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Low dose aerosol infection of mice with virulent type A *Francisella tularensis* induces severe thymus atrophy and CD4⁺ CD8⁺ thymocyte depletion

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Abstract

Francisella tularensis is a gram-negative facultative intracellular bacterium and the causative agent of tularemia. Two subspecies (type A and B strains) of the pathogen exist, the former being much more virulent than the latter for humans and other higher mammals. In this study, we examined the effect of virulent strains of *F. tularensis* infection on the thymus and thymocytes and the potential mechanisms involved. Low-dose aerosol exposure of C57BL/6 mice with type A, but not type B, *F. tularensis* caused severe reduction in thymus weight and destruction of thymocytes, particularly CD4⁺ CD8⁺ thymocytes, by day 4 after infection. The depletion of thymocytes was accompanied by a significant increase in circulating cortisone levels and could be partially prevented by adrenalectomy. Moreover, thymus atrophy and thymocyte depletion following infection were abolished in mice deficient in tumor necrosis factor receptors 1 and 2, but not in FasL-deficient mice. The severe destruction of the thymus and selective depletion of immature thymocytes during type A *F. tularensis* infection may represent a key pathogenic mechanism in tularemia and could hinder the development of an effective primary immune response against this highly virulent pathogen.

Keywords

Francisella tularensis; Thymus; Pathogenesis; Mouse model; Host response

1. Introduction

Francisella tularensis is a facultative intracellular bacterium and the causative agent of human tularemia [1]. Naturally, it can initiate infection by inhalation, ingestion, or skin contact and arthropod bites [1]. Two sub-species of *F. tularensis*, *F. tularensis* subspecies tularensis (type A) and *F. tularensis* subspecies holarctica (type B), exist and both are highly infectious for humans [2]. However, only type A strains of *F. tularensis* routinely cause fatal infection in people, especially following exposure to infectious aerosols of the pathogen [1]; inhalation of as few as 10 virulent type A bacilli is sufficient to initiate severe disease. Thus, type A *F. tularensis* has been classified as a Category A biological warfare agent by the CDC [3]. Despite

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its clinical and biosecurity importance, the pathogenic mechanisms of virulent strains of *F. tularensis* remain largely unknown since, most research work in this area during the last two decades has utilized the attenuated, live vaccine strain of *F. tularensis* (LVS) (reviewed in [4]).

One of the common features occurring in acute natural infections in humans and experimental animals is a thymus atrophy defined by loss of thymus weight and cellularity (reviewed in [5]). In this regard, experimental infection of mice with a number of bacteria (*Listeria monocytogenes* [6], *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Streptococcus pneumoniae* [7]), viruses (murine cytomegalovirus [8], murine leukemia virus [9], HIV [10], and mouse hepatitis virus [11]), and protozoa (*Toxoplasma gondii* [12], and *Trypanosoma cruzi*) induces a severe lymphocytopenia and marked thymus atrophy and loss of cortical lymphocytes. In addition, certain bacterial products and toxins, such as lipopolysaccharide (LPS) [13], enterotoxigenic *E. coli* enterotoxin [14] and mycobacterial cord factor (trehalos 6,6'-dimycolate) [15], also cause severe thymus damage. Furthermore, infection of mice with a large dose (~100 LD₅₀) of type A *F. tularensis* through a unnatural route (i.p.) for initiating tularemia resulted in a marked thymus atrophy and T cell depletion [16]. However, the impact of type A *F. tularensis* infection via other more natural routes (such as intradermal and aerosol) on thymus anatomy and physiology has not been examined although we have recently shown that low-dose aerosol infection with virulent type A *F. tularensis* induced high mortalities and severe destruction of peripheral lymphoid tissues in mice [17]. Since, respiratory tularemia initiated via inhalation of small numbers of pathogens is the most likely form of the disease in the event of bioterror attacks we were, therefore, interested in determining the effect of low-dose aerosol infection with virulent type A *F. tularensis* on the thymus in a mouse model of inhalation tularemia [17], and to characterize the potential underlying mechanisms. We report here that low-dose aerosol infection of mice with type A, but not type B, *F. tularensis* induces severe thymus atrophy and marked depletion of CD4⁺ CD8⁺ thymocytes, which is mediated at least partially by tumor necrosis factor (TNF) and corticosteroid, but is independent of Fas ligand (FasL) signaling.

2. Results and discussion

2.1. Low-dose aerosol infection of mice with type A *F. tularensis* induces severe thymus atrophy and selective depletion of CD4⁺ CD8⁺ thymocytes

Our previous studies have shown that most C57BL/6 (B6) mice died by day 5 following a low-dose (lung retention dose 10-20 cfu) aerosol exposure to type A *F. tularensis* strain FSC033 [17]. Infected B6 mice showed mild weight loss, but otherwise remained asymptomatic until a few hours before death. To determine the effect of low-dose aerosol infection with type A *F. tularensis* on the thymus, groups of B6 mice were killed on day 0, 2 and 4 post-infection (dpi), and the changes in the mean body weights and relative weights of the thymus over the course of infection were assessed. As can be seen in Table 1, the thymus size decreased slightly at dpi 2 and drastically by dpi 4 (24-48 h prior to expected death). Concurrent with the decrease in the thymus size, the total number of thymocytes in the infected mice dropped by 85% at dpi 4 (Table 1). Histological examination of the thymuses from infected mice revealed a marked disappearance of lymphocytes from the thymic cortex (Fig. 1). Thus, in mice infected with type A *F. tularensis* by aerosol, the thymus involutes during the infectious process to the same extent as that reported after high dose i.p. infection with another type A strain of the pathogen [16] and during infections initiated by other pathogenic microbes such as *L. monocytogenes* [6], *K. pneumoniae* [7]), *T. gondii* [12] and *Trypanosoma cruzi* [18].

To further evaluate the effect of type A *F. tularensis* infection on the thymus and to determine whether type A *F. tularensis* selectively affects certain thymocyte subpopulations, thymocytes were isolated from mice on dpi 0, 2 and 4, and subsets of immature CD4⁺ CD8⁻ and CD4⁺

CD8⁺ T cells, as well as more mature single-positive CD4⁺ CD8⁻ and CD4⁻ CD8⁺ cells, were analyzed by flow cytometry. *F. tularensis* infection drastically reduced the percentage of CD4⁺ CD8⁺ cells from 76% at dpi 0 to 31% at dpi 4 (Table 1 and Fig. 2). When combined with the significant decrease in the total number of thymocytes (Table 1), the absolute number of CD4⁺ CD8⁺ cells decreased to <5% of normal levels. In contrast, the absolute number of CD4⁻ CD8⁻ cells decreased only moderately in spite of the substantial increase in the relative proportion (percentage) of this subpopulation (Table 1). Similarly, both the CD4⁺ CD8⁻ and the CD4⁻ CD8⁺ subpopulations were also affected, although not significantly, by the infection. Thus, aerosol infection with type A *F. tularensis* affected all subsets of thymocytes but the CD4⁺ CD8⁺ subset was the most severely affected.

The massive decline in CD4⁺ CD8⁺ thymocytes during infection with type A *F. tularensis* may reflect impaired thymocyte maturation from CD4⁻ CD8⁻ precursor cells. In this regard, stem cells produced in the bone marrow enter the bloodstream and are transported to the thymus. In the earliest stage, these cells express neither CD4 nor CD8, then they differentiate to become CD4⁺ CD8⁺ thymocytes, which undergo a rigorous process of selection. Cells that survive this process down-regulate either their CD4 or CD8 molecules to become single-positive, immunocompetent cells, which are released into the periphery and capable of recognizing foreign antigens presented by cell surface molecules of the MHC [19].

2.2. Effect of adrenalectomy on type A *F. tularensis*-induced thymus atrophy

Many pathogens and their products have previously been shown to affect thymus development (reviewed in [5]). Several mechanisms have been implicated in the process of thymocyte death and thymus atrophy during the course of an infection. While some pathogens can interact directly with thymocytes and thymic epithelial cells, others cause cortical depletion of the thymus in an indirect way, mediated either by the secretion of adrenal glucocorticosteroid hormones resulting from the acute stress of the infection or by pathogen-induced release of certain cytokines [5].

To better understand the process of thymocyte death after *F. tularensis* infection, we first assessed the potential contribution of stress-mediated corticosteroid activity. Low-dose aerosol infection of B6 mice with type A *F. tularensis* significantly increased the serum cortisol level (Fig. 3(A)), which was evident as early as dpi 2 ($p < 0.05$) and peaked at dpi 4 ($p < 0.01$), the kinetics of which coincided with the appearance of the severe thymus atrophy and onset of thymocyte death (Table 1). Moreover, adrenalectomy, but not sham-surgery, prevented both the increase in the serum cortisone level (Fig. 3(B)) and thymocyte depletion (Table 2). Taken together, these data suggest that glucocorticoids are partially involved in the development of thymus atrophy and thymocyte death associated with type A *F. tularensis* infection. In this regard, it is well recognized that all lymphocytes express functional corticosteroid receptors [20], and murine and human immature thymocytes are sensitive to cortisone-induced apoptosis [21]. However, the role of corticosteroids in mediating thymocyte death in response to infection appears to vary from pathogen to pathogen. Thus, associations between increased adrenal corticosteroid secretion and thymus lymphoid depletion have been established in the early phase of *L. monocytogenes* infection [22], in high dose i.p. challenge with type A *F. tularensis* [16] and in LPS-treated mice [13]. On the other hand, high levels of circulating cortisone were detected in acute *T. cruzi* infection but adrenalectomy did not prevent the development of thymus atrophy or thymocyte subset depletion [18]. Finally, thymus atrophy seen in *T. gondii* infection [12] or in mice treated with mycobacterial cord factor administration [15] appears to be independent of corticosteroid activity since the thymus atrophy was not prevented in infected adrenalectomized mice [12].

It is worthy noting that the thymus weight index and total thymocyte number were higher in the mice used in this experiment (Table 2) as compared with these used in other experiments

(Tables 1 and 3). We have no obvious explanation for this difference except that the mice used in this experiment were younger than those used in other experiments (Tables 1 and 3) because the vendor preferred to perform the adrenalectomy at 6 week of age and mice were used 2 weeks after the surgical recovery. However, despite the initial difference in the thymus weight index and total numbers of thymocytes between these experiments, the magnitudes of thymus atrophy and thymocyte reduction were nevertheless similar between experiments (43-66% reduction in thymus index and 72-89% reduction in thymocyte number).

2.3. Role of TNF and FasL signaling in type A *F. tularensis*-induced thymus atrophy

Both Fas and TNF have been shown to induce apoptosis either by engaging their respective ligands or by cross-linking with an agonist antibody [23]. Low dose aerosol infection with type A *F. tularensis* induced TNF- α mRNA expression in the lung and a moderate increase in serum TNF- α (data not shown). To determine the potential role of TNF and FasL signaling pathway in the development of type A *F. tularensis* infection-associated thymus atrophy, TNFR1R2-/- mice and gld (generalized lymphoproliferative disease) mice with alterations in the Fas signaling pathway due to a point mutation in the Fas ligand [24] were challenged by aerosol with low-dose type A *F. tularensis*. While gld mice developed thymus atrophy and CD4⁺ CD8⁺ thymocyte depletion, which was comparable to infected conventional B6 mice at dpi 4, TNFR1R2-/- mice did not show any thymus changes (Table 3), suggesting that the presence of TNF receptors, but not FasL, is required for *F. tularensis* infection-associated thymocyte death. In this regard, TNF is a common denominator released during both Gram-+ve and -ve bacteria-induced thymus atrophy and could induce thymocyte death [7,13]. TNF- α plays a very prominent and probably critical role in mediating cyclic AMP-induced apoptosis of CD4⁺ CD8⁺ cells [25]. Although serum TNF- α levels in mice treated with mycobacterial cord factor were not significantly elevated, administration of anti-TNF- α antibody almost completely inhibited thymus atrophy [15]. Moreover, lymphotoxin inhibits intrathymic T cell differentiation, thymus atrophy and death of CD4⁺ CD8⁺ thymocytes [26]. On the other hand, thymocyte depletion during acute *T. cruzi* infection in B6 mice coincided with increased serum levels of TNF- α but neutralization of TNF- α with Mab failed to prevent the thymus damage [27]. It is also interesting to note that the thymocyte death induced by monoglyceride was independent of Fas/FasL interactions [28].

2.4. Presence of *F. tularensis* in the thymus tissue

The effective prevention of thymocyte death in *F. tularensis* infected adrenalectomized mice and TNFR1R2-/- mice implies that the *F. tularensis* infection-associated thymocyte death is unlikely the consequence of a direct bacterial cytotoxicity effect. In this regard, *F. tularensis* was not detected by TEM and bacterial culture in severe atrophic thymuses of mice i.p. infected with high dose of type A *F. tularensis* [16]. However, in our mouse model we found that the infected mice develop severe bacteremia at dpi 4 [17], thereby raising the possibility that some circulating organisms might deposit in the thymus tissue. Indeed, in contrast to the results of Ito et al. [16], we have cultured large numbers of *F. tularensis* ($\log_{10}$ 6.07 $\pm$ 0.35 cfu) consistently from the thymuses of our infected mice at dpi 4. The *F. tularensis* cultured from the thymus was unlikely due to the potential contamination by trace amounts of blood retained in the tissue because the *F. tularensis* burden in the thymus was equivalent to the number of *F. tularensis* in one ml of blood ($\log_{10}$ 6.52 $\pm$ 0.56 cfu/ml) at the same time point. Moreover, the presence of *F. tularensis* and its association with thymus cells was confirmed by TEM (Fig. 4). However, in vitro exposure of cultures of thymocytes, isolated from untreated mice, to *F. tularensis* failed to induce thymocyte death (data not shown). Thus, thymus atrophy seen in *F. tularensis* infected mice appears to be mediated by an indirect mechanism.

2.5. Apoptosis is not involved in the depletion of CD4⁺ CD8⁺ thymocytes

It is well documented that a number of bacterial, viral and parasitic infections induce preferential decreases of CD4⁺ CD8⁺ thymic lymphocytes through the apoptosis pathway [9, 11, 14]. In this regard, it has been reported that LVS, an attenuated live vaccine strain of type B *F. tularensis*, induces apoptosis of the macrophage-like cell line J774 in vitro [29]. To determine whether apoptosis contributes to the selective loss of CD4⁺ CD8⁺ thymocytes during type A *F. tularensis* infection, thymuses were collected from infected and control mice on dpi 4 and examined for evidence of apoptosis. Although, the thymic cortex had a 'starry-sky' appearance histologically, apoptotic bodies were found only occasionally in both the cortex and medulla. TEM examination showed that the majority of thymocytes appeared necrotic with disrupted nuclear and plasma membranes. Cells with characteristic features associated with apoptosis (nuclear condensation and fragmentation) were rarely seen, implying that thymocyte apoptosis was not a common feature in type A *F. tularensis*-associated thymocyte death. The lack of apoptosis was further confirmed by examining thymocyte DNA by electrophoresis for the characteristic laddering pattern associated with apoptosis and by in situ end labeling of fragmented DNA in thymocyte cytospin slides (data not shown). Taken together, these results unequivocally demonstrate that type A *F. tularensis* induces thymocyte death by a mechanism distinct from apoptosis following inhalation of low doses of the pathogen. In this regard, bacterial induced cell death, independent of apoptosis, was reported in macrophages infected by *Salmonella enterica* serovars *Typhimurium* and *Dublin*, by a mechanism involving caspase 1 but not caspase 3 [30]. In addition, it has been recently shown that necrosis may be less 'accidental' or more regulated than previously thought, with some involvement of caspases and with regulatory links to apoptosis [30, 31]. Further study of the mechanism of *F. tularensis* induced thymocyte lysis will shed light on the regulation and mechanism of apoptosis-independent cell death, which might also be induced by other pathogens.

2.6. Clinical relevance of thymocyte death in the pathogenesis of *F. tularensis* infection in mice

We next determined whether the thymus damage induced by aerosol infection with type A *F. tularensis* contributes to the high mortalities and rapid death of infected mice since, previous studies have shown that morbidity and mortality from severe bacterial infections may be directly related to sepsis-induced death of lymphocytes in the thymus and other lymphoid organs [7]. To examine this possibility, we first compared the clinical outcome of thymectomized C57BL/6 mice, C.B.-17 *scid* mice (lack of functional T and B lymphocytes), and control C57BL/6 mice following low-dose aerosol infection with type A *F. tularensis*. There was no significant difference in the mortality or the median time to death among these mouse strains (Fig. 5), implying that thymocyte death per se does not significantly contribute to the high mortality seen in conventional mice infected with type A *F. tularensis*. This conclusion is further supported by the finding that mice infected with similar numbers of type B *F. tularensis* by aerosol did not develop thymus atrophy or thymocyte death at dpi 5 (data not shown) although all the mice, as expected, died of the infection between dpi 6 and 7 [17]. Although, the disassociation between the thymus atrophy and clinical outcome observed in this study was somewhat surprising and disappointing, detailed characterization of the thymus damage and the potential mechanism involved remains important for our understanding of the pathogenesis of this devastating disease since the thymus appears to be one of the major target organs of *F. tularensis* infection and could still play an important role in the development of tularemia.

In summary, the present study has shown that, similar to high-dose i.p. infection [16], low-dose aerosol infection with type A *F. tularensis* caused severe thymus atrophy, thymocyte destruction, and increased circulating cortisone levels. Furthermore, we have identified that the CD4⁺ CD8⁺ thymocytes were the cell subtype predominantly affected and that the

development of thymus atrophy was mediated by corticosteroid and TNF, but was independent of FasL signaling. Unlike high-dose i.p. type A infection [16], *F. tularensis* were identified in the thymus tissue of our infected mice by both bacterial culture and TEM methods.

F. tularensis may kill thymocytes or their progenitors either directly or indirectly. However, the precise mechanism remains obscure although, adrenal activity and TNF signaling appear to be contributing factors. A direct thymocytolytic effect by the pathogen is unlikely since *F. tularensis* lacks typical LPS or any other known cytotoxins [32] and none of the numerous *F. tularensis* organisms present in the thymus tissue of infected mice was found within thymic lymphocytes, which is consistent with our current knowledge that *F. tularensis* does not infect lymphocytes [1]. Indeed, experiments using cultured thymocytes failed to show any cytolytic effect of the organism (data not shown). On the other hand, *F. tularensis* infection of thymus macrophages, dendritic cells and/or epithelial cells may compromise their functions in regulation of thymopoiesis and facilitate the process of programmed cell death that normally occurs in the thymus [11].

Regardless of the underlying mechanisms, the severe destruction of the thymus and thymocytes seen in the present study may represent a key pathogenic mechanism in fatal tularemia and could have serious clinical and immunological consequences, as the thymus is the site of T cell differentiation and maturation throughout life. Deletion of thymocytes thereby eliminates the precursors of the cells that would otherwise help to maintain effective immunity against the pathogen. Besides the phenotypic changes identified in the present study, thymocytes may be also functionally affected (such as cytokine production and cell cycling) [5,12]. In this regard, although the thymus is not the site of memory cell generation and is probably not essential for the maintenance of memory cell pool, thymus destruction will nevertheless have the potential impact on the lymphocyte output and/or functional lymphocyte responses to foreign antigens. Thus, disruption of thymic tissues could hinder the development of an effective primary immune response against *F. tularensis* in naïve individuals. In addition, although in this study we have focused our attention on the thymus because of the limited human resources and biocontainment facility capacities future detailed study on the effect of aerosol *F. tularensis* infection on the peripheral lymph organs (such as spleens and regional lymph nodes) will be important for our further understanding of the immunopathogenesis of respiratory tularemia.

3. Materials and methods

3.1. Mice

Specific-pathogen-free adult female C57BL/6 (B6) mice (8-12 weeks of age), age- and sex-matched adrenalectomized B6 mice, and thymectomized B6 mice were purchased from Charles River Laboratories (St Constant, Que.). B6;129S-Tnfrsf1a^{tm1}Imx Tnfrsf1b^{tm1}Imx (TNFR1R2-/-) mice and B6Smn.C3H-FasI^{gld}(gld) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in microisolator cages under barrier conditions with free access to commercially available chow and water. All animal experimentation was performed in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals, and with the approval of the local institutional review board.

3.2. *F. tularensis* and aerosol infection

Stocks of type A *F. tularensis* strain FSC033/snMF, originally isolated from a squirrel in Georgia, USA [33], and type B *F. tularensis* strain FSC108/SBL R45/81, originally isolated in Sweden from an ulcer of a tularemia patient [33] were prepared as previously described [17]. For aerosol exposure, thawed *F. tularensis* stocks were diluted in Mueller Hinton broth containing 20% (v/v) glycerol to maintain infectivity at the high relative humidity employed.

Aerosols of *F. tularensis* strains were generated with a Lovelace nebulizer operating at a pressure of 40 psi to produce particles in the 4–6 μm range required for inhalation and retention in the alveoli [34]. Mice were exposed to these aerosols for 7 min using a customized commercial nose-only exposure apparatus (In-tox Products, Albuquerque, NM) resulting in the implantation of 10–20 organisms into the lungs (referred to as low-dose exposure).

At predetermined times, groups of 4–5 mice were individually weighed and then sacrificed by CO_2 asphyxiation. Blood was collected by cardiac puncture, and serum separated and stored at -80°C . The thymus and liver were removed and weighed in a balance with accuracy to 0.1 mg. The organ index was calculated as organ weight (in grams)/body weight (in grams) $\times 100$. In some experiments, the number of viable bacteria in the thymus and blood was determined by quantitative bacteriology.

3.3. Flow cytometry analysis of thymocytes

Single thymocyte suspensions were prepared by pressing individual thymus in cold PBS through a 75- μm pore sized Falcon disposable cell strainer. The total thymocyte number in each mouse was determined using a hemocytometer. The cells ($\sim 1.0 \times 10^6$) in 50 μl cold staining buffer were first incubated with Fc Block (clone 2.4G2) in the dark on ice for 5 min, and then incubated on ice for 30 min with FITC-conjugated anti-mouse CD8 α (clone 53-6.7), Cy-Chrome-conjugated anti-mouse CD4 (clone RM4-5), and phycoerythrin (PE)-conjugated anti-mouse Thy 1.2 (clone 30H12) (BD Biosciences, San Diego, CA). The samples were then fixed in 2% paraformaldehyde in PBS overnight at 4°C and analyzed on a Coulter EPICS XLTM unit (Beckman Coulter, Ville St-Laurent, QC, Canada). Background staining values obtained with fluorochrome conjugate isotype controls were subtracted.

3.4. Light and electron microscopy

Tissues for light microscopy were fixed immediately by immersion in 10% neutral buffered formalin, and processed for histopathology by standard paraffin embedding methods. Sections of 4 μm thickness were stained with hematoxylin and eosin (HE), and examined by light microscopy. Preparation for transmission electron microscopy (TEM) essentially followed the method of Austin et al. [35]. Tissues were fixed in 0.2 M cacodylate buffer pH 7.4 containing 2.5% (v/v) glutaraldehyde, post-fixed in 0.2 M cacodylate buffer pH 7.4 containing 1% OsO_4 , and dehydrated through a graded series of ethanol (15 min each in 50, 70, and 90% ethanol, followed by three 20 min incubations in 100% ethanol). Samples were then infiltrated and embedded in Taab 812 resin (Marivac, Halifax, NS, Canada). Thin sections were cut on a Reichert-Jung Ultracut E ultramicrotome (C. Reichert AG, Wien, Austria), stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Zeiss EM902 transmission electron microscope (Carl Zeiss, Thornwood, NY, 10594, USA) operating at 80 kV with the energy loss spectrometer in place.

3.5. Determination of serum TNF- α and corticosterone levels

Serum TNF- α levels were measured by specific sandwich enzyme-linked immunosorbent assay (ELISA) (BD Biosciences). Serum corticosterone levels were measured with an EIA kit (ALPCO Diagnostics, Windham, NH). The detection limits of the assays were 125 pg/ml for TNF- α and 2.3 ng/ml for corticosterone, respectively. All samples were assayed in duplicate and were diluted 1:4 for the TNF- α cytokine ELISA and 1:10 for the corticosterone assay.

3.6. Detection of thymocyte apoptosis

The degree of thymocyte apoptosis was assessed by both analysis of DNA fragmentation and the terminal deoxynucleotide transferase (TdT) dUTP-biotin nick-end labeling (TUNEL) assay. For DNA fragmentation analysis, DNA was extracted from 10^7 thymocytes using the

DNeasy kit (Qiagen, Valencia, CA) and 10 mg of such DNA was electrophoresed for 3 h at 90 V in 2% agarose containing 5 µg/ml ethidium bromide. For TUNEL assay, single-cell suspensions of thymocytes were prepared and fixed in cold acetone and stained using the In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Indianapolis, IN). Thymocytes from mice treated with dexamethasone (2.5 mg in 0.1 ml PBS) were included as positive control for the analysis.

3.7. Statistical analysis

Data are presented as means $\pm$ standard deviations. Differences in quantitative measurements were assessed by one-way analysis of variance (ANOVA) followed by Neuman-Keuls post-hoc multiple comparison tests. Differences were considered significant at $p < 0.05$.

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Fig. 1.

Thymus from 8-week-old female CB57BL/6 mice infected by aerosol with low-dose type A *F. tularensis*. (A) Normal appearance of the thymus from a mouse killed at dpi 1 showing a thick zone of densely staining cortex (C) and a narrow zone of lightly staining medulla (M). (B) Severe thymus atrophy from a mouse killed on dpi 4 showing substantial reductions in the thickness of the cortex and depletion of cortical lymphocytes. HE (bar=100 µm).

Fig. 2. Representative flow cytometry profiles of CD4 and CD8 expression on thymocytes from control and *F. tularensis* infected mice. Subset percentages are indicated in each quadrant.

Fig. 3.

Effect of aerosol infection with low-dose type A *F. tularensis* on serum cortisone levels in mice. (A) Significantly increased serum cortisone levels in 8-week-old female C57BL/6 mice at both day 2 ($p < 0.05$) and day 4 ($p < 0.01$) following the aerosol infection. (B) Compare to sham-operated mice (sham), adrenalectomy (Adrx) prevented the elevation of circulating cortisone levels in mice following the aerosol infection with low-dose type A *F. tularensis*.

Fig. 4.

Electron micrographs of the thymus from a mouse killed at day 4 post aerosol infection with low-dose type A *F. tularensis*. (a) Region showing the necrosis of a thymocyte (*) and the presence of an *F. tularensis* organism (rectangle box). (b) Higher magnification of area in box showing the presence of a relatively intact *F. tularensis* organism between two thymus cells. Bars=2.5 μ m in A, and 0.25 μ m in B.

Fig. 5. Survival rates of thymectomized, SCID and C57BL/6 mice after aerosol infection with low-dose type A *F. tularensis*.

Table 1

Effect of low-dose aerosol infection of mice with type A F. tularensis on the thymus weight and thymocyte number and subpopulations

DPI	Thymus weight index	Total no. of thymocytes ($\times 10^7$)	% Cells positive for (total cells/thymus, $\times 10^7$)			
			CD4 ⁺ CD8 ⁻	CD8 ⁺ CD4 ⁻	CD4 ⁺ CD8 ⁺	CD4 ⁻ CD8 ⁺
0	0.374 $\pm$ 0.164	11.81 $\pm$ 10.55	11.59 $\pm$ 3.33 (1.15 $\pm$ 0.82)	2.15 $\pm$ 1.23 (0.57 $\pm$ 0.09)	75.77 $\pm$ 7.61 (8.50 $\pm$ 7.05)	10.49 $\pm$ 9.49 (2.01 $\pm$ 3.08)
2	0.294 $\pm$ 0.040	7.50 $\pm$ 2.15	12.69 $\pm$ 1.99 (0.93 $\pm$ 0.21)	2.52 $\pm$ 1.12 (0.17 $\pm$ 0.07)	72.85 $\pm$ 9.60 (5.36 $\pm$ 1.33)	11.94 $\pm$ 11.79 (1.04 $\pm$ 1.29)
4	0.160 $\pm$ 0.058 ^{**}	1.77 $\pm$ 2.51 [*]	25.03 $\pm$ 14.88 (0.28 $\pm$ 0.21)	9.08 $\pm$ 5.27 (0.10 $\pm$ 0.08)	31.38 $\pm$ 25.44 ^{***} (0.39 $\pm$ 0.52)	34.52 $\pm$ 29.43 (1.00 $\pm$ 1.86)

Groups of 10-week-old C57BL/6 mice were inoculated by aerosol with ~20 cfu type A F. tularensis and killed at 0, 2 and 4 days post-inoculation (DPI). The organ weight index was calculated as organ weight (in grams)/bodyweight $\times$ 100. Data are compiled from two separate experiments and expressed as means $\pm$ standard deviations (n=8).

*
p<0.05

**
p<0.01

<p<0.001 significantly lower than dpi 0 by ANOVA and Neuman-Keuls post-test, respectively.

Table 2

Thymus weight and thymocyte number and subpopulations in adrenalectomized and sham-adrenalectomized mice following low-dose aerosol infection with type *AF. tularensis*

Group	Thymus weight index	Total no. of thymocytes ($\times 10^7$)	% Cells positive for (total cells/thymus, $\times 10^7$)			
			CD4 ⁺ CD8 ⁻	CD8 ⁺ CD4 ⁻	CD4 ⁺ CD8 ⁺	CD4 ⁻ CD8 ⁺
Adrex-infect	0.530 $\pm$ 0.027 ^{**}	20.3 $\pm$ 6.89 ^{***}	6.9 $\pm$ 0.5 (1.38 $\pm$ 0.37)	2.4 $\pm$ 0.2 (0.50 $\pm$ 0.18)	88.9 $\pm$ 0.4 [*] (18.10 $\pm$ 6.19)	1.8 $\pm$ 0.2 (0.37 $\pm$ 0.0.17)
Sham-infect	0.309 $\pm$ 0.111	3.62 $\pm$ 2.58	24.5 $\pm$ 19.7 (0.60 $\pm$ 0.45)	10.4 $\pm$ 8.3 (0.27 $\pm$ 0.24)	60.6 $\pm$ 29.6 (2.61 $\pm$ 2.40)	4.5 $\pm$ 1.7 (0.14 $\pm$ 0.07)
Adrex only	0.662 $\pm$ 0.089 ^{***}	22.4 $\pm$ 4.46 ^{***}	9.4 $\pm$ 0.7 (2.10 $\pm$ 0.44)	2.9 $\pm$ 0.3 (0.65 $\pm$ 0.16)	85.7 $\pm$ 0.8 [*] (19.2 $\pm$ 3.75)	2.1 $\pm$ 0.4 (0.47 $\pm$ 0.18)
Sham only	0.493 $\pm$ 0.089 [*]	10.1 $\pm$ 4.27 [*]	9.6 $\pm$ 1.0 (0.91 $\pm$ 1.0)	2.6 $\pm$ 0.3 (0.29 $\pm$ 0.13)	84.9 $\pm$ 1.5 [*] (8.63 $\pm$ 3.65)	2.8 $\pm$ 0.2 (0.28 $\pm$ 0.12)

Groups of 8-week-old adrenalectomized (adrex) or age-matched sham-operated (sham) mice were left uninfected (only) or inoculated (infect) by aerosol with ~20 cfu type A *F. tularensis* and killed at 4 days post-inoculation (DPI). The organ weight index was calculated as organ weight (in grams)/bodyweight $\times$ 100. Data are from one of two separate experiments and expressed as means $\pm$ standard deviations ($n = 5$).

* $p < 0.05$,

** $p < 0.01$,

*** $p < 0.001$ significantly higher than shaminfected mice by ANOVA and Neuman-Keuls post-test.

Table 3

Thymus weight and thymocyte number and subpopulations in TNFR1R2^{-/-} and *gld* mice following low-dose aerosol infection with type A *F. tularensis*

Group	Thymus weight index	Total no. of thymocytes (×10 ⁷)	% Cells positive for (total cells/thymus, ×10 ⁷)			
			CD4 ⁺ CD8 ⁻	CD8 ⁺ CD4 ⁺	CD4 ⁺ CD8 ⁺	CD4 ⁻ CD8 ⁻
TNFR1R2 ^{-/-}	0.276±0.054 **	5.41±1.76 ***	15.3±6.1 * (0.78±0.26)	7.0±5.7 (0.35±0.28)	74.8±10.0 * (4.13±1.57)	4.32±0.97 * (0.22±0.06)
Gld	0.105±0.031	0.36±0.29	31.6±13.7 (0.09±0.04)	16.7±7.4 (0.05±0.02)	33.6±25.6 (0.18±0.25)	18.21±7.4 (0.05±0.03)
WT	0.183±0.054	0.61±0.39	30.4±16.3 (0.17±0.11)	10.42±5.9 (0.06±0.04)	45.2±21.3 (0.31±0.36)	14.0±9.4 (0.08±0.04)

Groups of 12-week-old TNFR1R2^{-/-} mice, *gld* mice or wild-type (B6) mice were inoculated by aerosol with ~20 cfu type A *F. tularensis* and killed 4 days postinoculation. The organ weight index was calculated as organ weight (in grams)/bodyweight×100. Data are compiled from two separate experiments and expressed as means±standard deviations (*n* = 8 for TNFR1R2^{-/-} and *gld* mice and *n* = 5 for WT mice).

*
p<0.05,

**
p<0.01,

p<0.001 significantly higher than wildtype mice by ANOVA and Neuman-Keuls post-test.