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$CD4^+$ and $CD8^+$ T-Cell-Dependent and -Independent Host Defense Mechanisms Can Operate To Control and Resolve Primary and Secondary *Francisella tularensis* LVS Infection in Mice

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Immunity to experimental infection with the facultative intracellular bacterium *Francisella tularensis* is generally considered an example of T-cell-mediated, macrophage-expressed immunity. However, the results of the present study indicate that T-cell-independent mechanisms are also important in anti-*Francisella* defense. They show that mice selectively depleted of $CD4^+$, $CD8^+$, or both T-cell populations by treatment with T-cell subset-specific monoclonal antibodies remained capable of controlling and partly resolving a primary sublethal *Francisella* infection. Similarly, it was found that *Francisella*-immune mice depleted of either or both subsets of T cells retain a high degree of acquired immunity to reinfection. Together, these findings imply that resistance to primary and secondary tularemia can be mediated by cells other than $CD4^+$ and $CD8^+$ T cells.

Francisella tularensis is a gram-negative, facultative intracellular coccobacillus and is the etiological agent of tularemia, an often severe and sometimes fatal infection of humans and other mammals (reviewed in reference 27). Individuals who recover from primary tularemia have acquired specific protective immunity against *F. tularensis* (5). Similarly, vaccination with the attenuated live vaccine strain (LVS) of *F. tularensis* confers a long-lasting protection against infection with virulent strains of the pathogen (5). *F. tularensis* LVS, although attenuated for humans, remains virulent for mice, and murine tularemia caused by LVS has been used extensively to study mechanisms of anti-*Francisella* resistance (2, 3, 7, 12–14, 17, 21). Because *F. tularensis* survives and grows inside macrophages (1, 16), it is generally believed that host defense against this pathogen (27), as against other intracellular bacteria (18), is cell mediated. This implies that defense depends on the ability of *Francisella*-specific T cells to enhance the microbicidal capacity of macrophages, thereby enabling the latter cells to destroy the pathogen (17, 27). It has been shown in vitro (16), in support of this hypothesis, that murine peritoneal macrophages can kill ingested *F. tularensis* LVS, but only after exposure to gamma interferon (IFN-γ), a T-cell lymphokine. Moreover, mice treated with a monoclonal antibody (MAb) directed against IFN-γ quickly succumbed to an otherwise sublethal inoculum of *F. tularensis* LVS (21). However, because other leukocytes, including NK cells, can produce IFN-γ (28), it remains possible that these cells, rather than T cells, are required for expression of anti-*Francisella* resistance. In support of this possibility, it was recently demonstrated that congenitally athymic nude mice do not die from acute infection with *F. tularensis* (14) but instead develop a protracted infection to which they eventually succumb. This suggests that $\alpha\beta^+$ T cells are not required to restrict early progressive growth of *Francisella* organisms but are needed to completely resolve primary infection. However, a disadvantage of the nude mouse model of infection is that it does not reveal the relative contribution of different T-cell subsets to antibacterial resistance. Furthermore, nude mice cannot be used to determine the need for T cells for the expression of specific acquired immunity after resolution of primary infection. This immunity is also generally believed to be T-cell mediated (3, 17, 27), as evidenced by the demonstration that splenic lymphocytes from *Francisella*-immune mice can confer a greater degree of anti-*Francisella* resistance on normal recipients than immune serum can (3, 12). Moreover, depletion of Thy-1^+ cells from donor spleen cells was shown to eliminate their capacity to transfer immunity (17). However, because cells other than T cells can be Thy-1^+ (25), these results do not represent unequivocal evidence that T cells are necessary for resolution of secondary tularemia. Neither is it known what T-cell subsets are involved in anti-*Francisella* immunity.

The purpose of this study was to examine the roles of $CD4^+$ and $CD8^+$ T cells in anti-*Francisella* defense. To this end, the course of primary and secondary tularemia was monitored in control mice and in mice selectively depleted of $CD4^+$, $CD8^+$, or both subsets of T cells. The results show that mice depleted of $CD4^+$ and/or $CD8^+$ T cells remained capable of controlling a primary infection with *F. tularensis*, but not of completely resolving it. The results also show that, although immune mice depleted of T cells exhibit impaired resistance to reinfection, they still can survive a challenge inoculum that is lethal for naive mice.

MATERIALS AND METHODS

Mice. Female B6D2F1 (C57BL/6 × DBA/2) mice were used in all experiments. They were obtained from the Trudeau Institute Animal Breeding Facility and were free of common viral pathogens on the basis of the results of routine screening by the Research Animal Diagnostic Laboratory, University of Missouri, Columbia. Some mice were thymectomized at 5 to 6 weeks of age and housed for 2 to 3 weeks before use in cell-depletion studies. Thymectomy was performed to inhibit T-cell repopulation in mice depleted of these lymphocytes as detailed below.

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Bacteria. *F. tularensis* LVS (ATCC 29684), obtained from the American Type Culture Collection, Rockville, Md., was grown to the log phase in modified Mueller-Hinton broth as described previously (7), harvested, and frozen at −70°C in 1-ml aliquots (8×10^8 CFU/ml) in the presence of 10% (wt/vol) sucrose. For each experiment, inocula were prepared from frozen stocks by thawing an aliquot, washing it once in 0.9% (wt/vol) sterile saline, and diluting it to the required concentration in saline. Bacterial inocula were given intravenously (i.v.) in a volume of 200 μl in a lateral tail vein. The i.v. 50% lethal dose (LD_{50}) of *F. tularensis* LVS for normal mice is approximately 1.5×10^5 CFU (7). Mice that received a secondary challenge had been given an intravenous inoculation of 5×10^3 CFU of *F. tularensis* LVS 4 to 5 weeks earlier. *F. tularensis* in the livers, spleens, and lungs was enumerated by plating 10-fold serial dilutions of organ homogenates on cystine heart agar (Difco Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) hemoglobin. Colonies were counted after 48 h of incubation at 37°C.

MAbs and antisera. Anti-CD4 (GK 1.5), anti-CD8 (TIB.210), and anti-Thy-1.2 (30.H.12) MAbs were obtained from the American Type Culture Collection. An isotype-matched, immunoglobulin 2b (Ig2b) MAb directed against keyhole limpet hemocyanin (anti-KLH MAb) was produced in the Trudeau Institute by L. L. Johnson. MAbs were purified from ascites fluid by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-Sephacel (Pharmacia LKB, Piscataway, N.J.). The Ig concentrations of the purified MAbs were determined from their peak heights during elution from a high-performance liquid chromatography size-exclusion column. Normal rat IgG was purchased from Sigma Chemical Co., St. Louis, Mo. Depleting thymectomized mice of $CD4^+$ and/or $CD8^+$ T cells was achieved by intraperitoneal injection of the appropriate anti-T-cell subset MAb at 0.5 mg 3 days before, 0.25 mg 1 day before, and 0.25 mg at 5-day intervals after initiation of infection.

Cytofluorometry. Flow cytofluorometric analysis was carried out as previously described (10) on pooled unfractionated spleen cells obtained from two mice per group at indicated times of infection. Analysis was performed on cell suspensions labeled with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of anti-T-cell MAbs (anti-CD4, anti-CD8, or anti-Thy-1.2) prepared as described elsewhere (10). All samples were also stained with propidium iodide (2 μg/ml) to detect dead cells that were eliminated from the analysis. Stained cells were analyzed by using a FACScan cytofluorograph (Becton Dickinson, Sunnyvale, Calif.) equipped with Lysis II software. For analysis, cells were gated according to the known forward- and side-scatter light properties of lymphocytes. The percentage of positively staining cells was determined from fluorescent histograms. Cells with a mean fluorescent intensity of $\geq 1 \log_{10}$ above the background level were considered positive. The results were expressed as the percentage of lymphocytes staining positively for a given label. By these criteria, the percentages of $Thy\text{-}1^+$, $CD4^+$, and $CD8^+$ lymphocytes detected in the spleens of unthymectomized, uninfected control mice were 23.3, 15.4, and 8.5, respectively.

RESULTS

Mice depleted of $CD4^+$ and $CD8^+$ T cells retain most of their capacity to deal with primary *F. tularensis* infection. In a preliminary experiment, the ability of thymectomized mice to control infection initiated by i.v. inoculation of 3×10^3 CFU of *F. tularensis* was examined. Figure 1 shows that thymectomized mice were the same as normal mice in their ability to control and resolve tularemia caused by i.v. inoculation of a sublethal dose of *F. tularensis* LVS. Furthermore, this figure shows that treating thymectomized mice with normal rat IgG had no effect on their ability to resolve infection.

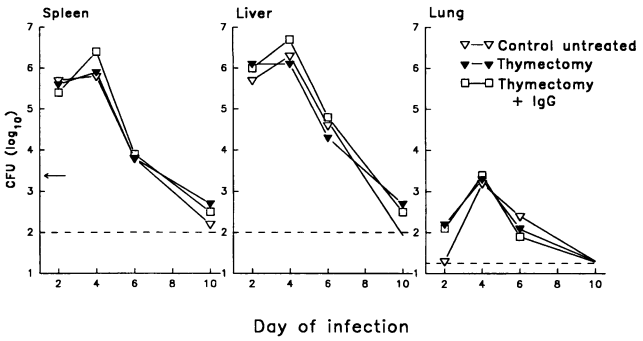

FIG. 1. Effect of thymectomy and control antibody treatment on the course of primary *F. tularensis* infection in mice. All groups of mice were inoculated with a sublethal i.v. inoculum (3×10^3 CFU) of *F. tularensis* LVS, and the course of infection in the liver, spleen, and lungs was monitored for a period of 10 days. Antibody-treated mice received 0.5 mg of normal rat IgG i.v. 3 days before and 0.25 mg intraperitoneally 1 day before initiation of infection. The arrow indicates the size of the bacterial inoculum. The broken line represents the detection limit of the assay. The means for five mice per group and time point are shown. The standard errors of the means were <0.4 $\log_{10}$ units.

To determine the requirements for T cells in controlling primary tularemia, thymectomized mice were given the control MAb or appropriate T-cell-depleting MAbs and then inoculated i.v. with a sublethal dose (5×10^3) of *F. tularensis* LVS. Figure 2 shows that the courses of infection were similar in all groups of T-cell-depleted mice. Compared with the situation in control mice, depletion of either or both T-cell subsets resulted in more, but insignificant, bacterial growth in the liver, spleen, and lungs during the first 4 days. However, after this time, infection underwent progressive resolution in the livers and spleens of all groups during the remaining 10-day period of the experiment. In the lungs, enhancement of infection lasted longer in T-cell-depleted mice and infection resolved more

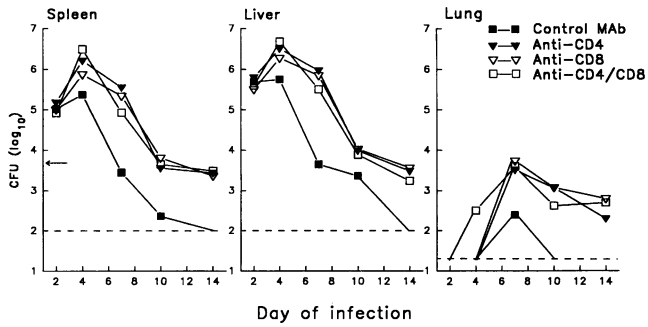

FIG. 2. Growth curves of *F. tularensis* LVS in the livers, spleens, and lungs of control and T-cell-depleted thymectomized mice. MAbs were administered before and during infection as described in Materials and Methods. Mice received a sublethal i.v. inoculum (5×10^3 CFU) of *F. tularensis*, and numbers of bacteria were determined over 14 days. The broken line represents the detection limit of the assay. The arrow indicates the size of the bacterial inoculum. The means for five mice per group and time point are shown. The standard errors of the means were <0.5 $\log_{10}$ units.

TABLE 1. Cytofluorometric analysis of the surface markers on splenic lymphocytes from thymectomized MAb-treated mice inoculated with a sublethal dose of *F. tularensis* LVS

Treatment[a]	% of positively stained cells[b]		
	Thy-1$^+$	CD4$^+$	CD8$^+$
Control MAb	10.1	5.1	2.7
Anti-CD4 MAb	4.2	<0.02	2.6
Anti-CD8 MAb	7.9	4.5	<0.02
Anti-CD4 plus anti-CD8 MAbs	3.5	<0.02	<0.02

[a] Thymectomized mice were given a control MAb or MAbs that deplete CD4 and/or CD8 T cells and were inoculated i.v. with 5×10^3 CFU of *F. tularensis*. Spleen cells were prepared and analyzed on day 7 of infection.
[b] The extent of depletion of T cells was determined by cytofluorometry with fluorescein isothiocyanate-conjugated F(ab')$_2$ anti-T-cell MAbs.

slowly. No mice in any group died during the 14-day period of the experiment.

Given the limited effect of treatment with T-cell-depleting MAbs on resistance to *F. tularensis* infection, it was necessary to show that T-cell depletion was adequate. Table 1 shows that by day 7 of the experiment illustrated in Fig. 2, mice given anti-CD4 MAb or anti-CD8 MAb had below detectable numbers (<0.02%) of CD4$^+$ and CD8$^+$ T cells, respectively, in their spleens. Likewise, CD4$^+$ and CD8$^+$ T cells were not detected in mice given both MAbs. Similar results were obtained on day 4 of infection (not shown). Interestingly, although mice depleted of either or both T-cell subsets showed a large decrease in Thy-1-bearing cells, they nevertheless retained a substantial number of Thy-1$^+$ CD4$^-$ CD8$^-$ cells (Table 1).

Mice depleted of CD4$^+$ and CD8$^+$ T cells fail to completely resolve a primary *F. tularensis* LVS infection. The foregoing results show that, although mice depleted of CD4$^+$, CD8$^+$, or both subsets of T cells remained capable of controlling *F. tularensis* growth, they failed to completely resolve primary tularemia during the 14-day period of the experiment. In view of these results and those published by others (14) showing that BALB/c athymic nude mice develop a protracted *F. tularensis* infection, an experiment was undertaken to monitor the course of tularemia in mice chronically depleted of CD4$^+$ and CD8$^+$ T cells. Thus, thymectomized mice were treated with both anti-CD4$^+$ plus anti-CD8$^+$ MAbs two times before infection as described above and at 5-day intervals during the course of infection for a period of 8 weeks. Table 2 shows that, whereas control mice treated with an irrelevant MAb resolved infection after 2 weeks, mice depleted of CD4$^+$ and CD8$^+$ T cells had substantial numbers of *F. tularensis* in their lungs for at least 4 weeks and in their livers and spleens for at least 8 weeks of infection. No mice died during this experiment, and all appeared healthy. Cytofluorometric analysis performed on spleen cells of mice from the same experiment at 4 weeks of infection showed that mice treated with anti-T-cell MAbs were almost completely depleted of CD4$^+$ and CD8$^+$ T cells (Table 3), although they possessed substantial numbers of Thy-1$^+$ CD4$^-$ CD8$^-$ cells.

TABLE 2. Recovery of *F. tularensis* from livers, spleens, and lungs of mice depleted of CD4$^+$ and CD8$^+$ T cells and of control mice

Time post-infection (wk)	Log_{10} CFU of *F. tularensis* LVS in[a]:					
	CD4$^+$- and CD8$^+$-depleted mice			Control mice		
	Spleen	Liver	Lung	Spleen	Liver	Lung
2	3.8 ± 0.2	4.1 ± 0.3	2.6 ± 0.3	<2	<2	<1.3
4	3.2 ± 0.2	3.0 ± 0.6	1.8 ± 1.1	<2	<2	<1.3
8	3.4 ± 0.4	3.7 ± 0.6	<1.3	<2	<2	<1.3

[a] Thymectomized mice were given a control MAb or anti-CD4 plus anti-CD8 MAbs as described in Materials and Methods. Numbers of bacteria were determined at the indicated time points after an inoculation of 5×10^2 CFU of *F. tularensis*. Values are expressed as means ± standard deviations of five mice per group.

TABLE 3. Cytofluorometric analysis of the surface T-cell markers on spleen cells from thymectomized, MAb-treated mice infected with a sublethal inoculum of *F. tularensis* LVS 28 days earlier

Treatment[a]	% of positively stained cells[b]		
	Thy-1$^+$	CD4$^+$	CD8$^+$
Control MAb	5.4	4.7	3.1
Anti-CD4 plus anti-CD8 MAbs	1.6	0.1	<0.02

[a] Thymectomized mice were given a control MAb or anti-CD4 plus anti-CD8 MAbs as described in Materials and Methods. Mice were inoculated i.v. with 5×10^2 CFU of *F. tularensis*, and spleen cells were prepared and analyzed 28 days later.
[b] The extent of depletion of T cells was determined by cytofluorometry with fluorescein isothiocyanate-conjugated F(ab')$_2$ anti-T-cell MAbs.

To determine the role of CD4$^+$ and CD8$^+$ T cells in the expression of acquired immunity to secondary infection, thymectomized mice given a sublethal i.v. inoculum (5×10^3) of *F. tularensis* 5 weeks earlier were given T-cell-depleting MAbs and then were challenged with 2×10^6 CFU of the organism. These mice showed levels of T-cell depletion similar to those achieved in the foregoing experiments (not shown). Figure 4 shows that immune mice depleted of CD4$^+$, CD8$^+$, or

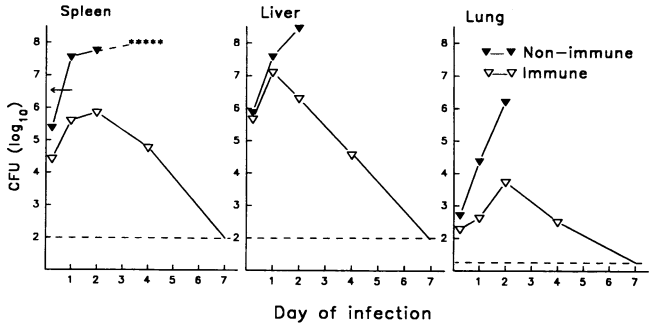

FIG. 3. Growth curves of *F. tularensis* LVS in the livers, spleens, and lungs of naive mice and immune mice that had received a sublethal inoculum of the pathogen 4 weeks earlier. Mice were inoculated i.v. with 3.5×10^6 CFU of *F. tularensis*, and infection was monitored for 7 days. The broken line represents the detection limit of the assay. The arrow indicates the size of the bacterial inoculum. The means for five mice per group and time point are shown. The standard errors of the means were <0.4 log_{10} units. Asterisks indicate the death of individual mice.

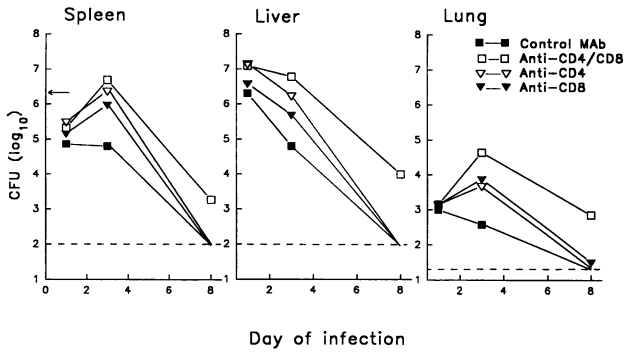

FIG. 4. Effect of T-cell depletion on secondary infection with *F. tularensis* LVS. Thymectomized mice that had received a sublethal i.v. inoculum 5 weeks earlier were depleted of various T-cell subsets and rechallenged with an i.v. inoculum of 2×10^6 CFU of *F. tularensis*. MAbs were administered 3 days and 1 day before reinfection and on day 5 of infection as described in Materials and Methods. The broken line represents the detection limit of the assay. The arrow indicates the size of the bacterial inoculum. The means for five mice per group and time point are shown. The standard errors of the means were <0.3 $\log_{10}$ units.

both T-cell subsets were able to control infection in the livers, spleens, and lungs initiated by an otherwise lethal inoculum of *F. tularensis*. However, immune mice depleted of both $CD4^+$ and $CD8^+$ T cells resolved infection at a slower rate than control mice and mice depleted of either T-cell subset. Regardless, it was clear that immune mice had acquired a $CD4^+$ and $CD8^+$ T-cell-independent protective immunity capable of dealing with an inoculum of 2×10^6 organisms (approximately 10 LD_{50}s for naive mice). In contrast, a group of age-matched, thymectomized naive mice all died within 4 days after the same challenge. On the other hand, when this experiment was repeated with a larger challenge inoculum (9×10^6 CFU), mice depleted of $CD4^+$, $CD8^+$, or both T-cell subsets all died within 4 days, whereas 80% of control immune mice survived (not shown). This suggests that T-cell-mediated immunity becomes critical for defense when numbers of bacteria exceed a certain threshold.

DISCUSSION

Immunity to *F. tularensis* infection is believed to be cell mediated, meaning that it is expressed by macrophages after their activation to a heightened antibacterial state by cytokines secreted by antigen-specific lymphocytes (reviewed in reference 27). In keeping with this view is the demonstration that mice treated with a MAb against IFN-γ, a cytokine produced by T cells, are rendered incapable of controlling an otherwise sublethal *F. tularensis* infection (21). In these mice, even the smallest inocula of *F. tularensis* grow unrestrictedly in target organs to reach lethal numbers. On the other hand, T cells are apparently not required to control the early progressive phase of primary tularemia in that athymic nude mice are remarkably capable of controlling *F. tularensis* infection (14). Similar studies have shown that nude mice are also highly resistant to infection with another facultative intracellular bacterium, namely, *Listeria monocytogenes* (6, 11, 15, 23, 24), which is also believed to be defended against by T-cell-mediated immunity. Together, these studies with nude mice suggest that mechanisms in addition to those dependent on T cells are important in host defense against facultative intracellular bacteria.

The results of the present study support and extend the findings with nude mice by showing that immunocompetent mice depleted of either $CD4^+$, $CD8^+$, or both subsets of T cells remained capable of controlling, but not resolving, *F. tularensis* infection. Consequently, T-cell-depleted mice, like nude mice, become chronically infected, thus showing that T cells are not required to prevent progressive bacterial growth during primary tularemia but apparently are required for complete destruction of bacteria in the tissues. In the present study, T-cell-depleted mice stayed healthy for at least 8 weeks, whereas a published study with nude mice reported that they succumb to *F. tularensis* infection within a month (14). Possibly, the different course of primary tularemia in T-cell-depleted B6D2 mice used here and BALB/c nude mice used elsewhere reflects strain differences in susceptibilities to infection with *F. tularensis* LVS. Certainly, B6D2 mice as used in the present study are relatively resistant to *F. tularensis* LVS, whereas BALB/c mice are reportedly susceptible (17). Additionally, the different routes by which infection was initiated in the two studies (i.v. versus intradermal) might have contributed to the observed susceptibility differences.

Precisely which host defense mechanisms operate to control primary *F. tularensis* infection in T-cell-depleted mice were not examined in the present study. However, it has been shown that mice depleted of $CD4^+$ and $CD8^+$ T cells retain various degrees of resistance to other intracellular pathogens such as *L. monocytogenes* and *Toxoplasma gondii* (10, 20, 22). In these situations, resistance to infection appeared to be mediated by $Thy\text{-}1^+$ $CD4^-$ $CD8^-$ cells because it was abolished by treating mice with the anti-Thy-1.2 MAb (10, 20, 22). This makes it possible that NK cells, $CD4^-$ $CD8^-$ $\alpha\beta^+$ T cells, $\gamma\delta^+$ T cells, or all three types of cells contribute significantly to Thy-1-dependent defense in mice depleted of $CD4^+$ and $CD8^+$ T cells (8, 10, 17, 19), possibly by acting as alternative sources of macrophage-activating cytokines, including IFN-γ (4, 9). Presumably, NK cells and $\gamma\delta^+$ T cells could serve a similar role in nude mice. Given that IFN-γ is required to control primary murine tularemia in euthymic (21) and nude (14) mice, it is possible that the aforementioned lymphocytes, rather than $\alpha\beta^+$ T cells, serve as a source of this critical cytokine during infection as others (14) have suggested. In this regard, it is important to remember that, in the present study, mice depleted of $CD4^+$ and $CD8^+$ T cells possessed substantial numbers of $Thy\text{-}1^+$ cells, because both NK cells and $\gamma\delta^+$ T cells can express this surface marker. However, whatever the nature of T-cell-independent defense, it is unable to expedite resolution of primary tularemia that is efficiently achieved by fully immunocompetent mice. It is possible that $CD4^+$ and $CD8^+$ T cells might be needed to lyse infected cells that are incapable of killing the *F. tularensis* bacteria they harbor. In this regard, it is important to remember that, in addition to macrophages, *F. tularensis* LVS can parasitize liver parenchymal cells that apparently have no antimicrobial defenses (7).

Surprisingly, *Francisella*-immune mice depleted of $CD4^+$ or $CD8^+$ T cells expressed a high degree of acquired resistance to reinfection, as evidenced by their ability to control and resolve a challenge dose of *F. tularensis* LVS that rapidly killed nonimmune immunocompetent mice. This result is in keeping with published findings showing that mice depleted of T cells can resolve secondary listeriosis (10). In the case of tularemia, Fortier et al. (16) showed that passively transferred *Francisella*-immune serum can confer significant protection on recipient mice, and others (3) showed that specific antibody greatly enhances the clearance rate of *F. tularensis* from the blood. The rapid ingestion of opsonized bacteria by professional phagocytes might prevent them from invading other more vulnerable target cells (e.g., hepatocytes). Furthermore, opso-

nized bacteria might be ingested via a mechanism that favors activation of the antimicrobial defenses of phagocytes. In addition to humoral immunity, specific antibody-independent and T-cell-independent enhanced protective immunity of unknown genesis is reportedly (reviewed in reference 14) rapidly generated during primary tularemia. This immunity might also be expressed by immune mice depleted of $CD4^+$ and $CD8^+$ T cells. Regardless, it should be remembered that immunocompetent mice expressed a higher level of acquired protective immunity than did T-cell-depleted mice. This T-cell-dependent immunity could complement or override other less effective T-cell-independent defense mechanisms during reinfection.

The nature of the $CD4^+$ and $CD8^+$ T-cell-dependent and -independent mechanisms of anti-*Francisella* resistance operating during primary and secondary tularemia is currently under investigation.

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