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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 *Rickettsia 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.

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 *Rickettsia 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.

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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 LD_{50} was determined experimentally in C3H/HeN mice using the Spearman-Karber method; LD_{50} and confidence intervals were: $LD_{50} = 8.13 \times 10^3$ PFU/ml, upper end point $=11.5 \times 10^3$ PFU/ml, and lower end point $=5.75 \times 10^3$ PFU/ml.

2.2. Immunoinformatics analysis

834 protein sequences from *R. 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 IC_{50} values $\leq$ 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 LD_{50} 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 toward 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 $\geq$60% and sequence identity $\geq$ 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-derive 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