N119A}$*,* $asp14_{T120A}$*,* and $asp14_{P121A}$ were produced by Genescript. *asp14* alanine substitution mutants $asp14_{Y116A}$*,* $asp14_{K122A}$*,* $asp14_{E123A}$*,* and $asp14_{S124A}$ were produced by PCR amplification. All of these sequences were inserted into the pEGFP-C1 (Clontech, Palo Alto, CA) plasmid by restriction digestion as previously described [183]. *PDI* was PCR amplified from Plasmid ID: ccsbBroadEn_01138 (DNASU plasmid repository, Tempe, Az) and inserted, by restriction digestion into pCMV 3xFLAG 7.1 as described previously [183]. *asp14* bearing a 6x histidine tag (His-tag) was produced by PCR amplification and ligation independent cloning into the pET46 Ek/LIC vector (MilliporeSigma), as described previously.[105] His-*PDI* and His-*dmPDI* ($PDI_{C55S,C58S,C399S,C402S}$), a gene encoding an enzymatically defective form of PDI, wherein all active site Cys were converted to serines, were kindly provided by Dr. Jaehyung Cho

Table 1. Oligonucleotides used for PCR and qPCR

Primer Name	Sequence (5'->3')
*Ap16S*_F	TGTAGGCGGTTCGGTAAGTTAAAG
*Ap16S*_R	GCACTCATCGTTTACAGCGTG
*asp14*_3LIC_F	ACGACGACAAGATGATACCATTAGCTCCTTGGAAGAG
*asp14*_372LIC_R	GAGGAGAAGCCCGGTTAGCTTTCTTTAGGAGTATTGGCACCGTAA
*asp14*_4EcoRI_F	ATCGGAATTCATACCATTAGCTCCTTGGAAGAGCATTTCGGTGGTGTA
*asp14*_372SalI_R	CGATGTCGACTTAGCTTTCTTTAGGAGTATTGGCACCGTAAACAGCCCT
*asp14*_Y116ASalI_R	CGATGTCGACTTAGCTTTCTTTAGGAGTATTGGCACCCGCAACAGCCCT
*asp14*_K122ASalI_R	CGATGTCGACTTAGCTTTCCGCAGGAGTATTGGCACCGTAAACAGCCCT
*asp14*_E123ASalI_R	CGATGTCGACTTAGCTCGCTTTAGGAGTATTGGCACCGTAAACAGCCCT
*asp14*_S124ASalI_R	CGATGTCGACTTACGCTTCTTTAGGAGTATTGGCACCGTAAACAGCCCT
*β-actin*_F	AGAGGGAAATCGTGCGTGAC
*β-actin*_R	CAATAGTGATGACCTGGCCGT
*Lys-Cre*_genotypeF	CCCAGAAATGCCAGATTACG
*Lys-Cre*_genotypeR	TT ACAGTCGGCCAGGCTGAC
*PDI*_4EcoRIF	ATCGGAATTCACTGCGCCGCGCTCTGCTG
*PDI*_1524SalIR	ATCGGTCGACCAGTTCATC TTTCACAGCTTTCTG
*PDI*_genotypeF	GGCTGAGTTATCTGGTGATTGACCAATG
*PDI*_genotypeR	TCCA GGCTCCACAAAAATTCCTTTAGC

(Department of Pharmacology and Anesthesiology, University of Illinois College of Medicine, Chicago, IL).[128,144,146]

Recombinant protein production

Recombinant 6xHis tagged protein expression was induced in *Escherichia coli* by the addition of IPTG as previously described.[184] His-wtPDI, His-Asp14 and His-dmPDI were isolated and purified by immobilized metal-affinity chromatography as previously described.[128,144,146,184,185,186]

Neutrophil isolation

Peripheral blood neutrophils were isolated from healthy human donors. Density gradient centrifugation was used to isolate human neutrophils from other cell types. Polymorph Prep (Axis-Shield, Frickenhausen, Germany) was applied over an equal volume of blood and centrifuged at 470 x *g* for 30 min at room temperature. The resulting band of neutrophils was recovered and mixed with an equal volume of 0.45% NaCl and RPMI 1640 with 0.5 mM ethylenediaminetetraacetic acid (EDTA, MilliporeSigma). Cells were isolated by centrifugation at 210 x *g* for 10 min, followed by resuspension in Red Blood Cell Lysis Buffer (ThermoFisher Scientific) to lyse erythrocytes. Cells were washed twice in RPMI 1640 with 0.5 mM EDTA, before a final resuspension in RPMI 1640. The protocol (HM11407) for obtaining donor blood for the isolation of neutrophils was reviewed and approved by the Virginia Commonwealth University Institutional Review Board with respect to scientific content and in compliance with applicable research and human subject regulations.

Yeast two-hybrid

ULTImate yeast two-hybrid analysis was performed by Hybrigenics Services (Paris, France) (http://www.hybrigenics-services.com) as previously described[187]**.** Yeast two-hybrid analysis screened the human codon-optimized *A. phagocytophilum* strain NCH-1 *asp14* (bait) against a human activated leukocyte cDNA library (prey).

Mammalian cell transfection, co-immunoprecipitation and Western blotting

HEK-293T cells were grown in six well plates until 80% confluency. These cells were co-transfected with a GFP-tagged protein and Flag-PDI via lipofectamine 2000 as per manufacturer's recommendation and as previously described.[187] Cells were harvested 24 h post transfection and lysed in immunoprecipitation lysis buffer (20 mM Tris base, 500 mM NaCl, and 0.7% Tween-20). FLAG-tagged proteins were immunoprecipitated and analyzed as described previously[187]. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and screened with antibodies to detect GFP (1:1000) and FLAG (1:1000). Unaltered lysates were used to confirm ectopic protein expression and equal loading using beta actin (1:2500) or GAPDH (1:750) as a loading control.

Co-immunoprecipitations (Co-IPs) were performed on the protein samples, wherein 100 µg of protein in 300 µl of lysis buffer were aliquoted for each sample and rotated for 4 h with 30 µl protein A/G resin at 4°C. The beads were pelleted by centrifugation at 300 x *g* for 1 min at 4°C. The resulting supernatant was mixed with 30 µl of anti-Flag resin and rotated overnight at 4°C. The resulting slurry was pelleted by centrifugation at 300 x *g* for 1 min at 4°C and resuspended in 1 ml of lysis buffer. Each pellet was washed 6 more

times and resuspended in 30 µl of Laemmli loading buffer with beta-mercaptoethanol. SDS-PAGE and Western blot analysis were performed on co-immunoprecipitation eluates and 30 µg of transfected cell lysate samples as previously described.[187]

Synchronous *A. phagocytophilum* infection assays

Competitive inhibition assays utilizing antibody were performed and analyzed by spinning-disk confocal microscopy as previously described[103,107,182]. To examine the contribution of PDI during infection, host cells were treated with bacitracin (3 mM) or a vehicle control (IMDM-10 media). Host cells were treated with 10 µg/ml BD34, polyclonal PDI antibodies, or isotype control antibody during antibody inhibition. For competitive inhibition assays using antisera raised against bacterial adhesin binding domain peptides, *A*. *phagocytophilum* DC bacteria were incubated with 100 µg/mL of the respective antiserum [105]. Preimmune rabbit serum (100 µg/ml) served as a negative control.

During TCEP rescue experiments the previous procedure was followed, wherein PDI was inhibited and cells were infected. At that point IMDM-10 with or without 0.01 mM TCEP was applied to the HL-60:*A. phagocytophilum* binding reaction following the removal of unbound bacteria. TCEP and vehicle-treated cells were incubated at 37°C for 30 min. Cells were washed twice with 1x PBS to remove TCEP. Recombinant PDI treatment was also used to rescue PDI inhibition. Enzymatically active or inactive PDI 40 µg/ml was applied to *A. phagocytophilum* bound to host cells at 37°C for 30 min. Cells were then washed twice as before to remove PDI. To determine the surface containing the target of

A. phagocytophilum-directed disulfide reduction, isolated DC bacteria or host cells treated with BD34 or an isotype control were treated with TCEP or a vehicle control for 30 min at 37°C. These cells were then washed twice with 1x PBS and host cells were infected with *A. phagocytophilum*.

Binding and uptake of Asp14-conjugated beads

Asp14-conjugated bead experiments were performed as previously described.[105] In brief, HL-60 host cells were exposed to Asp14-conjugated fluorescent carboxylate beads for 1 h with frequent agitation at 37°C. Following this incubation samples were washed thrice with 1X PBS. Samples were then taken for binding analysis via immunofluorescence. The remainder was incubated for 7 h at 37°C and 5% CO_2, after which samples were taken for immunofluorescence microscopy.

Immunofluorescence microscopy

Immunofluorescence assays were performed and analyzed by spinning disk confocal microscopy[103]. HL-60 cells adhered to glass microscope slides by cytospin (Shandon, ThermoFisher Scientific) at a concentration of ~30,000 cells per slide were fixed and permeablized using Quik-Diff fixative (ThermoFisher Scientific). HEK-293T cells were grown on 12-mm glass coverslips prior to infection. These cells were fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) for 20 min and permeablized with 0.5% Triton X-100 for 15 min. Cells were labeled for the presence of *A. phagocytophilum* as previously described[103,182].

siRNA knockdown

HEK-293T cells were grown to 80% confluency on 12-mm glass coverslips. ON-TARGETplus human *P4HB* (PDI mRNA) or non-targeting control siRNA were prepared with transfection reagent as previously described[120]. Cells were transfected as above 24 h after the initial transfection. At 72 h post transfection coverslips were taken for synchronous infection experiments and protein samples were taken for analysis via SDS-PAGE and Western blot analysis to confirm successful PDI knock-down.[112]

Quantitative PCR

Bacterial load was determined by qPCR analysis. DNA was isolated from infected culture cells and murine blood with the DNeasy Blood and Tissue Kit (Qiagen, Boston, MA). Bacterial load was determined as previously described by PCR amplification of *A. phagocytophilum 16S rDNA* and host *β-actin* with SsoFast EvaGreen Supermix (BioRad, Hercules, CA)[107]. Bacterial load was normalized on the basis of *16S rDNA* normalization to *β-actin* via the $2^{-\Delta\Delta CT}$ (Livak) method[188].

Determination of the target of PDI activity

The thiol-labeling biotin derivative was utilized to identify putative PDI substrates. DC bacteria, isolated as above, were exposed to 40 µg/mL wtPDI or dmPDI in 1x PBS. These reactions were incubated at 37°C for 30 min. MPB was added to this reaction to a concentration of 100 µM for 1 h, after which, the reaction was quenched with 0.6 mM glutathione. Following glutathione quenching, unlabeled thiols were alkylated with 1.2 mM iodoacetamide. The resulting MPB-labeled cell suspension was lysed in RIPA buffer and

MPB-labeled proteins were isolated by streptavidin-mediated pulldown. Streptavidin-conjugated beads were incubated with 100 µg of MPB lysate overnight at 4°C with constant rotation. The following day the resulting bead pellets were washed with 1x TBS six times, as described above. The resulting MPB-protein:bead pellet underwent trypsin digestion and the resulting peptides were analyzed by Mass-spectrometry as described previously.[105]

Production of PDI CKO mice

C57BL6/J mice encoding *loxP* sites flanking the PDI gene *(p4hb)* and transgenic C57BL6/J mice expressing *cre* recombinase under the control of the *LysM* promoter (*lys-cre)* were kindly supplied by Dr. Cho (University of Illinois School of Medicine) and produced as described.[128] Briefly, PDI CKO mice were produced by the selective breeding of homozygous *loxP* flanked *p4hb* mice and heterozygous *lys-cre* mice to produce heterozygous *loxP* flanked *p4hb* and heterozygous *lys-cre* mice[128]. These mice were then bred with homozygous *loxP* flanked *p4hb* mice to produce homozygous *loxP* flanked *p4hb* and heterozygous *lys-cre* mice (PDI CKO mice). Desired murine genotypes were confirmed by PCR are previously described.[128]

Murine infection of *A. phagocytophilum*

Littermate control C57Bl/6J or PDI CKO mice were inoculated with *A. phagocytophilum* as previously described [14,51]. Sex and age matched mice were intraperitoneally infected with 10^8 DC bacteria. Peripheral blood samples were collected from the tail vein on days 4, 8, 12, 16, and 21 post infection. Bacterial load was determined by qPCR. Additionally,

blood smears were prepared and stained with the commercial Romanowsky stain, Diff-Quik (ThermoFisher), and the percentage of neutrophils that contained ApVs was determined.[51]

Statistical Analysis

Prism 7.0 software package (GraphPad, San Diego, CA) was used to perform all statistical analysis. The student's t-test was used to determine the significant difference among two samples while a one-way analysis of variance (ANOVA) and Tukey's post hoc test tested the significant difference among more than two samples. P values <0.05 were deemed statistically significant.

Chapter 3: *A. phagocytophilum* Asp14 interacts with host surface PDI, co-opting its disulfide reductase activity to promote infection

3.1 Introduction

All obligate intracellular pathogens must adhere to and invade host cells in order to survive. If the internalization process of these pathogens were to be therapeutically targeted, these pathogens would be unable to replicate and cause disease. As such, the process of pathogen internalization into host cells is a vital area of study. In the case of *A. phagocytophilum,* it is known that DC bacteria bind to host cell surfaces at lipid raft membrane domains, after which they are internalized by receptor-mediated endocytosis into the host cell.[5,80] Binding to and invasion of host cells is induced by the cooperative actions of at least three bacterial surface proteins: OmpA, AipA, and Asp14. The mechanism by which OmpA contributes to bacterial adherence to host cells has been well studied by the Carlyon Lab, determining that it interacts with the sLe^x glycan of PSGL-1 on host surfaces to induce adherence to host cells[105,107]. In an effort to dissect the complete process of bacterial internalization, we have also examined the functions of AipA and Asp14. The functional domains of AipA and Asp14 that induce internalization by host cells have been determined. However, the cognate host receptors of these bacterial proteins has not been identified.[104-106]

As Asp14 has been shown to play a large role during the internalization process, it was chosen for further study. We have previously published that Asp14 primarily contributes to internalization. Asp14-mediated internalization and interaction with the host cell surface is dependent upon its C-terminal 12 amino acids, the Asp14 binding domain. To determine potential interacting partners of Asp14, yeast two-hybrid analysis was used.

This implicated PDI, a lipid raft-localizing host enzyme, as a putative interacting partner of Asp14.[167]

PDI has previously been shown to be utilized during the internalization process of several obligate intracellular pathogens, such as HIV, dengue virus, and *C. trachomatis*. However, the mechanism of how these pathogens exploit PDI is not well understood. It is known that all three utilize PDI disulfide reductase activity to promote internalization.[161,166,167,169-171,189] In the case of *C. trachomatis* a PDI-dependent host protein is also utilized as a receptor for adherence.[169] As *A. phagocytophilum* may utilize a similar strategy to mediate internalization, the Asp14:PDI interaction and the relevance of PDI and its enzymatic activity during bacterial infection was examined *in vitro* and *in vivo*.

In the case of HIV, the best studied of these pathogens, surface disulfide oxidoreductases, PDI, Trx-1, and glutaredoxin-1, are utilized to reduce disulfide bonds within the host receptor CD4. This induces a conformational change that improves the binding affinity of CD4 for the HIV adhesin gp120, securing viral adherence to the host cell. Subsequently, disulfide bonds within gp120 are reduced, resulting in a conformational change that allows for fusion of the viral envelope with the host cell membrane.[154,158,161,162] In the case of dengue virus, PDI-mediated disulfide reduction, likely of host β2 integrins promotes fusion of the viral envelope with the host membrane. *C. trachomatis,* the only other bacterium known to exploit surface PDI, utilizes PDI to reduce some host or bacterial surface protein to promote internalization. To date no

pathogen-derived protein has been shown to directly bind to PDI to promote the disulfide reduction of another protein.

Herein we confirmed the interaction between Asp14 and PDI. Furthermore we examined the benefit of PDI and its enzymatic capabilities during *A. phagocytophilum* infection. Finally, we confirm the importance of this interaction both in primary neutrophils and in a mouse model of granulocytic anaplasmosis.

3.2 Results

3.2.1 Asp14 interaction with PDI is dependent on the Asp14 C-terminus

Asp14 is essential to host cell invasion by *A. phagocytophilum*. To determine the cognate host receptor of Asp14, yeast two-hybrid analysis was performed utilizing Asp14 as a bait protein. An activated leukocyte transcriptome library of bait proteins was used. PDI was identified as a potential Asp14-interacting partner. Intriguingly, PDI has been previously shown to participate in the internalization of other obligate intracellular pathogens and is known to localize to lipid rafts, the host membrane domains that serve as sites of *A. phagocytophilum* internalization.[104,154,158,159,164,166,169-171,189,190]

Yeast two-hybrid analysis, while a powerful tool for identifying putative protein-protein interactions, can indicate false interactions.[191] In order to confirm the putative Asp14:PDI interaction indicated by yeast two-hybrid analysis, co-immunoprecipitation (Co-IP) experiments were performed. Co-IP is a method to directly confirm the physical interaction between proteins, and has been used extensively to validate interactions

between pathogen effector proteins and host interacting partners.[187,192-194] HEK-293T cells, immortalized cells that are amenable to transfection and *A. phagocytophilum* infection, were transfected to co-express Flag tagged PDI and either GFP or GFP tagged Asp14.[120] As it has been determined that Asp14 adherence to host cells is dependent on the Asp14 C-terminus (residues 113-124), HEK-293T cells co-expressing Flag-PDI and GFP Asp14 lacking the C-terminal binding domain (GFP-Asp14$_{1-112}$) were also examined [104,105]. Flag Co-IPs were performed wherein Flag-PDI was immunoprecipitated and the presence of co-precipitated GFP tagged proteins were examined. It was found that GFP-Asp14, but not GFP-Asp14$_{1-112}$ or GFP alone, was co-precipitated by Flag-PDI, confirming that Asp14 can interact with PDI and further suggesting that this interaction, like Asp14 host surface interactions, relies on the Asp14 C-terminal binding domain (Figure 5A).

3.2.2 *A. phagocytophilum* requires the presence of PDI for optimal infection of host cells

Having validated the interaction between Asp14 and PDI, we determined the role this interaction plays during *A. phagocytophilum* infection of host cells. In HEK-293T cells, which are susceptible to *A. phagocytophilum* infection, PDI transcript levels were knocked-down using siRNA targeting the mRNA encoding PDI, *P4HB*. Knock-down (KD) was confirmed by western blot (Figure 5B). Upon synchronous infection by *A. phagocytophilum,* it was found that bacterial binding to host cells was not altered by PDI KD (Figure 5C). However, PDI KD resulted in a 50% decrease in bacterial infection, relative to host cells treated with non-targeting siRNA controls (Figure 5D).

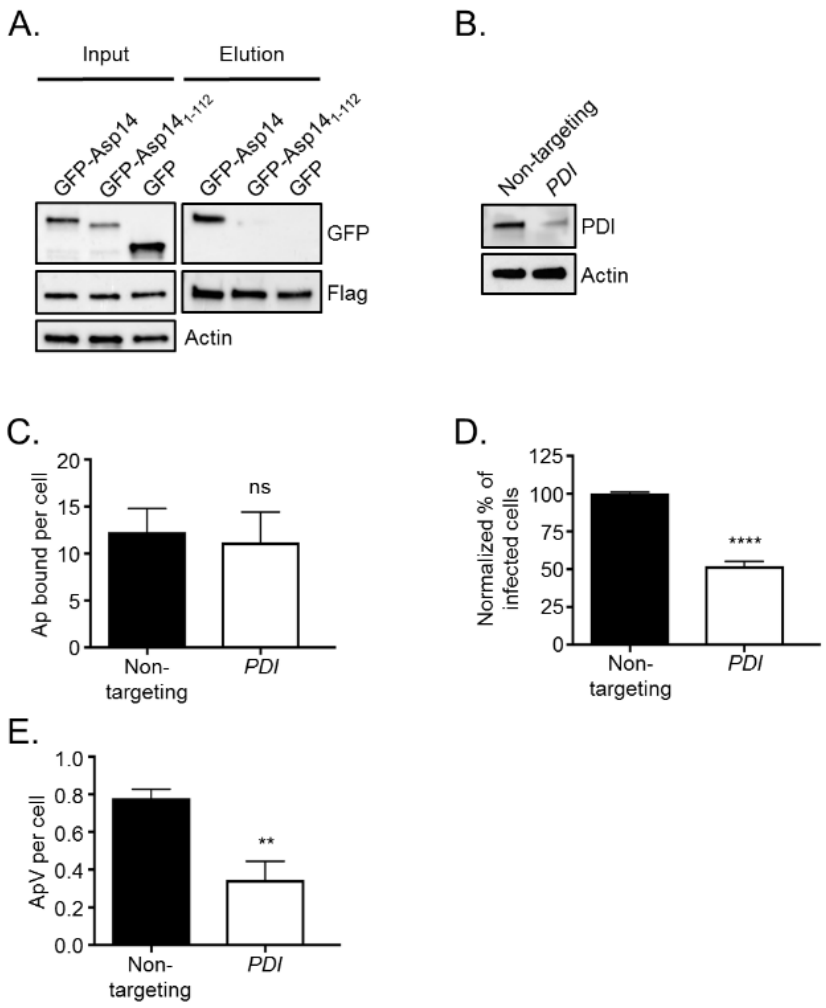


Figure 5: *A. phagocytophilum* Asp14 interacts with PDI, a host determinant required for optimal infection

(A) HEK-293T cells expressing Flag-PDI and GFP-Asp14, GFP-Asp14$_{1-112}$, or GFP were lysed and analyzed by Flag Co-IP. Transfected cell lysates and Co-IP eluates were analyzed by SDS-PAGE and Western blotting with Flag and GFP-targeting antibodies. β-actin was examined as a loading control. (B-E) HEK-293T cells were treated with non-targeting or *PDI*-targeting siRNA. At 24 h post KD, cells were either (B) lysed and examined by western blot utilizing PDI and β-actin antibodies to confirm KD, or (C-E) synchronously infected with DC bacteria. (C) At one hpi bacterial binding was determined by immunofluorescence microscopy (IFA). (D) Bacterial infection was examined by IFA at 24 hpi and recorded as (D) the % of cells infected, normalized to the control, or (E) the average number of ApVs observed per cell. Western blot data are representative of three experiments. IFA analysis was performed in triplicate. Statistically significant values are indicated. **,*P*<0.01; ****,*P*<.0001; ns, not significant. Statistical indicators are relative to the non-targeting treated control.

Bacterial load as determined by the average number of morulae per counted cell was similarly decreased (Figure 5E).

3.2.3 *A. phagocytophilum* requires PDI for optimal infection of mice

Having confirmed that the presence of PDI is vital for efficient *A. phagocytophilum* infection *in vitro*, it was important to determine if PDI is utilized *in vivo*. To examine PDI exploitation *in vivo,* phenotypically wild type littermate control mice (Wt) or PDI conditional knockout (CKO) mice, a C57BL6/J line wherein PDI is not expressed in myeloid lineage cells, neutrophils and monocytes, were infected with DC bacteria, by intraperitoneal inoculation (IP), and bacterial load was examined over the course of 28 days.[128] qPCR was used to determine relative bacterial load and the percentage of infected circulating peripheral neutrophils determined by microscopy. During normal murine *A. phagocytophilum* infection, bacterial load increases until reaching peak bacteremia between 8 and 16 days post infection (dpi), after which bacterial load quickly decreases, before being cleared by approximately 28 dpi.[51,122] It was found that PDI-expressing Wt mice experience peak bacteremia at 12 dpi (Figure 6A and B). PDI CKO mice experience a significantly decreased peak bacterial load compared to their wild type littermates, as confirmed by both detection of bacterial DNA and the percentage of peripheral neutrophils that were infected (Figure 6A and B). These data indicate that host PDI expression is critical for bacterial infection *in vivo.*

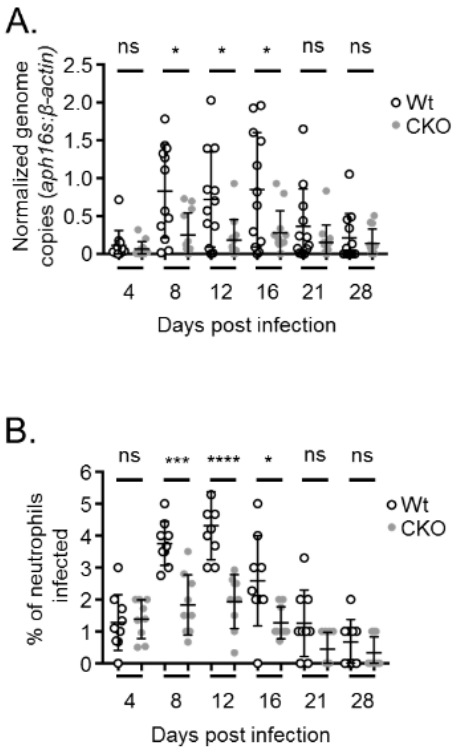


Figure 6: PDI knockout in neutrophils significantly inhibits *A. phagocytophilum* infection in a mouse model of granulocytic anaplasmosis

PDI CKO or littermate control mice (Wt) were infected with DC *A. phagocytophilum* by IP injection. (A and B) On 4, 8, 12, 16, 21, and 28 dpi blood samples were collected and bacterial DNA load of peripheral blood was determined. (A) Total DNA from blood was analyzed by qPCR wherein the Livak method was used to determine the relative expression of *Ap16S:*host *β-actin.* Data are the culmination of three different experiments (N=12). (B) Microscopic analysis of blood smears was used to determine the percentage of host neutrophils that harbored ApVs. Data represented are the culmination of two different experiments (N=9). Statistically significant values are indicated. *,*P*<0.05; ***,*P*<0.001; ****,*P*<0.0001; ns, not significant. Statistical indicators are relative to Wt control mice for each individual time point control.

3.2.4 *A. phagocytophilum* specifically requires disulfide reduction to promote internalization

As PDI-exploiting pathogens co-opt disulfide reduction to promote internalization, we examined *A. phagocytophilum* infection of host cells during inhibition of PDI enzymatic activity. Initially bacitracin, a chemical inhibitor of all surface disulfide reductases, was used to inhibit host disulfide reductases.[195] Bacitracin- or vehicle-treated host cells were synchronously infected with *A. phagocytophilum*. It was found that bacitracin treatment of host cells significantly reduced infection of but not binding to host cells (Figure 7A, B, and C). It is important to note that bacitracin treatment was not cytotoxic to host cells and did not affect bacterial infectivity. (Figure 7D and E).

Bacitracin is known to inhibit all surface disulfide reductases.[195,196] To determine the role of PDI during infection, host cells were treated with BD34, a monoclonal antibody that specifically inhibits the activity of PDI by preventing access to the “CxxC” catalytic motifs of PDI, or a polyclonal PDI-targeting antibody that does not catalytically inactivate PDI (non-CxxC-targeting). These cells were then synchronously infected. It was found that BD34-mediated inhibition of surface PDI activity, similarly to PDI KD, resulted in a 50% decrease in bacterial infection (Figure 8A and B). Polyclonal PDI-targeting antibody that does not specifically target the PDI active site resulted in minimal inhibition of PDI activity (Figure 8A and B). These results indicate that *A. phagocytophilum* specifically requires PDI enzymatic activity for optimal infection. Neither antibody treatment inhibited bacterial binding to host cells (Figure 8C).

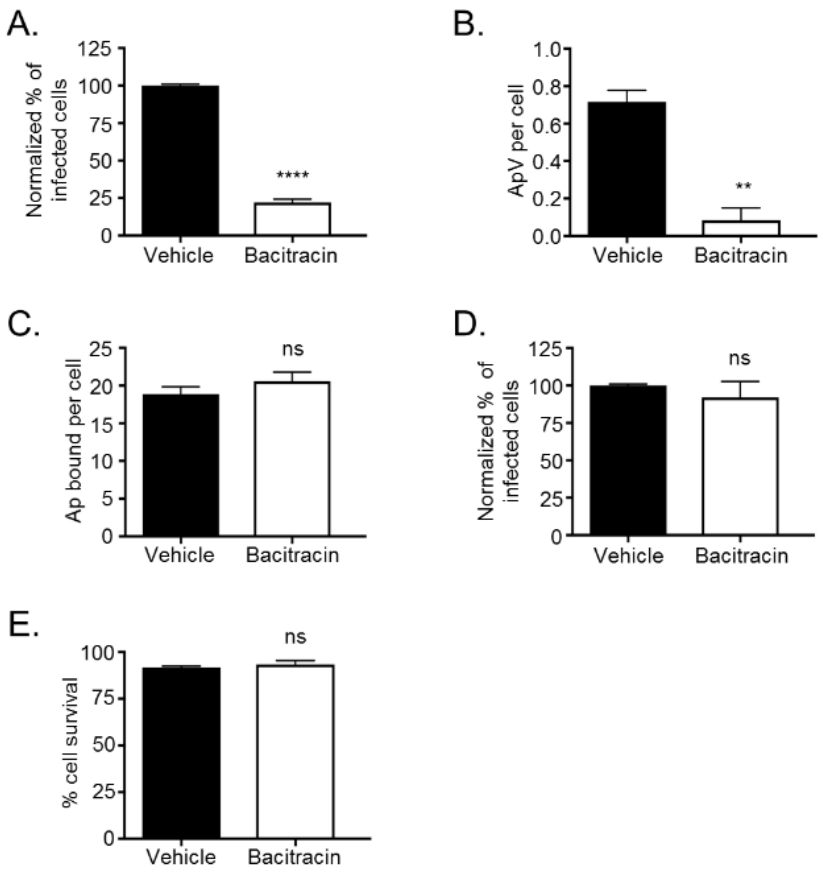
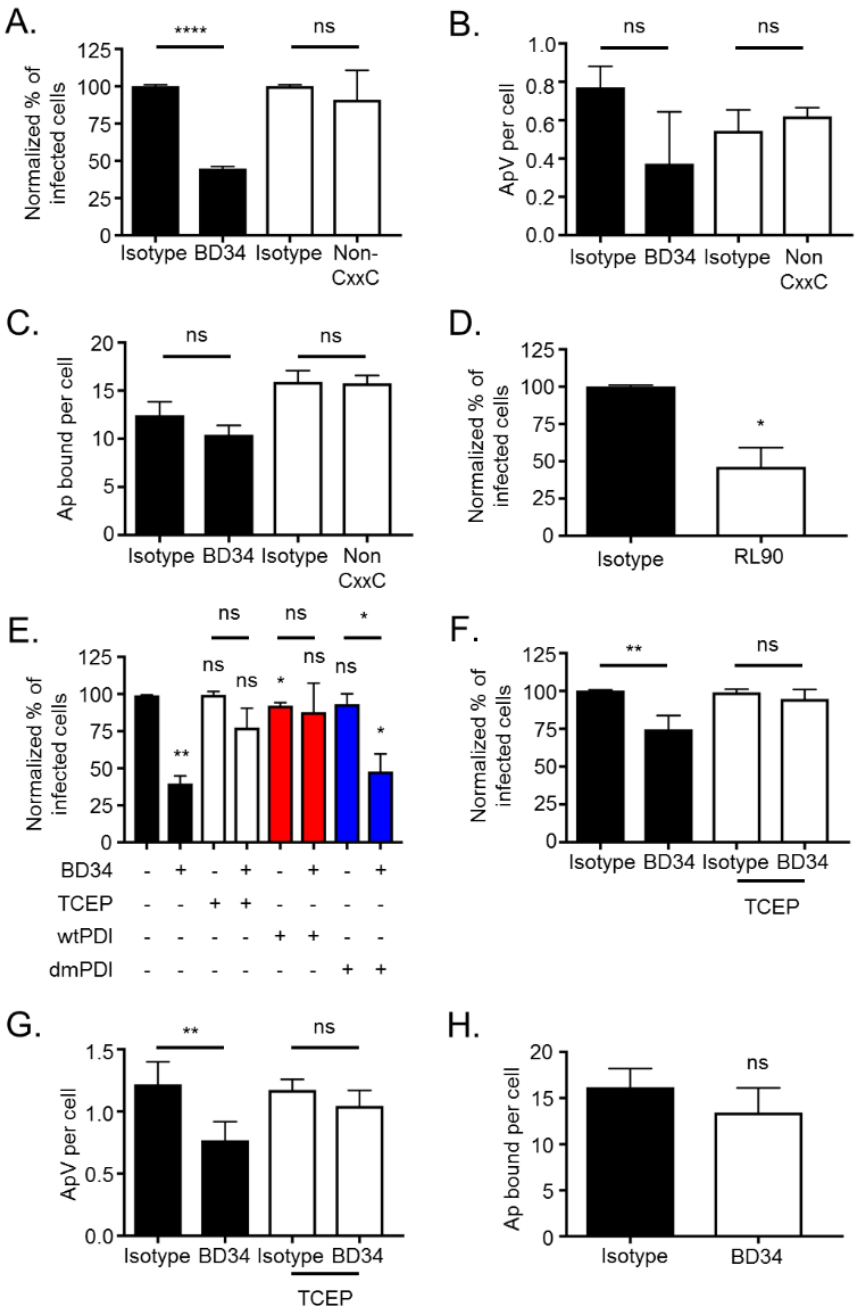


Figure 7: *A. phagocytophilum* exploits surface disulfide oxidoreductase activity to infect, but not bind to host cells

HL-60 cells treated with (A, B, and C) a vehicle control or bacitracin were synchronously infected with DC bacteria. At 24 hpi the (A) percentage of cells infected and (B) the ApV per cell were determined via IFA. (C) At one hpi bacterial adhesion to host cells was quantified by IFA. (D) HL-60 cells were synchronously infected with bacitracin- or vehicle-treated DC bacteria. At 24 hpi the percentage of infected cells, normalized to the vehicle control, was determined by IFA. (D) Bacitracin- or vehicle-treated HL-60 cells were incubated at 37°C and 5%CO_2 for 24 h. Cell viability was determined on the criteria of trypan blue exclusion. Data are representative of three experiments performed in triplicate. Statistically significant values are indicated. **,P<0.01; ****,P<.0001; ns, not significant. Statistical indicators are relative to the vehicle-treated control.


A.
Normalized % of infected cells
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ns
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B.
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Isotype
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C.
Ap bound per cell
ns
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Isotype
BD34
Isotype
Non CxxC
D.
Normalized % of infected cells
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Isotype
RL90
E.
Normalized % of infected cells
BD34
TCEP
wtPDI
dmPDI
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Figure 8: PDI-mediated disulfide reduction promotes bacterial infection

HL-60 cells treated with (A, B and C) an isotype control, BD34, a polyclonal PDI antibody, or (D) RL-90 were synchronously infected with DC bacteria. At 24 hpi the (A and D) percentage of cells infected and (B) the ApV per cell were determined via IFA. (C) At one hpi bacterial binding to host cells was determined by IFA. (E) Isotype control or BD34 inhibited HL-60 cells were synchronously infected with DC bacteria. Post DC binding, bacteria and host cells were treated with a vehicle control, TCEP, wtPDI, or dmPDI for 30 min at 37°C. Infection was measured at 24 hpi as the normalized percentage of cells infected. (F, G, and H)Isotype control or BD34 treated human neutrophils were synchronously infected with DC bacteria. At one hpi bacteria and HL-60 cells were treated with a vehicle control or TCEP. At 24 hpi bacterial infection was recorded as (F) the number of ApVs per cell and (G) the percentage of infected cells. (H) At one hpi DC adherence to neutrophils was determined via IFA. Data are representative of three experiments performed in triplicate. Statistically significant values are indicated. *,$P<0.05$; **,$P<0.01$; ****,$P<.0001$; ns, not significant. Statistical indicators are relative to the vehicle and isotype-treated control, except where relationships are indicated by underlining.

Another monoclonal antibody that inhibits PDI reductase activity, RL-90, was also examined. It was found that RL-90-mediated inhibition of PDI inhibited bacterial infection similarly to BD34 (Figure 8D). For the remainder of this work BD34 was utilized as a PDI inhibitor, as it is known to have a greater inhibitory effect on PDI than RL-90.[197]

To specifically confirm the requirement of disulfide reduction during bacterial infection, BD34 was used to inhibit host cell PDI, followed by synchronous *A. phagocytophilum* infection. After bacterial binding to host cells, host and bacterial cells were exposed to TCEP, a chemical disulfide reducing agent, recombinant PDI protein (wtPDI) or recombinant PDI that is enzymatically defective (dmPDI).[198] The active site Cys of both dmPDI active sites are mutated to serines, which are unable to catalyze disulfide bond reduction.[128,144,198,128,144,146,184,185,186] It was found that BD34 reduced bacterial infection as before (Figure 8E). TCEP treatment significantly rescued the ability of *A. phagocytophilum* to infect host cells (Figure 8E). wtPDI treatment restored bacterial infection similarly to the isotype control. Enzymatically defective dmPDI was unable to restore infection (Figure 8E). These data confirm that PDI enzymatic activity is critical during infection. Furthermore, TCEP-mediated rescue indicates that disulfide reduction alone, even in the absence of PDI is required for infection.

3.2.5 *A. phagocytophilum* exploitation of host surface PDI occurs in primary neutrophils

Having determined that disulfide reduction is required for optimal infection *in vitro,* we examined the utilization of PDI in a more physiologically relevant cell type. Human neutrophils, the cell type infected by *A. phagocytophilum in vivo,* were isolated from

healthy hosts and infected with DC bacteria post BD34 or isotype control treatment. It was found that BD34 significantly reduced bacterial infection of neutrophils, but to a lesser degree than is seen during bacterial infection of HL-60 cells (Figure 8F and G). Additionally, TCEP treatment rescued infection, similarly to what was seen in immortalized cell lines (Figure 8F and G). Bacterial binding to neutrophils was not affected by PDI inhibition (Figure 8H). These data indicate that PDI is required for optimal infection of human neutrophils.

As *I. scapularis* encodes a predicted PDI homolog that possesses the same CGHC active site as its mammalian counter parts, disulfide reductase exploitation was examined in the context of the tick vector.[199] ISE6 cells are immortalized tick cells derived from *I. scapularis* embryos and have since been utilized extensively to examine *A. phagocytophilum* infection.[180,200-204] ISE6 tick cells were treated with a vehicle control or bacitracin, which inhibits the “CxxC” motif common to both mammalian and arthropod oxidoreductases.[196] Tick cells were then synchronously infected with *A. phagocytophilum.* It was found that inhibition of surface disulfide reduction did not decrease bacterial infection (Figure 9). This indicates that *A. phagocytophilum* does not exploit host-mediated disulfide reduction during tick cell infection.

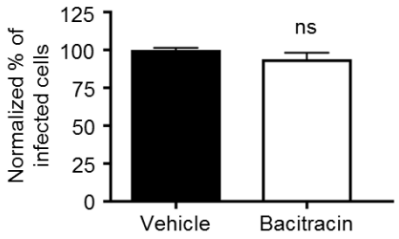


Figure 9: *A. phagocytophilum* does not exploit surface oxidoreductases during tick cell infection

Bacitracin or vehicle treated ISE6 tick cells were synchronously infected with DC bacteria. The percentage of infected cells was determined by IFA at 24 hpi. Data are representative of two experiments performed in triplicate. Statistically significant values are indicated. ns, not significant. Statistical indicators are relative to the vehicle-treated control.

Chapter 4: PDI is directed by Asp14 to reduce an *A. phagocytophilum* surface protein

4.1 Introduction

Having confirmed that *A. phagocytophilum* exploits disulfide bond reduction to promote infection, the mechanism of this exploitation was dissected during infection. As stated previously numerous obligate intracellular pathogens exploit PDI-mediated disulfide reduction during their internalization strategies. In most of these cases the identity of the protein being reduced is not known. In the case of HIV it is known that the host protein CD4 and the viral protein gp120 are both reduced to promote viral envelope fusion.[158,159,161,162] Dengue virus exploits PDI-mediated reduction of β integrins to promote viral fusion to the host membrane.[166,167] *C. trachomatis* interacts with a PDI-containing receptor complex, likely the estrogen receptor complex, to mediate bacterial adherence to the host membrane, after which it exploits PDI-mediated reduction of either a host or bacterial surface protein to mediate bacterial invasion.[169-171] It has been hypothesized that PDI may reduce proteins of the *Chlamydial* P-layer, a series of highly disulfide crosslinked outer membrane proteins that serve to provide outer membrane stability to the infectious extracellular *Chlamydial* elementary body. It has also been hypothesized that PDI may reduce disulfide bonds within the T3SS cap protein, CdsF.[169] In the case of *C. trachomatis* not only has no bacterial protein been shown to directly bind PDI, but the target protein of PDI-mediated reduction has not been identified.

Herein the importance of the Asp14:PDI interaction was examined during cellular infection and the target of *A. phagocytophilum*-directed PDI-mediated reduction was determined to be a bacterial outer membrane protein. Several methods were used to identify the target of PDI-mediated disulfide reduction during infection.

4.2 Results

4.2.1 Asp14 directs PDI-mediated disulfide reduction of a protein target

As Asp14 could not be studied in the absence of the other *A. phagocytophilum* adhesins, it was examined whether Asp14 directs PDI disulfide reduction during infection. Host cells were exposed to bacteria that had been treated with either a preimmune sera control, or antibodies raised against the binding domains of the *A. phagocytophilum* adhesin binding domains (OmpA 59-74, AipA 9-21, and Asp14 113-124). Following bacterial binding, the binding reactions were treated with TCEP or a vehicle control. Antibody blocking of the adhesin binding domains resulted in levels of infection inhibition similar to what has previously been reported.[104-107] TCEP treatment of the host cells and bound DC bacterial did not restore infection during OmpA and AipA inhibition (Figure 10A and B). However, Asp14 inhibition was rescued by TCEP, confirming that the Asp14:PDI interaction occurs during cellular infection (Figure 10A and B). Furthermore, this indicates that disulfide reduction rescues *A. phagocytophilum* infection when Asp14 is unable to interact with the host cell surface. This supports our hypothesis that Asp14 interacts with PDI to promote the reduction of disulfide bonds, which is the first reported instance of a bacterial protein doing so.

4.2.2 *A. phagocytophilum* can exploit Trx to reduce the disulfide bonds that promote infection

It has been reported that HIV is able to use other disulfide oxidoreductases to reduce gp120. Additionally, inhibition of all surface disulfide oxidoreductases by bacitracin results in more severe inhibition of *A. phagocytophilum* infection than inhibition of PDI alone

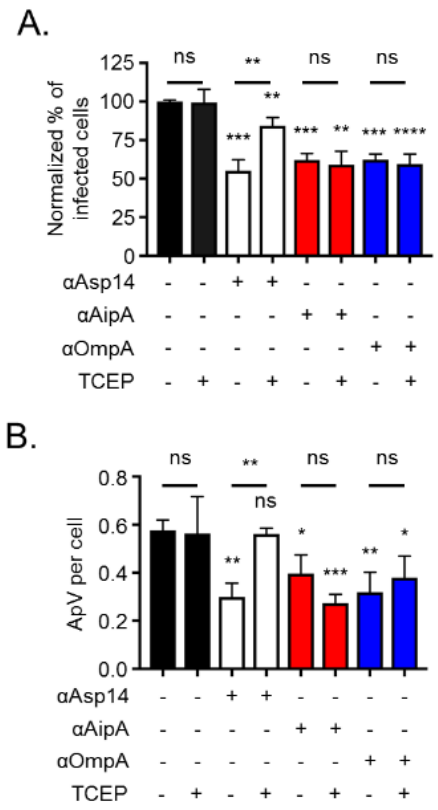


Figure 10: Asp14 directs PDI exploitation during *A. phagocytophilum* infection of mammalian cells

HL-60 cells were synchronously infected with DC bacteria that were treated with either preimmune sera or antisera targeting the binding domain of Asp14, AipA, or OmpA. At one hpi bacteria and host cells were treated with a vehicle control or TCEP l for 30 min at 37°C. At 24 hpi the (A) percentage of host cells that became infected and (B) the number of morulae per cell were determined by IFA. Data are representative of three experiments performed in triplicate. Statistically significant values are indicated. *,*P*<0.05; **,*P*<0.01; ***,*P*<0.001; ****,*P*<0.0001; ns, not significant. Statistical indicators are relative to the preimmune sera-treated control, except where relationships are indicated by underlining.

(Figure 7A and B, and Figure 8A and B, respectively). This could indicate that *A. phagocytophilum* utilizes other surface oxidoreductases in addition to PDI to reduce disulfide bonds. To determine whether Trx is exploited during *A. phagocytophilum* infection of host cells the Trx-targeting monoclonal antibody 2B1, which inhibits Trx-mediated HIV invasion of host cells, was used.[205] It was found that 2B1 treatment of host cells significantly inhibited bacterial infection of host cells. However, the degree of inhibition was less than that of BD34 (Figure 11A and B). Combined treatment of host cells with BD34 and 2B1 did not result in levels of infection inhibition that were greater than that of BD34 alone (Figure 11A and B). The infection defects associated with both host 2B1 and combined BD34 and 2B1 treatment of host cells were rescuable upon TCEP treatment (Figure 11A and B). These data suggest that while Trx may also provide disulfide reduction necessary for *A. phagocytophilum* infection of host cells, antibody-mediated inhibition of Trx and PDI is not sufficient to reduce infection to a greater degree than BD34-mediated inhibition alone.

Auranofin is a rheumatoid arthritis treatment that is known to specifically inhibit PDI and Trx, and has been used in the study of PDI exploitation by HIV. Auranofin inhibits the protein thioredoxin reductase-1, which maintains the reduced state of surface PDI and Trx.[161] This compound differentially impairs surface PDI and Trx activity in a dose-dependent manner. At concentrations of 100 nM PDI is maximally inhibited while Trx activity is unaffected. Both oxidoreductases are maximally inhibited at concentrations of 1000 nM. This makes auranofin a unique tool for the study of the individual contributions of these oxidoreductases. Upon treatment of HL-60 cells with auranofin, it was found that

this compound was highly cytotoxic, as determined by trypan blue exclusion and quantification of cell densities (Figure 12A and B). This indicated that further examination of auranofin as a therapy capable of targeting PDI-exploiting pathogens is not feasible.

4.2.3 The utilization of adhesin-conjugated microbeads is not a relevant model for examination of Asp14

As *A. phagocytophilum* internalization is the cooperative action of several different adhesins, examining the contribution of a single protein towards the process is difficult. This is made more difficult by the genetic intractability of *A. phagocytophilum*.[206] We have previously used OmpA-conjugated microbeads to examine the contribution of OmpA towards *A. phagocytophilum* binding and invasion, in the absence of other adhesins.[105] This tool relies on bacterium-sized beads (1 µm diameter) that are conjugated to an individual recombinant protein. This allows the examination of the contribution of a single surface protein to binding and internalization. As such this tool was used in the study of Asp14. Isotype control or BD34 treated HL-60 cells were exposed to *A. phagocytophilum* sized beads conjugated to His-Asp14 protein. Bead binding to and internalization by HL-60 cells were examined. It was found that BD34 treatment did not inhibit Asp14 bead binding to host cells (Figure 13A). PDI inhibition resulted in a decrease in the internalization of Asp14-conjugated beads (Figure 13B). However, TCEP was unable to rescue internalization after BD34-mediated inhibition (Figure 13B). This does not recapitulate previous results that TCEP rescues not only bacterial infection following PDI inhibition, but also infection deficits due to direct inhibition of Asp14. This indicates that

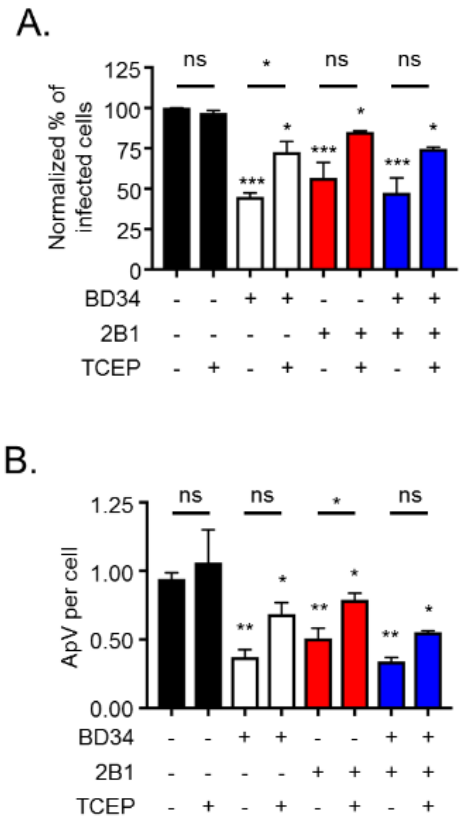


Figure 11: Trx can partially supply the disulfide reduction required for *A. phagocytophilum* infection

HL-60 cells treated were with An isotype control, BD34, or 2B1, or both BD34 and 2B1. These cells were synchronously infected with DC bacteria. At one hpi the bacteria and host cells were treated with a vehicle control or TCEP. At 24 hpi (A) the percentage of infected host cells and (B) ApV per host cell were determined by IFA. Data are representative of three experiments performed in triplicate. Statistically significant values are indicated. *,P<0.05; **,P<0.01; ***,P<0.001; ns, not significant. Statistical indicators are relative to the isotype-treated control, except where relationships are indicated by underlining.

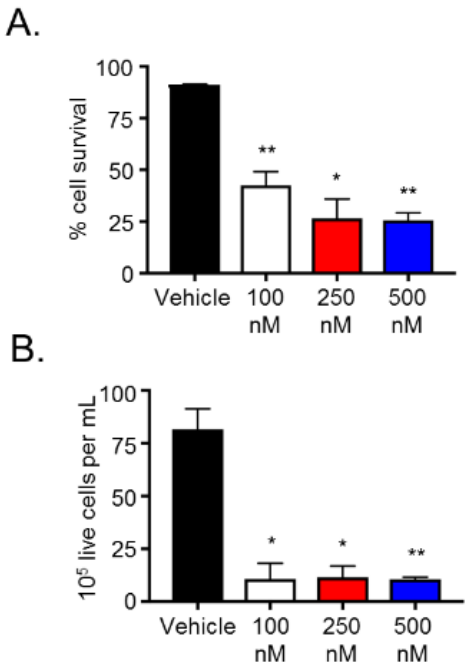


Figure 12: Auranofin, an inhibitor of PDI and Trx, is toxic to mammalian cells

HL-60 cells were treated with a vehicle control or increasing concentrations of auranofin. (A) At 24 hpi samples were examined for cell survival, as determined by trypan blue exclusion. (B) Additionally, the number of live cells per ml was determined. Data are representative of two experiments performed in triplicate. Statistically significant values, compared to the vehicle control, are indicated. *,$P<0.05$; **,$P<0.01$. Statistical indicators are relative to the vehicle-treated control.

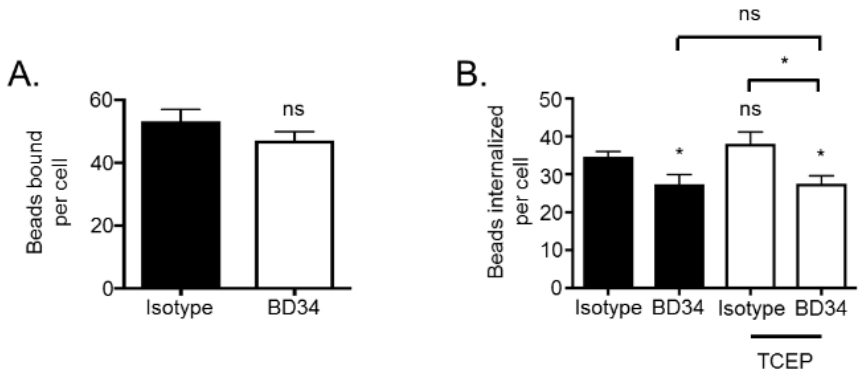


Figure 13: Asp14-conjugated beads do not recapitulate the phenotype of PDI exploitation documented during *A. phagocytophilum* infection

Asp14-conjugated beads were applied to isotype control or BD34 treated HL-60 cells. At one h post bead addition (A) the number of beads bound to the host surface were enumerated by IFA. (B) At that time the reaction mixture was treated with a vehicle control or TCEP. At eight h post bead addition the number of beads internalized per cell were determined by IFA. Data are representative of two experiments performed in triplicate. Statistically significant values are indicated. *,$P<0.05$; ns, not significant. Statistical indicators are relative to the isotype-treated control, except where relationships are indicated by underlining.

while this technique has proven useful for other adhesins in the past, it is not a representative model of Asp14-mediated internalization.

4.2.4 Disulfide reduction of an *A. phagocytophilum,* but not a host cell, surface protein promotes bacterial infection

Having identified a bacterial protein that directs PDI-mediated disulfide reduction, it is necessary to identify the protein being reduced. However, this is a daunting task given the expansive surface proteome of *A. phagocytophilum* and human neutrophils. To make this process more efficient we examined whether PDI was directed to reduce a host or bacterial surface protein. Host cells were treated with BD34 or a vehicle control. Prior to infection host cells or bacteria were treated with the disulfide reducing compound, TCEP or a vehicle control. These bacteria were used to infect TCEP or vehicle control and BD34 or isotype control treated host cells in various combinations. It was seen that when both bacterial and host cell surfaces were TCEP treated, BD34-mediated infection inhibition was rescued. When bacterial surfaces, but not host surfaces, were treated with TCEP, infection was significantly restored following PDI inhibition (Figure 14A and B). This indicates that an *A. phagocytophilum* surface protein is the target of Asp14-directed PDI-mediated disulfide reduction. This rescue was not observed when the HL-60 surface alone was treated with TCEP (Figure 14A and B). Thus, PDI is directed by Asp14 to reduce an *A. phagocytophilum* surface protein.

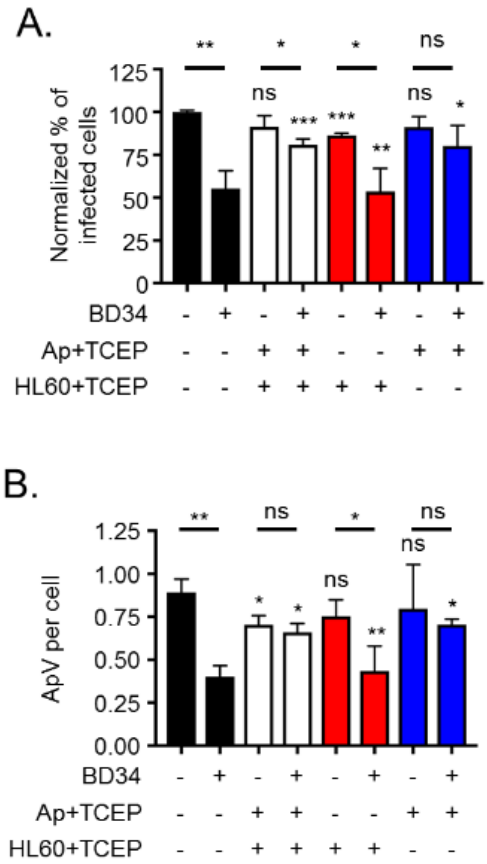


Figure 14: *A. phagocytophilum* directs PDI-mediated disulfide reduction of a bacterial surface protein to promote infection

HL-60 cells were treated with an isotype control or BD34. Following this, those host cells were treated with a vehicle control or TCEP. HL-60 cells were synchronously infected with TCEP or vehicle pretreated *Anaplasma.* At 24 hpi the (A) percentage of infected host cells and (B) the number of ApVs per host cell were determined by IFA. Data are representative of three experiments performed in triplicate. Statistically significant values are indicated. *,$P<0.05$; **,$P<0.01$; ns, not significant. Statistical indicators are relative to the isotype treated control, except where relationships are indicated by underlining. Statistical indicators are relative to the vehicle- and isotype-treated control, except where relationships are indicated by underlining.

4.2.5 APH_1235 is a potential substrate of PDI-mediated disulfide reduction

Having determined the surface reduced by PDI during bacterial infection, I endeavored to determine the protein target of PDI-mediated reduction. Identification of this protein would provide insight into the function of how the observed disulfide reduction promotes bacterial infection and internalization. N-(3-maleimidopropionyl) biocytin (MPB) was utilized to identify the target of PDI-mediated reduction. MPB is a thiol-labeling biotin derivative that has previously been used to label proteins reduced by PDI.[128,158] Isolated DC bacteria were treated with enzymatically functional wtPDI protein or enzymatically inactive dmPDI. After which, bacteria were treated with MPB, to label any revealed surface thiols. Glutathione and iodoacetimide were used to quench the MPB-labeling reaction and to block unlabeled thiols. These cells were lysed and MPB-labeled proteins were isolated by streptavidin pulldown of MPB-labeled proteins. These samples were then examined by mass-spectrometry (MS) to identify pulldown isolated MPB-labeled proteins. Those proteins present in the wtPDI treated samples but not in the dmPDI treated samples, or those proteins that were seen in greater abundances in the wtPDI treated samples compared to the dmPDI treated samples were identified as targets of interest. Several proteins of interest were identified by virtue of their increased prevalence in wtPDI treated samples (Table 2). Specifically APH_1235, DnaK (Hsp70), DNA binding protein HU, and the 10 kDa chaperonin protein (GroS), were identified as proteins of interest (Table 2).

Table 2: Putative PDI substrate proteins identified by MS of biotin-labeled Cys residues

Putative PDI substrate	Fold increase in wtPDI treated samples relative to dmPDI	Function	Cys residues within protein
APH_1235	1.6988	A highly expressed *A. phagocytophilum* DC surface protein. A putative adhesin.[61,111,112]	1 (Observed to dimerize)
DnaK (Hsp70)	1.5276	A heat shock protein. Mediates protein folding. A putative moonlighting adhesin in multiple bacterial spp. including *A. phagocytophilum.*[207-209]	6
DNA binding protein HU	3.3529	A histone like DNA binding protein. Stabilizes DNA.[210,211]	1 (Observed to dimerize)
10 kDa Chaperonin (GroS)	1.9282	Multimerizes with GroEL to mediate folding of substrate proteins.[212,213]	1 (Exists within a multimeric complex with GroEL)

As APH_1235 has been implicated as a potential adhesin, we chose to examine potential interactions between Asp14, APH_1235, and PDI.[111,112] Bacterial DCs were treated with either preimmune sera or antibodies targeting either the binding domains or Asp14, AipA, or OmpA, and either preimmune sera or antibodies targeting APH_1235. The bacteria were allowed to infect host cells prior to treatment with TCEP or a vehicle control. Infection was examined by IFA. It was found that bacterial infection did not respond consistently to dual APH_1235 antibody-targeting and TCEP treatment (Figure 15A and B). Inhibition of Asp14 and APH_1235 together also produced inconsistent infection restoration upon TCEP treatment (Figure 15A and B). This TCEP rescue inconsistency indicates that disulfide reduction may play a role in the function of APH_1235; however, this cannot be confirmed by the data herein presented. When dual inhibition of Asp14 and OmpA occurred TCEP restored bacterial infectivity and dual inhibition of Asp14 and AipA produced insignificant restoration of *A. phagocytophilum* infection levels. These do not indicate whether APH_1235 is a substrate of PDI; more study is needed to conclude this.

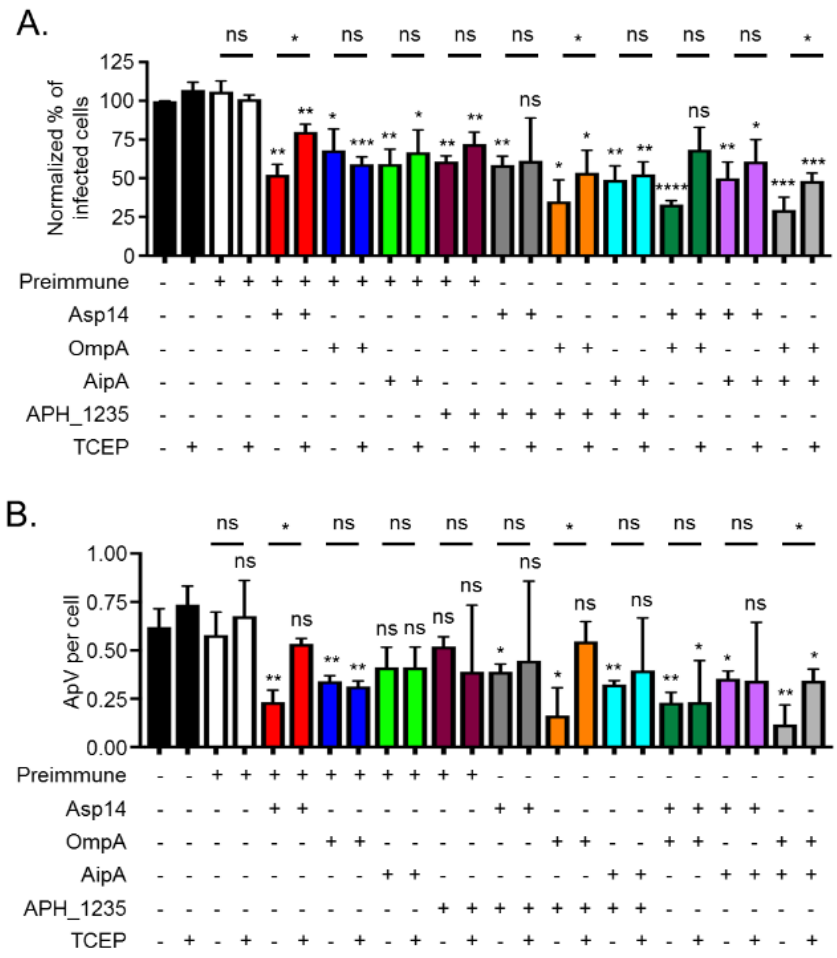


Figure 15: APH_1235 is potentially reduced by PDI during infection of mammalian cells

HL-60 cells were synchronously infected with DC bacteria that had been treated with various combinations of antisera targeting APH_1235 or the Asp14, OmpA, or AipA binding domains. At one hpi bacteria and host cells were treated with a vehicle control or TCEP. At 24 hpi the (A) percentage of infected host cells and (B) the mean number of ApVs per host cell were determined by IFA. Data are representative of two experiments performed in triplicate. Statistically significant values are indicated. *,P<0.05; **,P<0.01; ***,P<0.001; ****,P<0.0001; ns, not significant. Statistical indicators are relative to the vehicle- and isotype-treated control, except where relationships are indicated by underlining.

Chapter 5: Asp14 interacts with PDI via residues E123 and S124

5.1 Introduction

While it may be tempting to identify PDI as a therapeutic target that could treat several diseases, there are a few caveats. As PDI serves numerous essential functions throughout the cell, inhibition is often associated with severe toxicity. Bacitracin for instance is associated with severe renal toxicity. Auranofin, another inhibitor of PDI, has been used as a treatment for rheumatoid arthritis.[161] However, as we have shown in this work, it can be associated with significant cytotoxicity (Figure 12A and B). With this in mind, therapeutic targeting of pathogen-derived infection determinants is the best strategy for disease treatment and prevention.

Herein have documented the first identification of a bacterial protein that directly interacts with PDI to direct the disulfide reduction of another protein. While in the case of HIV, a protein substrate of HIV has been identified, a viral protein that directs this has not. Furthermore in the case of *C. trachomatis* no bacterial protein has been shown to directly interact with PDI. As such, this finding offers the unique opportunity to delineate a novel motif that mediates interactions with PDI.

Delineating the residues mediating the Asp14:PDI interaction serves not only to answer the basic scientific question of how Asp14 physically directs the exploitation of PDI, but also explains why inhibition of the Asp14 binding domain inhibits the ability of *A. phagocytophilum* to productively infect mice and human cells. Furthermore the identification of a novel PDI interaction motif has translational implications as well. The determination interaction mediating residues would allow a more focused and specific

design for Asp14-targeting therapeutics and prophylactics, such as an Asp14-targeting vaccine. Additionally, this would provide the ability to examine the proteomes of other pathogens to identify similar motifs, indicating potential targets that also manipulate PDI in this way.

Herein we examined the ability of the *Ehrlichia chaffeensis* Asp14 homolog ECH_0377 to interact with PDI, to identify conserved or disparate residues that are likely involved in the Asp14:PDI interaction. Furthermore, we produced Asp14:ECH_0377 fusion proteins in an attempt to further define the role of the Asp14 C-terminal binding domain in the Asp14:PDI interaction. Finally, we utilized alanine substitution to determine which residues of $Asp14_{113\text{-}124}$ mediate interaction with PDI.

5.2 Results

5.2.1 $Asp14_{113\text{-}124}$ is essential to interact with PDI, but is not sufficient to confer interaction

Having determined that Asp14:PDI interactions depend on the Asp14 binding domain, we wished to further define the amino acid determinants that mediate this interaction. Genomic analysis reveal that Asp14 is conserved in the related bacterium *E. chaffeensis*.[104] However, upon examination of their sequence identity it was found that while residues 101-112 are highly conserved, residues 113-124 vary considerably across different species (Figure 16A). It was hypothesized that examining the coprecipitation efficiency of each Asp14 homolog by Flag-PDI could implicate amino acids that mediate that Asp14:PDI interaction. HEK-293T cells co-expressing Flag-PDI and the GFP tagged

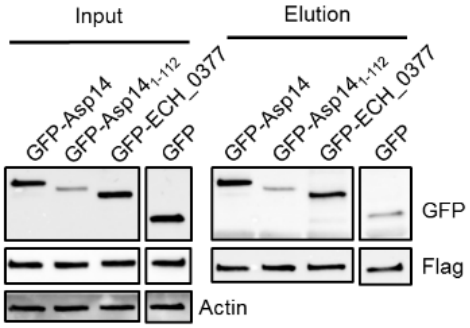
Asp14 homologues from *A. phagocytophilum* (GFP-Asp14) *and Ehrlichia chaffeensis* (GFP-ECH_0377) were co-immunoprecipitated as before (Figure 17B). Coprecipitation efficiencies were measured as normalized densitometric ratios. The densitometric ratio of coprecipitated GFP protein (normalized to the densitometric measurement of immunoprecipitated Flag-PDI) and expressed GFP protein of the input (normalized to the densitometric measurement of expressed Flag-PDI in the input) was normalized to the efficiency of *A. phagocytophilum* Asp14. It was found that GFP-ECH_0377 interacted with PDI half as efficiently as GFP-Asp14 (Figure 16B). A GFP control interacted negligibly with PDI (Figure 16B and C).

To further delineate the importance of the Asp14 C-terminus, chimeric constructs were produced wherein the N-terminal amino acids (1-112) of Asp14 were conjugated to the ECH_0377 C-terminal amino acids that correspond to the Asp14 residues 113-124 ($Asp14_{1\text{-}112}$:$ECH\_0377_{95\text{-}104}$). The reciprocal protein containing the ECH_0377 N-terminus and Asp14 binding domain was also utilized ($ECH\_0377_{1\text{-}95}$:$Asp14_{113\text{-}124}$). These constructs were analyzed by Co-IP with Flag-PDI as before. It was found that $ECH\_0377_{95\text{-}104}$ decreased the coprecipitation efficiency of Flag PDI for Asp14 (Figure 17A and B). The addition of $Asp14_{113\text{-}124}$ to ECH_0377 resulted in an insignificant increase in its interaction with PDI (Figure 17A and B). This increase was insignificant compared to both GFP-Asp14 and GFP-ECH_0377, indicating that while $Asp14_{113\text{-}124}$ is important to the Asp14:PDI interaction, it is not sufficient to produce an equally strong interaction.

A.


Asp14 1 MIPLAPWKSISVVYMSGSDEYKEIIKQCIGSVKEVFGEG-RFDDVVASIMKMQEKVLASS 59
ECH_0377 1 ----------MAEDDYKGVIKQYIDTVKEIVGDSKTFDQMFESVVRIQERVMAAN 45
Asp14 46 MQQDDTGTVGQIESGEGSGARLSDEQVQQLMNSIREEFKDDLRAIKRRILKLRAVYGAN 119
ECH_0377 60 AQNNEDGVIDNGDQVKRIG----SSTSESISNTEYKELMEELKVIKKRILRLRKILKPK 101
Asp14 120 TPKES 124
ECH_0377 102 EEV-- 104

B.


Input
Elution
GFP-Asp14
GFP-Asp14 1-112
GFP-ECH_0377
GFP
GFP-Asp14
GFP-Asp14 1-112
GFP-ECH_0377
GFP
GFP
Flag
Actin

C.

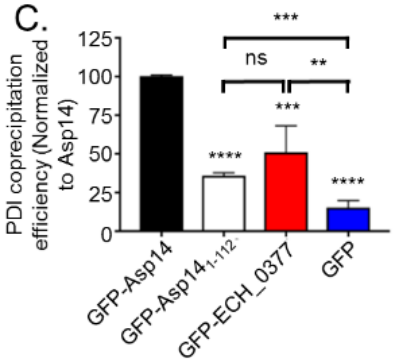

PDI coprecipitation efficiency (Normalized to Asp14)
125
100
75
50
25
0
***
ns
**
***
****
****
GFP-Asp14
GFP-Asp14 1-112
GFP-ECH_0377
GFP

Figure 16: The Asp14 homolog ECH_0377 interacts with PDI more weakly than Asp14

(A) An amino acid sequence alignment of Asp14 and ECH_0377. Hydrophobic, polar, basic, and acid residues are shown in red, green, pink, and blue, respectively. The residues corresponding to the Asp14 binding domain are indicated in a black square. (B) HEK-293T cells expressing Flag-PDI and GFP-Asp14, $Asp14_{1-112,}$ GFP-ECH_0377, or GFP were lysed and analyzed by Flag Co-IP. Transfected cell lysates and Co-IP eluates were analyzed by SDS-PAGE and Western blotting with Flag and GFP-targeting antibodies. Lanes containing irrelevant samples were cropped out. β-actin was examined as a loading control. (C) The flag coprecipitation efficiency was calculated from five experiments. The eluates densitometric GFP:Flag ratio was compared to the GFP:Flag ratio in the input to control for differences in expression. Data shown are representative of five experiments. Statistically significant values are indicated. **,$P<0.01$; ***,$P<0.001$; ****,$P<.0001$; ns, not significant. Statistical indicators are relative to GFP-Asp14, except where relationships are indicated by underlining.

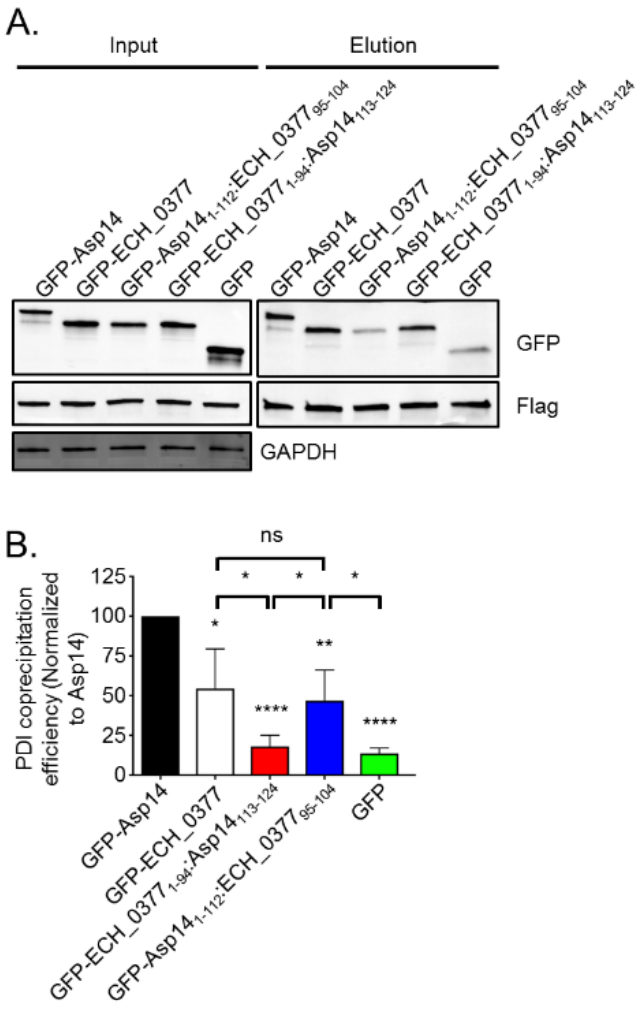
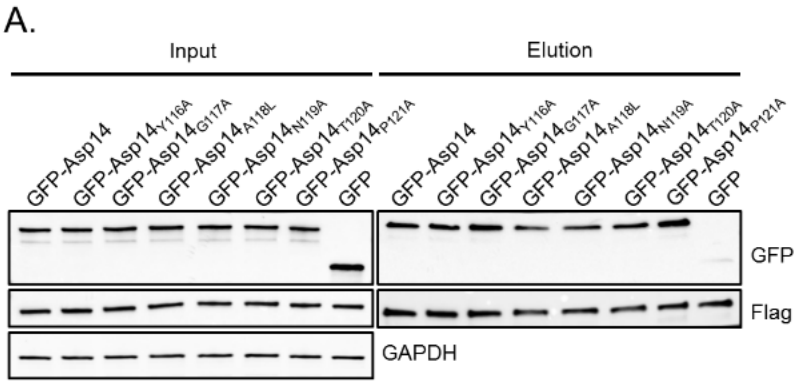


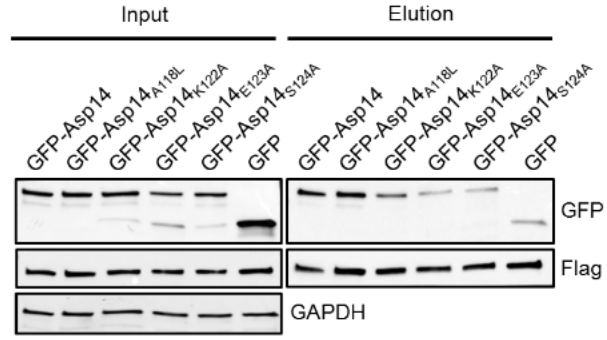
Figure 17: Asp14$_{113-124}$ is essential to interact with PDI, but is not sufficient to confer interaction

(A) HEK-293T cells expressing Flag-PDI and GFP-Asp14, GFP-ECH_0377, Asp14$_{1-112}$:ECH_0377$_{95-104}$, ECH_0377$_{1-94}$:Asp14$_{113-124}$, or GFP were lysed and analyzed by Flag Co-IP. Cell lysates and Co-IP eluates were analyzed by SDS-PAGE and Western blotting with Flag and GFP-targeting antibodies. GAPDH was examined as a loading control. (B) The flag coprecipitation efficiency was calculated from five experiments. The densitometric ratios of GFP:Flag in the eluate were compared to the ratio of GFP:Flag in the input to control for differences in expression. Data shown are representative of five experiments. Statistically significant values are indicated. *,P<0.05; **,P<0.01; ****,P<.0001; ns, not significant. Statistical indicators are relative to GFP-Asp14, except where relationships are indicated by underlining.

5.2.2 Asp14 residues E123 and S124 mediate interaction with PDI; K122, N119, and T120 contribute to this interaction

Having confirmed that $Asp14_{113\text{-}124}$ is critical to the stability of the Asp14:PDI interaction and that that $ECH\_0377_{95\text{-}104}$ is unable to simulate this interaction, we examined the amino acid similarities between Asp14 and ECH_0377 to implicate specific residues that mediate interactions with PDI. It was noted that Asp14 residues A114, Y116, G117, A118, N119, and T120 were unique to Asp14 (Figure 16A). GFP-Asp14 constructs were produced wherein the residues unique to Asp14 were mutated to an alanine, or leucine in the case of alanine. These constructs underwent Co-IP analysis with Flag-PDI as before. It was found that leucine substitution of A114 and alanine substitutions of Y116, G117, and P121 did not significantly alter the pulldown efficiency of Asp14 by PDI (Figure 18A and C). Leucine substitution of A118 and alanine substitution of N119 and T120 significantly reduced the interaction between Asp14 and PDI (Figure 18A and C). These results suggest that A118, N119, and T120 contribute to the interaction with PDI. Additionally, the alanine substitutions of residues K122, E123, and S124 were examined. It was found that alanine substitution of residues E123 and S124 consistently abrogated the Asp14:PDI to levels similar to that of GFP (Figure 18B and C). Alanine substitution of K122 significantly reduced the efficiency of the interaction, but less so than alanine substitution of E123 and S124, indicating that while E123 and S124 mediate the Asp14:PDI interaction, K122 is likely contributory (Figure 18B and C).


A.
Input
Elution
GFP-Asp14
GFP-Asp14Y116A
GFP-Asp14G117A
GFP-Asp14A118L
GFP-Asp14N119A
GFP-Asp14T120A
GFP-Asp14P121A
GFP
GFP
Flag
GAPDH


B.
Input
Elution
GFP-Asp14
GFP-Asp14A118L
GFP-Asp14K122A
GFP-Asp14E123A
GFP-Asp14S124A
GFP
GFP
Flag
GAPDH

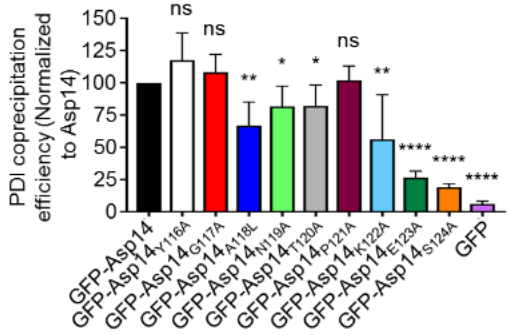

C.
PDI coprecipitation efficiency (Normalized to Asp14)
150
125
100
75
50
25
0
ns
ns
**
*
*
ns
**
****
****
****
GFP-Asp14
GFP-Asp14Y116A
GFP-Asp14G117A
GFP-Asp14A118L
GFP-Asp14N119A
GFP-Asp14T120A
GFP-Asp14P121A
GFP-Asp14K122A
GFP-Asp14E123A
GFP-Asp14S124A
GFP

Figure 18: Asp14 Residues E123 and S124 mediate interaction with PDI

(A) HEK-293T cells expressing Flag-PDI and GFP-Asp14, GFP-Asp14$_{Y116A}$, GFP-Asp14$_{G117A,}$ GFP-Asp14$_{A118L,}$ GFP-Asp14$_{N119A,}$ GFP-Asp14$_{T120A,}$ GFP-Asp14$_{P121A,}$ or GFP were lysed and analyzed by Flag Co-IP. (B) HEK-293T cells expressing Flag-PDI and GFP-Asp14, GFP-Asp14$_{A118L,}$ GFP-Asp14$_{K122A,}$ GFP-Asp14$_{E123A,}$ GFP-Asp14$_{S124A}$ or GFP were lysed and analyzed by Flag Co-IP. Transfected cell lysates and Co-IP eluates were visualized via Western blotting with Flag and GFP-targeting antibodies. GAPDH was examined as a loading control. (B) The flag coprecipitation efficiency was calculated from five experiments. The densitometric ratios of GFP:Flag in the eluate were compared to the ratio of GFP:Flag in the input to control for differences in expression. Data shown are representative of three experiments. Statistically significant values are indicated. *,P<0.05; **,P<0.01; ****,P<.0001; ns, not significant. Statistical indicators are relative to GFP-Asp14, except where relationships are indicated by underlining.

Chapter 6: Conclusions and potential future directions

6.1 Conclusions and future directions: Asp14 and PDI

6.1.1 Discussion: *A. phagocytophilum* Asp14 interacts with host surface PDI, which promotes infection via disulfide reduction

As an obligate intracellular pathogen, *A. phagocytophilum* survival is dependent upon host cell invasion. Thus, dissecting this process is an important avenue to discover new potential therapeutic and prophylactic targets. The *A. phagocytophilum* adhesin Asp14 was known to play a key role in mediating bacterial internalization through interaction with an unknown receptor.

To dissect the role of Asp14 during *A. phagocytophilum* internalization, its interacting partner was determined. PDI was identified and confirmed as an Asp14-interacting partner. Notably, this is the first report identifying a bacterial protein that directly interacts with PDI. In the case of *C. trachomatis,* a PDI-containing complex serves as a receptor promoting bacterial adherence, but no protein is known to interact directly with PDI.[169-171] It was discovered that the Asp14 C-terminus is critical for mediating interaction with PDI. As that same region is responsible for Asp14-mediated internalization and interaction between Asp14 and host cell surfaces, this data supports the hypothesis that PDI is a *bona fide* interacting partner that plays a role during *A. phagocytophilum* internalization.[104,105] Furthermore, this begins to explain why Asp14 internalization and host cell binding are dependent upon that domain—it mediates interactions with PDI, a requirement for efficient infection.

Having confirmed the interaction between Asp14 and PDI, it was necessary to determine its relevance during *A. phagocytophilum* infection. The presence of PDI was confirmed to be important during infection *in vitro* and *in vivo,* validating PDI as a determinant of *A. phagocytophilum* infection. It is important to note that this is the first confirmation of PDI exploitation by a pathogen *in vivo.* As other pathogens specifically utilize PDI-mediated disulfide reduction, the effect of PDI-inhibiting compounds on bacterial infection were examined. It was found that the disulfide reductase-inhibiting compound bacitracin, which inhibits all surface disulfide reductases, drastically inhibited *A. phagocytophilum* infection of mammalian host cells. Monoclonal antibodies that specifically inhibit PDI were used to examine the contribution of PDI to this activity. It was found that inhibition of PDI reduced bacterial infection similarly to PDI siRNA KD. The discrepancy between infection rates following bacitracin and BD34 inhibition may likely be explained by inspecting the disulfide reductase-exploiting pathogen HIV. HIV has been confirmed to utilize the surface oxidoreductases PDI, Trx, and glutaredoxin-1 to promote invasion.[161,162,189] As antibody targeting of Trx-1 reduces the efficiency of *A. phagocytophilum* infection, it is likely that in the absence of PDI, other surface oxidoreductases can be exploited to some degree. All oxidoreductases are inhibited by bacitracin, but not by antibody-mediated inhibition of PDI, supporting that other oxidoreductases may contribute to *A. phagocytophilum* internalization when PDI is inhibited.[196]

Bacterial binding to host cells was not altered by the absence of PDI or its enzymatic inhibition. This indicates the PDI exploitation is exclusively required for bacterial invasion and not binding. It is likely that OmpA, a bacterial protein that plays a major role during

adhesion to host cells, is sufficient to mediate binding to host surfaces when Asp14 cannot interact with PDI.[105,107] Other adhesins such as APH_1234, AipA, or possibly P44 could contribute to bacterial adherence to host cells in the absence of Asp14 binding to PDI. Furthermore, there are *A. phagocytophilum* host receptors, such as CD18 and GPI-anchored proteins that do not have confirmed *A. phagocytophilum* adhesins, it is possible that the interaction between these receptors and their unknown bacterial adhesins also contribute to bacterial binding under these conditions. Ultimately, the cooperative interactions between these adhesin:receptor pairs likely mediate bacterial binding, minimizing the deficit seen when a single adhesin is inhibited.

To further elucidate how *A. phagocytophilum* exploits PDI activity, TCEP and recombinant PDI protein were used to complement PDI inhibition. The chemical reducing agent, TCEP, and recombinant PDI protein, but not enzymatically inactive dmPDI, rescued PDI inhibition-mediated infection defects. This confirmed that *A. phagocytophilum* exploits PDI-mediated disulfide reduction during infection. Furthermore, this indicated that the structural presence of PDI is not truly necessary during bacterial infection. Rather, disulfide reduction is required for *A. phagocytophilum* infection, which can be supplied chemically even in the absence of functional PDI. This requirement is similar to other obligate intracellular pathogens that co-opt PDI, in that those pathogens exploit disulfide reduction to promote invasion.[158,159,164,166,169-171,189] This continues to support that PDI-mediated disulfide reduction during pathogen internalization is thematic across numerous obligate intracellular pathogens. Through the careful investigation of this phenomenon,

novel therapeutic and prophylactic countermeasures may be developed for the pathogens known to rely on this strategy.

Having confirmed the exploitation of disulfide reduction by *A. phagocytophilum,* we next examined its importance in a more physiologically relevant infection model. I first confirmed that PDI is exploited during human neutrophil infection, though to a lesser degree than is seen during HL-60 infection. This discrepancy is likely due to an inherent limitation of this neutrophil model. It is well documented that human neutrophils undergo apoptosis within 24 h of entering circulation.[89] Additionally, this bacterium is known to inhibit neutrophil apoptosis, resulting in an increased lifespan.[90,91,93,94,214] A significant portion of those neutrophils that did not become infected during BD34 treatment presumably apoptosed, altering the surviving population to over represent those cells that did become infected, increasing the apparent level of infection following BD34 treatment. While this discrepancy in the efficacy of BD34 for inhibition of bacterial infection may appear to lessen the importance of PDI during bacterial internalization in human neutrophils, the confirmation that PDI is required for *in vivo* infection of murine neutrophils*,* supports our model that *A. phagocytophilum* requires PDI for optimal infection of neutrophils*.* When this is considered with an understanding of the inherent limitations of examining *ex vivo* neutrophils these results become less detrimental.

Finally, disulfide reduction was examined in ISE6 tick cells, where we found that infection of tick cells is not dependent on surface disulfide reductases. We have previously shown that Asp14 is not expressed during *A. phagocytophilum* infection of ticks.[104] This would

indicate that Asp14 is not present to interact with surface oxidoreductases to promote internalization during tick infections, supporting our observations that surface oxidoreductases are not utilized during bacterial infection of tick cells, despite the presence of a PDI homolog.[199] The presence of surface PDI has not been confirmed on tick cells. It is possible that surface PDI is not present for Asp14 to interact with on tick cells, making Asp14 expression superfluous during the infection of ticks. This indicates another facet of *A. phagocytophilum* internalization—that cellular maintenance within the vector is mediated by different bacterial adhesins.

6.1.2 Discussion: PDI is directed by Asp14 to reduce an *A. phagocytophilum* surface protein

Having confirmed PDI utilization during *A. phagocytophilum* infection, the role of PDI was examined in the context of each major bacterial adhesin. Adhesin-inhibiting antibodies targeting Asp14, AipA, and OmpA, were applied to DC bacteria prior to TCEP treatment. TCEP rescued infection only after Asp14 inhibition, confirming that Asp14 directs the PDI-mediated disulfide reduction required by *A. phagocytophilum* during infection. It is unlikely that PDI directly reduces Asp14, as Asp14 does not contain intramolecular disulfide bonds. Furthermore, Asp14 contains a single Cys, which is unlikely to form intermolecular disulfide bonds due to the propensity of Asp14 to homotrimerize.[104] Additionally, as TCEP did not rescue OmpA and AipA inhibition, disulfide reduction likely does not play a role in their mechanisms of action. This implies that Asp14 interacts with PDI to direct the reduction of some protein on the host or bacterial surface that promotes infection. It is

important to note that this is the first report confirming the interaction between PDI and a bacterial protein during any infection model.

Given that HIV utilizes multiple disulfide reductases to promote internalization, we examined whether *A. phagocytophilum* can exploit non-PDI surface disulfide oxidoreductases.[161,162,189] Inhibition of the surface oxidoreductase Trx reduced infection; however, combined inhibition of Trx and PDI did not result in an additive reduction in infection. This confirmed our previous hypothesis that *A. phagocytophilum* is able to exploit other surface oxidoreductases to fulfil its need for disulfide reduction during infection. However, as Trx inhibition did not reduce infection as robustly as PDI inhibition, it is likely that PDI is preferentially exploited by *A. phagocytophilum.* Furthermore, while both PDI and Trx play a significant role during the infection processes it is likely that additional reductases such as glutaredoxin-1 may be exploited as well, as seen during HIV infection.[162] The PDI- and Trx-inhibiting drug auranofin was also examined to delineate the contribution of each oxidoreductase during *A. phagocytophilum* infection; however, it was found to be cytotoxic. With this in mind we determined that therapeutic targeting of this invasion mechanism is better served by targeting pathogenic determinants rather than PDI itself, as targeting the essential host protein PDI is likely not feasible.

Examination of Asp14-mediated internalization in the absence of the other *A. phagocytophilum* adhesins was attempted, as other interactions may obscure this process. To do this Asp14-conjugated beads were used. While adhesin-conjugated

beads have been used to examine the contribution of a single adhesin to binding, this model did not recapitulate infection studies examining the Asp14:PDI interaction.[105] While Asp14-conjugated beads did not reproduce infection studies, they were still internalized. It is likely that beads conjugated to enormous amounts of a normally lowly expressed protein (Asp14) results in significant non-specific interactions with host surface proteins that may allow internalization of these beads.[104]

Identifying the target of PDI-mediated disulfide reduction would greatly improve understanding of how this phenomenon promotes *A. phagocytophilum* infection. To limit the scope of this undertaking, the surface containing said target was first identified. It was determined that PDI reduces an *A. phagocytophilum,* but not a host surface protein to promote infection. This finding is especially encouraging given the failure of Asp14-conjugated beads to mimic infection studies. Without other *A. phagocytophilum* surface proteins there would be no substrate to reduce, eliminating the ability of TCEP to rescue internalization defects. The inability of Asp14 alone to mimic the Asp14:PDI interactions seen during infection supports our model that Asp14 directs PDI to reduce another bacterial surface protein, while Asp14 itself does not become reduced.

The confirmation that a bacterial surface protein is reduced leads to several possibilities for the function of disulfide reduction during *A. phagocytophilum* infection. The most likely use of disulfide reduction is that an as of yet unknown *A. phagocytophilum* adhesin is reduced by PDI, resulting in a conformational change that allows interaction with or increases the affinity for its substrate. This increased or allowed interaction would then

be suspected to mediate internalization. This is similar to what is seen during integrin functioning. Disulfide reduction converts integrins to an active conformation, allowing interaction with their substrate.[128,144,215] Furthermore, this is seen during HIV infection. During the internalization process gp120 becomes reduced, increasing its affinity for its co-receptor CXCR4/CCR5, thereby driving internalization.[154,158] Alternatively, the reduction of a bacterial protein may induce a signal into the bacterium that alters gene expression. In the case of *C. trachomatis* it has been suggested that PDI reduces disulfides within the heavily disulfide crosslinked P-layer to transduce a signal into the bacterial cytosol that results in transcriptional changes.[172,173,216] The *A. phagocytophilum* DC protein P44 has been shown to form large disulfide crosslinked hetero- and homomultimeric complexes, similarly to proteins of the *Chlamydial* P-layer.[216] Furthermore, we have reported that during the first four h of *A. phagocytophilum* infection, a period that corresponds to binding and internalization, expression of the adhesins OmpA and Asp14 are transcriptionally upregulated.[104,107]. It is possible that disulfide reduction of the *A. phagocytophilum* surface transduces a signal to alter *A. phagocytophilum,* resulting in the observed upregulation of adhesin expression, presumably to promote internalization. Alternatively, in *C. trachomatis,* it has been hypothesized that these transcriptional changes function to promote the transition from the infectious elementary body to the replicative reticulate body.[168] It is possible this hypothesized signal alters gene expression to initiate the DC to RC transition, serving as an indication of the bacteria being proximal to a host cell.

MS analysis of reduced DC surface proteins implicated several proteins of interest. DNAK, DNA binding protein HU, 10-kDa chaperonin protein, and APH_1235 were consistently more prevalent in wtPDI treated samples compared to dmPDI treated samples. DnaK is a heat shock chaperone that has been implicated as an adhesin that mediates *A. phagocytophilum* infection of tick cells. This may also be true in mammalian hosts, as vaccination of sheep with Hsp70 is slightly protective.[207,217] *Helicobacter pylori* and *Listeria monocytogenes* also use DNAK as an adhesin.[209,218] APH_1235 has previously been implicated as a potential *A. phagocytophilum* adhesin through antibody blocking studies. Additionally, APH_1235 expression is highly upregulated during the RC-to-DC conversion and in infectious DC bacteria indicating its importance to the infectious DC form.[111,112]

As we have implicated APH_1235 as an *A. phagocytophilum* adhesin, it was chosen for further investigation. Disulfide reduction did not rescue APH_1235 inhibition. Dual APH_1235:Asp14 inhibition did inhibit TCEP rescue of Asp14, indicating that dual inhibition could block the same pathway of PDI utilization. However, this result was inconsistent between experiments. This indicates that APH_1235 could be a target of PDI-mediated reduction, but more study is needed. It is possible that the polyclonal APH_1235 antisera utilized inconsistently prevents the ability of APH_1235 to perform its function after becoming reduced, obscuring the resolution of this study. Recent, unpublished work from our lab has identified the binding domain of APH_1235. Antisera targeting this binding domain would likely improve the resolution of this experiment.

6.1.3 Discussion: Asp14 interacts with PDI via residues E123 and S124

To further examine the importance of the Asp14 binding domain towards PDI interaction and the individual residues that mediate that interaction, the *E. chaffeensis* Asp14 homolog ECH_0377 was examined. It was found that ECH_0377 interacts with PDI more weakly than Asp14. These results indicated that the C-terminal amino acids shared between *Ehrlichia* and *A. phagocytophilum* likely do not participate in the Asp14:PDI interaction, implicating the differing residues mediating the interaction with PDI. To further delineate the importance of the Asp14 C-terminal residues, $Asp14_{1\text{-}112}$:$ECH\_0377_{95\text{-}104}$ and $ECH\_0377_{1\text{-}95}$:$Asp14_{113\text{-}124}$ were produced to examine the effects of the two C-termini on the ability of the other to interact with PDI. It was found that $ECH\_0377_{95\text{-}104}$ abrogated Asp14 interaction with PDI indicating that while ECH_0377 interacts with PDI, its C-terminus is not sufficient to confer any PDI interaction to $Asp14_{1\text{-}112}$. This indicates that the domain mediating PDI interaction with ECH_0377 is likely not within its C-terminus. Alternatively, ECH_0377 may interact with another surface disulfide reductase such as Trx, as HIV has been shown to do.[161,219,220] In this case $ECH\_0377_{95\text{-}104}$ would likely not be sufficient to confer a strong interaction with PDI. $Asp14_{113\text{-}124}$ did not significantly improve ECH_0377 binding to PDI. These data suggested that $Asp14_{113\text{-}124}$, while being critical, is not the sole determinant of the Asp14:PDI interaction. It is possible that other regions of Asp14 may play a role in stabilizing the interaction with PDI. Examination of the Asp14 and ECH_0377 sequence alignment shows that there are significant differences between Asp14 and ECH_0377 beyond the C-terminus. Specifically Asp14 residues 1-18 are completely unique to *A. phagocytophilum.* It is possible that this region may contribute to the interaction between Asp14 and PDI. Given the reduction in PDI

interaction when the Asp14 C-terminus is removed, it would be likely that this region functions to maintain the proper conformation of protein rather than directly interacting with PDI. Thus when ECH_0377 is fused to $Asp14_{113\text{-}124}$, interaction with PDI is not improved as $Asp14_{1\text{-}18}$ is not present maintain the functional conformation of the C-terminus.

Examination of sequence differences between $Asp14_{113\text{-}124}$ and $ECH\_0377_{95\text{-}104}$ indicated that Asp14 amino acids A114, Y116, G117, A118, N119, T120, and P121 may be critical to the ability of Asp14 and PDI to interact. Alanine-substituted point mutants of each residue were produced and examined to determine their impact on Asp14 binding to PDI. It was found that residues Y116, G117, and P121 do not contribute to this interaction. N119 and T120 minimally promote the interaction, whereas leucine substitution of A118 significantly disrupts the interaction. As none of these mutants abrogated interaction with PDI similarly to the loss of the C-terminus, additional C-terminal amino acids were examined. It was found that alanine substation of K122 reduces the efficiency of the Asp14:PDI interaction while alanine substitution of E123 and S124 nearly abrogate the interaction. In total these data indicate that E123 and S124 are critical for the Asp14:PDI interaction while K122, N119, and T120 contribute to the interaction.

In silico structural prediction of Asp14 indicated that $Asp14_{113\text{-}124}$ is disordered. Recently, disordered proteins and regions have become recognized as features that mediate protein-protein interactions.[221] Protein interactions mediated by disordered regions are described to occur via two different mechanisms. Interaction of the disordered region with

a region of the target protein, resulting in conformational changes producing a stable binding motif in the disordered region. Alternatively, disordered regions are thought to provide an otherwise unachievable level of flexibility that allows for interactions with hidden or buried binding sites on target proteins.[221] It is likely that the disorder of $Asp14_{113-124}$ is a structural feature that is critical for Asp14 binding to PDI. This is indicated by the inhibition of the interaction during leucine substitution of A118. It is known that large hydrophobic residues, such as leucine, disrupt disordered regions by promoting hydrophobic interactions with other regions of the protein.[221].[221] As such we hypothesize that E123 and S124 are critical to the Asp14 interaction with PDI, in a manner dependent upon the disordered nature of $Asp14_{113-124.}$ Furthermore N119, T120, and K122 play a contributory role to this interaction. The identification of these residues marks the first instance of a pathogen-derived PDI-interacting motif, which could potentially be used to identify PDI-interacting proteins in other pathogens.

6.2 Conclusion

In the context of *A. phagocytophilum* invasion of host cells, OmpA first interacts with sLe^x on the surface of host cells; this stage serves as a docking step to adhere the bacterium to the host surface.[105,107] This likely brings Asp14 into contact with PDI. The two proteins interact by virtue of a novel interacting domain within Asp14. This interaction then directs PDI-mediated disulfide reduction of an *A. phagocytophilum* outer membrane protein. It is suspected that the reduction of that protein results in a conformational change in that protein that presumably allows the protein to act as an adhesin, binding to a receptor and inducing internalization of the bacteria. The delineation of this model benefits future

studies as not only a road map for dissecting the exploitation of host PDI, but also through the identification of a novel PDI-interacting motif which could be used to identify putative PDI-interacting partners utilized by other pathogens.

6.3 Future directions

There are many avenues of research left to explore regarding *A. phagocytophilum* binding to and invasion of host cells. In these studies we meticulously explored the mechanism through which Asp14 exploits PDI. However, we were unable to conclusively identify the target of PDI-mediated disulfide reduction. The primary goal of any future Asp14 studies would be to determine the target of this disulfide reduction. The possibility that APH_1235 is reduced must be revisited. Recently, our lab has determined the specific region of APH_1235 that constitutes its binding domain. The use monoclonal antibodies against this region would be an ideal next step. Additionally, DnaK, another putative *A. phagocytophilum* adhesin, was implicated as a potential target of PDI-mediated reduction. Confirmation of this possibility must also be investigated.

While the PDI-interacting motif of Asp14 has been identified, there is still much that could be gained from further study. It would be beneficial to determine the specific biochemical requirements that mediate this interaction. This would be determined by additional mutational analysis of the interacting residues. Mutations determining the importance of residue charge to K122 and E123 and polarity to S124 would be the most beneficial. Additionally, it would be beneficial to identify the PDI residues that mediate the Asp14:PDI interaction. Yeast two-hybrid analysis of PDI and Asp14 indicated that Asp14 likely

interacts with PDI between residues 110-294. Deletion of amino acid stretches within this region along with Co-IP analysis could indicate the specific stretch of amino acids that interacts with Asp14.

The Asp14 homolog ECH_0377 offers a wealth of additional studies. Given that ECH_0377 interacts with PDI, if more weakly than Asp14, it may perform a similar function during *E. chaffeensis* invasion of host cells. It has been shown that *E. chaffeensis* invades host cells via the entry triggering protein of *Ehrlichia* (EptE) that interacts with host cell DNase X, resulting in actin polymerization that drives bacterial internalization.[222] Determining the role that PDI may play during infection in the context of this organism may offer further insight into PDI exploitation by other pathogens. Furthermore, as PDI utilization appears thematic across the internalization processes of obligate intracellular pathogens, other organisms could be examined for PDI exploitation, including members of the family *Rickettsiaceae*, specifically *Orientia tsutsugamushi,* which our lab also studies.

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Vitae

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Publications

1. Cockburn CL, **Green RS**, Damele SR, Martin RK, Ghahrai NN, Colonne PM, Fullerton MS, Conrad DH, Chalfant CE, Voth DE, Rucks EA, Gilk SD, and Carlyon JA 2019. Functional inhibition of acid sphingomyelinase disrupts infection by intracellular bacteria. Life Science Alliance. 2019 doi:10.26508/lsa.201800292

2. Naimi WA, **Green RS**, Cockburn CL, Carlyon JA. 2018. Differential Susceptibility of Male Versus Female Laboratory Mice to *Anaplasma phagocytophilum* Infection. *Trop Med Infect Dis*. doi:10.3390/tropicalmed3030078

3. Evans SM, Adcox HE, VieBrock L, **Green RS**, Luce-Fedrow A, Chattopadhyay S, Jiang J, Marconi RT, Paris D, Richards L, and Carlyon JA. 2018 Outer Membrane Protein A Conservation among *Orientia tsutsugamushi* Isolates Suggests Its Potential as a Protective Antigen and Diagnostic Target. *Trop Med Infect Dis*. doi:10.3390/tropicalmed3020063

4. Beyer AR, Rodino KG, L. VieBrock, **Green RS**, Tegels BK, Oliver LD, Marchoni RT, and Carlyon JA 2017. *Orientia tsutsugamushi* Ank9 is a multifunctional effector that utilizes a novel GRIP-like Golgi localization domain for Golgi-to-endoplasmic reticulum trafficking and interacts with COPB2. Cell Microbiol. doi:10.1111/cmi.12727.

Posters

1. **Green, R**., Naimi, W., Martin, R., Conrad, D., Cho, J., O'Beir, N., Oliver, L., Marconi, R., and Carlyon J. June 2019 Abstract for poster presentation, *Anaplasma phagocytophilum* Asp14 Exploits Host Cell Surface Protein Disulfide Isomerase Activity to Promote Infection, the American Society for Rickettsiology Annual Meeting. Santa Fe, NM.

2. **Green, R**., Naimi, W., Martin, R., Conrad, D., Cho, J., O'Beir, N., Oliver, L., Marconi, R., and Carlyon J. June 2018 Abstract for poster presentation, The *Anaplasma phagocytophilum* Invasin Asp14 Co-Opts Host Cell Surface Protein Disulfide Isomerase Activity to Promote Infection, the American Society for Microbiology Annual Meeting (Microbe 2018). Atlanta, Ga.

3. **Green, R**. and Carlyon J. Nov 2016 Abstract for poster presentation, The *Anaplasma phagocytophilum* adhesin Asp14 co-opts the activity of protein disulfide isomerase for the infection of host cells, Gordon Archer Research Day in ID and Microbiology and Immunology. Richmond, Va.

4. Beyer, A., **Green, R.,** and Carlyon, J. Nov 2015. Abstract for poster presentation, Orientia tsutsugamushi Ank9 interacts with COPB2 to Co-opt Host Cell Retrograde Traffic, Gordon Archer Research Day in ID and Microbiology and Immunology. Richmond, Va.

5. **Green, R.,** Beyer, A., and Carlyon, J. Nov 2015. Abstract for poster presentation, The Ank9 effector of the intracellular pathogen Orientia tsutsugamushi targets host COPB2 to co-opt Golgi-to-endoplasmic reticulum retrograde trafficking, American Society for Microbiology Virginia Branch 2015 Annual Meeting. Richmond, Va

6. **Green, R**. and Lewis, L. 2013. Abstract for poster presentation, Chloroquine's Anti-retroviral Properties. Virginia Academy of Science 91st Annual Meeting. Blacksburg, Va.

7. **Green, R.** and Lewis, L. 2012. Abstract for poster presentation, A Study of Chloroquine's Anti-retroviral Characteristics, American Society for Microbiology Virginia Branch 2012 Annual Meeting. Norfolk, Va.

8. **Green, R**. and Lewis, L. 2012. Abstract for poster presentation, A Study of Chloroquine's Antiretroviral Characteristics, Virginia Academy of Science Fall Undergraduate Research Meeting. Richmond, Va.

<u>Presentations and Seminars</u>

1. **Green, R**., Naimi, W., Martin, R., Conrad, D., Cho, J., O'Beir, N., Oliver, L., Marconi, R., and Carlyon J. June 2018 Abstract for oral presentation, *Anaplasma phagocytophilum* Asp14 Exploits Host Cell Surface Protein Disulfide Isomerase Activity to Promote Infection, the American Society for Rickettsiology Annual Meeting. Milwaukee, Wi.
2. **Green, R**., Naimi, W., Martin, R., Conrad, D., Cho, J., O'Beir, N., Oliver, L., Marconi, R., and Carlyon J. March 2016. Departmental Seminar. The *Anaplasma phagocytophilum* Adhesin Asp14 Exploits the Activity of Protein Disulfide Isomerase to Promote Infection. Department of Microbiology and Immunology. Virginia Commonwealth University. Richmond, Va.

3. **Green, R**., Naimi, W., Martin, R., Conrad, D., Cho, J., O'Beir, N., Oliver, L., Marconi, R., and Carlyon J. December 2017. Departmental Seminar. The *Anaplasma phagocytophilum* Invasin Asp14 Exploits the Activity of Protein Disulfide Isomerase to Promote Infection. Department of Microbiology and Immunology. Virginia Commonwealth University. Richmond, Va.

4. **Green, R**. and Carlyon J. March 2016. Departmental Seminar. The *Anaplasma phagocytophilum* Adhesin Asp14 Co-opts the Activity of Protein Disulfide Isomerase to Promote Infection. Department of Microbiology and Immunology. Virginia Commonwealth University. Richmond, Va.

Awards and Honors:

- Dean's List, University of Mary Washington, Fall 2009-Fall 2010, Spring 2012-fall 2012
- Presidents List, University of Mary Washington, Fall 2011 and Spring 2013
- Rebecca Culbertson Scholarship for Outstanding Biology Senior, University of Mary Washington, 2012
- Independent research grant from the University of Mary Washington, 2012-2013
- Virginia Academy of Sciences undergraduate research grant, 2012
- B.S. Awarded Summa cum Laude with Departmental Honors, 2013
- Phi Kappa Phi Nomination Award, Virginia Commonwealth University, 2016
- American society for Rickettsiology Travel Award, 2018
- American society for Rickettsiology Travel Award, 2019
- Mary P. Coleman Microbiology and Immunology award for extraordinary achievement in graduate studies and research, Virginia Commonwealth University,2019
- Professional Memberships

2014-Present	American Society for Microbiology
2015-2017	American Heart Association
2016-Present	American Society for Rickettsiology