Published in final edited form as:

Vaccine. 2014 September 3; 32(39): 4960–4967. doi:10.1016/j.vaccine.2014.07.032.

Phenotype of the Anti-*Rickettsia* CD8⁺ T cell Response suggests cellular correlates of protection for the assessment of novel antigens

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Abstract

The obligately intracellular bacteria *Rickettsia* infect endothelial cells and cause systemic febrile diseases that are potentially lethal. No vaccines are currently available and current knowledge of the effective immune response is limited. Natural and experimental rickettsial infections provide strong and cross-protective cellular immunity if the infected individual survives the acute infection. Although resistance to rickettsial infections is attributed to the induction of antigen-specific T cells, particularly CD8⁺ T cells, the identification and validation of correlates of protective cellular immunity against rickettsial infections, an important step towards vaccine validation, remains a gap in this field. Here, we show that after a primary challenge with *Rickettsia typhi* in the C3H mouse model, the peak of anti-*Rickettsia* CD8⁺ T cell-mediated responses occurs 7 days post-infection (dpi), which coincides with the beginning of rickettsial clearance. At this time point, both effector-type and memory-type CD8⁺ T cells are present, suggesting that 7 dpi is a valid time point for the assessment of CD8⁺ T cell responses of mice previously immunized with protective antigens. Based on our results, we suggest four correlates of cellular protection for the assessment of protective rickettsial antigens: 1) production of IFN- γ by antigen experienced CD3⁺CD8⁺CD44^{high} cells, 2) production of Granzyme B by CD27^{low}CD43^{low} antigen-experienced CD8⁺ T cells, 3) generation of memory-type CD8⁺ T cells [Memory Precursor Effector Cells (MPECs), as well as CD127^{high}CD43^{low}, and CD27^{high}CD43^{low} CD8⁺ T cells], and 4) generation of effector-like memory CD8⁺ T cells (CD27^{low}CD43^{low}). We propose that these

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Conflict of interest

None to declare.

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correlates could be useful for the general assessment of the quality of the CD8⁺ T cell immune response induced by novel antigens with potential use in a vaccine against *Rickettsia*.

Keywords

Rickettsia; CD8⁺ T cells; memory; vaccine; antigens

1. Introduction

Rickettsial agents are among the most virulent and lethal pathogens known to humans; in the absence of timely and proper antibiotic treatment, case fatality rates for the etiologic agents of epidemic typhus (*Rickettsia prowazekii*) and Rocky Mountain spotted fever (RMSF; *R. rickettsii*) can be as high as 60% [1, 2]. Moreover, *R. rickettsii* and *R. prowazekii* can potentially be used as bioweapons due to their high infectivity at low doses in aerosols [1, 3]. However, there are no prophylactic vaccines currently available for preventing any of the rickettsial diseases. Although antibodies were identified as the protective mechanism and correlate of protection in prior killed *Rickettsia* vaccines [4–8], it is also known that antibodies do not play a role in recovery from a primary infection [9], and that they are not cross-protective among phylogenetically distant rickettsiae [10]. In contrast, T cells can mediate cross-protection between rickettsiae as distantly related as *R. typhi* and *R. conorii* [11], suggesting that a T cell-mediated mechanism is partly responsible for the induction of long lasting cross-protective immunity and that T cell antigens should be included in the next generation of anti-rickettsial vaccines. To achieve this goal, the identification and validation of correlates of protective cellular immunity against rickettsial infections is a critical step that has yet to be addressed, and a particular focus on CD8⁺ T cells is necessary since their critical role over CD4⁺ T cells in resistance to rickettsial infections has been experimentally demonstrated [12,13]. Moreover, CD8⁺ T cells from convalescent individuals previously infected with *R. typhi* or *R. prowazekii* proliferate and are cytotoxic against typhus group rickettsial antigens [14–16]. Unfortunately, human data is not abundant because rickettsioses are underreported and underdiagnosed due to the lack of commercially available methods that can be implemented during the acute stage of the disease. For this reason, as in most neglected infectious diseases, the most sophisticated understanding of the immune response against rickettsiae derives from animal models. Nevertheless, the mouse models of rickettsioses are relevant models because they faithfully replicate most of the pathology and clinical behavior of human rickettsioses [17, 18].

Recently, it was shown that memory CD8⁺ T cells mediating strong recall responses display a “rested” phenotype consisting of CD127^{high}, CD43^{low}, CD27^{high}, and KLRG1^{low}; different combinations of these markers were proposed to be useful for the assessment of vaccine efficacy [19–21]. It was also proposed that the relative proportion of different subsets of antigen-specific CD8⁺ T cells defined by CD127 vs. KLRG1 could be a valuable predictor of vaccine efficacy; specifically, the induction of large numbers of memory precursor effector cells (MPECs), defined as CD127^{high} KLRG1^{low}, appears to be pivotal [21]. Since recovery from a natural or experimental rickettsial infection confers long-lasting protective immunity, it is reasonable to use the phenotype of this natural T cell response as a

paradigm to identify correlates of protection; however, the phenotypic transition of responding CD8⁺ T cells towards memory has not been characterized. Here, we explored the kinetics of memory-type CD8⁺ T cells after challenge with *R. typhi* and identified relevant time points and phenotypes that could be used to predict the protective potential of novel rickettsial antigens.

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. Yolk sacs were pooled and homogenized in a Waring blender, diluted to a 10% suspension in sucrose-phosphate-glutamate buffer (SPG; 0.218 M sucrose, 3.8 mM KH₂PO₄, 7.2 mM K₂HPO₄, 4.9 mM monosodium L-glutamic acid, pH 7.0). Rickettsiae were quantified by plaque assay [22], and the LD₅₀ was determined experimentally in C3H/HeN mice.

2.2. Animal model

The mouse model of endothelium-target typhus group rickettsioses has been previously described in detail [18]. Briefly, C3H/HeN mice (Charles River Laboratories, stock 025) were housed in an animal biosafety level-3 (ABSL3) facility and infected intravenously (i.v) through the tail vein with sublethal (0.3 LD₅₀) or lethal (5 or 10LD₅₀) doses of *R. typhi* in a volume of 300 µl of phosphate-buffered saline (PBS). 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).

2.3. Immunization with protective antigens

For immunization with protective antigens (RP884, RP778, RP739, RP403 and RP598) we used a cell-based system as reported by us [23, 24]. Briefly, the endothelial cell line SVEC4-10, modified to express the costimulatory molecules CD80 and CD137L through lentiviral transduction, was nucleofected with a PiggyBac transposon and a plasmid encoding the PiggyBac transposase in order to produce cell lines stably expressing *R. prowazekii* antigens in which the expression of the rickettsial protein is directly coupled to that of a fusion protein encoding resistance to hygromycin and GFP (see supplementary methods and supplementary figures 1 and 11). Each mouse was immunized intravenously (i.v.) and intramuscularly (i.m.) with 4.5×10⁵ cells expressing individual rickettsial proteins; 5 days later, mice received the same dose of cells i.m. and intraperitoneally (i.p.). After another seven days, mice were challenged i.v. with 5 LD₅₀ of *R. typhi* and euthanized on day 7 post-infection to measure rickettsial load and CD8⁺ T cell phenotypes.

2.4. Flow Cytometry

To analyze CD8⁺ T cell responses after challenge with rickettsiae, we used an *ex vivo* method that provides direct information about the response of T cells *in vivo* and allows the detection of cytokines that were being secreted by T cells at the time of the injection without

further *in vitro* stimulation [25, 26]. On the day of sacrifice, mice were injected intraperitoneally (i.p.) with brefeldin A (BFA; 250 µg/mouse) and monensin (500 µg/mouse); four hours later, spleens were collected and homogenized to a single-cell suspension in HBSS containing 2%BGS, 3mM HEPES, and Golgi Plug™ and Golgi Stop™ (BD Biosciences) at concentrations recommended by the manufacturer. Subsequently, mononuclear cells were purified by density gradient centrifugation with Lympholyte™-M (Cedarlane Laboratories). In order to minimize variability introduced by processing and flow cytometry acquisition, all cells were frozen in 90% fetal calf serum with 10% DMSO and subsequently processed and acquired at the same time. For assessment of the CD8⁺ T cell response, mononuclear cells were stained with Live/Dead Fixable Blue (Life Technologies), APC-Alexa Fluor 750 anti-CD8 (clone 5H10), FITC anti-CD3 (clone 17A2), BD Horizon V500 anti-CD44 (clone IM7), PE-Cy7 anti-IFN-γ (clone XMG1.2), Alexa Fluor 647 anti-Granzyme B (clone 16G6), PE-Cy5 anti-CD127 (clone A7R34), PE anti-CD43 (clone 1B11), PerCP-eFluor 710 KLRG1 (clone 2F1), eFluor 450 CD11a (clone M17/4), PE-Cy7 anti-CD62L (clone MEL-14) or BD Horizon V450 anti-CD27 (clone LG. 3A10). In addition to these markers, for recall responses, T cells were also stained with PE anti-IL-2 (clone JES6-5H4) or eFluor 450 anti-TNF-α (clone MP6-XT22). All antibodies and reagents were purchased from BD Biosciences, eBioscience, or Biolegend. CountBright™ Absolute Counting Beads (Life Technologies) were added to each sample prior to acquisition. All samples were acquired on a LSRII Fortessa cytometer (BD Biosciences); 500,000 events were captured and data were analyzed using FlowJo 9.5.3 software (Tree-Star Inc). All analyses were performed on live CD3⁺CD8⁺ single cells (Supplementary figure 2). Thresholds for positivity were determined with fluorescence-minus-one (FMO) control stains.

2.5. Statistics

Statistical significance analysis was performed using GraphPad Prism version 6.00, (GraphPad Software). Groups were compared by unpaired multiple t-tests followed by Holm-Sidak correction for multiple comparisons, or one-way analysis of variance (ANOVA) followed by Sidak's or Dunnett's multiple comparisons test. Pearson's correlation coefficient was used to calculate the correlation between *Rickettsia* load and the number of CD8⁺ T cells expressing a given activation trait.

3. Results

3.1. CD8⁺ T cell response after a primary sublethal challenge with *R. typhi*

Informative time points were selected based on the dynamics of the CD44^{high}CD62L^{low} effector subpopulation. The expression pattern of these two markers suggested that sublethal infection with *R. typhi* induces a biphasic effector phase that peaks at 7 and 11 dpi (Supplementary figure 3). Subsequently, we analyzed CD8⁺ T cell responses at 3, 5, 7, 11, and 14 dpi; 60 dpi was also included in order to capture the phenotype of resting memory CD8⁺ T cells. In agreement with the systemic nature of rickettsial infections, the *R. typhi* load had comparable kinetics in liver, lung, and spleen, peaking at 5 dpi (Supplementary figure 4). At 14 dpi, rickettsiae became undetectable by qPCR, indicating that C3H/HeN mice had cleared the infection. Due to the lack of tetramers to follow *Rickettsia*-specific

CD8⁺ T cell responses, we focused on the study of antigen-experienced cells expressing the CD3⁺CD8⁺CD44^{high} phenotype; all analyses were performed on this population. Since both memory precursors and terminally differentiated effector cells are present at the peak of primary CD8⁺ T cell-mediated immune responses [21], we determined critical time points of the primary anti-*Rickettsia* CD8⁺ T cell response by exploring the expression of the activated isoform of CD43, which discriminates quiescent from activated T cells, and KLRG1, a marker for terminally differentiated effector cells [19, 27]. After a sublethal challenge with *R. typhi*, effector CD8⁺ T cells (CD43^{high} KLRG1^{high}) were present from 7 to 14 dpi and the peak of expression occurred at 7 dpi (Fig. 1A and B); compared to uninfected controls, both markers remained high until 14 dpi and returned to basal conditions at 60 dpi. This pattern was further confirmed with an approach that relies on the synchronized downregulation of the CD8 α chain and upregulation of the integrin CD11a (LFA-1 α chain), which is driven by contact with antigens but not by bystander inflammation; thus, cells with the CD8 α ^{low}CD11a^{high} phenotype are considered authentic antigen-experienced CD8⁺ T cells as opposed to naïve cells, which are CD8 α ^{high}CD11a^{low} [28, 29]. This approach revealed an activation pattern similar to the one described for KLRG1, which is consistent with the concept that not only inflammation but also TCR signaling are required for the induction of KLRG1 expression on effector CD8⁺ T cells [30]. The peak of expression for the CD8 α ^{low}CD11a^{high} subset also included both 7 and 11 dpi (Fig. 1C and D). To confirm the effector potential of the highly activated cells that we identified at 7 and 11 dpi, we measured the expression of IFN- γ and Granzyme B. Compared to uninfected controls, significant expression of IFN- γ was observed at 7, 11, and 14 dpi (Fig. 1E and F). Although Granzyme B expression in total antigen-experienced CD3⁺CD8⁺CD44^{high} cells was not different from uninfected controls, a more detailed analysis showed significant expression of Granzyme B at 7 and 11 dpi in a subset of effector cells characterized by high expression of CD43 (Supplementary figure 5). The expression of IFN- γ and Granzyme B at 11 dpi could be related to elimination of residual bacteria since low copy numbers of rickettsiae were still detectable in some animals at this time point. Based on these data and the beginning of the elimination of rickettsiae at 7 dpi, we concluded that this time point represents the peak of effector CD8⁺ T cells responding to a primary *R. typhi* infection and that it is a critical window for the functional assessment, tracking, and potential identification of anti-*Rickettsia* CD8⁺ T cells induced by infection or vaccination.

3.2. Assessment of CD8⁺ T cell memory potential following a challenge with *R. typhi*

Previous studies showed that the adoptive transfer of memory CD8⁺ T cells protects against a lethal challenge with rickettsiae [12, 13]; however, the phenotypic changes associated with transition towards memory during the acute effector phase or the activation pattern of memory CD8⁺ T cells upon a secondary encounter with rickettsiae have not been described. Based on the analysis of CD127 vs. KLRG1 and CD127 vs. CD43 populations, we studied the effector-and memory-type CD8⁺ T cell subsets generated after a primary *R. typhi* challenge. At early time points, compared to uninfected controls, lower numbers of CD127^{high} cells were observed; this downregulation of CD127 is consistent with the instauration of the effector phase (Fig. 2). The peak of the effector cells, defined by the CD44^{high}CD127^{low}CD43^{high} phenotype, was observed at 7 dpi, returning to basal numbers by the end of the experiment on day 60 (Fig. 2A and B). Over time, we were expecting to

observe enrichment of the CD44^{high}CD127^{high}CD43^{low} subset, a phenotype representing memory-type CD8⁺ T cells [19]; however, compared to uninfected controls, lower numbers for this subpopulation remained even at 60 dpi. Interestingly, a ~2-fold increase in the CD44^{high}CD127^{low}CD43^{low} subset was observed at 60 dpi.

Expression analysis of CD127 vs. KLRG1 (Fig. 2C and D) indicated that MPECs (CD127^{high}KLRG1^{low}) first decreased after *R. typhi* infection followed by an increase on 7 dpi; similarly, the peak for short-lived effector cells (SLECs), defined as CD127^{low}KLRG1^{high}, was observed at 7 and 11 dpi. In fact, equivalent numbers of memory (MPECs) and effector (SLECs) type CD8⁺ T cells were observed at 7 and 11 dpi. Increased numbers of early effector cells (EECs), a subset suspected to have the potential for generating all effector lineages and defined as CD127^{low}KLRG1^{low} [21], were also observed beginning on 7 dpi and remaining high until the end of the experiment. Moreover, compared to uninfected controls, significantly higher numbers of SLECs, MPECs, and EECs were observed at 60 dpi. The observed enrichment of MPECs, a subset that has been proposed to predict CD8⁺ T cell long-term memory potential, is very significant because the production of these cells has been proposed as one of the goals for vaccines targeting CD8⁺ T cells [21].

Next, we explored the activation pattern of memory CD8⁺ T cells upon a secondary *Rickettsia* encounter. We challenged *Rickettsia*-immune mice at ~60 dpi with 10LD₅₀ of *R. typhi*, and explored the changes induced on antigen experienced CD3⁺CD8⁺CD44^{high} cells by a secondary rickettsial infection after 0, 6, 24, 72, and 120 hours postinfection (hpi). In addition to the memory subsets defined by CD127 vs. CD43 and CD127 vs. KLRG1, we also studied the subsets defined by CD27 vs. CD43 because they appear to be more informative than the classical central (T_{CM}) and effector memory (T_{EM}) phenotypes for the assessment of CD8⁺ T cell recall responses [19, 31]. Upon a secondary encounter with rickettsiae, compared to resting immune mice (0 hpi), a decrease in the CD127^{high}KLRG1^{low} and CD127^{high}CD43^{low} memory subpopulations was observed at 6 and 24 hpi (Supplementary figure 6). In contrast, a small (not statistically significant) increase in the CD27^{high}CD43^{low} subset, which represents the traditional dominant memory pool, was observed at 24 and 72 hpi (Supplementary figure 6). No major changes in the KLRG1^{high} or CD43^{high} effector subsets were detected and no significant changes in the production of Granzyme B, TNF-α, or IFN-γ were identified among CD3⁺CD8⁺CD44^{high} cells; only increased expression of IL-2 was observed at 72 and 120 hpi (Supplementary figure 7). Unexpectedly, after a secondary *Rickettsia* challenge, we observed increased numbers of the CD127^{low}KLRG1^{low}, CD127^{low}CD43^{low} and CD27^{low}CD43^{low} subpopulations (72 hpi), and the peak of expression of Granzyme B and IFN-γ was detected 72 hpi in CD127^{low}CD43^{low} cells (Supplementary figure 8). The significance of these findings is unclear since the CD127^{low}CD43^{low} and CD27^{low}CD43^{low} subsets have suboptimal recall capabilities in other models [19]; nevertheless, mice were healthy and rickettsiae were not detectable after a secondary challenge (data not shown).

3.3. Induction of IFN- γ and memory-type CD8⁺ T cells correlate with protection against *R. typhi* infection in mice immunized with novel protective rickettsial antigens

We developed a novel antigen screening method combining informatics predictions of proteasome processing and MHC class-I-binding with empirical testing using cell lines expressing predicted rickettsial antigens to discover five novel *R. prowazekii* protective antigens, RP884, RP778, RP739, RP403, and RP598. These antigens stimulate CD8⁺ T cell responses and result in cross-protection against a lethal challenge with *R. typhi*, the other member of the typhus group *Rickettsia* (supplementary table 1)[23, 24]. We decided to use the phenotype of the natural primary anti-rickettsial CD8⁺ T cell response on 7 dpi as a paradigm against which to compare the response against the newly discovered protective antigens for four reasons: 1) both memory and effector-type CD8⁺ T cells are present at this time (Figure 2); 2) our antigen screening method involves a rapid prime and boost immunization followed by lethal challenge, which is known to generate memory-type CD8⁺ T cells within 5 to 7 days after immunization [32, 33]; 3) this screening method does not allow for a resting period (i.e., primary T cells are continuously stimulated); and 4) the natural anti-rickettsial memory CD8⁺ T cell response, as characterized with our *ex vivo* methodology, is indistinct (supplementary figures 6–8).

We studied the CD8⁺ T cell responses of mice immunized with individual (RP778, RP739, RP403, and RP598) or pooled *R. prowazekii* protective antigens (RP884, RP739, RP403, and RP598) 7 days after a lethal challenge (5LD₅₀) with *R. typhi*; as controls, we immunized mice with an irrelevant protein (luciferase) or a non-protective rickettsial antigen (RP734). To confirm that immunization conferred protection, we measured the *Rickettsia* load in lung and liver. Mice immunized with the individual protective antigens showed reduced bacterial loads (supplementary figure 9). Compared to mice immunized with non-protective proteins (luciferase or RP734), mice that received protective antigens had increased numbers of memory-type CD8⁺ T cells (CD44^{high}CD127^{high}CD43^{low}) and reduced numbers of effector-type CD8⁺ T cells (CD44^{high}CD127^{low}CD43^{high}); however, these differences were only statistically significant for RP778 (Fig. 3A and B). Memory-type CD8⁺ T cells also had increased IFN- γ expression; at least a ~2-fold change was observed in mice immunized with RP778, RP403, or pooled protective antigens (Fig. 3C). In contrast, IFN- γ expression within effector-type cells was not different between groups (data not shown). In an independent experiment with only RP884, the first protective antigen reported by our group [23], we observed a similar pattern (Supplementary figure 10). For our most protective antigen, RP778, the CD127 vs. KLRG1 and CD27 vs. CD43 analyses also showed increased memory-type (CD127^{high}KLRG1^{low} or CD27^{high}CD43^{low}) CD8⁺ T cells (Fig. 3D–F). Interestingly, mice immunized with protective antigens also had increased numbers of CD27^{low}CD43^{low} and CD127^{low}CD43^{low} cells without an increase in the CD127^{low}KLRG1^{low} subpopulation (Fig. 4A–C). In fact, increased IFN- γ production in groups immunized with protective antigens, particularly those receiving RP778, RP403, or pooled protective antigens, was also observed in CD27^{low}CD43^{low} and CD127^{low}CD43^{low} CD8⁺ T cells (Fig. 4D and E). Since effector-like memory CD27^{low}CD43^{low} cells express Granzyme B, are cytolytic, and protect against *Listeria* [19, 31], we assessed Granzyme B expression and observed increased expression among CD27^{low}CD43^{low} cells from mice immunized with protective antigens (Fig. 4F).

Next we asked if the level of protection achieved after immunization with protective antigens (as assessed by rickettsial load) would correlate with the number of memory-type cells, and/or the number of cells producing IFN- γ or Granzyme B (Fig. 5). Calculation of Pearson's correlation coefficient showed statistically significant correlations between reduction in rickettsial load and the number of CD3⁺CD8⁺CD44^{high} cells producing IFN- γ , or the number of cells with any of the following phenotypes: CD127^{high}CD43^{low}, CD127^{low}CD43^{low}, CD127^{high}KLRG1^{low} (MPECs), CD27^{high}CD43^{low}, and CD27^{low}CD43^{low}, as well as with Granzyme B expression by CD27^{low}CD43^{low} cells. In contrast, the induction of effector subsets such as CD127^{low}CD43^{high} and CD127^{low}KLRG1^{high} (SLECs) was correlated with increased bacterial loads.

4. Discussion

CD8⁺ T cells from humans previously infected with typhus group rickettsiae respond to appropriate antigenic stimulation [14–16]. These cells are critical effectors of immunity against rickettsial infections in mouse models that closely mimic the pathophysiology of severe human rickettsioses [18]; mice that survive a rickettsial infection with low numbers of rickettsiae become solidly immune against subsequent lethal challenge [10, 11]. These data and the difficulty of obtaining human samples due to diagnostic limitations and underreporting justify the use of the mouse models for the definition and validation of potential correlates of protection for vaccine testing. Although previous studies have examined anti-*Rickettsia* CD8⁺ T cell responses [12, 13], there is still a large gap in the identification of antigens that provide strong protective T cell-mediated immunity and in the systematic definition of correlates or surrogates of protection in rickettsial diseases.

In this study, we analyzed the expression of activation markers and effector molecules that have been linked to the achievement of long-lasting protection after vaccination. We first studied the kinetics of primary and memory CD8⁺ T cell responses with the aim of identifying a combination of markers of cellular protective immunity that could be used for the validation of antigens for vaccine candidates against *Rickettsia*. We then asked whether this marker combination could serve as an immune correlate of protection in mice immunized with antigens that we recently identified as protective [23, 24]. We immunized mice using a cell-based system in which the APCs are derived from endothelial cells, which is relevant since the main targets of infection *in vivo* are endothelial cells. However, we do acknowledge that investigation of whether priming is mediated by the immunizing cells in our system or endogenous dendritic cells still needs to be addressed; this issue has not been our priority because our current platform was designed for antigen discovery only and we do not expect to use it for vaccine formulation.

Our detailed kinetic analysis showed that after a primary challenge with *R. typhi*, the peak of anti-*Rickettsia* CD8⁺ T cell-mediated responses occurs at 7 dpi (Fig. 1 and 2). We considered 7 dpi a valid time point for assessing CD8⁺ T cell responses in mice immunized with rickettsial vaccine antigens for two main reasons: Firstly, T cells with memory phenotypes appear early during the development of antigen-specific T cell responses (their magnitude has been correlated with protection) [21], and we observed a similar pattern here (Fig. 2). Secondly, we used a rapid prime and boost immunization protocol which has been

reported to accelerate the transition of effector cells into memory cells [32, 33]; as a consequence, CD8⁺ T cells with the phenotype and function of memory cells are generated within 5–7 days after immunization. In this study, mice were lethally challenged 12 days after the first immunization and the immune response was assessed 7 days after the challenge. With the rickettsial challenge provided on day 12, natural antigen presentation from rickettsia-infected cells continues the stimulation of the same primary antigen-specific T cells only if the same antigen is processed and presented as a consequence of rickettsial infection. Whenever that is not the case (such as with control antigens), T cells responding to the immunizing antigen stop contributing to the response and all that can be detected is the incipient primary response against the rickettsial challenge. We decided to use the natural primary T cell response as a paradigm not only because the natural memory response was subdued but also because our immunization and challenge strategy maintains the stimulation of primary T cells throughout the protocol; there is no resting time that would mimic the transition to memory observed after immunization with a sublethal dose of rickettsiae followed by rechallenge 8 weeks later. We hypothesize that the natural memory response against rickettsiae was restrained for two reasons: 1) antibodies are very effective at clearing rickettsiae during secondary challenges, which may result in much less antigenic stimulation for T cells; and 2) our analysis is truly *ex vivo* because brefeldin A and monensin (substances that provoke the intracellular accumulation of otherwise secreted proteins) are injected into animals 4 hours before sacrificing them to obtain splenocytes, as opposed to traditional methods that provide *in vitro* antigenic stimulation in the presence of brefeldin A and monensin after procurement of splenocytes. We believe that this true *ex vivo* analysis reveals the tightly regulated dynamics of T cell activation that occurs *in vivo*. This concept is supported by a recent report in which the role of glucocorticoids in the regulation of T cell responses was only evident when experiments were performed in the way we report here; T cells were hyperactive if analyzed after the traditional *in vitro* restimulation [34].

Based on our analysis of the anti-*R. typhi* CD8⁺ T cell response, we suggest four correlates of protection for the assessment of potential protective rickettsial antigens: 1) production of IFN- γ by antigen experienced CD3⁺CD8⁺CD44^{high} cells, 2) production of Granzyme B by CD27^{low}CD43^{low} antigen-experienced CD8⁺ T cells, 3) generation of memory-type CD8⁺ T cells (CD127^{high}CD43^{low}, MPECs, and CD27^{high}CD43^{low}), and 4) generation of effector-like memory CD8⁺ T cells (CD27^{low}CD43^{low}) (Fig. 5). These findings support the concepts that protective antigens identified through our antigen screening platform might have good “memory generation potential” and that immunization with these antigens can induce relevant memory phenotypes. Interestingly, we observed a correlation between decreased rickettsial load and increased expression of Granzyme B on effector-like memory CD8⁺ T cells (CD27^{low}CD43^{low}) but not with total antigen experienced cells (CD3⁺CD8⁺CD44^{high}). This finding and the detection of significant expression of Granzyme B among CD127^{low}CD43^{low} cells in the natural recall response of resting CD8⁺ T cells are aligned with the concept that memory CD8⁺ T cell subsets might have a specialized organization and distribution based on their effector functions and activation status [31].

There is evidence that the number of memory-type T cells generated after infection or vaccination strongly correlates with the degree of protection to subsequent challenge [33].

Our study supports this correlation since RP778, our strongest antigen as assessed by survival and rickettsial load [24], produced the largest increase in IFN- γ expression and generated the largest number of memory-like CD8⁺ T cells. Moreover, when we pooled RP884, RP739, RP403 and RP598, which includes antigens that were not as strong at inducing CD8⁺ T cell activation individually [23, 24], they provided an overall superior protection in terms of reduction of the rickettsial load (supplementary figure 9) and the induction of memory-type CD8⁺ T cells (Fig. 3 and 4), suggesting that some degree of synergy was achieved. Interestingly, mice immunized with protective antigens had increased numbers of CD27^{low}CD43^{low} and CD127^{low}CD43^{low}, but not of CD127^{low}KLRG1^{low} antigen experienced CD8⁺ T cells. Although in earlier studies CD27^{low}CD43^{low} effector-like memory CD8⁺ T cells were associated with inferior recall proliferation responses, compared to the CD27^{high} pool [19], it was recently demonstrated that this subset efficiently protects against challenge with *Listeria* or vaccinia [31]. Even though it is currently unknown if protection provided by the CD27^{low}CD43^{low} memory pool can be extended to other double negative memory CD8⁺T cell subsets (such as CD127^{low}CD43^{low} cells), the fact that a solid memory T cell response is generated after experimental sublethal infection with *Rickettsia* and the findings presented here support the speculation that rapid induction of the CD127^{low}CD43^{low} or CD27^{low}CD43^{low} subsets could mediate protection against infection with *Rickettsia* in immunized animals. In fact, it has been proposed that CD27^{low}CD43^{low} cells could represent the potential source of protection induced in rapid prime-boost vaccination approaches, which we used here, and that persistence of effector-like memory cells might account for immediate protection against acute infection [31, 35]. More studies are required in order to assess the protective capability of the CD27^{low}CD43^{low} subset upon recall compared to the classical T_{CM} and T_{EM} or the CD27^{high}CD43^{low} memory subsets that are known to mediate optimal protection in other models [19, 36–38].

In summary, this is the first report of a comprehensive characterization of the anti-*Rickettsia* CD8⁺ T cell immune response with implications for the identification of correlates of T cell-mediated protective immunity. Our findings provide useful paradigms that could assist in the assessment of the quality of the immune response induced by novel vaccine targets and, together with other *in vivo* protection measurements such as survival, could provide a rationale for the selection of rickettsial antigens for a subunit vaccine. The present results together with *in silico* predictions and *in vivo* protection testing as reported by us [23, 24] are being combined to identify and select relevant vaccine candidates for further characterization. In consequence, the best candidate antigens will encompass MHC class-I binding peptides (both mouse and human), provide protection against a rickettsial lethal challenge, and stimulate protective CD8⁺ T cell responses. It should be emphasized that the set of antigens presented herein does not represent a final vaccine formulation, but a step towards it. Further studies will seek to validate and expand the concepts presented here by testing novel vaccine candidates currently being identified in our laboratory. Also, we suggest that the general approach presented here could be useful in the assessment of the immune response and in the identification of markers that could serve as correlates/surrogates of T cell-mediated protection in other infections for which TCR-transgenic tools and/or tetramers are not yet available. This empirical approach fulfills the comprehensive

immunomonitoring strategy that has been advocated as a pivotal component in the effective development of T-cell vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Cesar Sanchez for his technical support and to Lynn Soong, Robin Stephens, Jere McBride, Gregg Milligan and Lenny Moise for helpful discussions. This publication was made possible by Grant Number U54 AI057156 from NIAID/NIH; its contents are solely the responsibility of the authors and do not necessarily represent the official views of the RCE Programs Office, NIAID, or NIH. Erika Caro-Gomez was also supported by a predoctoral fellowship from the McLaughlin fund and the Vale-Asche Foundation.

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Figure 1. CD8⁺ T cell activation after a primary sublethal challenge

Naïve mice were infected with 0.3 LD₅₀ of *R. typhi* and sacrificed at 0, 3, 5, 7, 11, 14 and 60 dpi (4 hours after i.p. injection of brefeldin A and monensin) to obtain splenocytes for flow cytometric analysis. (A) Number of antigen experienced CD3⁺CD8⁺CD44^{high} cells expressing CD43 and representative histograms showing CD43 expression. (B) Number of CD3⁺CD8⁺CD44^{high} cells expressing KLRG1 accompanied by illustrative histograms. (C). Representative density plots showing the kinetics of antigen experienced CD8⁺ T cells as assessed by the CD8α^{low}CD11a^{high} phenotype. (D) Number of CD8α^{low}CD11a^{high} cells at the indicated time points. (E) Representative density plots of the frequency of CD3⁺CD8⁺ cells expressing CD44 and IFN-γ. (F) Number of CD3⁺CD8⁺ cells co-expressing CD44 and IFN-γ. Data is presented as the mean ± standard error of the mean (SEM) from five mice per time point; each data point represents the mean value per 10⁵ CD8⁺ T cells. p values for comparisons against 0 dpi are represented as follows: **p<0.01; ***p<0.001; ****p<0.0001, non-significant (ns).

Figure 2. Kinetics of CD127 vs. CD43 and CD127 vs. KLRG1 subsets among antigen experienced CD3⁺CD8⁺CD44^{high} cells

Naïve mice were infected with 0.3 LD₅₀ of *R. typhi* and sacrificed at 0, 3, 5, 7, 11, 14 and 60 dpi (4 hours after i.p. injection of brefeldin A and monensin) to obtain splenocytes for flow cytometric analysis. (A) Based on the expression of CD127 and CD43, four different subpopulations were identified: CD127^{high}CD43^{low}, CD127^{high}CD43^{high}, CD127^{low}CD43^{high}, and CD127^{low}CD43^{low}; we show the mean value of each subset per 10⁵ CD8⁺ T cells from 5 mice per time point with the standard error of the mean. (B) Representative flow cytometry plots showing the frequency of the same subsets. (C) Numbers of cells expressing different combinations of CD127 and KLRG1: CD127^{high}KLRG1^{low}, CD127^{high}KLRG1^{high}, CD127^{low}KLRG1^{high}, and CD127^{low}KLRG1^{low}; each data point represents the mean value per 10⁵ CD8⁺ T cells from 5 mice per time point with the standard error of the mean. (D) Illustrative flow cytometry plots of the frequency of cells co-expressing CD127 and KLRG1.

Figure 3. Increased numbers of memory-type but not of effector-type CD8⁺ T cells are induced in mice immunized with protective rickettsial antigens

Mice were immunized with APCs individually expressing selected *R. prowazekii* protective antigens RP778, RP739, RP598 or RP403 or pooled *Rickettsia* proteins (RP739, RP598, RP403 and RP884; note that the pool does not include RP778). Animals immunized with an irrelevant protein (luciferase) or with a non-protective rickettsial antigen (RP734) were used as controls. Seven days after immunization, mice were challenged with $5 \times \text{LD}_{50}$ *R. typhi*. At 7 dpi, animals were sacrificed to obtain splenocytes for flow cytometric analysis. All analysis were performed on antigen experienced CD3⁺CD8⁺CD44^{high} cells. (A) Memory-type CD127^{high}CD43^{low} cells. (B) Effector-type CD127^{low}CD43^{high} cells. (C) IFN- γ expression in memory-type CD127^{high}CD43^{low} cells. (D) Memory precursor effector cells (MPECs, CD127^{high}KLRG1^{low}). (E) Short-lived effector cells (SLECs, CD127^{low}KLRG1^{high}). (F) Memory-type CD27^{high}CD43^{low} cells. We show individual data points with mean $\pm$ SEM. p values for comparisons against control mice are represented as follows: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; non-significant (ns).

Figure 4. CD27^{low}CD43^{low} and CD127^{low}CD43^{low} subsets, but not CD127^{low}KLRG1^{low} cells, are increased in mice immunized with protective rickettsial antigens

Mice were immunized and challenged with $5 \times \text{LD}_{50}$ *R. typhi* as described in figure 3. At 7 dpi, animals were sacrificed to obtain splenocytes for flow cytometric analysis. All analysis were performed on antigen experienced CD3⁺CD8⁺CD44^{high} cells. (A) CD27^{low}CD43^{low} cells. (B) CD127^{low}CD43^{low} cells. (C) CD127^{low}KLRG1^{low} cells. (D) IFN- γ expression in CD27^{low}CD43^{low} cells. (E) IFN- γ expression in CD127^{low}CD43^{low} cells. (F) Granzyme B expression in CD27^{high} and CD27^{low} memory-type cells. We show individual data points with mean $\pm$ SEM. p values for comparisons against control mice are represented as follows: **p<0.01; ***p<0.001; ****p<0.0001.

Figure 5. Induction of memory-type CD8⁺ T cells and CD27^{low}CD43^{low} cells, and expression of IFN- γ correlate with protection against rickettsia challenge in mice immunized with protective antigens

Mice were immunized and challenged with $5 \times \text{LD}_{50}$ *R. typhi* as described in figure 3. At 7 dpi, animals were sacrificed to obtain splenocytes for flow cytometric analysis. All analysis were performed on antigen experienced CD3⁺CD8⁺CD44^{high} cells. The protective effect of immunization with rickettsial vaccine targets was assessed as reduction of the *Rickettsia* load. (A) CD44^{high}IFN- γ ⁺ cells. (B) CD44^{high}GZMb⁺ cells. (C) CD27^{low}CD43^{low}GZMb⁺ cells. (D) CD127^{high}CD43^{low} cells. (E) CD127^{low}CD43^{high} cells. (F) CD127^{low}CD43^{low} cells. (G) CD127^{high}KLRG1^{low} cells. (H) CD127^{low}KLRG1^{high} cells. (I) CD27^{high}CD43^{low} cells. (J) CD27^{low}CD43^{low} cells. We show Pearson's correlation between rickettsial load (gltA copy number/ 10^5 copies of M-Idhal6b) and number of positive cells for each subset per 10^5 CD8⁺ T cells. (○) Mice immunized with control protein (luciferase). (◇) Mice immunized with non-protective rickettsial antigen RP734. (●) Mice immunized with protective antigens.