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Early cellular responses of germ-free and specific-pathogen-free mice to *Francisella tularensis* infection

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ABSTRACT

Bacteria that are highly virulent, expressing high infectivity, and able to survive nebulization, pose great risk to the human population. One of these is *Francisella tularensis*, the etiological agent of tularemia. *F. tularensis* is a subject of intense scientific interest due to the fact that vaccines for its immunoprophylaxis in humans are not yet routinely available. One of the substantial obstacles in developing such vaccines is our insufficient knowledge of processes that initiate and regulate the expression of effective protective immunity against intracellular bacteria. Here, we present data documenting the different pattern of cellular behavior occurring in an environment unaffected by microbiota using the model of germ-free mice mono-associated with *F. tularensis* subsp. *holarctica* strain LVS in comparison with a classic specific-pathogen-free murine model during early stages of infection.

1. Introduction

An organism's immune responses are achieved through cooperation among several types of immunocompetent cells that together ensure induction, regulation, and expression of the effector phase of immune reactions. In order to combat infections, the organisms are equipped with cellular and humoral components of the innate and adaptive immune systems that are closely interconnected by direct and indirect links [1]. The components of the innate immune system recognize microbe-associated molecular patterns or pathogen-associated molecular patterns and respond to them immediately. This recognition is a prerequisite (among others) for adaptive immune system activation and expression of protective immunity [2,3]. Gut-colonizing microbes play a substantial role in shaping and modulating immune responsiveness [4]. Collectively, these microbes are termed the microbiota. The primary interaction between a microorganism and its host changes intrinsic characteristics of the host cells based on epigenetic reprogramming and globally modulates the innate immune responses in a process recently given the name “trained immunity” [5–8]. This fact makes it difficult to study the early innate immune processes using conventional or specific-pathogen-free (SPF) animal models. Such animals have altered functional profiles of such antigen-recognizing cells as macrophages or dendritic cells, and they have non-negligible levels of cytokines and antibodies originating from the interactions with common microflora. For this reason, the concept of germ-free (GF) animals was developed and has recently been exploited successfully in many branches of laboratory animal research [9,10]. GF animals' almost-naïve innate immune systems provide a unique model for studying cellular and molecular events in the early stages of host–pathogen interactions. We recently published an overview on the contribution of gnotobiotic mouse models to understanding the host–pathogen interactions [11]. Data presented from GF as well as SPF animal models clearly demonstrate the dominant role played by the hierarchy of functional immune response modules that is based on the epigenetic reprogramming of innate immune cells after intercellular communication by cytokines and chemokines. In parallel, we have suggested the concept of innate immune recognition, a process that is critical for inducing and regulating expression of the adaptive immune response [12].

The original studies on GF animals mono-associated with bacteria presented the rapid colonization of the organs of GF animals infected with *Listeria monocytogenes* and greater sensitivity to this infection. It was also reported that conventionalization of *L. monocytogenes* mono-associated rats or di-association of mice with some representative of indigenous flora protects these animals against systemic *L. monocytogenes* infection [13,14]. A similar conclusion was drawn from studies on *Salmonella typhimurium* and *Vibrio cholerae* model infections [15,16]. The studies conclusively confirmed the importance of microbiota for the immune system's effective response to pathogenic bacteria. The data further demonstrated that the timing of onset, intensity, and composition of cellular and humoral immune responses are critical for GF animals' resistance to pathogenic bacteria.

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The decisive element within immune responses is an intercellular signaling and regulation of cellular effector functions. Impaired trafficking and activation of cell subpopulations is a general feature of the response of germ-free animal models to infections. Animals without endogenous microbiota have impaired activation and accumulation of phagocytes at the site of infection in response to primary infection with *L. monocytogenes*. Moreover, although they have enhanced activation of memory T cells the trafficking of those cells to inflamed sites is severely impaired. Altered regulation of cellular functions based on changed epigenetic reprogramming of cells thus contributes to increased susceptibility [17–19].

Even though microbiota have been declared to play a role in resistance to infections, the precise role of microbiota in modulating host immune response to pathogenic bacteria remains unknown. One of the reasons for this is that comparative studies of host defense in GF and SPF animals with normal microbial colonization are still rare. According to our experience, GF mice infected subcutaneously with one of the bacterial pathogens, *Francisella tularensis*, have reacted differently to attenuated and virulent strains in comparison with SPF mice. For this reason, we have used this infection model for a comparative study of early cellular response of GF and SPF mice to pathogenic infection.

F. tularensis is a small, facultative intracellular, and non-motile Gram-negative coccobacillus causing the potentially lethal illness tularemia in humans and animals. A low infectious dose, high infectivity, and easy transmission by biological aerosols qualify *F. tularensis* as a pathogen of Category A with the potential to be misused for terrorist and/or military acts. Moreover, *F. tularensis* has long been regarded as a suitable model for studies on the immune response to intracellular bacterial pathogens [20]. In the past, we have studied the interaction of *F. tularensis* with the cellular components of the immune system, as well as with macrophages [21], B cells [22], and dendritic cells [23]. We also have examined those interactions using *in vivo* models [24], and some of that work has been based on different genetic backgrounds [25,26]. Here, we use a model built upon germ-free animals mono-associated with *F. tularensis* subsp. *holarctica* strain LVS FSC155 to present data documenting the different pattern of cellular behavior in an environment unaffected by microbiota.

2. Materials and methods

2.1. Bacteria

The *F. tularensis* subsp. *holarctica* live vaccine strain FSC155 (LVS) was kindly provided by Åke Forsberg, FOI, Umeå, Sweden. *F. tularensis* was routinely cultured at 37 °C for 24 h on McLeod agar enriched with bovine hemoglobin (Becton Dickinson, San Jose, CA, USA) and IsoVitalex (Becton Dickinson). Following incubation, bacterial colonies were lifted from the plates and resuspended in saline to reach optical density 1.00 (corresponding to bacterial 5×10^9 CFU/mL). The actual number of bacteria in the suspension utilized for the experiments was determined by serial dilutions and the number of colony-forming units (CFU) was calculated.

2.2. Animals

Female specific-pathogen-free BALB/c (BALB/c-SPF) mice were purchased from Velaz (Unetice, Czech Republic). Female germ-free BALB/c (BALB/c-GF) mice were kindly provided by the Institute of Microbiology of the Czech Academy of Sciences, Department of Gnotobiology, Novy Hradek, Czech Republic. The BALB/c-GF mice were housed in micro-isolator cages under germ-free conditions and the BALB/c-SPF mice were housed under specific-pathogen-free conditions. The mice were placed into sterile boxes with air-conditioning and stabilized temperature of 22 ± 2 °C. Light mode was 12 h of light and 12 h darkness. All experiments on mice were conducted under supervision of the institution's Animal Unit and were approved by the Animal Care and Use Committee of the Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic under project number 35/17.

2.3. Infection of mice

BALB/c-SPF (SPF) and BALB/c-GF (GF) mice were intraperitoneally inoculated with an *F. tularensis*, subsp. *holarctica* strain LVS dose of 10^2 CFU in total volume of 0.2 mL of saline.

2.4. Collection of spleen and peritoneal cells

Spleen and peritoneal cells were collected at 0, 12, 24, and 48 h post infection. Three mice were used for the preparation of cell suspension for each time interval. After intraperitoneal infection, three SPF mice and three GF mice were killed by cervical dislocation, peritoneal lavage was performed, and the spleen was aseptically removed. Peritoneal cells were collected using peritoneal cavity washing with 3 mL of ice-cold phosphate-buffered saline (PBS). The peritoneum was gently massaged to dislodge attached peritoneal cells into the PBS solution. Subsequently, a beveled-up 25 g needle attached to a 5 mL syringe was inserted into the peritoneum and while moving the tip of the needle gently to avoid clogging by the fat tissue or other organs as much fluid was collected as possible. After removing the needle from the syringe, the aspirated peritoneal fluid was deposited into tubes kept on ice. Each individual spleen was immediately homogenized in 2 mL of PBS. Spleen cells were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Determination of cell subpopulations and expression of activating markers

Peritoneal and spleen cells were three times washed with ice-cold PBS and incubated with the following antibodies in order to characterize their phenotype and activation: CD19-Alexa Fluor 647 (clone 1D3; BD, USA), CD5-PerCP (clone 53–7.3; BD, USA), CD11b-PE (clone M1/70; BD, USA), CD3-Alexa Fluor 647 (clone 17A2; BD, USA), CD8-alphaRPE (Serotec, USA), F4/80-Alexa Fluor 647 (clone 6F12; BD, USA), Gr-1-APC (clone RB6-8C5; BD, USA), CD123-APC (clone EB11, eBioscience, USA), CD11c-PE (clone HL3; BD, USA), I-Ad-FITC (clone 39-10-8; BioLegend, USA), CD69-FITC (clone H1.2F3; BioLegend, USA), and CD80-FITC (clone 16-10A1; BioLegend, USA). Cells were analyzed using a CyAn ADP flow cytometer (Dako, Glostrup, Denmark). The data were analyzed using Summit version 4.3 (Dako).

2.6. Statistical analysis

Three biological replicates were used for each experiment. Graphs shown herein present the results of a representative experiment. An unpaired Student's two-tailed *t*-test was applied to all measured data to evaluate statistical significance between GF and SPF mice. P values $<$ 0.005 were accepted as indicating significant difference and were denoted by three asterisks (***), P values $<$ 0.01 were denoted by two asterisks (**), and P values $<$ 0.05 by one asterisk (*).

3. Results

It is generally accepted that the polarization of immune response to bacteria is mainly regulated by the immune microenvironment wherein the primary host–pathogen interaction occurs. At an infection site, the immune microenvironment is affected by “in situ” cells comprising the cellular basis of the microenvironment and by infiltrating immune cells expressing activation markers and producing molecular signals for intercellular communication. For this reason, we utilized immunophenotyping of cells, which constitutes a reliable tool for characterizing the different cell subpopulations coexisting at the site of