Transmission of *Anaplasma phagocytophilum* from endothelial cells to peripheral granulocytes *in vitro* under shear flow conditions

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For my family

Content

Figures and Tables

Figures and Tables in Appendix

Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
A. phagocytophilum	*Anaplasma phagocytophilum*
AipA	*Anaplasma phagocytophilum* invasion protein A
AL	autolysosomes
AmpA	*Anaplasma phagocytophilum* post-translationally modified protein A
AnkA	ankyrin-rich protein A
AP	autophagosomes
ASC	apoptosis-associated speck-like protein containing a CARD
Asp14	14-*Anaplasma phagocytophilum* surface protein
Atg	autophagy-related protein
Ats-1	*Anaplasma* translocated substrate-1
B. burgdorferi	*Borrelia burgdorferi*
Bcl-2 protein	B-cell lymphoma 2 protein
bfl-1	a member of antiapoptotic *bcl-2* genes family
bp	base pair(s)
CD62L	L-selectin
CG8	alpha-fodrin
CGA	canine granulocytic anaplasmosis
cIAP2	the cellular inhibitor of apoptosis protein 2
CXCR	CXC chemokine receptor
DC	dense-cored form of *Anaplasma phagocytophilum*
DEET	*N,N*-Diethyl-*meta*-toluamide
Depleted medium	hydrocortisone-free medium
DMSO	dimethylsulfoxide
E.	*Ehrlichia*
E. coli	*Escherichia coli*
EE	early endosome
EGA	equine granulocytic anaplasmosis
ELISA	the enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERC	endocytic recycling centre
Fuc-T	alpha-(1, 3) fucosyltransferase
GFP	green fluorescence protein

GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HGA	human granulocytic anaplasmosis
HL-60	human promyelocytic leukemia cell line
HME	human monocytotropic ehrlichiosis
HOCl	hypochlorous acid
IAP protein	inhibitors of apoptosis protein
IC	pre-Golgi intermediate compartment
iE-DAP	D-glutamyl-meso-diaminopimelic acid
IFA	indirect immunofluorescent assay
IFN-ɣ	interferon gamma
IL-10	interleukin 10
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-6	interleukin 6
IL-8	interleukin 8
IV	intravenously
JNK2	c-jun NH2-terminal kinase 2
kDa	Kilo-Dalton
kg	kilogram
LAMP-3	lysosome-associated membrane protein 3
LC3	light chain 3
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPS	lipopolysaccharides
Mac-1	Macrophage-1 antigen
Mb	Mega base pairs
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1 or CCL2
MDP	membrane-derived peptidoglycan components
mg	microgram
Msp2	major surface protein 2
N.	*Neorickettsia*
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase

NF-κB pathway	nuclear factor kappa-light-chain-enhancer of activated B cells pathway
NK cells	natural killer cells
NKT cells	natural killer T cells
NLR	Nod-like receptor
NOD	nucleotide-binding oligomerization domain
NOD1	nucleotide-binding oligomerization domain-containing protein 1
Ø	diameter
O_2^-	superoxide anion
-OH	hydroxyl radical
OmpA	outer membrane protein A
OMPs	outer membrane proteins
Pal	peptidoglycan-associated lipoprotein
PAMPs	pathogen associated molecular patterns
PBLs	peripheral blood leukocytes
PCR	Polymerase chain reaction
PI3K/Akt pathway	phosphatidylinositol 3-kinase/protein kinase-B pathway
PI3K	phosphatidylinositol 3-kinase
PM	plasma membrane
PMA	phorbol myristate acetate
PMNs	polymorphonuclear leukocytes
PO	by mouth – per oral
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1, CD162
PTMs	Post-translational modifications
PVMs	pathogen-occupied vacuolar membranes
Rabs	Ras-like GTPases
RE	recycling endosome
Rip2	receptor interacting protein-2
RNAi	RNA interference
ROCK1	Rho-associated protein kinase 1
ROS	reactive oxygen species
siRNA	small interfering RNA
sLe^X	sialyl Lewis^X, or sialyl Le^X, or CD15s
STAT	signal transducer and activator of transcription

Syk	spleen tyrosine kinase
T4SS	Type IV secretion system
TGF-β	transforming growth factor beta
TGN	*trans*-Golgi network
Th1 cells	type 1 of T helper cells
THP-1	an acute human monocytic leukemia cell line
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor alpha
XIAP	x-linked inhibitor of apoptosis protein

I INTRODUCTION

Anaplasma phagocytophilum (*Ap*) is a gram-negative, obligate intracellular bacterium that is able to infect different animal species and humans worldwide. Based on DNA sequencing, *Ap* has newly been reallocated from the genus *Ehrlichia* to the genus *Anaplasma* in the family Anaplasmataceae (DUMLER et al. 2001). In humans and animals, the clinical signs of *Ap* infection vary from mild symptoms to severe clinical outcomes, including death. However, the disease generally presents as undifferentiated fever accompanied by leucopenia, thrombocytopenia and increased serum transaminase activities (DUMLER et al. 2005; DUMLER et al. 2007; RIKIHISA 2011).

Hard-bodied ticks of the genus *Ixodes* (family Ixodidae) are the main vectors for *Ap* dissemination. Compared to other pathogens such as *Neorickettsia* and *Wolbachia* spp., which can be transmitted from adult ticks to their offspring, *Anaplasma* and *Ehrlichia* spp. are the only Rickettsiales that are not transmitted transovarially (RIKIHISA 2011). Thus, ticks need to acquire *Ap* through blood feeding from infected hosts to complete the life cycle of *Ap*. During attachment of the tick, the bacterium is released by salivary secretion and is transmitted to the host. It is known that *Ap* multiplies within membrane-bound vacuoles (or called 'morulae') in the cytoplasm of peripheral granulocytes. The binding and infection of bacteria depends on the tetrasaccharide sialyl Lewis^x (sLe^x or CD15s) of P-selectin glycoprotein ligand 1 (PSGL-1) on the surface of host cells, a factor expressed on peripheral granulocytes and HL-60 cells (GOODMAN et al. 1999; HERRON et al. 2000; RENEER et al. 2006; RENEER et al. 2008).

Only little information is known about the transmission pathway of *Ap* after tick bite in the very early stage of infection. It is described that *Ap* is able to evade and replicate within microvascular endothelial cells *in vitro* (MUNDERLOH et al. 2004), while endothelial cells lining the inner lumen of blood vessels allow them to easily interact with any circulating blood cells. Since granulocytes do not return back to the blood stream after extravasation, it is reasonable to postulate that *Ap* evades and replicates within microvascular endothelial cells in the initial transmission, and subsequently transmits into peripheral granulocytes for ongoing dissemination.

Therefore, the objective of the study was to establish a flow culture model that mimics the physiological environment in the blood vessel to study the possible transmission pathway of *Ap* between endothelial cells and polymorphonuclear leukocytes (PMNs). For this purpose, a

novel *ex vivo* flow culture system was established. For experimental setup, human microvascular endothelial cell line (HMEC-1) and primary human dermal microvascular endothelial cells (HDMEC) were used. Under static conditions, *Ap* evades endothelial cells within 24 h, supporting the hypotheses that endothelial cells might be the first infection site of the pathogen in the host. Thereby a high level of interleukin-8, a chemokine that is known to recruit PMNs, secreted by *Ap*-infected endothelial cells was detected. Using the investigated flow culture model, it was shown for the first time, that *Ap* is able to translocate from endothelial cells to PMNs under dynamic flow conditions. Furthermore, under defined shear stress, an increased binding of PMNs to *Ap*-infected endothelial cells monolayer was observed, resulting from the elevated expression of adhesion molecules associated with PMNs recruitment on endothelial cells.

The flow culture model investigated in this study can be used to study the interaction between *Ap*-infected endothelial cells and PMNs under physiological flow conditions, and is therefore helpful to study the infection mechanism in the early stage of *Ap* dissemination in the host.

II REVIEW OF THE LITERATURE

1 Historical Background of *Anaplasma phagocytophilum* (*Ap*)

A. phagocytophilum (*Ap*) was first recognized as the infectious entity of a distinct tick-transmitted disease of sheep in Scotland in 1932 by Macleod (MACLEOD 1932). Later the pathogen was demonstrated as the causative agent of the illness (tick-borne fever) in sheep by Gordon in 1940 and in cattle by Hudson in 1950 (WOLDEHIWET 1983). The first equine granulocytic anaplasmosis (formerly equine granulocytic ehrlichiosis) was reported as a disease of horses in California by Gribble and Stannard in 1969 (GRIBBLE 1969; STANNARD et al. 1969). Until today the epidemic areas of equine granulocytic anaplasmosis (EGA) include the United States, Europe, Asia and Northern Africa (DZIEGIEL et al. 2013). Dogs were first identified with *Ap* infection in California in 1982 (MADEWELL et al. 1982). Beside the United States, the canine granulocytic anaplasmosis (CGA) has been found in Germany, Italy, Poland, Spain, Sweden, Switzerland, UK and Japan (CARRADE et al. 2009). The first human granulocytic anaplasmosis (HGA) was described in 1990 in a patient from Wisconsin presenting a febrile illness accompanied by granulocytic cytoplasmic morulae in the peripheral blood (CHEN et al. 1994). HGA has become a notifiable disease in the United States since 1998, and the number of reported cases of anaplasmosis increased by approximately 50 %, from 1761 cases in 2010 to 2575 cases in 2011 (ADAMS et al. 2013).

2 Etiology

2.1 Cell Morphology and Development Stages

Ap is a small gram-negative, obligate intracellular bacterium. However, Gram staining is not suitable to visualize intracellular bacteria because of low contrast against the cytoplasm. Therefore Romanowsky staining is generally used, such as Wright-Giemsa staining dye that allows staining the bacteria in dark-blue to pale-blue in the infected cells (DUMLER et al. 2007; RIKIHISA 2011). *Ap* replicates in membrane-bound vacuoles within the cytoplasm of peripheral granulocytes (mainly neutrophils), and other eukaryotic host cells such as endothelial cells and tick cells. Such membrane-bounded vacuoles are generally 1.5 to 2.5 µm in diameter but can be as large as 6 µm (POPOV et al. 1998). The size of a single bacterium is generally 0.4 to 1.3 µm, and up to 2 µm in diameter (POPOV et al. 1998; RIKIHISA 2011).

Electron microscopy analysis shows two morphological cell forms of *Ap* in mammalian and tick cell cultures. First, the larger coccoid or elongated reticulate cells (RC[1]; 0.4 - 0.6 × 0.7 - 1.9 µm, Ø; Figure 1a) that are characterized by ribosomes and DNA strands that are spread over the entire cytoplasm. Second, the smaller coccoid dense-cored cells (DC[2]; 0.4 - 0.6 µm, Ø, Figure 1b) that are distinguished by ribosomes and DNA that are localized to the center of the cell (POPOV et al. 1998; MUNDERLOH et al. 1999; RAR et al. 2011). Both morphological cell forms have two layers of cell membranes comprising a smooth inner membrane and an irregular and loose outer membrane. The folding of the membranes leads to the enlargement of the periplasmic space (POPOV et al. 1998). The pathophysiology role of each form in the pathogenesis of *Ap* infection is unclear (TROESE et al. 2009). However, it is believed that both types are developmental stages similar to those described for other intracellular bacteria such as *Chlamydia* species (MUNDERLOH et al. 1999). Thus far, it is described that *Ap* undergoes a biphasic developmental cycle between an infectious DC form and a non-infectious replicating RC form (TROESE et al. 2009; TROESE et al. 2011).

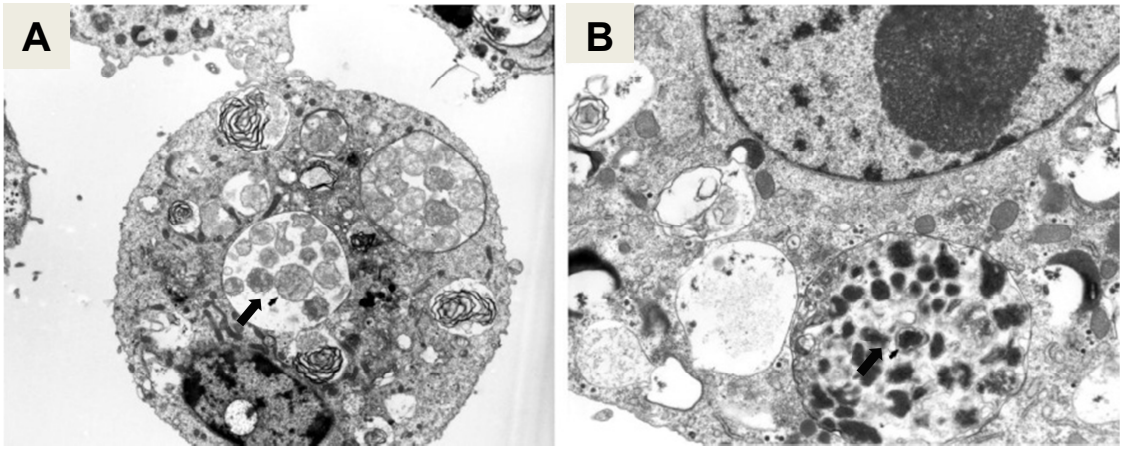

Figure 1: Two different morphotypes of *Ap* representing within tick cells

Cited from Dyachenko at al. (DYACHENKO et al. 2013)
a RC (5000×) and **b** DC (10000×) of *Ap* (*Ap*Muc01c, isolated from a canine blood sample) within the membrane-bound vacuoles in the cytoplasm of IRE/CTVM20 cells (*Ixodes ricinus*-derived cell line).

2.2 Taxonomy of *Ap*

Ap was previously referred to as *Ehrlichia phagocytophila* (mostly prevalent in Europe, the cause of tick-borne fever in ruminants), *Ehrlichia equi* (mostly prevalent in Western United Sates in horses) and the HGE agent in humans (the agent of human granulocytic ehrlichiosis

[1] RC: reticulate cell form
[2] DC: dense-cored cell form

worldwide) (WOLDEHIWET 2010). Based on molecular analysis of bacterial 16S rRNA and *groESL* gene sequences, *Ap* has been reclassified to the genus *Anaplasma*, in the family Anaplasmataceae in the order Rickettsiales (Figure 2) (DUMLER et al. 2001). The other bacterial species in the genus *Anaplasma* comprise: *A. marginale*, *A. centrale* and *A. bovis* causing diseases in bovine and wildlife animals, *A. platys* causing cyclic thrombocytopenia in dogs, *A. ovis* causing diseases in sheep, goats and wild ruminants (DUMLER et al. 2001; RAR et al. 2011; RIKIHISA 2011). However, the 16S rRNA gene sequences of *A. marginale*, *A. central* and *A. ovis* shown a minimum of 99.1 % similarity, suggesting the possibility that they represent different subspecies (THEILER 1911; DUMLER et al. 2001).

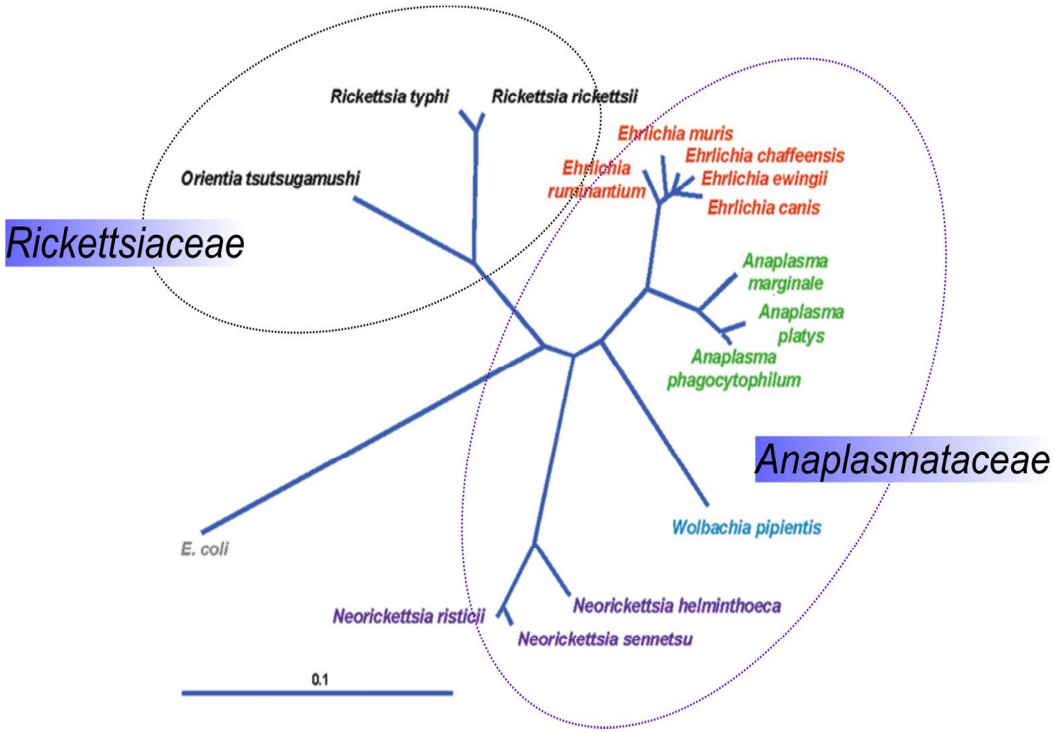

Figure 2: Current taxonomic classification of genera in the family Anaplasmataceae

Modified from Dumler et al. (DUMLER et al. 2005)
The phylogram is constructed based on the 16S rRNA sequences of these species. The distance bar represents substitution per 1,000 bp[3]. *E. coli, Escherichia coli*.

[3] bp = base pair(s)

2.3 Life Cycle of *Ap*

The life cycle of *Ap* is subdivided into the reproduction stages in the *Ixodes* ticks (hard-bodied ticks) and susceptible vertebrate animals and the transmission cycle, in which the pathogen is spread among different individuals by *Ixodes* ticks (RIKIHISA 1991; RAR et al. 2011). It was recently demonstrated that transplacental transmission in mammals occur and may play a role in the transmission pathway (REPPERT et al. 2013). That is underlined by experiments, in which a mother sheep was experimentally infected with *Ap*, resulting in a lamb that was also infected with *Ap* after birth (REPPERT et al. 2013). There is no evidence that transovarial transmission occurs during *Ap* reproduction in ticks (HOTOPP et al. 2006). Once *Ixodes* ticks acquire the bacterium from infected mammals, ticks are able to maintain the pathogen from the larva or nymph stage to adults. *Ap* first enters the tick midgut epithelium, where their primary replication takes place and then moves to the secretory salivary acini in the tick salivary glands (TELFORD et al. 1996). Afterwards, *Ap* is transmitted to the mammalian host from the salivary glands during tick feeding (REUBEL et al. 1998; FELEK et al. 2004). Experimentally, uninfected larval ticks begin to acquire *Ap* from infected mice within 24 h of attachment. Infected nymphal ticks are able to transmit *Ap* to the host within 24 h (DES VIGNES et al. 2001) to 48 h after attachment (HODZIC et al. 1998a; KATAVOLOS et al. 1998). *Ap* is naturally maintained in a cycle (Figure 3) between ticks and wild animals such as deer and rodents (RIKIHISA 2011).

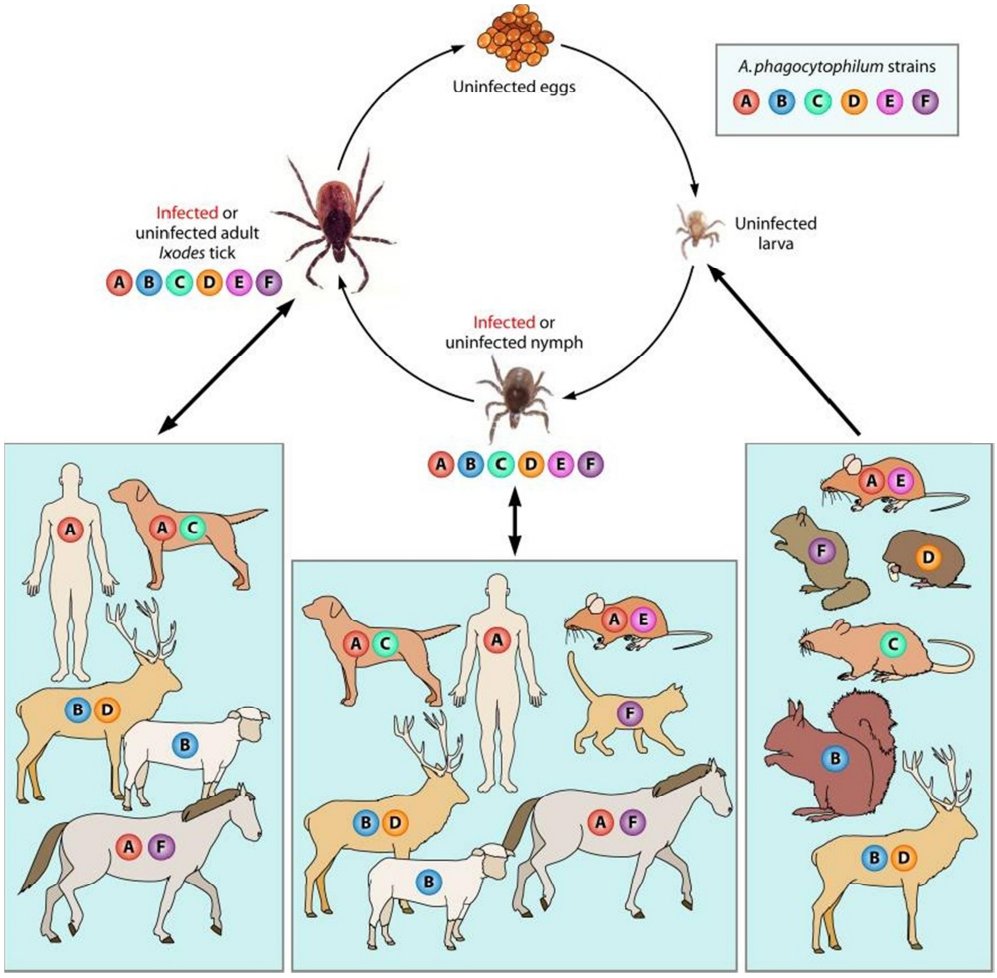

Figure 3: Proposed life cycle of *Ap*

Cited from Rikihisa (RIKIHISA 2011)
Diverse strains of *Ap* (e.g. A - F) exist in nature; susceptibilities of mammalian species to *Ap* vary. The animal species susceptibility to putative *Anaplasma* strains shown is a proposal, most of which has not been proven experimentally.
Humans are only susceptible to some strains. Therefore humans are considered dead-end host of *Ap*.

3 Epidemiology

3.1 Transmission Vectors, Global Distribution and Prevalence

Ticks in the genus *Ixodes* are the main vectors for transmission of *Ap* to mammalian hosts (BAKKEN et al. 2008). The worldwide distribution of *Ap* infection follows the local distribution of its primary tick vector *Ixodes* spp. (see Figure 4). Several studies used PCR methodology to detect *Ap* in different tick species. The DNA of *Ap* was detected in *I scapularis* (black-legged tick), *I. pacificus* (western black-legged tick) and *I. spinipalpis* in the United States, *I. ricinus* (castor bean tick) in Europe (VON LOEWENICH et al. 2003; SCHORN et al. 2011; WALLMENIUS et al. 2012) and *I. persulcatus* (taiga tick) in Russia

(RAR et al. 2005), China (CAO et al. 2003) and other Asian countries (OHASHI et al. 2005). Recently human-to-human transmission (nosocomial transmission) is reported to possibly occur in China in 2008 (ZHANG et al. 2008a). However, ticks are still the main transmission vector of *Ap* and naturally infected reservoir hosts are thought to be necessary to complete the life cycle of bacteria (RIKIHISA 2010). Different methods were developed to investigate the prevalence of *Ap* infection in mammals and ticks. In Germany, *Ap* recently was detected in tissue samples from red foxes (*Vulpes vulpes*, 8.2 %) and raccoon dogs (*Nyctereutes procyonoides*, 23 %) by real-time PCR (HARTWIG et al. 2014). In southern Germany, a significantly higher prevalence of HGE antibodies was present among humans at high risk areas for exposure to ticks compared with low risk areas (11.4 - 14 % vs 1.9 %) (FINGERLE et al. 1999). However, DNA of *Ap* was detected in only 1.6 % of the investigated ticks, indicating a low prevalence of *Ap* in ticks compared to relatively high prevalence of *B. burgdorferi* DNA (FINGERLE et al. 1999). For certain, the overall prevalence of *Ap* in ticks in Europe varies depending on different areas in which ticks are collected (MYSTERUD et al. 2013).

Ap infection in Eurasia was mainly reported as tick-borne fever in sheep, cattle, goats and horses (STUEN et al. 2013). Although being increasingly detected in animals, the number of clinically apparent human granulocytic anaplasmosis is small (HUHN et al. 2014). A latest research using multilocus sequence typing (MLST) method showed that 380 of investigated *Ap* strains from humans and animals from Europe belong to the same clonal complex (HUHN et al. 2014). While canine and equine granulocytic anaplasmosis occurs frequently in Europe, therefore, human granulocytic anaplasmosis in Europe is likely to be underdiagnosed (HUHN et al. 2014). In endemic areas, the seroprevalence of *Ap*-specific antibodies in humans is still high (STRLE 2004). *Ap* antibodies were found in 2 - 28 % of the examined people in Europe (STRLE 2004), and in 8.8 - 20 % of individuals at high risk for exposure to ticks and animals in China (ZHANG et al. 2008b; ZHANG et al. 2009). From public health considerations, dogs that have been infected with *Ap* should be carefully considered, even though the potential for dogs and other domestic animals to be zoonotic risk for human *Ap* infection is not known (GREENE 2012).

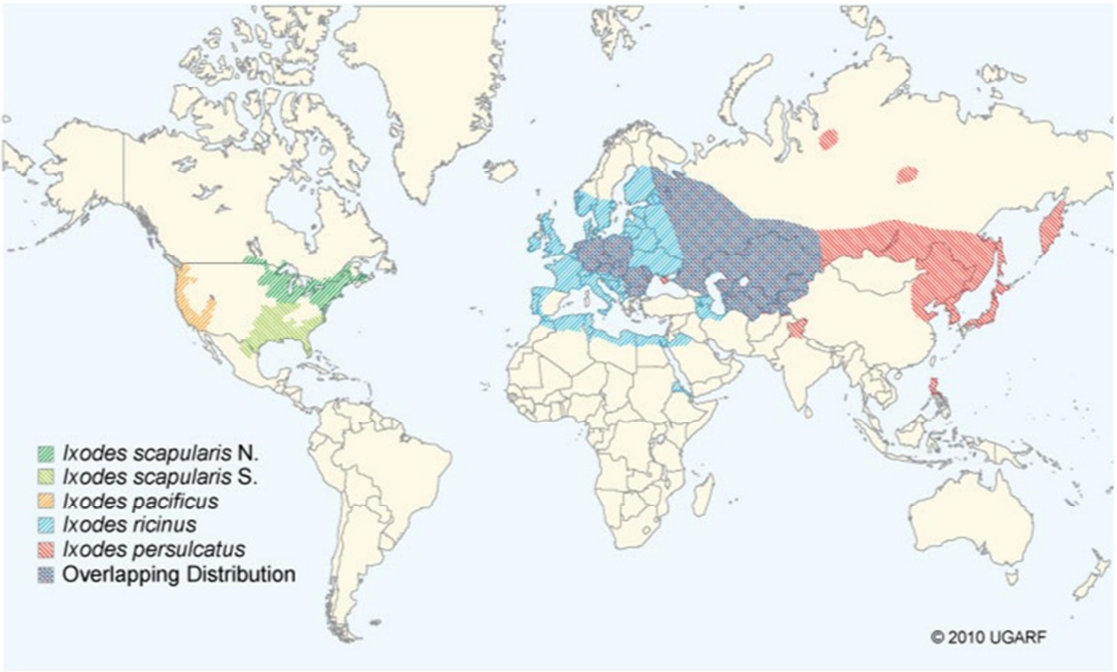

Figure 4: Approximate worldwide geographic distributions of four *Ixodes* spp. tick vectors of *Ap* and their overlapping regions

Cited from Greene CE (GREENE 2012)
In the United States, *I. scapularis* is found in the Northeast and upper Midwest, and *I. pacificus* in the western states. In Eurasia, *I. ricinus* and *I. persulcatus* are the main vectors of *Ap*.

3.2 Host range and Reservoirs of *Ap*

Since a transovarial transmission of *Ap* in *Ixodes* ticks has not been yet demonstrated, *Ap* cannot be passed effectively from infected adult *Ixodes* ticks to eggs. Consequently, tick larvae are not infected (RIKIHISA 2011). Therefore, reservoir hosts are essential to maintain the life cycle of the pathogen (RIKIHISA 2011). The host range of *Ap* varies in different geographical regions (STUEN et al. 2013). In the United States, mammalian reservoirs for *Ap* infection are considered to include white-footed mice (*peromyscus leucopus*), dusky-footed woodrats (*Neotoma fusipes*), raccoons (*procyon lotor*), gray squirrels (*Sciurus carolinensis*), gray foxes (*Urocyon cinereoargenteus*), redwood chipmunks (*Tamias ochrogenys*) and white-tailed deer (*Odocoileus virginianus*) (RAR et al. 2011; RIKIHISA 2011). The roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*) are considered as main reservoir hosts of *Ap* infection in Europe (LIZ et al. 2002). The detection of *Ap* by molecular methods in Europe has shown that a wide range of wildlife mammalian species are naturally infected with *Ap*, including voles (*Myodes glareolus*, *Microtus arvalis*,

Mi. agrestis and *Mi. oeconomus*), wood mice (*Apodemus sylvaticus*), yellow-necked mice (*Apodemus flavicollis*), common shrews (*Sorex araneus*) (LIZ et al. 2000; THOMAS et al. 2009; RAR et al. 2011), red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*) (HARTWIG et al. 2014), wild boars (*Sus scrofa*) (MICHALIK et al. 2012) and hedgehogs (*Erinaceus europaeus*) (SILAGHI et al. 2012). Interestingly, current research shows that wild boars are susceptible to *Ap* infection but do not show clinical signs, indicating their role as a source of *Ap* transmission (DE LA FUENTE et al. 2012). However, MLST revealed that strains from wild boars and hedgehogs belong to the same clonal complex. It indicates that wild boars and hedgehogs may serve as reservoirs and their harbored *Ap* strains are infectious for humans and domestic animals in Europe (HUHN et al. 2014). Moreover, four species of birds were assessed as potential reservoirs of *Ap*, including Veery (*Catharus fuscescens*), Gray catbird *(Dumetella carolinensis*), Wood thrush (*Hylocichla mustelina*) and American robin (*Turdus migratorius*). Uninfected larval black-legged ticks (*I. scapularis*) became infected when they fed on infected birds (KEESING et al. 2012). The role of birds in dispersing infected ticks in a long distance of migration has not been clearly investigated, but one study showed that *I. ricinus* nymphs on migrating birds in Sweden were infected with *Ap* (BJOERSDORFF et al. 2001).

4 Pathogenesis of *Ap* Infection

4.1 *Ap* Entry to Host Cells

4.1.1 *Ap* Adhesins and Invasins

Characteristic for obligatory intracellular parasites, *Ap* resides intracellularly within its own unique cytoplasmic membrane-bound vacuole and has a selective tropism for circulating neutrophils (DUMLER et al. 1996). For successful infection, *Ap* organisms must be able to attach and to enter host cells in order to survive (RIKIHISA 2011). The pathogens need to adhere to host cells, colonize the tissues, invade and multiply in the cells or disseminate to other tissues (PIZARRO-CERDA et al. 2006).

The intracellular pathogen internalization and colonization is mediated by multiple bacterial adhesins and invasins that cooperatively recognize host cell receptors and initiate signaling cascades to promote infection (TRUCHAN et al. 2013). Adhesins are specific surface proteins that mediate bacterial adhesion. They recognize defined receptors on the surface of target host

cells, determining tissue tropism of the pathogen (NIEMANN et al. 2004). Invasion allows bacteria to evade the cell-mediated or humoral immune response and to proliferate in a well-protected niche (NIEMANN et al. 2004). Several *Ap* outer membrane proteins (OMPs) have been identified and shown to play a role in mediating attachment to and invasion of mammalian host cells (PARK et al. 2003; OJOGUN et al. 2012; KAHLON et al. 2013; SEIDMAN et al. 2014).

Outer membrane protein A (OmpA), also known as peptidoglycan-associated lipoprotein (pal), is conserved in most Gram-negative bacteria (GODLEWSKA et al. 2009). One function of the protein OmpA is to maintain the integrity of the bacterial outer membrane by interacting with peptidoglycan (CASCALES et al. 2002). OmpA (APH_0338[4], strain: HZ) is described as a surface protein in *Ap* and *Ehrlichia chaffeensis* (HOTOPP et al. 2006). It is located on the cell surface and is transcriptionally induced in *Ap*-infected ticks during feeding on mice (OJOGUN et al. 2012). Pretreatment of *Ap* with anti-OmpA serum reduces the infection capability of the pathogen in HL-60 cells. Glutathione S-transferase (GST)-tagged full-length OmpA and $OmpA_{19\text{-}74}$ competitively inhibit the infection of myeloid cells (see Figure 5) (OJOGUN et al. 2012).

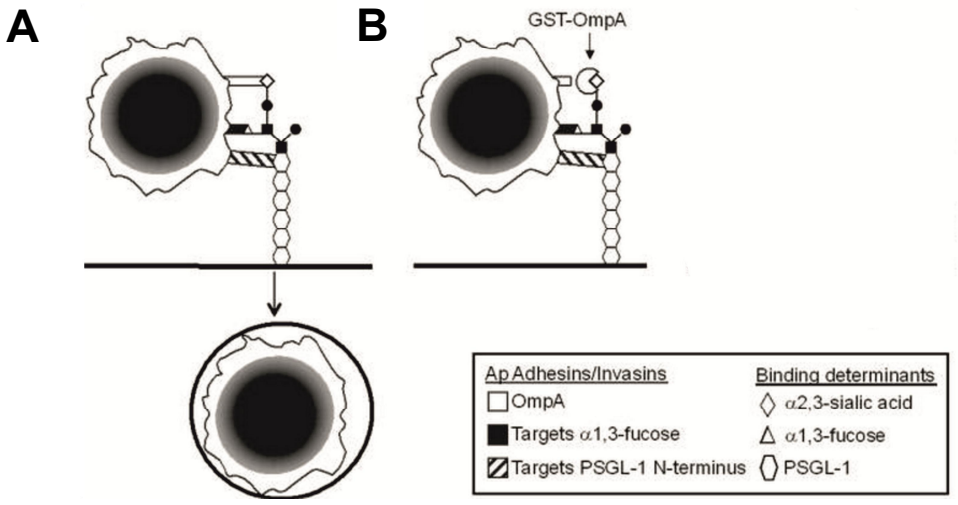

Figure 5: Models of *Ap* invasion and infection blocked by purified OmpA

Modified from Ojogun et al. (OJOGUN et al. 2012)
A *Ap* surface proteins cooperatively bind three determinants of Sialyl Lewis^X (sLe^X) -capped PSGL-1 receptor to promote bacterial adhesion and entry. **B** GST-OmpA binds to the α2,3-linked sialic acid determinant of sLe^x and competitively inhibits access of OmpA on the *Ap* surface resulting in a marked decrease in *Ap* infection.

[4] OmpA family protein, *Anaplasma phagocytophilum* strain HZ (HOTOPP et al. 2006; LIN et al. 2011)

Asp14 (14-kDa *Ap* surface protein, APH_0248[5], strain: HZ) is an outer membrane protein on the surface of dense-cored (DC) form of *Ap* (DUNNING HOTOPP et al. 2006; LIN et al. 2011). Asp14 is localized on the *Ap* surface and is expressed during *in vivo* infection (KAHLON et al. 2013). It is transcriptionally induced during transmission feeding of *Ap*-infected ticks on mice and is upregulated when the bacteria attach to host cells via the PSGL-1 receptor (KAHLON et al. 2013). The C-terminal domain at 12 - 24 amino acids of Asp14 protein plays a critical role in the cellular invasion (KAHLON et al. 2013). The combined use of GST-OmpA and GST-Asp14 results in a reduced infection rate of 90 % in HL-60 cells. The separate use of the proteins leads to a decrease of infection by 57 - 65 % (OJOGUN et al. 2012; KAHLON et al. 2013). Thus it is assumed that strategically targeting Asp14 and OmpA together may potentially protect against *in vivo* infection (KAHLON et al. 2013).

Msp2 (major surface protein 2, P44) proteins are major immunodominant surface antigens of *Ap* organism. The proteins consist of conserved N- and C-terminal domains and a central hypervariable region (ZHI et al. 1999; RIKIHISA 2011). This protein was widely used for target antigen in routine diagnostics (ZHI et al. 1997; IJDO et al. 1999; TAJIMA et al. 2000). The genome of *Ap* contains 113 of *msp2* (*p44*)-paralogous genes that encode for Msp2 (P44) proteins (HOTOPP et al. 2006). Except of the function as a major immunodominant outer-membrane protein, Msp2 (P44) also plays a role as an adhesin for *Ap* entry into granulocytes (PARK et al. 2003). Furthermore, pretreatment of *Ap* with Msp2 monoclonal antibody or pretreatment of host cells (HL-60 cells and neutrophils) with recombinant Msp2 protein reduced bacterial adhesion to HL-60 cells and neutrophils (PARK et al. 2003). Correspondingly, Msp2 (P44) is presumed to match the fucosylated (Fuc-VII) P-selectin glycoprotein ligand-1 (PSGL-1), a known ligand of granulocytes for *Ap* infection (Figure 6) (TRUCHAN et al. 2013). However, whether the actual binding occurs between Msp2 (P44) and fucosylated PSGL-1 or between PSGL-1 with a structurally related Msp2 (P44) protein is not known (PARK et al. 2003; TRUCHAN et al. 2013).

[5] Hypothetical protein, *Anaplasma phagocytophilum* HZ (strain: HZ) (HOTOPP et al. 2006; LIN et al. 2011)

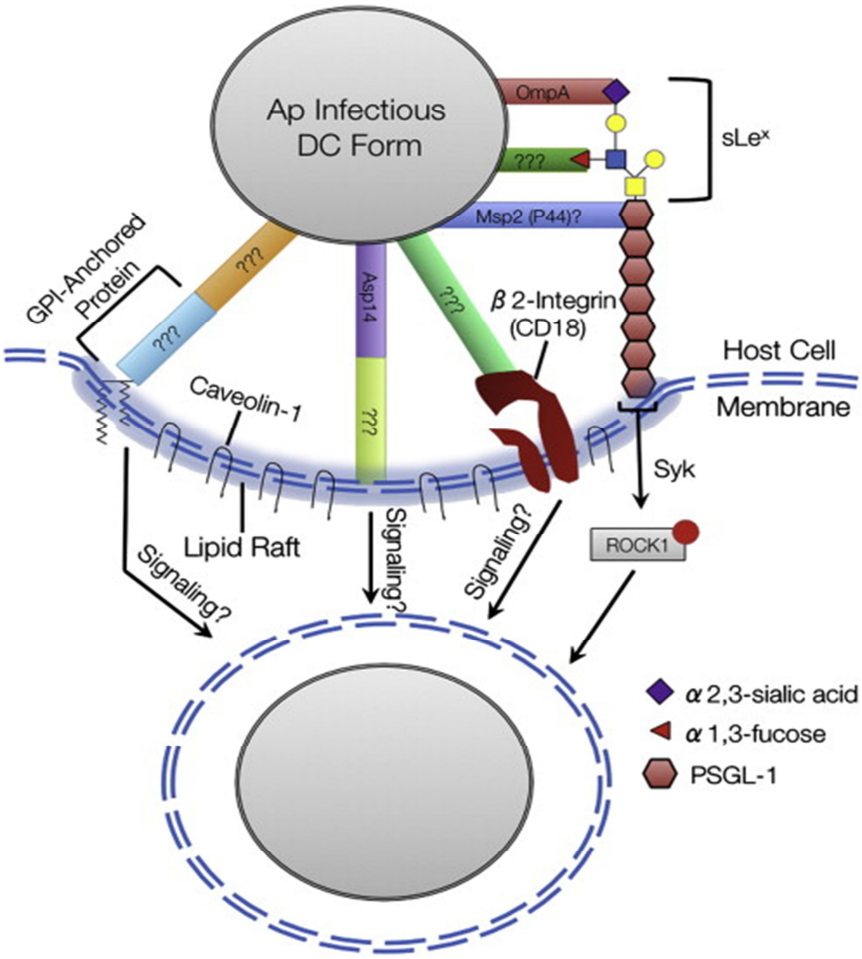

Figure 6: *Ap* cellular invasion

Cited from Truchan et al. (TRUCHAN et al. 2013)
The infectious dense-cored form of *Ap* utilizes multiple surface proteins to cooperatively bind PSGL-1. OmpA is the only identified *Ap* invasin that is known to bind α2,3-sialic acid of sLe^X tetrasaccharide that caps PSGL-1. Binding to PSGL-1 initiates a signaling cascade that involves spleen tyrosine kinase (Syk) and phosphorylation of ROCK1 and thus facilitates bacterial internalization. '???' in figure 6, unidentified *Ap* adhesins/invasins or host cell receptors.

AipA (*Ap* invasion protein A, APH_0915[6], strain: HZ), is one of the putative OMPs. It was recently shown to be important for bacterial entry into mammalian cells (NELSON et al. 2008; SEIDMAN et al. 2014). AipA is localized on the bacterial surface. Its expression is induced (10 - 20 fold) when *Ap* changes from the non-infectious RC morphotype to the infectious DC morphotype during infection of HL-60 cells (SEIDMAN et al. 2014). Pretreatment of *Ap* with AipA antibody inhibits the invasion of bacteria in host cells (SEIDMAN et al. 2014). Furthermore, a combination of antisera targeting AipA, OmpA and Asp14 was shown to abolish the infection (SEIDMAN et al. 2014).

[6] Hypothetical protein, *Anaplasma phagocytophilum* HZ (strain: HZ) (HOTOPP et al. 2006; LIN et al. 2011)

Other *Ap* hypothetical proteins such as surface-exposed Asp55 (APH_0405), Asp62 (APH_0404), and 'dense-cored' (DC)-associatedAPH_1235 are considered to be involved in bacterial adhesion and invasion, even though their receptors are not known so far (GE et al. 2007; TROESE et al. 2011; MASTRONUNZIO et al. 2012).

4.1.2 Host Cell Receptor and Internalization Signal

P-selectin glycoprotein ligand-1 (PSGL-1) is the best characterized *Ap* receptor on host cells. This receptor is found on neutrophils, bone marrow progenitors, and promyelocytic HL-60 cells (GOODMAN et al. 1999; HERRON et al. 2000). PSGL-1 is capped by an O-glycan that is terminally decorated with sialyl lewis^x (sLe^x), a tetrasaccharide that includes α1,3-flucose and α2,3-sialic acid (SOMERS et al. 2000). PSGL-1 is not required for binding and infection of murine neutrophils. However, sialylation and α1,3-fucosylation of neutrophils are essential for binding and infection of murine neutrophils. The infection rate of Fuc-TIV$^{-/-}$ / Fuc-TVII$^{-/-}$ mice with *Ap* is significantly reduced compared to wild-type mice (CARLYON et al. 2003a).

ROCK1 is a Rho kinase (ROCK) that belongs to the AGC (PKA/PKG/PKC) family of serine-threonine kinases and is a major downstream effector of RhoA that regulates the actin cytoskeleton (SURMA et al. 2011). In general, ROCK plays a central role in the organization of the actin cytoskeleton and is mainly involved in regulating of the morphology and movement of the cells (SURMA et al. 2011). Infection of promyelocytic HL-60 cells and neutrophils with *Ap* initiates a signaling cascade that involves tyrosine phosphorylation of ROCK1, which is associated with P-selectin glycoprotein ligand-1 (PSGL-1) and spleen tyrosine kinase (Syk) (THOMAS et al. 2007; TROESE et al. 2009). PSGL-1 blocking antibodies and siRNA targeting Syk interfere with ROCK1 phosphorylation in *Ap*-infected cells (THOMAS et al. 2007). Knockdown of either Syk or ROCK1 also markedly impaired *Ap* infection, suggesting that binding to PSGL-1 activates PSGL-1 signaling pathway through Syk and ROCK1 resulting in bacterial internalization (THOMAS et al. 2007). Downstream signaling pathways following tyrosine phosphorylation of ROCK1 remain to be explored in order to understand how bacteria enter into host cells (THOMAS et al. 2007).

It is also described that *Ap* adhesion and invasion take place through **sialic acid- and PSGL-1-independent ways** that involve β2 integrin and lipid rafts. PSGL-1 independent receptor has been shown to be involved during infection of two laboratory *Ap* strains in the mammalian cells (RENEER et al. 2006; SARKAR et al. 2007; RENEER et al. 2008). Strain NCH-1A is selected by cultivating strain NCH-1 in HL-60 sLe$^{X\ -/low}$ cells that defective in sialic acid but not fucose (RENEER et al. 2006), whereas strain NCH-1A2 is selected by cultivating strain

NCH-1A in HL-60 A2 cells (sLe^X-defective HL-60 cells) that are defective in the expression of sialylation and Fuc-TVII but not Fuc-TIV (GOODMAN et al. 1999; RENEER et al. 2006). Pretreatment of HL-60 cells with anti-sLe^X or anti-PSGL-1 antibodies reduces the infection of NCH-1A and NCH-1A2 compared to the wild-type NCH-1 strain (RENEER et al. 2006; RENEER et al. 2008). It is suggested that these two variants, NCH-1A and NCH-1A2, bind to HL-60 cells in a sialic acid- and PSGL-1 independent manner (RENEER et al. 2006). Furthermore, PSGL-1 independent entry does not alter NCH-1A2 replication in host cells and Syk (spleen tyrosine kinase) is not essential for NCH-1A2 infection of HL-60 cells (RENEER et al. 2008). To date, the genetic characterization of NCH-1A2 and the sialic acid- and PSGL-1-independent pathogen-host receptor interaction are still unclear.

4.2 Intracellular Survival Strategies

It has recently become evident that *Ap* has evolved several mechanisms to thwart the innate and adaptive immune response. To date, these mechanisms include down-regulation of reactive oxygen species (ROS) generation, inhibition of apoptosis, subversion of autophagy, antigenic variation of immunodominant surface protein Msp2/P44, nutritional virulence factor and manipulation of SUMOylation of host cells (TRUCHAN et al. 2013).

4.2.1 Down-regulation of Reactive Oxygen Species Generation

It is known that *Ap* lacks genes for the biosynthesis of Lipid A (an essential component of lipopolysaccharides, LPS) and the biosynthesis of peptidoglycan (LIN et al. 2003) that are two important pathogen associated molecular patterns (PAMPs) in most gram-negative bacteria (like *E. coli*). The absence of LPS and peptidoglycan contributes to the disability of Toll-like receptors (TLRs) to recognize foreign materials and the lack of a nucleotide-binding oligomerization domain (NOD) activation, that are expressed on/in host immune cells such as neutrophils (HEDAYAT et al. 2011; SORBARA et al. 2011). Neutrophils are the most abundant blood cells and exert their powerful and effective role in antimicrobial responses against invading pathogens (KOBAYASHI et al. 2009). Phagocytes such as neutrophils and monocytes/macrophages play a primary role in reactive oxygen species (ROS) generation such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (-OH) and hypochlorous acid (HOCl), aiding to the oxidative killing of microorganisms (EL-BENNA et al. 2005). *Ap* infection does not induce superoxide anion (O_2^-) generation in human neutrophils but requires HGE contact and protein synthesis in neutrophils (MOTT et al. 2000; WANG et al.

2002), HL-60 cells (BANERJEE et al. 2000; MOTT et al. 2002) and murine neutrophils (WANG et al. 2002). *Ap* infection also prevents the superoxide anion (O_2^-) by human neutrophils or HL-60 cells in response to various stimuli, such as phorbol myristate acetate (PMA) (BANERJEE et al. 2000; MOTT et al. 2000; MOTT et al. 2002; IJDO et al. 2004). This down-regulation of superoxide production is required for bacterial contact and protein synthesis by the neutrophils (MOTT et al. 2000). Contradictorily, another group reported that *Ap* does not inhibit the respiratory burst when PMA was added, but undoubtedly suppress the neutrophil respiratory burst (JW et al. 2004). Inhibition is specific in neutrophils, because human monocytes can respond to exogenous stimuli in the presence of *Ap* (MOTT et al. 2000). In fact, once internalization finished, the bacterium resides within a protective membrane-bound vacuole that excludes $gp91^{phox}$ and $p22^{phox}$ membrane proteins, which are essential for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase assembly in neutrophils (CARLYON et al. 2004; JW et al. 2004; EL-BENNA et al. 2005).

4.2.2 Inhibition of Host Cell Apoptosis

In multicellular organisms, cells that are damaged or are no longer needed are removed by a tightly regulated cell suicide process known as apoptosis or programmed cell death (ALBERTS 2002). This process is mediated by a serial of proteolytic enzymes called caspases, which are able to cleave specific proteins in the cytoplasm and nucleus (ELMORE 2007). Caspases exist in all cells as inactive precursors or pro-caspases. They are usually activated by a proteolytic caspase cascade that is induced through cleavage of other caspases (ALBERTS 2002). Caspase activation is mainly regulated by activity of Bcl-2 and IAP protein families in cell apoptosis (ALBERTS 2002).

Ap infection inhibits apoptosis of peripheral blood neutrophils by modulating the extrinsic as well as the intrinsic pathway of apoptosis in human (YOSHIIE et al. 2000; GE et al. 2006). This prolonged surviving time of neutrophils will benefit *Ap* dissemination to naive host cells (CARLYON et al. 2003b). Many apoptotic-related genes are up-regulated in human neutrophils within 1 - 3 h after infection (LEE et al. 2006). The PI3K/Akt[7] and NF-κB pathways are considered as important survival signaling pathways in neutrophils (ZHU et al. 2006). *Ap* infection activates the PI3K/Akt, NF-κB signaling pathways and enhances expression of the anti-apoptotic protein cIAP2 in human neutrophils (SARKAR et al. 2012). *Anaplasma* translocated substrate (Ats-1) was recently shown to prevent etoposide-induced apoptosis after translocation into mitochondria in mammalian cells (NIU et al. 2010). Ats-1 is secreted by the

[7] Phosphatidylinositol 3-kinase/protein kinase-B pathway

Type IV secretion system (T4SS) and translocated into mitochondria across five membranes (two bacterial membranes, inclusion membrane and two mitochondria membranes) via its cleavable N-terminal mitochondrion-targeting presequence by mitochondrial protein transport system (NIU et al. 2010). *bfl-1*, a member of antiapoptotic *bcl*-2 genes family, was shown to be increased in *Ap*-infected neutrophils and mediates the inhibition of human neutrophil apoptosis (GE et al. 2005). *Ap* infection inhibits caspase 3 enzyme activity and prevents the loss of mitochondrial membrane potential in human neutrophils (GE et al. 2005). P38 mitogen-activated protein kinase (MAPK) phosphorylation (activation) was shown to be involved in apoptotic inhibition of *Ap*-infected neutrophils, and this p38 MAPK signal transduction leading to delayed apoptosis is bypassed with active intracellular infection (CHOI et al. 2005). This anti-apoptotic effect of *Ap* infection in ovine neutrophils was also observed *in vivo* (SCAIFE et al. 2003).

Notably, unlike the anti-apoptotic effect in neutrophils, *Ap* promotes the apoptosis in HL-60 cells (BEDNER et al. 1998; KARKI et al. 2011). However, *Ap* inhibits the apoptosis of ISE6 tick cells (AYLLON et al. 2013). Reduced expression of spectrin alpha chain or alpha-fodrin (CG8) in tick salivary glands and voltage-dependent anion-selective channel or mitochondrial porin (T2) in both the gut and salivary glands were recently shown to be involved in the inhibition of tick cell apoptosis and *Ap* multiplication (AYLLON et al. 2013). An E3 ubiquitin ligase named x-linked inhibitor of apoptosis protein (XIAP) restricts *Ap* colonization in *I. scapularis* ticks (SEVERO et al. 2013).

4.2.3 Subversion of Autophagy

Autophagy is a ubiquitous eukaryotic cytoplasmic quality and quantity control pathway that is essential for survival, differentiation, development and homeostasis (DERETIC 2010). The key morphological feature of autophagy is the emergence of membranous organelles called autophagosomes (AP) that capture various cytoplasmic targets and deliver them for lysosomal degradation in autolysosomes (AL) within the cytosol (DERETIC 2010).

Several hallmarks of early autophagosomes have been identified in *Ap*-replicative inclusions, including a double-lipid bilayer membrane and colocalization with GFP-tagged LC3 (light chain 3) and Beclin 1, the human homologues of *Saccharomyces cerevisiae* autophagy-related proteins Atg8 and Atg6 respectively (NIU et al. 2008). These *Ap*-induced early autophagosomes do not fuse with lysosome to form mature autolysosome (AL) as the absence of LAMP-3 (lysosome-associated membrane protein 3), a late endosomal and lysosomal marker (NIU et al. 2008). Inhibition of autophagy with the class III PI3K (PI3KC3) inhibitor 3-

methyladenine (3-MA) does not enhance *Ap* infection but arrests its growth, indicating *Ap*-induced autophagosomes formation is not helpful for innate immune response to defense infection but helpful to aid bacterial replication (NIU et al. 2008). *Anaplasma* translocate substrate 1 (Ats-1) is one of two identified type IV secretion effectors of *Ap* (another is ankyrin-rich protein A, AnkA) (RIKIHISA et al. 2010; LIN et al. 2011). Ats-1 was recently shown to bind with Beclin 1, a subunit of the class III PI3K and Atg14L and subsequently hijack the Beclin 1-Atg14L autophagy initiation pathway for bacterial growth (NIU et al. 2012). Above all, *Ap* resides in a membrane-bound compartment and acquires nutrients from host cytoplasm in part by subversion of autophagy process (NIU et al. 2008; NIU et al. 2012).

4.2.4 Cholesterol Acquisition for Proliferation

As *Ap* lacks genes for synthesis of lipid A and peptidoglycan, *Ap* stabilizes its outer membrane by incorporating cholesterol from host cells in order to infect host cells (LIN et al. 2003). In general, cells acquire cholesterol via two ways, endogenous biosynthesis in the smooth endoplasmic reticulum (ER) or mostly low-density lipoprotein receptor (LDLR)-mediated uptake from exogenous lipoproteins via endocytosis (BROWN et al. 1986). Data have been shown that the uptake of fluorescence-labeled low-density lipoprotein (LDL) was enhanced and LDLR expression was upregulated at both mRNA and protein level in *Ap*-infected cells (XIONG et al. 2009). The total amount of host cell cholesterol is increased by 2-fold or more and enriched in *Ap* inclusion in infected HL-60 cells (XIONG et al. 2009). Additionally, LDL uptake blocking assay with a monoclonal antibody against LDLR or treatment with cholesterol transport inhibitors (like U18666A that blocks LDL-derived cholesterol egress from late endosomes or lysosomes) have shown that *Ap* replication was significantly inhibited (XIONG et al. 2009). Thus, *Ap* acquires cholesterol for its own replication through the low-density lipoprotein receptor (LDLR)-mediated uptake pathway (XIONG et al. 2009).

4.2.5 Selective Targeting of Rab GTPases

Ras-like GTPase (Rabs) constitute the largest family of small Ras-like GTPases of monomeric G proteins with eleven identified members in yeasts and approximately 70 members in humans (HUTAGALUNG et al. 2011). Rab GTPases serve as master regulators that regulate many steps of membrane transport mechanisms, including vesicle formation, vesicle movement, vesicle uncoating, vesicle tethering and membrane fusion (HUTAGALUNG et al. 2011). The disturbance of Rab-regulated pathways is involved in infection of several pathogens, like *Salmonella enterica* or *Chlamydia pneumonia* (CORTES et al. 2007). Rabs and their effectors

are known to be targets for infectious microorganisms to evade host defenses, obtain nutrients and replicate in an intracellular environment (BRUMELL et al. 2007).

Ap-occupied vacuole selectively targets Rab GTPases are primarily associated with the recycling endosomes (HUANG et al. 2010a). A list of Rab GTPases that have been examined and revealed to be recruited to *Ap*-occupied vacuoles is given in Table 1.

The hijack of Rab GTPases involved in recycling endosomes allows *Ap*-occupied vacuole to effectively disguises itself as a 'real recycling endosome', which is necessary for avoiding endosomal maturation and lysosomal fusion (HUANG et al. 2010a). Doing so, the *Ap*-containing vacuole provides a means of molecular camouflage and escapes the first wave of host defenses and bacterial-killing by neutrophils (HUANG et al. 2010a). This strategy may also likely contribute to the biogenesis of the vacuole, such as the acquisition of membrane materials, amino acids and cholesterol from the host cells (HUANG et al. 2010a).

Table 1: Location and transport functions of Rab GTPases associated with *Ap*-occupied vacuoles[a]

Rab GTPase	location(s)	function(s) in membrane transportation
Rab1	ER exit sites, IC	ER to Golgi, Golgi to ER, IC to PM
Rab4A	rapid RE	clathrin-dependent endocytic recycling
Rab10	EE to ERC, tubular RE, TGN	clathrin-independent endocytic recycling, TGN to PM
Rab11A	ERC, RE, tubular RE	clathrin-independent endocytic recycling
Rab14	EE, ERC, TGN	clathrin-independent endocytic recycling, TGN to EE
Rab22A	EE to ERC, tubular RE	clathrin-independent endocytic recycling, EE to TGN
Rab35	tubular RE, rapid RE	clathrin-dependent endocytic recycling

[a] Data summarized from reference by Huang et al. (HUANG et al. 2010a)

ER, endoplasmic reticulum; IC, pre-Golgi intermediate compartment; PM, plasma membrane; RE, recycling endosome; EE, early endosome; ERC, endocytic recycling center; TGN, *trans*-Golgi network.

4.2.6 Manipulation of SUMOylation of Host Cells

Protein post-translational modifications (PTMs), such as ubiquitination, phosphorylation and acetylation, are known as essential mechanisms used by eukaryotic cells to react rapidly to environment changes (ASHIDA et al. 2014). SUMOylation, the covalent attachment of a member of small ubiquitin-like modifier (or SUMO) proteins to lysines in target substrate proteins, is a reversible and essential post-translation modification step in eukaryotic cells (BEYER et al. 2014). SUMO proteins are biochemically similar to ubiquitin, but functionally distinct from that involved in ubiquitination and are involved in many different biological processes such as protein localization and stability, transcriptional activities, nucleocytoplasmic signaling and transport, genome replication, and regulation of gene expression (HAY 2005).

Pathogen-occupied vacuoles (PVs), derived from host cells and remodeled during internalization, are a special cytosolic compartment containing internalized pathogens and are optimal niches for intracellular survival (KUMAR et al. 2009). Pathogen-encoded proteins that localize to pathogen-occupied vacuolar membranes (PVMs) play a critical pathobiological role in providing structural integrity to the PVM, hijacking vesicular traffic, and intercepting host signal transduction pathways (HUANG et al. 2010c). Following internalization, *Ap* resides within a host cell-derived vacuole with a two layers membrane while avoiding fusion with lysosomes and NADPH oxidases (HUANG et al. 2010c). Based on the advantages of genomic sequencing, several *Ap*-encoded proteins, Ats-1, AptA, APH0032 and AmpA (*Ap* post-translationlly modified protein A; formerly APH1387) that are presented on the *Ap*-occupied vacuole membrane (AVM) have been identified (HUANG et al. 2010b; HUANG et al. 2010c; SUKUMARAN et al. 2011; HUANG et al. 2012; NIU et al. 2012). Of these few identified proteins, AmpA has been previously identified to be expressed throughout bacterial intracellular development and localized to the AVM in host cells (HUANG et al. 2010c). Recently, Beyer et al. showed that ectopically expressed green fluorescent protein (GFP)-tagged and endogenous AmpA molecules are poly-SUMOylated, which is consistent with the observation that AmpA colocalizes with SUMO2/3 at the AVM in infected cells (BEYER et al. 2014). Inhibition of SUMOylation by knockdown of Ubc9 (a necessary enzyme for SUMOylation) only slightly bolstered *Ap* infection (BEYER et al. 2014). However, ectopically expressed GPF-AmpA but not lysine-deficient GFP-AmpA was shown to serve as a competitive agonist against native AmpA in infected cells, implying an important role of modification of AmpA lysines during bacterial infection (BEYER et al. 2014).

5 Immune response to *Ap* infection

In vertebrates, the immune system defends the organism against infection through the activation of the innate and adaptive immune system (GIRARDIN et al. 2002). It is described that mice infected with *Ap* do not show clinical signs (HODZIC et al. 1998b; BUNNELL et al. 1999) but may develop histopathological injury, similar to those that are present in humans and horses (MARTIN et al. 2000; MARTIN et al. 2001). The use of laboratory mice to investigate host cell-pathogen interactions, infection kinetics, cellular alterations, cytokine profiles and immune response has expanded our understanding of *Ap* biology and pathogenesis (BORJESSON et al. 2002a).

During *Ap* infection, plasma IFN-γ levels are evaluated 4 h after inoculation in experimentally infected mice, implying a rapid proinflammatory response to occur in the meantime (MARTIN et al. 2000; MARTIN et al. 2001). A previous study has shown that production of IFN-γ is critical for the generation of protection against *Ap* infection, as its absence renders mice less able to control bacterial burden (CHOI et al. 2014). However, IFN-γ also plays an important role in the induction of severe immune mediated histopathological damage in *Ap*-infected mice, horses and humans (SCORPIO et al. 2005; DUMLER et al. 2007; SCORPIO et al. 2009; BUSSMEYER et al. 2010; DAVIES et al. 2011). IFN-γ is an important proinflammatory cytokine, which is necessary for innate immunity and mediates many biological actions such as macrophage activation, antimicrobial effector mechanisms and production of proinflammatory cytokines, chemokines and reactive oxygen species (PALUDAN 2000). Infection of $ifng^{-/-}$ (IFN-γ deficient) mice led to an increased bacterial load in the early phase of the infection and inhibition of immune induced inflammation reaction (AKKOYUNLU et al. 2000; MARTIN et al. 2001). Infection of NKT-deficient mice ($CD1d^{-/-}$) with *Ap* caused a complete loss of hepatic inflammatory lesions on days 4 - 7 postinfection compared with infection in wild-type mice (unpublished data), implying an involvement of natural killer T (NKT) cells in the early IFN-γ generation and further in the immunopathological injury (DUMLER 2012). Interestingly, a study using different gene-knockout mice showed that IFN-γ production by natural killer (NK) cells is important for initial early step of host immune response against *Ap*, but not critical for pathogen elimination (BIRKNER et al. 2008). In contrast, CD_4^+ T cells are strictly indispensable to subsequent bacterial clearance, while perforin, Fas/FasL, major cytokines from type 1 of T helper cells (Th1 cells) such as IL-12, IFN-γ, and MCP-1 (monocyte chemoattractant protein-1) are unexpectedly not important (BIRKNER et al. 2008). The IFN-γ production in *Ap* infected mice is induced through the signal transducer and activator of

transcription (Stat1) signaling pathway (CHOI et al. 2013). Compared to wild-type (WT) mice, *Ap* infected Stat1 knockout (KO) mice develop more severe disease resulting in >100-fold higher blood and splenic bacterial loads and a stronger proinflammatory reaction (CHOI et al. 2014). Nevertheless, various infection models with *Ap* provide evidence that the biological basis of the disease's inflammatory injury is not driven by bacterial load but rather by the host innate immune and/or inflammatory response (LEPIDI et al. 2000; MARTIN et al. 2001; SCORPIO et al. 2005; BROWNING et al. 2006; SCORPIO et al. 2006; CHOI et al. 2007). These results suggest that IFN-γ plays a critical role in the early eradication of *Ap*, and the same as a double-edged sword initiating immune response to play a key role in disease's immunopathological injury (DUMLER 2012).

The activation of the innate immune system relies on the recognition of pathogen-associated molecular patterns (PAMPs) by specific pattern-recognition receptors (PRRs) expressed by immune cells (GIRARDIN et al. 2002). Several classes of PRRs including Toll-like receptors (TLRs, see Table S1 in appendix) and recently identified cytoplasmic receptors recognize distinct microbial components and directly activate immune cells (AKIRA et al. 2006). It is shown that only TLR2, but not TLR4 is involved in NF-κB[8] translocation in TLR-transfected cells upon the infection with *Ap* in *in-vitro*-studies (CHOI et al. 2004). However, *in vivo* data demonstrate that mice lacking of TLR2 and TLR4, or even lacking of MyD88, TNF, iNOS are unimpaired in their ability to control a systemic infection with *Ap* (VON LOEWENICH et al. 2004). Thus the role of TLRs in *Ap* recognition needs to be further investigated.

Recently, NOD1[9] and NOD2, two cytoplasmic proteins containing an NBS-LRR[10] motif, have been identified as cytoplasmic pattern recognition receptor (PRR) with the function as intracellular sensors for 'inside-in' signaling following PAMPs recognition (GIRARDIN et al. 2002). Rip2 (receptor interacting protein-2) is a key adaptor molecule of NOD1 and NOD2 in the Nod-like Receptor (NLR) family (MAGALHAES et al. 2011). Sukumaran et al. showed that Rip2 transcription was induced in human primary neutrophils infected with *Ap* as early as 2 h post-infection and maintain a fourfold increase until 8 h post-infection (SUKUMARAN et al. 2005; SUKUMARAN et al. 2012). Moreover, *in vivo* infection assay using the Rip2 deficiency mice ($Rip2^{-/-}$ mice) showed an increased bacterial load and a delayed clearance (10-12 days *vs* ~20 days) of *Ap* in the peripheral blood, compared with those in wild-type mice (SUKUMARAN et al. 2012). Interestingly, the *Ap* genome does not encode genes for the

[8] Nuclear factor kappa-light chain-enhancer of activated B cells
[9] Nucleotide-binding oligomerization domain-containing protein 1
[10] Nucleotide-binding site (NBS) and leucine-rick repeat (LRR)

synthesis of D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide, which are two known bacterial membrane-derived peptidoglycan components (MDP) that interact with NOD1 and NOD2 in immune cells (INOHARA et al. 2003; HOTOPP et al. 2006; FRANCHI et al. 2009). Thus the key adaptor molecule 'Rip2' mediating cytoplasmic PRRs NOD1/2 signaling, activated by unknown component of bacterium, plays an important role during the early mid-phase of the immune control to *Ap* (SUKUMARAN et al. 2012).

It is known that adaptive immunity is important in elimination of pathogens in the late phase of infection as well as the generation of immunological memory (AKIRA et al. 2006). Given that immunocompetent mice control the infection, whereas immunocompromised severe combined immunodeficiency (SCID) mice become persistently infected, it is implied that the adaptive immune system provides protection against *Ap* (VON LOEWENICH et al. 2004). High titers of specific antibodies are induced in approximately 40 % of human patients and 44 % of equine patients (DUMLER et al. 1998; ARTURSSON et al. 1999). However, the protective role for antibody in clearing *Ap* infection is not demonstrated. One of these mechanisms may be due to antigenic variation of immunodominant proteins, Msp2/P44 of *Ap* (PALMER et al. 2009; BROWN 2012). The genome size of *Ap* strain HZ is 1.47 Mb[11], approximately one quarter of the size of the *E. coli* genome (DUNNING HOTOPP et al. 2006). The *Ap* genome contains three copies of *omp1*, one *msp2* locus, two *msp2* homologues (which are distinct from *P44*), one copy of *msp4* and 113 copies of *p44* (or *msp2*) genes, which are phylogenetically distinct from the *msp2* of *A. marginale* (DUNNING HOTOPP et al. 2006). The p44 (msp2) homologous genes consist of a single central hypervariable region of approximately 94 amino acid residues and N- and C-terminal regions highly conserved among the homologs (ZHI et al. 1999). During the course of infection, *Ap* utilizes gene conversion to shuffle about 100 functional pseudogenes into a single expression cassette of the *msp2* (*p44*) gene, which allows multiple P44 antigen variants to be rapidly exchanged at transcriptional regulation level (RIKIHISA 2010; REJMANEK et al. 2012). Therefore the evasion of specific protective antibody by the generation of differ Msp2/P44 variants allows *Ap* to survive in host cells and establish a long-term persistent infection (BROWN 2012).

A number of studies have examined the cytokine and chemokine responses to *Ap* infection (see Table 2). In humans with *Ap* infection, evaluated IFN-γ and IL-10 in serum from acute phase are significantly elevated compared with convalescent and normal serum, while tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and IL-4 levels are not elevated compared with

[11] Mb: Mega base paris

convalescent serum (DUMLER et al. 2000). *In vitro* study showed that IL-1β, TNF-α and IL-6 mRNA and protein in human peripheral blood leukocytes (PBLs) are induced by either recombinant 44-kDa major surface protein (rP44) of the HGA agent or viable HGA organism, while expression of IL-8, IL-10, IFN-γ, transforming growth factor beta (TGF-β) and IL-2 mRNA was not remarkable increased (KIM et al. 2000). The kinetics of induction of these three cytokines in PBLs is quite similar (KIM et al. 2000). Further analysis indicates that the monocytes present in the PBL preparation are responsible for expression of TNF-α and IL-6 mRNA, whereas IL-1β is generated by neutrophils, lymphocytes and monocytes in response to viable bacterium or rP44 of *Ap* (KIM et al. 2000). However, *in vivo* studies showed that infection with *Ap* does not affect IL-1β and TNF-α expression in humans and mice (MARTIN et al. 2000; THOMAS et al. 2001). IL-8, also known as neutrophil chemotactic factor, induces neutrophil migration to sites of infection. *Ap* or P44 protein induce IL-8 secretion in neutrophils and in a promyelocytic cell line (HL-60) that has been differentiated with retinoic acid into a neutrophil lineage (AKKOYUNLU et al. 2001). Expression of CXCR2 but not CXCR1, both are IL-8 receptors, are upregulated in neutrophils and in retinoic acid differentiated HL-60 cell line (AKKOYUNLU et al. 2001). Immunocompetent (BALB/c), severe combined immunodeficient (C_3H-*scid*) mice that are administered CXCR2 antisera, and IL-8 receptor ($CXCR2^{-/-}$) knockout mice (BALB/*c*-*Cmkar2*^{tm1Mwm}), showed much less susceptibility to *Ap* infection (AKKOYUNLU et al. 2001). Thus, these results indicate that IL-8 production is induced by *Ap* infection in host cell, and appears to be exploited in order to facilitate infection (AKKOYUNLU et al. 2001).

The horse is a valuable animal model of HGA (MADIGAN et al. 1995). Previously reported investigation of four horses experimentally infected with *Ap* showed that IL-1β and TNF-α mRNA expression were upregulated in peripheral blood leukocytes (PBLs) in all four horses, and IL-8 mRNA expression was up-regulated in three horses (KIM et al. 2002). None of IL-2, IL-4, IL-6 and IL-12p40 transcription was detected from any of the four horses (KIM et al. 2002). These data suggest that IL-1β, TNF-α and IL-8 play a primary role during the infection with *Ap* in horses (KIM et al. 2002).

Table 2: Alterations of cytokines and chemokines during *Ap* infection

cytokine/chemokine	kinetics	source	analyte	possible role(s) in *Ap* pathogenesis	reference(s)
IFN-γ	elevated	human serum from acute-phase HGA compared with convalescent serum	protein in serum	histopathological injury, early control infection	DUMLER et al. 2000
TNF-α	elevated from two h to 36 h p.i. (mRNA) and 24 h p.i. (protein)	human PBLs incubated with *Ap*	mRNA and protein in culture supernatant of PBLs	NM	KIM et al. 2000
	Increased during 20 days p.i.	PBLs from experimentally infected horse	mRNA	NM	KIM et al. 2002
IL-1β	increased during 20 days p.i.	PBLs from experimentally infected horse	mRNA	NM	KIM et al. 2002
	elevated from two h to 36 h p.i. (mRNA) and 24 h p.i. (protein)	human PBLs incubated with *Ap*	mRNA and protein in culture supernatant of PBLs	NM	KIM et al. 2000
IL-1β	increased	HL-60 cells differentiated with retinoic acid	mRNA	NM	CARLYON et al. 2002
IL-6	elevated from two h to 36 h p.i. (mRNA) and 24 h p.i. (protein)	human PBLs incubated with *Ap*	mRNA and protein in culture supernatant of PBLs	NM	KIM et al. 2000
IL-8	increased at 24 h p.i., until 120 h p.i.	HL-60 cells differentiated along the neutrophil lineage with retinoic acid incubated with *Ap* or HGA-44 antigen	mRNA and protein in culture supernatant	exploited to enhance infection	AKKOYUNLU et al. 2001
	increased at seven h p.i. and 24 h p.i.	human neutrophils incubated with *Ap*	protein in culture supernatant	exploited to enhance infection	AKKOYUNLU et al. 2001

	increased	serum from patient with confirmed HGA	protein in serum	NM	AKKOYUNLU et al. 2001
	increased from 24 h to 48 h p.i.	HL-60 cells differentiated along the neutrophil lineage with 1.25 % DMSO incubated with *Ap*	protein in culture supernatant	cytopenia and mediation of inflammatory response	KLEIN et al. 2000
	increased during 20 days of postinfection	PBLs from experimentally infected horse	mRNA	NM	Kim, H.Y. et al., 2002
IL-10	elevated	human serum from acute-phase HGA compared with convalescent serum	protein in serum	modulate histopathological injury triggered by IFN-γ	DUMLER et al. 2000
IL-18	elevated after four h p.i.	NB4 cells inoculated with *Ap*	mRNA	proinflammatory response, [a] Driving production of IFN-γ for *Ap* clearance	PEDRA et al. 2005; PEDRA et al. 2007
MCP-1, MIP-1α, MIP-1β, RANTES	increased from 24 h to 48 h p.i.	HL-60 cells differentiated along the neutrophil lineage with 1.25 % DMSO incubated with *Ap*	protein in culture supernatant	cytopenia and mediation of inflammatory response	KLEIN et al. 2000

p.i.: post infection
NM: not mentioned

6 PMNs Recruitment and Interaction with Microvascular Endothelial Cells

6.1 Leukocytes Migration and Cell Adhesion Molecules

The migration of leukocytes from circulating blood to specific sites is a crucial step in the inflammatory reaction against invasion of pathogens (KOLACZKOWSKA et al. 2013). Neutrophils are the first cells that cross the blood vessel to the site of infection (RAZAKANDRAINIBE et al. 2013). Even in the absence of infection, monocytes also continuously migrate into the tissue, where they differentiate into macrophages (JANEWAY 2001). The migration occurs through a multistep process (known as extravasation) in which neutrophils interact with the endothelium in postcapillary venules. The process is mediated by integrins, selectins, chemokines and their respective ligands or receptors (KOLACZKOWSKA et al. 2013). Traditionally, this cascade is dissected into a sequential process that includes the capture on, rolling along and firm adhesion to the microvascular endothelium, followed by transmigration through the vessel wall and further migration into the extravascular tissue (ULBRICH et al. 2003). However, this recruitment cascade has been augmented by several more steps, including slow rolling, adhesion strengthening, intraluminal crawling, and paracellular and transcellular migration (LEY et al. 2007).

Selectins are a family of transmembrane glycoprotein, including E-, L-, and P-selectin that bind to fucoslated and sialylated glycoprotein ligands to initiate the transmigration, and are found on endothelial cells, leukocytes and platelets (LEY 2003). Integrins are a group of heterodimeric transmembrane glycoprotein compromising one α- and one β-subunit, and they are mainly found on PMNs and other hematopoietic cells that serve as adhesion-mediating receptors to mediate cell-cell and cell-extracellular matrix adhesion (WAGNER et al. 2000). To date, mammals express 18 α-units and 8 β-units of integrin that combine to form 24 distinct receptor heterodimers (LARJAVA et al. 2014).

Firm adhesion of leukocytes to endothelial cells depends on interactions between integrins such as Macrophage-1 antigen (Mac-1) on neutrophils, and their protein ligands termed immunoglobulin superfamily (IgSF) cell adhesion molecules on endothelial cells (MULLER 2013). In the immunoglobulin superfamily, intercellular adhesion molecules (ICAMs) and vascular adhesion molecule-1 (VCAM-1) are two main surface proteins for PMNs recruitment. PECAM-1 (CD31) is an immunoglobulin superfamily member that is concentrated at the borders of endothelial cells as well as expressed diffusely on platelets and leukocytes (MULLER 2013). Homophilic interaction of leukocyte PECAM with endothelial PECAM is

required for transendothelial migration of leukocytes (MULLER et al. 1993; MULLER 2013). Several crucial adhesion molecules and their ligands involved in transendothelial migration of leukocytes are listed in Table 3.

Table 3: Adhesion molecules and their ligands generally involved in leukocyte transmigration

leukocyte recruitment	adhesion molecules	basic role(s)	name(s)	alternative name(s)	distribution	ligand(s)	distribution	reference(s)
capture, rolling, and slow rolling	selectins	initiate the migration	L-selectin	LAM-1, CD62L	leukocytes, mainly on lymphocytes	CD34[a], GlyCAM-1, MAdCAM-1, and PSGL-1 et al.	vein endothelial cells, leukocytes	WAGNER et al. 2000; JANEWAY 2001; LEY et al. 2007; NIMRICHTER et al. 2008
			E-selectin	ELAM-1, CD62E	activated ECs	E-selectin ligand 1 (in murine), unknown in human	PMNs	
			P-selectin	GMP-140, PADGEM, CD62P	activated ECs and platelets	PSGL-1(CD162), sialyl-Lewis x	PMNs, monocytes and platelets	
adhesion strengthening	integrins	bind the cell-adhesion molecules and extracellular matrix in order to strongly adhere	Mac-1	$\alpha_M\beta_2$, CR3, CD11b/CD18	monocytes, granulocytes, macrophages, and natural killer cells.	ICAM-1, iC3b, fibrinogen	endothelial cells	
			LFA-1	$\alpha_L\beta_2$, CD11a/CD18	T cells, monocytes, macrophages, neutrophils, dendritic cells	ICAMs	endothelial cells	
intravascular crawling	immunoglobulin superfamily	various roles in cell adhesion, transmigration, ligands for integrins	LPAM-1	$\alpha_4\beta_7$	lymphocytes	MAdCAM-1	endothelial cells	LEY et al. 2007
			ICAM-1	CD54	activated endothelial cells	Mac-1, LFA-1[b]	PMNs, lymphocytes	WAGNER et al. 2000; JANEWAY 2001 DAVENPECK et al. 1998; WAGNER et al. 2000
			ICAM-2	CD102	resting endothelial cells, dendritic cells	LFA-1	PMNs, lymphocytes	
			VCAM-1	CD106	activated endothelial cells	VLA-4 (integrin $\alpha_4\beta_1$)	activated human PMNs and rat PMNs	
paracellular and transcellular transmigration			PECAM-1	CD31	activated leukocytes, unstimulated endothelial cell-cell junctions	PECAM-1	activated leukocytes, unstimulated endothelial cell-cell junctions	WAGNER et al. 2000; JANEWAY 2001

[a] a number of glycosylated, fucosylated, sulfated sialylated glycoproteins
[b] less affinity to ICAM-1 than to ICAM-2
L-selectin: leokocyte selectin, E-selectin: Endothelial cell selectin, P-selectin: Platelet selectin, Mac-1: macrophage-1 antigen, LFA-1: lymphocyte-associated function antigen-1, LPAM-1: integrin alpha 4 beta 7 (α4β7), ICAMs: Intercellular adhesion molecules, VCAM-1: Vascular adhesion molecule-1, PECAM-1: Platelet endothelial cell adhesion molecule-1, LAM-1: leukocyte adhesion molecule-1, CD: Cluster of differentiation, ELAM-1: endothelial cell leukocyte adhesion molecule-1, GMP-140: Granule membrane protein 140, PADGEM: Platelet activation-dependent granule to external membrane protein, CR3: Complement receptor 3, GlyCAM-1: Glycosylation-dependent cell adhesion molecule-1, MAdCAM-1: Mucosal addressin cell adhesion molecule-1, PSGL-1: P-selectin glycoprotein ligand-1, VLA-4: Very late antigen-4

6.2 Endothelial Cells as Infection Target

The endothelium consists of a single layer of flat cells (endothelial cells) that line the vasculature. It has been demonstrated to be an infection target of several infectious pathogens, including viruses and bacteria (VALBUENA et al. 2006, 2009). Importantly, its critical location allows endothelial cells to easily interact with any circulating blood cells and be able to modulate host immune system, including initiating neutrophils recruitment, antigen presentation, regulating the passage of immune molecules and immune cells, in order to defense pathogenic invasion (ROTHERMEL et al. 2004; KOLACZKOWSKA et al. 2013; RAZAKANDRAINIBE et al. 2013). A limited number of intracellular bacteria, including *Rickettsia* spp., *Ehrlichia ruminantium*, *Bartonella* spp., *Orientia tsutsugamushi*, and *Anaplasma marginale* use endothelial cells as their main target host cells or as one type of target host cells during infection (VALBUENA et al. 2006, 2009). Besides erythrocytes, microvascular endothelial cells were also shown to be target cells of other related Anaplasma species, e.g. *Anaplasma marginale*, the agent of bovine anaplamosis (LEPIDI et al. 2000; CARRENO et al. 2007).

Only little information is available on *in vivo* infection of *Ap* in endothelial cells. Infection of microvascular endothelial cells by *Ap* has only been identified *in vitro* in bovine and human endothelial cell lines under culture conditions (MUNDERLOH et al. 2004). Interestingly, *Ap* transmission into granulocytes occurs, if co-culture with infected endothelial cells is allowed under static conditions (HERRON et al. 2005). Furthermore, blocking of the mostly utilized host cell receptor, PSGL-1, does not affect the binding of granulocytes to *Ap*-infected HMEC-1 cells, whereas it reduces infection of granulocytes (HERRON et al. 2005). Sialidase pretreatment increases the binding of *Ap* to HMEC-1 cells, while fucoidan as well as EDTA decrease the binding capacity. It is implied that a non-sialylated cell surface moiety different from PSGL-1 is utilized by *Ap* and a bivalent cations (such as Ca^{2+}) is involved during the adhesion process in the endothelial cells (HERRON et al. 2005). However, the evidence of infection of microvascular endothelial cells or other type of endothelial cells by *Ap* in a truly physiological context is still unclear so far.

7 Laboratory Diagnostics

7.1 Anaplasmosis

The manifestations of granulocytic anaplasmosis in humans and animals vary in severity from

non-clinical symptom, to severe clinical signs including death (DUMLER et al. 2005). According to clinical studies in 685 HGA patients across North America and Europe, the most common clinical manifestations in human patients are fever (92 %), headache (75 %), myalgia (77 %), and malaise (94 %). Other clinical manifestations include thrombocytopenia, leukopenia, anemia, and an elevated hepatic transaminase levels (DUMLER et al. 2005). The severity of clinical signs in animals is variable, based on different factors including ages, immune status, co-infections with other pathogens, natural or experimental infection. Despite those effects described above, the most prominent and consistent hallmark of infection is moderate to marked thrombocytopenia, with approximately 50 % decline of circulating platelet numbers (BAKKEN et al. 1996; BORJESSON et al. 2001). The pathophysiological mechanism of thrombocytopenia is not well understood, however it may results from decreased or ineffective hematopoiesis, increased intramedullary destruction (hemophagocytic syndrome), increased peripheral destruction (immune or non-immune mediated mechanism), decreased cell life span, or altered cellular distribution (endothelial or splenic sequestration) (BORJESSON et al. 2002a).

At least five species of bacteria in three genera in the family of Anaplasmataceae have been shown to cause human infection (DUMLER et al. 2007), namely *Ehrlichia chaffeensis* (*E. chaffeensis*), *E. ewingii*, *E. canis*, *Ap*, and *N. sennetsu* (see Table 4). Furthermore, other two important pathogens in veterinary health, *E. muris* and *E. ruminantium* (previously named *Cowdria ruminantium*), have been considered as emerging pathogenic agents for humans recently, while they are primarily reported in the United States and Africa, respectively (ALLSOPP et al. 2005; ESEMU et al. 2011; PRITT et al. 2011).

Table 4: Human infections by Anaplasmataceae and their host, host cells and distribution

genus	species	disease(s)	host cells	host(s)	distribution(s)	reference(s)
Anaplasma	*Ap*	HGA, EGA, CGA, TBRF	granulocytes, endothelial cells	humans, ruminants, horses, dogs, cats, rodents, deer etc.	United States, Europe, Asia, North Africa	RIKIHISA 2011
Ehrlichia	*E. chaffeensis*	HME	monocytes, macrophages	humans, deer, dogs	United States, South America, Asia	RIKIHISA 2011
	E. ewingii	HEE	granulocytes	dogs, deer, humans	United States, South America	NDIP et al. 2005; OLIVEIRA et al. 2009; RIKIHISA 2011
	E. canis	CME	monocytes, macrophages	canids, humans	Worldwide	RIKIHISA 2011
	E. muris	splenomegaly	monocytes, macrophages	rodents, humans	United States, Japan, Russia	PRITT et al. 2011; RIKIHISA 2011
	E. ruminantium	heartwater	endothelial cells, granulocytes	ruminants, humans	Africa, Caribbean	ALLSOPP et al. 2005; ESEMU et al. 2011; RIKIHISA 2011
Neorickettsia	*N. sennetsu*	Sennetsu fever, glandular fever	monocytes, macrophages	humans	Japan, Southeast Asia	RIKIHISA 2011

HGA: human granulocytic anaplasmosis, EGA: equine granulocytic anaplasmosis, CGA: canine granulocytic anaplasmosis, TBRF: tick-borne fever, HME: human monocytic/monocytotropic ehrlichiosis, HEE: human ewingii ehrlichiosis, CME: canine monocytic/monocytotropic ehrlichiosis.

7.2 Diagnosis of Anaplasmosis in Humans and Animals

Diagnostically much attention is devoted on peripheral blood samples from patient with acute HGA. The presence of neutrophilic morulae stained with Wright-Giemsa staining is indicative for an infection. However, the absence of morulae does not preclude infection. Therefore, polymerase chain reaction (PCR) analyses and evaluation of serological responses by indirect immunofluorescent antibody assays (IFA) are applied to confirm the diagnosis (BAKKEN et al. 1996). For serodiagnosis of HGA, *Ap* cultured in HL-60 cells is usually used especially in indirect immunoflurescent antibody assay (IFA) (GAOWA et al. 2014). However, *Ap* propagated in THP-1 cells (an acute human monocytic leukemia cell line) was recently recommended to be used as a supplementary antigen with antigen propagated in HL-60 cells for the serodiagnosis of rickettsiosis-like infections (OHASHI et al. 2013). Human monocytotropic ehrlichiosis (HME) is caused by *Ehrlichia chaffeensis*. HME is also a febrile tick-borne disease. HGA and HME have similar clinical manifestations and therefore need to be taken into consideration as a differential diagnoses. HME's clinical signs including fever, headache, leukopenia, thrombocytopenia and evaluated transaminase levels. Among patients with HME, morulae are rarely observed in peripheral monocytes (ISMAIL et al. 2010). Presumptive diagnosis of HME is based on clinical manifestation, medical history and specific hematologic abnormalities. However, a specific diagnostic test is needed to confirm HME in laboratory. These methods include specific antibodies titration by ELISA, detection of specific ehrlichial DNA in blood by PCR, direct detection of *Ehrlichia* spp. in tissue samples by immunohistochemistry, and bacterial isolation (ISMAIL et al. 2010).

Although no standardized procedure for *Ap* diagnosis in animals has been proposed, similar criteria of HGA diagnosis should be used. The identification of morulae in circulating neutrophils is the fastest and most cost-effective method of *Ap* diagnosis in animals (GREENE 2012). PCR is the most reliable method in the clinical laboratory for specific and early diagnosis of granulocytic anaplasmosis in animals (ENGVALL et al. 1996).

8 Therapy and Prevention

8.1 Therapy for Humans and Animals

Anaplasma and *Ehrlichia* species are sensitive to both tetracycline and doxycycline. Most patients with HGA or HME respond well, if treated with antibiotics early and properly in

illness (DUMLER et al. 2007). Because of fewer side effects, doxycycline is the recommended treatment for HGA or HME for both pediatric and adult cases (DUMLER et al. 2007). The recommended dose is 100 mg per dose administered twice daily (orally or intravenously) for adults or 2.2 mg/kg body weight per dose administered twice daily (orally or intravenously) for children weighing <45.4 kg. Duration of drug treatment is normally tenable for 3 to 5 days or longer (e.g. 10 - 14 days) after disappearance of fever (DUMLER et al. 2007). If co-infection with *B. burgdorferi* is apparent, doxycycline treatment should be continued for at least 10 days for adults (WORMSER 2006; WORMSER et al. 2006) or be continued for three days after disappearance of fever. Later the remainder 14-day course of treatment should be completed with an alternative antibiotic (e.g. amoxicillin or cefuroxime) against *B. burgdorferi* to minimize the risk of dental discoloration for children under eight years of age (CHAPMAN et al. 2006).

For treatment of canine *Ap* infection, antimicrobial therapy is a common and the most effective method. Most dogs respond rapidly to treatment and are frequently clinically healthy 24 to 48 hours after initiation of therapy (GREENE 2012). The recommended therapy for canine *Ap* infection is 5 - 10 mg/kg of doxycycline per os (PO) or intravenously (IV) per administration every 12 - 24 hours for 10 - 21 days. A total of 4 weeks of doxycycline treatment should be considered, if dogs are co-infected with *B. burgdorferi*. Chloramphenicol at 25 - 50 mg/kg, PO, three times per day for 14 - 21 days is recommended to treat puppies that under one year of age, in order to avoid teeth yellowing (GREENE 2012).

8.2 Prevention

So far, no vaccine is available to prevent *Ap* infection in both human and animals. The most effective strategies are (1) avoiding exposure to tick vectors during peak periods of activity (primarily April - September), (2) thorough inspection of the body and clothing for ticks after being in wooded or grassy areas, (3) immediate removal of attached ticks, (4) application of tick repellant before entering grassy or wooded areas (CHAPMAN et al. 2006).

III PUBLICATION

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ORIGINAL INVESTIGATION

Transmission of *Anaplasma phagocytophilum* from endothelial cells to peripheral granulocytes in vitro under shear flow conditions

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Abstract *Anaplasma phagocytophilum* (*Ap*) is a tick-borne pathogen, which can cause granulocytic anaplasmosis in humans and animals. In vivo this obligate intracellular pathogen is primarily located in circulating mature granulocytes, but it also infects endothelial cells. In order to study the interaction between *Ap*-infected endothelial cells and human granulocytes under conditions similar to those found naturally in the infected host, an in vitro model that mimics physiological flow conditions in the microvasculature was established. Cell-to-cell interactions were then visualized by microscopy, which showed that granulocytes adhered strongly to *Ap*-infected endothelial cells at a shear stress of 0.5 dyne/cm^2. In addition, *Ap*-transmission assays under flow conditions showed that the bacteria transferred from infected endothelial cells to circulating granulocytes and were able to establish infection in constantly moving granulocytes. Cell surface analysis showed that *Ap* induced up-regulation of the cell adhesion molecules ICAM-1 and VCAM-1 on infected endothelial cells in a dose-dependent manner. Furthermore, IL-8 secretion by endothelial cells indicated that the presence of *Ap* induced a pro-inflammatory response. In summary, the results of this study suggest that endothelial cells of the microvasculature (1) provide an excellent site for *Ap* dissemination to peripheral blood granulocytes under flow conditions and therefore may play a crucial role in the development of persistent infection, and (2) are stimulated by *Ap* to express surface molecules and cytokines that may lead to inflammatory responses at the site of the infection.

Keywords *Anaplasma phagocytophilum* · Transmission · Endothelial cells · Granulocytes adhesion · Shear flow

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Introduction

Anaplasma phagocytophilum (*Ap*) is a tick-borne pathogen that is able to infect many different animal species and humans worldwide. *Ap* can cause sometimes a severe clinical illness called granulocytic anaplasmosis in humans, domestic dogs, cats, horses or tick-borne fever in ruminants [3, 7, 8, 46]. The clinical signs are non-specific, including fever, leucopenia, thrombocytopenia and anorexia. During the acute phase of granulocytic anaplasmosis, the causative organism is visible in peripheral granulocytes and forms 'bacteria-filled vacuoles' known as morulae [4, 36].

Like other intracellular organisms, *Ap* is able to modulate host cell gene expression to favor its own survival. It uses differential gene expression to maintain the transmission cycle between tick vector and vertebrate host [29, 33, 40]. Feeding ticks carrying the organisms release bacteria into surrounding host tissue via salivary secretion. Interaction and invasion of mammalian cells are probably facilitated by salivary factors [20]. Polymorphonuclear leukocytes (PMNs) are recruited to the feeding lesion by pro-inflammatory cytokines, but the events leading to their invasion remain undefined. Adhesion to and infection of human neutrophil granulocytes by *Ap* during the acute stage of the disease are specifically mediated by tetrasaccharide sialyl Lewis^x (sLe^x or CD15s) on P-selectin glycoprotein ligand 1 (PSGL-1) [19, 22]. However, PMNs do not return to the circulatory

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system after extravasation into tissue. Consequently, these cells cannot serve as a source for subsequent *Ap* dissemination in the host. It has been suggested that endothelial cells can serve as reservoirs for the bacterium and to pass them on to PMNs under in vivo conditions. Microvascular endothelial cells probably represent the essential link between infectious *Ap* organisms and circulating PMNs [31]. Likewise, the closely related agent of bovine heartwater disease, *Ehrlichia ruminantium*, colonizes microvascular endothelium of the brain and heart in naturally infected ruminants and experimentally infected mice, respectively, as well as neutrophil granulocytes [10, 48]. Furthermore, *A. marginale* (the agent of bovine anaplasmosis) can infect endothelial cells in vivo [11, 30]. Needless to say, the physiological barrier formed by vascular endothelial cells (ECs), and particularly its breach, is important for the pathogenesis of infections with different representatives of the Anaplasmataceae family. This cell layer regulates the passage of immune molecules and immune cells from blood vessel into surrounding tissue with a complex system of molecules [34]. ECs also serve as important antigen-presenting cells for the immune system [17, 37]. Importantly, due to their access to the lumen of the blood vessels, endothelial cells easily interact with circulating blood cells. We therefore hypothesized that endothelial cells might be a well-suited niche for initial replication or that they could serve as a reservoir for *Ap* during persistent infection.

Over decades, most in vitro adhesion assays were performed under static conditions to analyze the interaction between ECs and PMNs. Static assays provide valuable information regarding the mechanisms of cell adhesion, but they are clearly limited models to understand adhesive processes in circulating fluids [6, 47]. Transmission of *Ap* from endothelial cells to PMNs was previously observed under static conditions [21]. However, if this behavior constitutes a key element of disease pathogenesis, it must also function under flow conditions. In this study, an in vitro model was utilized to mimic the microvascular environment at physiological shear stress. The aims of this project were (1) to investigate the adhesion of PMNs to *Ap*-infected ECs under flow conditions; (2) to evaluate the transmission of *Ap* between ECs and PMNs under flow conditions; and (3) to analyze the production of cell adhesion molecules and human interleukin-8 secretion by *Ap*-infected endothelial cells during the infection process.

Materials and methods

Ap culture, propagation and purification

The HL-60 (*Human promyelocytic leukemia cells*) cell line (ATCC® CCL-240) was obtained from American Type Culture Collection (LGC Standards GmbH, Wesel, Germany) and used to propagate the mCherry-transformed *Ap* strain HGE1 (mCherry/HGE1) [18]. All experiments described in this manuscript were performed with this *Ap* organism. Uninfected and infected HL-60 cells were cultured in RPMI-1640 medium (GE Healthcare Europe GmbH, Freiburg, Germany) buffered with 25 mM HEPES, 0.1 % $NaHCO_3$ and supplemented with 10 % heat-inactivated fetal bovine serum (Sigma-Aldrich Chemie GmbH, Munich, Germany), and 2 mM L-Glutamine in a humidified 5 % CO_2 atmosphere at 37 °C. Trypan blue (0.5 %) was used to determine cell viability. Giemsa staining was routinely used to check the percentage of *Ap*-infected cells in the cultures by counting 100 cells per slide using a light microscope (Leica DM5000; Leica Microsystems GmbH, Wetzlar, Germany) [9]. *Ap* cultures were harvested when ~80 % cells were infected.

Ap were purified from mechanically disrupted host cells. Briefly, infected HL-60 cells (1.0×10^6 or 1.0×10^7 cells) were concentrated in 1.5-ml culture medium in a 2.0-ml sterile tube containing 0.2 ml of autoclaved rock tumbler grit (60/90 grit silicon carbide; Lortone, Inc., Mukilteo, WA, USA). Cell suspensions were vortexed vigorously for 30 s, the grit was allowed to settle, and the supernatants were transferred to a 10-ml Luer lock syringe and passed through a 2.0-µm pore size filter (Puradisc™ 25 GD; GE Healthcare Europe GmbH) into a sterile 2.0-ml tube. Host cell-free *Ap* were collected by centrifugation at 11,000×*g* for 5 min at 4 °C. The *Ap* pellet was washed twice with 1× PBS containing 0.5 % fetal bovine serum and suspended in 150 or 200 µl of cold basic MCDB 131 medium (Life Technologies, Darmstadt, Germany).

Preparation of human PMNs and DMSO-differentiated HL-60 cells (dHL-60)

Heparin anticoagulated human peripheral blood was collected from volunteers who did not show any clinical symptoms of febrile disease. Informed consent was obtained from volunteers prior to acquisition of samples. Human PMNs were isolated by discontinuous density gradient centrifugation with Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich Chemie GmbH), as described elsewhere [16]. After isolation, the PMN pellet was resuspended in pre-equilibrated RPMI-1640 medium (37 °C, 5 % CO_2 overnight) and adjusted to 5.0×10^5 cells/ml. The viability and purity of PMNs were determined by trypan blue (0.5 %) stain and Diff-Quick stain, respectively.

HL-60 cells were induced to differentiate into mature granulocytes by incubating the cells in growth medium containing 1.25 % DMSO for 6–7 days as reported [14, 15]. The level of CD11b expression on DMSO-differentiated HL-60 cells was measured by flow cytometry.

DMSO-differentiated HL-60 cells were washed three times with 1× PBS prior tested. PMNs and DMSO-differentiated HL-60 cells were labeled with 20 µM CellTracker Green CMFDA dye (Life Technologies) at 37 °C for 30 min and then washed three times before they were used in transmission assays with endothelial cells.

Endothelial cell cultures under static and flow conditions

Two types of human microvascular endothelial cells were used in this study. Firstly, HMEC-1 cells (a human microvascular endothelial cell line) [1] were used between passages 20 to 35 and cultured in 25 mM HEPES and 0.25 % $NaHCO_3$-buffered MCDB 131 medium (pH 7.5) (Life Technologies) supplemented with 10 mM L-glutamine, 10 % heat-inactivated fetal bovine serum, 1.0 µg/ml hydrocortisone (all from Sigma-Aldrich Chemie GmbH) and 10 ng/ml epidermal growth factor (BD Biosciences, San Jose, CA, USA) in a humidified atmosphere with 5 % CO_2 in air at 37 °C. Secondly, primary HDMEC cells (human dermal microvascular endothelial cells) were purchased from PromoCell GmbH, Heidelberg, Germany, and cultured in Endothelial Cell Growth Medium 2 (ECGM2; PromoCell GmbH) according to instructions. The manufacturer had confirmed the presence of endothelial cell-specific markers (e.g., vWF, CD31). Endothelial cells were detached with trypsin/EDTA (0.5 mg/ml and 0.22 mg/ml) and subcultured into new flasks at a density of 5.0×10^4–1.0×10^5 viable cells/ml. Cell viability was assessed with 0.5 % trypan blue.

For cultivation of endothelial cells under flow conditions, a pump culture system to generate a controlled unidirectional shear flow was applied (ibidi GmbH, Martinsried, Germany). All devices were assembled according to the manufacturer's instructions and controlled by the Pump-Control software (version 1.5.0) to generate a laminar flow at defined shear stress in the channel slide. Briefly, 2×10^5 of HMEC-1 or HDMEC cells were seeded into a channel slide (µ-Slide $I^{0.6}$ Luer; ibidi GmbH) and incubated at 37 °C in a humidified atmosphere of air with 5 % CO_2 for 2.5 h to allow attachment prior to connection with the perfusion set. Endothelial cells were further grown overnight at a shear stress of 2.0 dyne/cm^2 and reached confluence after 24 to 48 h, which was suitable for infection or flow experiments.

Exposure of endothelial cells to *Ap*

Optimal infection time for Ap with endothelial cells under static conditions

To exclude the influence of hydrocortisone in MCDB 131 growth medium on endothelial cells adhesion expression, HMEC-1 endothelial cell layers were washed and cultured in hydrocortisone-free MCDB 131 growth medium in all infection assays in this study. At the beginning of this study, the optimal conditions to obtain low-level or high-level *Ap* infections of endothelial cell monolayers were assessed within a fixed time period under static conditions. HMEC-1 cells or HDMEC cells were seeded into a 4-well cell culture chamber (Sarstedt, Nümbrecht, Germany) and grown to 70–80 % confluency. Cell-free bacteria purified from 1.0×10^6 of infected HL-60 cells were then incubated with 2.0×10^5 HMEC-1 cells or HDMEC cells at a multiplicity of infection of 5:1 ('MOI' refers to the ratio of the number of *Ap*-infected HL-60 cells to the number of uninfected endothelial cells) for 24, 48 and 96 h in a humidified atmosphere with 5 % CO_2 in air at 37 °C. After removal of unbound bacteria with three 1× PBS washes, HMEC-1 or HDMEC cell layers were subjected to Giemsa staining and immunofluorescence in situ.

Preparation of Ap-infected HMEC-1 cells for adhesion assay under flow conditions

For the adhesion assay, 2×10^5 HMEC-1 cells in channel slides were incubated with cell-free *Ap* purified from 2×10^5 or 1×10^6 (MOI of 1:1 or 5:1) infected HL-60 cells for 24 h. As a positive control, human TNF-α (100 ng/ml rh TNF-α; R&D Systems, Inc., Minneapolis, MN, USA), a strong stimulator of cell adhesion molecules expression, was used to stimulate HMEC-1 cell monolayers for at least 8 h [27]. HMEC-1 cell monolayers inoculated with cell lysates prepared from uninfected HL-60 cells by using the same purification procedure as described under '*Ap* culture, propagation and purification' served as negative controls. HMEC-1 cells in slides for flow experiments were incubated in a humidified atmosphere of air with 5 % CO_2 at 37 °C. After 24 h, all slides were washed with pre-equilibrated hydrocortisone-free MCDB 131 medium (at 37 °C, 5 % CO_2 in air, overnight) and were then ready to be used for the adhesion assay under flow conditions.

PMN adhesion to Ap-infected HMEC-1 cell monolayer under flow conditions

Ap-infected HMEC-1 cell monolayers were prepared as described above. Similar experiments have been conducted under static conditions by one of the coauthors (UGM) [21]. The experiments described here, however, focused on PMN adhesion under flow conditions. A total volume of 8.0 ml RPMI-1640 medium containing 5×10^5 human PMNs/ml was added to the perfusion set, and PMNs were perfused over *Ap*-infected HMEC-1 cell monolayers at shear stresses ranging from 0.5 to 2.0 dyne/cm^2. Positive

and negative controls (see exposure of endothelial cells to *Ap*, part b) were handled in the same way.

The slides were placed in a heating chamber at constant 37 °C, which was controlled by a Temperature Controller (version 1.0.2; ibidi GmbH), and interactions were visualized using an inverted phase-contrast microscope equipped with a digital video camera (Leica Microsystems GmbH). Adherent PMNs were determined after 10 min of perfusion by photographing 10 randomly selected fields at 7× magnification. The numbers of adherent PMNs were counted using the software Image J (National Institute of Health, Bethesda, MD, USA), and the means values were calculated.

HMEC-1 cells in infection assays under static conditions with varying doses of Ap

Infection assays with varying doses of *Ap* were performed to verify dose-dependent effects of *Ap* organisms on cell adhesion molecule expression. A confluent monolayer of HMEC-1 in a 6-well tissue culture plate (approximately 8.0×10^5 cells/well) was incubated with different doses of *Ap* under static conditions. *Ap* were purified from 1.6×10^7 infected HL-60 cells and suspended in 200 µl of basic endothelial medium. Twofold serial dilutions of purified *Ap* were prepared and added to each well in order to obtain an MOI at 0.07:1–10:1. After 24 h, culture supernatants were collected and stored at −20 °C for IL-8 detection. ICAM-1 and VCAM-1 expression on HMEC-1 cells and the infection ratio of HMEC-1 cells were analyzed by flow cytometry.

Ap-transmission from endothelial cells to granulocytes

Primary HDMEC cells (PromoCell GmbH) were used in the *Ap*-transmission assay in order to maximally reflect physiological functions of endothelial cells. *Ap*-infected HDMEC cells were prepared as described for HMEC-1 cells under 'exposure of endothelial cells to *Ap*, part b' at an MOI of 1:1 or 5:1 for 24 h prior to the transmission assay. As negative control, uninfected HDMEC cells were incubated with lysates of uninfected HL-60 cells. The infection rates of HDMEC cells were measured by flow cytometry. Subsequently, 1.6×10^6 of uninfected human PMNs or DMSO-differentiated HL-60 cells (dHL-60 cells) in 8 ml of RPMI-1640 medium were added and co-cultured with uninfected (negative control) or *Ap*-infected HDMEC cells at 0.5 dyne/cm^2.

At the time points indicated (e.g., 1 h, 3.5 h, 4.5 h, 24 h, 3 days, 5 days, 7 days), 100 µl of suspended cells was harvested and subjected to fluorescence microscopy, Giemsa staining and immunofluorescence in order to detect *Ap* infection in PMNs or DMSO-differentiated HL-60 cells.

Immunofluorescence analysis of *Ap* infection

Immunofluorescence was used to visualize *Ap* infection in endothelial cells, PMNs and DMSO-differentiated HL-60 cells (dHL-60 cells). PMNs and dHL-60 cells were deposited on glass slides by centrifugation prior to fixation. Endothelial cell monolayers were gently washed twice with ice-cold Dulbecco's phosphate-buffered saline. Fixation was carried out with 2 % paraformaldehyde (PFA, in 1× PBS) at room temperature for 10 min. PMNs, dHL-60 or endothelial cell monolayers were permeabilized with 0.3 % (v/v) Triton X-100 for 10 min at room temperature followed by three 10-min washes with 1× Dulbecco's PBS [13]. Nonspecific binding sites were blocked using 5 % (w/v) bovine serum albumin (BSA) overnight at 4 °C followed by incubation with dog anti-*Ap* serum (diluted 1:200, pooled serum from naturally *Ap*-infected dogs) in 1× PBS with 1 % BSA for 1 h at room temperature. Afterwards, cells were washed three times and incubated with FITC-conjugated anti-dog serum (1:500; KPL, Inc., Gaithersburg, MD, USA) for 1 h at room temperature. After three washes with 1× Dulbecco's PBS, the cell nuclei were counterstained with DAPI nucleic acid stain (Life Technologies). Visualization was performed with a fluorescence microscope equipped with band-pass filters specific for DAPI, FITC and mCherry fluorophores, respectively (Leica Microsystems GmbH).

Cell adhesion molecule expression on HMEC-1 induced by *Ap* infection under flow and static conditions

Flow cytometric analysis was performed to detect intercellular cell adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD106) expression on HMEC-1 cells in adhesion assays (flow) and dose-dependent infection assays (static). After 24 h of incubation, uninfected and infected HMEC-1 cell monolayers were washed twice with 1× Dulbecco's PBS before enzymatic dissociation using Accutase™ (GE Healthcare Europe GmbH) at 37 °C for 5–10 min. Twenty microliters of normal human serum was added to 80 µl of cell suspensions (5×10^5–1×10^6 cells) in staining buffer (1× PBS containing 0.5–1.0 % (w/v) BSA and 0.09 % NaN_3 at pH 7.2) followed by incubation on ice for 20 min. Cell suspensions were then washed twice with staining buffer at 4 °C and 350×*g* for 5 min. Afterwards, 2 µl of undiluted FITC antihuman CD54 (clone: HCD54; BioLegend, Inc., London, UK) and 2 µl of undiluted phycoerythrin (PE)-conjugated anti-human CD106 (clone: STΛ; BioLegend, Inc.) were added to 100 µl cell suspensions in staining buffer and incubated on ice for 15–20 min in the dark, followed by washing twice with ice-cold 1× PBS. Corresponding fluorochrome-labeled mouse IgG1 and κ isotype-matched control antibodies (FITC or PE-labeled; BioLegend, Inc.)

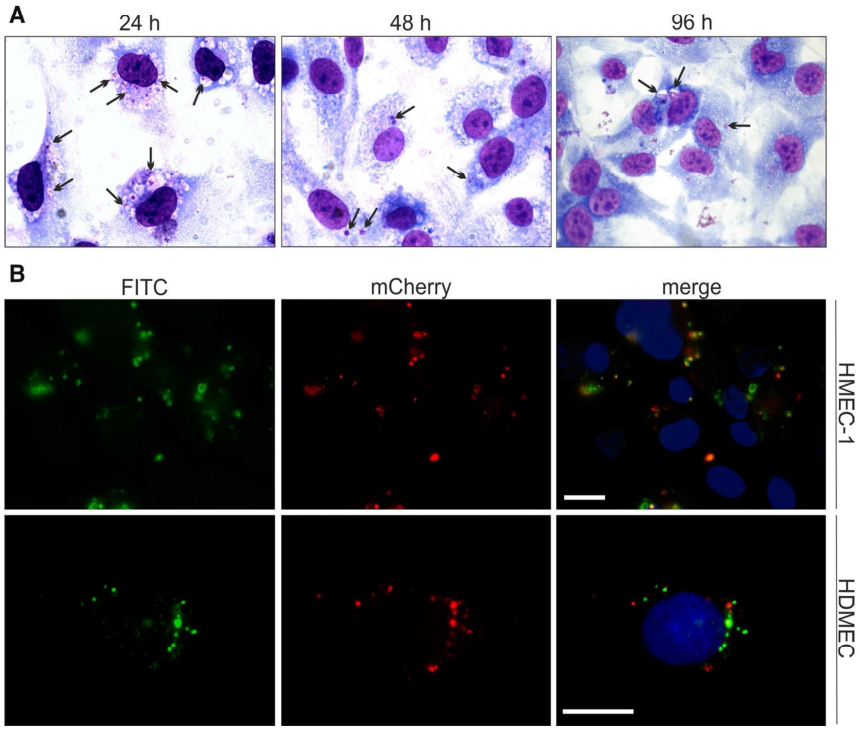

Fig. 1 Detection of *Ap* infection in endothelial cells by Giemsa staining and immunofluorescence. **a** Giemsa-stained *Ap*-infected HMEC-1 cell monolayers. HMEC-1 cells were incubated with isolated *Ap* at an MOI of 5:1 for 24, 48 and 96 h. Morulae in the cytoplasm of HMEC-1 cells are indicated with *black arrows*. **b** Immunofluorescence staining of *Ap*-infected HMEC-1 and HDMEC. The HMEC-1 or HDMEC cells were incubated with isolated *Ap* at an MOI of 5:1. After 24 h, cells were fixed with 2 % PFA and permeabilized with 0.3 % Triton X-100 in 1× PBS. Dog anti-*Ap* serum was added; this was followed by incubation with FITC-conjugated anti-dog serum. The cell nuclei were visualized by DAPI-staining (*blue*). Fluorescence microscopy was carried out using band-pass filters specific for FITC, mCherry and DAPI. *Scale bar* 20 µm. *Ap*: mCherry/HGE1 (color figure online)

were used to assess the level of background staining in cell–antibody binding. Dead cells were excluded with a fixable viability dye eFluor® 450 (eBioscience, Frankfurt am Main, Germany) using a violet laser (405 nm). After washing with staining buffer once, cells were fixed with ice-cold 2 % PFA (pH 7.2) before data acquisition in a MACSQuant® VYB (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). The infection ratio of HMEC-1 cells was evaluated by detecting mCherry-positive cells by flow cytometry. Ten thousand events were acquired and analyzed using FlowJo software (FlowJo, LLC., Ashland, OR, USA).

Measurement of interleukin-8 (IL-8) secretion by HMEC-1 cells infected with *Ap*

Culture supernatants of HMEC-1 cells infected with different doses of *Ap* as described under 'exposure of endothelial cells to *Ap*' were collected and stored at −20 °C. The concentration of IL-8 in the supernatants was measured with a commercial sandwich ELISA kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the instructions of the manufacturer. Each sample was measured in triplicate.

Statistical analysis

All statistical analyses were carried out using un-paired Student's *t* test with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Data were represented as the means ± SE. Mean differences between the groups were considered statistically significant and highly significant at a p value of <0.05 (*) and <0.01 (**), respectively.

Results

Optimal time period for *Ap* to infect endothelial cells under static conditions

Microscopic evaluation revealed that *Ap* was able to invade endothelial cells in vitro within 24 h of incubation (Fig. 1a, b). Between 60 and 80 % of HMEC-1 cells were infected after incubation with isolated *Ap* at an MOI of 5:1 when evaluated after 24 h. Classical inclusions, so-called 'morulae', were observed at 24, 48 and 96 h in the cytoplasm of Giemsa-stained HMEC-1 cells (Fig. 1a). The fraction of infected cells decreased to 20–30 % at 48 and 96 h p.i. The 24-h incubation period was used for further experiments.

In order to verify the observations obtained with Giemsa staining, immunofluorescence was used to assess *Ap* infection in HMEC-1 cells after 24 h of incubation (Fig. 1b). Morulae varying in size were visible around cell nuclei of HMEC-1 cells (Fig. 1b, merge-panel). Epifluorescence microscopy was used to visualize mCherry-labeled *Ap*

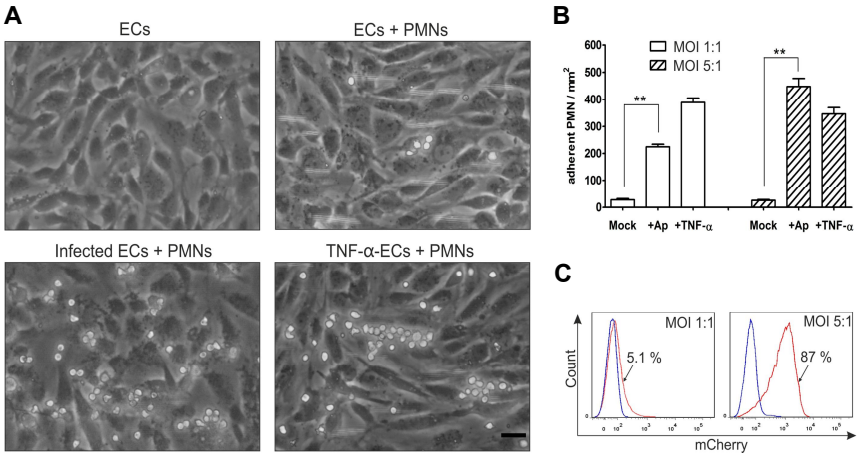

Fig. 2 PMN adhesion to *Ap*-infected HMEC-1 cell monolayers. 4×10^6 PMNs isolated from volunteers in 8 ml medium was added to uninfected or *Ap*-infected HMEC-1 cells or to HMEC-1 cells treated with 100 ng/ml TNF-α and then perfused for 10 min at 0.5 dyne/cm^2. Experiments were performed five times. **a** PMN adhesion after 10-min perfusion at 0.5 dyne/cm^2. *Scale bar* 25 μm. **b** Number of adherent PMNs per mm^2 on endothelial monolayer; means ± SE of three representative experiments are shown; * and ** indicate statistically significant differences: $p < 0.05$ and $p < 0.01$; Student's *t* test. **c** Flow cytometry analysis of *Ap* infection ratio in endothelial cells. HMEC-1 cells exposed to *Ap* at an MOI of 1:1 (*left panel*) and 5:1 (*right panel*) and cultured for 24 h were harvested and subjected to *Ap* detection (fluorescence mCherry positive). A fraction of 5.1 and 87 % of HMEC-1 cells visible in the *red spectrum* in both *panels* were shown to be infected. *Blue lines* show the fraction of uninfected cells. Representative results of one out of three experiments are shown. *ECs* endothelial cells; *Ap*: mCherry/HGE1 (color figure online)

(Fig. 1b, mCherry-panel), and results were compared to those found with immunofluorescence staining (Fig. 1b, FITC-panel). Comparable results were obtained with primary HDEMC cells (Fig. 1b).

PMN adhesion to *Ap*-infected HMEC-1 cell monolayers under flow conditions

In the flow culture system, HMEC-1 cells formed a confluent monolayer after overnight growth at a shear stress of 2.0 dyne/cm^2 (Fig. 2a, ECs). Infected HMEC-1 cells modified their morphology presenting enlarged cells or vacuolated cells; however, infected cells remained attached under shear stress (Fig. 2a, infected ECs). Harvested HMEC-1 cells analyzed with flow cytometry showed that 5.1 and 87.0 % of HMEC-1 cells carried *Ap* when exposed to the organisms at an MOI of 1:1 and 5:1, respectively (Fig. 2c). No tight adhesion of PMNs to the HMEC-1 cell monolayers was observed at 2.0 dyne/cm^2. When the shear stress was adjusted to 0.5 dyne/cm^2, tight adhesion of PMNs was observed on both *Ap*-infected HMEC-1 cell monolayers and TNF-α (100 ng/ml) stimulated monolayers. A shear stress of 0.5 dyne/cm^2 was used for further experiments.

Within 10 min of interaction, *Ap* infection of HMEC-1 cells (MOI of 1:1) significantly enhanced PMNs adhesion (224.9 ± 9.4 PMNs/mm^2, Fig. 2b) to the cells when compared to uninfected HMEC-1 cells (27.9 ± 4.5 PMNs/mm^2, $p < 0.01$). Many more PMNs adhering to high-level infected HMEC-1 cells (MOI of 5:1) were observed (448.3 ± 29.5 PMNs/mm^2, Fig. 2b, $p < 0.01$). PMN interaction with infected HMEC-1 (MOI of 5:1) was significantly more prominent than observed with cells stimulated with TNF-α (346.5 ± 23.8 PMNs/mm^2, Fig. 2b, $p < 0.01$).

Ap-transmission from endothelial cells to granulocytes

In order to maximally reflect physiological functions of endothelial cells, primary HDMEC cells were used in the transmission assay. Two HDMEC cell populations infected with varying doses of *Ap* were investigated under flow conditions. Before the *Ap*-transmission assay was initiated, flow cytometric analysis showed that approximately 10 % (low-level infection) or 70 % (high-level infection) of HDMEC cells carried *Ap* when exposed to the bacterium at an MOI of 1:1 or 5:1, respectively (data not shown).

PMN adhesion to the HDMEC monolayer was observed microscopically after leukocytes were added to the chamber slide. Less than 1 % of the PMNs isolated from volunteers were infected with *Ap* after 4.5 h co-culture with *Ap*-infected HDMEC cells under flow conditions (Fig. 3a, left panel). Interestingly, additional infected PMNs were not observed for up to 24 h, and PMNs isolated from volunteers lysed quickly within 24 h under flow conditions. This compares well to the reported half-life of 7 h for PMNs in peripheral blood [38].

DMSO-differentiated HL-60 cells (dHL-60 cells) mimicking functional PMNs [14, 15] were therefore used for

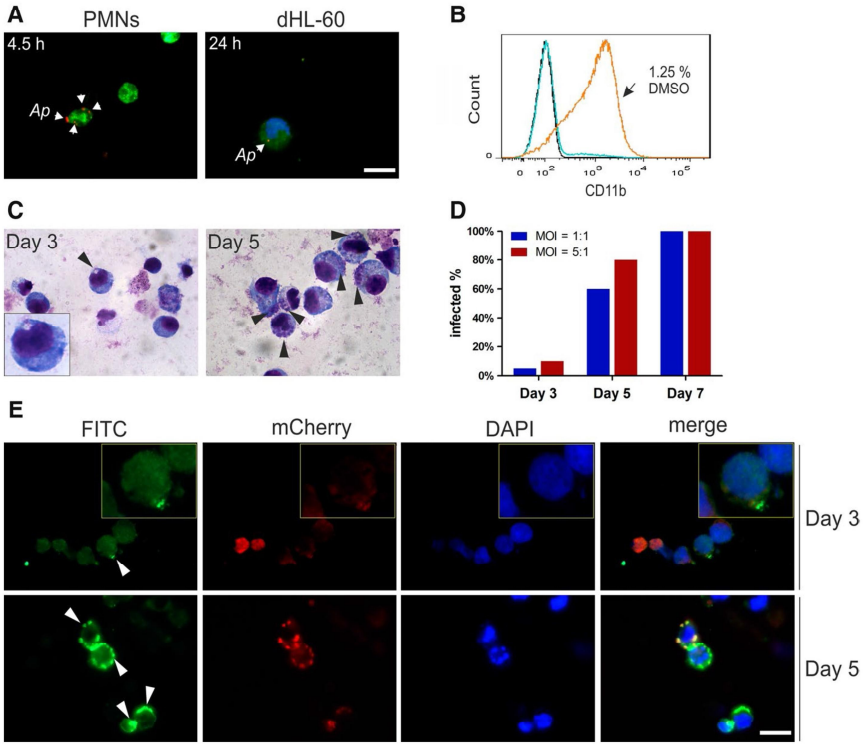

Fig. 3 *Ap* transmission from infected endothelial cells to PMNs and DMSO-differentiated HL-60 cells. dHL-60 cells were prepared by incubating the cells in the presence of 1.25 % DMSO in growth medium for 6–7 days. 1.6×10^6 CMFDA-labeled or CMFDA-unlabeled PMNs or dHL-60 cells in 8 ml of RPMI-1640 medium were added and co-cultured with uninfected or infected HDMEC cells (at 10 or 70 % infection level) at 0.5 dyne/cm^2. At time points indicated, PMNs or dHL-60 cells were harvested and subjected to fluorescence microscopy, immunofluorescence assay and Giemsa staining. **a** Representative fluorescence microscopy images for PMNs (4.5 h) and dHL-60 cells (24 h) from the high-level infection group (HDMEC cells at 70 % infection level) after co-culture with *Ap*-infected HDMEC cells. **b** Flow cytometric analysis of CD11b expression on DMSO-differentiated HL-60 cells (dHL-60 cells). Undifferentiated and differentiated HL-60 cells were washed and stained with FITC-conjugated anti-human CD11b antibody (*blue* and *orange line*) or FITC-conjugated mouse IgG1 (*black line*). **c** Images of Giemsa-stained dHL-60 cells in the high-level infection group at Day 3 and Day 5. **d** Proportions of *Ap*-infected dHL-60 cells at Days 0, 3, 5 and 7. Results of one of two independent experiments are shown. **e** Representative micrographs of immunofluorescence stained dHL-60 cells from the high-level infection group at Day 3 and Day 5. *Scale bar* 20 μm (color figure online)

additional co-culture experiments involving *Ap*-infected HDMEC-1 cells. Figure 3b shows that 92 % of all HL-60 cells expressed CD11b on their surface for 7 days in the presence of 1.25 % DMSO. After co-culture of endothelial and dHL-60 cells for 1 or 3.5 h, no morulae were observed in dHL-60 cells by Giemsa staining and fluorescence microscopy. After prolonged co-culture for 24 h, however, less than 1 % of dHL-60 cells interacting with the high-level infected HDMEC cells carried *Ap* (Fig. 3a, right panel). Two days later (day 3 after exposure to *Ap*), approximately 10 % of dHL-60 cells and at day 5, 80 % of the cells were infected with the bacterium (Fig. 3c). Most of dHL-60 cells were lysed by day 7. Comparable results were obtained when a HDMEC population was used as an *Ap* source and the bacteria were available at a low level (10 % infection rate in HDMEC cells; Fig. 3d). Immunofluorescence analysis was performed in addition to verify results seen after Giemsa staining (Fig. 3e).

Cell adhesion molecule expression on HMEC-1 cells induced by *Ap* infection: analyses under flow and static conditions

ICAM-1 and VCAM-1 expressions on HMEC-1 cells were evaluated under flow conditions and under static conditions. After 10 min of interaction with PMNs under flow conditions, high-level infected HMEC-1 cells (87.0 %) demonstrated a significant up-regulation of ICAM-1 from 24.7 % (baseline level expression of HMEC-1) to 91.7 % (Fig. 4a, ECs + *Ap*). In comparison, a slight increase to 37.7 % was observed when only 5.1 % of HMEC-1 cells were infected with *Ap* (Fig. 4a,

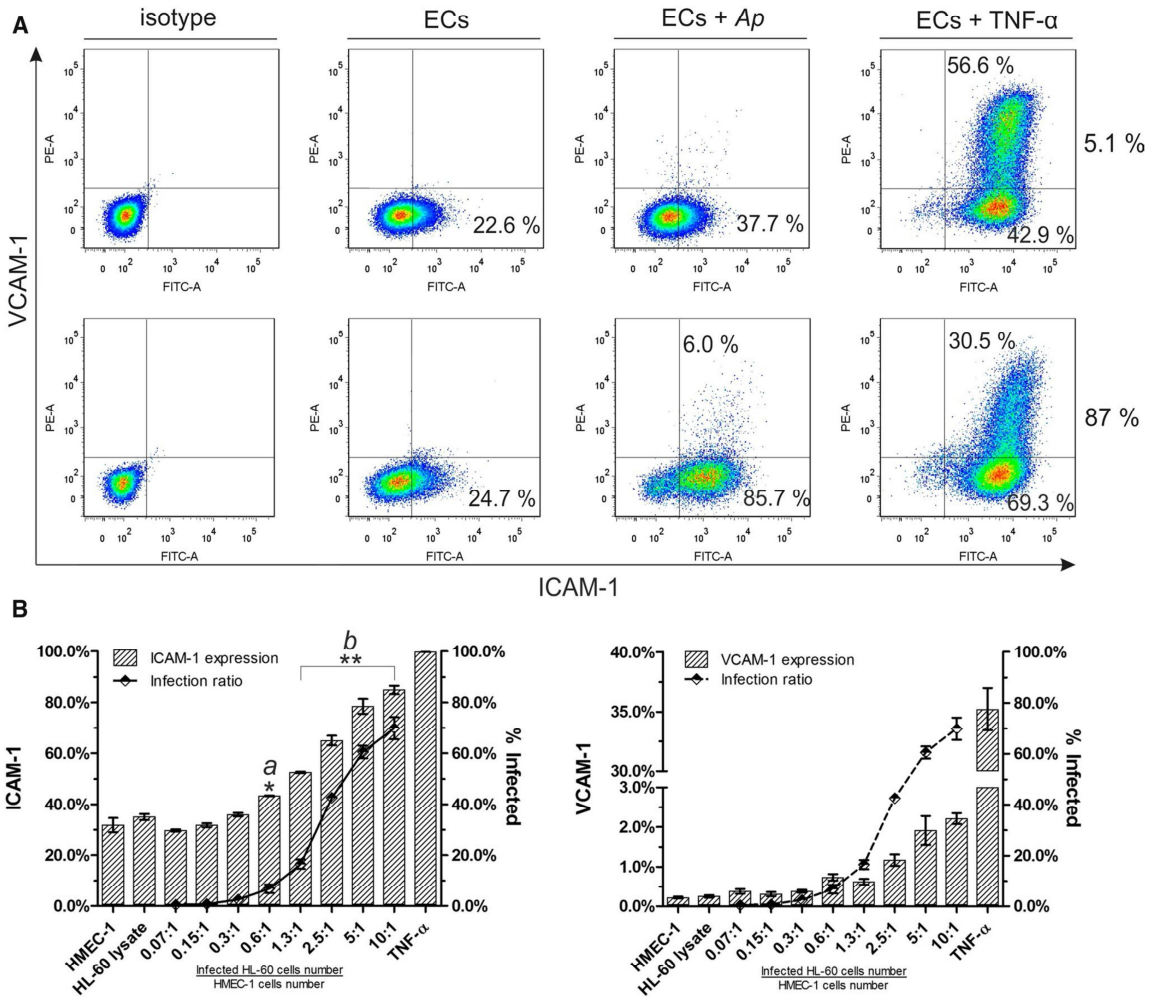

Fig. 4 ICAM-1 and VCAM-1 expression on HMEC-1 cells. **A** Flow cytometry analysis of ICAM-1 and VCAM-1 expression on HMEC-1 cells after interaction with PMNs under flow conditions. Uninfected, TNF-α (100 ng/ml) treated and *Ap*-infected HMEC-1 cells (5.1, 87.0 % infection rate) that were co-cultured with PMNs (5×10^5) at 0.5 dyne/cm^2 for 10 min were harvested and subjected to flow cytometric analysis. Results of one of three representative flow experiments are shown. **B** Expression of ICAM-1 and VCAM-1 on HMEC-1 cells by *Ap* infection under static conditions as function of the infection dose. Surface expressions are calculated as the percentages of positive cells in the gated cell population. Means ± SE from one of three independent experiments are shown. Each measurement was performed in triplicate. The infection ratios of HMEC-1 cells were determined by flow cytometry using mCherry-labeled *Ap*. * and ** indicate statistically significant differences: $p < 0.05$ and $p < 0.01$; Student's *t* test (*a*: 'group 0.6:1' vs. 'group HMEC-1 + HL-60'; *b*: any of 'group 1.3:1, 2.5:1, 5:1 and 10:1' vs. 'group HMEC-1 + HL-60'). *Ap*: mCherry/HGE1. ICAM-1: intercellular cell adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1

ECs + *Ap*). Almost 100 % of HMEC-1 cells were capable of expressing ICAM-1 in the presence of 100 ng/ml TNF-α (positive control) after 24-h incubation (Fig. 4a, ECs + TNF-α). In contrast to the baseline level of ICAM-1 expression, VCAM-1 was not constitutively expressed on the HMEC-1 cells. Moreover, VCAM-1 expression was only inducible on HMEC-1 cells with a high *Ap* infection rate (Fig. 4a, ECs + *Ap*). A fraction of HMEC-1 cells (30.5 or 56.6 %) was capable of expressing VCAM-1 after stimulation with 100 ng/ml TNF-α for 24 h (Fig. 4a, ECs + TNF-α).

Results of experiments under flow conditions showed that the number of *Ap* in the culture influenced ICAM-1 and VCAM-1 expression in a dose-dependent manner. To verify this effect, both ICAM-1 and VCAM-1 expressions were repeatedly examined under static conditions in a less complex in vitro system. As shown in Fig. 4b, ICAM-1 expression was induced in a dose-dependent way

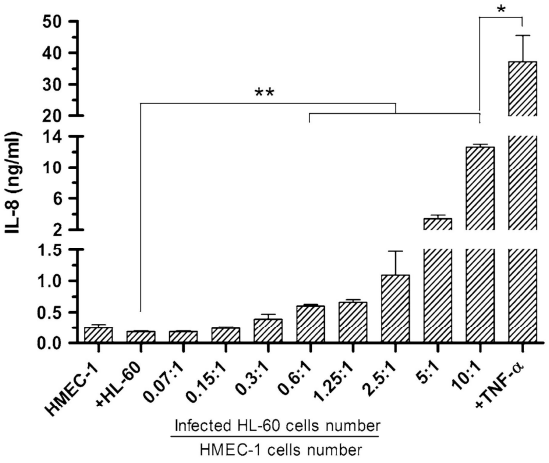

Fig. 5 Induction of IL-8 secretion by endothelial cells in response to *Ap* infection IL-8 concentration measured after 24 h exposure of HMEC-1 cells to different doses of *Ap* under static conditions. Each sample was measured in triplicate, and data are shown as means ± SE. *$p < 0.05$ and **$p < 0.01$ indicate statistically significant differences; Student's *t* test

on infected HMEC-1 cells when the infection ratio was increased (Fig. 4b, left panel). Similar results were obtained for VCAM-1 (Fig. 4b, right panel). The addition of HL-60 cell lysate had no effect on ICAM-1 and VCAM-1 expression when compared to untreated HMEC-1 cells (Fig. 4b, column HL-60 cell lysate).

Measurement of human IL-8 secretion by HMEC-1 cells infected with *Ap*

To verify whether IL-8 was released by endothelial cells, the concentration of IL-8 in the culture supernatants was assessed. ELISA results showed that secretion of IL-8 by HMEC-1 cells was dose-dependent and increased with the numbers of *Ap* organisms in the culture (Fig. 5). Again, the addition of HL-60 cell lysate had no effect on IL-8 secretion (184.3 ± 12.6 ng/ml, $p > 0.05$) when compared to untreated HMEC-1 cells (244.8 ± 58.9 ng/ml). When the multiplicity of infection was raised to 10:1, an extremely high level of IL-8 was detected in the supernatants (1,264.1 ± 377.2 ng/ml, $p < 0.01$) compared to untreated HMEC-1 cells. However, the level of IL-8 secretion by *Ap*-infected HMEC-1 cells was significantly lower than that observed after TNF-α stimulation (3,473.1 ± 539.9 ng/ml, $p < 0.05$).

Discussion

Besides PMNs, microvascular endothelial cells have been shown to be susceptible target cells for *Ap* infection in vitro [21, 31]. However, the role of microvascular endothelial cells in the pathogenesis, especially during the initial transmission period of *Ap* infection, is not fully understood. It is unknown how *Ap* migrates from tick attachment sites to peripheral blood granulocytes, which is the most commonly detected host cell type in vertebrates. The reason for the lack of information might be (1) due to *Ap*'s low-abundance in endothelial cells during the early stages of the infection; consequently, *Ap* is difficult to detect with conventional microscopic methods in clinical samples; thus, endothelial cells as a source for *Ap* dissemination have remained undetected, (2) and due to the lack of suitable in vitro models that allow a precise dissection of the interactions necessary to transfer *Ap* from endothelial cells to circulating PMNs.

In this study, a cell culture system mimicking physiological shear flow was established in order to explore the likely natural interaction between *Ap*-infected endothelial cells and PMNs as well as to analyze expression of human IL-8 and two cell adhesion molecules (ICAM-1 and VCAM-1) that are main contributors to the tight adhesion of PMNs in vivo [26, 39]. Using this flow culture system, different flow rates were applied to generate defined shear stress ranging from 0.5 to 2 dyne/cm^2. Interactions with endothelial cells such as rolling and tight adhesion of PMNs were clearly visible at the lowest chosen shear stress of 0.5 dyne/cm^2 (Fig. 2). Almost no tight adhesion was observed at 2.0 dyne/cm^2. Shear forces in the blood vessels should be considered when the interaction between *Ap*, endothelial cells and PMNs is investigated, as the forces exerted by moving fluids are capable of modulating cytoskeletal rearrangement, cell morphology and gene expression in cells as well as influencing leukocyte-endothelial adhesion [12, 44]. Typical values of shear stress in human blood vessels are in the range of 0.1–13 dyne/cm^2 depending on the type of blood vessel, e.g., large arteries, small arteries or, as in our case, capillaries [35].

Our transmission assays showed that *Ap* uptake by circulating PMNs occurred within 4.5 h after the addition of PMNs to the endothelial cell culture (Fig. 3b). Additional infected cells, however, were not observed in the following 19.5 h when PMNs isolated from volunteers were used. These results reflect the low number of infected PMNs seen in human patients. In order to obtain longer culture periods, dHL-60 cells, which have been shown to resemble mature neutrophils [14, 15], were used. The results of these transmission assays showed that *Ap* organisms transfer to circulating granulocytes from infected endothelial cells and finally establish infections in circulating, moving granulocytes under flow culture conditions. Infection in dHL-60 cells was detectable after 3 days, and subsequently, the infection level increased during the following 4 days of co-culture with infected endothelial cells (Fig. 3d). Given that

the replication cycle of *Ap* is roughly 24 h [43], it is likely that the bacteria replicated in infected host cells (endothelial cells or dHL-60 cells) and that released *Ap* organisms were the source for further ongoing infection of host cells. Interestingly, studies in sheep have revealed that each cycle of bacteremia is followed by a period of a few days in which *Ap* cannot be detected in blood [41]. This might indicate that the bacterium resides in tissue-bound cells only to return to the peripheral blood under optimal conditions. In this context, microvascular endothelial cells could serve as a 'safe-haven' for *Ap* during the early or chronic phase of the persistent infection.

Adhesion of circulating leukocytes to endothelial cells is an essential process of inflammatory responses of the innate immune system [26], which is mediated by specific endothelial-leukocyte adhesion molecules including selectins (e.g., P- and E-selectin), integrins (e.g., LFA-1, Mac-1) and the immunoglobulin superfamily (e.g., ICAM-1, VCAM-1 and PECAM-1) resulting in capture, rolling, tight adhesion of leukocytes, passage across the endothelial wall and subsequent migration to the inflammatory site [23, 26, 28]. The up-regulation of cell adhesion molecules on endothelial cells allows functional PMNs to reach the site of infection and exert their critical functions to eliminate foreign agents by phagocytic and cytotoxic activities [32, 45]. The results of this study showed that infected microvascular endothelial cells that up-regulated ICAM-1 and VCAM-1 molecules enabled PMNs to adhere to the endothelium under flow conditions. Furthermore, ICAM-1 and VCAM-1 up-regulation on HMEC-1 cells was induced by *Ap* infection in a dose-dependent manner and not by cell debris of other cultured cells. This suggests that endothelial cells can be activated by *Ap* organisms and that this process probably is the first step of an inflammatory response, which results in severe damage of the endothelial lining seen after infections with several rickettsial organisms [43].

IL-8, also known as neutrophil chemotactic factor (NCF), is a member of the CXC chemokine family. It attracts and activates neutrophils in inflammatory response [25]. A previous study demonstrated that IL-8 secretion is inducible in human neutrophils by *Ap* infection [2]. Our results showed a similar effect: *Ap* induced a substantial IL-8 secretion in cultured endothelial cells (Fig. 5). This indicated a strong inflammatory stimulation of endothelial cells triggered by *Ap* infection and might also be associated with tissue damage seen after infections with other rickettsial organisms [5, 24].

It is worth noting that the *Ap* load in HMEC-1 cells varied considerably in our experiments even when the same infection dose was used to infect the endothelial cells (Figs. 2c vs. 4b). Only dense-cored cell forms of *Ap* (DCs) are infectious for HL-60 cells as shown previously [42]. For our experiments, we determined the number of infected HL-60 cells prior to *Ap* purification. Because of the varying numbers of *Ap* organisms present in a single HL-60 cell, we assumed that the variability seen in our experiments was likely due to the varying numbers of infectious DCs found in different batches of infected HL-60 cell cultures.

In conclusion, data obtained with this in vitro model demonstrated that granulocytes strongly adhered to *Ap*-infected endothelial cells under flow conditions at shear stress of 0.5 dyne/cm^2. The close proximity of endothelial cells and PMNs resulting from the adhesion may further facilitate the transfer of *Ap* from endothelial cells to circulatory granulocytes. Up-regulations of the intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) on endothelial cells and the secretion of IL-8 by endothelial cells were induced by *Ap* infection in a dose-dependent manner. Here, we showed for the first time that transmission of *Ap* from microvascular endothelial cells to granulocytes occurred under flow conditions.

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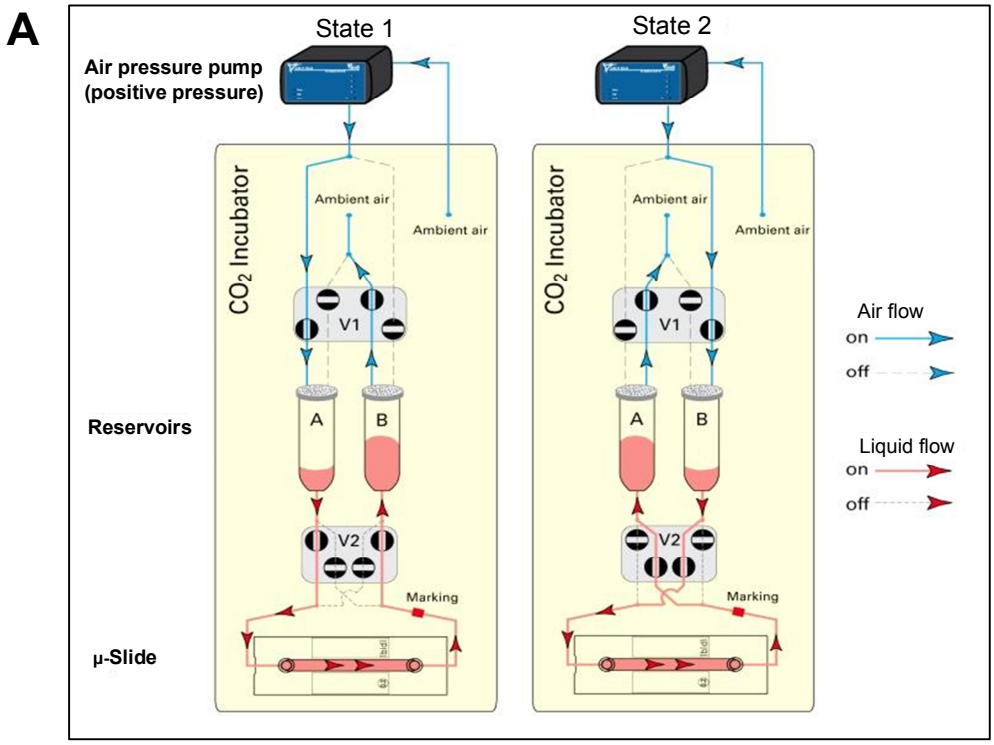
IV DISCUSSION

1 Establishment of the Flow Culture System

Endothelial cells cultivated *in vitro* are quite different from that *in vivo* due to dynamic blood flow-mediated regulation and tissue microenvironment-mediated regulation including the constantly changing environment of inflammation (VALBUENA et al. 2009). Dynamic shear forces are capable of modulating cytoskeletal rearrangement, cell morphology and gene expression in endothelial cells as well as influencing leukocyte-endothelial adhesion (WALPOLA et al. 1993; CHIU et al. 2005). Therefore, the endothelium cultured under shear stress becomes necessary when the interaction between *Ap*, endothelial cells and peripheral polymorphonuclear leukocytes (PMNs) is investigated. In this study, a flow culture system was initially established in order to mimic physiological shear stress *in vivo*. Defined shear stress is needed to simulate the dynamic interaction between *Ap*-infected endothelial cells and PMNs, and this allowed us to investigate the *Ap* transmission pathway between cells under physiological relevant flow conditions. In order to to generate a controlled unidirectional shear flow an air pump was used. The schematic of whole flow culture system driven by air pump is illustrated below.



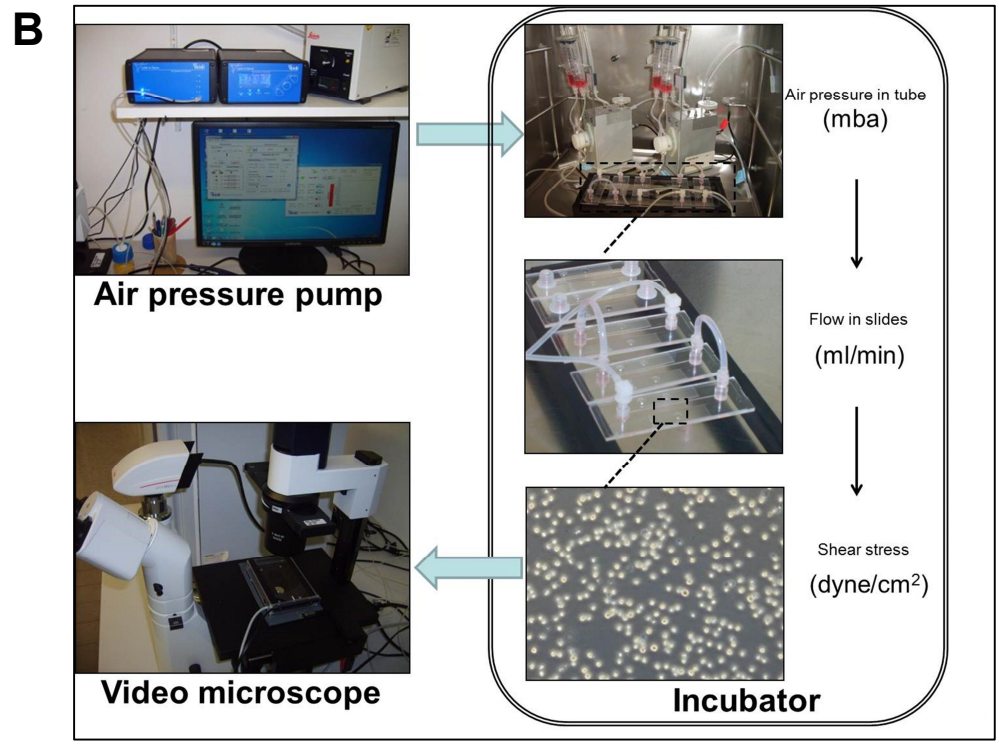


Figure 7: Schematic of the flow culture system

A Working principle of the pump system (ibidi GmbH, Martinsried, Germany) to ensure the unidirectional flow in the chamber slide; **B** schematic setup of the flow culture system inside and outside of incubator in lab.

Firstly, the feasibility of culturing endothelial cells under different shear stresses using this pump system was verified. Given that little information about the physiological shear stress in the microvasculature is available in the literature (PRIES et al. 1996; FRY et al. 2012) relevant values of shear stress can be only estimated *in vitro* by different models (RENEMAN et al. 2008). Typical values of shear stress in blood vessels are in the range of 0.1 - 13 dyne/cm^2 depending on the type of blood vessels in humans (RENEMAN et al. 2008). Considering that the minimal effective shear stress that can be used is 0.37 dyne/cm^2 (µ-Slide I$^{0.6}$ Luer, Handbook of Cell Analysis from ibidi, Martinsried, Germany), shear stresses at 0.5, 1.0 and 2.0 dyne/cm^2 were chosen and tested in this study. It was found that microvascular endothelial cells were able to form a confluent monolayer when shear stress was 0.5, 1.0 and 2.0 dyne/cm^2 (see Figure 2A in publication). The results are consistent with that from another group, which cultured HMEC-1 cells at shear stress levels from 0.25 to 4.0 dyne/cm^2 (GRUBB et al. 2009). Secondly, the endothelial cell culture medium (MCDB 131 medium) depleted of hydrocortisone was tested for its applicability for culturing HMEC-1 cells (data not shown) under shear stress to exclude hydrocortisone's influence on cell adhesion molecules expression.

Gluococorticoids exert their powerful anti-inflammatory effect through different mechanisms including inhibition of the expression of ICAM-1, VCAM-1 and LFA-3 as well as P- and E-selectin, inhibition of chemotaxis and adhesiveness of leukocytes. (SANCHEZ-MADRID et al. 2001; ULBRICH et al. 2003). It was found that no difference in the efficiency of proliferation was present when endothelial cells were freshly subcultured from cultured cells in MCDB 131 medium and subsequently cultured in hydrocortisone-free MCDB 131 medium under shear stress. Interestingly, preliminary results showed that hydrocortisone is able to decrease ICAM-1 expression in HMEC-1 cells, implying the expression of cell adhesion molecules is restrained by hydrocortisone and thereby impairs the adhesion of PMNs (data not shown). Therefore, hydrocortisone was not used when HMEC-1 cell monolayers were exposed to *Ap*, even though it is recommended to add 0.5 - 1.0 µg/ml of hydrocortisone to the endothelial cell culture medium (XU et al. 1994; SURMA et al. 2011). However, the effect of hydrocortisone on the other cell adhesion molecules and even on pathogenicity of *Ap* still needs to be investigated in greater detail.

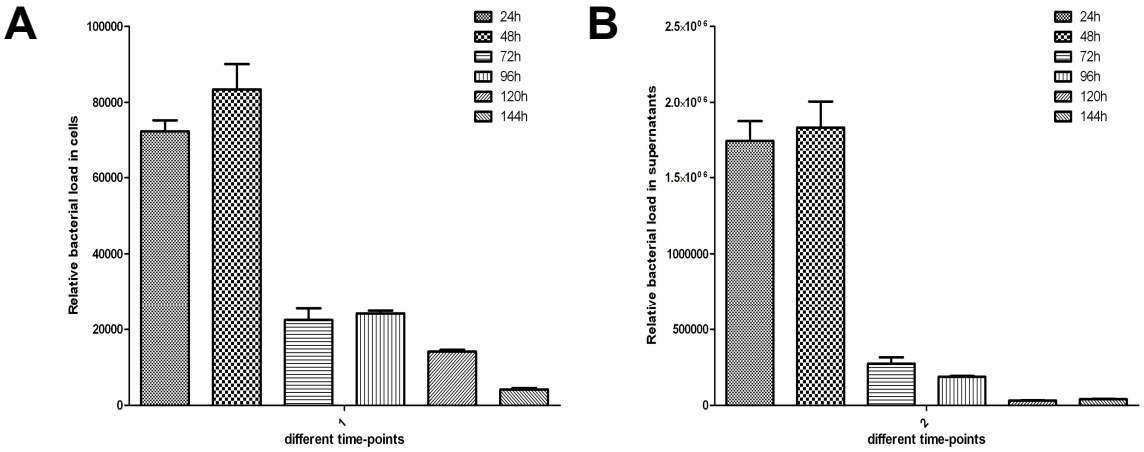
A large numbers of animal experiments were conducted via a direct intravenous injection of *Ap* organisms to study the infection pathway (AKKOYUNLU et al. 2001; SCORPIO et al. 2004). However, this infection approach does not mimic *Ap* infection under natural condition as it ignores the possible contribution of endothelial cells in the initial transmission of *Ap*. The data generated in the studies presented here proof that the flow culture system is an ideal platform to study the role of endothelial cell infection prior to interaction with neutrophils and consequently adds critical information to any new animal experiment.

2 Infection of Microvascular Endothelial Cells

It is known that microvascular endothelial cells are susceptible to *Ap* infection (MUNDERLOH et al. 2004; HERRON et al. 2005). *Ap* infects different relevant endothelial cells including rhesus (RF/6A), human (HMEC-1, MVEC) and bovine (BCEC/D-1b) endothelial cell lines *in vitro* (MUNDERLOH et al. 2004).

Due to a very fast proliferation of endothelial cells in nutrient-enriched culture medium, it is difficult to maintain the infected endothelial cells in the situation that holds a high infectious ratio but also retains a confluent monolayer. Therefore, the incubation time and infectious doses of *Ap* were optimized when HMEC-1 cells were inoculated with the bacteria. Given the proliferation of primary endothelial cells will stop once they are reaching a confluent stage,

primary HDMEC cells were also tested and used in the later *Ap* transmission assay. It was documented that *Ap* invades and replicates within HMEC-1 cells and primary HDMEC cells after 24 h post infection (see Figure 1 in publication). Numerous smaller inclusions or so called 'morulae' were observed at 24 h, 48 h and 96 h in the cytoplasmic vacuoles of Giemsa-stained HMEC-1 cells and HMDEC cells. It was also demonstrated that *Ap* invasion into endothelial cells occurred rapidly, in agreement with the result reported previously (MUNDERLOH et al. 2004). To determine the infection ratio, an immunofluorescent antibody test (IFAT) and Giemsa staining were used. It was shown that 60 % - 80 % of HMEC-1 cells were infected at 24 h, whereas the infection ratio decreased to 20 % - 30 % at 48 h and 96 h (see Figure 1 in publication). The results indicate that replication of *Ap* is not activated once the organisms are internalized into endothelial cells. Result of real-time PCR (COURTNEY et al. 2003; COURTNEY et al. 2004) also confirmed this phenomenon (see Figure 2 in publication). The phenomenon of decreased infection ratio along incubation time is also pointed out in a previous paper (MUNDERLOH et al. 2004). It was hypothesized, that surviving HMEC-1 cells in an infection milieu do replace cells that were lysed due to anaplasma growth, but subsequently are refractory to infection themselves. However, the mechanism of this phenomenon is not known so far. Hence, 24 h of incubation time was selected in order to observe the interaction between PMNs and *Ap*-infected HMEC-1 cells as this time point showed the highest infection rate.



1. Bacterial load in HMEC-1 cells

2. Bacterial load in culture supernatants

Figure 8: *Ap* load in HMEC-1 cells after different inoculation time

Bacterial load was evaluated by RT-PCR targeting *msp2* gene reported elsewhere. Culture media were changed at 48 h and 96 h. The bacterial load in cells (**A**), and in culture supernatants (**B**) decreased along the incubation time.

In this study, two different values of multiplicity of infection (MOI) (5 and 1) were applied in order to simulate different conditions of infection ratio in endothelial cells that may occur *in vivo*. Therefore, two different amounts of host-cell free *Ap* organisms were inoculated for 24 h with endothelial cells. Flow cytometric data showed that around 87 % of HMEC-1 cells were infected at a MOI of 5, while around 5.1 % of infected cells were obtained at a MOI of 1 (see Figure 2C in publication). However, this result was not reproducible between different batches of experiment when the same MOI was used. It is known that *Ap* transitions between an infectious DC form and a replicating RC form during replication in HL-60 cells (TROESE et al. 2009). Given the technical problem to exactly quantify the yield of such intracellular bacteria, it is explainable that the experiments did not show reproducible results concerning the infection ratio in endothelial cells. It is widely accepted to use a ratio of infected HL-60 cells to target cells (endothelial cells) for inoculum calculation in previous researches (CARLYON 2005). In this study, we used the same method to calculate inoculum dose. In fact, prior to *Ap* purification, only the number of infected HL-60 cells was counted even though different numbers of bacteria may be present in each HL-60 cell. Therefore when infected HL-60 cells were collected, it was not guaranteed that a same amount of infectious DCs was present in the same quantity of infected HL-60 cells. After *Ap* were liberated from infected HL-60 cells, varying numbers of infectious DCs were obtained between different batches of infected HL-60

cell cultures. Therefore, different numbers of *Ap* inoculating with endothelial cells leads to a diversity of infection ratio in endothelial cells.

The result above points out that the infection ratio in endothelial cells depends on the incubation time and infectious doses used. The fraction of infected endothelial cells apparently increased along with the infection doses, but likely decreased along with the incubation time.

3 Alterations of PMN Adhesion, Cell Adhesion Molecules

Neutrophils are the first wave of leukocytes to arrive at the site of inflammation to phagocytize pathogens (KOBAYASHI et al. 2009). To successfully exert their functions, neutrophils must firstly attach to the endothelium that is located to the interior of blood vessel. Previous study showed that PMN adhesion to *Ap*-infected endothelial monolayer is increased under static conditions (HERRON et al. 2005). However, no data is available in the context of flow conditions thus far. Therefore PMNs adhesion to *Ap*-infected endothelial cells under flow conditions was investigated for the first time in this study. It was found that PMN adhesion increased remarkably ($p < 0.01$) when naïve PMNs were perfused onto *Ap*-infected endothelial monolayer compared to uninfected endothelial monolayer at shear stress of 0.5 dyne/cm^2 (see Figure 2B in publication). This finding indicates that endothelial cells are activated via *Ap* infection. And because *Ap*-infected neutrophils and dimethyl sulfoxide (DMSO) differentiated-HL-60 cells displayed a reduced binding to cultured brain and systemic endothelial cells, which is accompanied with reduced surface expression of P-selectin glycoprotein ligand 1 (PSGL-1, CD162) and L-selectin (CD62L) under shear force conditions (CHOI et al. 2003). Such activation may be associated with the change of surface cell adhesion molecules expression (CHOI et al. 2003). It was also demonstrated that the susceptibility of mice to *Ap* depends on the presence of PMN (BIRKNER et al. 2008). Therefore, the enhanced recruitment of PMNs to endothelial cells may provide a new niche for *Ap* and might facilitate the bacterial dissemination by interacting with *Ap*-infected endothelial cells. Furthermore, PMN adhesion to endothelial monolayers that are highly infected with *Ap* was significantly more prominent than observed with cells stimulated with TNF-α ($p < 0.01$). It is supposed that a broad type of immune molecules such as CAMs and cytokines are activated and expressed by endothelial cells infected with high inoculum of *Ap*. Interaction between various factors thus exceeds the effect of single used TNF-α. However, this hypothesis needs to be investigated in further studies.

The migration of neutrophils to inflammatory sites is generally driven through a cascade reaction that is mediated by a series of cell adhesion molecules (CAMs) on neutrophils, endothelial cells and by the interaction with extracellular matrix (VOISIN et al. 2013). To characterize the enhanced PMN adhesion, two CAMs, intercellular adhesion molecule 1/ICAM-1 and vascular cell adhesion molecule 1/VCAM-1, on the surface of endothelial cells that are mainly responsible for firm adhesion of PMNs were studied. Flow cytometry analysis revealed that the expression of ICAM-1 and VCAM-1 were considerably upregulated on endothelial cells infected with *Ap* compared with uninfected endothelial cells. Compared with the result reported previously (MUNDERLOH et al. 2004), the established method of using flow cytometry allows to quantify the expression of ICAM-1 and VCAM-1 on *Ap*-infected endothelial cells. In this study, it was shown that the expression of ICAM-1 and VCAM-1 was induced by *Ap* infection in a dose-dependent manner (see Figure 4 in publication). Because ICAM-1 possesses a relative high level of basic expression (22.6 % - 24.7 %, see Figure 4A in publication) that is remarkably induced by *Ap* infection, while VCAM-1 is not constitutively expressed on HMEC-1 cells. Of these two cell adhesion molecules, ICAM-1 is likely to play a dominate role in mediating PMN recruitment during the *Ap* infection. In turn, VCAM-1 expression was only inducible when endothelial cells were infected with *Ap*. It is known that neutrophils express constitutively high levels of the β_2-integrins Mac1 (also known as $\alpha_M\beta_2$; CD11b/CD18) and LFA1 (also known as $\alpha_1\beta_{2;}$ β_2 integrin CD11a complexed with CD18), which bind to endothelial cell surface molecules, such as ICAM-1 and ICAM-2 (KOLACZKOWSKA et al. 2013). Especially the firm adhesion of neutrophil is mediated largely by the β_2-integrins LFA-1 and Mac1, by which binding to the same ligand, ICAM-1 (DIAMOND et al. 1991; ISSEKUTZ et al. 1992; PHILLIPSON et al. 2006). Therefore, increased levels of ICAM-1 and VCAM-1 upon *Ap* infection in this study can be interpreted as the phenomenon of enhanced PMN adhesion. On the other hand, the alteration of adhesion molecules was observed in the context of endothelium infection with other intracellular parasites, such as *Rickettsia conorii*, *Rickettsia rickettsii*, *Ehrlichia ruminantium*, *Orientia tsutsugamushi* and *Toxoplasma gondii* (TAUBERT et al. 2006; VALBUENA et al. 2006). Therefore, the alteration of adhesion molecules is considered as a consequence of host cells activation depending on the different cell tropism of intracellular parasites. Neutrophils infection with *Ap* also leads to increased level of β_2-integrin (Mac-1, CD11b/CD18) expression on their surface, indicating an activated condition of the neutrophils (CHOI et al. 2003). This activation of neutrophils augments organism clearance, as infection of CD11b/CD18-knockout C57/BL6 mice results in an early increase in bacteria burden in blood

compared to the wild-type mice (BORJESSON et al. 2002b). The evidences described above highlight an important role of manipulation of adhesion molecules expression in intracellular bacterial infection. Although membrane-bound forms of either adhesion molecules (ICAM-1, VCAM-1) are easy to measure *in vitro* culture conditions, soluble forms can be detected in the serum or plasma in many conditions with an inflammatory component (BALLANTYNE et al. 2002).It would be quite interesting to investigate such soluble adhesion molecules after tick bite but before appearance of intracellular inclusion within peripheral leukocytes *in vivo*. Doing so, it may provide a link between endothelial inflammatory response and *Ap* infection.

4 Alteration of IL-8

Cytokines and chemokines are proven to play a crucial role in the process of neutrophils migration (KOBAYASHI 2008). In this study, the expression level of IL-8 was measured in culture supernatant from endothelial cells infected with *Ap*. IL-8 is known as a key chemokine mediating neutrophils recruitment to the site of infection. It was previously shown that HGA bacteria and the HGA-44 protein (or P44) markedly induce IL-8 secretion in retinoic acid differentiated HL-60 cells (AKKOYUNLU et al. 2001). An increased IL-8 was detected in serum of humans, horses and mice infected with *Ap* (AKKOYUNLU et al. 2001; KIM et al. 2002; SCORPIO et al. 2004). However, the IL-8 secretion by endothelial cells upon *Ap* infection has not been investigated yet. In the adhesion assay of this study, it was found that adhering PMNs were evenly distributed on endothelial cell monolayers (see Figure 2A in publication). It indicates that the interaction between *Ap*-infected endothelial cells and PMNs is mediated by some soluble factors. Subsequently it was shown that IL-8 expression was significantly induced by *Ap*-infected endothelial cells compared with uninfected endothelial cells (see Figure 5 in publication). Increased secretion of IL-8 by *Ap*-infected endothelial cells attracts uninfected neutrophils attachment, subsequently increases the chance of *Ap* transmission back to neutrophils. In order to see whether the infection status of endothelial cells alters the level of IL-8 expression, different doses of cell-free *Ap* was added and co-cultured with endothelial cells. The infection ratio of endothelial cells showed a clear dose-dependent effect on IL-8 expression secreted by endothelial cells (see Figure 5 in publication). This finding demonstrates that *Ap* induces IL-8 expression and thereby facilitates the recruitment of PMNs to endothelial cell monolayer, in agreement with result from other researchers (AKKOYUNLU et al. 2001). A supportive role of IL-8 in *Ap* infectivity in host is also considered in other studies (AKKOYUNLU et al. 2001; SCORPIO et al. 2004). For

example, $CXCR_2^{-/-}$ mice that lack the human IL-8 receptor homologue showed a reduced susceptibility to *Ap* as also did the evidence of reduced pathogen load in tissues compared to control mice (SCORPIO et al. 2004). This result also supports the view that IL-8, as a crucial chemokine, is exploited by *Ap* to facilitate their replication in host cells.

Based on the communication networks of proinflammatory cytokines, it is quite interesting to investigate the profile of cytokines that secreted by endothelial cells and their roles in the context of *Ap* infection. It is known that recruitment of PMNs to endothelial monolayer can be blocked by pre-incubation with IL-8 antibodies (AKKOYUNLU et al. 2001). It can be assumed that the inhibition of PMNs recruitment will take place under flow conditions. Furthermore, that blockage of IL-8 receptor on neutrophils may inhibit or at least slow down *Ap* dissemination from endothelial cells to neutrophils using the established *in vitro* flow culture model. Nonetheless, this still needs intensive investigation in further studies.

5 The Role of Endothelial Cells in the Transmission of *Ap* Infection

Ap has developed mechanisms to facilitate its survival within its host. Even though the bacteria are majorly found in peripheral neutrophils in experimental infected animals and clinical cases (DUMLER et al. 1998; BUNNELL et al. 1999), it is still questionable whether neutrophils are the unique residence of bacteria for ongoing infection, or what other types of host cells are getting involved in the transmission route utilized by *Ap*. In mice, *Ap* is found mainly in blood, bone marrow, and tissue with high blood flow, especially in the spleen (HODZIC et al. 2001). Undoubtedly, tissues with good blood supply contain large numbers of peripheral leukocytes, which in turn are utilized by the bacterium for its growth. Plenty of *Ap* are released from infected neutrophils to interact with nearby endothelial cells of local blood vessel. On the other hand, *Ap* is able to invade and replicate within microvascular endothelial cells (MUNDERLOH et al. 2004). So it is reasonable to presume that microvascular endothelial cells harboring bacteria provide a well-suited niche for continuously infection in circulating peripheral leukocytes in blood.

To prove this hypothesis, the interaction between these two types of susceptible host cells (endothelial cells and neutrophils) were studied under shear flow conditions by using the flow culture system. Transmission assays showed that *Ap* uptake by PMNs only occurred in a short time (4.5 h) after fresh PMNs were added, but not increased during the following time (see Figure 3B in publication). Such relatively low number of infected PMNs acquired (see Figure

3B in publication) in the *in vitro* transmission assay may truly reflect the low amount of infected PMNs *in vivo*. By now, the transmission assay offers a hint that it is possible for *Ap* to transfer from endothelial cells to PMNs *in vivo*. Given the biphasic developmental stages and their multiplication time of *Ap* in host cells (POPOV et al. 1998; MUNDERLOH et al. 1999; TROESE et al. 2009), it is sensible to consider that the time for bacterial multiplication within endothelial cells is important for ongoing infection to other host cells. In order to achieve a longer culture time, a human promyelocytic leukemia HL-60 cell line that differentiated along neutrophil lineage with 1.25 % DMSO (dHL-60 cells) but also possessed normal functions of mature neutrophils (COLLINS et al. 1978, 1979) were used. As speculated, the results of transmission assays showed that *Ap* organisms were not only transferred to circulating granulocytes from infected endothelial cells, but also eventually established steady infections in circulating, moving granulocytes under flow culture conditions. Infection in dHL-60 cells was detectable after three days followed by an increased infection level for four days of co-culture with infected endothelial cells (see Figure 3D in publication). Given that the replication cycle of *Ap* is roughly 24 h (TROESE et al. 2009), it was likely that *Ap* replicated in infected host cells (endothelial cells or dHL-60 cells) and were released for further infection of uninfected host cells.

V SUMMARY

Anaplasma phagocytophilum (*Ap*) is a tick-borne and an intracellular pathogen that mainly replicates in neutrophils in humans and animals. Besides neutrophils, microvascular endothelial cells are also susceptible to *Ap in vitro*. Little information is available about the role of microvascular endothelial cells in the initial stage of *Ap* transmission. It is reasonable to hypothesize that endothelial cells might be a well-suited niche for initial replication or longer-lived endothelial cells could represent a tissue reservoir for *Ap* during persistent infection. The aims of this study were: 1. to establish a flow culture model to co-culture endothelial cells and polymorphonuclear leukocytes (PMN); 2. to investigate the adhesion of PMNs to *Ap*-infected endothelial cells and the changes of associated adhesion molecules; 3. to evaluate the feasibility of *Ap* transmission between endothelial cells and PMNs under flow conditions.

The recruitment of PMNs is a crucial step in the defense of *Ap* infection and is initiated by changes of surface adhesion molecules on endothelium that results in stimulation by inflammatory mediators. Over decades, most *in vitro* assays concerning PMNs adhesion were performed under static conditions. Static assays provide valuable information regarding the mechanisms of cell adhesion, but they are clearly limited models to understand adhesive process in circulating fluids. Therefore, in order to mimic the physiological flow conditions in microvasculature, a flow culture system was established in this study. The resulting data showed that microvascular endothelial cells grew very well and formed a confluent monolayer under shear stress of 2.0 dyne/cm^2. *Ap* replicated within HMEC-1 cells and primary HDMEC cells after 24 h post infection. The fraction of infected endothelial cells apparently increased along with infection doses, but likely decreased along with incubation time. In agreement with the results in other studies that were performed under static conditions, adhesion assay under flow conditions (e.g. 0.5 dyne/cm^2) showed a remarkable increase of PMNs adhesion to *Ap*-infected endothelial cell monolayers.

Intercellular adhesion molecules 1 (ICAM-1) and vascular cell adhesion molecules 1 (VCAM-1) are mostly presenting on endothelial cells that are mainly responsible for firm adhesion of PMNs. Flow cytometric analysis revealed that the expression of ICAM-1 and VCAM-1 are considerably upregulated on endothelial cells infected with *Ap* compared to uninfected endothelial cells. Of these two adhesion molecules, ICAM-1 was upregulated from 24.7 % (basic level of expression) on uninfected endothelial cells to 91.7 % on endothelial cells that were vastly infected with *Ap* (87.0 %). In contrast to the baseline level of ICAM-1 expression,

VCAM-1 was not constitutively expressed on endothelial cells (HMEC-1 cells) and was only inducible when the endothelial cells are infected with high dose of *Ap*. Moreover, ICAM-1 expression was induced in a dose-dependent manner on infected endothelial cells. Similar results were gathered for VCAM-1. This indicates that *Ap* induces the expression of the adhesion molecules and thereby enhances the recruitment of PMNs to infected endothelial monolayer under flow conditions. Concurrently, the concentration of IL-8 secreted by endothelial cells was measured by ELISA. A remarkable high level of IL-8 induced by *Ap* in endothelial cells was detected, and the induction was dose-dependently promoted by infectious doses of bacteria. Thus, IL-8 might be exploited by *Ap* to recruit more neutrophils and therefore to facilitate infection.

In the transmission assay, fresh human PMNs and HL-60 cells that differentiated into neutrophilic lineage with DMSO (dHL-60 cells) were used to be perfused with *Ap*-infected endothelial cells. It was clearly shown that *Ap* transmission occurred from endothelial cells to either fresh PMNs or dHL-60 cells. The result of Giemsa staining showed that approximately 10 % at day 3, and 80 % at day 5 of dHL-60 cells were infected with *Ap*. Most of dHL-60 cells were progressively lysed due to *Ap* infection in the next two days.

In summary, the flow culture model established in this study mimics the physiological environment in the microvasculature and allows a detailed observation of PMN adhesion to endothelial cells. The finding of *Ap* transmission from endothelial cells to PMNs in a dynamic flow conditions sheds light on the understanding of *Ap* pathogenicity mechanism, and this finding brings an insight into a situation of existence of *Ap* infection in microvasculature *in vivo*.

VI ZUSAMMENFASSUNG

Anaplasma phagocytophilum (*Ap)* ist ein durch Zecken übertragener, intrazellulärer Krankheitserreger. Das Bakterium vermehrt sich hauptsächlich in neutrophilen Granulozyten der Menschen und Säugetiere, wobei unter *in vitro* Bedingungen gezeigt wurde, dass *Ap* sich auch in Endothelzellen des mikrovaskulären Gefäßsystems replizieren kann. Über die Rolle des Endothels in der frühen Infektionsphase während einer *Ap*-Infektion ist nur wenig bekannt. Es besteht die begründete Annahme, dass Endothelzellen als primärer Replikationsort bei einer *Ap*-Infektion eine Rolle spielen oder sogar als „Gewebenische“ bei persistenten Infektionen dienen können. Daher war Ziel dieser Arbeit: 1. ein Kultivierungsmodell mit Endothelzellen und polymorphkernigen neutrophilen Granulozyten (PMN) unter dynamischen Flussbedingungen zu etablieren; 2. die Adhäsion von PMNs an *Ap*-infizierte Endothelzellen und die Veränderungen der damit in Verbindung stehenden Adhäsionsmoleküle zu untersuchen; 3. und zu analysieren, ob eine Übertragung des Erregers von infizierten Endothelzellen auf PMNs unter Flussbedingungen möglich ist.

Relevant für die effektive Abwehr bei einer *Ap*-Infektion ist, dass PMNs in ausreichender Menge angelockt werden. Dies wird durch die Änderung bestimmter Oberflächenproteine des Endothels initiiert, wodurch es zur Freisetzung proinflammatorischer Botenstoffe kommt. *In-vitro*-Untersuchungen zu Adhäsionsmechanismen der PMNs wurden bisher jahrzehntelang unter statischen Versuchsbedingungen durchgeführt. Durch Auswertungen konnten dabei sichere Erkenntnisse zu Zelladhäsionsmechanismen gewonnen werden. Diese Studien sind jedoch in ihrer Aussage limitiert, da keine Erkenntnisse über die Adhäsionsvorgänge unter physiologischen Flussbedingungen getroffen werden können. Daher wurde in dieser Arbeit ein dynamisches, steuerbares Kultivierungssystem etabliert, um die physiologischen Bedingungen im mikrovaskulären Gefäßsystem nachzuahmen. Das Wachstum der Endothelzellen in dem etablierten Kultivierungssystem war sehr gut. Bei Scherbedingungen von 2,0 dyne/cm^2 bildeten die Endothelzellen eine geschlossene Zellschicht. Vierundzwanzig Stunden *post infectionem* replizierte sich *Ap* in den beiden verwendeten Zelllinien HMEC-1 und der primären HDMEC Zelllinie. Der Anteil infizierter Endothelzellen stieg dabei mit Zunahme der Infektionsdosis, sank jedoch mit anhaltender Inkubationszeit. Diese Ergebnisse stimmen mit anderen Studien, die unter statischen Bedingungen durchgeführt wurden, überein. Unter Flussbedingungen (z.B. bei 0,5 dyne/cm^2) lag der Anteil an PMNs, die an *Ap*-infizierte Endothelzellen banden, jedoch deutlich höher als in den Studien, die unter statischen Bedingungen durchgeführt wurden.

Für eine sichere Adhäsion der PMNs an Endothelzellen sind hauptsächlich zwei Adhäsionsmoleküle, ICAM-1 (interzelluläres Adhäsionsmolekül) und VCAM-1 (vaskuläres Adhäsionsmolekül), die auf der Oberfläche von Endothelzellen exprimiert werden, verantwortlich. Verglichen mit nicht infizierten Endothelzellen waren die genannten Oberflächenmoleküle bei *Ap*-infizierten Endothelzellen in dem etablierten Infektionsmodell mit fliesenden Kulturmedium hochreguliert. Für ICAM-1 (ausgehend vom Basisexpressionslevel) wurde dabei ein Anstieg der Expression ausgehend von 24,7 % (nicht infizierten Endothelzellen) auf 91,7 % gemessen (bei einer Infektionsarte der Endothelzellen von 87,0 %). Für VCAM-1 konnte dies nicht gezeigt werden. Dieses Adhäsionsmolekül war lediglich bei hochgradig infizierten Endothelzellen exprimiert. Die Expressionsrate war jedoch sowohl im Falle von ICAM-1 als auch für VCAM-1 abhängig von der Infektionsrate in den Endothelzellen. Diese Ergebnisse deuten darauf hin, dass *Ap* durch Hochregulierung bestimmter Oberflächenmoleküle PMNs anlockt und dadurch auch unter dynamischen Kultivierungsbedingungen Endothelzellen infizieren kann. Mit Messungen mittels ELISA konnte zudem gezeigt werden, dass auch die Menge des freigesetzten Chemokines IL-8 direkt abhängig von der Bakterienmenge in den infizierten Endothelzellen ist. Dies lässt darauf schließen, dass *Ap* den Faktor IL-8 nutzt, um mehr neutrophile Granulozyten anzulocken und damit die Infektion auf weitere Zellen ausdehnt.

Um die Übertragung von *Ap* von infizierten Endothelzellen auf PMNs zu untersuchen, wurden frische PMNs von Versuchspersonen und zudem unter dem Einfluss von DMSO zu neutrophilen Granulozyten differenzierte HL-60 Zellen (dHL-60 Zellen) verwendet. An *Ap*-infizierte Endothelzellen wurden unter definierten Flussbedingungen die genannten, freibeweglichen Zellen vorbeigeleitet. Dabei zeigte sich, dass tatsächlich eine Übertragung von *Ap* entweder auf humane PMNs oder auf dHL-60-Zellen stattfindet. Giemsa-Färbungen betätigten einen prozentualer Anstieg der *Ap*-infizierten Zellen zwischen Tag 3 und Tag 5 *post infectionem* von 10 % auf 80 %. Durch die hohe Anzahl der *Ap* in den Endothelzellen lösten sich aber diese zunehmend während der folgenden zwei Tage auf.

Das in dieser Arbeit etablierte Kultivierungsmodell unter Flussbedingungen spiegelt die physiologischen Bedingungen im mikrovaskulären Gefäßsystem *in vitro* am besten wieder und gibt neue Informationen über die Anheftungsmechanismen von PMNs an Endothelzellen. Durch die im Flussmodel gezeigte Übertragung von *Ap* von Endothelzellen auf PMNs konnten detaillierte Erkenntnisse über mögliche Pathogenitätsmechanismen gewonnen werden, die bei einer *Ap*-Infektion eine Rolle spielen. Mit Hilfe des etablierten Modells sind nun differenzierte Einblicke in Mechanismen möglich, die *in vivo* während einer *Ap*-Infektion im mikrovaskulären Gefäßsystem geschehen.

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IX APPENDIX

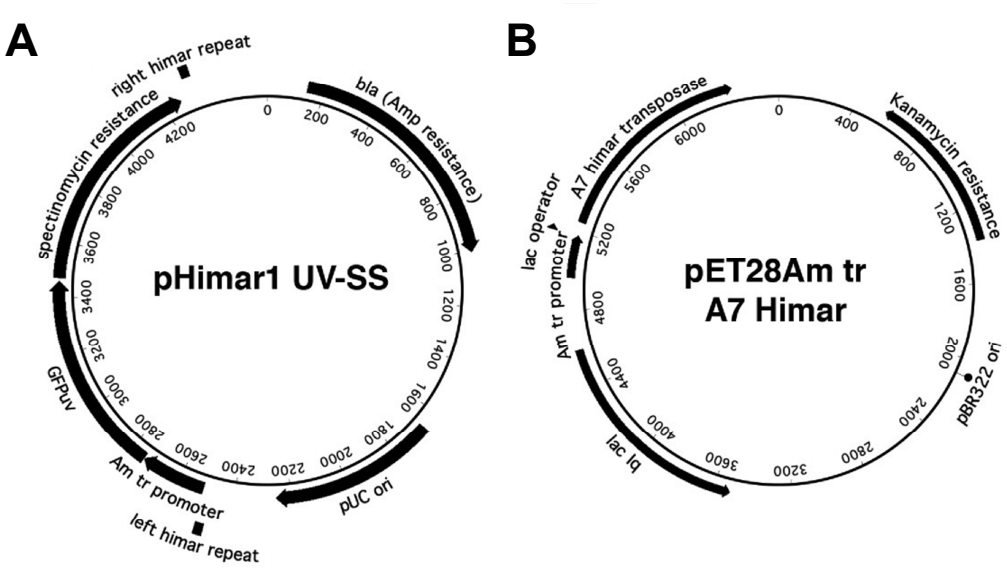

Figure A1: Physical maps of the Himar 1 transposon and transposase plasmid

From FELSHEIM et al. 2006
A pHIMAR1-UV-SS carries the *A. marginale* promotor tr driving expression of GFP and spectinomycin resistance. **B** pET28AMTR-A7-HIMAR contains the A7 hyperactive mutant of the Himar 1 transposase also driven by the Am tr promotor.

Table A1: Various microbial components recognized by mammalian Toll-like receptors

TLR	microbial components (ligands)	ligand location	cell types	reference(s)
TLR1	peptidoglycan and (Triacyl) lipopeptides with TLR2	bacteria and mycobacteria	macrophages and neutrophils	HAWN et al. 2007
TLR2	peptidoglycan, lipoprotein, MALP-2 [a], *Leptospira interrogans* LPS, diacyl lipopeptides (with TLR6), triacyl lipopeptides (with TLR1), LAM [b], LTA[c] (with TLR6), porins	bacteria, mycobacteria, *Mycoplasma* and *Neisseria*	macrophages, neutrophils and dendritic cells	GIRARDIN et al. 2002; AKIRA et al. 2006
	zymosan, phospholipomannan, and GXM[d]	*Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans*		
	tGPI-mutin	*Trypanosoma*		
	hemagglutinin protein, ND[e]	Measles virus and HCMV, HSV1		
TLR3	double-stranded RNA	viruses	dendritic cells, B lymphocytes	ALEXOPOULOU et al. 2001
TLR4	LPS, LTA	bacteria	macrophages, neutrophils, dendritic cells, B lymphocytes, Intestinal epithelium	GIRARDIN et al. 2002; AKIRA et al. 2006
	mannan and GXM	*Candida albicans* and *Cryptococcus neoformans*		
	glycoinositolphospholipids	*Trypanosoma*		
	envelope proteins, HSP60[f], HSP70 and fibrinogen	viruses and host		
TLR5	flagellin	flagellated bacteria	macrophages, a subset of dendritic cells and intestinal epithelium	GIRARDIN et al. 2002
TLR6	peptidoglycan (with TLR2), MALP-2 (with TLR2), LTA (with TLR2), Zymosan (with TLR2)	bacteria and *Saccharomyces cerevisiae*	lacrophages, B lymphocytes	GIRARDIN et al. 2002; AKIRA et al. 2006

TLR7	single-stranded RNA	RNA viruses	macrophages, plasmacytoid dendritic cells and B lymphocytes	AKIRA et al. 2006
TLR8	single-stranded RNA	RNA viruses	macrophages, plasmacytoid dendritic cells and B lymphocytes	AKIRA et al. 2006
TLR9	CpG-DNA hemozoin DNA	bacteria and mycobacteria *Plasmodium* viruses	macrophages, plasmacytoid dendritic cells and B lymphocytes	AKIRA et al. 2006
TLR10	unknown	unknown	unknown	AKIRA et al. 2006
TLR11	profilin	*Toxoplasma gondii*	macrophages, liver cells, kidney and epithelium in mice	YAROVINSKY et al. 2005
TLR12	profilin	*Toxoplasma gondii*	neurons and dendritic cells in mice	MISHRA et al. 2008; KOBLANSKY et al. 2013
TLR13	23S RNA sequence ‘CGGAAAGACC’	bacteria	macrophages and dendritic cells in mice	OLDENBURG et al. 2012

[a] MALP-2: mycoplasmal lipopeptide macrophage-activating lipopeptide-2
[b] LAM: lipoarabinomannan
[c] LTA: lipoteichoic acid
[d] GXM: glucuronoxylomannan
[e] ND: not determined
[f] HSP60: heat-shock protein 60