Published in final edited form as:

Vet Parasitol. 2008 December 20; 158(4): 344–359. doi:10.1016/j.vetpar.2008.09.028.

Defining the Immune Response to *Ehrlichia* species Using Murine Models

Stephen K. Chapes¹ and Roman R. Ganta²

¹ Division of Biology, College of Arts and Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS66506

² Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS66506

Abstract

Pathogenic bacteria belonging to the family *Anaplasmataceae* include species of the genera *Ehrlichia* and *Anaplasma*. *Ehrlichia chaffeensis*, first known as the causative agent of human monocytic ehrlichiosis, also infects several vertebrate hosts including white-tailed deer, dogs, coyotes and goats. *E. chaffeensis* is transmitted from the bite of an infected hard tick, such as *Amblyomma americanum*. *E. chaffeensis* and other tick-transmitted pathogens have adapted to both the tick and vertebrate host cell environments. Although *E. chaffeensis* persists in both vertebrate and tick hosts for long periods of time, little is known about that process. Immunological studies will be valuable in assessing how the pathogen persists in nature in both vertebrate and invertebrate hosts. Understanding the host immune response to the pathogen originating from dual host backgrounds is also important to develop effective methods of diagnosis, control and treatment. In this paper, we provide our perspective of the current understanding of the immune response against *E. chaffeensis* in relation to other related *Anaplasmataceae* pathogens.

E. chaffeensis belongs to the *Anaplasmataceae* family

The family *Anaplasmataceae* contains several species of obligate, intracellular, Gram-negative bacteria which include species of the genera *Ehrlichia* and *Anaplasma* (Dumler et al., 2001; Ristic and Huxsoll, 1984). A steady increase of potentially fatal human diseases caused by *Ehrlichia* and *Anaplasma* species that infect phagocytic cells has been reported in recent years (Dumler and Bakken, 1995; McDade, 1990; Schaffner and Standaert, 1996). These include human monocytic ehrlichiosis (HME) caused by the tick-transmitted rickettsial, *Ehrlichia chaffeensis* (Chen et al., 1997; Dawson et al., 1991; Maeda et al., 1987; Paddock et al., 1997), human granulocytic ehrlichiosis caused by *Ehrlichia ewingii* (Buller et al., 1999) and human granulocytic anaplasmosis (HGA) caused by *Anaplasma phagocytophilum* (Chen et al., 1994). *Ehrlichia canis* and *Ehrlichia (Cowdria) ruminantium* are responsible for causing canine monocytic ehrlichiosis and heartwater in dogs and ruminants, respectively (Kock et al., 1995; Perez et al., 1996; Perreau et al., 1980; Uilenberg, 1983). *E. canis* has also been reported to cause infections in people in Venezuela (Perez et al., 1996 and 2006). *E. canis* has a worldwide distribution except Australia, while *E. ruminantium* is distributed in all parts of the sub-Saharan Africa and in the Caribbean (McDade, 1990; Rikihisa, 1991; Uilenberg, 1983).

Using mice to understand *Ehrlichia* infections and host resistance

Ehrlichia species induce variable immune responses depending on the host species, their genetic backgrounds and the immunological status that may be altered by age, previous exposure to the bacteria and other defined or undefined environmental factors. It is these many confounding variables that make the study of *Ehrlichia* interesting.

The white-tailed deer is the natural vertebrate reservoir host for *E. chaffeensis* (Dawson et al. 1994; Lockhart et al., 1997). Dogs acquire infections with several *Ehrlichia* and *Anaplasma* species which include *E. chaffeensis*, *E. canis*, *E. ewingii*, *A. phagocytophilum* and *A. platys* (Breitschwerdt et al., 1998; Dawson et al., 1994; Sirigireddy and Ganta, 2005). The dog is also recognized as a host which can be infected by several rickettsiales and is reported to maintain a persistent infection for several months to several years (Harrus et al., 1998; Harvey et al., 1978; Mylonakis et al., 2004). Although dogs were used to show that *E. canis* does not induce immunosuppression (Hess et al., 2006), neither dog nor white-tailed deer are amenable to studies for understanding host immunity against *Ehrlichia* because of the lack of good immunological and genetic tools. Wild mice do not appear to acquire infections of *E. chaffeensis*, thus may not serve as the natural reservoir of infection. Very few rodents, including white-footed mice, have detectable antibody titers against *E. chaffeensis* (Lockhart et al., 1998). However, two *Ehrlichia* species, *E. muris* and a strain of *Ehrlichia* isolated from *Ixodes ovatus* ticks (commonly referred to as the IOE strain) infect mice with two different outcomes; persistent and lethal (Feng and Walker, 2004; Winslow et al., 2005). The mouse has been extensively used for experimental infection studies and has contributed significantly to the current understanding of host resistance against *E. chaffeensis* and other related *Ehrlichia* species, such as *E. muris* and IOE strain.

Ehrlichia species-specific pathogenesis

Experimental mouse challenges have revealed several levels of pathogenicity for different *Ehrlichia* species (Figure 1). For example, immunocompetent mice clear *E. chaffeensis* infections within 10–16 days (Ganta et al., 2002; Winslow et al., 1998). This contrasts the response to two other *Ehrlichia* species where immunocompetent mice are unable to resolve infections. Both C3H and C57BL/6 mice do not become sick after experimental challenge with *E. muris* (Feng and Walker, 2004) and AKR and C57BL/6 mice remain infected for up to 150 days or longer (Olano et al., 2004).

In 2000, Shibata et al. (2000) described the isolation of an *E. chaffeensis*-related bacteria from *Ixodes ovatus* ticks that caused lethal infection when injected i.p. into Balb/c mice. The mice displayed splenomegaly and died within 20 days after experimental challenge. *Ixodes ovatus Ehrlichia*, [IOE has been designated in two recent reports as *Candidatus Neoehrlichia mikurensis* (Naitou et al., 2006)], is also lethal in 129Sv mice (Winslow et al., 2005) and C57BL/6 mice (Olano et al., 2004) with mice surviving only when less than 100 bacteria are injected (Bitsaktsis et al., 2004). Therefore in the mouse system, different levels of pathogenicity can be induced depending on the species of *Ehrlichia* used: *E. chaffeensis* (curing), *E. muris* (persistent) and IOE (lethal) (Figure 1).

E. chaffeensis, *E. muris*, and IOE have approximately 98% identity at the GroEL and 16S ribosomal RNA genes (Shibata et al., 2000; Yu et al., 2001). Genomic comparisons amongst these three species may provide insight into the genetic basis of pathogenesis. Nevertheless, their distinct infection phenotypes have lead to serious debate about which type of infection in mice best reflects the human outcome. Some argue that because 3.1% of patients die with human monocytic ehrlichiosis disease (Paddock and Childs, 2003; Winslow et al., 2005), the study of IOE infections are critical. Alternatively, because immunocompetent mice resolve infections similarly to humans, and no human cases of IOE have been documented (Tabara et

al., 2007), we contend that the research carried out using *E. chaffeensis* in the mouse model is important to determine which components of the immune response are responsible for host resistance (Ganta et al., 2002, 2004, 2007). Indeed, recent comparisons of the pathogenicity of three *E. chaffeensis* isolates revealed that they did differ in pathogenicity and the severity in disease spanned the symptoms generally seen in humans (Miura and Rikihisa, 2007).

The presence of antibody titers for *E. chaffeensis* in many adults in the areas endemic to HME agent suggests that the immune responses in people following infections are protective and aid in resolving the infection (Winslow et al., 2000). Regardless of the model organism used, significant insights into how *Ehrlichia* species cause disease have been revealed in recent years.

Manipulating immune response genes to understand *Ehrlichia* resistance

The development of laboratory knockout mouse strains with assorted nonfunctional immune response genes has provided the most significant insights on how the host responds and resists *Ehrlichia* infections (Figure 2). It is clear that acquired immunity is necessary for resistance because both severe combined immunodeficiency (SCID) (Li et al., 2001; Winslow et al., 1998, 2000) and recombination-activating gene-deficient mice (Chapes and Ganta, unpublished data) develop severe infections and multi-organ pathology after infection with *E. chaffeensis*. Infected mice become moribund 24–30 days after infection and the infection progresses to death if the mice are left unattended.

CD4⁺ T cells

The role of CD4⁺ T cells in host resistance to *Ehrlichia* has come into question over the last few years. The ability of mice to clear bacteria or induce protective responses in the absence of CD4⁺ T cells suggests that helper T cells are not essential (Ganta et al., 2004; Bitsaktsis et al., 2007). Nevertheless, CD4⁺ T cells do appear to contribute to host resistance to *Ehrlichia*. The presence of CD4⁺ T cells in β_2 M-knockout mice (deficient for CD8⁺ T-cells), resulted in increased survival after an experimental *E. ruminantium* challenge (Byrom et al., 2000). Mwangi et al. (2002) found that *E. ruminantium* 28 kDa major outer membrane antigen protein 1 (MAP1)-specific CD4⁺ T cells produced interferon gamma (IFN γ) in response to infection. The depletion of helper T cells increased the number of fatalities of C3H mice when challenged with *E. muris* (Feng and Walker, 2004). Although IFN γ depletion only slightly increased mortality in this model, the combined depletion of IFN γ and TNF α resulted in significantly more mortality and exemplify the paradigm of helper T cell activation resulting in IFN γ production and subsequent processes. Bitsaktsis et al. (2004) demonstrated that mice did not survive an experimental challenge with IOE even at doses of approximately 100 bacteria. In that study, both IFN γ and IFN γ -receptor knockout mice were highly susceptible to infection and suggested that classic cell-mediated immunity orchestrated by CD4⁺ T cells is necessary for host resistance to the most lethal species of *Ehrlichia*. TNF α also played a role in controlling bacterial number in their study because TNF α and TNF-receptor knockout mice were highly susceptible to infection. Ismail et al. (2004) also discovered a role for TNF α in the host response to the IOE strain of *Ehrlichia*. The authors found that IFN γ production went down with a lethal experimental challenge and that CD8⁺ T cells produced high concentrations of TNF α which was not produced when animals were given a non-lethal challenge. Based on these observations, the authors suggested that TNF α may be essential early in the host for protection and survival. However, when TNF α or its receptors are nonfunctional, IL-10 concentrations are increased. The hypothesis is that TNF α may regulate IL-10. TNF α made early induces IL-10 which prevents overproduction of TNF α . This is an attractive hypothesis because neither cytokine appears to be produced in an exaggerated fashion in mice challenged with *Ehrlichia muris* (Ismail et al., 2004) and we have had difficulty detecting high concentrations of TNF α after experimental challenges with *E. chaffeensis* (Ganta et al., 2002). The hypothesis is also supported by recent data reported by Stevenson et al. (Stevenson et al., 2006). Mice

challenged with the IOE strain of *Ehrlichia* by intradermal route had a good Th1-type cell-mediated immune response and resulted in lower mortality rate, possibly due to lower systemic TNF α concentration. Interestingly, this study also shows that the route of inoculation plays an important role in determining the outcome of the infection. For example, Intradermal infection with IOE causes mild, self-limited disease, whereas intraperitoneal infection results in acute, severe toxic shock-like syndrome. The loss of CD4 $^{+}$ T cells in response to perforin made by CD8 $^{+}$ T cells also illustrates an additional level of immunoregulation during *Ehrlichia* infections (Ismail et al., 2007). These are both areas of *Ehrlichia* immunology that require more attention.

MHC II knockout and CD4 depletion

In our studies, we discovered that *E. chaffeensis* persists in MHCII-knockout mice (Ganta et al., 2002). These data indicate that MHCII molecules are essential for clearance of the bacteria but their function is not totally necessary for host survival. This hypothesis is supported by the observation that >75% of MHCII knockout mice also survive experimental challenge of *E. muris* (Feng and Walker, 2004). The absence of MHCII caused only minor suppression of the host response and did not overwhelmingly change a persistent infection into a lethal one. Although these data support the hypothesis that complete curing of this intracellular rickettsial infection requires the presence of functional CD4 $^{+}$ T-cells, we were surprised that CD4-knockout mice were still able to resolve an *E. chaffeensis* infection in 3–4 weeks (Ganta et al., 2004). Even so, the presence of CD4 $^{+}$ T cells led to the most rapid resolution of the experimental challenge (Ganta et al., 2004). Treating mice with anti-CD4 monoclonal antibody also delayed clearance in wild-type mice about one week (Figure 3). Therefore, there is redundancy in the immune response that allows for host resistance in the absence of CD4 $^{+}$ T cells. Unfortunately, as *Ehrlichia* species become more pathogenic (e.g. IOE strain), the less successful those compensatory mechanisms are. When CD4 $^{+}$ T cells were depleted prior to challenging with *E. muris* infection, there was a 44% increase in mortality compared to CD4 $^{+}$ T cell-bearing mice (Feng and Walker, 2004). Likewise there was a 100% increase in the mortality when CD4 $^{+}$ T cells were depleted before challenge with IOE strain (Bitsaktsis et al., 2004).

CD8 $^{+}$ T cells

Since CD4 knockout mice do cure *E. chaffeensis* infections, the logical question is “what host immune component is responsible for the resistance in those animals?” CD8 $^{+}$ T cells may be one of the effectors. Dierberg and Dumler (2006) reported that there are increased numbers of CD8 $^{+}$ T cells in persons who died of HME. However, the role of CD8 $^{+}$ T cells in humans and mice appears to be far from certain. As noted above, the production of TNF α by CD8 $^{+}$ T cells may be necessary early after infection for successful clearance of IOE but over stimulation of the CD8 $^{+}$ T cells can be detrimental (Bitsaktsis et al., 2004; Ismail et al., 2004). In fact, the adoptive transfer of memory CD8 $^{+}$ T-cells resulted in a fatal secondary response by C57BL/6 mice (Bitsaktsis and Winslow, 2006). The absence of CD8 $^{+}$ T cells also preserves lymphoid structure and tissue integrity after IOE challenge (Ismail et al., 2007). These data are consistent with the observation that β_2 M-knockout mice, deficient for CD8 $^{+}$ T cells, were less susceptible to *E. ruminantium* infection than normal or MHCII knockout mice (Byrom et al., 2000). When Feng and Walker (2004) depleted C3H mice of both CD4 $^{+}$ and CD8 $^{+}$ T cells, there was a 100% increase in mortality after *E. muris* challenge compared to CD4-depleted mice. These data suggest that CD8 $^{+}$ T cells may help cure the infection, but are not essential. In fact, in C3H mice, there was 81% mortality in MHCII knockout mice but the same impact was not seen when MHCII was absent in C57BL/6J mice (Feng and Walker, 2004). We also did not see any deaths in β_2 M-knockout mice on the C57BL/6 background after challenge with *E. chaffeensis*; although host resolution of the infection took slightly longer (Chapes and Ganta, unpublished data). Therefore, it appears that there may also be some mouse strain-specific impact on the contribution of CD8 $^{+}$ T cells. It does not appear that CD8 $^{+}$ T cells are important as cytotoxic

cells. Cytotoxic T lymphocytes (CTLs) were generated after *E. muris* challenge but perforin knockout mice were not more susceptible to infection (Feng and Walker, 2004). Our data show that CTLs were generated after *E. chaffeensis* challenge. However, activity could only be detected after a second injection of bacteria (Ganta et al., 2004). Moreover, even though MHCII knockout mice did not resolve *E. chaffeensis* infections, they too could generate CTL activity when challenged with bacteria a second time. Therefore, it does not appear that clearance is impacted by the presence of the CTL.

CD4⁻ CD8⁻ T cells

A number of intracellular, monocytotropic pathogens are cured in the absence of “classic” CD4⁺ T cell-mediated immunity. Hosts lacking CD4⁺ T cells contain CD4⁻ CD8⁻ T cells. These double negative T-cells are heterogenous. Some express classic $\alpha\beta$ T-cell receptors and occur naturally (Rahemtulla et al., 1991) and others appear to express the NK1.1 molecule (Skold and Cardell, 2000). The $\alpha\beta$ -T cells lacking CD4 and CD8 molecules may be necessary for the residual Ig-class switching present in CD4 knockout mice (Rahemtulla et al., 1993) and may be involved in host resistance to viruses (Rahemtulla et al., 1994). These cells have also been implicated in the control of the intracellular bacteria *Mycobacterium tuberculosis* and *Francisella tularensis* (Cowley et al., 2005; Cowley and Elkins, 2003) or may provide other “helper” functions since there appears to be a more mature helper T-cell population in CD4 knockout mice (Rahemtulla et al., 1994). These cells may also provide some level of recall and may function in the absence of CD4⁺ T cells (Cowley et al., 2005).

Natural killer T-cells

Natural killer T-cells (NKT cells) represent a second population of $\alpha\beta$ T-cell receptor-positive, CD4⁻CD8⁻ T cells that could be involved in host resistance to *Ehrlichia* species. There was an increase in the number of activated, CD1d-binding NKT cells in mice infected with *E. muris* (Mattner et al., 2005). These Va14-expressing T cells recognize glycolipids that are presented by CD1d (MHC Ib) molecules (Wu et al., 2005) and may be instrumental in activating the immune response via non-Toll-like receptor pattern recognition by producing IFN γ (Kinjo et al., 2005). Indeed, mice deficient for CD1d carried an 11.2 fold greater *E. muris* infection than CD1d heterozygotes (Mattner et al., 2005). NK cells also make IFN γ and perhaps that the early IFN γ production by NKT cells may be critical in the absence of CD4⁺ T cells.

In the absence of T cells in SCID mice, NK cells make IFN γ which activates macrophages against facultative intracellular bacteria (Bancroft et al., 1989; Hunter et al., 1994; Ramarathinam et al., 1993; Schafer and Eisenstein, 1992; Tripp et al., 1993; Wherry et al., 1991). *Ehrlichia* infection down regulated CD1d (Zhang et al., 2004b) and other molecules that regulate immune responses such as Tlr4 and MHCII (Figure 4). If there is a down regulation of other non polymorphic MHC I molecules, the absence of inhibitory receptors may allow infected cells to become targets (Salcedo et al., 1997). If this does occur, it does not appear to be a critical mechanism of host resistance. In our studies, we found that NK cells functioned normally in MHCII knockout mice (Chapes et al., 1993) indicating that functional NK cells alone are not capable of curing an *E. chaffeensis* infection based on the persistent infection in those MHCII knockout mice (Ganta et al., 2002, 2004). Our data are consistent with the findings by Winslow et al. (1998) showing that *E. chaffeensis* infection was more severe in SCID-beige mice but the absence of NK cells did not affect host resistance in the presence of T cells. Likewise, Hess et al. (2006) found no evidence for the presence of enhanced lymphokine activated killer cell activity in dogs infected with *E. canis*. Although the lymphocyte population was not pure NK cells in their study, the data are consistent with the hypothesis that NK cells may contribute but are not essential to host resistance against *Ehrlichia* species.

$\gamma\delta$ T cells

Another possible T cell that may be involved in *E. chaffeensis* host resistance is the $\gamma\delta$ -expressing T cell (Caldwell et al., 1996; Caldwell et al., 1995; Dunn and North, 1991). Caldwell et al. (1995 and 1996) found V γ 9, V δ 2 TcR-bearing $\gamma\delta$ T cells respond to *Ehrlichia* infection in humans. Macrophage-tropic, *Listeria*-elicited peritoneal mouse $\gamma\delta$ T cells make IFN γ which could be important *in situ* to activate host responses (Skeen and Ziegler, 1995) in the absence of CD4⁺ T cells. Interestingly, although the $\gamma\delta$ T cells are not MHC restricted they do recognize antigen-free, nonpolymorphic MHC molecules (Adams et al., 2005; Crowley et al., 2000; Wingren et al., 2000). Therefore, the role that NKT and $\gamma\delta$ T cells play in *E. chaffeensis* immunity must be distinct and need to be defined better.

Multigenic control [Tlr4, MHCII and Slc11a1 (formerly Nramp 1)]

We are interested in defining the contributions of multigenic control against *E. chaffeensis*. For that reason, we explored the role toll-like receptor-4 (Tlr4) played in resolving an *E. chaffeensis* infection (Ganta et al. 2002). We confirmed Telford and Dawson's report (Telford and Dawson, 1996) that the absence of Tlr4 causes a delay in clearance (Ganta et al., 2002). Tlr4 is involved in the recognition of lipopolysaccharide (LPS) (Vogel, 2000). Although *E. chaffeensis* is considered a Gram-negative organism, it does not have the ability to synthesize LPS (Lin and Rikihisa, 2003). Therefore, there is some question about what role Tlr4 plays in the activation of the host response against *E. chaffeensis*. It is possible that other molecular patterns found on *E. chaffeensis* interact with Tlr4. The Tlr4 gene not only regulates macrophage responsiveness to LPS, but also appears to regulate responsiveness to a number of substances including, *Propionibacterium acnes* (Jackson et al., 1986), calcium ionophore (Perera et al., 1996; Shinomiya and Nakano, 1987), taxol (Perera et al., 1996; Shinomiya and Nakano, 1987), and staphylococcal exoproteins (Chapes and Beharka, 1995; Fleming et al., 1991). Thus, the LPS^{Tlr4-d/d} genotype (mutant) has wide ranging consequences which may impact the host response to several pathogens. For example, it is possible that Tlr4 affects phagocytosis of *Ehrlichia* (Blander and Medzhitov, 2004). Alternatively, the absence of Tlr4 may not affect whether a host can clear *E. chaffeensis* infection, but it may affect the rate at which macrophages are activated and the kinetics of clearance. Tlr4 mutant mice (C3H/HeJ) were recently examined after infection with *A. phagocytophilum* (Blas-Machado et al., 2007). Although those mice appeared normal, analysis of their blood revealed monocytosis and neutropenia and necropsy revealed splenomegaly and interstitial pneumonia that could be detected in the mice as long as 20 days after infection. Therefore, a defective Tlr4 response may impact the host longer than has been appreciated. Adoptive transfer of Tlr4-expressing macrophages but not immature bone marrow cells enhanced the number of inflammatory PMNs present in the peritoneum early in infection (Figure 5). It indicates that Tlr4 regulates the early inflammatory response. It is also clear that macrophages that carry the mutant alleles for Tlr4 did not make as much nitric oxide in response to experimental challenge with *E. chaffeensis* as wild-type macrophages (Ganta et al., 2002). In fact, when we analyzed the impact of three genes [MHCII, Slc11a1 (formerly Nramp 1) and Tlr4] on the macrophage IL-6, IL10 and nitric oxide responses after experimental challenge with *E. chaffeensis*, we found that only the Tlr4 gene significantly affected the macrophage cytokine responses (Table 1–3) (Ganta et al. 2002).

Delayed clearance of *E. chaffeensis* could occur because pattern recognition molecules regulate T cell responses. Tlr4 affects dendritic cell presentation of bacterial antigen which affects the kinetics of the T cell response (Blander and Medzhitov, 2006). Engagement of Tlr2 with bacterial lipoprotein activated CD25⁻ CD4⁺ T cells as well as CD25⁺ CD4⁺ T cells, T regulatory cells (Liu et al., 2006). More importantly, the presence of MyD88, a major adaptor molecule in Tlr signaling (Kawai et al., 1999), affected the development of Th1 responses to several macrophage-tropic, intracellular pathogens (Jankovic et al., 2002). B cells may also be

regulated by Tlrs independently of the Tlr impact on Th cells (Pasare and Medzhitov, 2005). However, the impact of Tlr molecules may depend on the antigen or may not be absolutely necessary (Gavin et al., 2006). Therefore, Tlr expression on lymphocytes and Tlr activation results in some “cross-talk” between the innate signaling pathway and lymphocyte activation pathways (Suzuki et al., 2006). This hypothesis is supported by two studies (Jordan et al., 2008, von Loewenich et al., 2004). In a study by von Loewenich et al., although there was considerable variation in the data, Tlr2/Tlr4 knockout mice had higher average splenic bacterial loads than wild type mice at 3 and 14 days after infection with *A. phagocytophilum* (von Loewenich et al., 2004). Moreover, the absence of MyD88, affected the average blood and splenic bacterial loads in mice at 14 and 21 days after infection (von Loewenich et al., 2004). More recently, Jordan et al. (2008) found a reduced Th1 cytokine response and higher bacterial loads after experimental challenge with *Rickettsia conorii* in Tlr4-deficient, HeJ mice (Huang et al., 2008). Several *Ehrlichia* lipoproteins were shown to elicit cell mediated immune responses in dogs (Jordan et al., 2008). Those molecules have not been defined. It is also not clear whether they function through Tlr2 or Tlr4. However, it is clear that Tlr4 and possibly other Tlr molecules contribute to host *Ehrlichia* resistance in both innate and acquired immune responses. Although the mechanism of how Tlr4 affects the host response to *E. chaffeensis* and other related rickettsiales is unknown, it is clear that those molecules do contribute to host resistance.

The antibody response to *Ehrlichia* species

People who acquire *E. chaffeensis* infection from an infected tick bite develop fever and produce antibodies specific to the pathogen (Fishbein et al., 1994; Pearce et al., 1988; Standaert et al., 2000). Surveys of people and mice in Japan show an approximate seropositive rate of 0.6–1% to *E. muris* (Kawahara et al., 1999). Therefore, specific antibody production is part of the host response to infection. It is clear that cell-mediated immunity plays an important role in host resistance to *Ehrlichia* species. This is consistent with the paradigm that intracellular bacteria require T cells to produce IFN γ (Bitsakis et al., 2004; Feng and Walker, 2004; Winslow et al., 2005) and that IFN γ -activated macrophages inhibit *E. chaffeensis* replication (Barnewall and Rikihisa, 1994). Although antibodies can be made against several different *Ehrlichia* proteins (Luo et al., 2008; Nethery et al., 2007), the most compelling evidence that antibody has a protective role during *Ehrlichia* infections comes from the work published by the Winslow group on p28 OMP molecules (Winslow et al., 1998, 2000, Bitsakis et al., 2007). For example, *E. chaffeensis* infections are fatal in SCID mice but adoptive transfer of *E. chaffeensis*-specific immunoglobulins to SCID mice induced transient protection and survival. Ohashi et al. (Ohashi et al., 1998) found that adoptive transfer of antibody against the p28 outer membrane protein (p28-Omp) into Balb/c mice caused significantly faster clearance compared to the control group. Li et al. (2001) found that p28-Omp-specific IgG2a and 3 antibodies reacted against the first hypervariable region of the Omp and could eliminate infection in some instances. Both antibodies fix complement and experiments by Yagar et al. (2005) indicate that complement and Fc γ are required for the antibodies to provide protection. They speculated that antibodies are able to clear or lyse *E. chaffeensis* when bacteria are released from infected cells before the bacteria can infect other cells. Although these antibodies were able to offer transient protection against fatal infection with *E. chaffeensis* in SCID mice, some mice still had bacteremia 70 days after infection (Li et al., 2001). It is not clear if this means that antibodies are not sufficient for complete clearance, the immunogens are not very good or if adoptive transfer is not as efficient as an active response. There are some suggestions that natural crosslinking of the bacterial outer membrane proteins may make some of the outer membrane protein species less immunogenic individually (Macmillan et al., 2006). Therefore, immunogenicity may be an issue. Mice infected with IOE strain of *Ehrlichia* have significantly lower antibody titers against p28-Omp 19 paralog than mice challenged with *E. muris* (Yager et al., 2005). Ismail et al. (2004) showed that mice primed with *E. muris* could be protected

from an IOE challenge. Later, Nandi et al. (2007) found that mice could be immunized with IOE OMP-19 and later protected against a fatal IOE challenge. Therefore, the p28-OMP molecules can induce protective responses and the T cell epitopes that may control the response appear to be conserved between *Ehrlichia* species (Nandi et al., 2007). Ismail et al. (2004) suggested that host resistance to IOE was at least partially dependent on antibody. Bitsaktsis et al. (2007) used B-cell-deficient mice, T cell subpopulation-knockout mice and passive transfer experiments to convincingly demonstrate that mice can be protected against IOE by antibody in the absence of helper T cells. However, there appears to be some limits to the ability to induce protective memory responses to IOE (Thirumalapura et al., 2008). When high numbers of bacteria were used in secondary IOE experimental challenges mice could not be protected (Thirumalapura et al., 2008). Therefore, there are still mechanisms of the host response to *Ehrlichia* that need to be defined. However antibody responses appear critical to host resistance.

Since host clearance of *E. chaffeensis* is faster in the presence of CD4⁺ T cells (Ganta et al., 2004), it would appear that T cells contribute to antibody responses through their ability to mediate class switching and affinity maturation (Berek 2004). However, in that absence of T cells, Racine et al. (2008) have described the presence of B cells that contribute to host resistance during T-independent responses to *E. muris*. These cells resembled B1 B cells and marginal zone B cells and were present even in MHCII knockout mice, and were specific for OMP-19 (Racine et al., 2008). These data raise some intriguing issues about the role of the immunodominant outer membrane proteins in host resistance to *Ehrlichia*. Perhaps their induction of innate immune responses interferes with the development of memory and explains why there are limits to how well mice can be primed to respond to IOE (Thirumalapura et al., 2008). Additional experiments will be necessary to sort out these different resistance mechanisms.

Slc11a1 expression and antibody response

Winslow et al. (2005) suggested that Slc11a1 (Blackwell et al., 2001) had a modest impact on an experimental challenge by the IOE strain. Although we found no apparent effect of the Slc11a1 gene on *E. chaffeensis* clearance, there was a notable correlation between Slc11a1 expression and the IgG subclasses made during an experimental challenge with *E. chaffeensis* (Figure 6). Mice with a functional Slc11a1 gene had predominant expression of IgG2a antibody. Contrary to this, Slc11a1 mutant mice made more of IgG2b and IgG3. Because these three IgG subclasses are the Th1-type, complement fixing antibodies (Germann et al., 1995; Kuhn et al., 1991), one would not predict that the different isotype makeup of the host response to have a major impact on the pathogen clearance. Nevertheless, evaluation of the isotype-specific monoclonal antibodies against p28-Omp of *E. chaffeensis* revealed that the IgG2a isotype was the most effective antibody in protecting SCID mice from fatal infections compared to IgG2b and IgG3 (Li et al., 2001; Winslow et al., 2003). These data suggest that IgG2a is more effective in promoting host resistance. It appears that Slc11a1 protein influences isotype expression by affecting the type of cytokines made by helper T cells. The expansion of IgG2a, IgG2b and IgG3 secreting B cells is IFN- γ dependent (Snapper and Paul, 1987; Stevens et al., 1988). Moreover, recent data by Caron et al. (2006) suggest that Slc11a1 promotes a Th1 response to intracellular pathogens. We also saw a low concentration of IgG1, a Th2 antibody (Yamamoto et al., 2001) in all mouse strains that we screened (Ganta et al., 2002). The Slc11a1 mutant mice may produce more IgG2b and IgG3 because of the absence of IL-4 (Kuhn et al., 1991) and because IgG subclass responses can be influenced at different IFN- γ concentrations (Snapper et al., 1995; Snapper and Paul, 1987). Our hypothesis for why we did not see an impact of Slc11a1 on clearance probably has to do with the experimental system. Our mice had complete immune systems that masked the minor impact of the Slc11a1 gene. In SCID mice, the “noise” of a complete immune system did not mask the minor

contribution of *Slc11a1*. As noted above, a protective immune response against *E. chaffeensis* may be associated with IFN- γ secretion and a Th1 response which may be the most important component in host resistance. Caron et al. (2006) recently demonstrated that *Slc11a1* affects the Th1 response to *Salmonella* infection. Therefore, the correlation between our observation of a skewed IgG2a response (Figure 5) and the efficacy of IgG2a in protecting SCID mice clearing *E. chaffeensis* is consistent with the *Slc11a1* gene role in showing a host response toward a Th1 type outcome (Li et al., 2001; Winslow et al., 2003). This also demonstrates how multiple immune response genes contribute to host resistance and the value of immune response gene knockout mice. Only in the absence of the dominant immune response did one see the impact of *Slc11a1* in resistance against an *Ehrlichia* species.

The *E. chaffeensis* mouse model; provocative and neglected aspects, but entering a new discovery period

About 97% of the population that contracts HME has a successful immune response and resolves the infection. We reasoned that a model that mimics the human response is relevant to understanding how the host deals with the pathogen. In this aspect, the mouse *E. chaffeensis* infection model is very similar to natural infections in people. Analysis of *E. chaffeensis* and closely related *Ehrlichia* and *Anaplasma* pathogens (Dunning Hotopp et al., 2006) indicates that there is a conserved chromosomal gene order amongst these pathogens. There are also homologous, immunodominant outer membrane protein antigens that included 28 kDa proteins (Crocquet-Valdes et al., 2005; Lin et al., 2004; Lohr et al., 2004; Ohasi et al., 1998; Reddy et al., 1998; Rikihisa et al., 1994). The outer membrane proteins are actively pursued as targets for serodiagnostic test and vaccine development. Using an *E. ruminantium* 28 kDa Omp gene-based naked DNA vaccine, Nyika et al. (1998 and 2002) suggested that it offered significant protection from a homologous challenge. As discussed above, Winslow *et al.* demonstrated that the 28 kDa OMP-specific monoclonal antibodies offered significant transient protection against fatal infection with *E. chaffeensis* in SCID mice (Li et al., 2001; Winslow et al., 2003). Ehrlichiosis is a zoonotic disease that includes ticks and vertebrates as hosts. However, few studies have examined the expression of the outer membrane proteins by bacteria growing in different hosts. Unver et al. (2001) found 10 paralogous *E. canis* genes, that were transcribed at different levels in dogs, but were not expressed when the bacteria grew in *Rhipicephalus sanguineus* ticks. Similarly, only *E. chaffeensis* Omp 14 was expressed in *Amblyomma americanum* ticks or tick cell lines (Singu et al., 2005; Unver et al., 2001). However, there is a considerable discussion about how many p28-Omp paralogs are transcribed when the bacteria grow in dog macrophages (Unver et al., 2001). Our recent proteomic data suggest that only a subset of proteins were expressed in the canine macrophages (Singu et al., 2005). *E. chaffeensis* had a higher expression of p28 Omp 19 when it grew in dog macrophages than when it grew in tick cells (Singu et al., 2005). Conversely, *E. chaffeensis* had a higher expression of p28 Omp 14 when it replicated in vector and non-vector tick cell lines (ISE6 and AAE2, respectively) (Singu et al., 2005, 2006). Ijdo et al. (2002) found that *A. phagocytophilum* expressed different p44 genes when growing in actively feeding ticks than when growing in mice. There have been some suggestions that variability in the expression of paralogous outer membrane proteins may serve as a mechanism of pathogen resistance (Reddy and Streck, 1999). However, the kinetics of the production of antibody in dogs against 21 *E. chaffeensis* outer membrane proteins over a period of 462 days suggests that there may not be sequential/differential expression of the proteins during the course of the infection (Zhang et al., 2004a). Recent proteome studies by Ge and Rikihisa (2007) and Seo et al., (2008) found that all but a few p28-Omp proteins were expressed in macrophage cells, while the major expression in tick cells is limited to one p28-Omp protein 14 (Seo et al. 2008). It is clear that a better understanding of when these genes are expressed and how the host deals with them is important to understanding *Ehrlichia* immunity.

Recently, we discovered that the antibody response by mice to *E. chaffeensis* grown in tick cells is significantly different than the antibody response to bacteria grown in the DH82 macrophage cell line (Ganta et al., 2007). Although we do not know which of the antibodies contributed to host resistance to infection, there were distinct antigens recognized by mice challenged with DH82 macrophage-grown bacteria compared to tick cell-grown bacteria. Moreover, our study suggests that tick cell-derived bacteria might be a better model or source of bacteria for infection experiments to assess the host immune responses against *E. chaffeensis*. It is possible that macrophage-grown bacteria may not express the appropriate array of molecules to allow accurate analysis of vertebrate host responses. Our proteomics analysis supports this hypothesis (Singu et al., 2005), because about 50% of the *E. chaffeensis* proteins were expressed in a host cell-specific manner. Thus, a significant change in the model paradigm currently being used in the *Ehrlichia* research community is needed to assess the host response against *E. chaffeensis*. Most investigators use the canine macrophage cell line, DH82, derived *E. chaffeensis* for *in vivo* experimental challenge studies. If the antigenic make up is not representative of the antigens that the host might see during the “natural” infection, many of the conclusions made about host resistance to date may not be relevant to what actually happens in nature. Thus, future studies to map the host immunity should utilize vector tick cells and tick cell-grown bacteria.

Acknowledgements

This work was supported in part by the National Institutes of Health grants AI050785, AI055052, AI070908, AI052206, RR016475, RR017686, and NASA grant NAG2-1274, the Howard Hughes Foundation and the Kansas Agricultural Experiment Station. We thank Chuanmin Cheng, Elizabeth C. Miller, Kamesh R. Sirigireddy and other members of our research group for their technical assistance and help. The manuscript is Kansas Agricultural Experiment Station contribution # 07-50-J.

References

- Adams EJ, Chien YH, Garcia KC. Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. *Science* 2005;308:227–231. [PubMed: 15821084]
- Bancroft GJ, Sheehan KC, Schreiber RD, Unanue ER. Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in scid mice. *J Immunol* 1989;143:127–130. [PubMed: 2499625]
- Barnewall RE, Rikihisa Y. Abrogation of gamma interferon-induced inhibition of *Ehrlichia chaffeensis* infection in human monocytes with iron-transferrin. *Infect Immun* 1994;62:4804–4810. [PubMed: 7927758]
- Berek C. T-cell-dependent immune responses, germinal center development, and the analysis of V-gene sequences. *Methods in Molecular Biology* (Clifton, NJ) 2004;271:127–138.
- Bitsaktsis C, Huntington J, Winslow G. Production of IFN-gamma by CD4 T cells is essential for resolving *Ehrlichia* infection. *J Immunol* 2004;172:6894–6901. [PubMed: 15153508]
- Bitsaktsis C, Nandi B, Racine R, MacNamara KC, Winslow G. T-cell-independent humoral immunity is sufficient for protection against fatal intracellular *Ehrlichia* infection. *Infect Immun* 2007;75:4933–4941. [PubMed: 17664264]
- Bitsaktsis C, Winslow G. fatal recall responses mediated by CD8 T cells during intracellular bacterial challenge infection. *J Immunol* 2006;177:4644–4651. [PubMed: 16982903]
- Blackwell JM, Goswami T, Evans CA, Sibthorpe D, Papo N, White JK, Searle S, Miller EN, Peacock CS, Mohammed H, Ibrahim M. SLC11A1 (formerly NRAMP1) and disease resistance. *Cell Microbiol* 2001;3:773–784. [PubMed: 11736990]
- Blander JM, Medzhitov R. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* 2006;440:808–812. [PubMed: 16489357]
- Blander JM, Medzhitov R. Regulation of phagosome maturation by signals from toll-like receptors. *Science* 2004;304:1014–1018. [PubMed: 15143282]

- Blas-Machado U, de la FJ, Blouin EF, Almazan C, Kocan KM, Mysore JV. Experimental Infection of C3H/HeJ Mice with the NY18 Isolate of *Anaplasma phagocytophilum*. *Vet Pathol* 2007;44:64–73. [PubMed: 17197625]
- Breitschwerdt EB, Hegarty BC, Hancock SI. Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, or *Bartonella vinsonii*. *J Clin Microbiol* 1998;36:2645–2651. [PubMed: 9705408]
- Buller RS, Arens M, Hmiel SP, Paddock CD, Sumner JW, Rikhisa Y, Unver A, Gaudreault-Keener M, Manian FA, Liddell AM, Schmulewitz N, Storch GA. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *N Engl J Med* 1999;341:148–155. [PubMed: 10403852]
- Byrom B, Barbet AF, Obwolo M, Mahan SM. CD8(+) T cell knockout mice are less susceptible to *cowdria ruminantium* infection than athymic, CD4(+) T cell knockout, and normal C57BL/6 mice [In Process Citation]. *Vet Parasitol* 2000;93:159–172. [PubMed: 11035234]
- Caldwell CW, Everett ED, McDonald G, Yesus YW, Roland WE. Lymphocytosis of gamma/delta T cells in human ehrlichiosis. *Am J Clin Pathol* 1995;103:761–766. [PubMed: 7785663]
- Caldwell CW, Everett ED, McDonald G, Yesus YW, Roland WE, Huang HM. Apoptosis of gamma/delta T cells in human ehrlichiosis. *Am J Clin Pathol* 1996;105:640–646. [PubMed: 8623774]
- Caron J, Larivière L, Nacache M, Tam M, Stevenson MM, McKerly C, Gros P, Malo D. Influence of Slc11a1 on the outcome of *Salmonella enterica* serovar *Enteritidis* infection in mice is associated with Th polarization. *Infect Immun* 2006;74:2787–2802.
- Chapes SK, Beharka AA. Lipopolysaccharide is required for the lethal effects of enterotoxin B after D-galactosamine sensitization. *J Endotoxin Res* 1995;2:263–271.
- Chapes SK, Hoynowski SM, Woods KM, Armstrong JW, Beharka AA, Iandolo JJ. *Staphylococcus*-mediated T-cell activation and spontaneous natural killer cell activity in the absence of major histocompatibility complex class II molecules. *Infect Immun* 1993;61:4013–4016. [PubMed: 8359928]
- Chen SM, Dumler JS, Bakken JS, Walker DH. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. *J Clin Microbiol* 1994;32:589–595. [PubMed: 8195363]
- Chen SM, Yu XJ, Popov VL, Westerman EL, Hamilton FG, Walker DH. Genetic and antigenic diversity of *Ehrlichia chaffeensis*: comparative analysis of a novel human strain from Oklahoma and previously isolated strains. *J Infect Dis* 1997;175:856–863. [PubMed: 9086141]
- Cowley SC, Elkins KL. Multiple T cell subsets control *Francisella tularensis* LVS intracellular growth without stimulation through macrophage interferon gamma receptors. *J Exp Med* 2003;198:379–389. [PubMed: 12885873]
- Cowley SC, Hamilton E, Frelinger JA, Su J, Forman J, Elkins KL. CD4-CD8- T cells control intracellular bacterial infections both in vitro and in vivo. *J Exp Med* 2005;202:309–319. [PubMed: 16027239]
- Crocquet-Valdes PA, McBride JW, Feng HM, Ismail N, Small MA, Yu XJ, Walker DH. Analysis of *ehrlichial* p28 gene expression in a murine model of persistent infection. *Ann N Y Acad Sci* 2005;1063:420–424. [PubMed: 16481552]
- Crowley MP, Fahrner AM, Baumgarth N, Hampl J, Gutgemann I, Teyton L, Chien Y. A population of murine gammadelta T cells that recognize an inducible MHC class Ib molecule. *Science* 2000;287:314–316. [PubMed: 10634788]
- Dawson JE, Anderson BE, Fishbein DB, Sanchez CY, Goldsmith CY, Wilson KH, Duntley CW. Isolation and characterization of an *Ehrlichia* sp from a patient diagnosed with human ehrlichiosis. *J Clin Microbiol* 1991;29:2741–2745. [PubMed: 1757543]
- Dawson JE, Stallknecht DE, Howerth EW, Warner C, Biggie K, Davidson WR, Lockhart JM, Nettles VF, Olson JG, Childs JE. Susceptibility of white-tailed deer (*Odocoileus virginianus*) to infection with *Ehrlichia chaffeensis*, the etiologic agent of human ehrlichiosis. *J Clin Microbiol* 1994;32:2725–2728. [PubMed: 7852563]
- Dierberg KL, Dumler JS. Lymph node hemophagocytosis in rickettsial diseases: a pathogenetic role for CD8 T lymphocytes in human monocytic ehrlichiosis (HME). *BMC Infect Dis* 2006;6:121. [PubMed: 16859547]
- Dumler JS, Bakken JS. Ehrlichial diseases of humans: emerging tick-borne infections. *Clin Infect Dis* 1995;20:1102–1110. [PubMed: 7619983]

- Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 2001;51:2145–2165. [PubMed: 11760958]
- Dunn PL, North RJ. Resolution of primary murine listeriosis and acquired resistance to lethal secondary infection can be mediated predominantly by Thy-1+ CD4- CD8- cells. *J Infect Dis* 1991;164:869–877. [PubMed: 1682394]
- Dunning Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, Eisen J, Seshadri R, Ren Q, Wu M, Utterback TR, Smith S, Lewis M, Khouri H, Zhang C, Niu H, Lin Q, Ohashi N, Zhi N, Nelson W, Brinkac LM, Dodson RJ, Rosovitz MJ, Sundaram J, Daugherty SC, Davidsen T, Durkin AS, Gwinn M, Haft DH, Selengut JD, Sullivan SA, Zafar N, Zhou L, Benahmed F, Forberger H, Halpin R, Mulligan S, Robinson J, White O, Rikihisa Y, Tettelin H. Comparative genomics of emerging human ehrlichiosis agents. *PLoS Genet* 2006;2:e21. [PubMed: 16482227]
- Feng HM, Walker DH. Mechanisms of immunity to *Ehrlichia muris*: a model of monocytotropic ehrlichiosis. *Infect Immun* 2004;72:966–971. [PubMed: 14742542]
- Fishbein DB, Dawson JE, Robinson LE. Human ehrlichiosis in the United States, 1985 to 1990. *Ann Intern Med* 1994;120:736–743. [PubMed: 8147546]
- Fleming SD, Iandolo JJ, Chapes SK. Murine macrophage activation by staphylococcal exotoxins. *Infect Immun* 1991;59:4049–4055. [PubMed: 1937764]
- Ganta RR, Cheng C, Miller EC, McGuire BL, Peddireddi L, Sirigireddy KR, Chapes SK. Differential clearance and immune responses to tick cell-derived versus macrophage culture-derived *Ehrlichia chaffeensis* in mice. *Infect Immun* 2007;75:135–145. [PubMed: 17060466]
- Ganta RR, Cheng C, Wilkerson MJ, Chapes SK. Delayed clearance of *Ehrlichia chaffeensis* infection in CD4+ T-cell knockout mice. *Infect Immun* 2004;72:159–167. [PubMed: 14688093]
- Ganta RR, Wilkerson MJ, Cheng C, Rokey AM, Chapes SK. Persistent *Ehrlichia chaffeensis* infection occurs in the absence of functional major histocompatibility complex class II genes. *Infect Immun* 2002;70:380–388. [PubMed: 11748204]
- Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, Nemazee D. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* 2006;314:1936–1938. [PubMed: 17185603]
- Ge Y, Rikihisa y. Surface-exposed proteins of *Ehrlichia chaffeensis*. *Infect Immun* 2007;75:3833–3841. [PubMed: 17517859]
- Germann T, Bongartz M, Dlugonska H, Hess H, Schmitt E, Kolbe L, Kolsch E, Podlaski FJ, Gately MK, Rude E. Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses in vivo. *Eur J Immunol* 1995;25:823–829. [PubMed: 7705414]
- Harrus S, Waner T, Aizenberg I, Foley JE, Poland AM, Bark H. Amplification of ehrlichial DNA from dogs 34 months after infection with *Ehrlichia canis*. *J Clin Microbiol* 1998;36:73–76. [PubMed: 9431923]
- Harvey JW, Simpson CF, Gaskin JM. Cyclic Thrombocytopenia Induced by a Rickettsia-Like Agent in Dogs. *J Infect Dis* 1978;137:182–188. [PubMed: 627738]
- Hess PR, English RV, Hegarty BC, Brown GD, Breitschwerdt EB. Experimental *Ehrlichia canis* infection in the dog does not cause immunosuppression. *Vet Immunol Immunopathol* 2006;109:117–125. [PubMed: 16169601]
- Huang H, Lin M, Wang X, Kikuchi T, Mottaz H, Norbeck A, Rikihisa Y. Proteomic analysis of and immune responses to *Ehrlichia chaffeensis* lipoproteins. *Infect Immun* 2008;76:3405–3414. [PubMed: 18490460]
- Hunter CA, Subauste CS, Van CV, Remington JS. Production of gamma interferon by natural killer cells from *Toxoplasma gondii*-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. *Infect Immun* 1994;62:2818–2824. [PubMed: 7911785]
- IJdo JW, Wu C, Telford SR 3rd, Fikrig E. Differential expression of the p44 gene family in the agent of human granulocytic ehrlichiosis. *Infect Immun* 2002;70:5295–5298. [PubMed: 12183586]

- Ismail N, Crossley EC, Stevenson HL, Walker DH. Relative importance of T-cell subsets in monocytotropic ehrlichiosis: a novel effector mechanism involved in *Ehrlichia*-induced immunopathology in murine ehrlichiosis. *Infect Immun* 2007;75:4608–4620. [PubMed: 17562770]
- Ismail N, Soong L, McBride JW, Valbuena G, Olano JP, Feng HM, Walker DH. Overproduction of TNF- α by CD8 $^{+}$ type 1 cells and down-regulation of IFN- γ production by CD4 $^{+}$ Th1 cells contribute to toxic shock-like syndrome in an animal model of fatal monocytotropic ehrlichiosis. *J Immunol* 2004;172:1786–1800. [PubMed: 14734762]
- Jackson RA, Lill PH, Gangemi JD. Role of pulmonary macrophages in resistance to experimental metastasis. *J Leukoc Biol* 1986;40:575–587. [PubMed: 3464675]
- Jankovic D, Kullberg MC, Hieny S, Caspar P, Collazo CM, Sher A. In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(–/–) setting. *Immunity* 2002;16:429–439. [PubMed: 11911827]
- Jordan JM, Woods ME, Olano J, Walker DH. The absence of Toll-like receptor 4 signaling in C3H/HeJ mice predisposes them to overwhelming rickettsial infection and decreased protective Th1 responses. *Infect Immun* 2008;76:3717–3724. [PubMed: 18490467]
- Kawahara M, Ito T, Suto C, Shibata S, Rikihisa Y, Hata K, Hirai K. Comparison of *Ehrlichia muris* strains isolated from wild mice and ticks and serologic survey of humans and animals with *E. muris* as antigen. *J Clin Microbiol* 1999;37:1123–1129. [PubMed: 10074536]
- Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999;11:115–122. [PubMed: 10435584]
- Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, Tsuji M, Kawahara K, Wong CH, Kronenberg M. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 2005;434:520–525. [PubMed: 15791257]
- Kock ND, VanVliet AHM, Charlton K, Jongejan F. Detection of *Cowdria ruminantium* in blood and bone marrow samples from clinically normal, free-ranging Zimbabwean wild ungulates. *J Clin Microbiol* 1995;33:2501–2504. [PubMed: 7494060]
- Kuhn R, Rajewsky K, Muller W. Generation and analysis of interleukin-4 deficient mice. *Science* 1991;254:707–710. [PubMed: 1948049]
- Li JS, Yager E, Reilly A, Freeman C, Reddy GR, Reilly AA, Chu FK, Winslow GM. Outer membrane protein specific monoclonal antibodies protect SCID mice from fatal infection by the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis*. *J Immunol* 2001;166:1855. [PubMed: 11160232]
- Lin M, Rikihisa Y. *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. *Infect Immun* 2003;71:5324–5331. [PubMed: 12933880]
- Lin Q, Rikihisa Y, Felek S, Wang X, Massung RF, Woldehiwet Z. *Anaplasma phagocytophilum* has a functional msp2 gene that is distinct from p44. *Infect Immun* 2004;72:3883–3889. [PubMed: 15213131]
- Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4 $^{+}$ CD25 $^{+}$ regulatory T cells. *Proc Natl Acad Sci U S A* 2006;103:7048–7053. [PubMed: 16632602]
- Lockhart JM, Davidson WR, Stallknecht DE, Dawson JE. Lack of seroreactivity to *Ehrlichia chaffeensis* among rodent populations. *J Wildl Dis* 1998;34:392–396. [PubMed: 9577793]
- Lockhart JM, Davidson WR, Stallknecht DE, Dawson JE, Little SE. Natural history of *Ehrlichia chaffeensis* (*Rickettsiales: Ehrlichieae*) in the piedmont physiographic province of Georgia. *J Parasitol* 1997;83:887–894. [PubMed: 9379294]
- Lohr CV, Brayton KA, Barbet AF, Palmer GH. Characterization of the *Anaplasma marginale* msp2 locus and its synteny with the omp1/p30 loci of *Ehrlichia chaffeensis* and *E. canis*. *Gene* 2004;325:115–121. [PubMed: 14697516]
- Luo T, Zhang X, Wakeel A, Popov VL, McBride JW. A variable-length PCR target protein of *Ehrlichia chaffeensis* contains major species-specific antibody epitopes in acidic serine-rich tandem repeats. *Infect Immun* 2008;76:1572–1580. [PubMed: 18212082]
- Macmillan H, Brayton KA, Palmer GH, McGuire TC, Munske G, Siems WF, Brown WC. Analysis of the *Anaplasma marginale* major surface protein 1 complex protein composition by tandem mass spectrometry. *J Bacteriol* 2006;188:4983–4991. [PubMed: 16788207]

- Maeda K, Markowitz N, Hawley RC, Ristic M, Cox D, McDade JE. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *N Engl J Med* 1987;316:853–856. [PubMed: 3029590]
- Mattner J, Debord KL, Ismail N, Goff RD, Cantu C III, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N, Hoebe K, Schneewind O, Walker D, Beutler B, Teyton L, Savage PB, Bendelac A. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 2005;434:525–529. [PubMed: 15791258]
- McDade JE. Ehrlichiosis - A disease of animals and humans. *J Infect Dis* 1990;161:609–617. [PubMed: 2181027]
- Miura K, Rikihisa Y. Virulence potential of *Ehrlichia chaffeensis* strains of distinct genome sequences. *Infect Immun* 2007;75:3604–3613. [PubMed: 17438035]
- Mwangi DM, McKeever DJ, Nyanjui JK, Barbet AF, Mahan SM. Immunisation of cattle against heartwater by infection with *Cowdria ruminantium* elicits T lymphocytes that recognise major antigenic proteins 1 and 2 of the agent. *Vet Immunol Immunopathol* 2002;85:23–32. [PubMed: 11867164]
- Mylonakis ME, Koutinas AF, Breitschwerdt EB, Hegarty BC, Billinis CD, Leontides LS, Kontos VS. Chronic Canine Ehrlichiosis (*Ehrlichia canis*): A Retrospective Study of 19 Natural Cases. *J Am Anim Hosp Assoc* 2004;40:174–184. [PubMed: 15131097]
- Naitou H, Kawaguchi D, Nishimura Y, Inayoshi M, Kawamori F, Masuzawa T, Hiroi M, Kurashige H, Kawabata H, Fujita H, Ohashi N. Molecular identification of *Ehrlichia* species and '*Candidatus Neoehrlichia mikurensis*' from ticks and wild rodents in Shizuoka and Nagano Prefectures, Japan. *Microbiol Immunol* 2006;50:45–51. [PubMed: 16428872]
- Nandi B, Hogle K, Vitko N, Winslow GM. CD4 T-cell epitopes associated with protective immunity induced following vaccination of mice with an ehrlichial variable outer membrane protein. *Infection and immunity* 2007;75:5453–5459. [PubMed: 17698576]
- Nethery KA, Doyle CK, Zhang X, McBride JW. *Ehrlichia canis* gp200 contains dominant species-specific antibody epitopes in terminal acidic domains. *Infect Immun* 2007;75:4900–4908. [PubMed: 17682040]
- Nyika A, Barbet AF, Burridge MJ, Mahan SM. DNA vaccination with map1 gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. *Vaccine* 2002;20:1215–1225. [PubMed: 11803084]
- Nyika A, Mahan SM, Burridge MJ, McGuire TC, Rurangirwa F, Barbet AF. A DNA vaccine protects mice against the rickettsial agent *Cowdria ruminantium*. *Parasite Immunol* 1998;20:111–119. [PubMed: 9568614]
- Ohashi N, Zhi N, Zhang Y, Rikihisa Y. Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. *Infect Immun* 1998;66:132–139. [PubMed: 9423849]
- Olano JP, Wen G, Feng HM, McBride JW, Walker DH. Histologic, serologic, and molecular analysis of persistent ehrlichiosis in a murine model. *Am J Pathol* 2004;165:997–1006. [PubMed: 15331423]
- Paddock CD, Childs JE. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. *Clin Microbiol Rev* 2003;16:37–64. [PubMed: 12525424]
- Paddock CD, Sumner JW, Shore GM, Bartley DC, Elie RC, McQuade JG, Martin CR, Goldsmith CS, Childs JE. Isolation and Characterization of *Ehrlichia chaffeensis* Strains from Patients with Fatal Ehrlichiosis. *J Clin Microbiol* 1997;35:2496–2502. [PubMed: 9316896]
- Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. *Nature* 2005;438:364–368. [PubMed: 16292312]
- Pearce CJ, Conrad ME, Nolan PE, Fishbein DB, Dawson JE. Ehrlichiosis: a cause of bone marrow hypoplasia in humans. *Am J Hematol* 1988;28:53–55. [PubMed: 3369436]
- Perera PY, Qureshi N, Vogel SN. Paclitaxel (Taxol)-induced NF-kappaB translocation in murine macrophages. *Infect Immun* 1996;64:878–884. [PubMed: 8641795]
- Perez M, Rikihisa Y, Wen B. *Ehrlichia canis*-Like Agent Isolated from a Man in Venezuela: Antigenic and Genetic Characterization. *J Clin Microbiol* 1996;34:2133–2139. [PubMed: 8862572]
- Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. *Ann N Y Acad Sci* 2006;1078:110–117. [PubMed: 17114689]

- Perreau P, Morel PC, Barre N, Durand P. Existence de la coudriose (heartwater) a *Cowdria ruminantium* ches les ruminants des Antilles francaises (La Guadeloup) et des Mascareignes (La Reunion et Ile Maurice). Rev Elev Med Vet Pays Trop 1980;33:21–22. [PubMed: 7455277]
- Racine R, Chatterjee M, Winslow GM. CD11c Expression identifies a population of extrafollicular antigen-specific splenic plasmablasts responsible for CD4 T-independent antibody responses during Intracellular bacterial infection. J Immunol 2008;181:1375–1385. [PubMed: 18606692]
- Rahemtulla A, Fung-Leung WP, Schilham MW, Kundig TM, Sambhara SR, Narendran A, Arabian A, Wakeham A, Paige CJ, Zinkernagel RM. Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4. Nature 1991;353:180–184. [PubMed: 1832488]
- Rahemtulla A, Kundig TM, Narendran A, Bachmann MF, Julius M, Paige CJ, Ohashi PS, Zinkernagel RM, Mak TW. Class II major histocompatibility complex-restricted T cell function in CD4-deficient mice. Eur J Immunol 1994;24:2213–2218. [PubMed: 7916297]
- Rahemtulla A, Shahinian A, Kundig T, Zinkernagel R, Mak TW. CD4 negative mice as a model for immunodeficiency. Philos Trans R Soc Lond B Biol Sci 1993;342:57–58. [PubMed: 7904347]
- Ramarathinam L, Niesel DW, Klimpel GR. Salmonella typhimurium induces IFN-gamma production in murine splenocytes. Role of natural killer cells and macrophages. J Immunol 1993;150:3973–3981. [PubMed: 8473744]
- Reddy GR, Streck CP. Variability in the 28-kDa surface antigen protein multigene locus of isolates of the emerging disease agent *Ehrlichia chaffeensis* suggests that it plays a role in immune evasion. Molecular Cell Biology Research Communications 1999;1:167–175. [PubMed: 10425222]
- Reddy GR, Sulsona CR, Barbet AF, Mahan SM, Burridge MJ, Alleman AR. Molecular characterization of a 28 kDa surface antigen gene family of the tribe *Ehrlichiae*. Biochem Biophys Res Commun 1998;247:636–643. [PubMed: 9647746]
- Rikihisa Y. The tribe *Ehrlichiae* and ehrlichial diseases. Clin Microbiol Rev 1991;4:286–308. [PubMed: 1889044]
- Rikihisa Y, Ewing SA, Fox JC. Western immunoblot analysis of *Ehrlichia chaffeensis*, *E. canis*, or *E. ewingii* infections in dogs and humans. J Clin Microbiol 1994;32:2107–2112. [PubMed: 7814533]
- Ristic, M.; Huxsoll, DL. Tribe II. *Ehrlichiae* Philip 1957, 948^{AL}. In: Krieg, NR.; Holt, JG., editors. Bergey's Manual of Systematic Bacteriology. 1. Williams and Wilkins; Baltimore: 1984. p. 704-711.
- Salcedo M, Diehl AD, Olsson-Alheim MY, Sundback J, Van KL, Karre K, Ljunggren HG. Altered expression of Ly49 inhibitory receptors on natural killer cells from MHC class I-deficient mice. J Immunol 1997;158:3174–3180. [PubMed: 9120271]
- Schafer R, Eisenstein TK. Natural killer cells mediate protection induced by a *Salmonella* aroA mutant. Infect Immun 1992;60:791–797. [PubMed: 1541553]
- Schaffner W, Standaert SM. Ehrlichiosis- in pursuit of an emerging infection. N Engl J Med 1996;334:262–263. [PubMed: 8532005]
- Seo G, Cheng C, Tomich J, Ganta RR. Total, membrane, and immunogenic proteomes of macrophage- and tick cell-derived *Ehrlichia chaffeensis* evaluated by LC-MS/MS and MALDI-TOF methods. Infect Immun. 2008In press
- Shibata S, Kawahara M, Rikihisa Y, Fujita H, Watanabe Y, Suto C, Ito T. New *Ehrlichia* species closely related to *Ehrlichia chaffeensis* isolated from *Ixodes ovatus* ticks in Japan. J Clin Microbiol 2000;38:1331–1338. [PubMed: 10747103]
- Shinomiya H, Nakano M. Calcium ionophore A23187 does not stimulate lipopolysaccharide nonresponsive C3H/HeJ peritoneal macrophages to produce interleukin 1. J Immunol 1987;139:2730–2736. [PubMed: 3116092]
- Singu V, Liu H, Cheng C, Ganta RR. *Ehrlichia chaffeensis* expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. Infect Immun 2005;73:79–87. [PubMed: 15618143]
- Singu V, Peddireddi L, Sirigireddy KR, Cheng C, Munderloh UG, Ganta RR. Unique macrophage and tick cell-specific protein expression from the p28/p30 Omp multigene locus in *Ehrlichia* species. Cell Microbiol 2006;8:1475–87. [PubMed: 16922866]
- Sirigireddy KR, Ganta RR. Multiplex detection of *ehrlichia* and *anaplasma* species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. J Mol Diagn 2005;7:308–316. [PubMed: 15858156]

- Skeen MJ, Ziegler HK. Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. *J Immunol* 1995;154:5832–5841. [PubMed: 7538532]
- Skold M, Cardell S. Differential regulation of Ly49 expression on CD4+ and CD4-CD8- (double negative) NK1.1+ T cells. *Eur J Immunol* 2000;30:2488–2496. [PubMed: 11009081]
- Snapper CM, Kehry MR, Castle BE, Mond JJ. Multivalent, but not divalent, antigen receptor cross-linkers synergize with CD40 ligand for induction of Ig synthesis and class switching in normal murine B cells. A redefinition of the TI-2 vs T cell-dependent antigen dichotomy. *J Immunol* 1995;154:1177–1187. [PubMed: 7529792]
- Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 1987;236:944–947. [PubMed: 3107127]
- Standaert SM, Yu T, Scott MA, Childs JE, Paddock CD, Nicholson WL, Singleton JJ, Blaser MJ. Primary isolation of *Ehrlichia chaffeensis* from patients with febrile illnesses: clinical and molecular characteristics. *J Infect Dis* 2000;181:1082–1088. [PubMed: 10720534]
- Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, Vitetta ES. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 1988;334:255–258. [PubMed: 2456466]
- Stevenson HL, Jordan JM, Peerwani Z, Wang HQ, Walker DH, Ismail N. An intradermal environment promotes a protective type-1 response against lethal systemic monocytotropic ehrlichial infection. *Infect Immun* 2006;74:4856–4864. [PubMed: 16861674]
- Suzuki N, Suzuki S, Millar DG, Unno M, Hara H, Calzascia T, Yamasaki S, Yokosuka T, Chen NJ, Elford AR, Suzuki J, Takeuchi A, Mirtsos C, Bouchard D, Ohashi PS, Yeh WC, Saito T. A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. *Science* 2006;311:1927–1932. [PubMed: 16574867]
- Tabara K, Arai S, Kawabuchi T, Itagaki A, Ishihara C, Satoh H, Okabe N, Tsuji M. Molecular Survey of *Babesia microti*, *Ehrlichia Species* and *Candidatus Neoehrlichia mikurensis* in wild rodents from Shimane Prefecture, Japan. *Microbiol Immunol* 2007;51:359–367. [PubMed: 17446675]
- Telford SR, Dawson JE. Persistent infection of C3H/HeJ mice by *Ehrlichia chaffeensis*. *Vet Microbiol* 1996;52:103–112. [PubMed: 8914255]
- Thirumalapura NR, Stevenson HL, Walker DH, Ismail N. Protective heterologous immunity against fatal ehrlichiosis and lack of protection following homologous challenge. *Infect Immun* 2008;76:1920–1930. [PubMed: 18285501]
- Tripp CS, Wolf SF, Unanue ER. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc Natl Acad Sci U S A* 1993;90:3725–3729. [PubMed: 8097322]
- Uilenberg G. Heartwater (*Cowdria ruminantium* infection): current status. *Adv Vet Sci Comp Med* 1983;27:427–480. [PubMed: 6359836]
- Unver A, Perez M, Orellana N, Huang H, Rikihisa Y. Molecular and antigenic comparison of *Ehrlichia canis* isolates from dogs, ticks, and a human in Venezuela. *J Clin Microbiol* 2001;39:2788–2793. [PubMed: 11473993]
- Vogel SN. Lps: another piece in the puzzle. *J Endotoxin Res* 2000;6:295–300. [PubMed: 11521048]
- von Loewenich FD, Scorpio DG, Reischl U, Dumler JS, Bogdan C. Frontline: control of *Anaplasma phagocytophilum*, an obligate intracellular pathogen, in the absence of inducible nitric oxide synthase, phagocyte NADPH oxidase, tumor necrosis factor, Toll-like receptor (TLR)2 and TLR4, or the TLR adaptor molecule MyD88. *Eur J Immunol* 2004;34:1789–1797. [PubMed: 15214027]
- Wherry JC, Schreiber RD, Unanue ER. Regulation of gamma interferon production by natural killer cells in scid mice: roles of tumor necrosis factor and bacterial stimuli. *Infect Immun* 1991;59:1709–1715. [PubMed: 1902195]
- Wingren C, Crowley MP, Degano M, Chien Y, Wilson IA. Crystal structure of a gammadelta T cell receptor ligand T22: a truncated MHC-like fold. *Science* 2000;287:310–314. [PubMed: 10634787]
- Winslow GM, Bitsakis C, Yager E. Susceptibility and resistance to monocytic ehrlichiosis in the mouse. *Ann N Y Acad Sci* 2005;1063:395–402. [PubMed: 16481547]

- Winslow GM, Yager E, Li JS. Mechanisms of humoral immunity during *Ehrlichia chaffeensis* infection. *Ann N Y Acad Sci* 2003;990:435–443. [PubMed: 12860671]
- Winslow GM, Yager E, Shilo K, Collins DN, Chu FK. Infection of the Laboratory Mouse with the Intracellular Pathogen *Ehrlichia chaffeensis*. *Infect Immun* 1998;66:3892–3899. [PubMed: 9673277]
- Winslow GM, Yager E, Shilo K, Volk E, Reilly A, Chu FK. Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. *Infect Immun* 2000;68:2187–2195. [PubMed: 10722619]
- Wu D, Xing GW, Poles MA, Horowitz A, Kinjo Y, Sullivan B, Bodmer-Narkevitch V, Plettenburg O, Kronenberg M, Tsuji M, Ho DD, Wong CH. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc Natl Acad Sci U S A* 2005;102:1351–1356. [PubMed: 15665086]
- Yager E, Bitsakis C, Nandi B, McBride JW, Winslow G. Essential role for humoral immunity during *Ehrlichia* infection in immunocompetent mice. *Infect Immun* 2005;73:8009–8016. [PubMed: 16299294]
- Yamamoto M, McGhee JR, Hagiwara Y, Otake S, Kiyono H. Genetically manipulated bacterial toxin as a new generation mucosal adjuvant. *Scand J Immunol* 2001;53:211–217. [PubMed: 11251876]
- Yu XJ, Zhang XF, McBride JW, Zhang Y, Walker DH. Phylogenetic relationships of *Anaplasma marginale* and '*Ehrlichia platys*' to other *Ehrlichia* species determined by GroEL amino acid sequences. *Int J Syst Evol Microbiol* 2001;51:1143–1146. [PubMed: 11414267]
- Zhang JZ, Guo H, Winslow GM, Yu XJ. Expression of members of the 28-kilodalton major outer membrane protein family of *Ehrlichia chaffeensis* during persistent infection. *Infect Immun* 2004a; 72:4336–4343. [PubMed: 15271888]
- Zhang JZ, Sinha M, Luxon BA, Yu XJ. Survival strategy of obligately intracellular *Ehrlichia chaffeensis*: novel modulation of immune response and host cell cycles. *Infect Immun* 2004b; 72:498–507. [PubMed: 14688131]

Figure 1. Outcome of different *Ehrlichia* species infections in immunocompetent mice
E. chaffeensis is cured, *E. muris* causes a persistent infection and *Ixodes ovatus Ehrlichia* causes fatal infections in immunocompetent, normal mice.

Figure 2. Immune response gene control of *E. chaffeensis* infections

E. chaffeensis is cured in about two weeks by normal, immunocompetent mice. However, MHCII knock out mice are persistently infected. The absence of *CD4* or *Tlr4* genes delays curing of the disease. The absence of T and B cells makes an *E. chaffeensis* infection fatal in SCID or recombinae knock out mice.

Figure 3. CD4⁺ T cells promote host resistance

Mice were injected i.p. every three days with 750 ug of rat anti mouse CD4 antibody (ATCC TIB207) or rat IgG2b isotype control antibody. Injection of antibody was initiated 1 week before i.p. experimental challenge with *Ehrlichia chaffeensis* ($\sim 5 \cdot 10^5$ infected DH82 cells). Mice were sacrificed weekly and infections were determined by sampling spleen RNA for the presences of bacteria.

Figure 4. Expression of host immune response genes after *E. chaffeensis* infection

Mouse macrophage cell line, C2D, was infected with purified *E. chaffeensis*. Cellular RNA was assessed for the presence of bacterial RNA or for the expression of the MHCII *IAβ* *Tlr4* and *c-fos* genes by RT-PCR at various times after infection. Semi quantitative data are presented by comparison to the level of expression of β actin RNA from the same cells.

Figure 5. Tlr4 control of neutrophil inflammation after *E. chaffeensis* infection

C2D macrophages (Tlr4-positive), or freshly harvested bone marrow cells from C57BL/6J mice were adoptively transferred i.p. into C57BL/10ScN (Tlr4 gene deletion mutant mice) 1 week before i.p. experimental challenge with *Ehrlichia chaffeensis* ($\sim 5 \cdot 10^5$ infected DH82 cells). Peritoneal exudates cells were plated into 96-well microtiter plates and were incubated with tetramethyl benzidine (TMB). The number of PMNs was assessed by amount of peroxidase present. Peroxidase concentration was determined spectrophotometrically from standard curves constructed by incubating TMB with different concentrations of horse radish peroxidase. 4 mice per treatment group.

Figure 6. Plasma isotype distribution in mice after *E. chaffeensis* infection

Plasma samples from mice were assessed by quantitative ELISA from 16–92 days after infection. The data are presented by days post-infection relative to the presence (r/r genotype) and absence (s/s genotype) of a functional Slc11a1 gene. Each bar represents median individual IgG concentrations determined from samples analyzed from six mice.

Table 1
Influence of MHCII, *Slc11a1* (*Nramp1*), and *Tlr4* genes on peritoneal macrophage IL-6 secretion after infection with *E. Chaffeensis*

Day ¹	MHCII			Slc11a1			Tlr4		
	+/- (8) ²	-/- (12) ²	Stat. ³	r/r (8) ²	s/s (12) ²	Stat. ³	n/n (10) ²	d/d (10) ²	Stat. ³
3	3,294 (2,274;4,112)	4,872 (791;6,161)	NS	3,811 (2,306;4,102)	5,504 (2,306;4,102)	NS	4,277 (3,720;5,196)	1,808 (652;3,643)	NS
8	3,361 (1,764;7,063)	4,594 (1,080;7,270)	NS	4,454 (2,014;7,364)	3,703 (998;7,170)	NS	6,251 (4,575;7,552)	1,396 (574;2,749)	p<0.02
16	1,191 (502;2,616)	2,515 (506;5,413)	NS	873 (264;1900)	2,517 (748;6,118)	NS	4,673 (2,931;6,363)	532 (211;1,387)	p<0.01
23	1,565 (277;2,887)	868 (488;3,011)	NS	868 (427;2,443)	1,804 (375;3,557)	NS	3,010 (2,802;3,557)	501 (301;647)	p<0.001
30	1,073 (502;2,615)	1,737 (850;3,593)	NS	1,445 (919;1,841)	1,959 (629;3,685)	NS	2,722 (2,097;4,027)	656 (250;1,055)	p<0.001
51	2,458 (635;3,598)	2,287 (829;5,925)	NS	2,050 (644;2,658)	3,960 (821;5,925)	NS	4,936 (2,888;5,924)	792 (350;1,143)	p<0.01

¹ Peritoneal exudate cells were recovered from mice at the indicated times after i.p. infection with *E. chaffeensis* and were cultured *in vitro* for 20 hrs. Supernatants were assayed for IL-6 using a capture ELISA assay.

² Numbers in parenthesis indicates the number of mice included in the analysis for each genotype.

³ Number represent median IL-6 concentration (pg/ml). First and last quartile value presented in parenthesis. Statistical comparison between functional and nonfunctional alleles at each gene using the Mann Whitney test. NS indicates not statistically significant, p>0.1. The analysis was performed as previously reported in (Ganta et al. 2002).

Table 3
Influence of MHCII, *Slc11a1*, and *Tlr4* genes on peritoneal macrophage nitrite secretion after infection with *E. Chaffeensis*

	MHCII			SIcIIaI			Tlr4		
Day ¹	+/ r (8) ²	-/(12) ²	Stat. ³	r/r (8) ²	s/s (12) ²	Stat. ³	n/n (10) ²	d/d (10) ²	Stat. ³
3	0 (0, 5)	0 (0, 23)	NS	0 (0, 20)	0 (0, 0)	NS	10 (0, 29)	0 (0, 0)	$p < 0.06$
8	90 (71, 99)	57 (36, 79)	$p < 0.06$	67 (50, 99)	67 (43, 90)	NS	87 (65, 100)	55 (37, 77)	$p < 0.05$
16	90 (50, 132)	66 (20, 144)	NS	56 (25, 98)	108 (31, 155)	NS	143 (120, 155)	24 (16, 64)	$p < 0.01$
23	37 (22, 66)	41 (10, 107)	NS	23 (9, 41)	72 (21, 120)	NS	105 (55, 120)	15 (7, 24)	$p < 0.01$
30	13 (0, 38)	41 (30, 58)	NS	17 (7, 33)	46 (20, 81)	$p < 0.1$	40 (25, 77)	23 (0, 43)	$p < 0.1$
51	0 (0, 9)	68 (8, 84)	NS	0 (0, 58)	44 (2, 83)	NS	48 (2, 83)	16 (0, 54)	NS
93	0 (0, 8)	54 (0, 112)	NS	0 (0, 12)	28 (0, 111)	NS	32 (0, 92)	0 (0, 7)	NS

¹ Peritoneal exudate cells were recovered from mice at the indicated times after i.p. infection with *E. chaffeensis* and were cultured *in vitro* for 20 hrs. Supernatants were assayed for nitrite using the Griess reagent.

²Number in parenthesis indicates the number of mice included in the analysis for each genotype.

3. Number represent median nitrite concentration (μM). First and last quartile value presented in parenthesis. Statistical comparison between functional and nonfunctional alleles at each gene using the Mann Whitney test. NS indicates not statistically significant, $p > 0.1$, except where indicated. The analysis was performed as previously reported in (Ganta et al. 2002).