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Identification of a dominant CD4 T cell epitope in the membrane lipoprotein Tul4 from *Francisella tularensis* LVS

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ABSTRACT

Francisella tularensis is a Gram-negative intracellular bacterium that is the causative agent of tularemia. Small mammals such as rodents and rabbits, as well as some biting arthropods, serve as the main vectors for environmental reservoirs of *F. tularensis*. The low infectious dose, ability to aerosolize the organism, and the possibility of generating antibiotic resistant strains make *F. tularensis* a prime organism for use in bioterrorism. As a result, some strains of *F. tularensis* have been placed on the CDC category A select agent list. T cell immune responses are thought to be a critical component in protective immunity to this organism. However, investigation into the immune responses to *F. tularensis* has been hampered by the lack of molecularly defined epitopes. Here we report the identification of a major $CD4^+$ T cell epitope in C57Bl/6 (B6) mice. The murine model of *F. tularensis* infection is relevant as mice are a natural host for *F. tularensis* LVS and exhibit many of the same features of tularemia seen in humans. Using T cell hybridomas derived from B6 mice that had either been inoculated with *F. tularensis* and allowed to clear the infection or which had been immunized by conventional means using purified recombinant protein in adjuvant, we have identified amino acids 86–99 of the lipoprotein Tul4 (RLQWQAPEGSKCHD) as an immunodominant CD4 T cell epitope in B6 mice. This epitope is a major component of both the acute and memory responses to *F. tularensis* infection and can constitute as much as 20% of the responding CD4 T cells in an acute infection. Reactive T cells can also effectively enter the long-term memory T cell pool. The identification of this epitope will greatly aid in monitoring the course of *F. tularensis* infection and will also aid in the development of effective vaccine strategies for *F. tularensis*.

1. Introduction

Francisella tularensis is a Gram-negative facultative intracellular bacterium that can infect a variety of species and is the cause of the disease known as tularemia or rabbit fever (Sjostedt, 2007). Infection can result by exposure to the bacterium by contact with the skin, by ingestion, or by inhalation of aerosolized organisms (Ellis et al., 2002; Sjostedt, 2007). The precise course and kinetics of the disease varies with the *Francisella* strain and route of inoculation (Chen et al., 2003; Conlan et al., 2003). However, all routes of exposure can ultimately result in sepsis and widespread dissemination of the bacteria in the host (Conlan et al., 2003; Elkins et al., 2003). Inoculation with microorganisms in aerosolized form for some strains of *F. tularensis* has a remarkably low infectious dose (10 organisms or less) with a significant fatality rate if left untreated (Conlan et al., 2003; Twine et al., 2006). Tularemia can be treated with antibiotics if detected early (Hepburn and Simpson, 2008; Kman and Nelson, 2008). However, the possibility that the organism could be made antibiotic resistant, either through classic microbiologic means or by using recombinant DNA technology, is a considerable concern if the modified organism were then intentionally spread. The extreme virulence of certain strains, the ability to aerosolize the organisms and the ability of the organism to persist in the environment make it a potent potential bioweapon (Altman, 2002; Ellis et al., 2002). Indeed, both the former Soviet Union, as well as the United States, reportedly had a bioweapons program employing *F. tularensis* (Dennis et al., 2001; Fong and Alibek, 2005). Unfortunately, in comparison to other pathogenic microorganisms, the host response to *F. tularensis* is not yet well understood.

The immune response to *F. tularensis* appears complex. As might be anticipated for an intracellular organism, classical

Abbreviations: DHFR, dihydrofolate reductase; 6× His, 6× histidine; APC, antigen presenting cell; bp, base pairs; aa, amino acids; LVS, live vaccine strain; PCR, polymerase chain reaction; ISCOM, immunostimulating complexes; B6, C57Bl/6J.

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cellular immune responses appear to be critical. Studies using lymphocyte-deficient (CD4$^-$, β_2m$^-$, TCR-γ^-, TCR-β^-, *scid*, *nude*) or lymphocyte-depleted (by using specific antibodies) mice have illustrated an important role for both CD4 and CD8 T cells (Conlan et al., 1994; Elkins et al., 1993, 1996; Rhinehart-Jones et al., 1994; Yee et al., 1996). Interestingly, there is also an unusual Thy1$^+$ $\alpha\beta$ TCR$^+$ CD4$^-$ CD8$^-$ NK1.1$^-$ T cell subset that has been shown to contribute to protection against *F. tularensis* challenge (Cowley and Elkins, 2003; Cowley et al., 2005). Recent studies have also suggested that IgA antibodies as well as CD4$^+$ T cells may also play a role in the context of intranasal immunization with an inactivated strain of *F. tularensis*, in conjunction with IL-12 as an adjuvant (Baron et al., 2007). Thus, while the immune response against *F. tularensis* is clearly multifactorial, it seems that a cellular response, including CD4$^+$ and CD8$^+$ T cells, plays a critical role in protection.

There is no FDA approved vaccine for *F. tularensis*. During the 1940s an attenuated subsp. *holarctica* live vaccine strain (LVS) was developed (Eigelsbach and Downs, 1961) as a vaccine candidate. This strain has proven invaluable for examining aspects of the *F. tularensis*–host interaction (Elkins et al., 2007). While LVS has greatly aided our understanding of *F. tularensis* biology and microbial host interactions, there are significant side effects to the use of LVS as a vaccine and the protection it affords is incomplete (Griffin et al., 2007; Saslaw et al., 1961a, 1961b). The drawbacks of the current LVS vaccine, and the possibility that *F. tularensis* might be used in a bioterror weapon, have added impetus to the identification of antigens recognized by the immune system. Currently, the nature of the protective antigens, indeed the molecular definition of any antigens in the cellular immune response, is limited. There have been only a few reports of immunostimulatory molecules for T cells in mice or humans (Golovliov et al., 1995; Lee et al., 2006; McMurry et al., 2007; Sjostedt et al., 1991, 1992). Perhaps the best-characterized response is to the lipoprotein Tul4. Tul4 can be a target of the cellular and humoral immune response in both mice and humans (Golovliov et al., 1995; Sjostedt et al., 1991, 1992). Mice are a natural host for *F. tularensis* infection and exhibit many of the same aspects of the infection in humans (Fortier et al., 1991). Interestingly, mice immunized with *Salmonella typhimurium* expressing Tul4 appeared to give partial protection as assessed by a decreased bacterial burden in spleen and liver (Sjostedt et al., 1992). It would be extremely valuable to define epitopes at the molecular level in mice so that the immune response could be quantitatively and qualitatively assessed. This would be a great aid in understanding the host immune response in the context of infection as well as helping to develop and assess vaccine vectors and immunization strategies. In the current study, we have defined in Tul4 a potential immunodominant epitope in B6 mice using a novel strategy and shown that it is an important epitope in the context of a *F. tularensis* infection in both the acute and memory immune response.

2. Materials and methods

2.1. Mice, cell lines, and bacteria

C57BL/6J (*H2^b*) (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). MHC class II knockout mouse B6.129-*H2Ab^{tm1Gru}* was generously provided by Dr. Andrea Sant (University of Rochester). The T cell fusion partner BWZ.36/CD8$^+$, which can be used to make MHC class II and class I restricted hybridomas, was kindly provided by Dr. Nilabh Shastri and maintained as previously described (Sanderson and Shastri, 1994). The *F. tularensis* live vaccine strain was used in these studies (Cowley and Elkins, 2003). Bacterial viability was quantified by serial dilution on chocolate agar.

2.2. Fusion protein constructs and peptides

Recombinant proteins were generated using the bacterial expression vector pQE40, which contains a 6× histidine coding region (6× His) followed by murine dihydrofolate reductase (DHFR). The DHFR–Tul4 construct was constructed by cloning full length Tul4 from *F. tularensis* live vaccine strain genomic DNA using polymerase chain reaction (PCR) and the specific primers described below. All primers were constructed with 15–18 base pairs complimentary to the sequence of interest and included an enzyme restriction site and a GC clamp. The 3′reverse primers also included a stop codon upstream of the restriction site. The Tul4 insert was cloned into the pQE40 vector using BglII and PstI restriction sites designed into the primers. DHFR–OVA encoded in the pQE40 vector was created in a similar fashion. Tul4 fusion protein deletion constructs were created by using the same 5′ primer and substituting with nine individual 3′ primers that recognize sequences starting at 384 base pairs (bp), 369 bp, 345 bp, 324 bp, 300 bp, 282 bp, 183 bp, and 89 bp. The Tul4 transplant epitope construct (DHFR–Tul4 86–110) was created by cloning out a 25 amino acid (aa) region from Tul4 using a specific primer set. This region was cloned into pQE40 using BglII and PstI sites engineered into the primers yielding a 'transplanted' region of Tul4 fused directly behind DHFR. Tul4 transplant epitope deletion constructs were created by using a constant 5′ primer recognizing a region found before the DHFR sequence and substituting individual 3′ primers producing the segments of Tul4 86–99 (14 aa), 86–98 (13 aa), 86–97 (12 aa), and 86–95 (10 aa) fused to DHFR. Site directed mutagenesis of a cysteine to serine at residue 97 by an additional 3′ primer produced the DHFR–Tul4 86–(C97S)–99 construct. All synthetic Tul4 peptides used in this paper Tul4 86–99 (RLQWQAPEGSKCHD), Tul4 86–(C97S)–99 (RLQWQAPEGSKSHD), Tul4 86–98 (RLQWQAPEGSKCH), Tul4 86–97 (RLQWQAPEGSKC), and Tul4 86–95 (RLQWQAPEGS) were synthesized by SynBioSci (Livermore, CA). The NP 118–126 peptide derived from LCMV (RPQASGVYM) and OVA 323–339 peptide (ISQAVHAAHAEINEAGR) served as irrelevant peptide negative controls and were synthesized by Macro-Molecular Resources (University of Colorado, Ft. Collins, CO).

2.3. Protein production

Production of DHFR–OVA and all DHFR–Tul4 fusion proteins were performed in *Escherichia coli* strain M15 as described by manufacturers specifications (Qiagen, Valencia, CA). Recombinant proteins were isolated by lysing the bacterial pellets with 8 M urea (pH 7.5) and purified over Ni–NTA column (Qiagen). The DHFR–Tul4 was dialyzed with PBS to remove urea before injection. To couple tosylactivated M280 magnetic Dynabeads (Invitrogen, Carlsbad, CA), 1×10^7 beads with 8 μg protein were mixed overnight at 37 °C in 0.1 M borate buffer, washed three times with PBS using magnetic separation, and diluted to working volume in PBS (2×10^5 beads/μl). Tul4 deletion construct production and characterization were confirmed by SDS-PAGE techniques and Coomassie Blue staining as described previously (Turner et al., 2001).

2.4. Generation of T cell hybridomas

T cell hybrids were produced in two ways. To ensure that the epitopes recognized by the hybrids were generated in a natural infection, mice were inoculated intradermally at the base of the tail with 1×10^5 viable *F. tularensis* LVS and allowed to clear the infection. At least 3 weeks later, mice were sacrificed and spleen cells re-stimulated *in vitro* using *F. tularensis* LVS infected spleen cells. Restimulation was performed for 5 days and the cells harvested (FACS analysis confirmed cells were predominantly T cells) and fused with