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Minimal Requirements for Murine Resistance to Infection with *Francisella tularensis* LVS

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Intraperitoneal or intravenous infection of mice with *Francisella tularensis* LVS is lethal, with an intraperitoneal 50% lethal dose (LD_{50}) approaching a single bacterium. Intradermal (i.d.) LVS infection has a much higher LD_{50}, about 10^6 bacteria in BALB/cByJ mice, and survival of i.d. infection leads to solid generation of immunity against lethal challenge. To define the minimal requirements for both initial and long-term survival of i.d. infection, we characterized the nature of i.d. LVS infection in lymphocyte-deficient BALB/cByJ*.scid* (*scid*) mice. *scid* mice infected i.d. with strain LVS survived for about 20 days and then died from overwhelming disseminated infection. However, *scid* mice treated with monoclonal antibodies to gamma interferon, tumor necrosis factor alpha, or neutrophils-granulocytes all died within 1 week of infection, indicating that these were essential for early control of infection. Studies using GKO (gamma interferon knockout) mice emphasized that gamma interferon is absolutely required for initial survival of i.d. LVS infection. *scid* mice could be reconstituted for long-term survival of i.d. LVS infection and clearance of bacteria by intravenous transfer of splenic lymphocytes or purified $B220^-/T^+$ lymphocytes but not *nu*/*nu* lymphocytes. T cells are therefore required for long-term clearance and survival of i.d. LVS infection; efforts to determine whether $CD4^+$ T cells, $CD8^+$ T cells, or both are involved are ongoing.

Francisella tularensis is a small, gram-negative facultative intracellular bacterium that is responsible for the rare but highly pathogenic disease tularemia (43). An attenuated live vaccine strain denoted LVS was developed as a human vaccine, but this strain is virulent for laboratory mice and causes a fulminant infection with a histopathology quite similar to that of human tularemia (15, 17). Previous studies have characterized the nature of the murine infection with LVS and demonstrated that intraperitoneal (i.p.) or intravenous (i.v.) infection of mice with *F. tularensis* LVS has an i.p. 50% lethal dose (LD_{50}) approaching a single bacterium (15, 17). Intradermal (i.d.) LVS infection has a much higher LD_{50}, about 10^6 bacteria in BALB/cByJ mice, and survival of i.d. infection leads to generation of very strong specific protective immunity against lethal challenge by any route with up to 10^6 LD_{50}s (14, 15, 17). This discrepancy in murine i.p. and i.d. LD_{50}s may have a counterpart in human tularemia infection, in that the case fatality rate has been estimated to be about 5% when the skin is the route of entry but approaches 60% when aerosol exposure leads to untreated systemic disease (42, 43). Thus, there are strong similarities between humans and mice in disease pathology, mice are readily susceptible to both lethal and sublethal infection, and survival of sublethal infection leads to a very strong and easily measurable protective immune response. These features make LVS infection in mice a very attractive model with which to address questions on the basic mechanisms of protection against intracellular bacterial infections.

Previous studies have emphasized a critical role for T cells in long-term resolution of LVS infection. Normal mice resolve a sublethal dose of i.d. LVS over 2 to 3 weeks (15, 35). However, athymic T-cell-deficient *nu*/*nu* mice are able to survive and control LVS infection for about 30 days but then succumb to fulminant systemic infection (13, 14). This initial survival was dependent on both gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (1, 14, 26). Since both normal and *nu*/*nu* mice also exhibit an unusual T-cell-independent protective mechanism that wanes after about 30 days (13, 14), it is difficult to further examine the contributions of T cells with *nu*/*nu* mice as either a model or for recipients in cell transfer studies.

scid mice, which lack both mature B and T lymphocytes, have been quite useful in studying initial infection with other bacteria. For example, studies of *Listeria monocytogenes* using *scid* mice have emphasized the importance of both natural killer cells and cytokines such as IFN-γ and TNF-α in initial survival of infection (4, 5, 45). Here we characterize the nature of LVS infection in *scid* mice to define requirements for initial and long-term survival of this intracellular bacterial infection.

MATERIALS AND METHODS

Animals. Specific-pathogen-free, male BALB/cByJ, BALB/cByJSmn-*scid*/J, and C57B1/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used at 6 to 12 weeks of age. C.B17 normal and C.B17-*scid* male mice were purchased from Taconic Farms (Germantown, N.Y.) and used with very similar results. Male BALB/c *nu*/+ and *nu*/*nu* mice were purchased from the Biological Resources Branch, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, Md.). GKO (gamma interferon knockout) mice on a C57B1/6J background ($N = 7$) were a generous gift from Ronald Schwartz and Elizabeth Majane, National Institute of Allergy and Infectious Diseases (Bethesda, Md.), through a material transfer agreement with Genentech, Inc. (South San Francisco, Calif.). Mice were barrier housed in sterile microisolator cages and routinely tested for common murine pathogens through the Division of Veterinary Services, Center for Biologics Evaluation and Research, and under a protocol approved by the Animal Care and Use Committee of the Center for Biologics Evaluation and Research. Mice were given 0.5 ml i.p. or 0.1 ml i.d. of the indicated dilution of LVS; the actual doses of bacteria inoculated were simultaneously determined by plate count. The LD_{50} was calculated by the method of Reed and Muench as discussed by Lennette (27). LD_{50}

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TABLE 1. Time course of i.d. infection of BALB/cByJ.*scid* mice with LVS[a]

Expt no.	i.d. LVS dose	No. of deaths/total no. of mice	MTD[b]
1	1.2×10^0	3/5	18.0 ± 1.2
	1.2×10^1	4/5	18.0 ± 0.0
	1.2×10^2	5/5	16.2 ± 1.6
	1.2×10^3	6/6	15.5 ± 3.3
2	1.3×10^3	5/5	17.8 ± 2.6
	1.3×10^4	5/5	20.1 ± 2.8
	1.3×10^5	5/5	18.5 ± 2.4
	1.3×10^6	5/5	19.2 ± 3.1
	1.3×10^7	5/5	10.4 ± 3.9

[a] BALB/cByJ.*scid* mice were infected with the indicated number of LVS bacteria (determined by plate count at the time of infection) and observed for morbidity and mortality through day 60. The two experiments are representative of seven total experiments of similar design.
[b] MTD, time to death (mean ± standard deviation) in days.

determinations, antibody treatments, and transfer experiments involved the use of groups of four to six mice, as indicated.

Bacteria and growth conditions. *F. tularensis* LVS (ATCC 29684; American Type Culture Collection, Rockville, Md.) was cultured on modified Mueller-Hinton agar plates or in modified Mueller-Hinton broth as described previously (3, 15, 17) and frozen in broth alone at −70°C; 1-ml aliquots were periodically thawed for use. The number of CFU after thawing varied less than 5% over a 6-month period.

Treatment of mice with anti-cytokine or anti-granulocyte antibodies. To deplete mice of circulating cytokines, mice were treated i.p. with 500 μg of anti-IFN-γ (39), anti-TNF-α (40), or control hamster immunoglobulin G (IgG) 1 h before infection with LVS, as described previously (14, 26). To deplete mice of neutrophils, mice were treated i.p. with 250 μg of RB6-8C5 (44) at 3 days and again at 4 hours before infection with LVS, as described previously for depletion of Gr-1$^+$ cells (41). Purified anti-IFN-γ and anti-TNF-α were the generous gift of Robert Schreiber (Washington University, St. Louis, Mo.). Purified RB6-8C5 was purchased from Pharmingen (San Diego, Calif.). Hamster IgG was purchased from Jackson Immunochemicals (West Grove, Pa.).

Reconstitution of *scid* mice with lymphocyte subpopulations. Single-cell suspensions were prepared from spleens from the indicated donor mice, erythrocytes were lysed with ammonium chloride, dead cells were removed by centrifugation through a 40% Percoll gradient, and the resulting cell suspensions were counted after trypan blue staining. For depletion of B cells, surface-activated T-25 or T-150 flasks purchased from Applied Immune Sciences (San Diego, Calif.) were coated with 50 μg of anti-B220 antibody (RA3-6B2; purchased from Pharmingen, San Diego, Calif.) per ml. For depletion of T cells, flasks were coated with a mixture of 50 μg of anti-CD3 (145-2C11), anti-CD4 (RM4-4, RM4-5, and H129.19), and anti-CD8 (53-6.7; all purchased from Pharmingen) per ml; a cocktail was determined to be optimal in initial experiments. The remainder of the depletion procedure was carried out as described in the manufacturer's instructions. For the enrichment of total T cells, splenic lymphocytes were applied to T-cell enrichment columns (R&D Systems, San Diego, Calif.) as described in the manufacturer's instructions. For the enrichment of CD8$^+$ T cells and CD4$^+$ T cells, splenic lymphocytes were prepared from normal BALB/cByJ mice that were treated in vivo on days −4 and −1 with 500 μg of anti-CD4 (GK1.5) or anti-CD8 (2.43) monoclonal antibodies, respectively, to deplete the corresponding T-cell subpopulation. Both the GK1.5 and 2.43 antibodies were produced as ascites, precipitated with 50% ammonium sulfate, and quantitated by an enzyme-linked immunosorbent assay (ELISA) with monoclonal rat IgG2b as a standard (Pharmingen). The depleted spleen cells were then applied to either CD4 or CD8 enrichment columns (R&D Systems). In all cases, aliquots of both the starting spleen cell populations and the final populations were analyzed by flow cytometry with a FACScan, with gates set for viable lymphocytes and monocytes according to forward- and side-scatter profiles. Cells were stained with a panel of monoclonal antibodies, including fluorescein isothiocyanate-anti-B220, phycoerythrin (PE)-anti-CD4, PE-anti-CD8, PE-anti-CD3, and PE-anti-γ/δ T-cell receptor (all purchased from Pharmingen) in both one- and two-color staining protocols. Optimal concentrations for staining with each lot of each fluorochrome-labeled antibody were carefully determined in preliminary experiments.

Characterization of antibody response. Titers of specific anti-LVS serum antibodies were determined as described previously (35). Briefly, Immulon 1 plates were coated overnight with live LVS, washed, and blocked with 10% calf serum, and serum samples were serially diluted. Horseradish peroxidase-labeled antibodies (goat anti-mouse immunoglobulin, anti-IgM, and anti-IgG that detect IgG1, IgG2a, IgG2b, and IgG3; Southern Biotech, Birmingham, Ala.) were added, and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used for color development. The endpoint titer was defined as the lowest dilution of immune serum that gave an optical density at 405 nm greater than the optical density at 405 nm plus three times the standard deviation of the value of the matched dilution of normal prebleed mouse serum and also greater than 0.050.

RESULTS

Initial survival of i.d. LVS infection by *scid* mice. To determine the resistance of *scid* mice to infection with LVS, both i.p. and i.d. LD_{50} determinations were made. Groups of five BALB/cByJ *scid* mice were inoculated i.p. with 10-fold dilutions of LVS bacteria ranging from 10^0 to 10^5 per mouse. As previously observed for normal and *nu*/*nu* mice, i.p. infection of *scid* mice with LVS was lethal. In one determination (of three total determinations), the i.p. LD_{50} of normal BALB/cByJ mice was 3.5 bacteria, while the i.p. LD_{50} of BALB/cByJ.*scid* mice (with the same dilutions of bacteria) was 1.9 bacteria. Unlike normal mice, however, *scid* mice cannot survive i.d. LVS infection (Table 1). When infected i.d. with any dose of LVS up to about 10^6 bacteria, *scid* mice lived for 15 to 20 days. At the highest doses tested (1.3×10^7 [Table 1] and 2×10^8 [data not shown]), *scid* mice died sooner, between 7 and 10 days. Evaluation of organ burdens in *scid* mice infected i.d. with 10^3 LVS showed that the numbers of bacteria in the spleen, liver, and lung achieved a plateau within about 5 days, remained static through about day 15, and dramatically increased shortly before death (data not shown), similar to the time course of infection in *nu*/*nu* mice (14).

Previous results have demonstrated that IFN-γ and TNF-α are critical to the early survival of i.d. LVS infection by normal and *nu*/*nu* mice (14, 26). These two cytokines are also required for early survival of i.d. LVS infection by *scid* mice, as shown in Table 2. In vivo depletion of either IFN-γ or TNF-α by treatment of mice with monoclonal antibodies to these cytokines lowered the mean time to death from i.d. LVS infection from 19 days to 7 to 9 days. The absolute dependence of early survival on IFN-γ is also shown by experiments using knockout mice that lacked the ability to make IFN-γ. When 14 GKO mice were infected with doses of LVS ranging from 1.8×10^1 to 1.8×10^4, they died within about a week of i.d. LVS infection (time to death [mean ± standard deviation], 8.0 ± 1.1 days for all doses); five of five control C57B1/6J mice survived the highest dose (1.8×10^4).

Previous results have also shown that neutrophils are important in initial survival of i.d. LVS infection by normal mice (41). This is also the case for *scid* mice: depletion of all granulocytes by in vivo treatment with an anti-Gr1 antibody (41, 44) prior to i.d. infection lowered the time to death from 18.7 ± 4.0 days

TABLE 2. Requirement for IFN-γ and TNF-α or initial survival of i.d. infection of *scid* mice with LVS[a]

Mice	Treatment	i.d. LVS dose	No. of deaths/total no. of mice	MTD[b]
C.B17 *scid*	Hamster IgG	10^3	4/4	18.8 ± 1.0
	Anti-TNF	10^3	4/4	7.0 ± 0.0
	Anti-IFN-γ	10^3	4/4	7.6 ± 0.5
C.B17 normal	Hamster IgG	10^3	0/4	
	Anti-TNF	10^3	4/4	9.2 ± 0.8
	Anti-IFN-γ	10^3	4/4	7.6 ± 1.1

[a] C.B17 normal or *scid* mice were treated with 500 μg of the indicated antibody and then infected with 10^3 LVS bacteria i.d. 1 h later; the actual infection dose was confirmed by plate count at the time of infection. Mice were observed for morbidity and mortality through day 60. This experiment is representative of three total experiments of similar design.
[b] MTD, time to death (mean ± standard deviation) in days.

TABLE 3. Reconstitution with normal, but not *nu/nu*, lymphocytes of long-term survival by *scid* mice of i.d. infection with LVS[a]

Cells transferred	i.d. LVS dose	No. of deaths/total no. of mice	MTD[b]
PBS	10^3	5/5	23.6 ± 2.6
Normal spleen cells	10^3	0/5	
nu/nu spleen cells	10^3	5/5	29.5 ± 6.7

[a] BALB/cByJ.*scid* mice were given either diluent PBS i.v., 2×10^7 normal BALB/cByJ spleen cells i.v., or 1×10^7 BALB/c *nu/nu* spleen cells i.v. on day 0. One day later, they were infected with 10^3 LVS bacteria i.d.; the actual infection dose was confirmed by plate count at the time of infection. Mice were observed for morbidity and mortality through day 60. This experiment is representative of three experiments of similar design.

[b] MTD, time to death (mean ± standard deviation) in days.

for six phosphate-buffered saline (PBS)-treated *scid* mice days to 7.0 ± 0.9 days for five RB6-8C5-treated mice. This is consistent with data demonstrating that i.d. infection of normal BALB/cByJ mice with LVS results in an accumulation of neutrophils in the spleen and liver within 2 to 3 days after infection (9 and data not shown).

Reconstitution of *scid* mice for long-term survival of i.d. LVS infection. To determine the lymphocytes required for long-term survival and clearance of LVS infection directly, spleen cells from normal, histocompatible BALB/cByJ mice were transferred i.v. to BALB/cByJ.*scid* mice, which were then infected i.d. 1 day later with 10^3 LVS bacteria. Initial experiments showed that transfer of 2×10^7 normal spleen cells was sufficient to reconstitute long-term survival of i.d. LVS infection by *scid* mice (termed reconstituted mice) (Table 3 and data not shown). Mice that received spleen cells from *nu/nu* mice only survived i.d. infected LVS infection for about 30 days (Table 3), similar to survival times of intact *nu/nu* mice that are i.d. infected (14). Reconstituted mice were observed through day 60, and then the survivors were sacrificed and analyzed for clearance of bacteria as well as cell engraftment in the spleen. All mice reconstituted with normal spleen cells contained $B220^+$, $CD4^+$, $CD8^+$, and $CD3^+$ cells in their spleens 6 weeks after transfer and had no detectable LVS bacteria (data not shown).

Two approaches were used to examine the contributions of the lymphocyte subpopulations present in normal spleen that were responsible for successful transfer of protection. First, spleen cells were depleted of either total $B220^+$ B cells or total T cells through specific adherence to anti-B220 antibodies or a cocktail of anti-T-cell antibodies on a solid surface (see Materials and Methods). This method permitted very efficient removal of the target cell population. All mice that received $B220^-$ cells survived i.d. LVS infection, while only two of five mice that received T^- cells survived (Table 4). Those mice that received normal spleen cells made small amounts of both IgM and IgG anti-LVS-specific antibodies, while those that received $B220^-$ cells had no detectable anti-LVS antibody, and those that received T^- cells made only IgM anti-LVS antibodies (Table 4). Surviving *scid* mice were sacrificed after 60 days and analyzed. Mice that received normal spleen cells contained both T and B cells, as above, and no detectable bacteria; cell recovery from these reconstituted mice averaged 10^7 total cells per spleen, about double the number usually found in (unreconstituted) *scid* spleens. Mice that received $B220^-$ cells averaged 3×10^6 T^+ cells per spleen but had small numbers of $B220^+$ cells as well, i.e., about 3.9×10^5 per spleen. This indicates that, despite apparently excellent depletion in the selection technique, B cells nonetheless repopulated mice. These surviving recipients had no detectable bacteria in their spleens. Conversely, surviving mice that received T^- cells averaged about 3.1×10^6 $B220^+$ cells but also had about 6.6×10^5 $CD4^+$ and 3.6×10^5 $CD8^+$ T cells (for a total of 1×10^6 T cells). There were no detectable bacteria in the spleens of these two surviving mice. This is in contrast to recipients of *nu/nu* spleen cells, which never survived i.d. LVS infection for more than about 30 days (Table 3).

In repeated experiments of similar design and comparable effectiveness of depletion, 16 of 17 recipients of $B220^-$ cells survived i.d. LVS infection, 1 of 15 recipients of *nu/nu* spleen cells survived infection, and 8 of 15 recipients of T^- cells survived; all recipients of T^- cells that survived contained moderate numbers of $CD4^+$ and $CD8^+$ T cells in their spleens when examined after day 60. All surviving $B220^-$ recipients cleared bacteria, but 3 of 8 surviving recipients of T^- cells still contained small numbers of LVS in their spleens, despite having survived through day 60 and being apparently asymptomatic.

Similar limitations in cross-contamination of subpopulations were encountered in experiments attempting to deplete $CD4^+$ or $CD8^+$ T cells from spleen cells, and therefore an alternate approach was used. Normal spleen cells were enriched for T^+ cells, $CD4^+$ cells, or $CD8^+$ cells by in vivo depletion (see Materials and Methods) followed by application to columns designed to remove B cells, macrophages, and the unwanted $CD4^+$ or $CD8^+$ cells from the final population. The number of cells transferred to *scid* mice was chosen to approximate the numbers of total T cells, $CD4^+$ T cells, and $CD8^+$ T cells in 2×10^7 normal spleen cells. All recipient mice survived i.d. LVS infection for 60 days. When sacrificed and analyzed, all mice contained no detectable LVS in their spleens. Four T^+-cell recipients (from the experiment shown in Table 5) contained an average of 21.1% B cells, 25.8% $CD4^+$ T cells, and 9.0% $CD8^+$ T cells. Four $CD4^+$ recipients contained an aver-

TABLE 4. Reconstitution with T lymphocytes of long-term survival by *scid* mice of i.d. Infection with LVS[a]

Cells transferred	i.d. LVS dose	No. of deaths/total no. of mice	MTD[b]	Anti-LVS IgM titer	Anti-LVS IgG titer
PBS	10^3	5/5	20.8 ± 2.0	ND[c]	ND
Normal spleen cells	10^3	0/5		1:160	1:160
$B220^-$ spleen cells	10^3	0/5		<1:10	<1:10
T^- spleen cells	10^3	3/5	15.3 ± 0.6	1:80	<1:10

[a] BALB/cByJ.*scid* mice were given either diluent PBS i.v., 2×10^7 normal BALB/cByJ spleen cells i.v., 1×10^7 BALB/cByJ $B220^-$ spleen cells i.v., or 1×10^7 BALB/cByJ T^- spleen cells i.v. on day 0. Flow cytometry analysis demonstrated that the transferred normal spleen cells were 42.8% T cells ($CD4^+$ plus $CD8^+$) and 47.3% B cells ($B220^+$); $B220^-$ cells were 93.5% T cells and 0.2% B cells; and T^- cells were 0.4% T cells and 92.2% B cells. One day later, the mice were infected with 10^3 LVS bacteria i.d.; the actual infection dose was confirmed by plate count at the time of infection. Sera were collected on day 56. Mice were observed for morbidity and mortality through day 60. This experiment is representative of three experiments of similar design.

[b] MTD, time to death (mean ± standard deviation) in days.

[c] ND, not determined.

TABLE 5. Reconstitution with $CD4^+$ or $CD8^+$ T lymphocytes of long-term survival by *scid* mice of i.d. infection with LVS[a]

Cells transferred	i.d. LVS dose	No. of deaths/total no. of mice	MTD[b]
PBS	10^3	5/5	23.2 ± 1.3
T^+ spleen cells	10^3	0/4	
$CD4^+$ spleen cells	10^3	0/4	
$CD8^+$ spleen cells	10^3	0/4	

[a] BALB/cByJ.*scid* mice were given either diluent PBS i.v., 1×10^7 BALB/cByJ T^+ spleen cells i.v., 5×10^6 BALB/cByJ $CD4^+$ spleen cells i.v., or 2×10^6 $CD8^+$ spleen cells i.v. on day 0. Flow cytometry analysis demonstrated that the T^+ cells were 87.7% T cells ($CD4^+$ plus $CD8^+$) and 2.4% B cells ($B220^+$); the $CD4^+$ cells were 93.2% $CD4^+$, <0.5% $CD8^+$, and 0.9% $B220^+$; and the $CD8^+$ cells were 94.6% $CD8^+$, <0.5% $CD4^+$, and 0.6% $B220^+$. One day later, they were infected with 10^3 LVS bacteria i.d.; the actual infection dose was confirmed by plate count at the time of infection. Mice were observed for morbidity and mortality through day 60. This experiment is representative of two experiments of similar design.

[b] MTD, time to death (mean ± standard deviation) in days.

age of 17.2% B cells, 29.4% $CD4^+$ T cells, and 9.8% $CD8^+$ T cells, while four $CD8^+$ recipients contained an average of 14.2% B cells, 12.4% $CD4^+$ T cells, and 26.8% $CD8^+$ T cells. Thus, populations enriched for either $CD4^+$ or $CD8^+$ T cells can reconstitute long-term survival and clearance of i.d. LVS infection in *scid* mice, but over time, reconstituted recipients contain all subpopulations.

DISCUSSION

By the use of, first, athymic *nu/nu* mice and, more recently, *scid* mice, an early lymphocyte-independent phase and a late T-cell-dependent phase of resistance to infection with intracellular bacteria such as *L. monocytogenes* have been recognized for almost 20 years (reviewed in reference 33). This distinction has also been made with a variety of other intracellular pathogens, including *Mycobacterium* (21), *Salmonella* (29, 30), and *Leishmania* (6, 25) spp., *Toxoplasma gondii* (20), and even *Yersinia enterocolitica* (2). Early-phase resistance probably permits time for development of a specific (T-dependent) immune response that is ultimately responsible for control of infection and clearance, concurrent with development of secondary immunity. In all models, both IFN-γ and TNF-α have been recognized as critical cytokines in initial survival of infection. Mice treated with anti-TNF-α antibodies (5) or knockout mice lacking functional TNF-α receptors (32, 37) are extremely susceptible to *Listeria* infection. Similarly, GKO mice infected i.v. or by aerosol with *Mycobacterium tuberculosis* succumb to infection (8, 16), as do IFN-γ receptor knockout mice infected with *Listeria* spp. (19). The experiments using knockout mice unequivocally demonstrate that there is no compensatory activity available during bacterial infection for IFN-γ and TNF-α, despite the remarkable functional redundancy generally observed in the cytokine network. The studies reported here add *F. tularensis* to the list of intracellular pathogens that demonstrate an early-phase resistance to infection that is absolutely dependent on non-lymphocyte-derived IFN-γ and TNF-α for initial survival of infection. We note that *scid* mice survive i.d. infection with doses of LVS ranging from 10^0 to 10^6 for about 20 days; it is possible that bacteria replicate until a sufficient threshold induces cytokine secretion, which then limits further short-term growth. This control is eventually lost, and T lymphocytes must be available for clearance of bacteria and long-term survival.

Previous studies demonstrated that normal mice treated with the anti-granulocyte antibody RB6-865 died from a sublethal infection with LVS (41). Here we found that elimination of neutrophils in vivo dramatically affected survival of sublethal LVS infection by *scid* mice as well. Neutrophils may be a source of cytokines, since they clearly respond to bacterial products such as LPS for the production of a variety of inflammatory factors such as interleukin-1α (IL-1α), IL-1β, TNF-α, IL-3, IL-6, and IL-8; they are also activated by IFN-γ (reviewed in reference 7). Other studies suggested the intriguing possibility that the function of neutrophils is to kill bacterium-infected hepatocytes in the liver very early after infection (10), and human polymorphonuclear leukocytes can kill *Francisella* bacteria directly by an oxygen-dependent mechanism (38). The mechanism of their contribution to early survival may therefore be multifactorial.

The study of both *nu/nu* and *scid* immunodeficient mice clearly indicated that mature T cells, if not also B cells, were required for late-phase clearance and survival of i.d. LVS infection (Table 1) (14). Our efforts to determine precisely the lymphocyte populations required for long-term survival using this transfer model were somewhat limited by technical considerations; we were unable to selectively reconstitute *scid* mice with individual subpopulations and maintain selectivity in recipient mice for the entire time needed to study resolution of infection. These problems were also noted in previous studies attempting very long-term reconstitution of *scid* mice with selected subpopulations, in which very low numbers (10^3 to 10^5 cells per mouse) of highly purified $CD4^+$ or $CD8^+$ T cells were transferred to *scid* mice and recipients were analyzed for engraftment 4 to 5 months later (reviewed in reference 34). In these studies, transferred $CD4^+$ T cells did not function well and appeared to have a skewed repertoire but reproducibly induced significant B-cell leakiness. Both these long-term studies and our shorter-term studies here demonstrate that selective T-cell reconstitution of *scid* mice will result in B-cell repopulation as well. Future studies may address these limitations both by the use of knockout mice and by treatment of recipients with antibodies to maintain depletion.

Nonetheless, a number of conclusions can be drawn from the available data. First, we attribute the partial success (eight deaths among 15 total recipients) of T^- cells in reconstituting *scid* mice for survival of i.d. LVS infection to occasional development of enough T^+ cells to effect survival (but not always clearance). Very small numbers must be sufficient, since we detected only about 10^6 total T cells in the spleens of surviving mice; the lymph nodes were too small to be studied. Reconstitution of *scid* mice with $B220^-/T^+$ cells invariably led to survival of i.d. LVS infection. We are therefore confident of the T-cell requirement for clearance of infection and long-term survival. We cannot exclude the possibility that B cells contribute to this process as well; despite the use of the most stringent depletion technique available to us, all surviving $B220^-$ recipients had readily detectable B cells in their spleens after 60 days. This is particularly important in light of our previous description of an unusual, rapidly generated protective mechanism in LVS infection that is T cell independent and may be a function of B cells (13). Furthermore, there was a slight but reproducible difference in the mean time to death from i.d. LVS infection between BALB/cByJ *nu/nu* mice (30 days) and *scid* mice (20 days), suggesting a subtle contribution of either B cells, γ/δ T-cell-receptor-positive T cells, or both. However, clearance is clearly not dependent on the production of anti-LVS antibody, since $B220^-$ cell recipients had no detectable specific antibodies in their sera and all other surviving recipients had very low levels of antibodies (Table 4).

Since all surviving recipients that cleared bacteria contained both $CD4^+$ and $CD8^+$ T cells in their spleens, including those that originally received highly enriched $CD4^+$ or $CD8^+$ T cells,

we cannot determine at this time whether both are needed to clear bacteria and survive i.d. LVS infection. Results from other ongoing studies in our laboratory using either selectively depleted normal mice or various T-cell knockout mice (46) will also address this question. To date, the studies of Conlan et al. have demonstrated that mice depleted of either $CD4^+$, $CD8^+$, or total T cells were able to control but not clear sublethal LVS infection for up to 8 weeks (11), implying that both subpopulations are necessary for clearance. The relative contributions of each subpopulation have been a subject of much debate in other infection models and are of interest not only for the basic immunobiology but also for implications for vaccine development. For instance, $CD8^+$ T cells have been considered much more important than $CD4^+$ T cells in *Listeria* infection (12, 18), and $CD4^+$ T cells have been considered more important than $CD8^+$ T cells in *Mycobacterium* infections (22). These simplistic conclusions have been called into question by results of both time course studies of *Mycobacterium* infection (31) and recent investigations using knockout mice lacking either $CD4^+$, $CD8^+$, α/β^+ T cells, or γ/δ^+ T cells (23, 24, 28, 36). Collectively, these studies suggest that while one α/β^+ T-cell subpopulation may have a moderately stronger role than the other, both are heavily involved in optimal control of infection and may take precedence at difference time points after bacterial infection. $\gamma\delta^+$ T cells appear to have a somewhat subtler, but nonetheless significant, role throughout bacterial infection. The results seen here with *Francisella* infection are consistent with this developing perspective and further indicate that the LVS model is quite useful for future studies on the nature of infection and protective immunity to intracellular bacteria.

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