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The biological basis of severe outcomes in *Anaplasma phagocytophilum* infection

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Abstract

Anaplasma phagocytophilum causes granulocytic anaplasmosis, an acute disease in humans that is also often subclinical. However, 36% are hospitalized, 7% need intensive care, and the case fatality rate is 0.6%. The biological basis for severe disease is not understood. Despite *A. phagocytophilum*'s mechanisms to subvert neutrophil antimicrobial responses, whether these mechanisms lead to disease is unclear. In animals, inflammatory lesions track with IFN γ and IL-10 expression, and infection of *Ifng*^{-/-} mice leads to increased pathogen load but inhibition of inflammation. Suppression of STAT signaling in horses impacts IL-10 and IFN- γ expression, and also suppresses disease severity. Similar inhibition of inflammation with infection of NKT-deficient mice suggests that innate immune responses are key for disease. With severe disease, tissues can demonstrate hemophagocytosis, and measures of macrophage activation/hemophagocytic syndromes (MAS/HPS) support the concept of HGA as an immunopathologic disease. MAS/HPS are related to defective cytotoxic lymphocytes that ordinarily diminish inflammation. Pilot studies in mice show cytotoxic lymphocyte activation with *A. phagocytophilum* infection, yet suppression of cytotoxic responses from both NKT and CD8 cells, consistent with the development of MAS/HPS. Whether severity relates to microbial factors or genetically-determined diversity in human immune and inflammatory response needs more investigation.

Keywords

Anaplasma; rickettsia; immunopathology; hemophagocytic syndrome; macrophage activation syndrome; cytotoxic lymphocyte

Introduction

Human granulocytic anaplasmosis (HGA) is a tick-transmitted zoonosis caused by the intraneutrophilic rickettsia, *Anaplasma phagocytophilum* (Dumler, *et al.*, 2007, Bakken & Dumler, 2008). The infection occurs broadly across mammalian species, but disease manifestations are in part species-dependent, with humans, horses, and dogs recognized to have mild to severe clinical signs and/or symptoms with infection. Small mammals, including laboratory mice, can sustain infection without clinical signs, yet develop inflammatory histopathologic lesions nearly identical to those observed in humans, horses, and dogs, underscoring their utility as models to study disease processes (Bunnell, *et al.*, 1999, Lepidi, *et al.*, 2000, Choi, *et al.*, 2007).

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In humans, HGA is now the 3rd most common vector-borne infection in the U.S. and Europe, and is increasingly recognized as an important vector-borne infection in Asia, especially in China (Zhang, *et al.*, 2008, Zhang, *et al.*, 2008). Among cross-sectional serosurveys conducted in North America, Europe and Asia, 3.7% have *A. phagocytophilum* antibodies, and that proportion increases with activities predisposing to or predicting tick exposures and bites (Dumler, *et al.*, 2005, Dumler, *et al.*, 2007). However, in areas with high seroprevalences, such as in the northeast and Upper Midwest USA, active surveillance identifies far fewer clinically-evident infections (Bakken, *et al.*, 1996, IJdo, *et al.*, 2000). Features of human disease include fever, myalgia, headache, malaise, thrombocytopenia, leukopenia, anemia, and mild to moderate hepatic injury sufficient to result in increased aspartate and alanine aminotransferase activities in serum (Dumler, *et al.*, 2007, Dumler, *et al.*, 2007, Bakken & Dumler, 2008). These undifferentiated clinical features result in a very broad differential diagnosis and make final definitive diagnosis of HGA difficult. This is confounded by the diversity of disease presentations in humans; while many patients have mild clinical signs and symptoms, infection results in hospitalization for 36%, ICU admission in 7%, and death in 0.6% of cases (Bakken, *et al.*, 1994, Bakken, *et al.*, 1998, Bakken & Dumler, 2000, Chapman, *et al.*, 2006). Severe complications include septic shock-like syndromes, acute respiratory distress syndrome, immune compromise and opportunistic infections, among others (Dumler, *et al.*, 2007).

A. *phagocytophilum* subversion and disease pathogenesis

The underlying pathogenetic events that lead to disease manifestations are not well understood for *A. phagocytophilum*. Early investigations linked disease severity with pathogen load, and disease manifestations are often easily reversed with specific antimicrobial treatment (Bakken, *et al.*, 1994, Bakken & Dumler, 2006, Wormser, *et al.*, 2006, Dumler, *et al.*, 2007, Bakken & Dumler, 2008). However, these observations were contradicted in subsequent studies where the degree of disease and pathologic injury did not correlate well with bacterial burden, and pathologic findings suggested the potential for a cytokine- or immune-mediated process (Walker & Dumler, 1995, Walker & Dumler, 1996, Jahangir, *et al.*, 1998, Bunnell, *et al.*, 1999, Lepidi, *et al.*, 2000). For example, the hallmark of infection that plausibly reflects direct pathogen-mediated cytolytic injury is leukopenia, especially neutropenia, since *A. phagocytophilum* infects almost exclusively neutrophils *in vivo*. However, among infected patients, the mean *infected* leukocyte count is 0.3×10^9 cells/L, whereas the mean total leukocyte count for HGA patients is 3.7×10^9 cells/L. Since the mean leukocyte count with infection is approximately 50% of that for uninfected patients, 4.1×10^9 cells/L, or 15-fold more leukocytes are lost than can be accounted for by direct *A. phagocytophilum* lysis alone (Bakken, *et al.*, 2001, Dumler, *et al.*, 2007). Thus, another explanation for the hematologic derangements is needed.

Considerable investigation regarding how *A. phagocytophilum* subverts neutrophil function to survive within this key host defense cell has been conducted (Carlyon & Fikrig, 2006, Rikihisa, 2011). However, enhanced intracellular survival or increased pathogen load does not necessarily belie clinical signs or disease. *A. phagocytophilum* uses several strategies (Table 1) that conceivably could also contribute to *disease* pathogenesis: 1) its ability to alter neutrophil mobilization of phagocyte oxidase by disorganized assembly, superoxide dismutase activity, and reduced transcription of key phagocyte oxidase genes such as *CYBB* and *RAC1*, appear to be involved (Choi & Dumler, 2003, Carlyon, *et al.*, 2004, IJdo & Mueller, 2004); 2) its ability to induce upregulated chemokine expression and neutrophil vesicle discharge leads to the recruitment of new neutrophil hosts to support pathogen population expansion (Klein, *et al.*, 2000, Akkoyunlu, *et al.*, 2001, Scorpio, *et al.*, 2004), whereas inhibition of this process by blockade of the key chemokine IL-8 receptor (CXCR1) or pharmacologic alteration of IL-10:IFN- γ balance by dexamethasone, leads to reduced

pathogen numbers in tissues, but does not appear to alter or reduce host pathologic responses (Scorpio, *et al.*, 2004, Davies, *et al.*, in press); 3) its ability to introduce microbial effectors, such as AnkA, into the neutrophil via a type IV secretion system, and to alter local signaling (ROCK1 and Syk) that facilitates *A. phagocytophilum* endocytic entry and over the course of infection, leads at minimum to reduced *CYBB* transcription and reduced microbial killing in the infected cell (Lin, *et al.*, 2007, Thomas & Fikrig, 2007, Garcia-Garcia, *et al.*, 2009, Garcia-Garcia, *et al.*, 2009); and 4) its ability to upregulate cellular histone deacetylase transcription and activity that likely promotes transcriptional program alterations associated with pathogen survival and population expansion (Garcia-Garcia, *et al.*, 2009). While each of these has been shown to play a role in pathogen survival in vitro, and for some, in vivo, their role in disease pathogenesis is poorly understood. For example, although blockade of CXCR1 leads to reduced pathogen load, the degree of histopathologic injury in the murine model, which correlates strongly with disease manifestations in humans and horses, is not altered, and infection in a phagocyte oxidase deficient condition does not lead to increased pathogen load but actually ameliorates inflammation (Scorpio, *et al.*, 2004, Scorpio, *et al.*, 2006).

Evidence for immunopathogenesis of disease with *A. phagocytophilum*

The immunologic responses to *A. phagocytophilum* include both humoral and cellular; however, immune control of *A. phagocytophilum* is still under investigation. *A. phagocytophilum* is cleared with kinetics that mimic rising antibody titers (Bakken, *et al.*, 1996, Bakken, *et al.*, 2002); yet there are clear examples of patients with high antibody titers, substantial bacteremia, and disease (Bakken, *et al.*, 1994, Bakken, *et al.*, 1996). While SCID mice cannot control infection, neither do they develop clinical signs, suggesting a dichotomous relationship between immune processes that protect and those that may cause injury (Hodzic, *et al.*, 1998, Bunnell, *et al.*, 1999). In contrast, *A. phagocytophilum* tissue and blood loads do not change with genetic deletion of iNOS (*Nos2*^{-/-}), TLR2 or MyD88, and only variably so for phagocyte oxidase (*Cybb*^{-/-}) (Banerjee, *et al.*, 2000, von Loewenich, *et al.*, 2004). Yet, activation of these same innate immune pathways by infection, leads to hepatic inflammatory injury, in part dependent upon mouse strain genetic background (Scorpio, *et al.*, 2006).

The cellular immune hallmark of *A. phagocytophilum* in humans, horses, and in mice is the early appearance of IFN- γ and IL-10 in serum or plasma (Dumler, *et al.*, 2000, Martin, *et al.*, 2000, Martin, *et al.*, 2001, Scorpio, *et al.*, 2008). While the proinflammatory cytokines TNF- α and IL-1 β can be induced from peripheral blood leukocytes after infection *ex vivo*, these cytokines are generally not detected at high quantities in serum or plasma with active infection (Dumler, *et al.*, 2000, Kim & Rikihisa, 2000, Dumler, *et al.*, 2007). In the murine model of *A. phagocytophilum* infection, both IFN- γ and IL-10 are present in plasma during active infection, and appear as early as several hours post infection, peaking between days 2 and 7, typically considered an interval prior to adaptive immune response (Akkoyunlu & Fikrig, 2000, Martin, *et al.*, 2001, Choi, *et al.*, 2007, Pedra, *et al.*, 2008). As anticipated, IFN- γ peaks shortly after maximum *A. phagocytophilum* bacteremia in mice; however, histopathologic injury, as reflected in hepatic inflammatory lesions, tracks more closely with IFN- γ levels than with bacteremia (Martin, *et al.*, 2001). Among IFN- γ knockout mice, the level of bacteremia is 7–8 times higher than in wild type mice, yet IFN- γ knockout mice lack hepatic histopathology that is present in wild type mice. Interestingly, IL-10 knockout mice do not have altered bacteremia compared to wild type mice, yet develop significantly worse hepatic inflammatory lesions, providing key evidence for an immunopathologic role in the balance between Th1 and Th2 responses, or with relative roles of both IFN- γ and IL-10.

Given the clear importance of IFN- γ and IL-10 in histopathologic inflammatory lesions in the murine model of HGA, and their early appearance post infection, potentially important cellular sources include CD8 T lymphocytes, and the innate immune effector cells, NK and NKT cells (Choi, *et al.*, 2007, Pedra, *et al.*, 2008). Splenic CD8 T lymphocytes from infected mice become more activated than from mock-infected mice by day 4 post infection, when IFN- γ is peaking in plasma. However, the NK1.1-labeled splenic lymphocyte population, that includes both NK and NKT cells, is even more significantly activated with infection vs. mock-infection, peaking in near precise concordance with plasma IFN- γ levels. Infection in *Ja18* $-/-$ (Choi, *et al.*, 2007) and *CD1d* $-/-$ (unpublished data) mice that lack NKT cells leads to complete loss of hepatic inflammatory lesions on days 4 to 7 post infection, and antibody depletion of NK cells also suppresses hepatic inflammation at day 7 compared to infection in wild type or control antibody-treated mice. These results clearly indicate that innate immune induction of inflammatory lesions, which correspond to disease manifestations, is a key component of disease pathogenesis with HGA.

A role for cytotoxic lymphocytes in disease pathogenesis with *A. phagocytophilum*?

How do cytotoxic cells, like NK, NKT, and CD8 cells contribute to inflammatory disease manifestations with activation? The underpinning for IFN- γ driven effector response is two-fold: induction of proinflammatory cytokines and chemokines that activate local effector cells for phagocytosis and enhanced cellular killing and for recruitment of more effector cells; and production of effector molecules for microbial killing, including the oxygen-dependent production of superoxide radicals and hypochlorite and of nitric oxide and peroxynitrates (Ismail, *et al.*, 2002, Mosser, 2003, Mantovani, *et al.*, 2004, Lang, 2005, Browning, *et al.*, 2006). When sequestered, these effector molecules and pathways are potent in their antimicrobial capacity; however, when excessive or exposed to the external host milieu, they can lead to “collateral” host inflammatory injury (Grom, 2004, Larroche & Mouthon, 2004, Janka & zur Stadt, 2005, Menasche, *et al.*, 2005, Ravelli, *et al.*, 2005).

The balance between microbial killing and host injury is a major determinant of disease outcome in many infections. One class of effector cells recruited and activated for many infectious agents, particularly intracellular infectious agents like viruses and intracellular bacteria is the cytotoxic lymphocyte. Cytotoxic lymphocytes have a potent capacity to both amplify the response by further release of IFN- γ , to directly kill cellular targets identified by specific cytotoxic cell-target cell interactions. This occurs as with CD8 T lymphocyte recognition of foreign peptides presented in the context of MHC class I molecules, or with NK lymphocyte recognition of altered HLA expression or TLR2 signaling, or with NKT cell recognition of specific glycolipids in the context of MHC class I CD1d (Scharton & Scott, 1993, Crowe, *et al.*, 2003, Davis & Dustin, 2004, Godfrey & Kronenberg, 2004, Kronenberg, 2005, Mattner, *et al.*, 2005, Voskoboinik, *et al.*, 2006).

When the typical homeostatic mechanism of inflammatory dampening occurs after the inflammatory stimulus has been controlled and reduced in quantity, inflammatory signaling ceases and the underlying pathologic processes is altered to that of repair and reconstitution of function. However, significant and unremitting inflammatory injury occurs in macrophage activation and hemophagocytic syndromes (Francois, *et al.*, 1997, Fisman, 2000, Grom, 2004, Larroche & Mouthon, 2004, Janka & zur Stadt, 2005, Menasche, *et al.*, 2005, Behrens, *et al.*, 2007, Arceci, 2008), genetic and acquired conditions that share the phenotype of i) active cytotoxic lymphocyte participation, ii) leukopenia, iii) thrombocytopenia, iv) anemia, and in hemophagocytic syndromes, v) the presence of hemophagocytic cells. Macrophage activation and hemophagocytic syndromes are believed to result from an underlying genetic or acquired defect in the ability of the cytotoxic

lymphocyte, whether a CD8 T lymphocyte, an NK cell, or an NKT lymphocyte, to mobilize or deliver functional secretory granules that contain both perforin (which creates a membrane pore in the target cell) and granzyme (which enters via the perforin pore and causes DNA degradation, inducing apoptosis and death of the target cell) (Fisman, 2000, Grom, 2004, Larroche & Mouthon, 2004, Janka & zur Stadt, 2005, Menasche, *et al.*, 2005, Arceci, 2008). Such genetic conditions include *PRF1* (perforin) mutations, *RAB27A* mutations (secretory vesicle trafficking; Griscelli syndrome), *LYST* mutations (lysosomal trafficking regulator; Chediak-Higashi syndrome), and *SH2D1A* mutations (development of NKT cells; X-linked lymphoproliferative syndrome). When the target is an infected cell, the pathogen is killed. However, these cytotoxic cells do not directly recognize intact pathogen; rather pathogen molecular patterns are the ligands in these immunologic synapses (Davis & Dustin, 2004, Voskoboinik, *et al.*, 2006). Thus, antigen presenting cells, whether MHC-I- or CD1d-restricted, are also targets. This process is believed to be a normal homeostatic mechanism to contain unrestrained inflammatory activation. Thus, when cytotoxic cells are activated, they release the macrophage activating, proinflammatory IFN- γ that drives further inflammatory injury, but killing of antigen presenting cell targets initiates homeostatic control; with defective vesicular trafficking or inactive perforin mutants, the inability to deliver perforin and granzyme and to kill antigen presenting cells permits an unrestrained, amplifying proinflammatory response without benefit of cytolytic homeostatic resolution (Fisman, 2000, Grom, 2004, Larroche & Mouthon, 2004, Menasche, *et al.*, 2005, Arceci, 2008). The result is a poorly controlled, in cases, relentless downward spiral of inflammatory injury and severe or fatal disease.

In fact, HGA patients have many features of macrophage activation, as evidenced by high levels of IFN- γ , IL-10, IL-12, and serum ferritin, as well as the key clinical features leukopenia, thrombocytopenia, anemia, and hepatic injury, and periodically including the detection of hemophagocytic cells in bone marrow, spleen, lymph node and liver, where examined (Lepidi, *et al.*, 2000, Dumler, *et al.*, 2007) (Figure 2). Moreover, HGA severity is correlated with the level of serum ferritin, serum IL-12, and the ratio of IL-10 to IFN- γ (Dumler, *et al.*, 2007). The latter feature is highly similar to observations in the murine model (Martin, *et al.*, 2001, Choi & Dumler, 2007). Since mice do not develop disease, the horse model, where clinical disease is strikingly similar to that in humans, is studied. Here, infected horses are studied after the IFN- γ :IL-10 axis is pharmacologically manipulated by dexamethasone treatment that alters STAT3 and STAT1 signaling to reduce propagation of effector signaling – functionally polarizing the response to favor anti-inflammatory IL-10 domination. As anticipated, horses treated with dexamethasone had increased IL-10:IFN- γ and IL-10:IL-12 ratios, higher overall bacteremias than untreated animals, but significantly less clinical signs and disease (Davies, *et al.*, in press).

It is reasonably understood why genetically-linked syndromes that result from specific mutations in key pathways important for secretory vacuole delivery or effector function result in a macrophage activation or hemophagocytic syndrome. However, why this occurs with acquired infections, especially those associated with bacteria is not well understood (Francois, *et al.*, 1997, Fisman, 2000, Larroche & Mouthon, 2004, Janka & zur Stadt, 2005). To determine whether such a principle might apply to *A. phagocytophilum*, the murine model was studied using ex vivo splenic lymphocytes from infected and mock-infected animals. These cells were examined for their ability to discharge secretory vacuole contents (CD107a) when activated in vitro, a well-recognized correlate of cellular cytotoxicity. Ex vivo splenic NKT cells from infected animals stimulate for degranulation were significantly less cytotoxic than mock-infected controls at days 7 and 10, as were CD8 T lymphocytes at day 4 (Dumler, unpublished data). While neither NKT nor CD8 T lymphocytes are targets of *A. phagocytophilum* infection, these preliminary results suggest the basis for an acquired cytotoxicity defect with *A. phagocytophilum* infection that plausibly provides a tool to

examine the periodic occurrence of severe inflammatory disease complications in HGA, and to investigate mechanisms of acquired cytotoxic lymphocyte function with non-viral infections.

Conclusions

In summary, HGA is an important emerging tick-borne infection that occurs world-wide. While most infections seem only moderate in severity and are easily treated with doxycycline, a minority will have very significant and debilitating disease, despite otherwise adequate antimicrobial treatment. The inflammatory basis for disease is most likely a result of immunopathologic injury after IFN- γ activation of effector cells not adequately tempered by the dampening effects of IL-10 (Martin, *et al.*, 2001, Dumler, *et al.*, 2007). A subset of patients that does poorly owing to serious inflammatory injury, could occur because of a higher degree of polarization in this IL-10:IFN- γ axis, resulting in a macrophage activation-like syndrome, that if accompanied by defects in cytotoxic effector molecule delivery, whether genetically-predisposed or acquired via infection, could lead to the most polar of severe infection complications (Dumler, *et al.*, 2007, Davies, *et al.*, in press). How this happens, what ligands drive the inflammatory process, the relative role of innate and adaptive immunity, and how they can be controlled are now important questions to address (Choi & Dumler, 2007). The ability to toggle disease progression by targeting anti-inflammatory treatment during infection in horses could provide additional guidance to help for those severely affected (Scorpio, *et al.*, 2006, Davies, *et al.*, in press). Moreover, the dissection of the fundamental biological processes that drive this syndrome with HGA at the level of cytotoxic lymphocyte dysfunction, offers a new possibility to study the pathogenesis of acquired non-viral hemophagocytic syndromes. Once again, obligate intracellular bacteria have lessons to teach, and as good students, we should pay careful attention.

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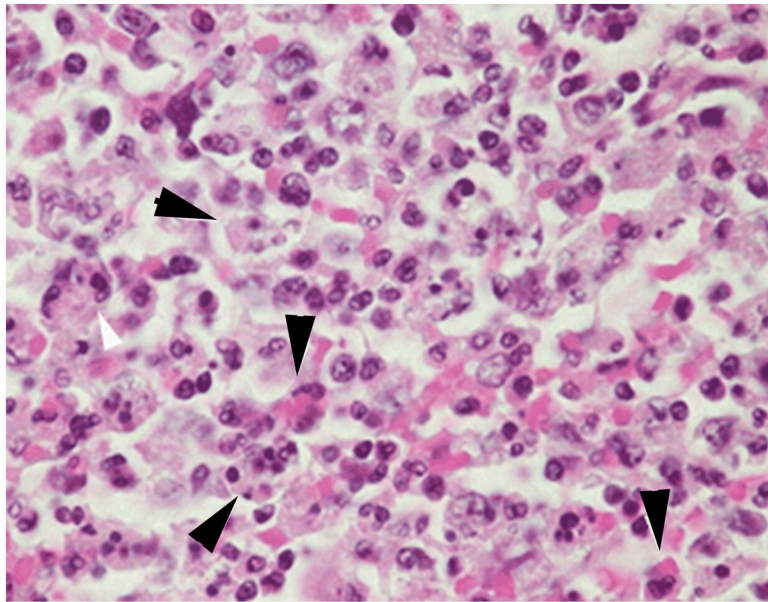


Figure 1. Splenic histopathology from a patient who died after HGA. Note the hemo- and leukophagocytic cells denoted by the white arrows. Hematoxylin & eosin stain; original magnification x 400.

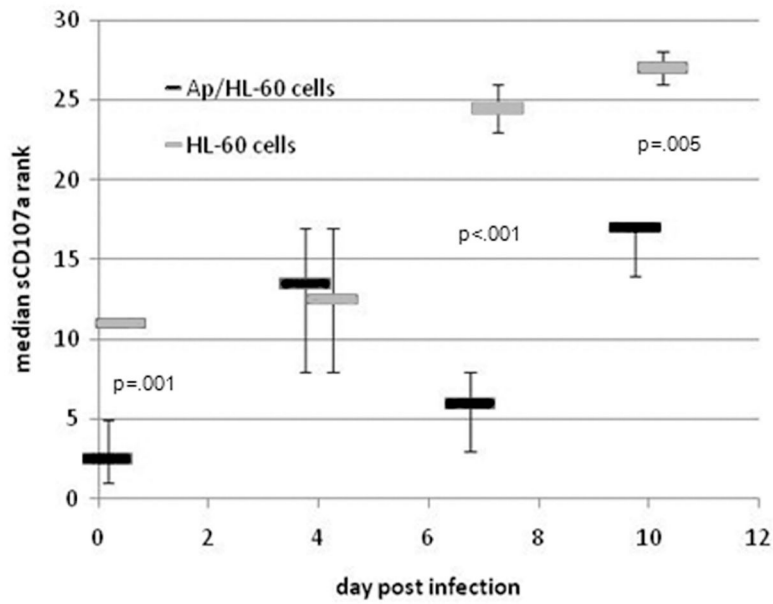


Figure 2.

Splenic NKT cells from *A. phagocytophilum*-infected mice (Ap/HL-60 cells) have abrogated cytotoxic responses, based on reduced delivery of vesicles (CD107a) to membranes after degranulation stimulated by ionomycin C, as compared with mock-infected (HL-60 cells) mice at days 7 and 10 post-infection. P values were calculated using the Wilcoxon test for nonparametric analysis, and a value <0.05 was considered significant.

A. phagocytophilum strategies for intracellular survival and propagation, and their effect on histopathology and disease.

Table 1

<i>A. phagocytophilum</i> -related pathogenic mechanism	Target or effector molecule	Effect on <i>A. phagocytophilum</i> load ^a	Effect of gene deletion/antibody inhibition on histopathologic lesions ^b	References
Phagocyte oxidase	Disorganized assembly or transcriptional repression of host <i>CYBB/RAC2</i>	0 (in vivo); ↑↑↑ (in vitro)	↓↓ (phox -/- B6 mice in vivo)	(Banerjee, <i>et al.</i> , 2000, Banerjee, <i>et al.</i> , 2000, Carlyon, <i>et al.</i> , 2002, von Loewenich, <i>et al.</i> , 2004, Scorpio, <i>et al.</i> , 2006)
Chemokine/cytokine expression	IL8, IL-10; IFN γ	↑↑	0 (CXCR1 antibody-treated vs. control antibody-treated in vivo); ↓↓ (in dexamethasone-treated horses)	(Akkoyunlu & Fikrig, 2000, Akkoyunlu, <i>et al.</i> , 2001, Scorpio, <i>et al.</i> , 2004, Davies, <i>et al.</i> , in press)
<i>A. phagocytophilum</i> granulocyte entry	<i>A. phagocytophilum</i> Anka Host ROCK1 and Syk <i>HDAC1</i> and 2	↑↑ to ↑↑↑	↓↓ to ↓↓↓ (vs. ROCK1 or Syk-silencing or vs. Anka control antibody in vitro)	(Lin, <i>et al.</i> , 2007, Thomas & Fikrig, 2007)
Histone deacetylase (HDAC)		↑↑ (vs. HDAC-silenced cells or NaB or trichostatin inhibition in vitro)	Not done	(Garcia-Garcia, <i>et al.</i> , 2009)

^a ↑ - increased bacterial load or survival

^b ↓ - decreased or "0" no change in histopathologic inflammation compared to unmodified control