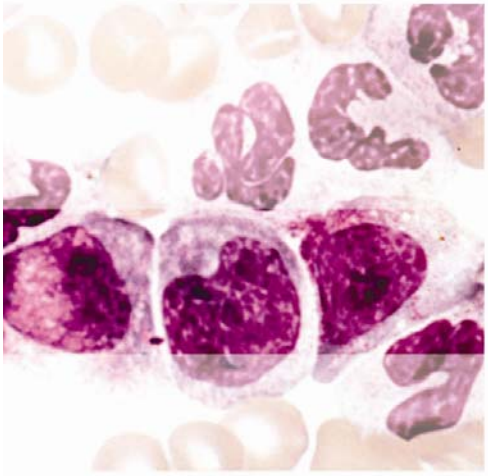

Pathogenesis of *Anaplasma phagocytophilum* infections

Master thesis Biology of Disease | Tryntsje Cuperus | Supervisor: Prof. Dr. A. Gröne

For my father
Foar heit

Table of contents

1. Introduction

Anaplasma phagocytophilum is a tick-borne, granulocytotropic bacterium causing a nonspecific febrile illness in humans and multiple domestic animals including cattle, sheep, horses and dogs. Though *A. phagocytophilum* was only recently described as a human pathogen, the organism has been known to cause animal diseases for over 70 years.

In 1940, the causative agent of tick borne fever (TBF), a disease affecting ruminants in the UK and mainland Europe, was described and named *Rickettsia phagocytophila* [114]. In the 1980's, the organism was included in the tribe Ehrlichieae and the name changed to *Ehrlichia phagocytophila* [112]. A similar disease in horses was recognized in 1969 in California and later other parts of the USA and Europe. The pathogen causing this so-called equine granulocytic ehrlichiosis was named *E. equi* [70]. In the 1980's, this bacterium was also shown to cause disease in dogs [19]. In 1990, the first human patient with granulocytic ehrlichiosis was described. Morphological and serological studies indicated a relationship between the human granulocytic ehrlichiosis (HGE) agent and the veterinary pathogens *E. phagocytophila* and *E. equi* [36]. In 2002, as part of a complete revision of the order Rickettsiales, the HGE agent, *E. phagocytophila* and *E. equi* were recognized as being a single organism and named *Anaplasma phagocytophilum* [35].

A. phagocytophilum is an obligatory intracellular Gram-negative bacterium. As a Gram-negative bacterium, it is unusual because it does not synthesize either LPS or peptidoglycan [6]. *A. phagocytophilum* infects cells of bone marrow derivation, primarily neutrophils. This makes *A. phagocytophilum* one of the very few bacteria able to survive and reproduce inside neutrophils [113]. *A. phagocytophilum* is a member of the family of the Anaplasmataceae in the order of the Rickettsiales and the class of the alpha-proteobacteria [35]. Close relatives of *A. phagocytophilum* are the canine pathogen *Anaplasma platys* and the bovine pathogen *Anaplasma marginale* (Fig.1).

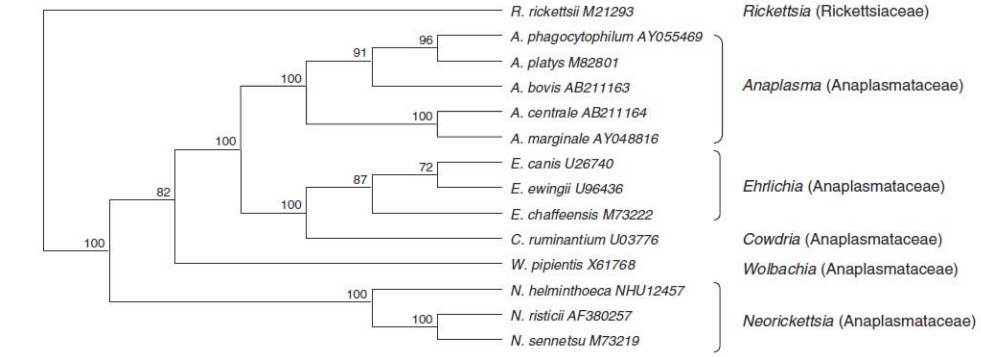

Fig.1 Phylogenetic tree, based on 16S rRNA gene sequences of several species in the order Rickettsiales [19]

The disease caused by *A. phagocytophilum* is known as TBF in ruminants, human granulocytic anaplasmosis (HGA) in humans, canine granulocytic anaplasmosis (CGA) in dogs and equine granulocytic anaplasmosis (EGA) in horses. The major symptoms of the disease are similar among hosts and comprise fever, malaise, muscle stiffness and headaches [8,36] (Fig.2). Commonly observed laboratory findings are a pancytopenia consisting of thrombocytopenia, leucopenia and a mild anemia [6,36]. Elevated levels of serum transaminases, indicative of liver damage, are also often found [6]. In horses, distal limb edema is frequently seen as a result of EGA, while in dogs polyarthritis is a common symptom [8]. In all hosts, infections with *A. phagocytophilum* are usually mild and self-limiting

and full recovery can be expected in 2 to 3 weeks. However, complications are not uncommon. Hospitalization is required in half of the recorded symptomatic cases of HGA [36]. Complications include toxic-shock like syndrome, respiratory insufficiency and invasive opportunistic infections. Opportunistic infections such as pasteurellosis and staphylococcal infections also complicate TBF in sheep. In addition, abortion storms can occur when pregnant ewes are transferred to tick infested pastures [114].

Prevalence of complaint	Symptom or sign	Published cases (%) N = 4–243
Common	Fever	99–100
	Headache	61–93
	Myalgias	40–83
	Malaise	47–93
	Rigors	27–39
Less common	Anorexia	6
	Nausea	53
	Arthralgias	27–78
	Cough	20
Uncommon	Abdominal pain	20
	Confusion	No published data
	Rash	2–16

Fig.2 Published signs and symptoms reported by patients treated for laboratory confirmed HGA in the USA and Europe [6]

In agreement with the common symptoms between hosts, pathological findings of granulocytic anaplasmosis also show many resemblances between species. The organs most affected are those of the lymphoreticular system: bone marrow, spleen, liver and lymph nodes. Perivascular inflammatory infiltrates enriched in lymphocytes and macrophages are frequently seen in those organs [32,70]. Erythrophagocytosis was observed in the spleens of human fatal cases and experimentally infected horses [70]. Other frequent observations include hepatitis with apoptotic hepatocytes and lymphoid depletion in lymph nodes and spleen. [36,70]. Bone marrow is generally normocellular, but both hypo- and hypercellular bone marrow have been described [36]. Variably, the lungs can also be involved in granulocytic anaplasmosis. The appearance is usually mild interstitial pneumonitis [32]. Infected neutrophils are not usually associated with the pathological lesions [36]. For research into the pathogenic mechanisms of *A. phagocytophilum*, mice are frequently used as a model. Mice do not show overt clinical signs, but their hematological and pathological responses mimic those of other mammalian hosts [8].

Because of the nonspecific symptoms, diagnosing *A. phagocytophilum* infections can be challenging. The organism can be detected in neutrophils on a Wright-stained peripheral blood smear. *A. phagocytophilum* grows as distinctive microcolonies called morulae in cytoplasmic vacuoles of neutrophils (Fig.3). Additional diagnostic tools to confirm granulocytic anaplasmosis are PCR amplification of *A. phagocytophilum* specific DNA from blood or serologic testing [6].

A. phagocytophilum is uniformly susceptible to tetracycline antibiotics [6]. Doxycycline is used to treat *A. phagocyphilum* infections in all hosts. In ruminants it can also be used as a long-acting prophylactic treatment against TBF [112]. Use of tick repelling agents and prompt removal of ticks can also prevent infection with the pathogen [19].

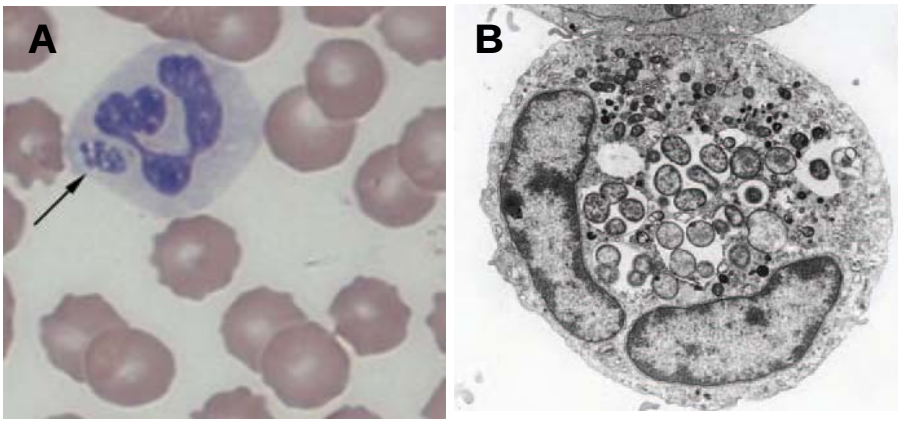

Fig.3 Canine neutrophil containing an *A. phagocytophilum* morula [19] (A) and the ultrastructure of morulae in an HL-60 cell [36] (B)

A. phagocytophilum is transmitted by Ixodes ticks, chiefly *Ixodes ricinus* in Europe, *Ixodes scapularis* in northeastern and midwestern USA and *Ixodes pacificus* in western USA [6]. The bacterium is maintained in a cycle between the tick vector and mammalian hosts. Wildlife probably fulfills an important reservoir function. Species believed to be important hosts for *A.phagocytophilum* are rodents such as the white-footed mouse (*Peromyscus leucopus*) and the wood mouse (*Apodemus sylvaticus*) in the USA and Europe respectively and larger mammals such as deer [32,112].

While human, canine and equine granulocytic anaplasmosis are found in both Europe and the USA, the occurrence of TBF seems to be limited to Europe [112]. There are no figures describing the incidence of TBF, but one report estimated that 300.000 lambs developed an opportunistic staphylococcal infection after TBF in the UK annually [114]. HGA has been described in 14 European countries and large parts of northern USA. In the USA, HGA is a reportable disease and until 2008, 2963 cases of HGA were reported at the Centers for Disease Control and Prevention (CDC) [6]. This makes HGA the third most common tick-borne infection in the USA, behind Lyme disease and Rocky Mountain spotted fever [36]. However, seroepidemiologic studies suggest that many infections may go unrecognized, as the seroprevalence of *A. phagocytophilum* in humans shows much higher rates than could be expected for the confirmed HGA cases. For example, a meta-analysis of seroepidemiologic studies of HGA showed a median seroprevalence of HGA of 5,6% in Europe and 3,0% in the USA [6]. The same discrepancy between seroprevalence and symptomatic rate was also shown in studies in dogs [19].

2. Pathogenesis I: Entry

2.1 Host and bacterial adhesins

As *A. phagocytophilum* is an obligatory intracellular organism, it must invade host cells to survive and replicate. To enter a host cell, a bacterium must bind to a cell-surface exposed receptor and set in motion a chain of signal transduction events. P-selectin glycoprotein ligand-1 (PSGL-1) and the sialic acid sialyl Lewis x (sLe^x) have been shown to be important receptors for *A. phagocytophilum* infection of neutrophils.

Goodman *et al* first identified the importance of sLe^x in *A. phagocytophilum* infection [51]. This carbohydrate moiety was chosen because of its rich expression on multiple cell types susceptible to invasion by *A. phagocytophilum*. Blocking of sLe^x with specific antibodies led to a significant reduction of *A. phagocytophilum* infection of both HL-60 cells and neutrophils (Box 1). Surprisingly, the antibodies did not prevent adhesion of the bacteria to HL-60 cells. Both adhesion and infection were however almost abolished by treatment of HL-60 cells with

neuraminidase. This enzyme, also known as sialidase, cleaves terminal sialic acids. The importance of sLe^x as an *A. phagocytophilum* receptor was also demonstrated by the fact that a HL-60 variant cell line known to be resistant to *A. phagocytophilum* infection, showed a very low expression of sLe^x. In addition, the efficiency of *A. phagocytophilum* adhesion to HL-60 cells shows a correlation to the level of sLe^x expression.

Box 1: HL-60 cells
A large amount of *in vitro* research with *A. phagocytophilum* is conducted using HL-60 cells. This cell line is also used to maintain laboratory colonies of *A. phagocytophilum*. The HL-60 cell line was established in 1976 from cells of a patient with promyelocytic leukemia [31]. HL-60 cells can be induced to differentiate into both the granulocytic and the monocytic direction [79]. Granulocytic differentiation following dimethyl sulfoxide (DMSO) or all-trans retinoic acid (ATRA) treatment is often used in *A. phagocytophilum* research.

As many proteins and lipids can be modified by sLe^x, a subsequent study examined multiple sLe^x-decorated proteins and identified PSGL-1 as the specific receptor for *A. phagocytophilum* [55]. Antibodies against PSGL-1 blocked *A. phagocytophilum* binding to and infection of HL-60 cells. To study the relative importance of both sLe^x and PSGL-1 in bacterial adhesion, the B lymphoblastoid cell line BJAB was used. This cell line does not express either PSGL-1 or Fuc-TVII, a fucosyltransferase important in the construction of sLe^x. When BJAB cells were transfected with either PSGL-1 or Fuc-TVII alone, no efficient binding or infection of the cells with *A. phaghocytophilum* took place. Only when the cells were made to express both PSGL-1 and sLe^x, the cells were susceptible to *A. phagocytophilum*, proving both protein and carbohydrate to be essential for infection of *A. phagocytophilum*.

PSGL-1 is a glycoprotein expressed on neutrophils, monocytes and subsets of lymphocytes. As a ligand for multiple selectins, PSGL-1 plays an important role in the early stages of inflammation. Interactions between PSGL-1 and P-, E- and L-selectin lead to leukocyte rolling on endothelium and subsequent transmigration [4]. The binding between P-selectin and PSGL-1 is a process which has been investigated in detail. The results of a study by Yago *et al* indicate that the characteristics by which *A. phagocytophilum* binds to PSGL-1 overlap with, but are clearly distinct from P-selectin binding to PSGL-1 [118]. P-selectin binds to a small *N*-terminal region of PSGL-1, which includes three sulfated tyrosines and a glycosylated threonine capped with sLe^x (Fig.4). Experiments with synthetic glycopeptides showed this region to be sufficient for binding of *A. phagocytophilum* as well. However, unlike P-selectin, binding of *A. phagocytophilum* does not require tyrosine sulfation. The orientation of the threonine-bound glycan is also of no importance to bacterial binding. *A. phagocytophilum* even readily binds to PSGL-1 without an sLe^x capped glycan at this specific threonine, as long as there is sLe^x presented on glycans at other locations on the peptide. But requirements for *A. phagocytophilum* binding to PSGL-1 seem to be even less stringent, as experiments with synthetic glycopeptides bound to microspheres revealed. The bacterium can even bind to non-glycosylated PSGL-1, as long as sLe^x capped glycans are presented on other cell-surface molecules.

Fig.4 The *N*-terminal P-selectin binding site of human PSGL-1 [118]

The importance of PSGL-1 for binding of *A. phaghocytophilum* to neutrophils does not seem to apply to all host species. Murine neutrophils devoid of PSGL-1 showed only marginally less binding of the bacterium compared to wildtype neutrophils [17]. SLe^x was however shown to be as important for *A. phagocytophilum* binding to mouse cells as it is for human cells. Experiments with modified PSGL-1 peptides revealed that bacterial interaction with PSGL-1 required a five-residue sequence (DFLPE) found in human, but not murine PSGL-1 [118]. The binding of *A. phagocytophilum* to PSGL-1 of other host species has not yet been investigated. Still, the *N*-terminal region of PSGL-1 does have a low homology between mammalian species [4]. It is thus very well possible *A. phagocytophilum* uses alternative receptors in other nonhuman host species such as cattle and horses as well.

Undoubtedly, PSGL-1 and sLe^x are important receptors for binding of *A. phagocytophilum* to human neutrophils. Nevertheless, multiple strains of the bacterium are able to bind to HL-60 cells low in or devoid of sLe^x or PSGL-1 [92]. The nature of the alternative receptor has not been investigated. Though *in vivo* this alternative binding might not have a large relevance for infection of neutrophils, it could be important in invading other host cells, such as endothelium.

While multiple studies have been conducted into the neutrophil receptors for *A. phagocytophilum*, the bacterial adhesin is less well studied. A study of Park *et al* showed evidence for a role of major surface protein-2 (Msp2) in bacterial adhesion [86]. Msp2 is the major immunodominant surface-exposed antigen of *A. phagocytophilum* [60]. The protein exists in many different variants, as it is encoded by a multigene family of over 100 genes. An antibody against Msp2 significantly reduced *A. phagocytophilum* adhesion and infection of both HL-60 cells and neutrophils. The antibody did not completely block binding and entry of the bacterium. This could mean the bacterial adhesin is a complex of multiple surface-exposed molecules, as is known for the related pathogen *A. marginale.* A model of multiple adhesins would also agree with the differing receptors between humans and mice. Msp2 might be involved in sLe^x independent entry of *A. phagocytophilum* as well. A recent experiment characterized the Msp2 variants expressed in *A. phagocytophilum* cultured in sLe^x-deficient HL-60 cells [107]. In bacteria cultured in these circumstances differential Msp2 variants turned out to be expressed and the glycosylation of the surface proteins changed compared to *A. phagocytophilum* cultured in conventional HL-60 cells.

2.2 Mode of entry

Following binding of a bacterium to a host cell, downstream signaling will commence and lead to uptake of the organism. *A. phagocytophilum* binding to PSGL-1 leads to rapid phosphorylation of ROCK1 in both HL-60 cells and neutrophils [104]. The serine/threonine kinase ROCK1 is known to phosphorylate proteins which tether the receptor PSGL-1 to the cytoskeleton. Also reported to be involved in the early PSGL-1 signaling cascade is the protein Syk. Silencing the genes encoding ROCK1 or Syk by siRNA leads to reduced *A. phagocytophilum* infection of HL-60 cells, emphasizing the importance of this signaling pathway for bacterial entry [104]. Other signaling molecules believed to be important for *A. phagocytophilum* invasion are protein kinase A (PKA), phospholipase C (PLC), transglutaminase and protein tyrosine kinases (PTK) [82,119]. Chemical inhibition of these proteins inhibited internalization of *A. phagocytophilum* in neutrophils. This corresponds with microarray results in HL-60 cells where transglutaminase 3, PLC and Tec protein tyrosine kinase were found up-regulated three days after infection with *A. phagocytophilum* [30].

A. phagocytophilum is believed to enter neutrophils via receptor-mediated endocytosis. As *A. phagocytophilum* inclusions do not co-localize with clathrin, the bacterial mode of entry is probably clathrin independent [80]. A study by Lin and Rikishisa examined the involvement of

caveolae in the entry of *A. phagocytophilum* [75]. Caveolae are specialized membrane regions formed from lipid rafts which play an important role in endocytic trafficking pathways. Disrupting lipid rafts or caveolae in HL-60 cells led to reduced binding and infection of *A. phagocytophilum*. Additional evidence for the importance of caveolae in the entry of *A. phagocytophilum* is the presence of the caveolae specific protein caveolin-1 in *A. phagocytophilum* inclusions. Multiple bacterial proteins could be found in lipid raft fractions of infected HL-60 cells, indicating a strong attachment between the bacteria and the lipid rafts. Surprisingly, PSGL-1 could not be found in these fractions. Apparently, bacterial binding to PSGL-1 does not directly lead to interactions with caveolae.

2.3 Residing vacuole

After entry into neutrophils, *A. phagocytophilum* resides and replicates within a membrane-bound cytoplasmic vacuole. As these vacuoles incorporated endocytosed colloidal gold particles, it was suggested they were part of the endocytic pathway [109]. However, the bacterial inclusions do not co-localize with markers of either early endosomes (rab5, annexins) or late endosomes (LAMP-1, CD36) [80]. A recent study by Niu *et al* postulated the *A. phagocytophilum* inclusions to be autophagosomes [85]. Autophagy mediates the degradation of cytosolic components and organelles by sequestering cytoplasm in vesicles named autophagosomes and delivering these to the lysosome. Several hallmarks of autophagosomes, such as a double membrane and the marker Beclin 1, can also be found in *A. phagocytophilum* inclusions. Though the exact nature of the bacterial inclusions may not yet be elucidated, it is clear these vacuoles do not fuse with lysosomes. The vesicles could not be labeled with the low pH indicators DAMP or acridine orange [80,109]. Vesicles containing myeloperoxidase were seen surrounding, but never fusing with the bacterial inclusions [80] (Fig.5). The absence of endosome-lysosome fusion was observed both in HL-60 cells and ovine neutrophils [50]. It does not seem to be the result of generalized lysosomal dysfunction, as inclusions with latex beads or *Candida albicans* in *A. phagocytophilum*-infected ovine neutrophils did show fusion with lysosomes [50]. The inhibition of endosome-lysosome fusion might be the result of the mode of entry of *A. phagocytophilum*, as vesicles derived from caveolae are known to bypass the lysosomal pathway [75].

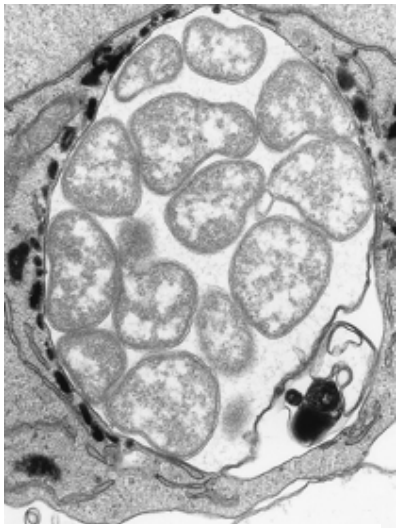

Fig.5 Myeloperoxidase containing granules (black) around *A. phagocytophilum* inclusion [80]

3. Pathogenesis II: Neutrophil functional changes

In living and replicating within neutrophils, *A. phagocytophilum* has chosen a dangerous residence. Neutrophils constitute the body's first line of defense against invading pathogens. These abundant leukocytes are equipped with a large array of microbicidal functions, including the respiratory burst, phagocytosis and granule release. To survive in this hostile environment, *A. phagocytophilum* has found numerous unique mechanisms to effect its host cell's behavior.

3.1 Reduction of the respiratory burst

A. phagocytophilum inhibits superoxide generation (Box 2). This inhibition has been shown *in vitro* in HL-60 cells, human and ovine neutrophils and *in vivo* in a murine model of infection and neutrophils of HGA patients [7,82,108,111].

The pathogen blocks NADPH oxidase activity after activation by multiple stimuli, such as IFNγ, PMA, fMLP and *E. coli*. Wang *et al* subjected *A. phagocytophilum*-infected neutrophils to tests used for the diagnosis of chronic granulomatous disease (CGD) which determine superoxide production on a cell-to-cell basis [108]. The results of these NBT test and Fc-Oxyburst assay showed that *A. phagocytophilum* infection reduced the number of positive cells to approximately 12-14%, while >90% of uninfected cells where positive. The time-frame in which *A. phagocytophilum* causes the inhibition of superoxide generation is a topic of discussion. While some studies state the inhibition starts immediately after contact between the host neutrophil and the bacterium [81], other experiments seem to indicate a late abrogation of the respiratory burst. Choi and Dumler measured superoxide generation of neutrophils 0, 3 and 5 hours after incubation with *A. phagocytophilum* [23]. The inhibition of LPS-stimulated respiratory burst was only seen after 5 hours of incubation. In the experiments of Ijdo and Mueller no inhibition was seen at all during the 120 minute incubation of neutrophils with the pathogen [59].

Box 2: NADPH oxidase

One of the major pathways by which neutrophils kill bacteria is the respiratory burst: production of toxic oxygen intermediates derived from superoxide (O_2^-). The production of O_2^- is catalyzed by NADPH oxidase [3]. In resting neutrophils, this multicomponent oxidase is unassembled. Two components are membrane bound ($gp91^{phox}$ and $p22^{phox}$) and four can be found in the cytoplasm ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac2). Rac2 functions as the molecular switch for assembly of the complex. Upon activation of the neutrophil, Rac2 dissociates from its inhibitor and migrates to the membrane, where it joins $gp91^{phox}$ and $p22^{phox}$. Concurrently, $p47^{phox}$ is phosphorylated and carries the cytosolic components to the membrane bound complex. Once the complex is assembled, the oxidase donates electrons from NADPH to oxygen, thereby creating O_2^-. From this initial superoxide, multiple other reactive oxygen species (ROS) are formed such as H_2O_2, HOCl, ozone and $OH^{\bullet}$. Next to the direct toxic effects of the ROS on the pathogen, the production of O_2^- causes a pH change in the phagosome, which leads to protease release and activation and additional bacterial killing [99]. The importance of NADPH oxidase as a neutrophil defense is exemplified by chronic granulomatous disease (CGD). Patients with this genetic disorder, caused by mutations in subunits of NADPH oxidase, suffer from severe opportunistic infections [3].

The inhibition of NADPH oxidase activity is specific to neutrophils, as *A. phagocytophilum* is not able to inhibit the respiratory burst in monocytes [81]. Contact between pathogen and host cell is necessary for inhibition, *A. phagocytophilum* separated from the neutrophils by a porous membrane did not reduce the respiratory burst [81]. Surprisingly, entry of the bacterium is no requisite for initial inhibition. Both antibodies against PSGL-1 and neuraminidase treatment, previously shown to inhibit entry of *A.phagocytophilum*, did not prevent inhibition of superoxide production during two hours of incubation [82]. New protein synthesis of the bacterium was not necessary for inhibition, in contrast to protein synthesis of the host cell [81]. When neutrophils were treated with cyclohexamide, which blocks eukaryotic protein synthesis and subsequently incubated with *A. phagocytophilum*, no inhibition of NADPH oxidase activity occurred. This indicates a newly formed neutrophil protein is involved in the inhibition of NADPH oxidase by *A. phagocytophilum*.

A. phagocytophilum seems to use multiple molecular strategies to inhibit NADPH oxidase activity. Firstly, $gp91^{phox}$ and $p22^{phox}$ are selectively excluded from the membrane of the bacterial inclusion [59]. In infected neutrophils, the percentage of *A. phagocytophilum* vacuoles positively labeled with $gp91^{phox}$ and $p22^{phox}$ was less than 20%, compared to >60% of *E. coli* vacuoles. Coupled to the absence of endosome-lysosome fusion, *A. phagocytophilum* has found two ways in which to make its inclusion a safe haven. *A. phagocytophilum* also influences gene expression and protein levels as a strategy to inhibit NADPH oxidase. Banerjee *et al* reported downregulation of $gp91^{phox}$ mRNA levels in infected HL-60 cells and

in splenic neutrophils of infected mice [7]. At the time-point measured, 5 days post infection, there was no indication of changes in the mRNA levels of other NADPH oxidase components. In contrast to this study, Mott *et al* showed a reduction in $p22^{phox}$ protein levels in both HL-60 cells and neutrophils, while $gp91^{phox}$ levels were not changed [82]. A subsequent study added Rac2 to the list of NADPH oxidase components *A. phagocytophilum* influences [18]. Rac2 gene expression was found down-regulated in a microarray of infected HL-60 cells. This observation was confirmed in human neutrophils. In addition, protein levels were measured, and infected HL-60 cells showed lower Rac2 protein levels compared to non-infected controls. A transfection experiment showed the importance of both $gp91^{phox}$ and Rac2 for *A. phagocytophilum* inhibition of the respiratory burst [18]. HL-60 cells were transfected with vectors to express Rac1, $gp91^{phox}$ or both. Rac1 and Rac2 have been shown to be interchangeable. The genes were not expressed with their native promoter, so *A. phagocytophilum* infection was believed not to influence expression of these vectors. *A. phagocytophilum* could still inhibit superoxide production in cells expressing either $gp91^{phox}$ or Rac1, though the inhibition was not as high as in non-transfected cells. In HL-60 cells transfected to express both genes, *A.phagocytophilum* was not able to inhibit the respiratory burst. In fact, these cells showed an increase in superoxide production. These data indicate *A. phagocytophilum* influences the gene expression of multiple subunits of NADPH oxidase, probably at different time-points. However, the multiple microarrays are not in agreement with these data (Box 3). None of the microarray experiments showed convincing changes in gene expression of NADPH oxidase subunits, despite the different cell types and time-points used [10,18,30,69,89,103].

The way in which *A. phagocytophilum* inhibits $gp91^{phox}$ gene expression has been investigated in some detail in HL-60 cells. The $gp91^{phox}$ gene is tightly regulated by competition between activator proteins and the repressor CCAAT displacement protein (CDP). In infected HL-60 cells, reduced nuclear protein levels and gene expression of two activators, IRF1 and PU.1 were reported [106]. In addition, binding of PU.1 and another activator Elf-1 to the $gp91^{phox}$ promoter was found reduced. Concurrent with this, binding of CDP was increased, which was attributed to reduced competition with the activators. However, binding of CDP to promoters of other neutrophil target genes was also increased during *A. phagocytophilum* infection [106]. Increased binding activity of CDP is associated with cleavage of the protein by cathepsin L. In *A.phagocytophilum*-infected HL-60 cells, cleavage of CDP and increased activity of cathepsin L were reported. The importance of cathepsin L activity for *A. phagocytophilum* infection was demonstrated by siRNA. When the gene encoding cathepsin L was silenced, *A. phagocytophilum* infection was significantly reduced as measured by decreased levels of *A. phagocytophilum* DNA. Thus, *A. phagocytophilum* increases cathepsin L activity as a means to alter expression of multiple neutrophil genes.

Box 3: Microarray studies of *A. phagocytophilum*

Microarray technology can be used to investigate expression changes of a large number of genes in a single experiment. Multiple microarray studies have been performed to determine the gene expression changes resulting from *A. phagocytophilum* infection in mammalian host cells. The different studies used several cell types: human neutrophils [10,69,103], ovine leukocytes [43] and the promyelocytic leukemic cell lines HL-60 [18,30,69] and NB4 [65]. Additionally, gene expression was measured at different time-points post infection, from 1 hour to three days. Between different cell types and time-points, very little correlation in gene expression changes was seen. This is no surprise, as undifferentiated leukemic cells generally show very different gene expression compared to neutrophils in microarray studies [79]. Changes in gene expression in HL-60 cells in response to *A. phagocytophilum* are generally slower and less intense compared to neutrophils [69]. Most of the differentially regulated genes could be placed in the categories cytokine/chemokine, apoptosis, cell adhesion/cytoskeleton, signaling molecules and differentiation, indicating these are the main processes affected by *A. phagocytophilum* infection.

3.2 Delay of neutrophil apoptosis

Mature neutrophils have the shortest life span among leukocytes. *In vivo* they die due to spontaneous apoptosis within 8-20 hours after their release into the peripheral blood (Box 4) [76]. Neutrophil death is essential for maintaining cellular homeostasis under physiological conditions and to prevent tissue damage when neutrophils invade sites of inflammation. *A. phagocytophilum* is able to prolong the life span of neutrophils. This is critical for bacterial survival, as it allows *A. phagocytophilum* to replicate before its host cell dies. The ability of *A. phagocytophilum* to inhibit neutrophil apoptosis was first reported by Yoshiie *et al* and later confirmed in several other studies [10,49,68,119]. Multiple apoptosis assays, such as morphology, Annexin V staining and TUNEL have consistently shown the prolonged life span of infected neutrophils. While 100% of uninfected neutrophils were apoptotic by morphological criteria after 24 hours of *in vitro* culture, only 20% of infected neutrophils showed signs of cell death at this point. Over 50% of infected cells survived for up to 48 hours and approximately 20% was not even apoptotic by 96 hours of culture [119]. This means *A. phagocytophilum* increases the half-life of its host cell up to 45 hours, in contrast to 12,2 hours for uninfected neutrophils.

While apoptosis inhibition by *A. phagocytophilum* has not been measured *in vivo*, Scaife *et al* isolated neutrophils from infected sheep and cultured these *ex vivo* [93]. The proportion of apoptotic cells in the isolated population of neutrophils was not different between infected and uninfected sheep. However, the infected neutrophils showed a significantly longer life span in culture. Entry of *A. phagocytophilum* and bacterial proteins were shown to be required for the anti-apoptotic effect, while bacterial carbohydrates, protein synthesis or intracellular survival are not essential [119]. As various cytokines and chemokines are known to inhibit neutrophil apoptosis, the effect of blocking of interleukin-1β (IL-1β) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) on apoptosis in *A. phagocytophilum*-infected neutrophils was tested. The absence of IL-1β and NF-κB had no effect on the bacterial ability to inhibit apoptosis [119]. Similarly, the anti-apoptotic effect of *A. phagocytophilum* does not seem to be mediated by the chemokines IL-8 and its receptors CXCR1 and CXCR2 [26].

Box 4: Mechanisms of neutrophil apoptosis

Neutrophil death is mediated by a complex network of signalling pathways, which are generally classified into two categories: the extrinsic pathway and the intrinsic pathway. Both pathways lead to the characteristic caspase cascade, thereby initiating apoptosis. Caspases are a family of cysteine proteases which play a critical role in apoptosis by cleaving various substrates. They are divided into initiator and effector caspases. The *extrinsic pathway* starts with stimulation of death receptors (eg Fas, TRAIL) found on the cell surface, with their respective ligands. This leads to the formation of a death-induced signalling complex (DISC) containing among other the initiator caspase 8. Caspase 8 is activated in the DISC and initiates the downstream caspase cascade. Mitochondria are central to the *intrinsic pathway*. Pro-apoptotic members of the Bcl-2 family permeabilize the mitochondrial membrane, leading to release of apoptotic factors, such as cytochrome c. A multimolecular apoptosome is formed containing cytochrome c. The apoptosome activates caspase 9 and the downstream caspase cascade. During infection, multiple inflammatory stimuli can influence the rate of neutrophil apoptosis. Reactive oxygen species enhance neutrophil death, while proinflammatory cytokines as interferons and IL-1β are pro-survival signals [76].

A. phagocytophilum influences both the intrinsic and the extrinsic pathway of apoptosis. The activities of caspase 3, caspase 8 and caspase 9 were all found down-regulated during *A. phagocytophilum* infection [26,48]. For caspase 3 and 8, decreased cleavage of inactive procaspase was reported. This is presumably an important mechanism by which the bacterium inhibits caspase activity. During neutrophil apoptosis, spontaneous clustering of the death receptor Fas takes place on the cell membrane. In neutrophils infected with *A. phagocytophilum*, Fas remained evenly distributed on the cell

surface after 16 hours of culture [48]. *A. phagocytophilum* also inhibits the loss of mitochondrial membrane integrity, a central event in the intrinsic pathway [49]. Mitochondrial membrane integrity is determined by the balance between pro- and anti-apoptotic members of the Bcl-2 family. In uninfected neutrophils, the protein levels of anti-apoptotic Bcl-2 members slowly decreases over time. *A. phagocytophilum* infection inhibits the decrease of Bfl-1, Mcl-1 and Bcl-2, all anti-apoptotic proteins [26,49]. The level of Bfl-1 is maintained by increased transcription. Bax and Bid, two pro-apoptotic members of the Bcl-2 family seem to be influenced at a post-translational level by *A. phagocytophilum* infection. In spontaneous neutrophil apoptosis, Bax is translocated and colocalizes with the mitochondria. In infected neutrophils however, Bax translocation was inhibited and no colocalization with mitochondria was shown [48]. In addition, *A. phagocytophilum* inhibited cleavage of Bid [48]. Truncated Bid (tBid) is known to activate Bax for translocation. This activation is prevented when the anti-apoptotic Bfl-1 sequesters tBid. Thus, translocation of Bax is inhibited by *A. phagocytophilum* in two ways: decreased Bid cleavage and increased tBid sequestration by the continuous presence of Bfl-1.

Transcription of the Bcl-2 family is influenced by the p38 MAPK signal pathway. Choi *et al* showed increased phosphorylation and thus activation of p38 MAPK in *A. phagocytophilum*-infected neutrophils [26]. Inhibition of p38 MAPK caused time dependent effects on neutrophil apoptosis. When infected neutrophils were incubated with a p38 MAPK inhibitor at the time of infection, this reversed the delayed apoptosis. However, when the inhibitor was added 3 or 6 hours post infection, it did not alter the inhibition of apoptosis by *A. phagocytophilum*. Thus, p38 MAPK initially plays an important role in the anti-apoptotic effect of *A. phagocytophilum* infection, but its role might later be taken over by other signaling pathways.

Microarrays confirmed the results of other studies. Up-regulation of Bfl-1 transcription was reported in infected neutrophils 4 hours post infection and in the promyelocytic cell line NB4 [89,103]. Many other anti-apoptotic genes, both of the Bcl-2 family and other classes of proteins where also found up-regulated [68]. Borjesson *et al* reported major differences in apoptosis related gene expression of neutrophils after phagocytosis of *Staphylococcus aureus* or infection with *A. phagocytophilum* [10]. Thus, next to actively inhibiting neutrophil apoptosis, *A. phagocytophilum* intracellular infection also fails to induce the apoptosis differentiation program normally activated following bacterial phagocytosis. This is probably a result of the unique non-phagocytic way in which *A. phagocytophilum* invades its host cell. A microarray study by Lee *et al* showed increased transcription of SOD2 [68]. This manganese superoxide dismutase clears reactive oxygen species. As ROS is known to accelerate neutrophil apoptosis, the up-regulation of SOD2 might be another way in which *A. phagocytophilum* facilitates increased life span of its host cell [76]. The inhibitory effect of *A. phagocytophilum* on NADPH oxidase activity can, in this way, also add to the bacterial ability to inhibit neutrophil apoptosis.

3.3 Cytokines and chemokines

Different studies investigating whether *A. phagocytophilum* induces neutrophil secretion of proinflammatory cytokines have shown conflicting results. Klein *et al* measured levels of IL-1β, IL-6 and tumor necrosis factor-alpha (TNF-α) in culture supernatants of HL-60 cells 48 hours after infection with *A. phagocytophilum* [66]. No measurable production of any of the three cytokines was found, either using undifferentiated HL-60 cells or cells treated with DMSO. A second experiment confirmed these findings and extended them with measurements in infected neutrophils. Supernatants of *A. phagocytophilum*-infected neutrophils did not show increased IL-1β or TNF-α protein levels compared to non-infected control cultures [2]. In contrast to these reports, Kim and Rikihisa found increased mRNA and

protein levels of IL-1β, IL-6 and TNF-α in human peripheral blood leukocytes (PBL) incubated with *A. phagocytophilum* [64]. The same increase in cytokine levels could be achieved by stimulation with recombinant Msp2. As PBLs are a complex mixture of white blood cells, cytokine levels were measured separately in populations of neutrophils, lymphocytes and monocytes. It appeared that in monocytes gene expression of all three cytokines was up-regulated, while neutrophils and leukocytes only induced IL-1β in response to *A. phagocytophilum*. IL-1β and TNF-α gene expression was also seen in PBLs of horses experimentally infected with *A. phagocytophilum* [62]. However, as this study did not include a non-infected control, the results should be viewed cautiously. More convincing evidence for the induction of IL-1β in *A. phagocytophilum*-infected neutrophils comes from microarray studies. IL-1β gene expression was found up-regulated in three different neutrophil microarrays [10,69,103]. Considering the large differences between the microarray studies performed with *A. phagocytophilum*-infected cells, finding IL-1β up-regulated in three studies is a strong argument that *A. phagocytophilum* does induce this proinflammatory cytokine. The contrasting results could be explained by the differential response between HL-60 cells and neutrophils and the fact that IL-1β was maximally induced at the early time-point of 2-4 hours post infection [64]. The mechanisms by which *A. phagocytophilum* induces IL-1β were investigated further by Kim *et al* [63]. The p38 MAPK pathway does not seem to be involved in the IL-1β up-regulation, in contrast to phospholipase C and protein tyrosine kinases. Blocking protein synthesis of either bacteria or neutrophils had no effect upon IL-1β induction, so a newly synthesized protein by *A. phagocytophilum* or the host cell is not required for the proinflammatory response.

In addition to proinflammatory cytokines, *A. phagocytophilum* also upregulates gene expression and secretion of the chemokine IL-8 in both neutrophils and differentiated HL-60 cells [2,66]. This response could also be obtained with recombinant Msp2. Neutrophils express two receptors for IL-8: CXCR1 and CXCR2. In *A. phagocytophilum*-infected HL-60 cells, the CXCR2 expression was up-regulated as measured by flow cytometry [2]. IL-8 is a potent neutrophil attractant and supernatants of *A. phagocytophilum*-infected cells showed neutrophil chemotactic activity. Thus, the bacterium seems to use the IL-8 up-regulation to attract susceptible cells to infected neutrophils and in this way facilitate cell-to-cell transfer of the bacterium. This is exemplified by diminished *A. phagocytophilum* infection in CXCR2 -/- mice [2,95]. In this respect, *A. phagocytophilum* shows an opposing reaction to other bacteria. As it happens, IL-8 also stimulates neutrophil bacteriocidal functions, including phagocytosis and granule release. Therefore, in many bacterial infections, an absence of CXCR2 makes animals more susceptible to disease. Apparently, for *A. phagocytophilum*, the beneficial effect of using IL-8 to attract new host cells offsets the increased killing capacity of the neutrophil, or these bacteriocidal effects are inhibited in themselves by the bacterium.

Another potent modulator of neutrophils is IFNγ. *A. phagocytophilum* impairs IFNγ signaling in neutrophils [15]. After 5 hours of *in vitro* infection, neutrophils showed decreased surface expression of the IFNγ receptor alpha chain, decreased production of the IFNγ inducible chemokines CXCL9 and CXCL10 and increased gene expression of the IFNγ negative regulators SOCS1 and SOCS3.

3.4 Additional functional changes

Next to the above described, well investigated changes *A. phagocytophilum* causes in its host cell, there are indications the bacterium effects many other functions as well. Both neutrophils and HL-60 cells differentiated with DMSO showed decreased phagocytosis of *Candida albicans* when infected with *A. phagocytophilum* [46]. Phagocytosis is mediated by opsonophagocytic receptors such as FcγRI, FcεRII and CR1. These receptors bind to opsonizing ligands on pathogens and particles, thereby initiating phagocytosis. In *A.*

phagocytophilum-infected neutrophils, surface levels of multiple of these receptors were found reduced [47]. Whether decreased phagocytic capacity is also a consequence of *in vivo* infections is unclear, as various studies show contradicting data. Woldehiwet *et al* showed decreased phagocytosis of *S. aureus* by neutrophils from infected sheep, but in a later study in sheep this was not confirmed [111,116]. A defect in phagocytosis was also not observed in neutrophils from dogs with CGA on multiple time points during infection [73].

Neutrophils are the first leukocytes to invade tissues during inflammation. Therefore, transmigration through endothelial cell barriers is an important neutrophil function. *A. phagocytophilum*-infected neutrophils were reported to have a decreased ability to transmigrate in an *in vitro* endothelial monolayer assay [87]. Transmigration is precluded by rolling and adhesion of neutrophils on the endothelium. It was shown adhesion was decreased in *A. phagocytophilum*-infected neutrophils [24]. Flow cytometry showed reduced expression of L-selectin and PSGL-1 on infected cells, important receptors in initial adhesion. The expression of β2-integrin and ICAM-1, receptors involved in tight adhesion, were up-regulated three days after *A. phagocytophilum* infection. This indicates that defective transmigration of *A. phagocytophilum*-infected neutrophils is mediated at the first step of the process. PSGL-1 loss from the neutrophil surface is probably the result of shedding, as an increased PSGL-1 level was found in supernatants of cultured neutrophils infected with *A. phagocytophilum*. Shedding of surface molecules is mediated by sheddases. These enzymes are released from neutrophils upon degranulation. A study by Choi *et al* reported increased and protracted degranulation of *A. phagocytophilum*-infected neutrophils [25]. Neutrophil granules are packed with antimicrobial peptides, oxidants and enzymes. The heterogenic content of the granules allows neutrophils to target bacteria in many different ways [40]. However, poorly controlled degranulation causes tissue damage and is a component of the pathogenesis of many inflammatory diseases. Increased activity of the metalloprotease MMP-9 has been shown in supernatants of *A. phagocytophilum*-infected neutrophils [25]. This enzyme might be one of the mediators of the tissue damage reported in granulocytic anaplasmosis.

In the previous paragraphs, the differential regulation of many neutrophil genes has been described. A study by Garcia-Garcia *et al* indicates *A. phagocytophilum* might use epigenetic regulators to globally control gene expression [44]. Histone modification is a major epigenetic regulator of chromatin structure and in turn gene expression. Histone modifying enzymes, such as histone deacetylases (HDAC) maintain patterns of histone modification. In acute monocytic leukemia THP-1 cells infected with *A. phagocytophilum* gene expression of HDAC1 and HDAC2 were increased from 24 hours post infection onward. A concomitant increase in protein expression and histone deacetylase activity was found. The chromatin structure of multiple neutrophil defense genes shown to be down-regulated upon *A. phagocytophilum* infection was investigated and decrease in histone acetylation was observed in nine of eleven down-regulated genes. The role of HDACs in *A. phagocytophilum* infection was confirmed by inhibiting these enzymes. After HDAC inhibition, bacterial infection of THP-1 cells was significantly reduced.

3.5 Summary

Neutrophils infected by *A. phagocytophilum* show a characteristic 'activated-deactivated' phenotype (Table 1). Infected cells are clearly activated, as shown by increased surface expression of the neutrophil activation markers CD11b/CD18 [11], expression of chemokines and cytokines and degranulation. However, some other important functions are impaired, including respiratory burst, phagocytosis and transmigration. Many neutrophil functional changes are essential for bacterial survival and replication. Still, an *A. phagocytophilum* infection leaves the host with a dysfunctional first line of defense. This immunosuppression

could explain the opportunistic infections which are often reported in *A. phagocytophilum* infections. The activation of the neutrophils and their prolonged presence due to delayed apoptosis could account for the inflammatory symptoms of granulocytic anaplasmosis, for example fever and malaise.

Table 1 Effects of *A. phagocytophilum* on neutrophils and consequences for bacterium and host

Functional change	**Consequence *A. phagocytophilum***	**Consequence host**
Reduced respiratory burst	Increased survival	Immunosuppression, increased susceptibility to infections
Delayed apoptosis	Prolonged time window for replication	Prolonged presence of activated neutrophils, inflammation
Up-regulated IL-1β expression	?	Inflammation
Up-regulated IL-8 expression	Increased cell-to-cell spread	?
Impaired IFNγ signaling	Increased survival	Immunosuppression
Reduced phagocytosis	Increased survival	Immunosuppression
Reduced transmigration	Sustained neutrophil presence in bloodstream, facilitates uptake by tick vector	Reduced capacity for neutrophils to invade infected tissues, immunosuppression
Protracted degranulation	?	Tissue damage

4 Pathogenesis III: Beyond the neutrophil

4.1 Hematopathology

Pancytopenia is a commonly observed laboratory finding of *A. phagocytophilum* infections in humans, animals and the murine model of granulocytic anaplasmosis. The cytopenia is somewhat variable between studies and host species, but usually characterized by thrombocytopenia, mild anemia and an early lymphopenia (Table 2) [5,72,111]. Neutropenia was observed in experimentally infected sheep and mice [11,111], but not in a retrospective study of HGA patients [5]. In the latter study, an increase in the proportion of band neutrophils was seen concurrent with a decrease of segmented neutrophils, evident of a left shift [5]. In later stages of the disease, lymphopenia can be followed by reactive lymphocytosis, manifesting a defensive response against the bacterial infection. Pathological evidence for this lymphocyte reaction is seen in lymph nodes and spleen of infected mice, where lymphoid hyperplasia can be observed [8]. In experimentally infected sheep, the contribution of specific lymphocyte subsets to lymphopenia has been investigated. A reduction of both B- and T-lymphocytes was reported, with CD4+ as well as CD8+ T cell subsets involved in the T-lymphopenia [115]. A subsequent article also examined γδ T-cells in an ovine model of *A. phagocytophilum* infection [110]. This subset of T-cells, which in ruminants plays an important role in the immune response to intracellular infections, was found decreased in infected sheep for the complete 8 week period of the study. Lymphocytosis was observed from the third week onwards in the ovine model of granulocytic anaplasmosis and consisted of increased B- and CD8+ T-cell subsets. In addition to the cytopenia, a dysfunction of human platelets has been described after association with *A. phagocytophilum in vitro* [9]. Whether this observation has *in vivo* significance is unknown.

Cytopenia can have multiple causes, either decreased bone marrow production, increased peripheral destruction, a decreased cellular lifespan or an altered distribution. Although neutrophils infected with *A. phagocytophilum* ultimately die due to cytolysis, the number of

infected cells is too low to cause significant neutropenia [5]. Additionally, the decreased numbers of thrombocytes, erythrocytes and lymphocytes cannot be explained by infection, as these cells are not susceptible to *A. phagocytophilum*. Peripheral destruction has been investigated as a possible cause of thrombocytopenia. Autoantibodies against platelets were found in 80% of serum samples from HGA patients, indicating involvement of the humoral immune system in the thrombocytopenia [117].

Table 2 Comparison of blood parameters (both absolute and relative) from HGA patients and matched controls from the upper Midwest of the USA [5]

Parameter	Value for Control patients	Value for Patients with HGE	*P*
Total WBC count, $\times 10^9$ cells/L	7.2 ± 3.8	5.0 ± 2.4	<.00001
Segmented neutrophils, $\times 10^9$ cells/L	4.8 ± 3.3	3.1 ± 2.0	<.0001
Segmented neutrophils, %	63.0 ± 15.5	59.0 ± 15.5	.057
Band neutrophils, $\times 10^9$ cells/L	0.42 ± 0.44	0.83 ± 0.65	<.00001
Band neutrophils, %	6.0 ± 6.7	18.3 ± 12.8	<.00001
Lymphocytes, $\times 10^9$ cells/L	1.4 ± 1.1	0.95 ± 0.92	.001
Lymphocytes, %	22.4 ± 14.6	19.5 ± 14.5	.14
Platelets, $\times 10^9$ cells/L	213 ±102	124 ± 69	<.00001

NOTE. Data are ± SD, unless otherwise indicated.

However, in HGA patients thrombocytopenia was already observed from the first day of infection, too early for a significant adaptive immune response [5]. In addition, thrombocytopenia was also observed in SCID mice infected with *A. phagocytophilum* to a similar degree as in immunocompetent mice [12]. Thus, at least in mice, thrombocytopenia occurs independently of B- and T-lymphocytes. Another mechanism of peripheral destruction of blood elements is intramedullary destruction by hemophagocytosis. Hemophagocytosis has been observed in fatal cases of HGA and experimentally infected horses [70]. In contrast to this observation, splenectomised mice infected with *A. phagocytophilum* also develop thrombocytopenia [12]. Whether these contrasting study results concerning peripheral destruction are the result of differences between host species is currently unknown. Additional experiments in larger host species as horses or ruminants could shed light on this phenomenon.

Both primary bone marrow progenitors of the myelomonocytic lineage and megakaryocytes are susceptible to infection with *A. phagocytophilum in vitro* [52,67]. The *in vivo* infection of bone marrow cells has not been definitely demonstrated. Though the bone marrow as a complete organ has been found PCR positive in several host species [56, 100], it is not clear whether this is the result of influx of infected neutrophils or infection of other cell types. The direct infection of megakaryocytes does not seem to be a cause of thrombocytopenia, as *in vitro* infected megakaryocytes produced similar amounts of proplatelets compared to non-infected cells [52]. Decreased bone marrow production was initially ruled out as a cause for the cytopenia in *A. phagocytophilum* infection, as bone marrow of infected hosts was usually normocellular [70]. However, pathogens can have large effects upon hematopoiesis with only limited morphological bone marrow alterations. Johns *et al* reported a marked decrease in colony formation of bone marrow cells from *A. phagocytophilum*-infected mice as measured by CFU assays [61]. The decrease was seen in both the granulocytic-macrophage and the erythroid lineage. In the granulocytic-macrophage lineage, the reduction in colony formation was significant until day 21 post infection. The reduced proliferative capacity of the bone marrow in *A. phagocytophilum* infection could be the result of alterations in the levels of cytokines and chemokines regulating hematopoiesis. *Ex vivo* culturing of bone marrow cells

from infected mice did indeed lead to an increased production of the myelosuppressive chemokines KC, MIP-2 and JE [61]. This is in agreement with a previous study where increased production of myelosuppressing chemokines such as MIP-1α, MIP-1β and RANTES was reported from HL-60 cells and human bone marrow cells infected with *A. phagocytophilum* [66]. The results of these studies indicate altered chemokine production to be a major reason for *A. phagocytophilum*-induced cytopenia. However, the *in vivo* situation seems to be more complex, as concurrent with the decrease in bone marrow proliferation, an increase in the number of splenic colony forming units was found [61]. Apparently, bone marrow progenitor cells are mobilized to the spleen or splenic progenitor cells are expanding. Splenomegaly is often observed in murine models of granulocytic anaplasmosis and was reported to coincide with extramedullary hematopoiesis.

The cytopenia resulting from *A. phagocytophilum* infection is a well-documented laboratory finding, consistent among hosts. However, the exact mechanism which leads to this decrease in thrombocytes, erythrocytes and multiple leukocytes is still unknown. Though in patients there are indications of peripheral destruction, in the mouse model of infection, thrombocytopenia occurs independent of this mechanism. In mice, there are good arguments for decreased bone marrow hematopoiesis. This phenomenon should be validated in other host species of granulocytic anaplasmosis, especially as a study by Lilliehöök *et al* seems to contradict the depressed bone marrow production. In this study, the bone marrow of experimentally infected dogs showed increased numbers of myeloid precursors and megakaryocytes [72].

4.2 Histopathology

A characteristic histopathological pattern has been reported in multiple natural host species of *A. phagocytophilum* and experimental infection studies [70,77]. Inflammatory infiltrates, mainly consisting of macrophages and lymphocytes are found in multiple organs, but most frequently in the liver and spleen (Fig.6). This infiltrates are often accompanied by hemophagocytosis, apoptotic cells, necrosis and edema. In most cases, infected neutrophils are not associated with inflammatory lesions [14,70]. In experimental infection studies with mice, the infiltrates can be found as early as two days after infection [77]. In recent years, the involvement of multiple elements of the immune system in causing these lesions has been investigated. As SCID mice also develop hepatic inflammatory lesions, the innate immune system seems to be the main effector [14]. This agrees with the early onset of lesions. IFNγ mRNA expression and production was found increased in splenocytes and serum of infected mice and a higher plasma level of this cytokine was also found in two independent studies of HGA patients [1,34,37,77]. IFNγ is an important activator of multiple macrophage functions, including the NF-κB response, nitric oxide production and lysosomal enzymes [94]. This cytokine seems to be critical for development of tissue pathology in *A. phagocytophilum* infections. Infected IFNγ -/- mice showed an absence of histopathologic lesions, despite an increased bacteraemia [78]. IL-10 is known to inhibit IFNγ effects. The levels of this cytokine increased 4-5 fold in the plasma of *A. phagocytophilum*-infected mice [77]. An increase of IL-10 was also found in the serum of HGA patients [37]. Increased severity of hepatic lesions was reported in *A. phagocytophilum*-infected IL-10 -/- mice and additionally lesions remained evident for a longer period [78]. Apparently, IL-10 is up-regulated upon *A. phagocytophilum* infection to inhibit the potentially damaging IFNγ response. Supporting this idea is the observation that disease severity in HGA patients is correlated with the IL-10 to IFNγ ratio [34].

Important sources of IFNγ are natural killer (NK) cells and T-lymphocytes. Choi *et al* examined the involvement of these cell types in *A. phagocytophilum* infection of mice and reported an increased level of NK1.1 expression on splenocytes of infected animals at day 4 and 7 post infection [29]. There was also an increased number of cells double-positive for both

NK1.1 and the activation marker FasL. As NK1.1 expressing cells can be either NK cells or NK T-lymphocytes, NK T-cells, as defined by NK1.1/TCR expression were examined separately on day 4. At this time point, the number of splenic NK T-cells was lower in infected mice compared to the non-infected controls. Although to a lesser degree than NK1.1 expressing cells, the number of CD8+ cytotoxic T-lymphocytes also increased in the spleen of *A. phagocytophilum*-infected mice. These results indicate an activation and expansion of the NK cell and cytotoxic T-cell population during *A. phagocytophilum* infection and a possible role for these cell types in the development of the inflammatory lesions. However, peak hepatic histopathology and IFNγ secretion often occur before day 4 post infection, when the expansion of NK and T-cells was first measured. Apparently, another cell type is involved in the early phases of infection. For this position, the NK T-cell should not be ruled out, despite the reduction of this cell type found in the spleen of infected mice. The population of NK T-cells should be measured at earlier time points and in the liver, where NK T-cells form a significant portion of the lymphocytes [39].

Dumler *et al* compared the phenotype of granulocytic anaplasmosis to macrophage activation syndromes (MAS). In these syndromes, as in *A. phagocytophilum* infection, infiltrates enriched in macrophages are seen in multiple organs [53]. The pathogenesis of MAS includes activation of macrophages by cytokines from cytotoxic cells (e.g. NK cells, CD8+ T-lymphocytes), leading to nonspecific phagocytic activity, including hemophagocytosis. Important diagnostic criteria of MAS are increased serum levels of ferritin and triglycerides. Although levels of ferritin and the macrophage cytokine IL-12 were found increased in the serum of HGA patients, triglyceride levels showed no difference with matching healthy controls [34]. Therefore, although infection with *A. phagocytophilum* does have some characteristics of macrophage activation, it is not a classic MAS, explaining the usually mild disease course.

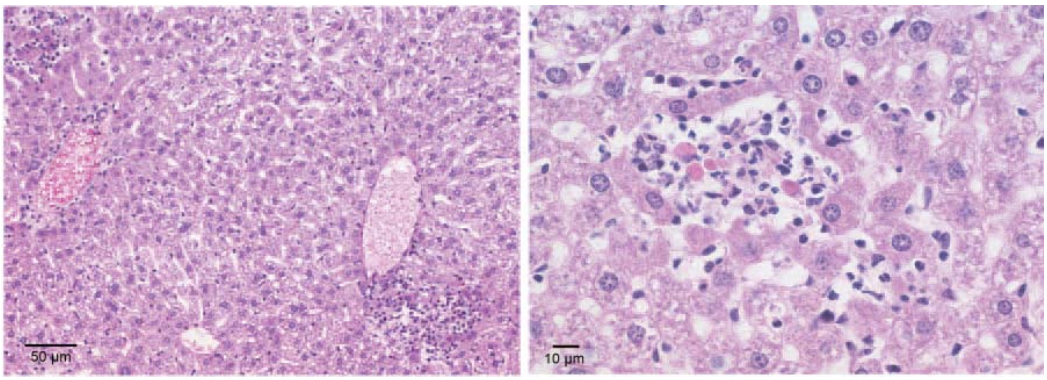

Fig. 6 Example of liver histopathology due to *A. phagocytophilum* infection in a murine model. Note the lobular infiltrates with predominantly lymphocytes and macrophages with edema, necrosis and apoptotic cells [29]

The role of several macrophage effectors in the *A. phagocytophilum*-induced tissue injury were examined by knockout murine models. Mouse deficient in TNFα showed significantly less severe hepatic lesions compared to wildtype controls at day 3 post infection. At day 7, a decrease in histopathologic severity was seen in TNFα -/- mice and mice deficient in $gp91^{phox}$ or nitric oxide synthase (NOS) [97]. This indicates TNFα and both reactive oxygen and nitrogen species are involved in tissue pathology during *A. phagocytophilum* infection. The role of reactive nitrogen species was confirmed by an experiment of Browning *et al* in which infected mice were treated with the NOS inhibitor L-NAME [13]. Mice treated with L-NAME showed less severe hepatic pathology compared to untreated mice upon infection with *A. phagocytophilum*. Though TNFα was never found increased in serum of infected mice or

patients with HGA, an increased expression of this cytokine was found *in vitro* in monocytes incubated with *A. phagocytophilum* [34,64,77]. Apparently not involved in the induction of inflammatory lesions are Toll-like receptor 2 (TLR2) and the signalling protein Myd88, used by all Toll-like receptors to activate NF-κB. No differences is hepatic pathology were noted between TLR2 -/- or Myd88 -/- and wildtype mice [98]. Thus, although *A. phagocytophilum* was previously reported to be able to activate macrophage NF-κB signalling through TLR2, this pathway is not significant in tissue injury induction [28].

In summary, IFNγ and activated macrophages play an important role in the induction of inflammatory tissue infiltrates in *A. phagocytophilum* infections. Macrophages are most likely activated by IFNγ originating from NK cells or T-lymphocytes. Multiple functions of the activated macrophage are crucial to the formation of the lesions. The bacterial load is not directly correlated to the severity of the inflammatory lesions. In IFNγ -/- the bacterial load was increased while hepatic histopathology was absent, whereas in IL-10 -/- the severity of the histopathology had increased while the bacterial burden was similar to wildtype mice [78]. The tissue pathology in granulocytic anaplasmosis is mediated mainly by the host's immune system, with the innate branch playing an important role. The infected neutrophils might initiate the formation of the tissue lesions. The direct link between the infected neutrophils and the inflammatory infiltrates is currently still unknown and warrants further research.

4.3 Bacterial effectors

In the previous sections, multiple consequences of *A. phagocytophilum* infection on neutrophils and other host cells have been described. The question remains which component of the bacterium is responsible for these effects.

One of the best studied *A. phagocytophilum* components is AnkA. This 160-kDa protein is known to be translocated from the bacterium to the host cell upon *A. phagocytophilum* infection of neutrophils or HL-60 cells [21]. Multiple bacteria are known to translocate bacterial effectors into host cells by the type IV secretion system (T4SS). Genes encoding this secretion system have been found in the genome of *A. phagocytophilum* and the expression of several components is up-regulated during infection of neutrophils [57,91]. AnkA was suspected to be a T4SS-secreted effector since a C-terminal sequence partially matched the consensus secretory motif of T4SS substrates. [58]. Using the Cre recombinase reporter assay for translocation this idea was verified [74]. AnkA translocation takes place within minutes of incubation of *A. phagocytophilum* with susceptible host cells, a period too short for bacterial entry. Using HL-60 variants not susceptible to *A. phagocytophilum* infection, it was shown that bacterial entry is not required for AnkA translocation [58]. Consistent with T4SS substrates from other bacteria, AnkA is believed to influence multiple host cellular functions. In addition, AnkA contains multiple ankyrin repeats. Ankyrin repeat proteins are found in both prokaryotes and eukaryotes where they mediate protein-protein interactions and influence a multitude of cellular processes [91]. Part of the mechanisms by which AnkA influences neutrophil functions have been elucidated in recent studies. After translocation, AnkA is rapidly phosphorylated at multiple tyrosine residues. Two different types of host tyrosine kinases, Src and Abl-1 have been shown to be responsible for AnkA phosphorylation [58,74]. While inhibition of Abl-1 decreased *A. phagocytophilum* infection, Src inhibition had no effect, indicating Src phosphorylation of AnkA is not required for successful bacterial infection [74]. In HL-60 cells, phosphorylated AnkA binds the phosphatase SHP-1 [58]. This protein is known to function as a negative regulator of cell activation. By recruiting SHP-1 to AnkA, *A. phagocytophilum* may suppress activation signals invariably triggered upon bacterial binding to host cells. In *A. phagocytophilum*-infected neutrophils and HL-60 cells, AnkA is found in both cytoplasm and nucleus. In the nucleus, AnkA binds to both DNA and proteins. Sequencing of DNA immunoprecipitated with AnkA produced sequences with various

functions, among others ATPase, tyrosine phosphatase and NADPH dehydrogenase-like functions [88]. This variety could be explained by a mechanism in which AnkA binds to DNA motifs widely distributed through the genome. In a subsequent study, AnkA was reported to bind to ATC-enriched regions of the genome, which are known to have a specific secondary structure [45]. The direct involvement of AnkA regulated gene expression has been shown in the *A. phagocytophilum* inhibition of NADPH oxidase activity [45]. HL-60 cells transfected with AnkA expressing plasmids showed decreased expression of $gp91^{phox}$. Using an electrophoretic mobility shift assay (EMSA), AnkA was shown to bind to regions in the $gp91^{phox}$ promoter were transcriptional regulators are also known to bind. In summary, the *A. phagocytophilum* protein AnkA is rapidly translocated to host cells in a T4SS- dependent manner and interferes with neutrophil functions through protein-protein and protein-DNA interactions. The fact that AnkA can be translocated to neutrophils without *A. phagocytophilum* entry explains how neutrophil functional changes such as respiratory burst inhibition are achieved without bacterial invasion [82]. Recently, a second *A. phagocytophilum* T4SS substrate has been discovered and named Ats-1 for Anaplasma translocated substrate [84]. Using double immunofluorescence, Ats-1 was reported to be colocalized with mitochondria in neutrophils and HL-60 cells. In experiments using isolated mitochondria, transport of Ats-1 into the interior of the mitochondria was shown. Ats-1 is involved in the *A. phagocytophilum*-induced delay of neutrophil apoptosis through inhibition of Bax translocation to the mitochondria.

Another important *A. phagocytophilum* component is major surface protein-2 (Msp2) also called p44. These 44-kDa surface proteins are the major immunodominant antigens of *A. phagocytophilum*, proving they are important effectors of the humoral immunity against the bacterium [60]. Msp2 proteins are encoded by a multigene family with over 100 paralogs in the *A. phagocytophilum* HZ strain genome [57]. The proteins consist of conserved *N*- and *C*-terminal domains and a central hypervariable region of approximately 280 bp (Fig.7). Multiple variants are expressed simultaneously and sequentially during *in vivo* infection. It was suggested differences in clinical severity of *A. phagocytophilum* infections could be linked to differential Msp2 expression. However, this idea was rejected in two successive studies using murine and equine infection models [27,96]. Differences in liver histopathology, fever or thrombocytopenia could not be correlated to specific Msp2 expression. In fact, six different recombinant Msp2 proteins did hardly cause any lymphoproliferation or IFNγ production from murine splenocytes [27]. Therefore Msp2 is unlikely to be responsible for immunopathological findings in *A. phagocytophilum* infections. Whole-cell *A. phagocytophilum* did cause lymphoproliferative activity in splenocyte cultures [27]. Choi and Dumler reported splenocyte proliferation driven by a non-protein component of *A. phagocytophilum* enriched in the polar lipid fraction [22]. Polar lipids are believed to be important ligands for NK cells and innate immune responses via TLR2. This is in agreement with the previously suggested involvement of NK cells and TLR2 in *A. phagocytophilum* immunopathology [28,29].

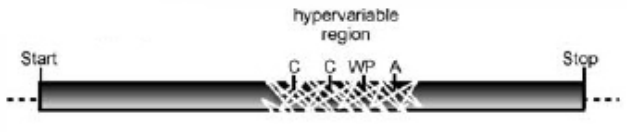

Fig.7 A full-length Msp2 gene containing *N*- and *C*-terminal conserved domains and a central hypervariable region with four conserved amino acids (C, C, WP,A) [57]

Despite being apparently insignificant for immunopathology, Msp2 might be an effector in several neutrophil responses during *A. phagocytophilum* infection. As described previously, Msp2 plays a role in bacterial adhesion to neutrophils [86]. Furthermore, recombinant Msp2 was shown to stimulate secretion of the proinflammatory cytokine IL-1β and the chemokine IL-8 from neutrophils and IL-1β, IL-6 and TNF-α from monocytes [2,64].

4.4 Other susceptible cells

Though neutrophils are undoubtedly the major type of host cell in which *A. phagocytophilum* resides, several other cell types can also be infected by the bacterium. *In vitro* infection has been reported of human primary bone marrow progenitors [67], human, rhesus and bovine endothelial cell lines [83] and megakaryocytes [52]. In the case of megakaryocytes, *A. phagocytophilum* entry relies on PSGL-1 and sLe^x expression, which has also been demonstrated for neutrophils [52]. In contrast to this, infection of endothelial cells was not diminished after the use of sialidase [54]. Thus, *A. phagocytophilum* might use either its defaul receptors or other routes of entry for the infection of non-neutrophil host cells. The infection of endothelial cells by *A. phagocytophilum* has also been demonstrated *in vivo* in a murine model of granulocytic anaplasmosis [54]. Endothelial infection was found in the heart and liver of infected SCID mice for seven weeks. The significance of endothelial infection is currently unknown, but the endothelium has been suggested as the first place of replication for *A. phagocytophilum*. After experimental infection, bacteraemia does not become evident until after several days. During this early stages of infection, *A. phagocytophilum* might replicate in endothelial cells and subsequently be passed on to neutrophils. *In vitro*, the transfer of *A. phagocytophilum* from endothelial cells to neutrophils is very efficient [54]. Within 30 minutes of co-culture between infected HMEC-1 cells (human microvascular endothelial cells) and neutrophils, 20% of neutrophils became infected with *A. phagocytophilum*. The infected HMEC-1 cells showed up-regulation of ICAM-1 and increased adherence of neutrophils, facilitating bacterial transfer. Infection of monocytes and eosinophils by *A. phagocytophilum* is reported several times, mainly in early articles on TBF [114,119]. However, monocytic infection is probably an incidental event. In an experiment with HL-60 cells, induction of monocytic differentiation by TPA for as little as one hour led to resistance against *A. phagocytophilum* infection [65]. This might partly be caused by the absence of sLe^x expression of TPA differentiated HL-60 cells.

4.5 Persistence

In general, infection with *A. phagocytophilum* is short and self-limited. Clinical symptoms usually abate within a few weeks in all host species described, even without antibiotic treatment [38,42,114]. In laboratory mice, which do not show overt clinical signs, blood counts return to normal and inflammatory tissue infiltrates disappear by two weeks post-infection [11,77]. Still, early studies on TBF in ruminants reported the occurrence of a persistent carrier state in animals recovered from acute *A. phagocytophilum* infection. In one study, the blood of an *A. phagocytophilum*-infected sheep was shown to transfer infection to a naïve sheep 25 months after primary infection [112]. The persistent *A. phagocytophilum* infection in sheep was confirmed by recent studies. Six months after a successful experimental infection, blood from infected sheep was inoculated into healthy sheep and transfer of infection was seen in all but one sheep [102]. During the study period, infection was monitored by the presence of bacterial inclusions in blood smears. After the acute phase of infection, sheep showed intermittently positive blood smears during the entire six months of the study. Though the persistence of infection with *A. phagocytophilum* in ruminants is generally accepted, its occurrence in other host species is less well described. Egenvall *et al* reports intermittently positive PCR results from blood of *A. phagocytophilum*-infected dogs for up to 5,5 months after primary infection [38]. A similar result could be found in a recent study on *A. phagocytophilum* infection in horses monitored for 18 weeks [42]. No long-term studies using PCR or blood smears have been performed in infected laboratory mice. However, in a study investigating tick acquisition of *A. phagocytophilum*, ticks were reported to become infected with the bacterium when feeding on a mouse 12 weeks after primary infection [71]. When the experiment was repeated at 15 weeks, none of the feeding ticks became infected. In a study determining *A. phagocytophilum* infection in wild rodents in California, one wood rat was PCR positive six times over a period of 14 months [20]. However, as the rat was released and

recaptured in between blood sampling, the consistent positive PCR results could also be caused by reinfection. Human patients of *A. phagocytophilum* infection are usually treated with antibiotics, making investigation of persistence in human subjects a difficult undertaking. Humans can remain infected for at least a month, as Dumler and Bakken reported a PCR positive blood sample from 30 days after the onset of illness in a HGA patient [33]. Persistent infections are believed to be sub-clinical. In experimental infection studies using sheep, horses or dogs, no clinical signs were reported after the acute phase of infection, except for fever recurrences in young sheep [38,42,102]. It must however be noted, there was no clear correlation between the periods of fever and the occurrence of positive blood smears. Ramsey *et al* investigated the health perception of treated HGA cases 10-40 months after the onset of illness [90]. Compared to controls, HGA patients were more likely to report fever, chills, sweats and fatigue. Whether this is a result of a persistent *A. phagocytophilum* infection or a so-called post-infectious syndrome cannot be stated without further examinations of blood samples.

It is unknown where *A. phagocytophilum* survives during persistent infection. Stuen *et al* investigated multiple tissues of sheep three months after infection with *A. phagocytophilum* [100]. Over 150 tissue samples were tested and only six were found positive, consisting of one sample of sternal bone marrow, small intestine, kidney, mediastinal lymph node, bladder wall and thymus. This random organ distribution indicates peripheral neutrophils, the preferred host cells during acute infection, might also be the reservoirs during persistence. Another option could be infection of endothelial cells, which has been reported both *in vitro* and *in vivo* in laboratory mice for up to seven weeks post-infection [54].

5. Conclusion

Since the discovery of *A. phagocytophilum* as a human pathogen approximately 15 years ago, a large effort has been made to investigate the pathogenesis of this intriguing bacterium. Making use of different leukemic cell lines and knock-out mouse models many cellular and molecular pathogenic mechanisms have been unearthed. The picture arising from this research is one of a bacterium employing unique mechanisms to survive in its dangerous neutrophilic host cell. By entering via receptor-mediated endocytosis and residing in a vacuole which does not fuse with lysosomes, *A. phagocytophilum* evades the default pathway of bacterial phagocytosis and lysosomal destruction. Subsequently, the bacterium downregulates microbicidal functions of the neutrophil, while at the same time prolonging its life span and using some activated functions for its own benefit. This leaves the infected host with dysregulated neutrophils, on the one hand unable to act as microbial killers, while on the other hand participating in proinflammatory reactions. The symptoms of the diseases caused by *A. phagocytophilum* consist of immunosuppression as well as inflammation, agreeing with the actions of the 'activated-deactivated' neutrophils. Also beyond the neutrophil, infection with *A. phagocytophilum* causes disorder. Both hematological pathology and inflammatory tissue lesions seem to be caused by a poorly regulated inflammatory process. Bacterial burden does not directly correlate with severity of pathology, therefore the question arises to what degree disease in *A. phagocytophilum* infection is caused by direct bacterial mechanisms or the host's own immune responses. Most likely, cytokine secretion of infected neutrophils plays an initiatory role in pathology, with subsequent steps in pathogenesis being caused by the dysregulated immune system of the host. However, the link between the infected neutrophil and the pathology of granulocytic anaplasmosis is an under-researched area and the above mentioned assumption is not based on conclusive research.

A. phagocytophilum infects and causes diseases in multiple hosts. As the diseases are clinically and pathologically similar, it can be expected the same pathogenic mechanisms apply in different host species. However, this is hard to ratify, as the main body of research into *A. phagocytophilum* pathogenesis has been performed on cells of human origin or in murine model systems. Some neutrophil functional changes have also been reported in ovine neutrophils, for example absence of endosome-lysosome fusion, reduction of the respiratory burst and delay of apoptosis. However, no molecular experiments have been performed beyond these observations. Research on pathogenesis in horses and dogs is even more scarce. While appreciating that working with large host species is expensive and technical opportunities are limited, *in vitro* research with neutrophils of all species is possible and should be performed. Also hindering comparative research of *A. phagocytophilum* infections are different genetic variants of the bacterium. It has been observed that bacterial strains pathogenic to humans, dogs and horses, are unable to exert clinical disease in ruminants [41,112]. Different clinical responses can also occur following infection with genetic variants within a single host species [101]. Caution is thus needed when comparing experimental results obtained with different *A. phagocytophilum* variants.

Research performed mainly in leukemic cell lines, human neutrophils and mouse models has given us a large body of knowledge about molecular mechanisms in *A. phagocytophilum* pathogenesis. To convert this into knowledge applicable to the medical and veterinary practice, future research should concentrate on extending knowledge beyond the molecular scale to the whole organism, establishing the link between the infected neutrophils and the clinical symptoms and carefully planned experiments into comparative pathogenesis.

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