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Recombinant *Ehrlichia* P29 protein induces a protective immune response in a mouse model of ehrlichiosis

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Abstract

Ehrlichioses are emerging tick-borne bacterial diseases of humans and animals for which no vaccines are available. The diseases are caused by obligately intracellular bacteria belonging to the genus *Ehrlichia*. Several immunoreactive proteins of ehrlichiae have been identified based on their reactivity with immune sera from human patients and animals. These include the major outer membrane proteins, ankyrin repeat proteins and tandem repeat proteins (TRP). Polyclonal antibodies directed against the tandem repeats (TRs) of *Ehrlichia chaffeensis* TRP32, TRP47 and TRP120 have been shown to provide protection in mice. In the present study, we evaluated *E. muris* P29, which is the ortholog of *E. chaffeensis* TRP47 and *E. canis* TRP36, as a subunit vaccine in a mouse model of ehrlichiosis. Our study indicated that unlike *E. chaffeensis* TRP47 and *E. canis* TRP36, orthologs of *E. muris* (P29) and *E. muris*-like agent (EMLA) do not contain tandem repeats. Immunization of mice with recombinant *E. muris* P29 induced significant protection against a challenge infection. The protection induced by *E. muris* P29 was associated with induction of strong antibody responses. In contrast to development of P29-specific IgG antibodies following immunization, development of P29-specific IgG antibodies, but not IgM antibodies, was impaired during persistent *E. muris* infection. Furthermore, our study indicated that CD4+ T cells target P29 during *E. muris* infection and differentiate into IFN-γ-producing Th1 effector/memory cells. In conclusion, our study indicated that orthologs of *E. muris* P29 showed considerable variation in the central tandem repeat region among different species, induction of P29-specific IgG antibody response was impaired during persistent *E. muris* infection, and rP29 induced protective immune responses.

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Conflict of Interests
The authors have no financial conflicts of interest

Keywords

Ehrlichia; intracellular bacteria; vaccine; protective immunity; antigen; antibody

Introduction

Ehrlichioses are emerging tick-transmitted human zoonoses caused by obligately intracellular bacteria belonging to the genus *Ehrlichia*. Human ehrlichioses are caused by *E. chaffeensis*, the etiologic agent of human monocytotropic ehrlichiosis (HME), *E. ewingii*, which causes *E. ewingii* ehrlichiosis, and the newly discovered *E. muris*-like agent (EMLA) from the upper midwestern USA [1–3]. Animal pathogens include *E. canis* and *E. ruminantium*, which cause canine monocytic ehrlichiosis and heartwater in ruminants, respectively [4]. *E. chaffeensis* and *E. ewingii* also infect dogs. Currently human or veterinary vaccines are not commercially available for ehrlichiosis [5;6].

Several antigens of *Ehrlichia* spp. have been identified based on their reactivity with immune sera from infected hosts that include the major outer membrane proteins (OMP-1/P28) encoded by a multi-gene family, ferric ion-binding protein (Fbp), disulfide bond formation (Dsb) protein, ankyrin repeat proteins, and tandem repeat proteins (TRP) [7–12]. 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 [13–16]. Ehrlichial TRPs are secreted, serine/threonine-rich, and acidic, which results in higher electrophoretic mobility than their predicted molecular masses [17]. The TRPs contain varying numbers of TRs in different ehrlichial species and strains [18;19]. The TRPs interact with a diverse group of host proteins, suggesting functional importance in establishment of a productive infection [20–22]. The protective role of antibodies directed against the *E. chaffeensis* P28-19 was demonstrated in SCID mice [23]. Recently, we demonstrated the protective roles of *Ehrlichia* heat-shock protein 60 and the OMPs: P28-9, P28-12, and P28-19 in the *E. muris*-C57BL/6 mouse model [24;25]. Furthermore, antibodies directed against the major epitopes in the TR regions of *E. chaffeensis* TRP120, TRP47 and TRP32 inhibit ehrlichial replication *in vitro* and reduce the bacterial burden *in vivo*. [26].

Neither *E. chaffeensis* nor *E. ewingii* causes disease in immunocompetent mice; thus, surrogate ehrlichial pathogens that infect mice have been used in animal models [24;27–30]. In the present study, we evaluated the recombinant *E. muris* P29, which is an ortholog of *E. chaffeensis* TRP47 and *E. canis* TRP36, as a subunit vaccine candidate in the *E.muris*-C57BL/6 mouse model. Our study indicated that, unlike *E. chaffeensis* TRP47 and *E. canis* TRP36, their orthologs in *E. muris* (P29) and EMLA do not contain tandem repeats. Immunization with recombinant *E. muris* P29 conferred significant protection against challenge infection.

Materials and Methods

Mice

Six to eight-week old female C57BL/6 mice used in the study were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed and cared for in the Animal Research Center at the University of Texas Medical Branch. All experiments were carried out in accordance with the protocol (No. 95-09-066) approved by the Institutional Animal Care and Use Committee.

Bacteria

E. muris AS145 strain was cultured in the canine macrophage-like cell line DH82. For infection of mice, ehrlichial stocks were prepared from the spleens of syngeneic mice inoculated by the intraperitoneal (i.p.) route with *E. muris* grown in DH82 cells as described previously [31].

PCR amplification, cloning and expression of recombinant *Ehrlichia* proteins

We amplified the *E. muris p29* gene by PCR using primers P29F1 – CACCAATATTCATAGTGGGGACAGG and P29R1S – CTAAGCAGCTATTTGTTCACG, which covered the entire ORF except for 17 codons on the 5' end and 9 codons on the 3' end. The amplified PCR product was cloned into the pET151/D-TOPO vector (Invitrogen, CA) and expressed as a recombinant protein with an N-terminal tag containing the V5 epitope and a 6xHis-tag. The recombinant histidine-tagged protein was purified by immobilized metal ion affinity chromatography using HisTrap HP columns packed with Ni sepharose (GE Heathcare Life Sciences, NJ). The purified protein was dialyzed against PBS to remove detergents and salts. The N-terminal fusion tag was removed from the recombinant *E. muris* P29 (rP29) using the Tobacco Etch Virus protease (Invitrogen, CA). The recombinant protein purity was tested by SDS-PAGE, and concentration was determined by the Bradford method.

Bioinformatic analysis

Multiple protein sequences were aligned by the ClustalW method, and similarity index was calculated following pairwise alignment of protein sequences by the Lipman-Pearson method (MegAlign program; DNASTAR Inc.,WI). We used the Tandem Repeats Finder program to identify tandem repeats in the sequences [32].

Animal immunizations and challenge infections

Mice were immunized with recombinant proteins (50 μg per mouse) in complete Freund's adjuvant (CFA) by the i.p. route, followed by a booster immunization in incomplete Freund's adjuvant (IFA) 30 days after primary immunization. Mice immunized with recombinant *Chlamydia pneumoniae* MOMP and mice previously infected with *E. muris* (*E. muris*-immune mice) served as controls. All immunized and control mice were challenged with the splenic ehrlichial stock containing ~ 2×10^3 bacteria (low dose) or 1×10^4 *E. muris* (high dose) by the i.p. route 60 days after the booster immunization.

Determination of ehrlichial copy numbers in splenic stocks and quantification of ehrlichial load in organs

Ehrlichial copy numbers in stocks and organs were determined by a quantitative real-time PCR as described previously [33].

Splenocyte cultures and *in vitro* assay of CD4+ T cell responses

Frequencies of *Ehrlichia*-specific IFN-γ-producing CD4+ T cells in the splenocyte population from separate groups of mice infected with *E. muris* were determined by flow cytometry as described previously [25;34].

Indirect antibody ELISA

Indirect antibody ELISA was performed using a Protein Detector ELISA Kit (KPL, Inc. Gaithersburg, MD). ELISA plates were coated with rP29 (without the N-terminal fusion tag) or *E. muris* lysate antigen at a concentration of 10 μg/ml (100 μl per well). The plates were

incubated with serum samples diluted 1:300. Wells without coated antigen that were reacted with primary and secondary antibodies were used as blanks.

Western immunoblot analysis

Western blot analysis was carried out as described previously [35]. Blots were incubated with serum samples diluted 1:1000 (Figure 2B) or 1:300 (Figure 2C). All gel and western blot images were analyzed using the MyImageAnalysis software version 1.0 (Thermo Fisher Scientific Inc., Rockford, IL).

Statistical analysis

The data were square root transformed and analyzed by one way ANOVA with Bonferroni post test for comparison of multiple groups using the GraphPad Prism software. Statistical significance was determined at 95 % ($P < 0.05$). Data presented are expressed as means plus standard deviations and are representative of two to three independent experiments.

Results

Comparison of orthologs of *E. muris* P29 protein

We identified the open reading frame (ORF) of *E. muris p29* gene from a draft genome of *E. muris* based on homology to *E. chaffeensis trp47* and *E. canis trp36* and the synteny. *E. muris p29* gene is flanked by the tRNA(Pro)GGG gene on the 5' end and the 5-amino-6-(5-phosphoribosylamino) uracil reductase (*ribD*) gene on the 3' end on the same strand. The *E. muris p29* gene is 687 bp long and is predicted to encode a 228 amino acid protein with a predicted molecular mass of 25.17 kDa.

We compared the *E. muris* P29 with the orthologs in the other ehrlichial genomes (Table 1). *E. muris* P29 orthologs show high variation in size, which corresponds to differences in the repeat region. Both *E. muris* P29 and its ortholog in EMLA do not contain tandem repeats compared to the presence of tandem repeats of varying length and number in the orthologs (Table 1). Determination of the similarity index (SI) indicated that *E. muris* P29 has greatest sequence similarity to EMLA P29 protein followed by *E. chaffeensis* TRP47 and *E. canis* TRP36. The calculated isoelectric point (pI) of *E. muris* P29 was low (4.22) indicative of its acidic nature, which is similar to orthologs in other *Ehrlichia* (Table 1). It was reported that the tandem repeat region of *E. chaffeensis* TRP47 is more acidic than the flanking N-terminal and C-terminal non-repeat regions [17]. The predicted pI of the central region of *E. muris* P29 was 3.71 compared to the predicted pI of 4.76 for the combined N-terminal and C-terminal regions, suggesting the more acidic nature of the central region than the flanking N-terminal and C-terminal regions (data not shown).

Multiple sequence alignment by ClustalW method indicated that the N-terminal region (first 112 aa) of *E. muris* P29 has high amino acid sequence homology to the pre-repeat regions of *E. chaffeensis* TRP47 and *E. canis* TRP36 (Figure 1A). The central region (88 aa) of *E. muris* P29 showed little homology to repeat regions of orthologous *E. chaffeensis* TRP47 and *E. canis* TRP36 (Figure 1B). The post-repeat region of *E. chaffeensis* TRP47 showed some degree of homology to the C-terminal region (28 aa) of *E. muris* P29 (Figure 1C). Conservation of amino acid usage was noted in the repeat regions of *E. chaffeensis* TRP47, *E. canis* TRP36 and orthologous *E. ruminantium* mucin-like proteins [7]. The repeats in these proteins utilized a total of 10 amino acids with predominant use of serine, threonine, alanine, proline, valine and glutamic acid [7]. Examination of the amino acid composition of *E. muris* P29 indicated that the central region of *E. muris* P29 utilized a total of 16 amino acids with occurrence of higher frequencies of serine, threonine, valine and glutamate residues in the central region compared to the combined N-terminal and C-terminal regions.

Analysis of recombinant *E. muris* P29 and identification of native P29

SDS-PAGE analysis indicated that rP29 without the N-terminal tag exhibited migration mobility of an approximate molecular mass of 29 kDa compared to the predicted molecular mass of 22.71 kDa, suggestive of abnormal electrophoretic mobility similar to orthologs in other *Ehrlichia* due to acidic nature of these proteins (Figure 2A; lane 2)[17]. Western blot analysis indicated that anti-rP29 immune sera and an *E. muris*-immune serum containing a detectable concentration of anti-rP29 IgG antibodies by ELISA recognized the rP29 without the N-terminal tag (Figure 2B). Western blot analysis of *E. muris*-lysate antigen using anti-rP29 immune serum identified a 29 kDa native *E. muris* protein (Figure 2C; lane 2). The predicted molecular mass of native *E. muris* P29 is 25.17 kDa compared to observed electrophoretic mobility of 29 kDa. An *E. muris*-immune serum containing detectable concentration of anti-rP29 IgG antibodies by ELISA also recognized the native P29 on Western blot (Figure 2C; lane 3).

Mice immunized with recombinant *E. muris* P29 developed protective immune responses against challenge infection

We assessed the development of protective immune responses in mice immunized with rP29 by challenge with a low dose (2×10^3 bacteria) or a high dose (1×10^4 bacteria) of *E. muris* (Figure 3). *E. muris* does not cause lethal disease in C57BL/6 mice, and our preliminary studies indicated the bacteremia peaked around day 10 post-infection. Therefore, we used reduction in bacterial burden on day 10 after challenge infection as an indicator of protective immunity. Mice immunized with rP29 or *E. muris*-immune mice had significantly reduced bacterial loads in the liver, spleen, lung and blood after the low dose *E. muris* challenge infection compared to naïve unimmunized control mice or mice immunized with *Chlamydia pneumoniae* MOMP (Figure 3A). However, immunization with rP29 significantly reduced bacterial burdens in the liver and spleen, but not in the lung and blood, following high dose challenge infection compared to significant reductions in bacterial burdens in all the organs examined in *E. muris*-immune mice (Figure 3B). The reduced bacterial burdens in the liver after the high dose challenge infection were comparable in mice immunized with rP29 and *E. muris*-immune mice (Figure 3B).

Protection induced by rP29 is associated with induction of antibody responses

Antibodies have been demonstrated to play a significant role in protection against *Ehrlichia* [36– 38]. Mice immunized with the rP29 had high levels of rP29-specific IgG antibodies in sera before and after *E. muris* challenge (Figure 4A). However, immune sera from only one out of ten mice after primary *E. muris* infection and only two out of eight mice after secondary *E. muris* infection had detectable concentration of rP29-specific IgG antibodies (Figure 4A). Immune sera from mice immunized with rP29, and *E. muris*-immune mice showed strong reactivity with the *E. muris* lysate antigen (Figure 4B). Examination of immunoglobulin isotypes indicated that mice immunized with rP29 developed high concentrations of anti-rP29 serum IgG1, IgG2c and IgG2b, and low concentrations of IgG3 antibodies (Figure 4C). 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 (Figure 4D). All groups had antigen-specific IgM antibodies.

P29-specific effector/memory CD4+ Th1 responses are induced during *E. muris* infection

We examined by flow cytometry whether CD4+ Th1 cells target P29 during *E. muris* infection. Splenocytes from mice infected with *E. muris* at day 30 post-infection were stimulated *in vitro* with rP29 or *E. muris* lysate antigen for 24h. 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 (Figure 5).

Discussion

Orthologs of *Ehrlichia* tandem repeat proteins show considerable genetic variation in the repeat region, presumably due to host immune pressure or host adaptation, and some lack tandem repeats. The present study revealed that *E. muris p29* gene and its ortholog in EMLA lack tandem repeats and show considerable genetic variation in the central region from the corresponding repeat regions of orthologous *E. chaffeensis trp47* and *E. canis trp36* genes, which is consistent with the divergence of repeat regions of TRP47 and TRP36 [7]. Analysis of the amino acid composition and predicted isoelectric point indicated that the central region of *E. muris* P29 has similar properties to that of the repeat regions of orthologs in related *Ehrlichia*.

Our study indicated that recombinant *E. muris* P29 induced significant protection, which was comparable to protection observed in *E. muris*-immune mice, against low dose challenge infection in the *E. muris*-C57BL/6 mouse model. However, immunization with rP29 reduced the bacterial burden in only some of the organs examined following high dose challenge infection compared to *E. muris*-immune mice. The reasons for the observed differences in protection against the high dose challenge infection in different organs of mice immunized with rP29 are not known, and these could be due to differences in the bacterial burdens or due to differences in the number and composition of the cells of the immune system and their functional heterogeneity in different organs [39–41]. The data suggested that immunity generated by natural *E. muris* infection is superior to protection induced by rP29. Thus, a multicomponent vaccine mimicking infection-induced immunity may provide better protection against high dose challenge. Our results indicate that P29 could be an important subunit vaccine component against ehrlichiosis.

Immunization with rP29 induced strong IgG antibody responses in all mice examined. In contrast, *E. muris* infection generated inconsistent P29-specfic IgG antibody responses in C57BL/6 mice, indicating that the infection does not effectively stimulate the development of IgG antibody responses to P29. However, all mice infected with *E. muris* developed P29-specific IgM responses (Supplementary Figure S1). In addition, all mice infected with *E. muris* developed IgG responses to *E. muris*-lysate antigen and to *E. muris* P13 protein (Supplementary Figure S1). *E. muris* P13 is an ortholog of *E. chaffeensis* TRP32 and *E. canis* TRP19 with a predicted molecular mass of 13 kDa. A previous study reported the lack of development of specific IgM antibodies in BALB/c mice and IgG antibodies in C57BL/6 mice to *Trypanosoma cruzi* proline racemase (TcPRAC) during experimental *T. cruzi* infection in contrast to development of IgG antibodies to other *T. cruzi* antigens [42]. In addition, genetic immunization with recombinant TcPRAC DNA resulted in generation of high-titer recombinant TcPRAC-specific IgG antibodies [42]. The mechanisms that contribute to impaired IgG responses to *E. muris* P29 during persistent *E. muris* infection or its biological significance are not clear.

In contrast to impaired IgG responses to *E. muris* P29 in mice, previous studies indicated that all 15 dogs experimentally infected with *E. canis* developed IgG antibodies to TRP36, and 24 out of 31 serum samples (77.4%) from HME patients with detectable antibodies to *E. chaffeensis* by IFA had IgG antibodies reactive with TRP47 [7;8;14]. In addition, C57BL/6 and AKR mice infected with *E. muris* developed a high titer of anti-*E. muris* IgG antibodies in contrast to the development of poor antibody responses in BALB/c mice infected with *E. muris* [29;43]. These studies indicate the potential host differences in antibody responses to *Ehrlichia*.

The protection induced by the rP29 was associated with induction of a broad IgG isotype response (Figure 4C). As reported previously, our study indicated that immunization with antigens, which generally involves use of adjuvants, induces a broader isotype response than antibody responses induced by *Ehrlichia* infection [24;27]. A previous study indicated that antibodies directed against the tandem repeat regions of *E. chaffeensis* TRP120, TRP47 and TRP32, which mainly consisted of IgG1 isotype, were effective in reducing bacterial burdens and splenomegaly in C57BL/6-*scid* and C57BL/6 mice infected with *E. chaffeensis* [26]. It has been suggested that antibodies directed against *E. chaffeensis* TRP47 or its orthologs could potentially interfere with their interactions with the host cell proteins and thereby prevent the establishment of infection [26].

In addition to antibody responses, T cells play an important role in protection against *Ehrlichia* [36;44;45]. Our data indicated that 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 activates macrophages and enhances their microbicidal activity [46]. Taken together, these studies suggest that orthologs of *E. muris* P29, although exhibit considerable variation in the central tandem repeat regions, are targeted by the host immune responses and could serve as potential vaccine candidates.

In conclusion, our study indicated that (i) orthologs of *E. muris* P29 showed considerable variation in the central tandem repeat region among different species, (ii) induction of P29-specific IgG antibody response was impaired during persistent *E. muris* infection, and (iii) rP29 induced protective immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Immunization with recombinant *E. muris* P29 induced protective immune responses
- Protection induced by rP29 was associated with development of IgG antibodies
- P29-specific effector/memory CD4+ Th1 responses induced during *E. muris* infection

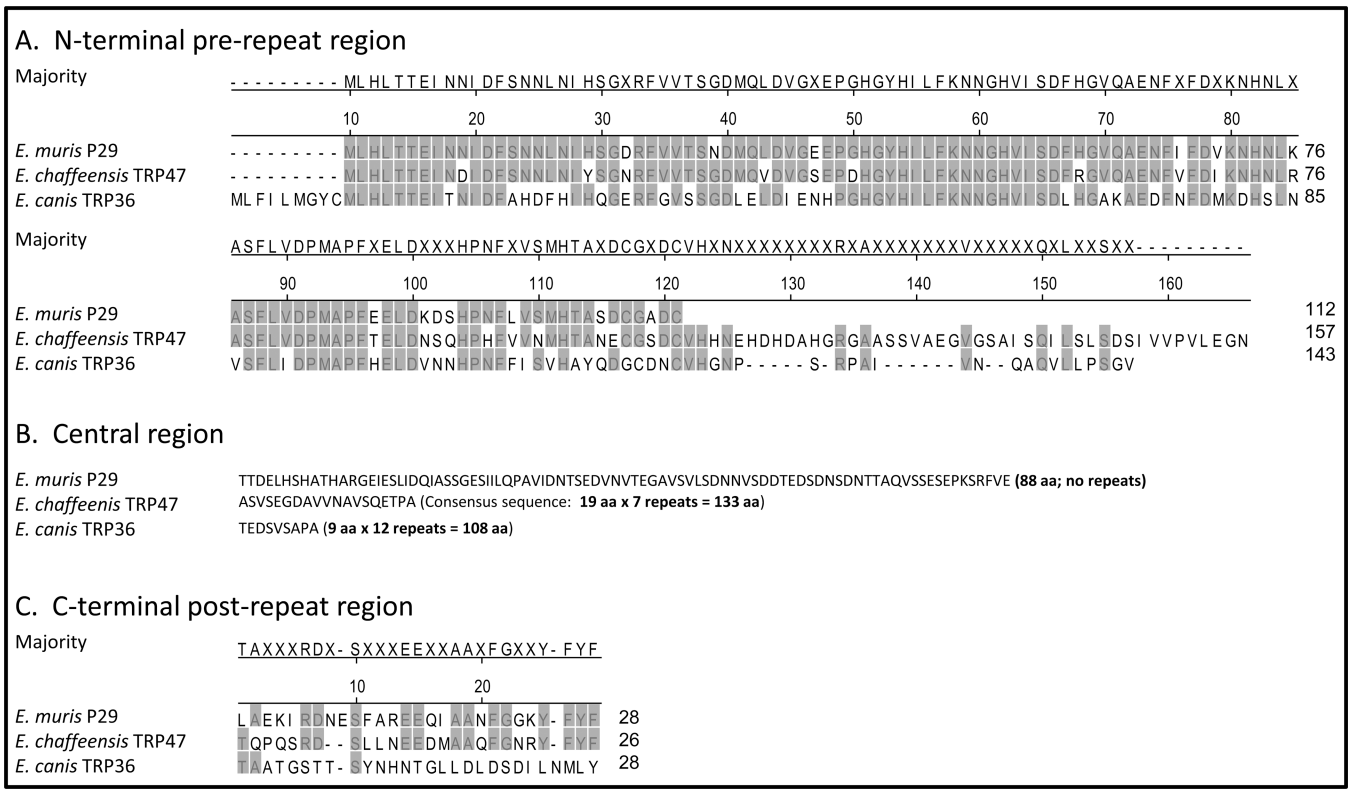

Figure 1. Orthologs of *Ehrlichia muris* P29 show high variation in the central region

The N-terminal regions (A) and C-terminal regions (C) of *E. muris* P29 and its orthologs of *E. chaffeensis* Arkansas strain (TRP47) and *E. canis* Jake strain (TRP36) were aligned by ClustalW method. The central region of *E. muris* P29 and the repeat regions of orthologous *E. chaffeensis* TRP47 and *E. canis* TRP36 (B). The amino acid residues that match the consensus sequence are highlighted in grey.

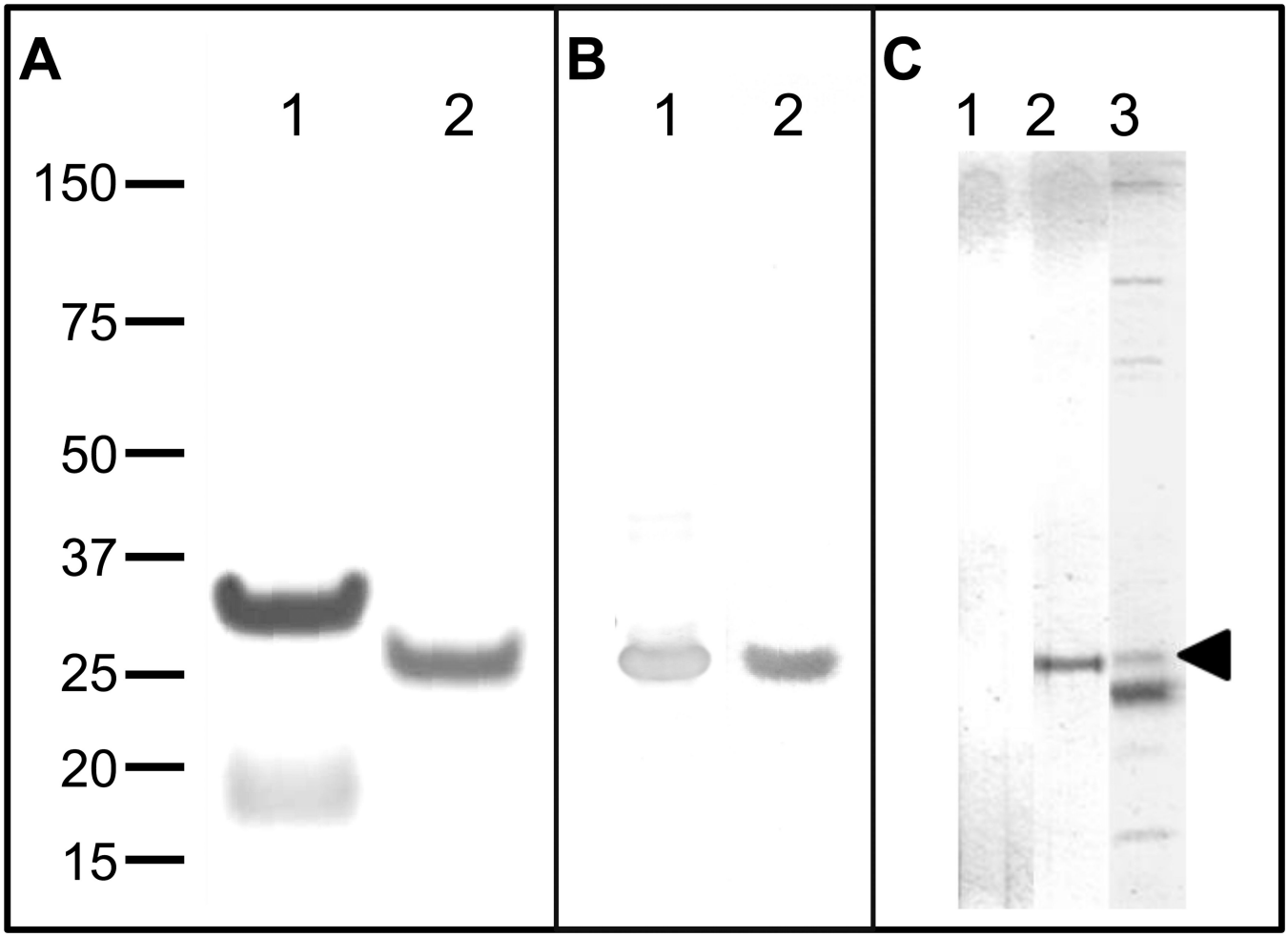

Figure 2. Analysis of recombinant *E. muris* P29 protein, and identification of native P29 protein

(A) Purified recombinant *E. muris* P29 protein with N-terminal fusion tag (lane 1) or without N-terminal fusion tag (lane 2) was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue G-250. (B) Western-blot analysis of rP29 protein. An anti-rP29 immune serum (lane 1) and an immune-serum from *E. muris*-infected mice containing detectable concentration of anti-rP29 IgG antibodies by ELISA (lane 2) reacted specifically with rP29. (C) Identification of native *E. muris* P29 protein. *E. muris* lysate antigen was separated on a NuPAGE Zoom gel containing an IPG well (Invitrogen) by SDS-PAGE and developed with naïve serum (lane 1), anti-rP29-serum (lane 2) or *E. muris*-immune serum containing detectable concentration of anti-rP29 IgG antibodies by ELISA (lane 3) using a Mini-PROTEAN II multiscreen apparatus (Bio-Rad laboratories). The anti-rP29-serum recognized a 29 kDa native *E. muris* protein (lane 2). The arrow head indicates the native P29 protein recognized by the *E. muris*-immune serum (lane 3). All images were analyzed using the MyImageAnalysis software (Thermo Scientific).

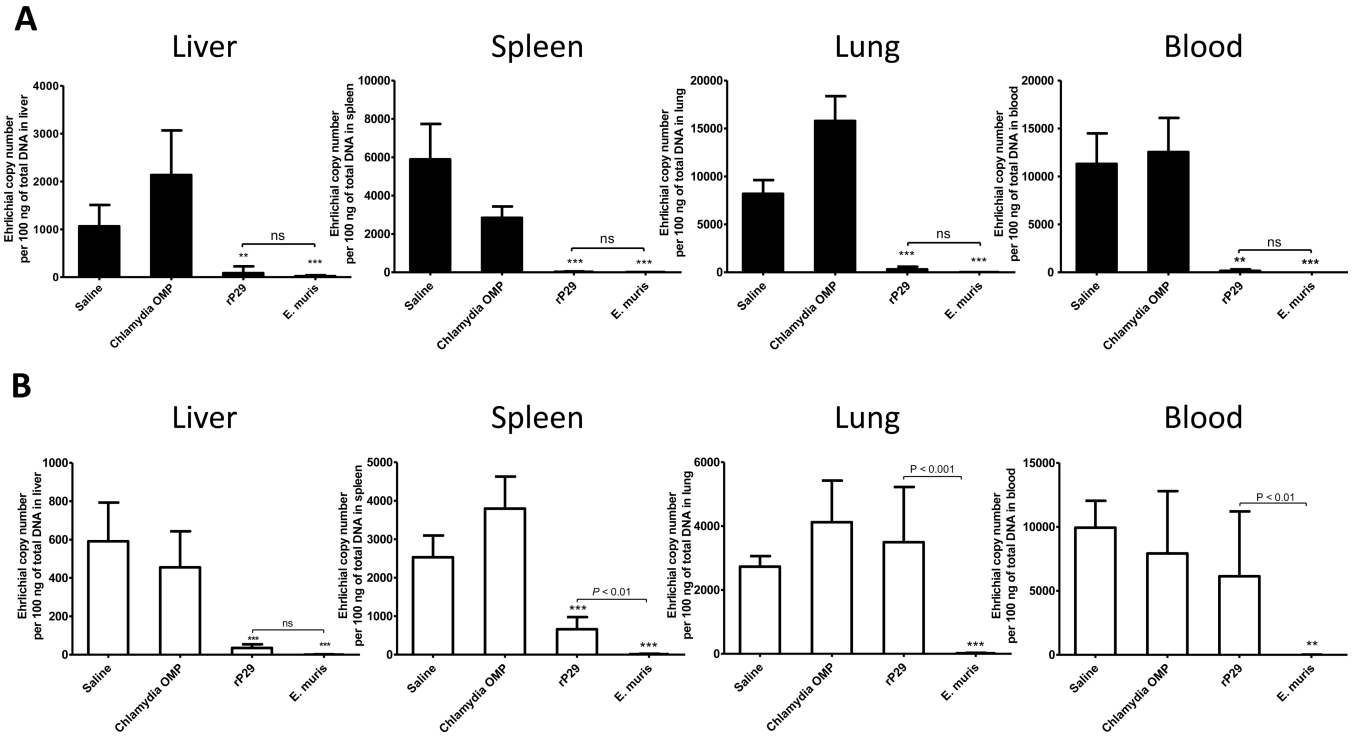

Figure 3. Immunization with recombinant *E. muris* P29 induces significant protection against challenge infection

C57BL/6 mice were immunized with two doses of rP29 and challenged with a low dose (A; 2×10^3 bacteria) or a high dose (B; 1×10^4 bacteria) of *E. muris* by the i.p. route 60 days later. Mice were sacrificed on day 10 post-challenge, and the bacterial burdens in the liver, spleen, lung, and blood were determined by quantitative real-time PCR. Unimmunized mice (saline), mice immunized with recombinant *Chlamydia pneumoniae* MOMP, and *E. muris*-immune mice served as controls. Ehrlichial copy numbers were normalized to the total DNA. Each group contained three to four mice, and data were square-root transformed and analyzed by one way ANOVA with Bonferroni post-test for comparison of multiple groups. The error bars represent the standard deviation. **, $P < 0.01$ and ***, $P < 0.001$ compared to the saline control group. ns – not significant

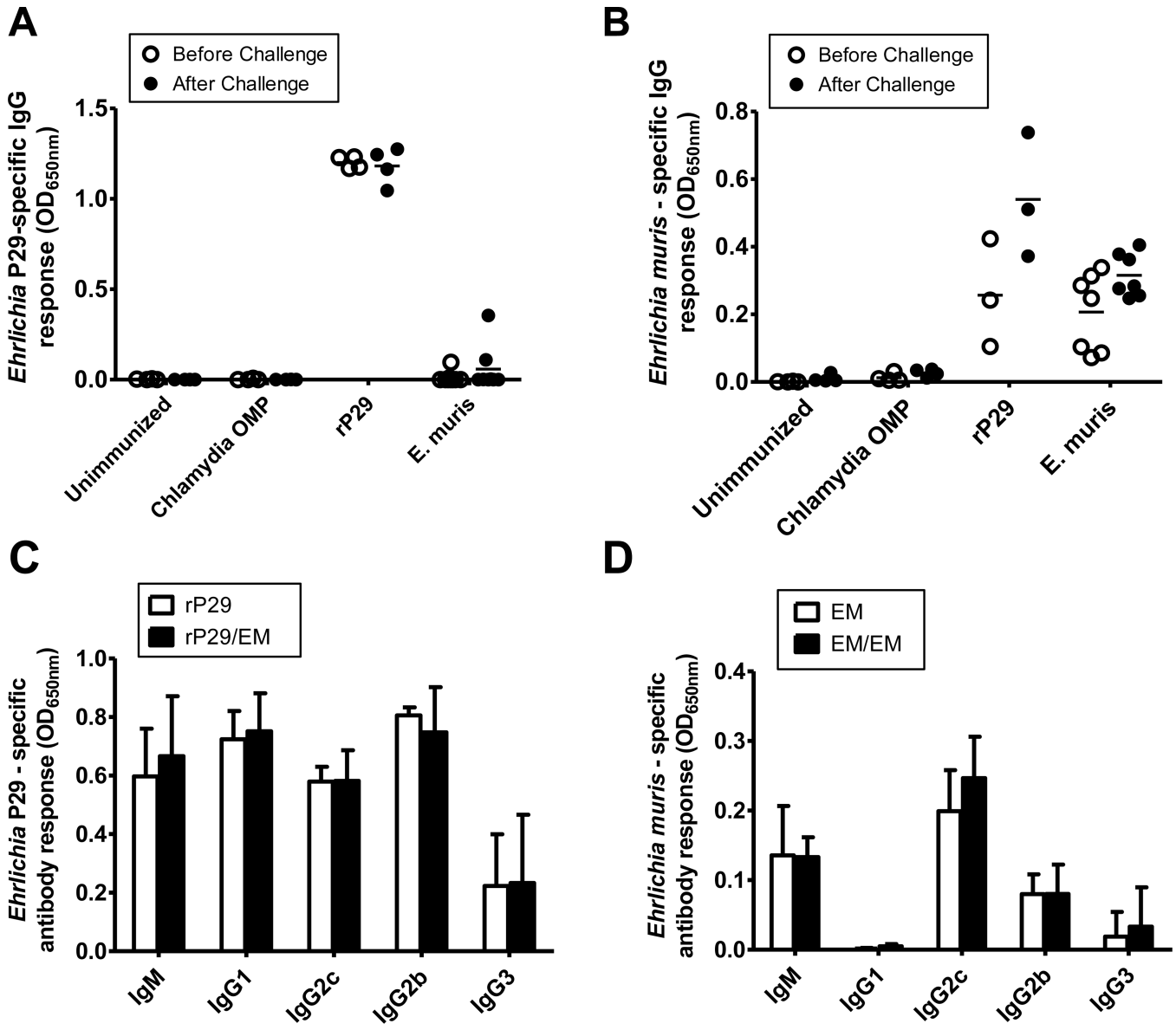

Figure 4. Protection induced by recombinant *E. muris* P29 is associated with induction of a broad IgG isotype responses

Serum IgG antibody responses specific to rP29 (A) and *E. muris* lysate antigen (B) in experimental groups before and on day 10 after *E. muris* challenge were determined by ELISA. (C) Serum immunoglobulin isotype responses specific to rP29 in mice immunized with rP29 before (rP29) and on day 10 after *E. muris* challenge (rP29/EM). (D) Serum immunoglobulin isotype responses specific to *E. muris*-lysate antigen in mice after primary (EM) and on day 10 after secondary *E. muris* infection (EM/EM). Data are representative of two independent experiments. (A & B) Each data point represents an individual immunized animal before *E. muris* challenge infection (open circles) or on day 10 after *E. muris* challenge (closed circles) and the horizontal solid bars represent the means. (C & D) Each bar represents the average of four immunized mice before *E. muris* challenge infection (open bars) or on day 10 after *E. muris* challenge (solid bars) and the error bars represent the standard deviation.

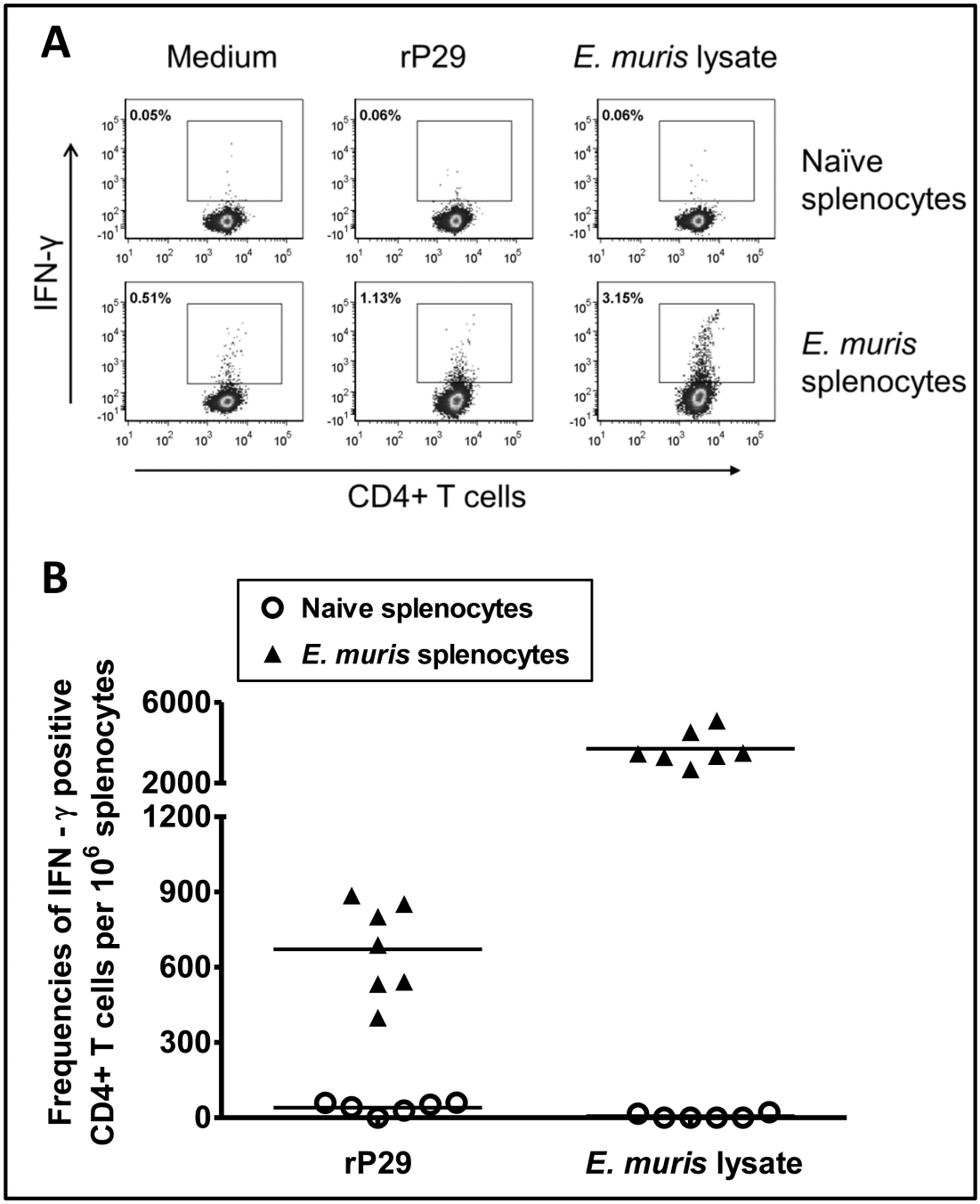

Figure 5. *E. muris* P29 induces effector/memory Th1 CD4+ T cells in infected mice

Frequencies of antigen-specific IFN-γ-producing CD4+ T cells in the spleens from separate groups of mice infected with *E. muris* were determined by flow cytometry. (A) Dot plots showing the percentages of P29-specific IFN-γ-producing CD4+ T cells in the spleens on day 30 after *E. muris* infection. (B) Frequencies of P29-specific IFN-γ-producing CD4+ T cells per million splenocytes in mice infected with *E. muris* on day 30 after infection. Each data point represents an individual animal, and data from two independent experiments are combined. Splenocytes from uninfected naïve mice stimulated with rP29 or *E. muris* lysate antigen served as controls. Background values from wells containing unstimulated splenocytes (medium only controls) were subtracted from antigen-stimulated wells for each

mouse. Frequencies of IFN-γ-producing CD4+ T cells responding to *E. muris* lysate antigen from the same mice are presented for comparison.

Table 1

Comparison of orthologs of *Ehrlichia muris* P29 protein

Organism /strain	Length (aa)	Similarity Index (%)[a]	Repeats[b]		Percent of protein in repeats (%)[d]	pI[e]	GenBank Accession Number
			No	Length (aa)			
E. muris P29	228	-	None	-	0	4.22	KC595883
E. muris-like agent P29	252	62.4	None	-	0	4.26	K523728
E. chaffeensis TRP47							
Arkansas	316	53.5	7	19	42.08	4.03	DQ085430.1
Sapulpa	328	56.7	4.5	33	40.24	4.04	DQ085431.1
E. canis TRP36							
Jake	279	41.7	12.2	9	38.70	3.93	DQ085427.1
Oklahoma	218	39.3	5.2	9	20.64	4.26	DQ085428.1
E. ruminantium mucin-like protein							
Gardel	530	29.9	16.9[c]	22	66.41	3.75	CR925677
Welgevonden	625	29	51.8[c]	9	73.44	4.19	CR925678.1

[a] Similarity index was calculated following pairwise alignment of *E.muris* P29 protein sequences with or thologs in other ehlichial genomes by the Lipman-Pearson method using the MegAlign program (DNASTAR Inc., Madison, WI)

[b] Tandem Repeats Finder program was used to identify the tandem repeats in the sequences

[c] Short four-amino acid tandem repeats of less than three numbers present in these sequences are not included in the analysis

[d] Proportion of protein in repeats expressed in percent

[e] Calculated isoelectric point (pI)