RESEARCH PAPER

Human Vaccines & Immunotherapeutics 11:3, 642–649; March 2015; © 2015 Taylor & Francis Group, LLC

Rickettsia rickettsii outer membrane protein YbgF induces protective immunity in C3H/HeN mice

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Keywords: cell-mediated immune response, *Rickettsia rickettsii*, Rocky Mountain spotted fever, YbgF, protective antigen

Rickettsia rickettsii is the etiological agent of Rocky Mountain spotted fever (RMSF). YbgF and TolC are outer membrane-associated proteins of *R. rickettsii* that play important roles in its interaction with host cells. We investigated the immunogenicity of YbgF and TolC for protection against RMSF. We immunized C3H/HeN mice with recombinant *R. rickettsii* YbgF (rYbgF) or TolC (rTolC). Rickettsial burden and impairment in the lungs, spleens, and livers of rYbgF-immunized mice were significantly lower than in rTolC-immunized mice. The ratio of IgG2a to IgG1 in rYbgF-immunized mice continued to increase over the course of our experiments, while that in rTolC-immunized mice was reduced. The proliferation and cytokine secretion of $CD4^+$ and $CD8^+$ T cells isolated from *R. rickettsii*-infected mice were analyzed following antigen stimulation. The results indicated that proliferation and interferon (IFN)-γ secretion of $CD4^+$ or $CD8^+$ T cells in *R. rickettsii*-infected mice were significantly greater than in uninfected mice after stimulation with rYbgF. YbgF is a novel protective antigen of *R. rickettsii*. Protection conferred by YbgF is dependent upon IFN-γ-producing $CD4^+$ and $CD8^+$ T cells and IgG2a, which *act in synergy to control R. rickettsii* infection.

Introduction

Rickettsia rickettsii is the etiological agent of Rocky Mountain spotted fever (RMSF), a life-threatening tick-transmitted infection.[1] Infection with *R. rickettsii* damages blood vessels throughout the body, causing increased vascular permeability, diminished serum oncotic pressure, and reduced perfusion of various organs.[2] The case fatality rate of RMSF in untreated patients is as high as 60%, with a range of 5–10% in treated patients. Fatality rates tend to rise if treatment with antibiotics (tetracycline or chloramphenicol) is delayed.[3] There have been concerted efforts to develop an effective vaccine against RMSF.

Three different vaccines containing nonviable *R. rickettsii* have been prepared from *R. rickettsii*-infected tick material,[4] egg-grown *R. rickettsii*,[5] and chicken embryonic cell-grown *R. rickettsii*.[6] These vaccines have been tested in human volunteers but have only been proven to be minimally effective in preventing RMSF.[7,8] Furthermore, preparation of these rickettsia vaccines involves large-scale culturing of *R. rickettsii* cells, which is difficult and hazardous because *R. rickettsii* is highly infectious. A good alternative would therefore be subunit vaccines against RMSF comprising *R. rickettsii* protective antigens that can be efficiently and safely produced using modern techniques.

In recent years, many surface proteins have been recognized in the spotted fever group (SFG) rickettsiae. The Sca0 (OmpA) and Sca1 proteins are involved in the attachment of rickettsiae to host cells,[9,10] while Sca5 (OmpB) is associated with the rickettsial invasion of host cells.[4] Sca2 serves as a formin mimic that is associated with actin-based motility of rickettsiae in host cells.[4] Sca4 can activate vinculin and interacts with the actin cytoskeleton of host cells.[11] Both OmpA and OmpB have the ability to elicit efficient protection against *R. rickettsii* infection.[12,13] Other Sca proteins have not been demonstrated to be protective antigens of *R. rickettsii*.

We previously showed that YbgF, a highly abundant surface-exposed protein (SEP) of *Rickettsia heilongjiangensis*, could confer protection against *R. heilongjiangensis* infection in a murine model.[14] TolC, a bacterial membrane-associated protein, is recognized as a major SEP through the proteomic analysis of biotinylated cell surface proteins of *R. rickettsii*. In the current study, we used recombinant YbgF (rYbgF) and TolC (rTolC) to immunize C3H/HeN mice and determine their efficacies in protecting against *R. rickettsii* infection.

Results

Immunoblotting

Purified rYbgF (44 kDa) and rTolC (63 kDa) were recognized by immunoblotting of sera collected from *R. rickettsii*-infected mice at 28 days post-infection (dpi; **Fig. 1**).

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Submitted: 10/05/2014; Revised: 11/11/2014; Accepted: 11/22/2014
http://dx.doi.org/10.1080/21645515.2015.1011572

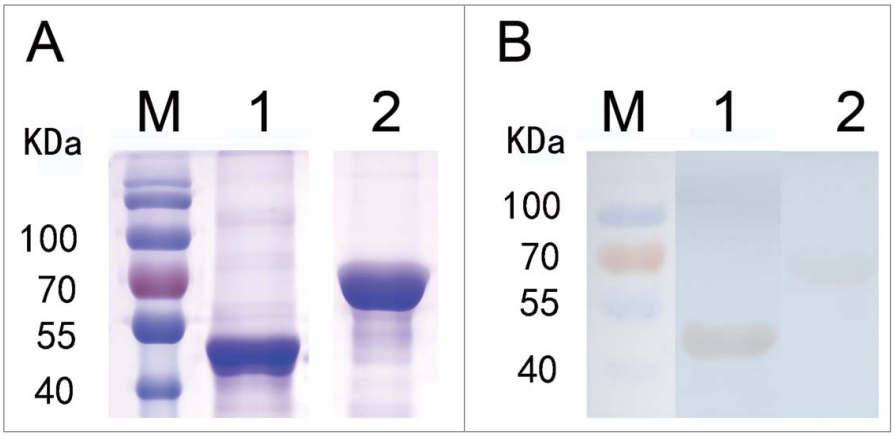

Figure 1. Immunoblotting analysis of rYbgF and rTolC. Recombinant YbgF and TolC purified from *E. coli* cell lysates were separated by 12% SDS-PAGE and stained with G-250 Coomassie brilliant blue (**A**). Immunoblotting analysis of rYbgF and rTolC. Lane M, protein molecular mass markers; lane 1, rYbgF; lane 2, rTolC (**B**).

Microscopy detection of YbgF and TolC

The presence of *R. rickettsii* YbgF or TolC was determined by indirect immunofluorescence assays (IFA) and transmission electron microscopy (TEM). Distinct fluorescent spots were observed in rickettsial cells stained with sera collected from mice immunized with rYbgF (**Fig. 2A**) or rTolC (**Fig. 2B**). Fluorescence was not observed in rickettsial cells stained with sera from mock-immunized mice (**Fig. 2C**). From our TEM results, the outer membrane (OM) and inner membrane (IM) of *R. rickettsii* cells were covered with colloidal gold particles when sera from mice immunized with rYbgF (**Fig. 2D**) or rTolC (**Fig. 2E**) were used.

Immune protection against *R. rickettsii* challenge

Using quantitative polymerase chain reaction (qPCR) assays, the loads of rickettsia in the spleens (**Fig. 3A**), livers (**Fig. 3B**), or lungs (**Fig. 3C**) of mice immunized with rYbgF or whole cell antigen (WCA) were significantly lower than those in mock-immunized mice. The rickettsial loads in the spleens or lungs of mice immunized with rTolC were not significantly different from those in PBS-immunized mice.

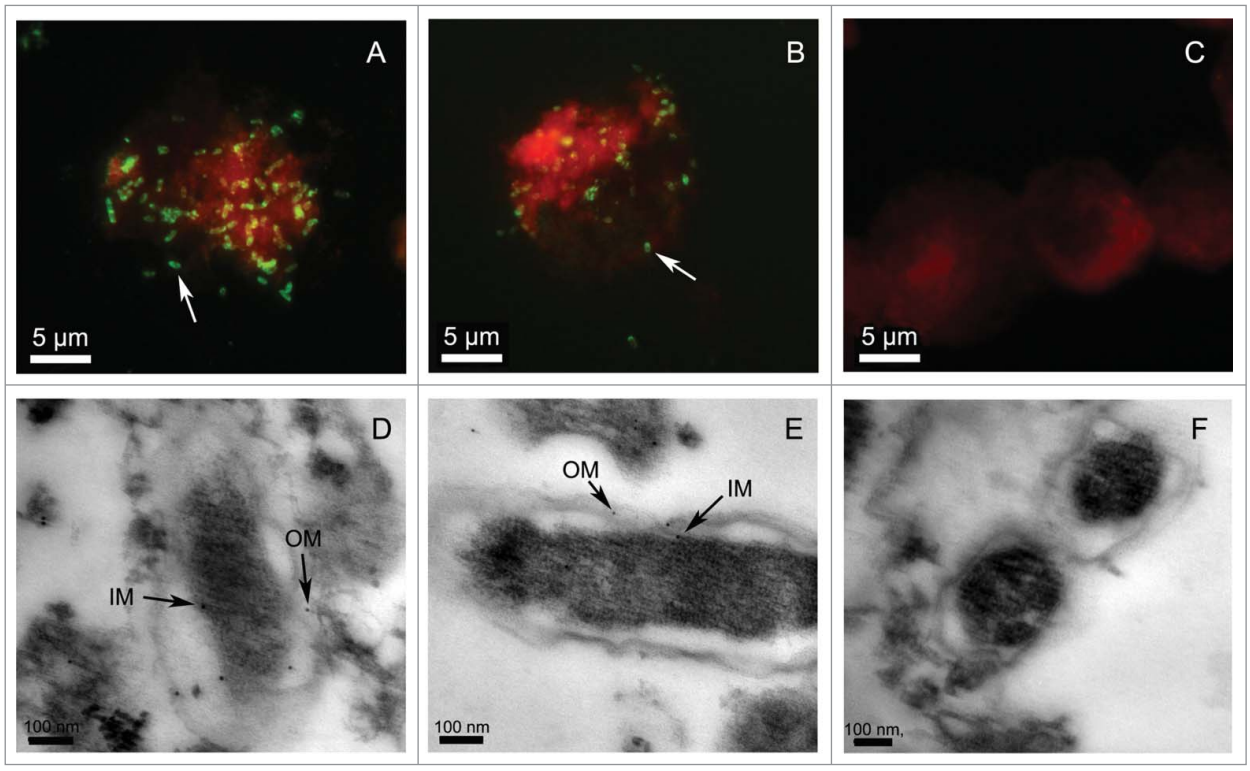

Figure 2. Microscopy analysis of YbgF and TolC in *R. rickettsii* cells. *Rickettsia rickettsii* were cultured in Vero cells and incubated with antibodies against rYbgF (**A**) or rTolC (**B**), or with naïve (**C**) serum. Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) were then applied. The white arrows indicate YbgF or TolC in *R. rickettsii* cells. TEM analysis of *R. rickettsii* in Vero cells involved staining with antibodies against rYbgF (**D**), rTolC (**E**), or with naïve (**F**) serum. A goat anti-mouse IgG labeled with colloidal gold particles was then added to samples. The black arrows indicate the locations of YbgF or TolC in the inner membrane (IM) and outer membrane (OM) of *R. rickettsii* cells.

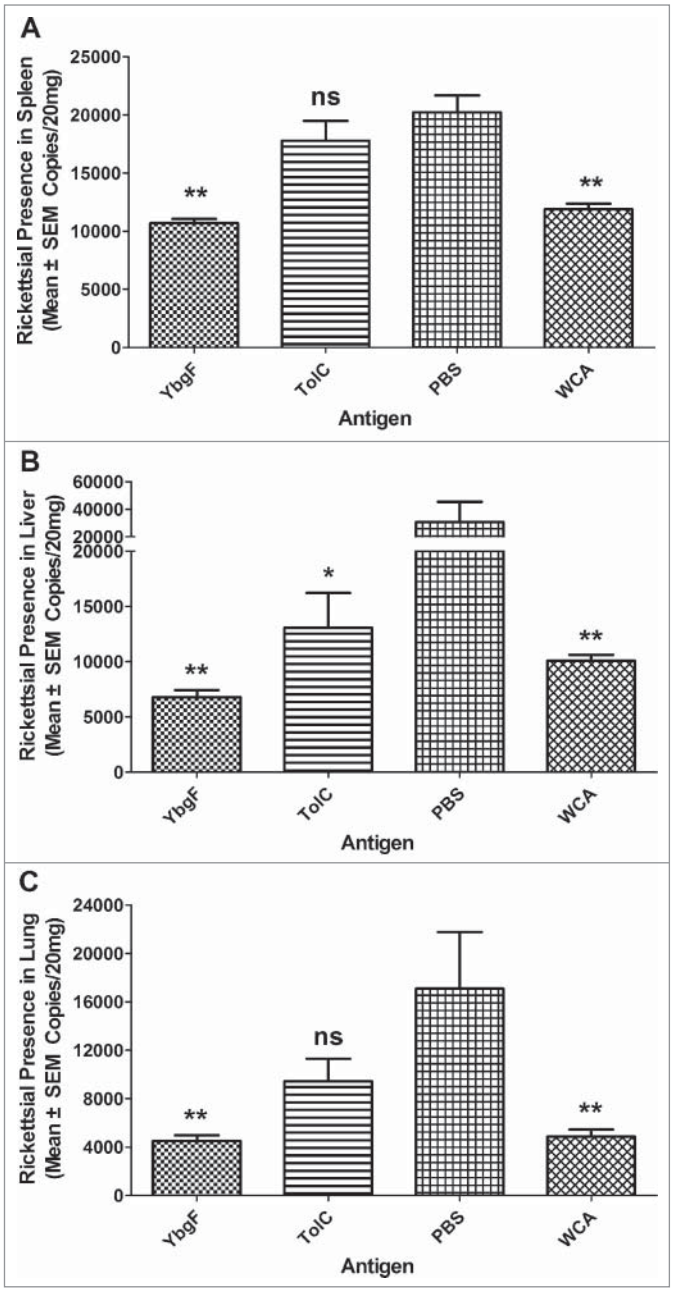

Figure 3. Immune protection against *R. rickettsii* infection. C3H/HeN mice were immunized 3 times with rYbgF, rTolC, WCA, or PBS. At day 14 after the last immunization, mice were challenged with *R. rickettsii*. At day 6 after challenge, mice were sacrificed and their spleens (**A**), livers (**B**), and lungs (**C**) were collected for use in qPCR assays. Rickettsial DNA copies among groups are expressed as mean copies + standard deviations. The statistically significant differences between PBS immunized mice and other groups were analyzed using the *T* test or Wilcoxon Two-Sample Test based on their normality and equality of variances and are indicated as follows: *, $P < 0.05$; ***, $P < 0.001$; ns, no significance. $^{*}P < 0.05$; $^{**}P < 0.01$; ns, no significance, respectively.

Serious pathological damages were observed in rTolC- and PBS-immunized mice (**Fig. 4**). In liver tissues, we observed hydropic degeneration, nuclear pyknosis, inflammatory infiltrates consisting of mononuclear cells and polymorphonuclear leukocytes that were focused on the portal area of the liver. In spleen tissues, large numbers of macrophages were observed. For lung lesions, interstitial pneumonia was characterized by numerous inflammatory infiltrates of mononuclear cells, polymorphonuclear leukocytes, alveolar interstitial thickening, and alveolar hemorrhage. The lesions in the organs of mice immunized with rYbgF or WCA were not as severe as those in other mice (**Fig. 4**).

Humoral immune responses

The mean $\log_{10}$ of IgG titers for rYbgF-immunized sera were 4.01, 4.23, 5.26, and 5.26, while those for rTolC-immunized sera were 3.40, 3.32, 4.53, and 4.01 on 14, 28, 42, and 56 dpi (**Fig. 5**). The titer ratio of IgG2a to IgG1 in rYbgF-immunized mice continued to increase over the course of our experiments, while that in rTolC-immunized mice was decreased on 42 dpi (**Fig. 5**).

Serum neutralization of R. rickettsii

The quantities of rickettsia for groups treated with sera from mice immunized with rYbgF (0.45 rickettsia/cell) or rTolC (0.43 rickettsia/cell) were lower than those treated with sera from PBS-immunized mice (0.61 rickettsia/cell; $P < 0.05$; **Fig. 6**). The amount of rickettsia in the group treated with sera from mice immunized with *R. rickettsii* WCA was higher than that in the group treated with sera from PBS-immunized mice.

Proliferation and cytokine production of T cells

$CD4^+$ or $CD8^+$ T cells from *R. rickettsii*-infected or uninfected mice were stimulated *in vitro* by rYbgF, rTolC, WCA, or ConA. Their proliferation and levels of cytokine secretion were analyzed (**Table 1**). After stimulation with rYbgF, the proliferation of $CD4^+$ and $CD8^+$ T cells from infected mice was significantly higher than that of uninfected cognate T cells ($P < 0.01$). The secretion of interferon (IFN)-γ and tumor necrosis factor (TNF)-α by $CD4^+$ T cells from infected mice stimulated with rYbgF was significantly higher ($P < 0.05$) than that seen in $CD4^+$ T cells from uninfected mice. Secretion of interleukin (IL)-6 and IL-10 from T cells was not dramatically different between infected and uninfected mice ($P > 0.05$). Secretion of IFN-γ ($P < 0.05$), IL-6 ($P < 0.001$), and IL-10 ($P < 0.001$) by $CD4^+$ T cells from infected mice was significantly greater compared with those from uninfected mice. The secretion levels of cytokines by $CD4^+$ T cells were not significantly different between infected and uninfected mice stimulated with ConA ($P > 0.05$).

The secretion of IFN-γ by $CD8^+$ T cells from infected mice and stimulated with rYbgF was significantly higher ($P < 0.05$) compared with those from uninfected mice. Secretion of TNF-α or IL-6 by $CD8^+$ T cells from infected mice was not significantly different ($P > 0.05$) compared with those from uninfected mice. Secretion of IL-10 by $CD8^+$ T cells from infected mice was lower ($P < 0.05$) compared with those from uninfected mice. After stimulation with *R. rickettsii* WCA, cytokine secretion by $CD8^+$ T cells from infected mice was greater than that from the $CD8^+$ T cells of uninfected mice ($P < 0.05$). There was no significant difference in the secretion of these cytokines from $CD4^+$

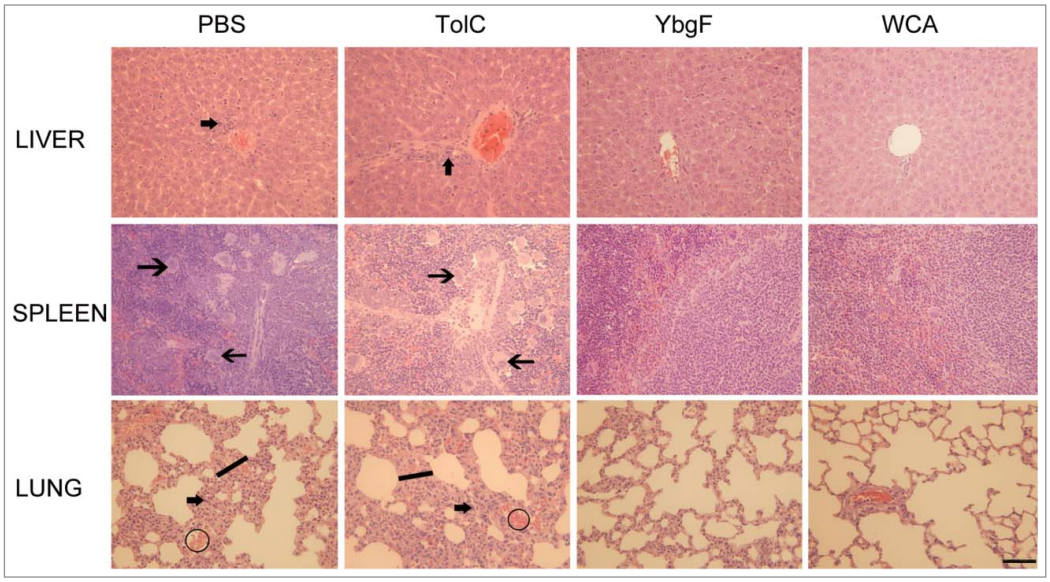

Figure 4. Pathological lesion in the organs of immunized mice after challenge. Infiltration of inflammatory cells is shown with thick arrows in the livers and lungs, macrophages in spleen tissues are shown with thin arrows, severe interstitial *thickened alveolar walls* are shown with short lines, and alveolar hemorrhage in lungs is shown with circles (400× magnification, bar = 200 μm).

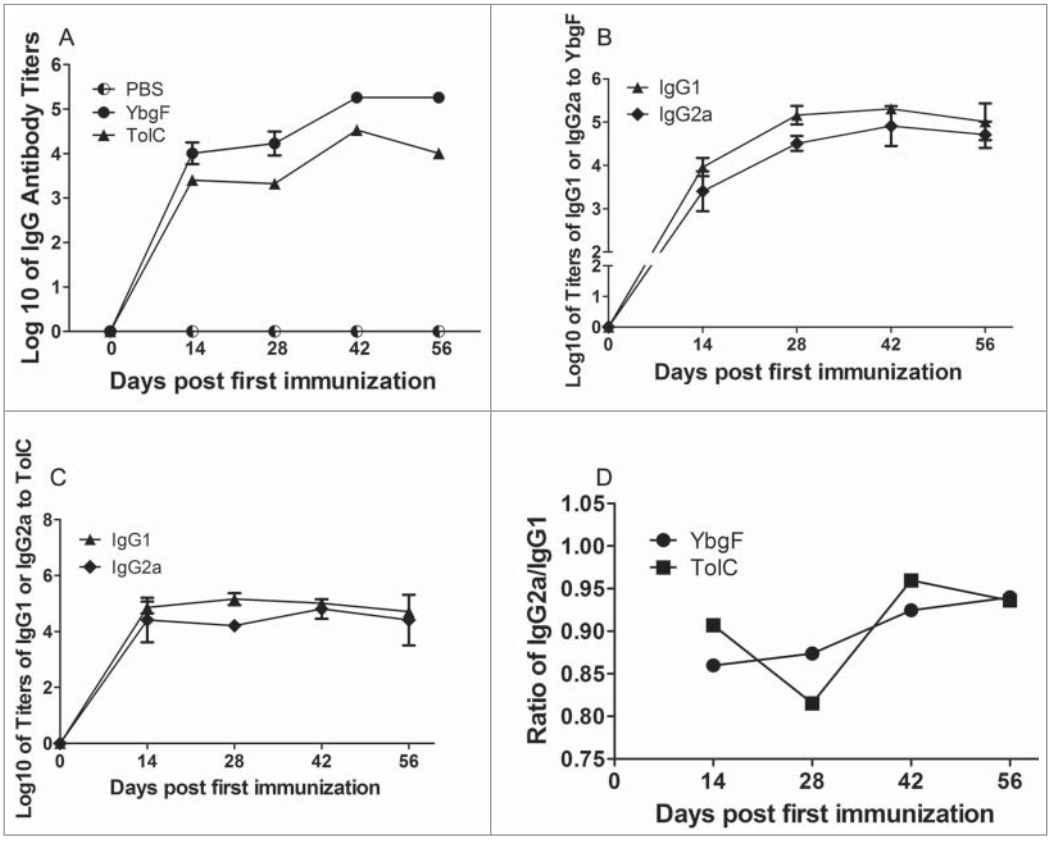

Figure 5. Humoral immune responses induced by different antigens. Log_{10} of titer kinetics for IgGs against rYbgF and rTolC (**A**). Titer kinetics of IgG1 and IgG2a against rYbgF (**B**). Titer kinetics of IgG1 and IgG2a against rTolC (**C**). IgG2a:IgG1 ratios for rYbgF and rTolC (**D**). Antibody titers were determined by ELISA and presented as log_{10}, with the standard deviation indicated by an error bar.

and $CD8^+$ T cells between infected and uninfected mice after rTolC stimulation. Additionally, the secretion of IL-2, IL-4, and IL-5 was too low to be detected except after stimulation with ConA (data not shown).

Discussion

Two abundant and high-molecular-weight outer membrane proteins, OmpA and OmpB, of the SFG rickettsiae are well-known protective antigens. Passively administered antibodies against OmpA or OmpB protect guinea pigs from *R. rickettsii* infection, and protect severe combined immunodeficient mice from lethal infection by *Rickettsia conorii*, which is closely related to *R. rickettsii*. These findings suggest that antibodies against OmpA or OmpB are important in host defense against rickettsial infection.[15] OmpA- and OmpB-induced protective immunity involves both complement-mediated killing in mammalian blood [12] and the activation of antigen-specific memory $CD4^+$ and $CD8^+$ T cells.[13]

YbgF and TolC have been previously identified as major SEPs in rickettsiae. YbgF, a periplasmic protein, is an abundant surface protein and major sero-reactive antigen of rickettsiae.[16,17] It is thought to function in maintaining the cell envelope integrity of Gram-negative bacteria.[18] TolC is thought to be a bioactive transport molecule that couples with different inner membrane/periplasmic proteins. These are associated with virulence or antimicrobial resistance in

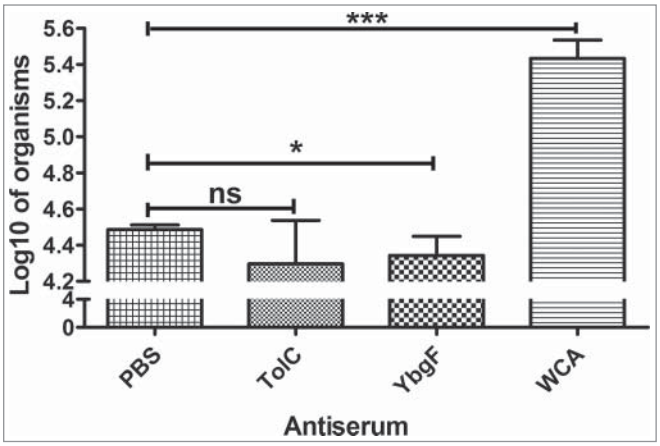

Figure 6. Neutralization of *R. rickettsii* by sera from mice immunized with various antigens. Purified *R. rickettsii* were incubated with sera from mice immunized with rYbgF, rTolC, WCA, or PBS for 60 min. Rickettsiae treated with these sera were then inoculated into EA.hy 926 cells. The total amount of *R. rickettsii* in host cells was determined by qPCR. Values are presented as the mean with standard deviations ($n = 3$). The statistically significant differences between anti-PBS immune serum and other immune serum groups were analyzed using the *T* test or Wilcoxon Two-Sample Test based on their normality and equality of variances and are indicated as follows: *, $P < 0.05$; ***, $P < 0.001$; ns, no significance.

Gram-negative bacteria.[19] From our TEM analysis, YbgF and TolC were shown to be associated with both the inner and outer membranes of *R. rickettsii*, suggesting that specific antibodies could prevent *R. rickettsii* from interacting with host cells. To validate this, sera from mice immunized with rYbgF or rTolC were used to neutralize *R. rickettsii in vitro*. Our findings showed that both sera could significantly reduce *R. rickettsii* invasion of vascular endothelial cells, suggesting that YbgF and TolC are both factors that mediate the interaction of *R. rickettsii* with vascular endothelial cells. Further studies regarding their receptors on vascular endothelial cells are warranted.

For the protective efficacy assay, our results showed that the rickettsial load in the lungs, spleens, or livers of mice immunized with rYbgF were significantly lower than those in mock-immunized mice. Additionally, organ impairment in rYbgF-immunized mice was lower than in rTolC- or mock-immunized mice. These results demonstrate that YbgF is a protective antigen of *R. rickettsii*, while rTolC is not. In a previous study, YbgF was also recognized as a protective antigen of *R. heilongjiangensis*.[14] A comparison of the amino acid sequences of YbgF from the 2 species showed that only 6 of 245 amino acids were different (**Fig. S1**), suggesting that YbgF is a conservative antigen, at least among the SFG rickettsiae.

To explore the potential protective mechanisms of YbgF, the immune response elicited by rYbgF was compared with that elicited by rTolC. Our results showed that high levels of specific antibodies were produced after immunization with both rYbgF and rTolC. This indicates that both proteins could elicit a specific humoral response. Results from the *in vitro* neutralization tests indicated that rYbgF- and rTolC-immunized sera could significantly reduce the number of *R. rickettsii*

Table 1. Cytokine secretion and proliferation of $CD4^+$ or $CD8^+$ T cells after antigen stimulation. $CD4^+$ or $CD8^+$ T cells isolated from mice injected with *R. rickettsii* or PBS were stimulated with rYbgF, rTolC, WCA of *R. rickettsii*, ConA, or PBS. TNF-α, IFN-γ, IL-6, and IL-10 in supernatants of $CD4^+$ T cells or TNF-α, IFN-γ, IL-6, and IL-10 in supernatants of $CD8^+$ T cells were detected using a BD Cytometric Bead Array and Mouse Th1/Th2 cytokine kit. Proliferation of $CD4^+$or $CD8^+$ T cells was measured using Cell Counting Kit-8 and expressed as a percent of the control group (stimulated with PBS). The data are expressed as the mean ± standard deviation. Significant differences between infected and uninfected mice were analyzed by the *T* test or Wilcoxon Two-Sample Test based on their normality and equality of variances and are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significance

			Cytokines											
T cells	**Stimulations**	**Groups**	**TNF (pg/ml)**		**IFN-γ (pg/ml)**		**IL-6 (pg/ml)**		**IL-10 (pg/ml)**		**Proliferation (%)**	
$CD4^+$	ConA	Uninfected	1045.6 ± 582.79	ns	2677 ± 490.73	ns	45.48 ± 4.45	ns	579.8 ± 49.22	ns	533.5 ± 35.22	ns
		Infected	317.33 ± 12.6		5462.3 ± 106.36		533.39 ± 391.99		1138.55 ± 588.25		673.34 ± 155.37	
	WCA	Uninfected	1287.83 ± 49.4	ns	21.7 ± 2.71	*	302.64 ± 33.33	***	144.34 ± 21.59	***	172.63 ± 19.65	ns
		Infected	1380.93 ± 65.49		1805.53 ± 49.65		602.76 ± 31.75		422.44 ± 28.95		195.48 ± 26.1	
	rYbgF	Uninfected	110.8 ± 1.94	*	2.1 ± 0.37	*	181.06 ± 102.95	ns	260.05 ± 152.79	ns	221.32 ± 11.68	**
		Infected	128.67 ± 8.36		18.97 ± 4.66		221.35 ± 16.47		431.63 ± 23.72		416.13 ± 55.91	
	rTolC	Uninfected	391 ± 82.13	ns	1.2 ± 0.22	ns	54.12 ± 13.35	ns	47.46 ± 10.62	ns	56.8 ± 4.93	ns
		Infected	361.23 ± 16.43		11.1 ± 12.59		78.36 ± 9.5		68.44 ± 8.57		92.26 ± 18.22	
	PBS	Uninfected	0.22 ± 0.31	ns	0.03 ± 0.05	ns	4.18 ± 0.25	ns	10.54 ± 0.96	ns	97.94 ± 14.63	ns
		Infected	0.16 ± 0.22		1.5 ± 2.12		4.59 ± 0.15		11.84 ± 0.61		117.18 ± 18.78	
$CD8^+$	ConA	Uninfected	346.77 ± 31.7	***	3348.33 ± 184.58	***	51.5 ± 6.99	ns	253.73 ± 43.55	ns	510.38 ± 10.08	ns
		Infected	588.63 ± 12.3		5497.9 ± 110.62		65.58 ± 4.68		292.54 ± 45.88		693.03 ± 67.81	
	WCA	Uninfected	971.67 ± 68.51	**	12.37 ± 3.36	**	321.33 ± 68.13	***	273.47 ± 41.75	***	246.96 ± 17.82	ns
		Infected	1529.27 ± 125.81		1716.77 ± 170		810.8 ± 77.98		565.02 ± 78.27		235.82 ± 32.57	
	rYbgF	Uninfected	303.27 ± 10.23	ns	1.8 ± 0.17	*	165.22 ± 14.45	ns	457.19 ± 61.63	*	291.17 ± 24.34	**
		Infected	327.13 ± 68.81		15.77 ± 2.48		196.75 ± 23.52		312.19 ± 29.93		472 ± 19.77	
	rTolC	Uninfected	363.77 ± 24.16	ns	0.93 ± 0.16	ns	56.35 ± 7.91	ns	94.45 ± 19.63	ns	152.08 ± 17.88	ns
		Infected	455.33 ± 84.25		11.73 ± 2.59		97.05 ± 3.78		131.72 ± 10.98		135.94 ± 7.85	
	PBS	Uninfected	0.09 ± 0.06	ns	0.11 ± 0.16	ns	3.97 ± 0.17	ns	10.93 ± 0.61	ns	100.81 ± 1.53	ns
		Infected	0.39 ± 0.55		0.64 ± 0.91		5.51 ± 0.41		15.33 ± 1.22		106.42 ± 13.9	

in vascular endothelial cells, suggesting that these sera contained neutralizing antibodies that could efficiently counteract *R. rickettsii* invasion of host cells. *R. rickettsii* WCA-immunized sera were able to enhance the number of *R. rickettsii* in host cells. This was observed in a previous neutralization assay with *R. heilongjiangensis* WCA-immunized sera.[14] The reason for this might be that antibodies against numerous rickettsia surface molecules in WCA-immunized sera interact with multiple Fc receptors expressed by vascular endothelial cells, thereby promoting entry of rickettsia.[14] Our results showed that rYbgF induced a more rapid increase in IgG2a levels compared with IgG1 levels during immunization. For rTolC, the ratio of IgG2a to IgG1 levels decreased during the intermediate and final phases of immunization. The IgG2a molecule has been proven to be involved in the elimination of intracellular pathogens from hosts *via* Fc and *complement receptors* of macrophages.[20] It has also been shown to mediate antibody-dependent cell-mediated cytotoxicity [21] and opsonophagocytosis of macrophages.[22] A high level of specific IgG2a has been shown to be involved in T-helper cell type 1 (*Th1*)-immune responses and enhanced efficacy of protein vaccination.[23] Eliciting high levels of specific IgG2a could be considered an important property of rYbgF in inducing protection against *R. rickettsii* infections.

Although specific antibodies can counteract rickettsial invasion of host cells, cell-mediated immunity plays a critical role in killing intracellular rickettsiae.[24] Our results showed that rYbgF but not rTolC could elicit antigen-specific $CD4^+$ and $CD8^+$ T cells to rapidly proliferate and differentiate into IFN-γ-producing $CD4^+$ *Th1* cells and $CD8^+$ cytotoxic T lymphocytes (Tcl) cells, respectively. Antigen-specific $CD4^+$ *Th1* cells and $CD8^+$ Tcl cells are well known to play key effector functions in the control of intracellular bacterial infection.[25] Antigen-specific $CD8^+$ Tcl cells are critical for the clearance of intracellular bacteria.[26] During *Toxoplasma gondii* infections, IFN-γ-producing $CD8^+$ T cells are the major effectors, and IFN-γ-producing $CD4^+$ T cells play a synergistic role in protection against this pathogen.[27]

Previous studies have also suggested that cytokine-activated intracellular killing of rickettsiae within endothelial cells, the major target cells of rickettsiae, could occur *via* nitric oxide- and hydrogen peroxide-mediated killing. These are induced by a combination of IFN- γ, TNF-α, IL-1, and CCL5.[28-30] Our results showed that rYbgF could elicit antigen-specific $CD4^+$ T cells to produce high levels of IFN-γ and TNF-α, while antigen-specific $CD8^+$ T cells produced high levels of IFN-γ. These findings suggest that the protective antigen, rYbgF, could efficiently activate antigen-specific $CD4^+$ and $CD8^+$ T cells to rapidly produce and secrete large quantities of TNF-α and/or IFN-γ, which function synergistically to activate endothelial cells and other target cells to kill intracellular rickettsiae.[31] In addition, secretion of TNF-α by infected and uninfected $CD8^+$ T cells was not significantly different following stimulation with rYbgF or rTolC, but was dramatically different after stimulation with WCA of *R. rickettsii*, suggesting that there were other components in WCA of *R. rickettsii* that could efficiently stimulate the antigen-specific $CD8^+$ T cells to generate high levels of TNF-α.

In conclusion, YbgF and TolC are surface proteins of *R. rickettsii* associated with the inner and outer membrane. YbgF is a protective antigen, and its use in a vaccine dramatically reduces the severity of *R. rickettsii* infection in mice. This protection is mainly dependent upon rYbgF-induced, Th1-type cell-mediated immune responses, manifesting as rapid proliferation and high levels of IFN-γ secretion from YbgF-specific $CD4^+$ and $CD8^+$ cells. The rYbgF-induced Th1-type cell-mediated immune response also mediates the rise of YbgF-specific IgG2a levels, synergistically activating and opsonizing host cells to kill intracellular rickettsiae.

Materials and Methods

Mice

Female C3H/HeN mice at 6 to 7 weeks of age were purchased from Vital River Laboratories (Beijing, China). The experiments were carried out according to the guidelines of the authors' institution. The protocol was approved by the Institute of Animal Care and Use Committee at the Academy of Military Medical Sciences (AMMS). All facilities were accredited by the AMMS Animal Care and Ethics Committee, and all efforts were made to minimize mice suffering.

Bacterial strains and plasmids

We cultured *R. rickettsii* (Sheila Smith strain) in Vero cells (ATCC). Rickettsia were purified from cells using isopycnic density gradient centrifugation as described previously.[32] The quantity and viability of organisms was measured by qPCR assays specific for *R. rickettsii* [33] and plaque assays,[34] respectively. Purified rickettsiae were inactivated at 90°C for 20 min [32] and then used as WCA. The prokaryotic expression plasmid pET32a(+) (Novagen) was used to construct recombinant plasmids containing *R. rickettsia* genes of interest. Recombinant plasmids were transformed into *Escherichia coli* BL21 cells (Novagen).[17]

Preparation of recombinant proteins and immunoblotting

The open reading frames of *ybgF* (GenBank Accession Number: ABV75922.1) and *tolC* (ABV75915.1) were amplified by PCR from the genomic DNA of *R. rickettsii* with specific primers (5′-GGG GAT CCA TGA AAC TGA TTG TCT TA-3′ and 5′-GGC TCG AGT TTA ATC TTA GCA TCT TC-3′ for *ybgF* with respective *Bam*HI and *Xho*I sites underlined; and 5′-CCG GAT CCA CTG AAG GGT ATA AGA A-3′ and 5′-CCG TCG ACA AAC TCT TCT TCA GGA CTA-3′ for *tolC* with respective *Bam*HI and *Sal*I sites underlined). Recombinant YbgF and TolC proteins were tagged with histidine and purified as described previously.[14] Purified proteins were analyzed by immunoblotting from pooled sera that had been collected from C3H/HeN mice on day 28 after *R. rickettsii* infection, as described previously.[35] Endotoxins in purified recombinant protein fractions were removed with Toxin Eraser™ (GenScript).[14]

Evaluation of protective efficacy

We subcutaneously injected 8 mice with 30 μg of rYbgF, rTolC, or *R. rickettsii* WCA (positive control) in 200 μL of phosphate-buffered saline (PBS), or PBS mixed with complete Freund's adjuvant (negative control; Sigma-Aldrich). Mice were intraperitoneally (i.p.) boosted with 20 μg of cognate antigen in 200 μL of PBS or PBS mixed with incomplete Freund's adjuvant 28 and 42 days after the primary immunization. On day 14 after the last immunization, each mouse was challenged i.p. with 6×10^5 plaque forming units (PFU) of *R. rickettsii*. Six days after challenge, 2 mice were sacrificed and their spleens, livers, and lungs collected for analysis. Tissues were fixed with formalin and embedded in paraffin. Sections were routinely stained with hematoxylin and eosin and examined by light microscopy. For the other 6 mice, each spleen was weighed and the rickettsial load in 20 mg of spleen, liver, or lung was determined using qPCR assays.[33]

Humoral immune analysis

Levels of IgG1 and IgG2a were determined by indirect enzyme-linked immunosorbent assays (ELISAs) using cognate antigens.[14] Blood samples were collected from the tail veins of 6 mice per group on day 0 and then days 14, 28, 42, and 56 after primary immunization. Each serum sample was serially diluted 2-fold for the determination of specific antibody titers by ELISA.[17] The cutoff value was set according to a previous study.[14]

Serum neutralization of R. rickettsii

The human endothelial hybrid cell line EA.hy926 was used as host cells of *R. rickettsii*. Cells were previously cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) containing 1% heat-inactivated fetal bovine serum (FBS; Hyclone) at 33°C/5% CO_2. At 14 days after the last immunization, the sera from 6 mice were collected and the pooled sera inactivated at 56°C for 30 min and filter sterilized.[36] We mixed 150 μL of sera with *R. rickettsii* cells in 150 μL of DMEM (1.0×10^6 PFU/mL) at room temperature for 60 min. This mixture was added to 9.7×10^5 cells in 2.7 mL of DMEM containing 1% heat-inactivated FBS; the mixture was then aliquoted into 3 wells of a 24-well plate (Corning) and cultured at 33°C for 6 h.[36] The supernatant was removed and DNA was extracted from the remaining cells using a DNeasy Blood & Tissue Kit (Qiagen). The purified DNA samples were then subjected to *R. rickettsii*-specific qPCR assays.[33]

Proliferation and cytokine production of T cells

Fifteen mice were injected i.p. with a sublethal dose (5×10^6 PFU/mouse) of *R. rickettsii* (infected mice) or PBS (uninfected mice). Fifteen days after injection, the mice were sacrificed and their spleens collected for isolation of $CD4^+$ or $CD8^+$ T cells using mouse $CD4^+$ or $CD8^+$ T cell isolation kits, respectively (Miltenyi). Mononuclear cells isolated from 5 naïve mouse spleens were used as antigen-presenting cells.[37] Isolated $CD4^+$ or $CD8^+$ T cells were suspended in DMEM; 6×10^5 T cells and 3×10^5 mononuclear cells were added to each well of 24-well plates. Cells in each well were stimulated with 10 μg of each antigen or 2.5 μg of concanavalin A (ConA; positive control), with 3 replicate wells used per sample. After a 48-h incubation at 37°C/5% CO_2, cytokines TNF-α, IFN-γ, IL-2, -4, -5, -6, and -10 in 500 μL of supernatant aspirated from each well were measured using a CBA Mouse Th1/Th2 cytokine kit (BD PharMingen). The remaining cells in each well were used to detect the proliferation of $CD4^+$ or $CD8^+$ T cells using a Cell Counting Kit-8 (CCK-8; Dojindo).

Microscopic detection of YbgF and TolC in R. rickettsii

R. rickettsii were cultured in Vero cells, and immune sera were prepared in C3H/HeN mice as previously described.[17] YbgF and TolC on the surface of *R. rickettsii* were detected using IFA as previously described.[17]

For TEM analysis, *R. rickettsii* cultured in Vero cells were fixed (0.5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate, 0.3% picric acid, pH 7.4) for 4 h on ice. After four washes in 0.1 M PBS, fixed cells were dehydrated with alcohol and permeabilized with a mix of LR White (Spi Supplies) and alcohol. Cells were then embedded in SPI-Pon 812 resin (Spi Supplies) and transferred to a 200-mesh nickel gird (BeiJingZhongXingBaiRui Technology Co., Ltd.) following standard methods [38]. Grids were incubated with sera (diluted 1:100) from mice immunized with rYbgF, rTolC, or PBS (negative control) for 2 h. Grids were then incubated with goat anti-mouse IgG conjugated to 10-nm colloidal gold particles (1:20; Aurion; EMS) for 2 h at room temperature. The grids were fixed in 1% glutaraldehyde for 10 min and then stained with uranyl acetate (Spi Supplies) and lead citrate (Spi Supplies) according to standard methods.[38] Grids were examined using an electron microscope (Hitachi H-7650; Hitachi Chemical Co., Ltd.) at 80 kV.

Statistical analysis

All statistics were computed using SAS statistical software (version 9.1, SAS Institute Inc.). The statistical significances of *the* differences in rickettsial numbers produced by qPCR and the levels of proliferation or cytokine secretion of T cells were assayed using the *T* test or Wilcoxon 2-sample test according to their normality and homogeneity of variance. A value of $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the National Basic Research Program of China (Grants 2010CB530200/2010CB530205), the National Natural Science Foundation of China (Grants 81371767 and 31170161), and the Natural Science and Technology Major Project of China (Grant 2013ZX10004803).

References

1. Walker DH. The realities of biodefense vaccines against *Rickettsia*. Vaccine 2009; 27:D52-D5; PMID:19837287; http://dx.doi.org/10.1016/j.vaccine.2009.07.045
2. Harrell GT, Aikawa JK. Pathogenesis of circulatory failure in Rocky Mountain spotted fever: alterations in the blood volume and the thiocyanate space at various stages of the disease. Arch Int Med 1949; 83:331; PMID:18112395; http://dx.doi.org/10.1001/archinte.1949.00220320085007
3. Walker DH. Biology of rickettsial diseases. Vol. I and Vol. II. CRC Press, Inc., 1988.
4. Parker R. Rocky Mountain spotted fever: results of fifteen years' prophylactic vaccination. Am J Trop Med Hygiene 1941; 1:369-83.
5. Cox HR. Cultivation of Rickettsiae of the Rocky Mountain Spotted Fever, typhus and Q Fever groups in the embryonic tissues of developing chicks. Science (New York, NY) 1941; 94:399.
6. Kenyon RH, Pedersen C. Preparation of Rocky Mountain spotted fever vaccine suitable for human immunization. J Clin Microbiol 1975; 1:500-3; PMID:809483
7. Clements M, Wisseman C, Woodward T, Fiset P, Dumler J, McNamee W, Black RE, Rooney J, Hughes TP, Levine MM. Reactogenicity, immunogenicity, and efficacy of a chick embryo cell-derived vaccine for Rocky Mountain spotted fever. J Infect Dis 1983; 148:922-30; PMID:6415182; http://dx.doi.org/10.1093/infdis/148.5.922
8. DuPont H, Hornick R, Dawkins A, Heiner G, Fabrikant I, Wisseman CL Jr, Woodward TE. Rocky Mountain spotted fever: a comparative study of the active immunity induced by inactivated and viable pathogenic *Rickettsia rickettsii*. J Infect Dis 1973; 128:340-4; PMID:4199563
9. Li H, Walker DH. rOmpA is a critical protein for the adhesion of *Rickettsia rickettsii* to host cells. Microb Pathog 1998; 24:289-98; PMID:9600861
10. Riley SP, Goh KC, Hermanas TM, Cardwell MM, Chan YG, Martinez JJ. The *Rickettsia conorii* autotransporter protein Sca1 promotes adherence to nonphagocytic mammalian cells. Infect Immun 2010; 78:1895-904; PMID:20176791; http://dx.doi.org/10.1128/IAI.01165-09
11. Park H, Lee JH, Gouin E, Cossart P, Izard T. The Rickettsia surface cell antigen 4 applies mimicry to bind to and activate vinculin. J Biolog Chem 2011; 286:35096-103; PMID:21841197; http://dx.doi.org/10.1074/jbc.M111.263855
12. Chan YG-Y, Riley SP, Chen E, Martinez JJ. Molecular basis of immunity to rickettsial infection conferred through outer membrane protein B. Infect Immun 2011; 79:2303-13; PMID:21444665; http://dx.doi.org/10.1128/IAI.01324-10
13. Crocquet-Valdes P, Dıaz-Montero C, Feng H, Li H, Barrett A, Walker D. Immunization with a portion of rickettsial outer membrane protein A stimulates protective immunity against spotted fever rickettsiosis. Vaccine 2001; 20:979-88; PMID:11738766; http://dx.doi.org/10.1016/S0264-410X(01)00377-2
14. Qi Y, Xiong X, Duan C, Jiao J, Gong W, Wen B. Recombinant protein YbgF induces protective immunity against *Rickettsia heilongjiangensis* infection in C3H/HeN mice. Vaccine 2013; 31:5643-50; PMID:24113261; http://dx.doi.org/10.1016/j.vaccine.2013.09.064
15. Feng H-M, Whitworth T, Olano JP, Popov VL, Walker DH. Fc-dependent polyclonal antibodies and antibodies to outer membrane proteins A and B, but not to lipopolysaccharide, protect SCID mice against fatal *Rickettsia conorii* infection. Infect Immun 2004; 72:2222-8
16. Deringer JR, Chen C, Samuel JE, Brown WC. Immunoreactive *Coxiella burnetii* Nine Mile proteins separated by 2D electrophoresis and identified by tandem mass spectrometry. Microbiology 2011; 157:526-42; PMID:21030434
17. Qi Y, Xiong X, Wang X, Duan C, Jia Y, Jiao J, Gong W, Wen B. Proteome analysis and serological characterization of surface-exposed proteins of *Rickettsia heilongjiangensis*. PLoS One 2013; 8:e70440; PMID:23894656; http://dx.doi.org/10.1371/journal.pone.0070440
18. Dubuisson J-F, Vianney A, Hugouvieux-Cotte-Pattat N, Lazzaroni JC. Tol-Pal proteins are critical cell envelope components of *Erwinia chrysanthemi* affecting cell morphology and virulence. Microbiology 2005; 151:3337-47; PMID:16207916
19. Zgurskaya HI, Krishnamoorthy G, Ntreh A, Lu S. Mechanism and function of the outer membrane channel TolC in multidrug resistance and physiology of enterobacteria. Front Microbiol 2011; 2:189; PMID:21954395
20. David MM, Xia Z. Measuring opsonic phagocytosis via Fcγ receptors and complement receptors on macrophages. Curr Protoc Immunol 2011; Chapter:Unit-14.27; PMID:22048802; http://dx.doi.org/10.1002/0471142735.im1427s95
21. Kipps TJ, Parham P, Punt J, Herzenberg L. Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. J Exp Med 1985; 161:1-17; PMID:3918141; http://dx.doi.org/10.1084/jem.161.1.1
22. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR γ chain deletion results in pleiotrophic effector cell defects. Cell 1994; 76:519-29; PMID:8313472; http://dx.doi.org/10.1016/0092-8674(94)90115-5
23. Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, Makarova N, Perez DR, Macdonald GH, McCullers JA. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol 2006; 13:981-90; PMID:16960108; http://dx.doi.org/10.1128/CVI.00156-06
24. Xiong X, Meng Y, Wang X, Qi Y, Li J, Duan C, Wen B. Mice immunized with bone marrow-derived dendritic cells stimulated with recombinant *Coxiella burnetii* Com1 and Mip demonstrate enhanced bacterial clearance in association with a Th1 immune response. Vaccine 2012; 30:6809-15; PMID:23000126; http://dx.doi.org/10.1016/j.vaccine.2012.09.017
25. Crocquet-Valdes PA, Thirumalapura NR, Ismail N, Yu X, Saito TB, Stevenson HL, Pietzsch CA, Thomas S, Walker DH. Immunization with Ehrlichia P28 Outer Membrane Proteins Confers Protection in a Mouse Model of Ehrlichiosis. Clin Vaccine Immunol 2011; 18:2018-25; PMID:22030371; http://dx.doi.org/10.1128/CVI.05292-11
26. Walker DH, Olano JP, Feng H-M. Critical role of cytotoxic T lymphocytes in immune clearance of rickettsial infection. Infect Immun 2001; 69:1841-6; PMID:11179362; http://dx.doi.org/10.1128/IAI.69.3.1841-1846.2001
27. Gazzinelli R, Hakim F, Hieny S, Shearer G, Sher A. Synergistic role of $CD4^+$ and $CD8^+$ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. J Immunol 1991; 146:286-92; PMID:1670604
28. Feng H-M, Walker DH. Mechanisms of intracellular killing of *Rickettsia conorii* in infected human endothelial cells, hepatocytes, and macrophages. Infect Immun 2000; 68:6729-36; PMID:11083788
29. Feng H-M, Popov VL, Walker DH. Depletion of gamma interferon and tumor necrosis factor alpha in mice with *Rickettsia conorii*-infected endothelium: impairment of rickettsicidal nitric oxide production resulting in fatal, overwhelming rickettsial disease. Infect Immun 1994; 62:1952-60; PMID:8168962
30. de Sousa R, Ismail N, Dória NS, França A, Amaro M, Anes M, Poças J, Coelho R, Torgal J, Bacellar F, et al. Intralesional expression of mRNA of interferon-γ, tumor necrosis factor-α, interleukin-10, nitric oxide synthase, indoleamine-2, 3-dioxygenase, and RANTES is a major immune effector in Mediterranean spotted fever rickettsiosis. J Infect Dis 2007; 196:770-81; PMID:17674321; http://dx.doi.org/10.1086/519739
31. Feng H, Walker DH. Interferon-gamma and tumor necrosis factor-alpha exert their antirickettsial effect via induction of synthesis of nitric oxide. Am J Pathol 1993; 143:1016; PMID:8213997
32. Ammerman NC, Beier-Sexton M, Azad AF. Laboratory maintenance of Rickettsia rickettsii. Curr Protoc Microbiol 2008; Chapter 3:Unit 3A.5.; PMID:19016440; http://dx.doi.org/10.1002/9780471729259.mc03a05s11
33. Niu DS CM, Wen BH, Li QF, Qiu L, Zhang JB. Development of quantitative PCR specific for *Ricekttsia rickettsii*. Chinese J Epidemiol 2006 6:526-9
34. Weinberg EH, Stakebake JR, Gerone PJ. Plaque assay for *Rickettsia rickettsii*. J Bacteriol 1969; 98:398-402
35. Balraj P, Nappez C, Raoult D, Renesto P. Western-blot detection of RickA within spotted fever group rickettsiae using a specific monoclonal antibody. FEMS Microbiol Lett 2008; 286:257-62; PMID:18657112; http://dx.doi.org/10.1111/j.1574-6968.2008.01283.x
36. Hanson BA. Effect of immune serum on infectivity of *Rickettsia tsutsugamushi*. Infect Immun 1983; 42:341-9; PMID:6137458
37. Kruisbeek AM. Isolation of mouse mononuclear cells. Current Protocols in Immunology 2001:[chapter 3: Unit 3.1]. doi: 10.1002/0471142735.im0301s39
38. Vellaiswamy M, Campagna B, Raoult D. Transmission electron microscopy as a tool for exploring bacterial proteins: model of RickA in *Rickettsia conorii*. New Microbiologica 2011; 34:209-18; PMID:21617834