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Molecular basis of antibody mediated immunity against *Ehrlichia chaffeensis* involves species-specific linear epitopes in tandem repeat proteins

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Abstract

Humoral immune mechanisms are an important component of protective immunity to *Ehrlichia* species. However, the molecular basis of antibody mediated immunity is not completely defined, and the role of other molecularly characterized major immunoreactive proteins is unknown. In previous studies, we mapped major species-specific continuous epitopes in three surface exposed and secreted tandem repeat proteins (TRP32, TRP47 and TRP120). In this study, we report that protection is provided by antibodies against these molecularly defined TRP epitopes using *in vitro* and *in vivo* models. Protection was demonstrated *in vitro* after prophylactic and therapeutic administration of epitope-specific anti-TRP antibodies, suggesting that the protective mechanisms involve extracellular and intracellular antibody-mediated effects. *In vivo* passive transfer of individual epitope-specific TRP sera significantly reduced the ehrlichial load and splenomegaly, and protected mice against lethal infection. Moreover, the combination of antibodies to all three TRPs provided enhanced reduction in ehrlichial load similar to that of *E. chaffeensis* immune sera. IgG1 was the predominant antibody isotype in the epitope-specific TRP mouse sera. These results demonstrate that antibodies against linear epitopes in TRP32, TRP47 and TRP120 are protective during *E. chaffeensis* infection and involves extracellular and intracellular antibody-mediated mechanisms.

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Keywords

EHRlichia CHAFFEENSIS; TANDEM REPEAT PROTEINS; IMMUNOREACTIVE PROTEINS; EPITOPE; ANTIBODY

1. Introduction

Ehrlichia chaffeensis is an obligately intracellular bacterium that is the causative agent of human monocytotropic ehrlichiosis, an emerging life-threatening zoonosis. Major immunoreactive proteins of *E. chaffeensis* and *E. canis* include a small subset of proteins strongly recognized by antibody [31]. Humoral immunity is essential for protection against active *E. chaffeensis* infection, and antibody specific for a linear epitope located in a hypervariable region of the major outer membrane protein (OMP-1g) is involved [20;44]. Antibody-mediated Fc γ receptor (FcR) dependent phagocytosis has been identified as a mechanism contributing to *E. chaffeensis* clearance during active infection [44]. However, the role of other major immunoreactive proteins in immunity to *E. chaffeensis* is unknown. The majority of the major immunoreactive proteins of *E. chaffeensis* and *E. canis* have been molecularly characterized, and many of these proteins contain tandem repeats. Molecularly defined tandem repeat protein (TRP) orthologs in *E. chaffeensis* and *E. canis* include TRP120/TRP140, TRP75/TRP95, TRP47/TRP36, and TRP32/TRP19 [6;24–26;32;33]. Several TRP ortholog pairs have similar characteristics including the fact that they are secreted, are serine/threonine-rich and highly acidic, and have a major molecularly distinct continuous antibody epitope (~20 amino acids) located within the tandem repeat regions [6;24;26].

The role of TRPs in pathogenesis is emerging, and it is well established that TRP47 and TRP120 are differentially expressed on the surface of dense-cored (infectious) ehrlichiae and TRP32 is extracellularly associated with the morular fibrillar matrix and the morula membrane and is expressed on both dense cored and reticulate cells [4;24;26]. Additionally, *TRP47* and *TRP32* transcripts are hyper-expressed during infection of the macrophage [16]. Recent studies have also demonstrated that *E. chaffeensis* TRPs are secreted effector proteins that interact with many host cell targets [22;40]. Molecular interactions recently reported between host cell proteins and TRP47 and TRP120 include targets associated with distinct cellular functions including signaling, transcriptional regulation, vesicle trafficking and cellular proliferation and differentiation [22;40]. The TRP120 is also involved in the binding and internalization of *E. chaffeensis*, and its expression is regulated by the second messenger cyclic di-GMP and protease HtrA [15;38]. Moreover, the TRP120 is a DNA binding protein with transcriptional activator function that targets host genes associated with biological processes known to be altered during *E. chaffeensis* infection [47]. Molecular host pathogen interactions between TRP32, and host targets associated with TRP47 and TRP120, have also been recently described [23].

Antibodies against a number of intracellular pathogens have been shown to mediate protection [3]. Recently, protection against intracellular bacteria, *Mycobacterium* and *Legionella*, was demonstrated by antibody mediated FcR signaling for lysosomal targeting [11]. During *Legionella* infection, antibodies block the downstream functions of the type 4 secretion system effectors, which include subversion the host cell trafficking system to prevent lysosomal fusion [11]. Prophylactic administration of immune serum or purified antibodies has been shown to reduce the severity and duration of disease caused by *Francisella tularensis* and *Mycobacterium tuberculosis* [9;39], and antibodies are required for protection, but not clearance of, *Salmonella typhimurium* [34]. Antibodies against outer membrane proteins of *Rickettsia conorii* and secreted *Listeria monocytogenes* listeriolysin O

(LLO) are also protective during infection [7;8], and in the case of *Listeria*, neutralization of LLO by antibodies occurs intracellularly [7].

Studies to determine protection provided by major immunoreactive proteins and experimental vaccine development for *Ehrlichia* spp. have focused primarily on the OMP family. The objective of this investigation was to determine protection provided by epitope-specific antibody against the major immunoreactive proteins TRP 32, TRP47 and TRP120 during *E. chaffeensis* infection. In the present study, we demonstrate that antibodies directed at major linear epitopes of three secreted *E. chaffeensis* TRPs reduce ehrlichial load through extracellular and intracellular antibody mediated mechanisms. In addition, we identified IgG1 as the predominant TRP epitope-specific antibody isotype in anti-TRP sera. Furthermore, an *in vitro* model was developed to test the antibody-mediated protection stimulated by the major antibody epitopes of the TRPs that correlated with protection during active infection in the animal model.

2. Materials and methods

2.1. Antisera and antibody purification

Mouse and rabbit (TRP32, TRP47, TRP120 and control) antisera were generated against the synthetic keyhole limpet hemocyanin-conjugated peptides located in the epitope-containing tandem repeat regions by a commercial vendor (Bio-Synthesis, Lewisville, TX). IgG was purified from the rabbit TRP32, TRP47, TRP120, control and pre-immune antisera using Melon Gel IgG purification kit (Thermo Fisher, Rockford, IL) and were used in the *in vitro* experiments. A convalescent-phase anti-*E. chaffeensis* dog serum was derived from an experimentally infected dog (no. 2251).

2.2. *E. chaffeensis* and cell culture

E. chaffeensis (Arkansas strain) was propagated in DH82 (canine macrophages) cells in DMEM as previously described [30]. Cells were prophylactically treated with 0.5µg/ml of purified IgG (anti-TRP or control) for 2 hours prior to infection or therapeutically 2 and 24 hours post-infection in serum-free medium (1µg/ml). Host cell-free *E. chaffeensis* was purified by sonication and differential centrifugation. Briefly, infected DH82 cells were harvested and pelleted (5000 × g) for 15 min. The pellet was resuspended in sterile PBS and sonicated twice for 10 s on ice at 40W using an Ultrasonic Processor (Sonics, Newtown, CT). The suspension was centrifuged for 10 min (1500 × g) to pellet the cell debris. The supernatant containing the host cell-free ehrlichiae was pelleted for 15 min (10,000 × g). Monolayers of DH82 cells in serum-free medium were prophylactically treated with purified IgG (0.5µg/ml) for 2 hours and the cells were infected with host cell-free *E. chaffeensis* in the presence of IgG. The recombinant TRP32 (rTRP32) and the TR domain (first two repeats) of TRP120 (rTRP120) were expressed and purified as previously described [26;46]. In the competition experiments, recombinant proteins (1µg/ml) were added in addition to TRP IgG. The therapeutic effects of the anti-TRP IgG were tested by adding the IgG after internalization of *E. chaffeensis*. Cells were washed three times with sterile phosphate-buffered saline to remove extracellular *E. chaffeensis* and were treated with IgG (0.5µg/ml) 2 and 24 hours post-infection. Three control groups were used including IgG purified from sera generated against a control peptide, pre-immune sera and PBS. Monolayers were infected with same amount of host cell-free *E. chaffeensis* and were harvested 1, 2, and 3 days post infection.

2.3. Western immunoblotting

E. chaffeensis whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and western immunoblotting was performed as previously described [31]. The

dog anti-*E. chaffeensis* serum was diluted 1:100, and mouse anti-TRP sera were diluted 1:500 (Fig. 1). Bound primary antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG (H+L) secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and visualized after incubation with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium) substrate.

2.4. Mice and infection

C57BL/6-*scid* (B6.CB17-*Prkdc^{scid}*) and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed and cared for in the Animal Research Center at the University of Texas Medical Branch in accordance with the Institutional Animal Care and Use Committee guidelines under whose review and approval the experiments were conducted. Six to 12-week-old, sex-matched mice were infected intraperitoneally with approximately 2×10^6 infected DH82 cells (>95% infected) as previously described [43]. Immune serum was produced in C57BL/6 mice as previously described [44]. Mice were intraperitoneally administered mouse anti-TRP (individually or in combination), *E. chaffeensis*-immune, or control sera (0.1 ml) on days 7 and 17 post-infection for the C57BL/6-*scid* mice and on day 3 for the C57BL/6 mice. Mice were sacrificed 21 days (C57BL/6-*scid*) or 5 days (C57BL/6) post-infection and the liver and spleen were collected. The control groups were administered sera generated against a control peptide, pre-immune sera or PBS. Three mice were used per group, and each experiment was repeated at least three times.

2.5. Quantification of *E. chaffeensis*

Total DNA was extracted from infected DH82 cells or homogenized mouse liver with DNeasy Blood and Tissue Kit (Qiagen). Recombinant plasmids were constructed by cloning the *E. chaffeensis dsb* gene using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The size of the plasmid (with the insert) was determined, and the concentration of the double-stranded DNA was calculated with a NanoDrop ND-1000 spectrometer (Wilmington, DE). The plasmid copy number for the standards was calculated using the following formula: $\text{plasmid copy}/\mu\text{l} = [(\text{plasmid concentration g}/\mu\text{l}) / (\text{plasmid length in basepairs} \times 660)] \times 6.022 \times 10^{23}$. *E. chaffeensis* was amplified with primers Dsb-321 forward, 5' TTG CAA AAT GAT GTC TGA AGA TAT GAA ACA 3', and Dsb-671 reverse, 5' GCT GCT CCA CCA ATA AAT GTA TCT CCT A 3' [5]. The thermal cycling protocol consisted of an initial denaturation step of 95°C for 2 min and 40 cycles of 95° for 10 s, 55°C for 30 s, and 65°C for 30 s. The absolute *E. chaffeensis dsb* copy number in the cells (*in vitro*) and liver was determined by real-time qPCR and plotted against the standard curve.

2.6. ELISA

Plates (MaxiSorp; NUNC, Roskilde, Denmark) were coated with synthetic peptides (1 µg/well; 50 µl) suspended in phosphate-buffered saline (pH 7.4) as previously described [24]. Plates were blocked with 100 µl of 10% equine serum (Sigma) in TBST for 1 h at room temperature with agitation and washed. Mouse immune sera or anti-TRP sera (1:100 or 1:6400) diluted in 5% equine serum-TBST were added to each well (50 µl) and incubated at room temperature for 1 h with gentle agitation. The plates were washed four times and incubated in 50 µl of rat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (BD Biosciences, Sparks, MD) (1:1000) for 1 h at room temperature. The plates were washed and incubated in 50 µl of horseradish peroxidase-labeled goat anti-rat IgG antibody (Southern Biotech, Birmingham, AL) (1:2000) for 1 h at room temperature. Substrate (100 µl, TMB Liquid Substrate System, Sigma) was added to each well, and plates were incubated in the dark for 30 min with agitation. Optical density was determined on a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA) at A₆₅₀ and data analyzed by SoftmaxPro v4.0

(Molecular Devices). Optical density (OD) readings represent the mean OD for three wells (\pm standard deviations) after subtracting the OD value of the negative peptide control.

2.7. Statistical Analysis

Data are expressed as means \pm standard deviations (SD), and the statistical significance was determined using a one-way analysis of variance followed by Tukey's multiple comparison tests were performed using Prism 5 software (GraphPad). *P* values of < 0.05 were considered significant.

3. Results

3.1. Antibodies against TRP32, TRP47 and TRP120

Major immunoreactive epitopes of tandem repeat proteins 32, 47, 120 have been previously determined [6;24;26]. Mouse and rabbit antisera against these epitopes were generated and tested to confirm recognition of the ehrlichial proteins (Fig. 1, Table 1).

3.2. Prophylactic protection by TRP32 and TRP120 antibody in vitro

To determine the effect of antibodies prior to ehrlichial entry, DH82 monolayers were treated with purified rabbit anti-TRP32 and anti-TRP120 IgG prior to infection. A lower ehrlichial load was observed in anti-TRP32 and anti-TRP120 IgG treated cells compared to the controls with a significant reduction ($p < 0.05$) on day three (Fig. 2A). However, there was no difference observed with anti-TRP47 IgG. The reduction in bacterial load observed with anti-TRP32 and anti-TRP120 IgG was completely reversed by addition of recombinant TRP32 or TRP120 as a competitor (Fig. 2B). No significant difference in bacterial copy number was observed in the control peptide IgG, pre-immune IgG or PBS treated cells; thus, pre-immune sera or PBS were used in all subsequent experiments.

3.3. Therapeutic protection of TRP32 and TRP120 antibody in vitro

To determine the effect of antibodies post ehrlichial entry, infected DH82 cells were treated with TRP32, TRP120 or control IgG 2 and 24 hrs post-infection. A significant ($p < 0.05$) reduction in bacterial load was observed when TRP120 IgG was added 2 hrs post-infection and TRP32 IgG was added 24 hrs post-infection (Fig. 2C).

3.4. Passive protection of TRP antibodies individually in vivo

To determine the *in vivo* protection of anti-TRP sera, we used a previously developed murine model of *E. chaffeensis* infection [43]. In several initial experiments, no significant differences were observed in control peptide sera, pre-immune sera or PBS-treated mice (data not shown); therefore, in subsequent experiments, pre-immune sera or PBS were used as controls. Immunocompetent C57BL/6 mice develop a transient infection and inflammation but clear ehrlichiae within two weeks and immunocompromised SCID mice, lacking T and B lymphocytes develop persistent infection and disease and become moribund within three weeks after infection [43]. Immunocompetent C57BL/6 mice and susceptible SCID mice were infected with *E. chaffeensis* and were treated with anti-TRP32, TRP47 or TRP120 sera to test their protective capacity. Ehrlichial burden in the liver, one of two principal sites of ehrlichial infection in mice [43], was determined by qPCR. Since *E. chaffeensis*-infected mice exhibit pronounced splenomegaly, the spleen weights of the anti-TRP sera-treated mice were compared to controls.

SCID mice were infected and treated with sera on days 7 and 17 post-infection, and the livers and spleens were harvested 21 days post-infection. A significant ($p < 0.005$) reduction in ehrlichial copy number was observed when TRP32, TRP47 or TRP120 antisera were

administered to SCID mice during the course of the infection (Fig. 3A). Furthermore, anti-TRP antibody treated mice had significantly ($p < 0.05$) lower spleen weights than the controls (Fig. 3B).

To examine the anti-TRP protection in immunocompetent mice, C57BL/6 mice were infected and treated with TRP32, TRP47 or TRP120 antisera three days post infection, and organs were harvested five days post-infection. Similar to the SCID mice, significant reductions in the *E. chaffeensis* burden ($p < 0.005$) and spleen weights ($p < 0.05$) were observed in the immunocompetent mice compared to controls (Fig. 3C and D).

3.5. Passive protection with TRP antibodies and *E. chaffeensis* immune sera

In a previous study, significant protective effect was observed in SCID mice that received immune sera from immunocompetent mice during *E. chaffeensis* infection [44]. To compare the protection mediated by antibodies directed at TRP epitopes compared to the protection previously observed with immune sera, immunocompetent and SCID mice were treated with a combination of anti-TRP32, TRP47 and TRP120 sera or *E. chaffeensis*-immune sera. SCID mice treated with the combination of TRP antisera and immune sera had a significantly ($p < 0.005$) lower ehrlichial burdens compared to the control group (Fig. 4A). Furthermore, the mice that received *E. chaffeensis* immune sera had fewer than 10 ehrlichiae/250 ng of total DNA. The spleen weights of mice that received immune sera were similar to the mice treated with the combination of anti-TRP sera, and both groups' spleen weights were significantly ($p < 0.05$) lower than the control group (Fig. 4B). In the immunocompetent mice, immune sera-treated mice and the mice treated with the combination of TRP antisera had significantly lower ehrlichial loads ($p < 0.005$) than controls. Most notable were the similar bacterial loads and spleen weights observed in mice treated with immune sera and combination of TRP antisera (Fig. 4C and D).

3.6. IgG isotypes in anti-TRP and immune sera

The TRP32, TRP47 and TRP120 epitope-specific IgG isotypes in the *E. chaffeensis*-immune sera were quantified by ELISA (Fig. 5A). Previously described protective OMP-1g (p28-19) epitope was also measured for comparison [20]. In the *E. chaffeensis*-immune sera, all IgG isotypes (IgG1, 2a, 2b and 3) were detected against the three protective TRP epitopes and IgG1 was the predominant isotype. TRP32 has the highest antibody levels of the three TRPs in immune serum obtained from *E. chaffeensis*-infected mice and used in passive transfer experiments. In hyperimmune mouse serum generated against peptides representing TRP epitopes, IgG1 was also the predominant isotype. The concentration the other isotypes (IgG2a, 2b, and 3) were detected in similar concentrations, but lower than IgG1 (Fig. 5B).

4. Discussion

It is well established in that antibodies against the obligately intracellular pathogen, *E. chaffeensis*, are essential for immunity [18;44]. However, during *E. chaffeensis* infection, many proteins elicit strong antibody responses, yet only antibodies against a linear epitope in the hypervariable region of OMP-1g protein primarily of the isotype IgG2a have been demonstrated to provide protection [20;44]. This antibody mediated protection appears to involve Fc receptor-dependent mechanisms [45]. The purpose of this study was to expand our knowledge of immunoprotective components of *Ehrlichia* and have a better understanding of the antibody-mediated immune mechanisms involved. We have previously identified and molecularly characterized a subset of immunodominant ehrlichial proteins that contain tandem repeats and determined that each of these TRPs contains a single major continuous species-specific antibody epitope within the TR region [4;24;26]. In this study, we conclusively demonstrated antibody-mediated protection against *E. chaffeensis* infection

by passively transferring TRP epitope-specific antibody against three *E. chaffeensis* TRPs (TRP32, TRP47 and TRP120).

Humoral immunity to bacteria has been associated with proteins localized on the cellular surface such as OspA of *Borrelia burgdorferi* and PsaA of *Yersinia pestis* [1;17]. In *Anaplasma marginale*, antibodies against the highly immunogenic multifamily major surface proteins (Msps) that are components of the outer membrane provide protection against infection, anemia and high-level bacteremia [36;37]. In addition, the alpha C protein of the group B *Streptococcus* is a surface-expressed TRP that elicits protective antibody-mediated immunity [35]. Monoclonal antibodies to the alpha C protein induce opsonic killing of the bacterium and protect mice from lethal challenge, and altered tandem repeat numbers are associated with immune evasion [14;27;28]. However, unlike the MSPs of *A. marginale*, many of the major immunoreactive proteins of *E. chaffeensis* are secreted TRPs that are involved in molecular interactions with the host cell. Secreted effector proteins of several pathogens have been shown to be immunogenic, such as the recently described *Chlamydia trachomatis* Tarp protein or LLO from *Listeria monocytogenes* [7;42]. Although the *C. trachomatis* Tarp is a secreted TRP effector that is strongly recognized by antibodies in immune patient sera, its role in inducing protective antibodies is unknown [41;42]. In contrast, antibodies against the secreted effector LLO have been demonstrated to provide protection when administered prophylactically [7]. Prophylactic and therapeutic administration of antibodies is also protective against respiratory infection with *F. tularensis* [39].

In this investigation, the effect of *E. chaffeensis* TRPs linear epitope specific antibodies during infection was determined using an *in vitro* model which was validated and supported using immunocompetent and SCID murine models. In the *in vitro* model, anti-TRP120 IgG was protective when administered prophylactically and therapeutically at two hours post-infection, but not at 24 hours post infection. TRP120 is differentially expressed on the infectious dense-cored ehrlichiae and has been previously associated with ehrlichial binding and internalization [15;38]. Moreover, it has recently been identified as a DNA binding protein and interacts with other defined host cell targets [22;47]. The prophylactic efficiency of the anti-TRP120 IgG during the early stages of infection in the *in vitro* model when the antibody is in direct contact with the host cell-free ehrlichiae suggests that the antibody is involved in neutralization or other extracellular antibody mediated mechanism. Recently, antibodies were shown to protect by altering the intracellular trafficking of *Legionella pneumophila* into lysosomal compartments via Fc receptor (FcR) mediated signaling in macrophages [12]. Antibody mediated activation of the FcR blocks the functions of bacterial effector proteins secreted into the host cell cytoplasm which include avoiding fusion to the lysosome, making the host cell nonpermissive for intracellular replication, and this was observed both *in vitro* and *in vivo* [12]. During ehrlichial infection of immunocompetent mice, FcR common γ chain and Fc γ RI have been previously demonstrated to be required for clearance of bacteria [45], consistent with the FcR mediated clearance of *L. pneumophila*.

A reduction in bacterial burden was observed when anti-TRP32 IgG was administered prophylactically and therapeutically at 24 hours post-infection, but not two hours post-infection. TRP32 is expressed on both dense-cored and replicating reticulate cells and is associated with morular fibrils and membrane [26]. TRP32 was recently identified as a secreted ehrlichial effector protein that interacts with several host cell proteins involved in protein synthesis, iron acquisition, immune signaling and transcriptional regulation [23]. The protection mediated by therapeutically administered anti-TRP32 IgG also likely involves extracellular neutralization, since both TRP32 and TRP120 are found on the surface of DC ehrlichiae. However, the therapeutic protection mediated by anti-TRP32 and anti-TRP120

antibodies two and 24 hours post-infection, after ehrlichiae have entered the host cell, suggests that intracellular antibody-mediated immune mechanisms are involved. TRP32, TRP47 and TRP120 are found extracellularly in inclusion matrix and associated with the inclusion membrane [6;26;38]. Additionally, TRP47 and TRP32 are highly upregulated during ehrlichial infection of the macrophage and TRP120 is translocated to the host cell nucleus [16;47]. The subcellular localization of and TRP120 and the molecular interactions between TRP32, TRP47 and TRP120 with host proteins [22;23;40] suggest that the TRPs and associated effector functions may be neutralized by cytoplasmic antibodies that block the effector functions of these proteins at different stages of infection. Antibody directly transfected into cells blocked interactions of type IV secreted effector protein, AnkA, resulting in reduction of the *A. phagocytophilum* burden [21]. Moreover, antibodies against LLO, a secreted effector protein of *L. monocytogenes* provided protection through intracellular mechanisms post-internalization of the bacterium [7]. Additionally, monoclonal antibodies against intracellular oncoproteins have been shown to enter host cells and inhibiting tumor cell growth [10]. Cytosolic IgG receptors such as the recently described TRIM21 could be involved in the intracellular antibody mediated immunity [13]. TRIM21 is expressed on all cell types, binds all IgG isotypes, and mediates intracellular immunity by recruiting antibody-bound antigen for proteasomal degradation [29].

In the *in vivo* mouse model, the three TRP antisera were protective when therapeutically administered individually and in combination to immunocompetent and SCID mice after establishment of infection. In the SCID mice, anti-TRP32 was the most protective of the three TRP antisera. Interestingly, in the immunocompetent mice, when treated with the combination of the three antisera, mice had similar correlates of protection as immune sera treated mice. Similarly, Winslow *et. al.* previously reported that extremely low copy numbers of bacterial DNA was detected in immune sera-treated immunocompetent mice four and seven days post-infection [44]. These results suggest that the TRP epitopes contribute to a significant fraction of antibody mediated protection in the immune sera in the immunocompetent mice in addition to previously characterized outer membrane protein epitopes.

The *in vivo* studies were supportive of the *in vitro* model data for TRP32 and TRP120 antibodies. The difference in protection mediated by TRP47 antibody *in vitro* and *in vivo* is potentially due to differences in antibody titer (as measured by IFA), or suggestive of a mechanistic deficiency that is reconstituted *in vivo*. Higher concentrations of anti-TRP47 antibody were not tested to determine if *in vitro* protection could be achieved, but based our *in vivo* findings, anti-TRP47 antibodies are protective. Results from the *in vitro* model of antibody protection correlated well with the *in vivo* model of protection for the *E. chaffeensis* TRPs, supporting the *in vitro* model as a valid screening alternative for defining immunoprotective proteins. During infection with *A. marginale*, an intracellular bacterium closely related to *E. chaffeensis*, a similar assay was used to demonstrate the inhibition of infection mediated by antibodies against immunogenic major surface proteins (Msp) of *A. marginale* during infection of tick cells [2].

Murine *E. chaffeensis*-specific monoclonal antibodies generated in a previous study were of the IgG2a, 2b and 3 isotypes [20], and protective antibodies identified were directed at the hypervariable region of OMP-1g and were of the IgG2a isotype [18]. IgG2a is capable of activating complement and binding to Fc receptors. Thus, consistent with antibody mediated mechanisms associated with bacterial OMPs and protective ehrlichial OMP IgG2a antibodies have been proposed to opsonize *E. chaffeensis* and involve Fc γ RI-mediated phagocytosis of antibody opsonized bacteria that either escaped from infected host cells or were released during cell lysis [19;45]. In this study, we demonstrated that TRP-specific antibodies provide protection similar to OMP antibodies demonstrating that both TRP and

OMP specific antibodies are important for protection. However, the functional differences in these proteins and the cellular localization (membrane versus secreted) suggest that protective antibodies may confer protection through distinct mechanisms.

In the present study, IgG1 was the predominant isotype detected in the protective TRP-peptide antisera, but other antibody isotypes (IgG2a, 2b, and 3) were also present in substantially lower concentrations. Interestingly, although we detected similar antibody concentrations of all four isotypes in sera from mice infected with *E. chaffeensis*, monoclonal antibodies developed by Winslow *et. al* represented primarily IgG2a and 2b isotypes, and IgG1 monoclonal antibodies were not obtained [18]. The predominance of the IgG1 isotype (which does not activate complement or bind Fc receptors) in the murine epitope-specific anti-TRP antisera, suggests that mechanisms other than complement activation and Fc receptor mediated killing could also be involved. Furthermore, higher concentrations of IgG1 antibody in anti-TRP peptide serum and immune serum (TRP32) from *E. chaffeensis*-infected mice suggest that antibodies to TRPs are more likely to be of the IgG1 isotype. Nevertheless, multiple antibody isotypes are likely to play a role in TRP specific antibody mediated immunity through different mechanisms. Characterization of antibody isotypes directed at TRPs and OMPs during *E. chaffeensis* human infections would also help define that antibody isotypes produced during infection and the antibody mediated mechanisms are associated with anti-TRPs and anti-OMP antibodies.

Our findings support the role of antibodies in host defense during ehrlichial infection. In this study we have demonstrated that antibodies against the linear epitopes of immunodominant TRPs (32, 47 and 120) are protective and appear to involve extracellular and intracellular antibody-mediated mechanisms. These epitopes should be included in development of vaccines and therapies for ehrlichial diseases and further studies need to be done to elucidate the mechanism of anti-TRP antibody-mediated protection during *E. chaffeensis* infection.

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Abbreviations

TRP	tandem repeat protein
OMP	outer membrane protein

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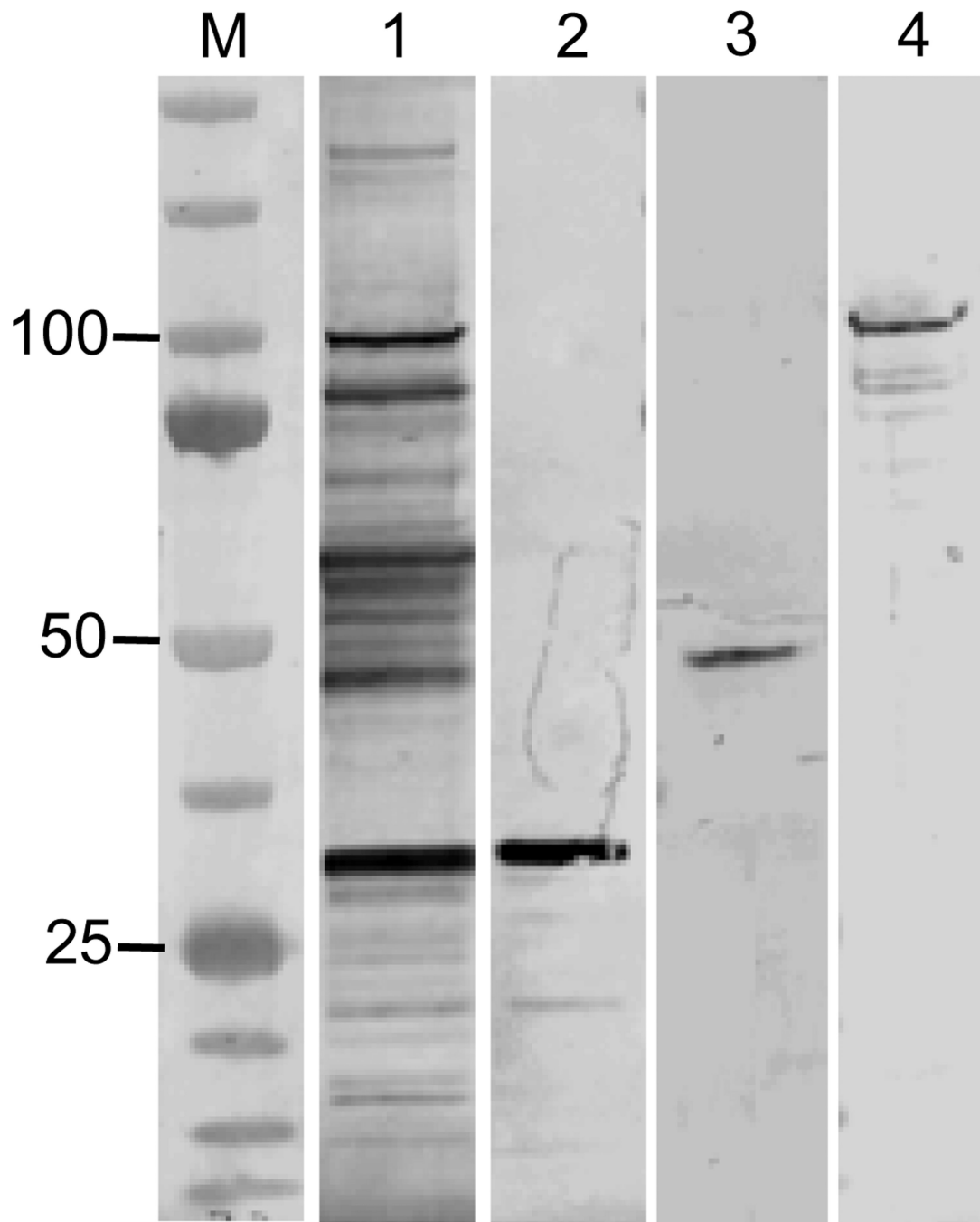
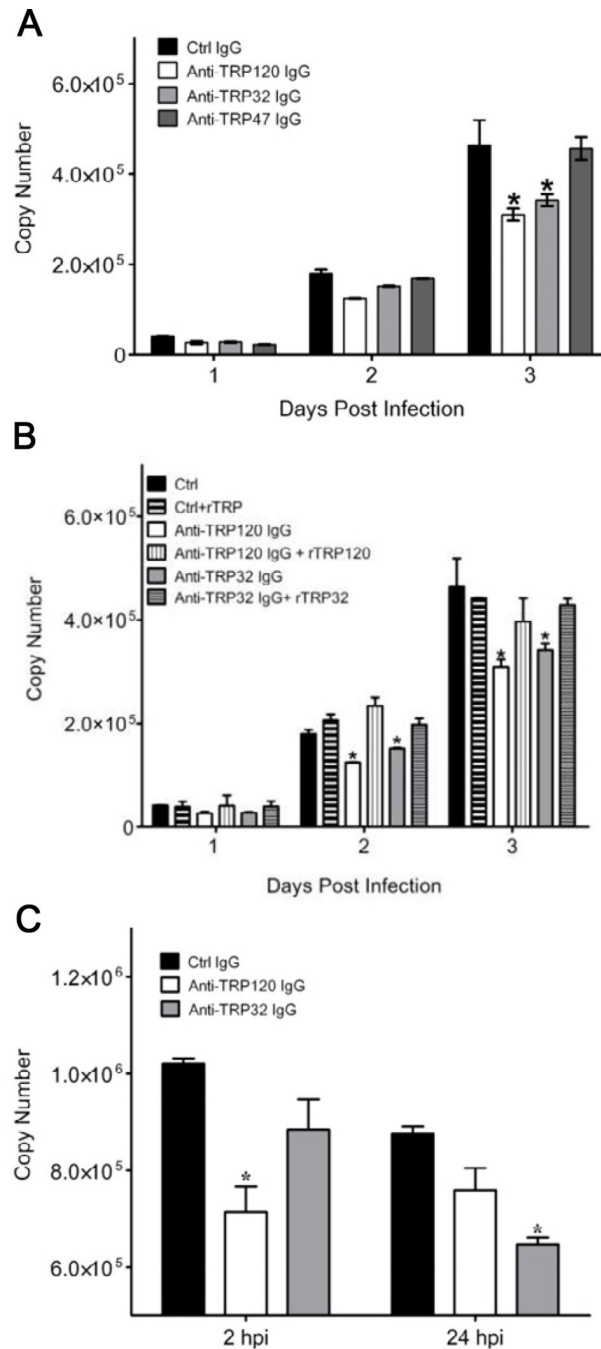
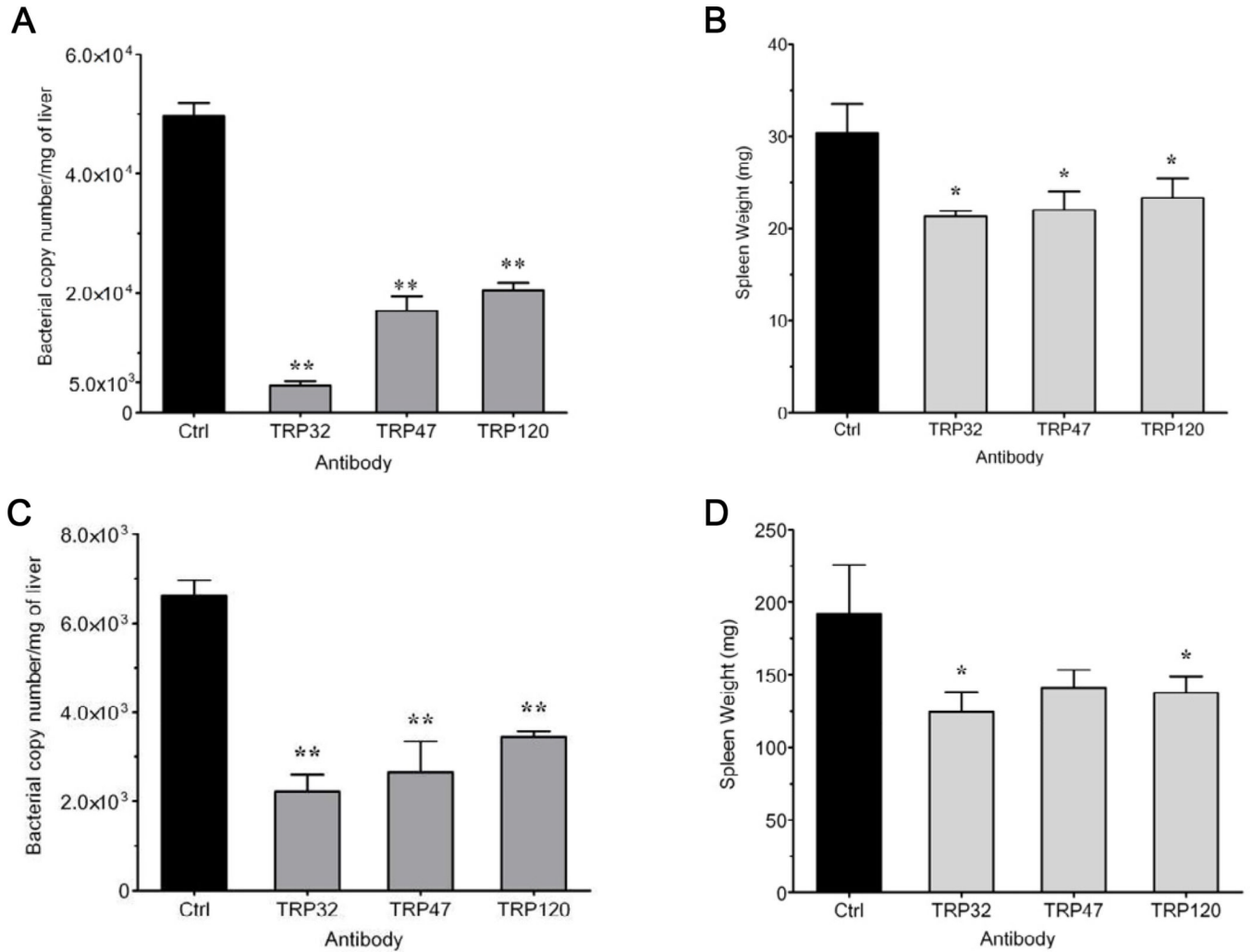


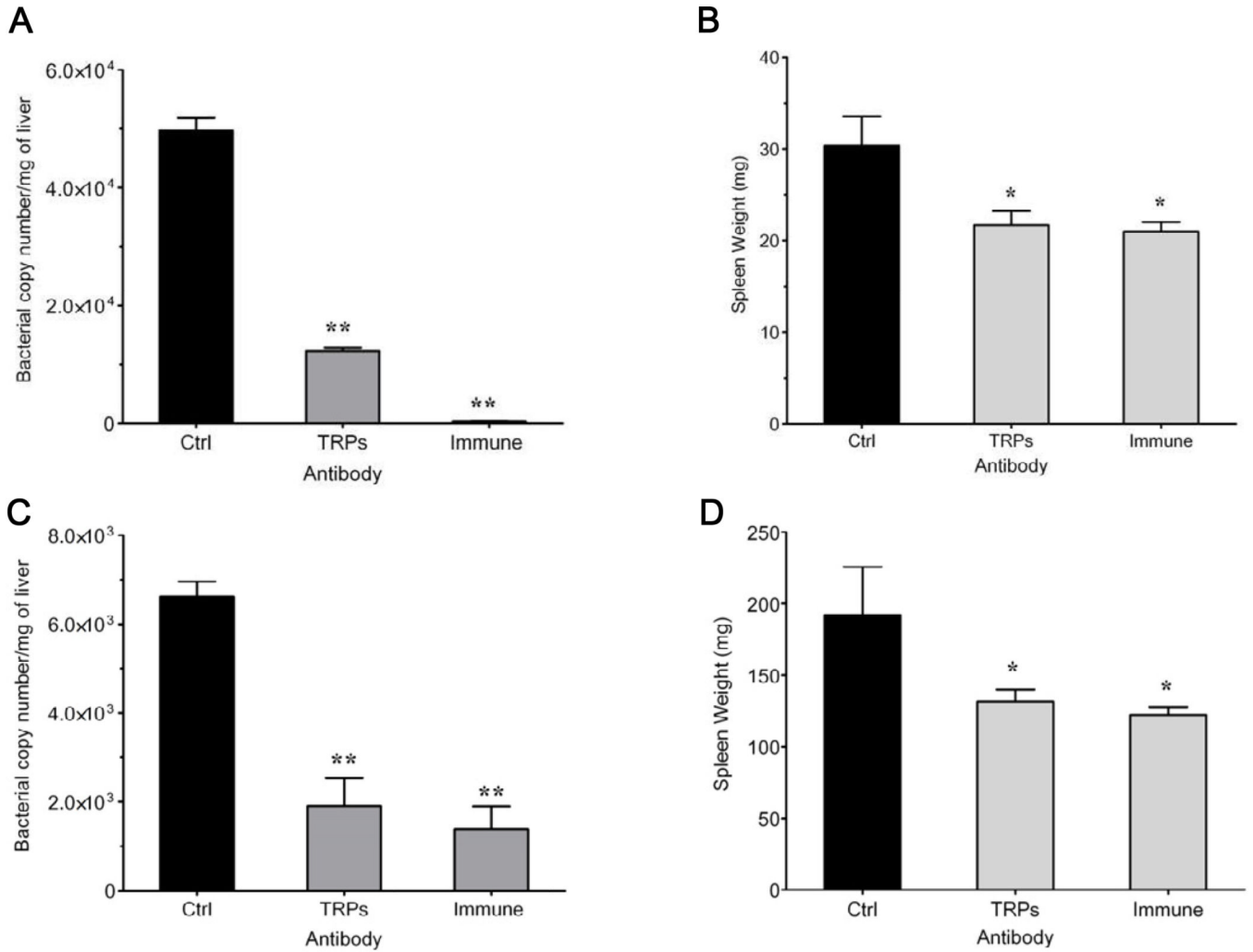
FIG. 1. Identification of immunodominant *E. chaffeensis* TRPs with epitope-specific mouse antisera used for passive protection experiments. Western immunoblot of whole-cell lysate from *E. chaffeensis*-infected DH82 cells probed with anti-*E. chaffeensis* dog (no. 2251) serum (lane 1; positive control) and, mouse anti-TRP32 (lane 2), anti-TRP47 (lane 3) and anti-TRP120 (lane 4) sera. M, molecular mass marker (kilodaltons).

**FIG. 2.**

Prophylactic and therapeutic effect of anti-TRP IgG on *E. chaffeensis* infection *in vitro*. (A) Cells were treated with IgG (anti-TRP or control) 2 hrs prior to infection and ehrlichial loads determined 1, 2, and 3 days post infection by qPCR. (B) Reduction in ehrlichial load in the presence of anti-TRP32 and anti-TRP120 IgG is protein-specific. Cells were incubated with anti-TRP IgG alone or in combination with recombinant protein (rTRP32 and rTRP120) to neutralize the effect of specific antibody for 2 hrs prior to infection and ehrlichial loads determined by qPCR. (C) Infected cells were treated with IgG (anti-TRP or control) 2 and 24 hrs post-infection, and ehrlichial load determined 3 dpi by qPCR. Bar graphs represent means \pm SD, (* $p < 0.05$).

**FIG. 3.**

Reduction of ehrlichial burden in *C57BL/6-scid* and *C57BL/6* mice treated with anti-TRP32, anti-TRP47 or anti-TRP120 sera. Mice (SCID and immunocompetent) were inoculated intraperitoneally with 2×10^6 *E. chaffeensis*-infected DH82 cells on day 0. SCID mice were administered anti-TRP32, anti-TRP47, anti-TRP120 or control sera on days 7, 17, and mice were sacrificed 21 days post-infection. Immunocompetent *C57BL/6* mice were administered anti-TRP32, anti-TRP47, anti-TRP120 or control sera on day 3 and sacrificed 5 days post-infection. *E. chaffeensis dsb* copy number in the liver was determined by qPCR (A and C) and spleen weights (B and D) were compared to controls. Three mice were used per group and data are representative of one of three independent experiments. Graphs represent means \pm SD, (* $p < 0.05$, ** $p < 0.005$).

**FIG. 4.**

Reduction of ehrlichial burden in C57BL/6-*scid* and C57BL/6 mice after treatment with a combination of anti-TRP sera compared to immune sera. Mice were infected intraperitoneally with 2×10^6 *E. chaffeensis* infected DH82 cells on day 0. SCID mice were administered a combination of anti-TRP32, anti-TRP47 and anti-TRP120 sera (TRPs), immune or control sera on days 7, 17, and mice were sacrificed 21 days post-infection. Immunocompetent C57BL/6 mice were administered a combination of anti-TRP32, anti-TRP47 and anti-TRP120 sera (TRPs), immune or control sera on day 3 and sacrificed 5 days post-infection. *E. chaffeensis dsb* copy number in the liver was determined by quantitative real-time PCR (A and C) and spleen weights (B and D) were compared to control mice. Three mice were used per group, and data are representative of one of three independent experiments. Graphs represent means \pm SD, (* $p < 0.05$, ** $p < 0.005$).

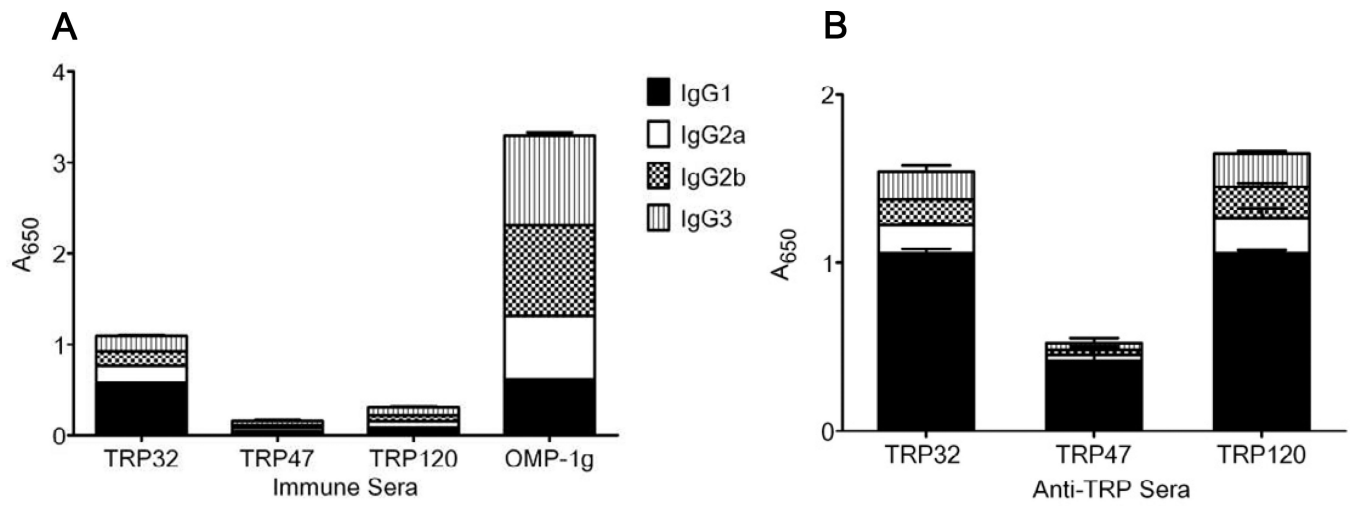


FIG. 5. Quantification of antibody isotypes detected in *E. chaffeensis*-immune and TRP epitope-specific sera by ELISA. (A) Epitope-specific TRP32, TRP47, TRP120 and OMP-1g IgG isotypes in *E. chaffeensis*-immune sera and (B) mouse anti-TRP32, TRP47 and TRP120 serum.

Table 1

Antibody titers of TRP mouse and rabbit sera generated against epitope-containing peptides of TRP32, TRP47 and TRP120 as determined by three immunoassays.

Sera	IFA		ELISA		Western Blotting	
	Mouse	Rabbit	Mouse	Rabbit	Mouse	Rabbit
Anti-TRP32	1:1000	1:500	1:6400	1:25600	nd	1:5000
Anti-TRP47	1:100	1:100	1:12800	1:512000	nd	1:100
Anti-TRP120	1:1000	1:1000	1:12800	1:6400	nd	1:1000
Immune Sera	1:1000		nd		nd	

nd, not determined.