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Discovery of novel cross-protective *Rickettsia prowazekii* T-cell antigens using a combined reverse vaccinology and *in vivo* screening approach

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Abstract

Rickettsial agents are some of the most lethal pathogens known to man. Among them, *Rickettsia prowazekii* is a select agent with potential use for bioterrorism; yet, there is no anti-*Rickettsia* vaccine commercially available. Owing to the obligate intracellular lifestyle of rickettsiae, CD8⁺ T cells are indispensable for protective cellular immunity. Furthermore, T cells can mediate cross-protective immunity between different pathogenic *Rickettsia*, a finding consistent with the remarkable similarity among rickettsial genomes. However, *Rickettsia* T cell antigens remain unidentified. In the present study, we report an algorithm that allowed us to identify and validate four novel *R. prowazekii* vaccine antigen candidates recognized by CD8⁺ T cells from a set of twelve *in silico*-defined protein targets. Our results highlight the importance of combining proteasome-processing as well as MHC class-I-binding predictions. The novel rickettsial vaccine candidate antigens, RP778, RP739, RP598, and RP403, protected mice against a lethal challenge with *R. typhi*, which is indicative of cross-protective immunity within the typhus group rickettsiae. Together, our findings validate a reverse vaccinology approach as a viable strategy to identify protective rickettsial antigens and highlight the feasibility of a subunit vaccine that triggers T-cell-mediated cross-protection among diverse rickettsiae.

Keywords

Rickettsia; vaccine; reverse vaccinology; CD8⁺ T cells

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Conflicts of interest

None to declare.

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1. Introduction

Rickettsia prowazekii, a louse-borne obligate intracellular bacterium, is the agent of epidemic typhus, which is one of the most lethal pathogens known to humans [1]. Due to lethality as high as 60% and its prior use as a bioweapon [1, 2], it is classified as a category B priority pathogen and a CDC select agent. Unfortunately, an effective vaccine, a deterrent to its weaponization, is not currently available for this or any of the other rickettsial diseases. The potential impact of vaccines against these pathogens is highlighted by two facts: 1) there are no commercial methods for the acute diagnosis of rickettsioses, and 2) all rickettsial diseases present with non-specific initial clinical symptoms. In appropriate animal models, CD8⁺ T cells are critical effectors of protective anti-*Rickettsia* immunity [3–5]. Also, previous work explored rickettsial surface proteins OmpA [6, 7] and OmpB [8, 9] as potential targets for CD8⁺ T cells; however, the selection of these proteins for testing was based on the fact that they are immunodominant for the humoral immune response, which we now know is unlikely to be a good guide to select antigens recognized by CD8⁺ T cells [10, 11]. No other antigens that trigger T-cell-mediated protective immunity have been identified since. To address this gap, we recently reported the proof-of-principle of an *in vivo* screening approach to identify antigens recognized by CD8⁺ T cells [12]. Herein, we extend this work through refinement of our screening platform and the identification of new antigens of *R. prowazekii* that stimulate a cross-protective response against the closely related *R. typhi*, the agent of flea-borne murine typhus, which is the most prevalent and neglected of the rickettsioses [13]. We performed an *in silico* analysis of the entire *R. prowazekii* ORFeome (834 proteins) to identify and prioritize potential targets for CD8⁺ T cells. From a set of twelve *in silico*-defined antigenic targets, we identified and validated four novel cross-protective vaccine candidates.

2. Materials and Methods

2.1. Bacteria

R. typhi (Wilmington strain) working stock was produced in a CDC-certified biosafety level 3 (BSL3) laboratory by cultivation in specific pathogen free embryonated chicken eggs. *Rickettsia* present in the stock was quantified by plaque assay [14], and the LD₅₀ was determined experimentally in C3H/HeN mice using the Spearman-Kärber method; LD₅₀ and confidence intervals were: LD₅₀= 8.13 ×10³ PFU/ml, upper end point=11.5×10³ PFU/ml, and lower end point= 5.75 ×10³ PFU/ml.

2.2. Immunoinformatics analysis

834 protein sequences from *Rickettsia prowazekii* strain Madrid E (Gene Bank ID AJ235269.1) were analyzed for the prediction of 9-mer peptides restricted to MHC class-I mouse allele H-2K^k using the following servers: NetMHCpan (<http://www.cbs.dtu.dk/services/NetMHCpan/>), IEBD-ANN (http://tools.immuneepitope.org/main/html/tcell_tools.html), and SYFPEITHI (<http://www.syfpeithi.de/>) [15–18]. Only proteins containing peptides predicted to be strong binders were considered for further analysis. For predictions performed using NetMHCpan and IEBD-ANN, only peptides with IC₅₀ values 50 nM were considered; for SYFPEITHI, only peptides with an S-score of 21 and higher

were included; this score was arbitrarily chosen and it represents 70% of the influenza A matrix protein epitope GILGFVFTL S-score. Rickettsial proteins were further analyzed using RANKPEP (<http://imed.med.ucm.es/Tools/rankpep.html>), which combines MHC class-I-binding affinity and proteasome processing [19]. We used RANKPEP to evaluate the likelihood of peptides predicted by NetMHCpan, IEBD-ANN and SYFPEITHI to be generated via proteasome-processing as we previously described [12]. Analysis of similarity to human and mouse proteins, as well as HLA class-I binding, were performed using Vaxign and Vaxitope, respectively; these programs are available through the Vaccine Investigation and Online Information Network (VIOLIN, <http://www.violinet.org/>) [20]. HLA class-I-binding data was used to calculate a score to re-rank the selected *in silico* defined targets previously ranked with the mouse MHC class-I binding predictions; the score resulted from dividing the number of HLA class-I epitopes predicted by the number of amino acids in the rickettsial protein (length adjustment).

2.3. Screening and validation of *in silico* vaccine targets

We used an established mouse model of typhus [5] consisting of C3H/HeN mice infected intravenously through the tail vein (i.v.) with *R. typhi*, which is phylogenetically closely related to *R. prowazekii*, the other member of the typhus group *Rickettsia*. This model has a consistent dose-dependent lethality, which is critical for vaccine testing, and faithfully replicates the clinical and pathological characteristics of epidemic typhus in humans. C3H/HeN mice were housed in an animal biosafety level-3 (ABSL3). For immunization, we followed a short immunization protocol that enhances CD8⁺ T cell responses [21] as we did in our previous report [12]. Generation of antigen presenting (APCs) cell lines expressing rickettsial proteins is described in the supplementary methods section. Each mouse received 4.5×10^5 cells of cell suspension i.v. and intramuscularly (i.m.); 5 days later, mice received the same dose of cells i.m. and intraperitoneally (i.p.). Seven days after the second immunization, mice were infected i.v. with 5 or 6 LD₅₀ of *R. typhi*. Animals were monitored for clinical symptoms and mortality for 21 days or euthanized after 7 days for rickettsia load assessment in the lungs. Rickettsiae were measured by quantitative real-time PCR using a validated assay previously described [12]. We followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch (protocol number: 0903026).

2.4. Statistics

The proportion of surviving animals was analyzed with the Log-rank (Mantel-Cox) test. Mean absolute counts of CD8⁺ T-cell subpopulations were compared using one-way analysis of variance (ANOVA) followed by Dunnett's correction for multiple comparisons (GraphPad Prism, version 6).

3. Results

3.1. Rank of proteins encompassing MHC class-I-binding peptides

We recently described an *in silico* analysis strategy for the discovery of antigens with immunogenicity potential towards CD8⁺ T cells based on the identification of proteins

encompassing predicted high-affinity proteasome-derived MHC class-I-binding peptides [12]. In the present study, this *in silico* approach was extended to the entire *R. prowazekii* ORFeome (the collection of all ORFs from a microbe) (Fig. 1). A total of 834 *R. prowazekii* protein sequences were analyzed, and rickettsial proteins were ranked according to the described algorithm, which includes information about the number of predicted peptides per protein, their likelihood of being generated by the proteasome, and their predicted affinity for MHC class I (H-2K^k, which is the haplotype of C3H mice). Analysis of the relative frequency of high-affinity proteasome-derived MHC class-I-binding peptides among rickettsial proteins showed the following: 21.5% (179/834) of all *R. prowazekii* proteins did not include any peptide that fulfilled our inclusion criteria; 30% (250/834) contained one; 25% (209/834) contained two; 14.9% (124/834) contained three; 6.8% (57/834) contained four; and 1.9% (16/834) contained five proteasome-derived peptides.

3.2. Selection of *in silico*-defined antigen candidates

Further *in silico* analysis was restricted to the top 100 rickettsial proteins (Supplementary table 1). To limit the number of proteins to be tested as vaccine candidates with potential for cross-protection, we first used BLASTp to search for the presence of orthologs in other pathogenic *Rickettsia* species: *R. typhi* strain Wilmington, *R. conorii* strain Malish 7, and *R. rickettsii* strain ‘Sheila Smith’ (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Proteins with a query coverage ≥ 60% and sequence identity ≥ 60% were considered orthologs to *R. prowazekii* proteins; only one protein did not meet these inclusion criteria, and three others were absent in spotted fever group (SFG) *Rickettsia*. Next, we excluded proteins with similarities to human and mouse proteins. This component of the analysis was performed using Vaxign, available through the Vaccine Investigation and Online Information Network (VIOLIN, <http://www.violinet.org/>) [20]. We found 45 proteins with homology to mouse or human; since the area of homology of 8 of these proteins was of only 16 amino acids or less (with 4 or fewer regions like this per protein), these proteins were also included in our final ranking list; the rationale was that, if they are protective, the epitopes conferring protection might be outside the regions of homology. Until this point of our analysis, *in silico* vaccine targets were defined based on the presence of proteasome-derived peptides that are also strong binders to the MHC class-I mouse allele H-2K^k. In a final step, we assigned an HLA class-I-binding score to the 63 *in silico*-defined antigen candidates. This new score incorporated the capability of the selected proteins to bind HLA class-I alleles; the score resulted from dividing the number of HLA class-I epitopes predicted by Vaxitope from VIOLIN, regardless of the allele or binding affinity, by the number of amino acids in the rickettsial protein (length adjustment). Compared to the original ranking, which was only mouse-based, several proteins had striking changes in their ranking positions when binding to HLA class-I alleles was incorporated (Table 1).

3.3. *In vivo* testing of candidate antigens

To assess the predictive power of our algorithm prior to future comprehensive testing of all candidates, we first looked for proteins that were also predicted to encompass MHC class-II-binding peptides since CD4⁺ T cell responses are likely to be important in memory generation; this analysis (described in the supplementary methods section) resulted in 21 proteins (table 1). We then selected 12 of those 21 proteins that had already been transferred

to our new destination vector for *in vivo* testing. These 12 *R. prowazekii* ORFs represent the range of immunogenic potential predicted after applying the HLA class-I-binding score since six proteins were ranked among the top 20 and the rest were ranked at position 22 or below as shown in table 1. For the identification of protective antigens, we followed our recently described screening strategy [12]. Mice were immunized with APCs expressing individual ORFs (1.5×10^5 APCs per ORF/mouse), pooled in sets of three. Reduction of *Rickettsia* load at 7 days post infection (dpi) was used as surrogate of protection (Fig. 2A); although no statistically significant differences between control and immunized groups were observed, two potentially protective pools were suspected based on a lower mean value of the rickettsial load in lungs (pool #1 and pool #3). These pools were deconvoluted in order to identify ORF(s) responsible for this potential protective effect. Mice were immunized with 4.5×10^5 APCs individually expressing RP739, RP403, RP734, RP598, RP778 or RP559, and challenged as described above. None of the tested ORFs induced significant reduction of *Rickettsia* load; yet, we did observe remarkable differences in the clinical status of mice in different immunization groups at the time when we sacrificed animals to procure samples for bacterial load determination (7 dpi): some animals were found dead, some others were moribund (unresponsive to stimuli), while others were active. In figure 2B, we labeled individual mice accordingly to show that, unexpectedly, the rickettsial load was not a predictor of clinical behavior by itself; for example, 6 out of 7 mice from the RP739 vaccinated group were active and clinically healthy. These observations questioned the rickettsial load reduction as the most appropriate single readout for screening vaccine targets. We then proceeded to test whether survival analysis would be more informative.

3.4. *In silico* defined vaccine candidates confer protection against a lethal challenge and stimulate CD8⁺ T cell responses

To test antigen screening through survival analysis, we selected a subgroup of antigens from those in figure 2B with more active mice and lower mean values of rickettsial load among mice classified as moribund (RP739, RP403, RP734, RP598); RP778 was also included as a control not meeting those criteria (with the expectation that it would not be protective). Bacterial load formed part of the selection criteria in this case because it allowed us to select a subset of candidates when combined with clinical criteria (not by itself). We immunized mice with RP739, RP403, RP734, RP598 or RP778 and challenged them with 5LD₅₀ of *R. typhi*. Mice were monitored for signs of illness and death for 21 days. As shown in figure 3A and 3B, 92% to 100% of control animals succumbed to the rickettsial infection by day 9; in contrast, all tested ORFs decreased mortality and provided significant protection against a lethal challenge with *R. typhi*. RP778 and RP739 provided 100% protection while RP403 protected 62.5% of mice; immunization with RP598 or RP734 resulted in 37.5% and 25% survival, respectively. Thus, it appears that bacterial load at a fixed time point (Fig. 2) is not always sufficiently informative as a surrogate for protection. This concept is further supported by the fact that RP778 demonstrated to be one of the best antigens when assessed through survival analysis while the rickettsial load analysis would have discarded it as a relevant protective antigen.

Since our *in silico* approach for identification of potential targets and our immunization strategy were designed to be biased towards MHC class-I presentation, we analyzed

CD3⁺CD8⁺ cells from animals immunized with APCs expressing RP739, RP403, RP734, RP598 or RP778 at 7 dpi. Antigen-experienced CD8⁺ T cells (CD3⁺CD8⁺CD44^{high}) from immunized animals showed increased expression of IFN- γ (Fig. 4A), ~1.5 fold-increase or higher, after *Rickettsia* challenge compared to control animals immunized with APCs expressing an irrelevant antigen (luciferase); however, this change was only statistically significant for RP778 ($p < 0.01$) (Fig. 4B). Memory-type CD8⁺ T-cells (CD44^{high}CD127^{high}) were also increased, particularly in RP778-immune mice (Fig. 4C), and significantly larger numbers of IFN- γ -producing cells were observed for this subpopulation (Fig. 4D and E). Although tested in a context that favors CD8⁺ T cell responses, RP778 also induced significant IFN- γ expression in antigen-experienced CD4⁺ T cells (CD3⁺CD4⁺CD44^{high}) (Supplementary figure 1).

3.5. Protective antigens are not necessarily synergistic or even additive

Intrinsically, our strategy for discovery of protective antigens allows for the detection of cross-protective responses since the rickettsial proteins used for immunization are derived from *R. prowazekii* but mice are challenged with *R. typhi*, the other member of the typhus group rickettsiae. Next, we asked whether the novel protective antigens could be combined to produce larger T-cell responses to support the complete protection afforded by RP739 and RP778. We immunized mice with a pool of the best candidates found in the present study (those protecting more than 1/3 of challenged mice), RP778, RP739, RP403 and RP598, together with RP884, a protective antigen that we previously reported [12]. Animals were then challenged with 5LD₅₀ of *R. typhi*. After 21 days of observation, only 62.5% of mice were protected (Fig. 5), which suggests that some antigenic combinations may not be synergistic and may actually result in diminished protection. In support of this hypothesis, immunization of mice with a similar antigen cocktail without RP884 increased the protection against lethal challenge (Fig 5).

4. Discussion

This study describes the first application of a reverse vaccinology approach [22] to the discovery of protective *Rickettsia* antigens that are recognized by CD8⁺ T cells. The entire *R. prowazekii* ORFeome (834 proteins) was analyzed for the prediction of proteins encompassing high-affinity, proteasome-derived, MHC class-I-binding peptides. From 63 top-ranked proteins meeting these criteria, we added another layer of selection (presence of predicted epitopes for MHC class-II) to decrease the number of candidates to be tested (Table 1) to 21 proteins. From this smaller group, we tested 12 proteins whose genes had already been cloned in our new destination vector; we identified four of these proteins as novel protective antigens (RP778, RP739, RP403, RP598). The critical role of CD8⁺ T cells in the recovery from rickettsial infections has been previously demonstrated in murine models [3–5]. Since CD8⁺ T cells play a key role in protective immunity against many intracellular pathogens, including respiratory viruses, CMV, HIV, *Plasmodium* spp., and *M. tuberculosis*, among others [23–29], it has been proposed that the rational design of the next generation of vaccines should include T cell epitopes [30]. However, finding an efficient approach to identify protective T cell antigens remains one major challenge in the development of T cell-based vaccines [30, 31]. We began to address this challenge by

combining *in silico* predictions with an *in vivo* screening method that we recently reported. In that work, we used our empirical antigen discovery data to formulate an *in silico* strategy that better predicted that empirical data. The resulting method highlights the importance of combining proteasome-processing as well as MHC class-I-binding predictions [12]. In agreement with this concept, 7 out of 16 rickettsial proteins containing all 5 of the top 5 proteasome-derived peptides predicted by RANKPEP were included in our final top-ranked 63 potential antigen targets. This observation is also reinforced by the fact that all rickettsial protective antigens identified thus far (RP884, RP778, RP739, RP403, and RP598) have at least 4 predicted proteasome-derived peptides. To further support the potential relevance of the selected antigens for human application, we added another layer of information to our *in silico* analysis by incorporating human MHC (HLA class-I) binding data. In this regard, RP739, one of our best candidates as assessed by survival (Fig. 3A), provides an interesting scenario as its position in the protein rank changed significantly when the HLA-binding score was introduced, moving from position 21 in the mouse-only ranking to position 2 in the mouse-human combined ranking (Table 1). This finding suggests that, notwithstanding target vaccine proteins are being tested using a relevant mouse model of rickettsiosis [5], the inclusion of HLA binding data not only seems to help refine the predictive power of the algorithm, but could also contribute to the prioritization of *in silico*-defined vaccine targets that may be more readily applicable to humans. Predictions for class-II epitopes were not included in earlier steps of our algorithm because those tools are not as developed as those for epitopes that bind MHC class-I. However, including that information as a measure to further narrow down candidates to be tested appears to be justified by our results. Although it could be argued that including peptides presented to CD4⁺ T cells would favor antibody responses, this was not the case in our studies. As supportive evidence that T cells, and not antibodies, are the effectors of protection, antibodies against protective antigens were assessed by indirect immunofluorescence but were not detected (data not shown). Furthermore, only one of the four novel protective antigens (RP739) is predicted to be a membrane protein and, therefore, the only possible target of protective humoral immunity. These data also supports the notion that subcellular localization is not as critical for T cell antigens as it is for B cell antigens.

Although the accuracy of computer algorithms for prediction of CD8⁺ T cell epitopes has notably improved in recent years, some drawbacks persist such as underestimation of the breadth of CD8⁺ epitopes [32] and the fact that antigen discovery across entire proteomes using overlapping peptides is not feasible for all pathogens, especially those with large genomes [22]. Whole-protein-based approaches for T cell antigen discovery have been proposed as a cost-effective alternative [31], and our strategy is in line with this proposal. The novelty of our approach lies in the use of *in silico* predictions of MHC class-I-binding peptides for the selection of target antigens to be tested as whole proteins using APCs modified to stably express candidate proteins. Although, the mechanism by which our immunization strategy results in T-cell priming and protection is yet to be addressed, we hypothesize that our platform is allowing the APC's intrinsic cellular machinery to process and select the antigenic determinants to be presented to T cells, which is in agreement with the concept that the actual array of critical antigenic determinants driving protective T cell responses might be revealed only if antigens are naturally processed [30].

An important finding of our study is the fact that antigens that are individually protective are not necessarily additive or synergistic; they may in fact be detrimental as shown for the addition of RP884 to an antigen cocktail (Fig. 5). Analogous findings have also been published [33]. Such data may be explained by changes in immunodominance of the most protective antigen when other antigens that are not as protective are also present; conceivably, they may even compete for the same MHC molecules. For this reason, in the near future, we plan to screen candidate antigens individually from the beginning, as opposed to pools. It will also be important to complete the screening of the remaining *in silico*-defined vaccine targets in order to verify the predictive power of our algorithm and to identify sufficient novel antigens that can be assessed in multiple permutations to define antigenic combinations that result in protective immune synergy.

Rickettsiae in the two major pathogenic groups, SFG and typhus, have many similar genes; the genomes *R. prowazekii*, *R. typhi*, and *R. conorii* share 775 genes [34, 35]. The similarity of the *R. prowazekii* protective antigens tested here in comparison with their orthologs in *R. conorii* is significant: 90% for RP884, 89% for RP778, 93% for RP739, 73% for RP403 and 88% for RP598. When tested as a pool, these *R. prowazekii* antigens protected 62.5% of immunized mice against a lethal challenge with *R. conorii*, a SFG *Rickettsia* (data not shown). Our plan now is to further validate this level of cross-protection across different groups and to identify novel cross-protective antigens for a subunit vaccine that triggers T-cell-mediated cross-protection among phylogenetically distant *Rickettsia*. Such a vaccine would have wider applicability since it would simultaneously address the emergence and re-emergence of SFG rickettsiae in the Americas, Europe, Asia, Africa, and Australia [36–39] in addition to the highly neglected murine typhus and the need for a vaccine against epidemic typhus as a deterrent to the weaponization of *R. prowazekii*.

We believe that our approach might have broader application beyond the *Rickettsia* field; *in vivo* screening with APCs expressing microbial proteins selected based on the prediction of high-affinity MHC class-I-binding peptides that are also predicted to be processed by the proteasome should be applicable to the rational discovery of T cell protective antigens of other intracellular pathogens. Future experiments will seek to further characterize the four novel rickettsial vaccine targets identified here in order to generate tools such as MHC tetramers for tracking antigen-specific protective responses, and to identify protective epitopes relevant for human vaccination. Initial steps towards the preclinical validation of *Rickettsia*-vaccine targets derived from this and future studies will involve assessment of the *in vivo* protection and comprehensive delineation of human *Rickettsia*-specific CD8⁺ T cell responses using the humanized BLT mouse model, which, unlike the CH3 mouse model, supports infection by highly pathogenic *R. prowazekii* and *R. rickettsii*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Flow diagram of the discovery process for antigens recognized by CD8⁺ T cells.

Figure 2. *In vivo* testing of *in silico*-defined vaccine targets

(A) Antigen presenting cell (APC) lines individually expressing selected *R. prowazekii* open reading frames (ORFs) were combined in 4 pools as follows: Pool 1 (RP739, RP403, RP734), Pool 2 (RP540, RP006, RP246), Pool 3 (RP598, RP778, RP559), and Pool 4 (RP146, RP858, RP226). Mice were immunized with pooled APCs expressing *Rickettsia prowazekii* proteins (8 mice per group) or an irrelevant protein (luciferase, 5 mice per group). Seven days after immunization, mice were challenged with $6 \times \text{LD}_{50}$ of *R. typhi*. At 7 dpi, animals were terminated and rickettsial load in the lungs was measured using quantitative real time PCR (Q-PCR) targeting the mouse gene *Idhal6b* and the rickettsial gene *gItA*. Although no significant reduction of *Rickettsia* load was observed, pools 1 and 3 showed lower mean values. (B) *R. prowazekii* ORFs in pool 1 and 3 were individually tested as described above. No significant differences in the rickettsia load were detected after the deconvolution step; however, differences in clinical findings were observed among groups; they were active, moribund, or dead. We show individual data points with mean $\pm$ standard error of the mean (SEM).

Figure 3. Survival analysis allowed the identification of novel *R. prowazekii* protective antigens (A) Mice immunized with APCs expressing luciferase (control, n=8) or selected rickettsial proteins (n=8) were challenged with $5 \times \text{LD}_{50}$ of *R. typhi* and followed for 21 days to determine survival. 100% of RP739-immune mice ($p < 0.0001$); 62.5% of RP403-immune mice ($p = 0.0014$); 37.5% of RP598-immune mice ($p = 0.0413$); and 25% of RP734-immune mice ($p = 0.0015$) survived the lethal challenge while none of the control animals did. (B) RP778 was separately tested (because it did not meet our initial criteria for selection from analysis of the pools) as in A (12 mice per group); 100% of RP778-immune mice survived while only 8.3% of control animals did ($p < 0.0001$).

Figure 4. Novel rickettsial protective antigens are recognized by CD8⁺ T cells

Mice immunized with APCs expressing luciferase or *R. prowazekii* proteins were challenged with $5 \times \text{LD}_{50}$ of *R. typhi* and sacrificed at 7dpi (4 hours after i.p. injection of brefeldin A and monensin) to obtain splenocytes for flow cytometric analysis. Cells were stained with CD3, CD8, CD44, CD127, and IFN- γ to determine (A) the frequency and (B) the number of antigen experienced CD8⁺ T cells producing IFN- γ *ex vivo*. (C) Number of memory-type (CD127^{hi}CD44^{hi}) CD8⁺ T cells. (D) Number of memory-type CD8⁺ T cells producing IFN- γ . (E) Illustrative density plot of CD127 vs. CD44 expression with IFN- γ expression overlaid on the plot (black dots). Data in (B), (C), and (D) is presented as mean $\pm$ SEM from five mice per group. p values for comparisons against control mice are represented as follows: **p<0.01.

Figure 5. Inclusion of RP884 in an antigen cocktail decreases protection

APCs individually expressing selected *R. prowazekii* open reading frames (ORFs) RP778, RP739, RP598 and RP403 were combined in 2 pools based on the presence or absence of RP884. Mice were immunized with pooled rickettsial proteins (n=8) or with APCs expressing luciferase (control, n=8), challenged with $5 \times \text{LD}_{50}$ of *R. typhi* and followed for 21 days to determine survival. In the presence of RP884, the survival rate was 62.5% ($p=0.0090$); when RP884 was excluded from the pool, the survival rate was 87.5% ($p=0.0008$).

Table 1

Antigens from *R. prowazekii* predicted *in silico* to encompass MHC class-I-binding peptides.

Rickettsial protein	Annotated function	Predicted subcellular localization [*]	HLA class I- ranking	Proteasome /H2K ^k ranking	Number of proteasome-peptides
RP216	cytochrome D ubiquinol oxidase subunit I (cydA)	Cytoplasmic Membrane	1	45	3
🔴 RP739 ¹	ADP-ATP carrier protein	Cytoplasmic Membrane	2	21	4
RP047	hypothetical protein RP047	Cytoplasmic Membrane	3	51	4
🔴 RP403 ¹	hypothetical protein RP403	Cytoplasmic	4	2	5
RP884	Ferrochelatase	Cytoplasmic	5	1	4
RP718	lipid A biosynthesis lauroyl acyltransferase	Cytoplasmic	6	58	3
RP876	Lipoyltransferase	Cytoplasmic	7	42	5
🔴 RP734 ¹	ATP-dependent nuclease subunit A (addA)	Cytoplasmic	8	55	3
RP329	hypothetical protein RP329	Cytoplasmic Membrane	9	27	4
RP027	hypothetical protein RP027	Cytoplasmic	10	47	2
🔴 RP042	cell cycle protein MESJ (mesJ)	Cytoplasmic	11	7	4
RP339	minor teichoic acids biosynthesis protein ggab (ggab)	Cytoplasmic Membrane	12	31	3
RP864	hypothetical protein RP864	Cytoplasmic	13	39	4
🔴 RP540 ²	primosome assembly protein PriA	Cytoplasmic	14	28	4
🔴 RP006 ²	hypothetical protein RP006	Cytoplasmic	15	16	4
🔴 RP512	ribonucleotide-diphosphate reductase subunit beta	Cytoplasmic	16	60	3
RP120	hypothetical protein RP120	Unknown	17	56	3
RP804	F0F1 ATP synthase subunit delta	Cytoplasmic	18	62	3
🔴 RP246 ²	hypothetical protein RP246	Cytoplasmic Membrane	19	41	4
RP731	dephospho-CoA kinase	Cytoplasmic	20	4	5
RP048	putative inner membrane protein translocase component YidC	Cytoplasmic Membrane	21	24	3
🔴 RP598 ³	transcription-repair coupling factor	Cytoplasmic	22	12	4
🔴 RP778 ³	DNA polymerase III subunit alpha	Cytoplasmic	23	23	4
🔴 RP849	glycyl-tRNA synthetase subunit beta	Cytoplasmic	24	38	3

Rickettsial protein	Annotated function	Predicted subcellular localization*	HLA class I- ranking	Proteasome /H2K ^k ranking	Number of proteasome-peptides
RP029	recombination protein F	Cytoplasmic	25	14	3
RP431	hypothetical protein RP431	Cytoplasmic	26	17	4
RP572	excinuclease ABC subunit C	Cytoplasmic	27	34	3
🔴 RP758	hypothetical protein RP758	Cytoplasmic	28	37	3
RP367	hypothetical protein RP367	Cytoplasmic Membrane	29	29	4
🔴 RP559 ³	hypothetical protein RP559	Unknown	30	40	3
RP622	3-demethylubiquinone-9 3-methyltransferase	Cytoplasmic	31	49	3
RP071	transcriptional activator protein CZCR (czcR)	Cytoplasmic	32	44	4
RP170	acriflavin resistance protein D	Cytoplasmic Membrane	33	18	3
RP531	translation initiation factor IF-3	Cytoplasmic	34	57	3
RP441	hypothetical protein RP441	Cytoplasmic	35	54	4
RP014	hypothetical protein RP014	Periplasmic	36	11	5
RP822	hypothetical protein RP822	Cytoplasmic Membrane	37	5	4
🔴 RP203	excinuclease ABC subunit B	Cytoplasmic	38	9	3
RP635	DNA-directed RNA polymerase subunit alpha	Cytoplasmic	39	20	4
🔴 RP492	pyruvate phosphate dikinase	Cytoplasmic	40	10	4
🔴 RP146 ⁴	hypothetical protein RP146	Cytoplasmic	41	30	2
RP505	arabinose-5-phosphate isomerase	Cytoplasmic	42	8	5
RP753	aspartate kinase	Unknown	43	61	3
RP404	hypothetical protein RP404	Cytoplasmic	44	43	3
RP589	inorganic pyrophosphatase	Cytoplasmic	45	22	4
RP306	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	Cytoplasmic	46	26	4
RP320	ATP-dependent protease ATP-binding subunit	Cytoplasmic	47	32	3
RP030	hypothetical protein RP030	Cytoplasmic	48	53	3
RP314	alkaline protease secretion protein AprE	Cytoplasmic Membrane	49	52	3
RP848	hypothetical protein RP848	Cytoplasmic	50	33	4
RP341	hypothetical protein RP341	Cytoplasmic Membrane	51	19	3
🔴 RP347	outer membrane assembly protein (asmA)	Unknown	52	48	3

Rickettsial protein	Annotated function	Predicted subcellular localization*	HLA class I- ranking	Proteasome /H2K ^k ranking	Number of proteasome-peptides
🔍 RP858 ⁴	RNA polymerase sigma factor RpoD	Cytoplasmic	53	13	3
RP627	co-chaperonin GroES	Cytoplasmic	54	6	5
RP522	cytidylate kinase	Cytoplasmic	55	46	3
RP300	outer membrane antigenic lipoprotein B precursor (mlpD)	Unknown	56	63	3
RP391	hypothetical protein RP391	Periplasmic	57	35	4
RP228	tail-specific protease precursor (CTP)	Unknown	58	36	3
🔍 RP226 ⁴	hypothetical protein RP226	Unknown	59	50	4
🔍 RP498	cell surface antigen (sca4)	Cytoplasmic	60	25	3
🔍 RP704	cell surface antigen (sca5)	Outer Membrane	61	3	4
RP585	preprotein translocase subunit YajC	Cytoplasmic Membrane	62	15	4
RP763	acyl carrier protein	Cytoplasmic	63	59	5

* Subcellular localization was predicted using PSORTb 3.0.2 (<http://www.psorth.org/psorth/>).

- I* *R. prowazekii* proteins in pool #1;
- ² *R. prowazekii* proteins in pool #2;
- ³ *R. prowazekii* proteins in pool #3;
- ⁴ *R. prowazekii* proteins in pool #4.
- 🔍 Rickettsial proteins predicted to encompass MHC class-I and MHC class-II binding peptides.