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Original article

Murine survival of infection with *Francisella novicida* and protection against secondary challenge is critically dependent on B lymphocytes

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Abstract

Respiratory infection of mice with *Francisella novicida* has recently been used as a model for the highly virulent human pathogen *Francisella tularensis*. Similar to *F. tularensis*, even small doses of *F. novicida* administered by respiratory routes are lethal for inbred laboratory mice. This feature obviously limits study of infection-induced immunity. Parenteral sublethal infections of mice with *F. novicida* are feasible, but the resulting immune responses are incompletely characterized. Here we use parenteral intradermal (i.d.) and intraperitoneal (i.p.) *F. novicida* infections of C57BL/6J mice to determine the role of B cells in controlling primary and secondary *F. novicida* infections. Despite developing comparable levels of *F. novicida*-primed T cells, B cell knockout mice were much more susceptible to both primary i.d. infection and secondary i.p. challenge than wild type (normal) C57BL/6J mice. Transfer of *F. novicida*-immune sera to either wild type C57BL/6J mice or to B cell knockout mice did not appreciably impact survival of subsequent lethal *F. novicida* challenge. However, *F. novicida*-immune mice that were depleted of T cells after priming but just before challenge survived and cleared secondary i.p. *F. novicida* challenge. Collectively these results indicate that B cells, if not serum antibodies, play a major role in controlling *F. novicida* infections in mice.
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Keywords: *Francisella tularensis*; *Francisella novicida*; B lymphocytes; T lymphocytes; Protective immunity

1. Introduction

Francisella tularensis is a Gram negative intracellular bacterium that is highly virulent for humans. Two biovars, *F. tularensis* subsp. tularensis and *F. tularensis* subsp. holarctica, cause the potentially lethal disease tularemia, and *F. tularensis* subsp. tularensis has been used for bioterrorism [1,2]. *F. tularensis* infects a wide variety of mammals by many routes, and can be introduced through inhalation, ingestion, skin wounds, and wounds to the conjunctiva of the eye; respiratory infections of humans with the most virulent *F. tularensis* strains (*F. tularensis* subsp. tularensis) have a particularly poor prognosis [3]. Its classification as a Tier 1 Select Agent in the U.S. severely restricts research studies, and prompts interest in identifying research surrogates that are not BSL-3 pathogens or select agents. Surrogates invoked to date include the attenuated Live Vaccine Strain (LVS) of *F. tularensis* subsp. holarctica, and *Francisella novicida* [4,5]. *F. novicida* has long been more amenable to genetic manipulations, and the U112 strain is specifically exempt from U.S. Select Agent registration. Thus this species has become an attractive option for studies of pathogenesis, cell biology, and vaccine development.

Although *F. novicida* and *F. tularensis* are closely related [6], there are a number of important differences. To date, only a handful of human cases of *F. novicida* infection have been reported in the medical literature, most in people with underlying health problems [5,7]. Unlike people, *F. novicida* is

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quite pathogenic for laboratory mice: similar to *F. tularensis*, the respiratory LD_{50} of *F. novicida* in C57BL/6J mice approaches a single bacterium [8,9]. In animal models of infection, both similarities and differences between the two species have been recognized. For example, the cell biology of murine macrophage infection with both species appears similar [10,11]. However, innate immune responses and pathology appear to differ [8,12,13]. While both bacteria readily infect and replicate in mammalian macrophages, there are well-documented differences in the cytokine responses of infected macrophages. Some of the differences are related to efficient inflammasome activation in rodent macrophages infected with *F. novicida*, compared to inflammasome activation by *F. tularensis* that is actively restricted and delayed [14]. Further, differences in immune responses may be partially related to subtle structural differences between the O antigens of LPS from *F. novicida* compared to those from *F. tularensis* strains [15−17].

In nature, people who survive infections with *F. tularensis* appear to have strong and long-lasting resistance to subsequent exposures, prompting interest in developing live attenuated strains as vaccines. For example, the former Soviet Union developed a number of attenuated strains from *F. tularensis* subsp. holarctica [18], one of which was the predecessor of the LVS strain subsequently developed in the U.S [19]. Further, the field observations suggest that the nature of protective immune responses may be studied productively by invoking models of infection-induced immunity. However, respiratory *F. novicida* infection of mice is too virulent for such an approach to be practical or informative.

Instead, we have explored the use of parenteral infection of mice with *F. novicida*. We previously found that intraperitoneal (i.p.) infection of BALB/cByJ mice with very low numbers of bacteria was lethal, similar to respiratory infection [20]. In contrast, BALB/cByJ mice exhibit an intradermal (i.d.) LD_{50} of about 2×10^2 CFU. Here, we further develop a parenteral model of sublethal *F. novicida* infection using C57BL/6J mice, and apply it to examine the role of B cells and T cells in responding to both primary *F. novicida* vaccinating infection and secondary lethal challenge. Unlike LVS infections in C57BL/6J mice, we find that B cells play a major role in murine control of *F. novicida* infections. These results have implications for the relevance of murine *F. novicida* infection models to understanding of human tularemia.

2. Materials and methods

2.1. Experimental animals

Wild type (WT) BALB/cByJ and C57BL/6J male mice, as well as male mice deficient in mature B cells or mature α/β TCR^+ T cells (B6.129S2-*Ighm*^{tm1Cgn}/J or B6.129P2-*Tcrb*^{tm1Mom}/J, respectively; both on a C57BL/6J background), were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were age-matched within an experiment. Animals were housed in a facility at the Center for Biologics Evaluation and Research, fed autoclaved food and water *ad libitum*, and used between eight and twelve weeks of age. All experiments were performed under protocols approved by the CBER/FDA Institutional Animal Care and Use Committee.

2.2. Bacteria, growth conditions, and preparation of infection stocks

F. novicida strain U112 (originally obtained from Dr. Francis Nano, University of Victoria, British Columbia, CA) was cultured on modified Mueller-Hinton (MH) agar plates or in modified MH broth (Difco Laboratories, Detroit, MI) supplemented with ferric pyrophosphate and IsoVitalex (Becton Dickinson, Cockeysville, MD) as described previously [21]. Aliquots of bacteria were frozen in broth alone at −80 °C and thawed for individual experiments. Viable *F. novicida* bacteria were quantified by plating serial dilutions on MH agar plates that were incubated for 1−2 days at 37 °C in 5% CO_2. All stocks used for *in vivo* infections were confirmed as "fully virulent" by determining that the i.p. LD_{50} in BALB/cByJ male mice was <3 CFU [22].

2.3. F. novicida *parenteral infections*

Mice were infected by administering the indicated numbers of bacteria in 100 μl at the base of the tail, or i.p. in 500 μl, diluted in sterile phosphate buffered saline (PBS; Lonza, Walkersville, MD) containing < 0.01 ng/ml of endotoxin. Actual bacterial doses were determined by simultaneous plate count. Infected mice were observed for at least 30 days, as indicated.

2.4. *Determination of* F. novicida *bacterial loads in infected mice*

Numbers of CFU in the organs of infected mice were determined by removing spleens, livers, and lungs aseptically into sterile PBS, and homogenizing organs using a Stomacher (Tekmar, Cincinnati, OH). Appropriate dilutions were plated on MH agar plates, CFU determined, and bacterial loads calculated.

2.5. Characterization of antibody responses

Blood was obtained via the lateral tail vein from the indicated mice and pooled within groups of similarly treated mice, taking care to combine approximately equal amounts of blood. Sera were prepared and stored at −20 °C until testing. Titers of specific anti-*F. novicida* antibodies in sera were determined by an ELISA optimized in initial experiments, and essentially as described previously for antibodies to *F. tularensis* LVS [23,24]. Briefly, Immulon 1 plates were coated overnight with 5×10^6 CFU per well live *F. novicida*, blocked with 10% calf serum, and serum samples serially diluted using 2-fold intervals. Purified antibody from the Fn8.2 hybridoma (IgG_3 anti-*F. novicida* LPS; Immuno-Precise Antibodies Ltd., Victoria, British Columbia, Canada), and sera from *F. novicida*-

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