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Lethal pulmonary infection with *Francisella novicida* causes depletion of $\alpha\beta$ T cells from lungs¹

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Abstract

Respiratory *Francisella* infections induce a delayed innate immune response followed by a severe sepsis like condition. In this study, mice infected intranasally with *F. novicida* showed a depletion of $\alpha\beta$ T cells in lungs while exhibiting large accumulations of other leukocytes correlating with disease severity. The depleted T cells were predominantly CD4+. The $\alpha\beta$ T cells in infected mice showed significantly higher levels of Annexin V binding than those in mock control mice suggesting increased apoptosis of T cells. These results suggest that lack of transition from an innate to adaptive host response is associated with lethality of respiratory tularemia.

Keywords

Francisella; tularemia; respiratory infection; severe sepsis; T cell depletion; apoptosis

1. Introduction

Francisella tularensis is a CDC category A Gram negative coccobacillus which causes respiratory tularemia [1]. Respiratory infections by murine the model organism *F. novicida* (F.n.) present the deadliest form of disease leading to severe lung pathology and death of experimental host within 3–5 days [2]. No exo- or endotoxins have been identified which may contribute to the severity of this disease. However, deregulated host immune responses are proposed to play a role in mortality associated with respiratory tularemia [2;3].

Innate immune responses in lethal respiratory *Francisella* infection in mice show an initial delay of 36–48 h followed by severe sepsis like condition where a robust upregulation of host mediators and a massive influx of innate immune cells are observed [2;4]. The effect of this deregulation of innate immune responses on adaptive immunity, if any, is not known. Sublethal infection and vaccination studies have shown that effector mechanisms of both CD4+ and CD8+ T cells are required for protection against infection [5]. However, no studies have been reported showing the status of T cells in lungs of mice following primary pulmonary infection with a lethal dose of *Francisella*.

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In the current study lethal pulmonary infection of mice with F.n. resulted in depletion of $\alpha\beta$ T cells from lungs at 72 h post-infection (p.i.). In-situ immunofluorescence (IF) staining and FACS analysis further showed that the depleted T cells were mainly CD4 positive and that $\alpha\beta$ T cell loss in lungs of infected mice was due to increased apoptosis. The results represent the first report of T cell depletion during lethal pulmonary *Francisella* infection and raise the possibility that *Francisella* interferes with the normal transition of an innate to adaptive immune response contributing to the lethality associated with respiratory tularemia.

2. Materials and methods

2.1. Bacterial strains and Mice

The F.n. strain U112 was grown on Trypticase Soy Agar (TSA) medium at 37°C. After an overnight growth, the bacteria were harvested and suspended in a freezing medium (250 mM sucrose, 10 mM sodium phosphate pH 7.2 and 5 mM glutamic acid). Stocks were aliquoted and frozen at -80°C for further use.

All experiments were performed using 6–8 weeks old female C57BL/6 purchased from Charles River Laboratories, Wilmington, MA. The animal usage protocols were approved by the Institutional Animal Care and Usage Committee at University of Texas at San Antonio and followed federal guidelines.

2.2. Infection of Mice

Mice were infected intranasally with 3×10^2 CFUs of F.n. in 20 μ l of PBS as previously described [2]. In our hands, this dose of bacteria resulted in 100% mortality rate by 96–120 h p.i. The mock inoculated mice received 20 μ l of PBS alone intranasally.

2.3. Immunofluorescence staining

For in-situ IF staining lung tissues were obtained at 72 h p.i. and were processed as previously described [2]. An R-phycoerythrin (R-PE) conjugated $\alpha\beta$ T-cell receptor β chain monoclonal antibody (BD Pharmingen, NJ) for $\alpha\beta$ T-cells and an R-PE conjugated rat anti-mouse CD11b monoclonal antibody (BD Pharmingen, NJ) for CD11b+ cells was used. The images were acquired using a Leica DMR epifluorescent microscope (Leica Microsystems, Wetzlar, Germany) with an attached cooled CCDSPOT RT camera (Diagnostic Instruments Inc., Sterling Heights, MI).

2.4. FACS Analysis and apoptosis assay

Lungs were harvested from mice at 72 h p.i. after perfusion with PBS and treated with collagenase to obtain lung cells as previously described [6]. Lung cells were first treated with Fc receptor blocking antibodies for 15 minutes at 4°C, followed by staining with fluorescently conjugated antibodies at 4°C for 15 minutes. FACS analysis was performed using an BD LSR II (Becton Dickinson) FACS analysis machine equipped with 488, 633, 355 and 405-nm lasers. Cells were stained singly for TCR β and CD11b, or doubly for TCR β and CD4 (APC-Cy7 conjugated monoclonal rat anti-mouse CD4 antibody, BD Pharmingen, NJ) or TCR β and CD8 (Alexa-647 conjugated rat anti-mouse CD8 antibody, BD Pharmingen, NJ). For analysis of apoptotic $\alpha\beta$ T cells, the lung cells were successively stained with TCR β antibody (APC conjugated hamster anti-mouse TCR β chain, BD Pharmingen, NJ), FITC-conjugated Annexin V and Propidium iodide according to manufacturer's instructions (BD Pharmingen). FlowJo (Tree Star) software was used to analyze the FACS data.

2.5. Statistical Analysis

Statistical comparison between FACS data of different experimental groups was performed by Student's *t* test using Sigma Plot 8.0.

3. Results and discussion

3.1. Intranasal infection with *F. novicida* results in depletion of $\alpha\beta$ T cells from lungs

Studies from our laboratory have shown that respiratory infection with *F.n.* causes little cellular infiltration in mouse lungs until 72 h p.i. [2]. In this study, *in-situ* IF analysis of mouse lungs infected with *F.n.* at 72 h p.i. showed that the number of $\alpha\beta$ T cells in these lungs were substantially lower as compared to those in mock control lungs (figure 1A, *upper panel*). As a positive control for infection, infiltration of CD11b+ cells was also analyzed. In contrast to $\alpha\beta$ T cells, extensive infiltration of CD11b+ cells in lungs of infected mice was observed indicating a selective depletion of $\alpha\beta$ T cells upon infection. FACS analysis of lung cells confirmed the observations made by IF staining which showed the number of $\alpha\beta$ T cells in infected lungs was > 3 fold less than those in mock control lungs. The absolute number of $\alpha\beta$ T cells in lungs of mock control (app. 0.6×10^6 cells per mouse) and infected mice (app. 0.1×10^6 cells per mouse) reflected even more pronounced differences despite influx of other cell types upon infection. In contrast, the number of CD11b+ cells in *F.n.* infected lungs was 10 fold higher than their mock control counterparts (figure 1A, *lower panel*). This confirmed a significant loss of $\alpha\beta$ T cells in the lungs upon respiratory infection with *F.n.* For further analysis, lung cells from mock control and *F.n.* infected animals were stained simultaneously for $\alpha\beta$ T cells and CD4 or $\alpha\beta$ T cells and CD8 markers and analyzed by FACS. Mock control lungs displayed 7.7 ± 0.5 % of lung cells to be $\alpha\beta$ + CD4+ and 2.9 ± 0.3 % to be $\alpha\beta$ + CD8+ (figure 1B). On the other hand, *F.n.* infected lungs showed 2.0 ± 0.3 % $\alpha\beta$ + CD4+ and 1.1 ± 0.3 % $\alpha\beta$ +CD8+ cells at 72 h p.i.. This indicated that although the T cell population in lungs was predominantly CD4+, the infection resulted in depletion of both CD4+ and CD8+ T cells from lungs.

Respiratory infection with *Francisella* results in a late stage hyper-active host immune response characterized by wide-spread upregulation of inflammatory immune mediators and influx of innate immune cells [2;4]. In light of the increased infiltration of these leukocytes upon infection, reduced numbers of $\alpha\beta$ T cells in lungs of infected mice was intriguing. In-vitro infection of innate immune cells like macrophages and dendritic cells with *Francisella* results in aberrant maturation affecting their cytokine secretion and antigen presentation abilities [7; 8]. It is possible that, in-vivo, due to absence of proper antigen presentation by these cells, the T cells in lungs of infected mice fail to proliferate. However, a decrease in the number of T cells to levels even lower than those in mock control animals in the current study suggested depletion of T cells either by cell death or by trafficking to other organs. To test this possibility, staining for Annexin V, an apoptosis marker, was performed.

3.2. $\alpha\beta$ T cells in lungs undergo apoptosis during pulmonary *F. novicida* infection

Total lung cells from mice at 72 h p.i. were simultaneously stained for TCR β and Annexin V and were analyzed by FACS. Propidium iodide was used to exclude necrotic/dead cells. The gating scheme is described in figure 2A. As depicted in figure 2B, 62% of $\alpha\beta$ T cells were apoptotic upon *F.n.* infection at 72 h p.i. which was significantly higher than those in mock control lungs.

In experimental models of sepsis, the hyper-inflammatory state of the immune system is coupled with apoptosis of cells of innate and adaptive immune system as a mechanism for the host to control overt inflammation [9]. In addition, respiratory infections by *Mycobacterium* [10], *Streptococcus pneumoniae* [11] as well as HIV infections [12] have been shown to cause

T cell apoptosis. In both conditions, a profound depletion of T cells manifests in an inability of the host to eradicate the primary infection and/or prevent the development of new secondary infections resulting in death [13;14]. Whether the T cell apoptosis observed during respiratory tularemia in this study is a result of a virulence mechanism of *Francisella* or a bystander effect of hype-immune responses caused by infection is currently being investigated in our laboratory. Our preliminary in-vitro experiments with human Jurkat T lymphocytes showed that *F. novicida* does not infect T cells (data not shown). Thus T cell apoptosis observed in current study may not be due to direct infection of the cells by the organism. A previous study had shown TNF receptors 1 and 2 dependent depletion of CD4+CD8+ thymocytes from thymus upon aerosol infection of mice with virulent Type A strain of *Francisella* [15]. Whether similar mechanisms are responsible for T cell depletion in lungs of *F. novicida* infected mice needs to be determined. Regardless, the loss of T cells may be further contributing to the lack of the host's ability to control the infection either due to lack of T lymphocytes tempering the strength of innate immune response [16] or due to the loss of the normal transition from an innate to adaptive response.

The current study shows a significant loss of CD4+ and CD8+ T cells from lungs during respiratory tularemia. This finding of T cell depletion at a critical time in host immunity may have wide-spread implications in designing future therapeutics and vaccination remedies for this deadly infection.

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Figure 1.

Intranasal infection with *F. novicida* results in depletion of $\alpha\beta$ T cells from lungs. Lungs from mock control and mice infected intranasally with F.n. were processed for in-situ IF staining or for FACS analysis as described in Materials and Methods. (A, upper panel) Lungs from infected mice showed reduced number of $\alpha\beta$ T cells (red) while the number of CD11b+ cells (red) was substantially higher in infected lungs in comparison to mock lungs at 72 h p.i. Nuclei (blue) are stained with 4'6' diamidino-2-phenylindol-dilactate (DAPI). Images are from one representative experiment of three performed ($n = 3-4$ mice per group for each experiment). Magnification X 200. Lower panel shows FACS analysis of lung cells from mock control or mice infected with F.n. at 72 h p.i. The cells were stained for $\alpha\beta$ T cells and CD11b+ cells as described in Methods. Appropriate isotype matched negative controls were used to set the gates. The positively stained cells were expressed as % of total lung cells. An average of % positive cells from three mice per group of one representative experiment is shown. The total number of lung cells ($\times 10^6$) in mock mice was 7.9 ± 1.9 and in *F. novicida* infected mice was 10.2 ± 1.2 in this representative experiment. Experiment was repeated three times with similar results. ***, $p < 0.0005$. (B). Upper panel shows FACS analysis of lung cells from mock control or infected mice at 72 h p.i. stained simultaneously for CD4+ and TCR β for CD8+ and TCR β markers. The numbers on upper right box of each image depict % of double positive cells. Lower Panel shows graphical representation of these results. The positively stained cells were

expressed as % of total lung cells. An average of % positive cells from three mice per group is shown. Experiment was repeated two times with similar results. ***, $p < 0.0005$.

Figure 2.

$\alpha\beta$ T cells in lungs undergo apoptosis during pulmonary F.n. infection. (A) Gating scheme for identification of apoptotic $\alpha\beta$ T cells in mice lungs is shown. The lung cells from mock control or infected mice at 72 h p.i. were stained simultaneously with APC conjugated rat anti-mouse TCR β antibody for $\alpha\beta$ T cells, FITC conjugated Annexin V and propidium iodide. Lymphocyte population in lungs (P1) was gated to identify $\alpha\beta$ T cells (P2). P2 was then gated to identify propidium iodide positive necrotic cells (P3). P3 was then excluded to identify Annexin V positive apoptotic $\alpha\beta$ T cell population. B, Upper panel shows a representative image of FACS analysis of apoptotic $\alpha\beta$ T cells in mock control and F.n. infected mice lungs at 72 h p.i. The positively stained cells are expressed as % of $\alpha\beta$ T cell population in lower panel. An average of % positive cells from one representative experiment with three mice per group is shown. The experiment was repeated three times with similar results. *, $p < 0.05$; **, $p < 0.005$, ***, $p < 0.0005$.