Chapter 9
Development of Vaccines for Ehrlichiosis

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1 Introduction

The obligate intracellular bacterium *Ehrlichia chaffeensis* that resides in mononuclear phagocytes is the etiologic agent of human monocytotropic ehrlichiosis (HME). HME is an emerging and often life-threatening zoonotic, tick-transmitted infectious disease in the United States (Paddock and Childs 2003; Thomas et al. 2010). Lack of early diagnosis and treatment of HME are the main factors that lead to severe and fatal disease. *Ehrlichia* also causes diseases in companion animals and domesticated ruminants. *E. chaffeensis* and *E. canis* cause canine ehrlichioses in dogs, whereas *E. ruminantium* causes heartwater in cattle, sheep, and goats. Vaccines are required for these tick-transmitted pathogens, but are hindered by many obstacles that exist in their development. These include knowledge of genetic and antigenic variability, identification of the ehrlichial antigens that stimulate protective immunity or elicit immunopathology, development of animal models that reflect the immune responses of the hosts and understanding molecular host–pathogen interactions involved in immune evasion or that may be blocked by the host immune response. Until recently, several strains of ehrlichiae were observed only in animals. However, recent studies demonstrated that many strains of *Ehrlichia* earlier confined to animals are now also observed to infect humans.

In April 1986, a medical intern scanning the peripheral blood smear of a severely ill man with an unexplained illness observed peculiar intracytoplasmic inclusions in several of the patient's monocytes. The patient-described multiple tick bites sustained approximately 2 weeks earlier during a visit to a rural area in northern Arkansas. The disease was initially diagnosed as Rocky Mountain spotted fever.

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Clinicians and scientists subsequently identified these inclusions as clusters of bacteria belonging to the genus *Ehrlichia* (it was initially called *E. canis*), previously known in the United States solely as veterinary pathogens (Maeda et al. 1987). The sequence comparisons indicated that the human ehrlichiosis agent was a new species most closely related to *E. canis* (98.2%) and more distantly related to other *Ehrlichia* spp. Anderson et al. (1991) proposed that this species be named *Ehrlichia chaffeensis* with the Arkansas strain as the type strain. Thus *E. chaffeensis* was the first *Ehrlichia* described as a human pathogen in 1986 (Paddock and Childs 2003). Later, *E. ewingii* (Buller et al. 1999) and *E. muris*-like agents (Pritt et al. 2011), pathogens associated with animals, were observed to infect humans. As yet there are no commercially available vaccines to protect against ehrlichiosis in humans and animals (Thomas et al. 2011, 2016). There is a lack of interest by commercial entities to develop vaccines for ehrlichiosis as there are several strains of *Ehrlichia*. Hence, there is a need to develop novel vaccines that protect against multiple strains of *Ehrlichia*.

Vaccines are considered as one of the most successful medical intervention against infectious diseases. Vaccines include killed or attenuated organisms or purified products derived from them. One of the drawbacks of killed or attenuated vaccines is the potential side effect of some of the antigenic proteins. This led to the design of recombinant vaccines based on whole antigens. As whole antigenic proteins are not essential in inducing immunity, it led to the emergence of a new branch of vaccine design termed "structural vaccinology" (Dormitzer et al. 2008; Nuccitelli et al. 2011). Structure-based vaccines have the rationale that protective epitopes are enough to induce immune responses and provide protection against pathogens (Koide et al. 2005). Structure-based peptide antigens induce antibodies which recognize the denatured form of a protein from which their sequences are derived (Rowlands 1992). It is difficult to predict the strategy to develop an efficacious vaccine; hence, multiple strategies have to be tested to develop an efficacious vaccine for ehrlichiosis.

2 Antigenic Proteins of *Ehrlichia*

The *Ehrlichia* species that are associated with human or veterinary diseases include: *E. chaffeensis*, *E. ewingii*, *E. canis*, *E. ruminantium*, *Ixodes ovatus* ehrlichia, *E. muris*, and *E. muris-like agent*. Development of a murine model of persistent ehrlichiosis has greatly facilitated our understanding of the pathogenesis and mechanisms of host defenses against ehrlichial infections. Mildly virulent *Ehrlichia muris* infection in mice results in persistent infection and mimics *E. chaffeensis* infection in its natural host, white-tailed deer (Olano et al. 2004). Murine models of systemic infection associated with the mildly virulent *E. muris* or the highly virulent IOE (*Ixodes ovatus Ehrlichia*) have provided knowledge of immunological mechanisms involved in host defenses against ehrlichial infection (Sotomayor et al. 2001; Bitsaktsis et al. 2004; Olano et al. 2004). Protective immunity against ehrlichiae involves both humoral and cell-mediated immunity.

The ehrlichial antigenic proteins are selected for vaccines based on reactivity of the proteins with antibodies from sera of infected animals. This is based on the rationale that immunoreactive proteins induce antibodies which could protect against bacterial pathogens. The major immunoreactive proteins of *E. chaffeensis* include 200-, 120-, 88-, 55-, 47-, 40-, 28-, 23-, and 19-kDa proteins (Luo et al. 2008). Analysis of immunoreactive antigens for peak intensity and relative quantity identified major immunoreactive *E. canis* antigens recognized early in the infection as the 19-, 37-, 75-, and 140-kDa proteins. Later in infection, additional major immunoreactive *E. canis* proteins were identified, including the 28-, 47-, and 95-kDa proteins (McBride et al. 2003).

The major antigenic proteins of *Ehrlichia* are outer membrane proteins (OMP-1/P28) encoded by a multi-gene family, gp19, p29, gp36, gp140, gp200, ferric ion-binding protein (Fbp), disulfide bond formation (Dsb) protein, GroEL (*Ehrlichia* Hsp60), MAP2, VLPT, ankyrin repeat proteins, and tandem repeat proteins (TRP) (Alleman et al. 2001; Cárdenas et al. 2007; Luo et al. 2008; Thomas et al. 2011; Thirumalapura et al. 2013). Ortholog tandem repeat proteins of *E. chaffeensis* and *E. canis*, TRP120/TRP140, TRP75/TRP95, TRP47/TRP36, and TRP32/TRP19 contain major antibody epitopes in the tandem repeat regions (Luo et al. 2008, 2009; Luo and McBride 2012; McBride et al. 2011). Ehrlichial TRPs are secreted, serine/threonine-rich, and acidic, which results in higher electrophoretic mobility than their predicted molecular masses (Wakeel et al. 2009). The TRPs contain varying numbers of tandem repeats in different ehrlichial species and strains. Host cell proteins that are targeted by TRPs include proteins involved in signaling, vesicle trafficking, and transcriptional regulation. The interactions between TRPs and host targets cause the redistribution of some host proteins to ehrlichial morula or cytoplasm adjacent to the morulae in *E. chaffeensis*-infected cells, further indicating the profound effects of TRPs on host cell protein recruitment (Wakeel et al. 2009; Luo et al. 2011; Luo and McBride 2012).

Ankyrin repeat protein orthologs (p200) are large 200-kDa proteins that have been characterized in *E. chaffeensis* and *E. canis* (Zhu et al. 2009). The p200 target genes for tumor necrosis factor alpha, Stat1, and CD48 and these are strongly upregulated during ehrlichial infection.

The outer membrane proteins (OMPs) of *Ehrlichia* play a crucial role in virulence and pathogenesis. These antigenic proteins are immunoreactive and aids in vaccine development (Moumène et al. 2015). The chaperone protein GroEL (Hsp60) is found on the outer membrane and plays a role in the folding of a large number of proteins; they are also involved in bacterial adhesion (Kusukawa et al. 1989).

The strongly acidic 19-kDa major immunoreactive glycoprotein (gp19) elicits an early *Ehrlichia*-specific antibody response in infected dogs. *E. canis* gp19 has substantial carboxyl-terminal amino acid homology (59 %) with *E. chaffeensis* VLPT and the same chromosomal location; however, the *E. chaffeensis* VLPT gene (594 bp) has tandem repeats that are not present in the *E. canis* gp19 gene (414 bp). *E. canis* gp19 composition consists of five predominant amino acids, cysteine, glutamate, tyrosine, serine, and threonine, concentrated in the STE-rich patch and a carboxyl-terminal domain predominated by cysteine and tyrosine (55 %).

The amino-terminal STE-rich patch contained a major species-specific antibody epitope strongly recognized by serum from an *E. canis*-infected dog. The gp19 protein is present on reticulate and dense-cored cells and is localized predominantly in the cytoplasm of ehrlichiae; it is also found extracellularly in the fibrillar matrix and associated with the morula membrane (McBride et al. 2007).

All members of *Rickettsiales* have limited biosynthetic capabilities due to the loss of genes required by free-living bacteria during reductive genome evolution (Dunning Hotopp et al. 2006). These bacteria, therefore, cannot survive extracellularly and are obliged to import most nutrients and metabolic products from their host cells. In order for small hydrophilic compounds, such as sugars, amino acids, or ions to pass through, the outer membrane of Gram-negative bacteria have β-barrel proteins called porins that function as passive diffusion channels (Kumagai et al. 2008). The immunodominant P28/OMP-1 family of proteins is the most abundant outer membrane proteins in *E. chaffeensis* (Ohashi et al. 1998). Different alleles from this multigene family are expressed in different host cell types. P28 family members are the most studied *E. chaffeensis* outer membrane proteins (OMPs). They have multiple predicted transmembrane β strands and are encoded by an antigenically variant multigene family composed of 22 paralogous genes clustered in a 27-kb gene locus of the *E. chaffeensis* genome. All 21 *p28* genes of *E. muris* are transcriptionally active in vivo on day 9 post-infection in mice (Crocquet-Valdes et al. 2011). Differential expression of the p28 family of outer membrane genes (OMP-1) occurs, with OMP-1b (p28-14) upregulated in tick cells, and p28-19, an integral membrane protein with porin activity that is upregulated in mammalian cells (Kuriakose et al. 2013).

The outer membrane protein of *E. muris*, P29 is an ortholog of *E. chaffeensis* TRP47 and *E. canis* TRP36. Unlike *E. chaffeensis* TRP47 and *E. canis* TRP36, orthologs of *E. muris* (P29) and *E. muris*-like agent (EMLA) do not contain tandem repeats (Thirumalapura et al. 2013).

E. chaffeensis p120 is an outer membrane protein that is preferentially expressed on the dense-core ultrastructural form of *E. chaffeensis* but not on the reticular cell (Popov et al. 2000). The p120 is an adhesin of *E. chaffeensis* (Popov et al. 2000; Yu et al. 2000). p120 is expressed on the surface of the microorganism and free in the morula space; however, the role of this protein in pathobiology or in eliciting a protective immune response is unknown (Popov et al. 2000). *E. chaffeensis* p120 has two to five nearly identical serine-rich 80-amino-acid TRs; similarly, orthologous *E. canis* p140 contains 12 or 14 nearly identical serine-rich 36-amino-acid TRs. The TR regions of the p120 and p140 proteins are immunoreactive; however, the specific molecular immunodeterminant(s) is not defined (Luo et al. 2009). The glycoprotein genes of *Ehrlichia chaffeensis* (1644 bp) and *Ehrlichia canis* (2064 bp) encode proteins of 548–688 amino acids with predicted molecular masses of only 61 and 73 kDa but with electrophoretic mobilities of 120 kDa (P120) and 140 kDa (P140), respectively. The 120-kDa protein gene of *E. chaffeensis* contains four identical 240-bp tandem repeat units, and the 140-kDa protein gene of *E. canis* has 14 nearly identical, tandemly arranged 108-bp repeat units. Antibodies against the recombi-

nant P120 and P140 proteins reacted with *E. chaffeensis* P120 and *E. canis* P140, respectively. Carbohydrate was detected on the *E. chaffeensis* and *E. canis* recombinant proteins, including the two-repeat polypeptide region of *E. chaffeensis* P120. A carbohydrate compositional analysis identified glucose, galactose, and xylose on the recombinant proteins. The presence of only one site for N-linked (Asn-Xaa-Ser/Thr) glycosylation, a lack of effect of *N*-glycosidase F, the presence of 70 and 126 Ser/Thr glycosylation sites in the repeat regions of P120 and P140, respectively, and a high molar ratio of carbohydrate to protein suggest that the glycans may be O linked (McBride et al. 2000).

gp200 is the largest major immunoreactive ehrlichial protein ortholog of *E. canis* and *E. chaffeensis* (Nethery et al. 2007). The native and recombinant *E. chaffeensis* and *E. canis* gp200 orthologs exhibit molecular masses larger than those predicted by their amino acid sequences but lack serine-rich tandem repeats present in other ehrlichial proteins (McBride et al. 2003). The gp200 is a secreted nuclear translocated ankyrin repeat-containing protein that numerous ankyrin repeats that may mediate protein–protein interactions. gp200 has five major species-specific epitopes that are primarily located in terminal acidic domains (Nethery et al. 2007). The protein has been shown to elicit strong antibody responses in the acute phase of the infection (Zhang et al. 2008). Though the function of gp200 is unknown, the protein is translocated to the nucleus of infected monocytes. gp200 exhibits homology with *Anaplasma phagocytophilum* AnkA, which is a type IV secretion substrate and is phosphorylated by host Abl-1 and Src tyrosine kinases (Caturegli et al. 2000; Lin et al. 2007). Initially, p43 was thought to be an antigenic protein of *E. canis* (McBride et al. 2001). Later studies demonstrated that *E. canis* p43 represents the N-terminal portion of the largest immunoreactive protein described in *Ehrlichia* spp. with a predicted molecular mass of 153 kDa (McBride et al. 2003). A native *E. canis* protein with a molecular mass of 200 kDa reacted with antisera produced against the N-terminal region (p43) of the p153, suggesting that the native protein was post-translationally modified. Similarly, recombinant constructs of *E. chaffeensis* p156 migrated larger than predicted (approximately 200 kDa), and carbohydrate was detected on the recombinant proteins. The chromosomal location, amino acid homology, and biophysical properties support the conclusion that the p153 and p156 glycoproteins (designated gp200s) are species-specific immunoreactive orthologs.

3 The Importance of Cellular and Humoral Immunity in Bacterial Clearance

Ehrlichia cause persistent infection in their natural hosts (e.g., *E. ruminantium* in certain ruminants and *E. chaffeensis* in white-tailed deer); whereas, infection of some accidental hosts results in a severe toxic shock-like illness (e.g., *E. chaffeensis* in humans and *Ixodes ovatus* ehrlichia [IOE] in experimentally inoculated mice)

(Feng and Walker 2004). Studies of the immune response to *E. chaffeensis*, *E. ruminantium*, and *E. canis* as well as the host response to *E. muris* and IOE have all contributed to the overall understanding of the cell-mediated and humoral host responses to *Ehrlichia* spp. (Thomas 2016).

The importance of antibodies in the control of *E. canis* was demonstrated initially by Lewis and colleagues in 1978 (Lewis et al. 1978; Lewis and Ristic 1978). The authors demonstrated that specific antibodies could inhibit the growth of *E. canis* in vitro. The importance of humoral immunity in ehrlichiosis was demonstrated when passive transfer of antibodies could prevent fatal disease during *Ehrlichia chaffeensis* infection of immunodeficient SCID mice (Winslow et al. 2000). Further studies have concluded that antibodies against specific p28 linear epitopes located in a hypervariable region mediate this protection (Li et al. 2001, 2002). The antibody-mediated bacterial clearance, at least in part, by opsonizing bacteria released from infected host cells (Li and Winslow 2003). These findings demonstrated a possible therapeutic role for antibodies during ehrlichial infections (Yager et al. 2005).

There are well documented studies on the importance of cell-mediated involvement in protective immunity. MyD88-dependent signaling is required for controlling ehrlichial infection by playing an essential role in the immediate activation of the innate immune system and inflammatory cytokine production, as well as in the activation of the adaptive immune system at a later stage by providing for optimal Th1 immune responses (Koh et al. 2010). Numerous studies with multiple *Ehrlichia* spp. indicate that IFN-γ is an essential mediator of protection (Totté et al. 1993, 1994). Moreover, $CD4^+$ and $CD8^+$ T cells both contribute to IFN-γ production (Esteves et al. 2004). Notably, similar conclusions regarding the importance of MHC class I, $CD4^+$, and $CD8^+$ T cells, and the synergistic roles of IFN-γ and TNF-α have been reported in mice infected with *E. muris* (Feng and Walker 2004). An important role for $CD4^+$ T cells in immunity to *E. ruminantium* and IOE has been suggested (Totté et al. 1998; Bitsaktsis et al. 2004). Similarly, mice lacking functional MHC class II genes are unable to clear *E. chaffeensis* infection, suggesting that $CD4^+$ T cells are essential for ehrlichial clearance (Ganta et al. 2002, 2004). The route of administration of *Ehrlichia* influences cellular immunity. Stevenson et al. (2006) demonstrated that the intradermal environment (natural route of inoculation) appears to promote the induction of protective type-1 responses characterized by increased $CD4^+$ and $CD8^+$ T cells and IFN-γ producing $CD4^+$ T cells. However, B cells are also involved in immune responses against the pathogenic *Ehrlichia*.

Immunity to the highly pathogenic IOE revealed that B cells are essential for protection in immunocompetent mice following a low-dose sublethal infection (Yager et al. 2005). However, low-dose IOE-infected wild-type mice generated relatively poor antibody responses and were not protected from a subsequent fatal high-dose IOE challenge infection. In contrast, infection with the low pathogenic *E. muris* was shown to generate effective immunity to IOE challenge. Protection against high-dose IOE was mediated by B cells and antibodies and can be generated in the absence of CD4 T-cell-mediated help (Bitsaktsis et al. 2004). The studies

demonstrated the importance of antibodies in providing protection against *Ehrlichia* infection. Overall, studies demonstrated that both the host humoral and cell-mediated immunity is essential in the control of *Ehrlichia* bacteria during infection.

4 Development of Vaccines for Ehrlichiosis

Though several antigenic proteins of *Ehrlichia* are defined and characterized, there are only very few vaccines that have been generated based on these antigenic proteins. Vaccines that are reported for ehrlichiosis are based on the antigenic proteins *Ehrlichia* Hsp60 (GroEL), P28-9, P28-12, P28-19, and P29. There are no vaccines based on *Ehrlichia* TRPs; however, it has been reported that passive transfer of antibodies against *E. chaffeensis* TRPs provides protection against challenge in mice (Kuriakose et al. 2012). As yet there are no effective vaccines available commercially, either for humans or animals, and all of them are in the preclinical stages of development.

4.1 Attenuated Strain of E. ruminantium *as Vaccines*

Heartwater, the tick-borne disease of ruminants, is caused by the intracellular *Ehrlichia ruminantium*. Current immunization procedure involves infecting animals with cryopreserved sheep blood containing virulent *E. ruminantium* organisms, followed by treatment with tetracyclines when fever develops. Attenuated strain of *E. ruminantium* is also used as an immunogen. The virulent Welgevonden strain of *E. ruminantium* was attenuated by continuous propagation of the organisms in DH82 cell line, followed by re-adaptation to grow in a bovine endothelial cell line, BA 886. Sheep and goats inoculated with the attenuated organisms followed by lethal needle challenge with the virulent homologous stock were protected (Zweygarth et al. 2005). A study in Burkina Faso demonstrated that immunization of sheep with the Gardel strain of *E. ruminantium* was more efficacious compared to other strains in providing protection against heartwater (Adakal et al. 2010).

4.2 The Outer Membrane Protein P28 as a Vaccine Candidate

OMPs are immunodominant B-cell antigens and that passive transfer of anti-OMP antibodies can protect mice from fatal ehrlichial infection. Nandi et al. (2007) demonstrated that recombinant P28-19 (OMP 19) of IOE could elicit strong humoral and cellular responses in mice and they induced significant protection against lethal challenge.

Use of DNA vaccines is another strategy to provide protection against infectious diseases. A naked-DNA vaccine based on *p28* was found to protect mice against challenge with a lethal dose of *Ehrlichia ruminantium* (Nyika et al. 1998). Vaccination strategies using pathogen DNA priming followed by administration of homologous recombinant proteins have demonstrated enhanced immune responses compared with vaccines using DNA vaccination alone. Our group had recently used a DNA-prime/protein boost vaccination strategy to control *Ehrlichia* infection (Crocquet-Valdes et al. 2011). In the DNA-prime/protein boost vaccination strategy, the initial immunization is with DNA and the final immunization with the corresponding protein. C57BL/6 mice were immunized with recombinant DNA plasmids carrying the *p28-9*, *p28-12*, *p28-14*, and *p28-19* genes or a mixture of all four in combination with IL-12 DNA on days 0 and 28 followed by two homologous recombinant protein booster immunizations with P28-9, P28-12, P28-19, and the P28 mixture days 56 and 84. IL-12 is known to enhance cellular immunity. Combining DNA vaccine with immune stimulatory molecules delivered as genes (IL-12) significantly enhance Ag-specific immune responses in vivo (Sin et al. 1999). Immunized mice were challenged intraperitoneally with a high dose of *E. muris* 28 days after the last booster immunization, and the bacterial burden in the spleen was determined on day 7 after challenge. A high bacterial burden was detected in the spleens of mice immunized with empty vector/protein (mock-vaccinated mice). In contrast, the spleens of mice vaccinated with P28-9, P28-12, P28-19, and the P28 mixture exhibited significantly reduced bacterial loads on day 7 post-infection than those of the mock-vaccinated control group (Crocquet-Valdes et al. 2011).

As the P28-19 DNA/protein immunization was found to reduce the ehrlichial burden significantly, we further evaluated recombinant P28-19 (rP28-19) as a vaccine candidate. We immunized mice with two doses of rP28-19 (15 days apart) and challenged them with *E. muris* 15 days after the last dose of immunization. We assayed the bacterial burden in the spleens and livers of mice vaccinated with the P28-19 protein and unvaccinated mice harvested on days 7 and 14 after *E. muris* challenge by quantitative real-time PCR. The spleens of rP28-19-vaccinated mice had significantly lower bacterial loads on day 7 than the spleens of unvaccinated mice. Furthermore, on day 14, there were no detectable bacteria by qPCR in either the spleens or livers of the rP28-19-vaccinated mice (Crocquet-Valdes et al. 2011).

Analysis of P28-19-specific antibody responses by ELISA on days 7, 14, and 21 after *E. muris* challenge demonstrated that the IgG response in vaccinated mice challenged with *E. muris* was highest on day 14 compared to day 7 or 21. We further analyzed the isotypes of P28-19-specific antibodies in sera from mice. Both vaccinated and unvaccinated mice challenged with *E. muris* had higher concentrations of P28-19-specific IgM and IgG2b antibodies on day 7 after the challenge. The rP28-19-vaccinated mice challenged with *E. muris* had substantially higher levels of IgG1, IgG2b, and IgG3 antibodies on day 14 after *E. muris* challenge than mice infected with *E. muris* alone. In contrast, mice infected with *E. muris* alone developed substantial levels of P28-19-specific IgG2c, IgG2b, and IgG3 by day 21 post-infection.

To determine cellular immune response to the vaccine, we determined by flow cytometry whether P28-19-specific T cells are induced during *E. muris* infection. Splenocytes from *E. muris*-infected mice were harvested on day 45 post-infection

and stimulated in vitro with the recombinant P28-19 protein for 18 h. Compared to uninfected naïve mice, *E. muris*-infected mice had significantly higher frequencies of P28-19-specific IFN-γ-producing $CD4^+$ Th1 cells in their spleens and they develop into memory cells (Crocquet-Valdes et al. 2011).

4.3 P29 as a Vaccine Candidate

Our group had evaluated the efficacy of recombinant *E. muris* P29 (an ortholog of *E. chaffeensis* TRP47 and *E. canis* TRP36), as a subunit vaccine candidate (Thirumalapura et al. 2013). Immunization with recombinant *E. muris* P29 conferred significant protection against challenge infection. Mice were immunized with recombinant *E. muris* P29 proteins in the presence of adjuvant by the i.p. route, followed by a booster immunization 30 days after primary immunization. The immunized and control mice were challenged with the *E. muris* 60 days after the booster immunization.

Mice immunized with rP29 had significantly reduced bacterial loads in the liver, spleen, lung, and blood after the *E. muris* challenge infection compared to naïve unimmunized control mice. Mice immunized with the rP29 had high levels of rP29-specific IgG antibodies in sera before and after *E. muris* challenge. Examination of immunoglobulin isotypes indicated that mice immunized with rP29 developed high levels of anti-rP29 serum IgG1, IgG2c and IgG2b, and low concentrations of IgG3 antibodies. In contrast, immune sera collected from mice after primary or secondary *E. muris* infection had high concentrations of IgG2c and lower concentration of IgG2b and IgG3 with no detectable concentration of IgG1 directed against the *E. muris*-lysate antigen as determined by ELISA.

To determine cellular immunity, the splenocytes from mice infected with *E. muris* at day 30 post-infection were stimulated in vitro with rP29 or *E. muris* lysate antigen for 24 h. Flow cytometric analysis indicated that *E. muris*-infected mice had significantly higher frequencies of P29-specific CD4+ Th1 cells in the spleen compared to naïve uninfected mice. CD4+ T cells target P29 during *E. muris* infection and differentiate into IFN-γ-producing Th1 effector/memory cells. CD4+ Th1 cells mediate immune responses against intracellular pathogens, and IFN-γ produced by CD4+ Th1 cells could activate macrophages and enhance their microbicidal activity (Thirumalapura et al. 2013).

4.4 Development of Structure-Based Vaccines for Ehrlichiosis

The genetic diversity of microorganisms, coupled with the high degree of sequence variability in antigenic proteins, presents a challenge to developing broadly effective conventional vaccines. The observation that whole protein antigens are not necessarily essential for inducing immunity has led to the

emergence of a new branch of vaccine design termed "structural vaccinology". Structural vaccinology combines elements of structural biology and bioinformatics into a promising new method for the identification of antigenic protein elements of interest based on the protein amino acid sequence and the resulting secondary and tertiary structure. The enabling principle is that the entire antigenic protein is not essential for inducing an immune response as only the epitope sequence per se actually induces the immune response and provide protection against pathogens. Recent studies demonstrated that designing structure-based vaccine candidates with multiple epitopes induce a higher immune response. Structural vaccinology is quickly emerging as a viable strategy for the rational design of vaccine candidates because structure-based vaccines based on epitopes appear to be more specific, inherently safer and easier and less costly to produce (Thomas and Luxon 2013).

Analysis of *E. muris* splenocyte lysate by polyclonal antibodies from *E. muris*-infected mice demonstrated *Ehrlichia* Hsp60 (GroEL) and OMP-1 (P28) as the major antigenic protein of *E. muris* (Thomas et al. 2009). Using eastern blotting, we determined that these proteins are also post-translationally modified. We used the probes concanavalin A, Cholera Toxin B, and nitrophospho–molybdate complex for the detection of glucose, lipid, and phosphate residues, respectively (Thomas et al. 2009).

To determine a protein sequence for potential antigenic epitopes, sequences that are hydrophilic, surface-oriented, and flexible are selected. Most naturally occurring proteins in aqueous solutions have their hydrophilic residues on the protein surface and hydrophobic residues buried in the interior. Three regions of the *E.* muris P28-19 and Hsp60 protein sequence had good hydrophilicity predicted by the Lasergene software (DNAStar, WI, USA). The hydrophilic sequences of both the *Ehrlichia* P28-19 and Hsp60 proteins with no hydrophobic residues were selected. The hydrophilic regions of P28-19 correspond to amino acids 55–75, 91–103, and 124–145 (Fig. 9.1). The hydrophilic regions of *Ehrlichia* Hsp60 correspond to amino acids 43–63, 179–199, and 387–406 (Fig. 9.2). The sequences showed homology to other *Ehrlichia* species. The peptides (underlined) were synthesized and conjugated to KLH and used as probes to detect antibodies to *E. canis* and *E. chaffeensis* or to raise antibodies.

The 3D structure of P28-19 in Fig. 9.1 was modeled using the online I-TASSER (iterative threading assembly refinement) server. The 3D structure of *Ehrlichia* Hsp60 in Fig. 9.2 was modeled using the online Phyre2 server (Thomas et al. 2011). The P28-19 $_{55–75}$ and *Ehrlichia* Hsp60 $_{43–63}$ epitope peptides induced antibodies (Thomas et al. 2010, 2011); hence, we reasoned that they also could provide protection against *Ehrlichia* thereby functioning as potential vaccine candidates.

Mice were immunized i.p., with two doses P28-19 $_{55–75}$ peptide or *Ehrlichia* Hsp60 $_{43–63}$ peptides conjugated to KLH 15 days apart (the first immunization with complete Freund's adjuvant and the second immunization with incomplete Freund's adjuvant). Thirty days after the first immunization, mice were challenged intraperitoneally (i.p.) with a high dose of *E. muris* ($\sim 1 \times 10^4$ bacterial genomes) and

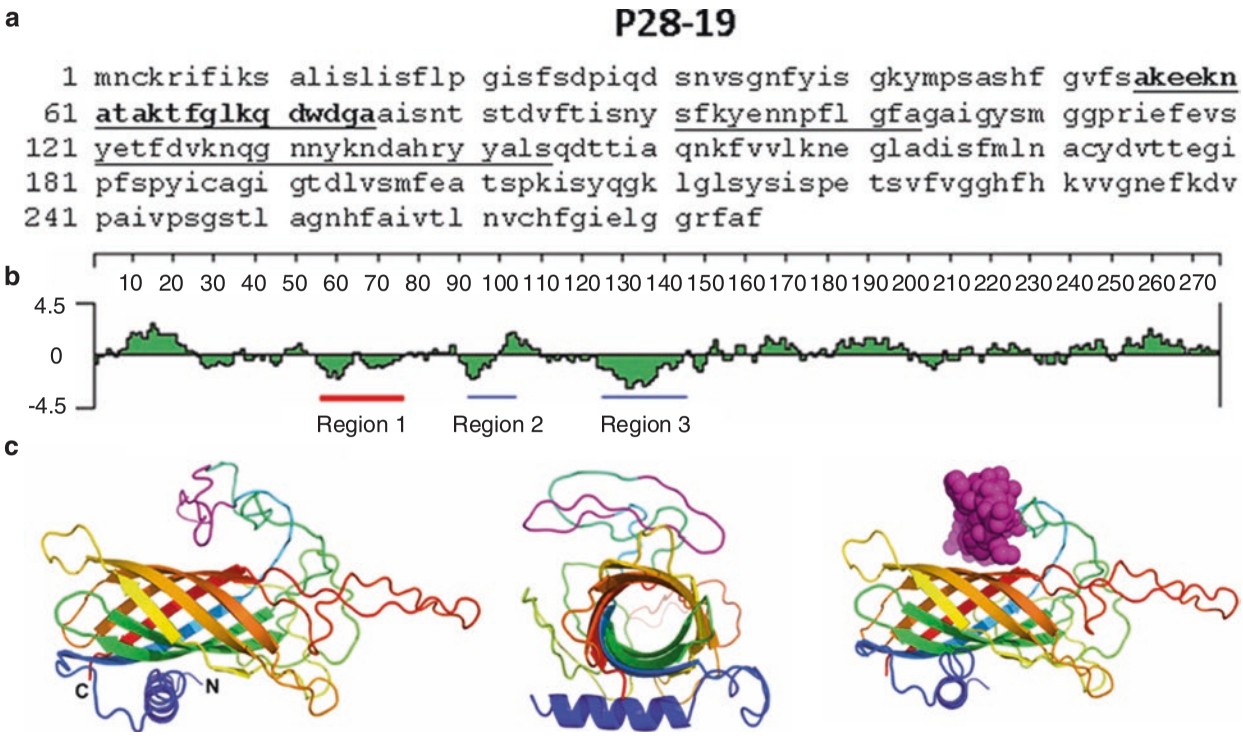

Fig. 9.1 Amino acid sequence of P28-19. (**a**) P28-19 peptides corresponding to the *underlined* predicted hydrophilic sequence were synthesized. The peptide corresponding to the *bold underlined* (55–75) sequence was found to react with antibodies to *Ehrlichia* as well as to induce antibody production. (**b**) Hydrophobicity plot of P28-19. The sequences *underlined* (in *red* and *blue*) were used for synthesizing peptides; however, the best peptide sequence selected is *underlined* in *red*. (**c**) (*Left*) Predicted 3D structure of P28-19 (side view), (*Middle*) predicted 3D structure of P28-19 (basal view), (*Right*) predicted 3D structure of P28-19 with the Van der Waals radii of the heavy atoms highlighting the region of interest (P28-19 $_{55–75}$)

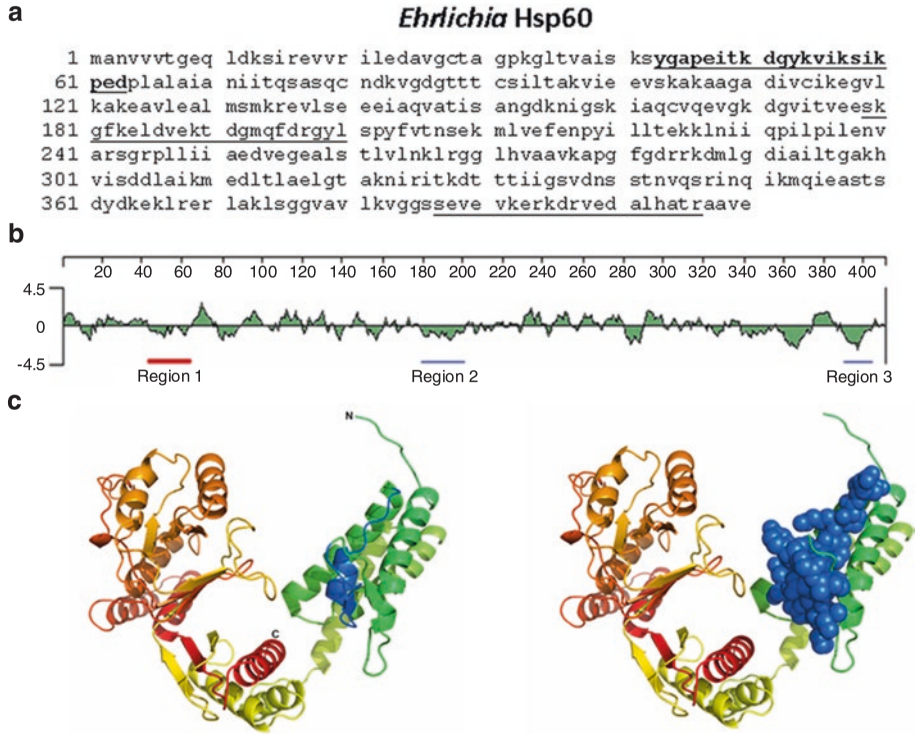

Fig. 9.2 Amino acid sequence of *Ehrlichia* Hsp60. (**a**) Hsp60 peptides corresponding to the *underlined* predicted hydrophilic sequence were synthesized. The peptide corresponding to the *bold underlined* (43–63) sequence was found to react with antibodies to *Ehrlichia* as well as to induce antibody production. (**b**) Hydrophobicity plot of *Ehrlichia* Hsp60. The sequences *underlined* (in *red* and *blue*) were used for synthesizing peptides; however, the best peptide sequence selected is *underlined* in *red*. (**c**) (*Left*) Predicted 3D structure of *Ehrlichia* Hsp60, (*Right*) predicted 3D structure of *Ehrlichia* Hsp60 with the Van der Waals radii of the heavy atoms highlighting the region of interest ($Hsp60_{43–63}$)

observed daily. Controls included unchallenged naïve mice as well as unvaccinated mice injected with *E. muris* alone. Mice were sacrificed on days 7, 14, and 21 after ehrlichial challenge, and spleen and liver were harvested and sera collected. The ehrlichial load in spleen and liver was determined by quantitative RT-PCR. Sera were assayed for determination of antibody titers.

We immunized mice with P28-19 $_{55–75}$ or *Ehrlichia* Hsp60 $_{43–63}$ epitope peptides and challenged 30 days later with *E. muris*. The spleen and liver were collected at different days after bacterial challenge and the bacterial copy number determined by quantitative real-time PCR. We observed lower bacterial load in both spleen and liver on days 7 and 14 after bacterial infection in the vaccinated mice compared to unvaccinated controls (Fig. 9.3). The data demonstrated that P28-19 $_{55–75}$ and *Ehrlichia* Hsp60 $_{43–63}$ peptides functioned as vaccine candidates and provided protection against *Ehrlichia* infection.

Immunization with vaccines stimulates the immune system to produce a robust antibody response that can provide protection against pathogens. To determine the antibody responses against the *Ehrlichia* Hsp60 $_{43–63}$ peptide vaccine, blood was collected from vaccinated mice on days 7 and 14 and performed ELISA. There was a significant difference in the antibody response between unvaccinated and *Ehrlichia* Hsp60 $_{43–63}$ vaccinated mice after challenge with *E. muris*. However, there was no difference between the antibody levels in vaccinated mice between days 7 and 14. The *Ehrlichia* Hsp60 $_{43–63}$-specific antibody levels in infected unvaccinated mice were highest on day 14 compared to day 7. To determine the antibody responses against the P28-19 $_{55–75}$ peptide vaccine, blood was collected from immunized mice on days 7 and 14 and subjected the samples to ELISA. There was a significant difference in the antibody response between unvaccinated and P28-19 $_{55–75}$ vaccinated mice after challenge with *E. muris*. Antibody levels were higher on day 14 compared to day 7 (Fig. 9.4).

As antibody isotype responses can be useful indicators of immune bias during infection, we determined the antibody isotypes after vaccination with the peptide epitopes. The level of antibody isotypes increased by day 14 compared to day 7 after bacterial challenge. The *Ehrlichia* Hsp60 $_{43–63}$-vaccinated mice had higher levels of IgG1, IgG2c, IgG2b, IgG3, and IgM after bacterial challenge compared to unvaccinated mice on day 14. By ELISA, we analyzed the isotypes of the antibodies of P28-19 peptide in vaccinated and unvaccinated mice after challenge with *E. muris* (day 14 post challenge). The P28-19 $_{55–75}$ vaccinated mice challenged with *E. muris* had higher levels of IgG1, IgG2b, IgG3, and IgM compared to unvaccinated mice infected with the pathogen.

Flow cytometry was used to determine whether *Ehrlichia* Hsp60 $_{43–63}$ and P28-19-specific memory T cells are induced during *E. muris* infection. Splenocytes from *E. muris*-infected mice were harvested on day 45 post-infection and stimulated in vitro with the *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$ for 18 h. Compared to uninfected naïve mice, *E. muris*-infected mice had significantly higher frequencies and absolute numbers of *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$-specific IFN-γ-producing CD4+ Th1 cells in their spleen (Fig. 9.5). As both the antigens *Ehrlichia* Hsp60 as well as P28-19 could induce B cells and T cells, our studies conclude that both the antigenic proteins are highly efficient vaccine candidates to protect against *Ehrlichia* infection.

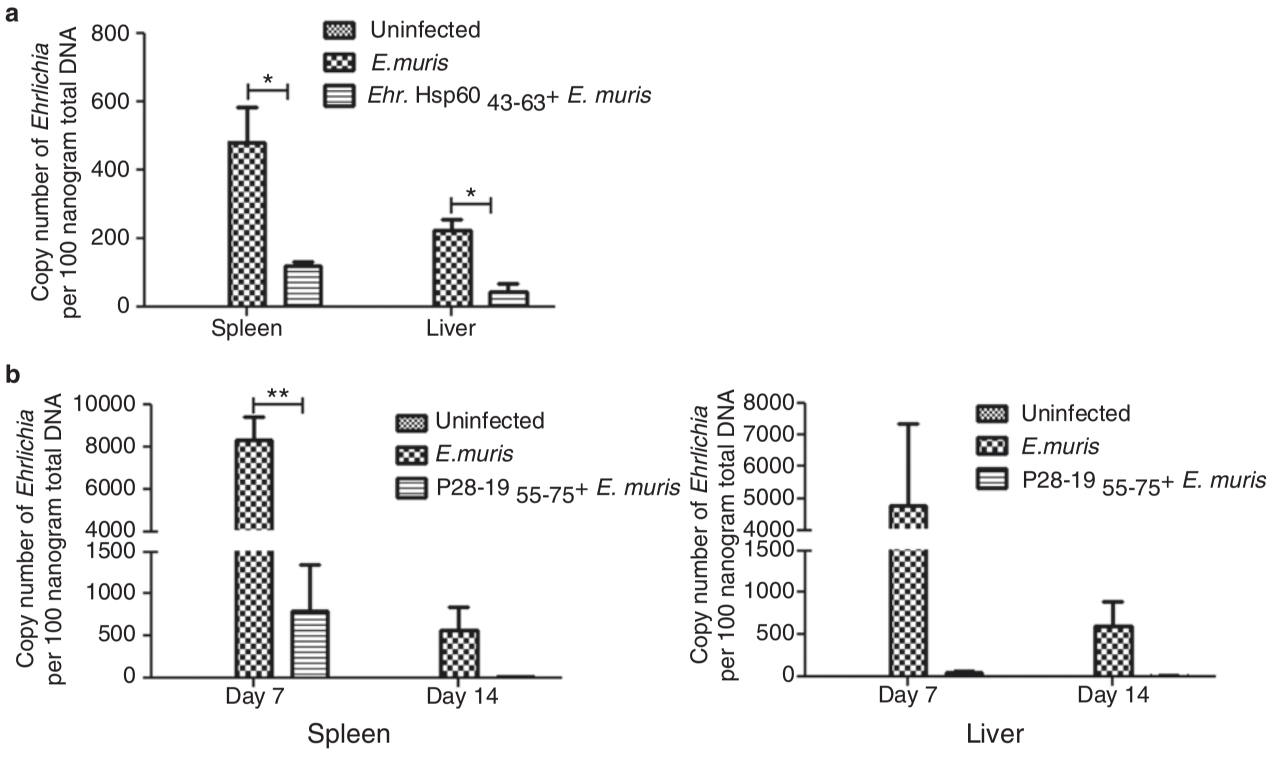

Fig. 9.3 Immunization with *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$ peptides protected mice from *Ehrlichia* infection. (**a**) Mice immunized with *Ehrlichia* Hsp60$_{43–63}$ were protected against *E. muris* challenge as determined by the bacterial load measured by quantitative real-time PCR on day 14 after *E. muris* challenge (*$p<0.05$ as determined by *t* test). (**b**) Mice immunized with P28-19 $_{55–75}$ peptide was protected against *E. muris* challenge as determined by the bacterial load measured by quantitative real-time PCR on days 7 and 14 after *E. muris* challenge (**$p<0.01$ as determined by *t* test)

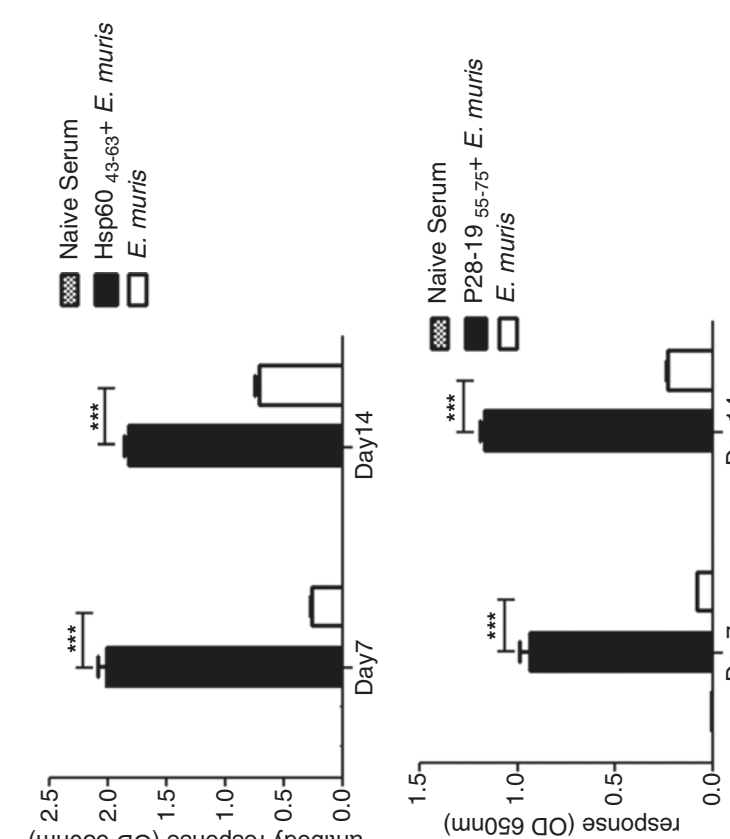

Fig. 9.4 Protection induced by *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$ peptides was associated with induction of *Ehrlichia*-specific IgG antibody. (**a**) *Ehrlichia* Hsp60 $_{43–63}$ vaccinated mice induced higher IgG antibody levels after challenge with *E. muris* compared to unvaccinated *E. muris*-infected mice (***$p<0.001$ as determined by *t* test). (**b**) P28-19 $_{55–75}$ peptide vaccinated mice induced higher IgG antibody levels after *E. muris* challenge compared to unvaccinated *E. muris*-infected mice (***$p<0.001$ as determined by Student *t* test)

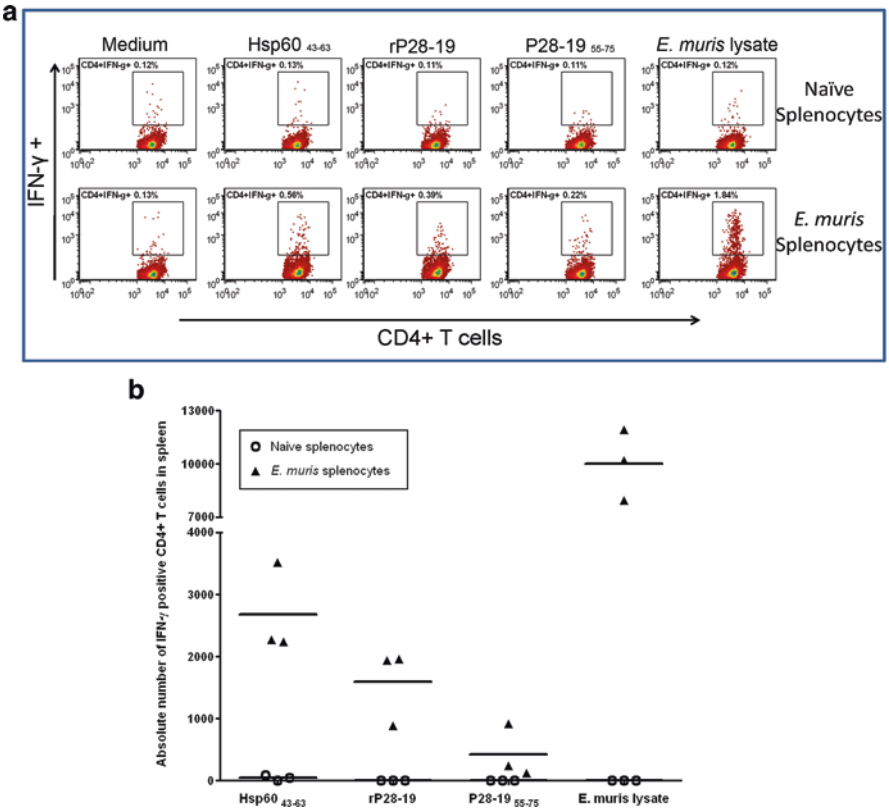

Fig. 9.5 *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$-specific memory CD4+ T cells develop during *E. muris* infection. By flow cytometry, the frequencies and absolute numbers of *Ehrlichia* Hsp60 $_{43–63}$- and P28-19-specific IFN-γ-producing CD4+ T cells in the spleen of mice infected with *E. muris* was determined. (**a**) Mice infected with *E. muris* had higher frequency of *Ehrlichia* Hsp60 $_{43–63}$- and P28-19 $_{55–75}$-specific IFN-γ-producing CD4+ T cells in the spleen on day 45 after infection compared to naïve uninfected mice. Representative dot plots were gated on live cells followed by CD3+ T cells (**b**) Absolute numbers of *E. muris*-specific IFN-γ-producing CD4+ T cells in the spleen of the same mice detected following in vitro stimulation with the *Ehrlichia* Hsp60 $_{43–63}$, P28-19 $_{55–75}$ peptides; rP28-19 and *E. muris* whole cell lysate are shown for comparison. Horizontal bars represent the mean; data are representative of two independent experiments ($n = 3$ animals per group)

5 Conclusion

As yet there are no vaccines for the intracellular Gram-negative bacterium causing ehrlichiosis in animals and humans (Thomas et al. 2016). Though many antigenic proteins of *Ehrlichia* are described, as yet there are very few vaccine candidates even in preclinical stages. Another factor that is preventing a lack of interest by vaccine manufacturers in developing vaccines for *Ehrlichia* is the presence of several ehrlichial strains that infects animals and humans, which may require multiple

vaccines. Development of a recombinant or structure-based vaccine incorporating several antigenic proteins or epitopes may lead to the development of novel vaccines that protect against multiple strains of *Ehrlichia*.

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