

Original Research

Mice Housed at Elevated Vivarium Temperatures Display Enhanced T-cell Response and Survival to *Francisella tularensis*

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The inability to translate findings from studies performed in mouse models to the corresponding human condition is well known, especially those involving infectious, atherosclerotic, and other inflammatory diseases. We hypothesize that mice fail to mount robust or adequate immune response to infectious agents because of physiologic effects of cold stress due to housing temperatures below the mouse thermoneutral zone (TNZ). This hypothesis was tested by comparing the immune response to the *Francisella tularensis* live vaccine strain in mice housed at a typical vivarium temperature, which is below the TNZ, with that of mice housed at a temperature near their TNZ. Mice maintained at 28 °C displayed elevated antigen-specific T-cell responses compared with mice housed at 22 °C and survived intranasal challenge that was fatal to immunized mice at 22 °C. These results demonstrate that cold stress due to housing below the mouse TNZ results in a blunted immune response and may compromise their translational value as models for infectious diseases and vaccine development.

Abbreviations: APC, antigen-presenting cells; *Ft*, *Francisella tularensis* subsp. *holarctica*; LVS, live vaccine strain; TNZ, thermoneutral zone

The appropriate temperature for housing mice in animal research facilities has been the subject of numerous studies and reviews, but few studies have addressed the effect of ambient temperature on protective immunity in mice. Guidelines from the United States' National Institutes of Health dictate that mice should be housed at 20 to 26 °C.¹⁵ However, to maintain a core temperature of approximately 37 °C, mice begin to produce additional body heat when the ambient temperature drops below approximately 30 °C. Studies on thermal physiology have established that the thermoneutral zone (TNZ) of various mouse strains is 30 to 32 °C, the temperature range of minimal metabolic energy expenditure.^{11,13} The discrepancy between standard vivarium housing temperatures and the mouse TNZ has largely been ignored by the research community and regulatory agencies but may have profound implications in the translation of mouse models to human disease.

Chronic cold stress results in production and recruitment of brown adipose tissue for adaptive thermogenesis, as a result of norepinephrine release from the sympathetic nervous system.⁴ This then may result in corticosteroid release from the adrenal cortex, which engages β -adrenergic receptors on many immune cells.²⁶ Consequently, global suppression of the immune system may occur, particularly of proinflammatory cells involved in immune protection⁸ as well as the expansion of immunosuppressive M2 macrophages.²⁵ Suppressed immune responses due to cold stress in mice housed below their lower critical temperature may account for the high mouse mortality in and failure

of numerous mouse models of bacterial, viral, and protozoal infections.¹⁹

Several recent studies are consistent with this hypothesis. Mice inoculated intraperitoneally with *Klebsiella pneumoniae* and housed at 34 °C displayed as much as a 10⁵-fold lower bacterial load¹⁸ and a 400-fold lower pulmonary pathogen burden after intratracheal inoculation²⁸ than control infected mice maintained at 23 °C. In a pulmonary influenza virus infection model, mice maintained at 30 °C avoided excessive production of proinflammatory cytokines in lung tissue compared with mice maintained at 22 °C or 26 °C.¹⁶

Although these results suggest that better immune protection occurs in the absence of cold stress, these studies were all short-term (3 to 7 d), so only innate immune responses would likely be engaged. In addition, with the exception of one study,¹⁸ survival did not improve at elevated ambient temperatures, possibly due to collateral inflammation-mediated damage from a robust immune response. In a recent study of immunity to solid tumors, mice maintained at 31 °C developed increased numbers of tumor-infiltrating CD8⁺ T cells, reduced tumor volume, and increased survival compared with mice housed at 23 °C,²¹ suggesting that adaptive immunity is improved when cold stress is avoided. Qualitatively similar findings were obtained in a graft-versus-host disease mouse model.²³

It is hypothesized that mice commonly fail to mount protective immune responses to infectious agents because of the physiologic effects of cold stress brought on by housing temperatures below the mouse TNZ. Because mice housed well below their TNZ maintain an average core body temperature of 36 to 37.5 °C^{12,35} due to adaptive thermogenesis,⁴ the environmental temperatures of both the infectious agent and the mediators of protective immunity are essentially the same regardless of the

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ambient vivarium temperature. The current study compares the T- and B-cell responses to an attenuated strain of *Francisella tularensis* (*Ft* LVS) as well as survival to a potentially lethal dose of *Ft* LVS in mice maintained at typical vivarium temperatures compared with housing within the mouse TNZ.

Materials and Methods

Mouse husbandry. Animal housing conditions and experimental design were approved by the IACUC of the University of New Mexico Health Sciences Center (Albuquerque, NM) in accordance with the NIH guidelines¹⁵ and AALAS principles in an AAALAC-accredited SPF facility on a 12:12-h light:dark cycle. Every 3 to 4 mo, the facility submits blood from sentinel mice maintained in soiled bedding as well as fecal pelt and cage swabs from research rodent cages for serology and pathogen genetic testing (IDEXX BioResearch, Columbia, MO), respectively. The PCR panels include *Citrobacter rodentium*, *Pasteurella pneumotropica*, fur mites (for example, *Miobia*, *Radfordia*), and pinworms (*Aspicularis*, *Syphacia*). The annual comprehensive serology panel from sentinels screens for antibody to approximately 16 viruses as well as to *Mycoplasma pulmonis*, *Encephalitozoon cuniculi*, and *Clostridium piliforme*. Female BALB/cAnNHsd mice (age, 4 to 5 wk) were obtained from Harlan Sprague–Dawley (now Envigo, Livermore, CA) and acclimated for 1 wk in an ABSL2 facility at approximately 22 °C. Mice were randomly assigned to cages maintained at the normal ambient temperature or to adjacent cages (Wessels induction/warming chambers, WW-CL, Braintree Scientific, Braintree, MA), in which a heating element and temperature probe is imbedded in the center of the cage acrylic floor and thermostatically controlled by using a VE-100 controller (Vivarium Electronics, High Point, NC), modified by the manufacturer for elevated temperatures. Cages were covered with HEPA-filtered microisolation lids in a static airflow environment. Bedding (Teklad TEK-Fresh, Envigo) at an approximate depth of 0.64 cm was changed weekly. Temperature and relative humidity were monitored and recorded continuously at 30-min intervals by using a TR300 sensor (Amprobe Test Tools) protected by a cage of 23-gauge galvanized wire mesh with 0.6-cm² openings that was placed on the cage floor such that the sensor element was elevated 1.3 cm above the bedding, at approximately mouse nose height. The temperature within the acrylic cage floor adjacent to the heating element was set approximately 20 °C higher than the measured air temperature in the cage. Food (Teklad Global Irradiated, Envigo) and water were provided without restriction, supported by the wire cage top. Conscious mice were weighed (PB602-S balance, Mettler Electronics, Toledo, OH) at regular intervals by using 10-s dynamic weighing method.

A preliminary 'phase 1' study was undertaken, in which groups of 10 mice were maintained for 18 wk in large cages (23 cm × 46 cm × 18 cm) at an ambient room temperature (mean ± 1 SD) of 21.3 ± 0.2 °C for the control group or in heated cages at 27.5 ± 0.2 °C or at 32.2 °C ± 0.8 °C. However, because the internal measured cage temperature of the 10 control mice cohoused in the unheated cage averaged 23.2 ± 0.2 °C (approximately 2 °C higher than the room air temperature) over the 18-wk period, the housing condition of the 10 control mice was changed to 5 cages of 2 mice per cage for the 'phase 2' study, to minimize ambient temperature elevation from body heat and group huddling behavior. These smaller cages (17 cm × 28 cm × 13 cm) provided a floor area of 238 cm²/mouse. In addition, because mice maintained at 32 °C showed similar features to those of mice at 28 °C, only 28 °C was used in the phase 2 study. In the phase 2 study, 12 mice (2 cages of 6 mice per cage) were maintained at 28

°C, providing a floor area of 176 cm²/mouse. Unless otherwise noted, the data reported in the Results section are those from the phase 2 study or from both studies when the 2 protocols can be considered replicate experiments.

Protocol design. Mice were acclimated to their designate temperatures for 6 wk prior to immunization and individually identified by ear punch. Mice lightly anesthetized with isoflurane underwent intranasal immunization with approximately 200 cfu *Ft* LVS in 50 µL, a dose calculated from a serially diluted aliquot of stock *Ft* LVS stored at -70 °C that grew 3.6 × 10⁹ cfu after thawing, as previously described.³³ One cage of 4 unimmunized mice was used as controls. At 3 wk after inoculation, 0.2 mL of blood was collected by retroorbital bleeding from the medial canthus of isoflurane-anesthetized mice by using heparinized glass Natelson tubes, which was transferred into 10 U heparin (Sigma, St Louis, MO) and processed as described later. After another 4 wk (13 wk at test temperatures), mice were challenged intranasally with a suspension of 2.6 × 10⁷ cfu *Ft* LVS in 50 µL PBS; this dose is greater than 10⁴ times the dose of *Ft* LVS that is fatal to 100% of unimmunized Balb/c mice, for which the LD₅₀ is 300 cfu.¹⁰ Bacterial concentration in the challenge suspension was confirmed by plating on cysteine heart agar containing rabbit blood and antibiotics (Remel, Fisher Scientific, Lenexa, KS). Mice were monitored daily for another 2 to 3 wk for morbidity, weight, and mortality. Because immunized mice challenged with this high-dose *Ft* LVS may recover from a moribund condition, surviving mice were euthanized by CO₂ inhalation only after 4 wk of observation.

Isolation of peripheral WBC. For logistical reasons associated with handling 26 to 28 mice, half of the mice in each group were bled 2 d later than their counterparts, to set up the cell-based assays. Blood was centrifuged at 800 × g for 10 min, and plasma was removed, recentrifuged, and stored frozen. Washed blood cells were suspended in 0.5 mL RPMI1640 medium (serum-free, HyClone, San Angelo, TX) and underlaid with 0.5 mL Lympholyte-M (Cedarlane, Burlington, NC), followed by centrifugation at 1048 × g for 20 min. WBC at the polysucrose–medium interface were removed, washed 3 times as described earlier, and suspended in 0.9 mL medium containing 10% fetal bovine serum (HyClone). Viable PBMC were enumerated using a hemocytometer after diluting 1:5 in PBS containing 0.1% trypan blue. A mean ± 1 SD of 2.2 ± 0.9 × 10⁶ PBMC was obtained from 0.2 mL blood.

Immunoassays. *F. tularensis*-specific T cells were determined by using ELISpot assays (Mabtech, Cincinnati, OH), in which response to specific antigen was enumerated by counting the number of IFN γ - and IL2-secreting T cells. The day before each assay, splenocytes for use as antigen-presenting cells (APC) were prepared as previously described³⁰ from an untreated, typically housed Balb/c mouse, γ -irradiated at 30 Gy, and stored at 0 °C. The next day, these APC were pelleted and resuspended in complete medium, counted, and diluted to 3 × 10⁶ viable cells/mL. A portion of the APC preparation was loaded with heat-killed (60 °C, 30 min) *Ft* LVS at 2 × 10⁷ bacterial cells/mL for 1 h at 37 °C. For each WBC sample, 3 × 10⁵ antigen-loaded APC were added to triplicate wells, and single wells containing control APC were included as the negative control and for monitoring the maximal response to concanavalin A (10 µg/mL; Boehringer Mannheim, Phoenixville, PA). An equal volume (0.1 mL) of each WBC preparation containing an average of 2.2 × 10⁵ cells was added to each well except for the concanavalin A control, to which 2.2 × 10⁴ cells were added; the final volume of all wells was brought to 0.2 mL by using medium. After overnight incubation in 10% CO₂ at 37 °C, the IFN γ and

IL2 plates were processed according to the manufacturer's instructions. The number of developed spots in each well was counted by using an automated EliSpot reader (Autoimmunity Diagnostika, Strasburg, Germany).

Antibody to *Ft* LVS was assessed by ELISA, based on methodology previously described.¹ Briefly, a bacterial pellet was washed twice in PBS followed by centrifugation at $16,000 \times g$ for 20 min and suspended in PBS at 1.0×10^9 cells/mL. Bacteria were heat-killed as described earlier and stored in aliquots at -20°C . Polystyrene microtiter plates (Immulon 2HB, Fisher ThermoScientific, Waltham, MA) were coated overnight with 0.2 mL of bacterial solution (5.0×10^7 bacteria/mL, in PBS) followed by postcoating with 1 mg/mL gelatin (JT Baker, Phillipsburg, NJ). Plates were gently agitated for 2 h with 0.2 mL mouse test sera (diluted 1:1500) followed by peroxidase-conjugated goat antimouse IgG (H+L; diluted 1:6000; Jackson ImmunoResearch, West Grove, PA) for 1.5 h. Color development after addition of ABTS (Boehringer Mannheim) and H_2O_2 was measured intermittently for a maximum of 1 h by optical absorbance at $405 \mu\text{m}$, and results are reported as OD at 1 h, with samples producing greater than 3.0 OD calculated by extrapolation from earlier time points, as previously described.¹ For measuring IgA anti-*Ft* LVS antibodies, mouse sera were diluted 1:200, and the secondary antibody was peroxidase-conjugated goat antimouse IgA (diluted 1:500; Southern Biotech, Birmingham, AL).

Plasma corticosterone was determined by using an ELISA kit (Arbor Assays, Ann Arbor, MI) according to the manufacturer's instructions. Plasma samples were pretreated with dissociating solution, and the serum was diluted 1:100 for the assay. Absorbance readings were converted to ng/mL by using the corticosterone standard curve developed in parallel.

Statistical analysis. Two-tailed student's *t* tests were used to evaluate the statistical significance of data, in which values within a group were averaged. *P* values less than 0.05 were considered to indicate significant differences. Survival data were analyzed by using SPSS Statistics, version 23 (IBM SPSS, Armonk, NY), with log rank (Mantel-Cox) for pairwise comparisons and the Fisher exact test. Data are reported as means \pm 1 SD in the text and figures.

Results

Figure 1 shows the air temperatures in the control and heated cages as measured just above the bedding floor and recorded every 30 min throughout the entire protocol period. Short interruptions in data acquisition are due to removal of the temperature-humidity recording device every few weeks to upload the accumulated data. Regular diurnal temperature fluctuations occurred, with a peak temperature at 0100 to 0400 and a nadir at approximately 1600, spanning approximately 1.9°C for both the low- and elevated-temperature cages. This difference presumably reflects nocturnal animal activity, consistent with a similar percentage increase in relative humidity during the 0100 to 0400 period (data not shown). The average temperature throughout the entire 15-wk study was 28°C in the 2 heated cages and was 22°C in the monitored unheated cage.

The weight of mice for the duration of the protocol is shown in Figure 2 A. After 4 wk, mice maintained at 28°C had 6.8% (1.3 g) lower weight than mice at 22°C ($P < 0.006$, 95% confidence interval, 0.39 to 2.21 g difference). The lower weight of the mice maintained at elevated temperatures reached a 10.9% difference by 12.7 wk, just prior to challenge with a potentially lethal dose of *Ft* LVS. In the phase 1 study, mice maintained at 28°C for 6.4 wk also weighed less than mice at 23°C ($P < 0.04$), although a significant difference in weight between these 2 groups was

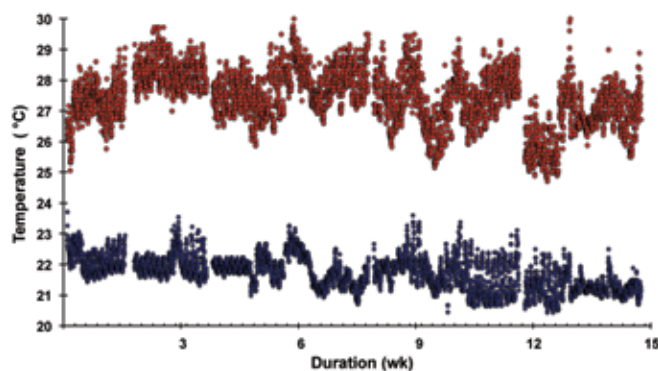


Figure 1. Ambient temperature in unheated (control) and heated mouse cages. The internal cage temperature was measured and recorded continuously every 30 min during the 15-wk exposure period. The 2 warmed cages each contained 6 mice (upper data), and the 5 cages at room temperature each had 2 mice (lower data). The temperature over the course of the study averaged over approximately 2-wk intervals was $27.5 \pm 0.5^\circ\text{C}$ and $27.6 \pm 0.6^\circ\text{C}$ (not shown) for the 2 heated cages; the average temperature was $21.8 \pm 0.3^\circ\text{C}$ for the monitored unheated cage.

not sustained for the duration of the observation period (data not shown). However, starting at 6.4 wk in the phase 1 study, mice maintained at 32°C were significantly lighter (0.9 to 1.6 g) than mice at 23°C (average *P* value through 18 wk was $P < 0.02$; data not shown). After mice were challenged with *Ft* LVS at 12.9 wk, weight dropped precipitously by 2 d after bacteria exposure, with a nadir at 4 to 6 d after challenge for mice that survived (Figure 2 B).

Because maintaining mice below their TNZ was expected to induce a metabolic stress response through activation of the hypothalamic-pituitary-adrenal axis, culminating in secretion of glucocorticoids, plasma corticosterone levels were measured after mice had spent 9 wk at the various temperatures (Figure 3). The average corticosterone level of immunized mice at 28°C was 1.5-fold higher than for the immunized mice held at 22°C ($P < 0.04$; 95% confidence interval, 0.1 to 2.8 ng/mL difference) and 2.8-fold higher than in unimmunized mice (at 22°C ; $P < 0.004$; 95% confidence interval, 1.0 to 4.5 ng/mL difference). The immunized mice at 22°C had a 1.9-fold higher corticosterone level than the unimmunized mice held at the same temperature, but this difference did not achieve statistical significance. In the phase 1 study, corticosterone levels did not differ between any of the groups (data not shown), perhaps because the temperatures in the control and heated cages were not separated as widely.

Tularensis-specific cellular and humoral immune responses were measured 3.4 wk after immunization with *Ft* LVS. Plasma was removed from heparinized blood and stored at -20°C for subsequent measurement of specific antibodies. Lymphocytes were enriched from a fixed volume of blood by density step-gradient centrifugation and quantified by hemocytometry. Individual cell release of $\text{IFN}\gamma$ during overnight incubation in the presence or absence of killed *Ft* LVS was measured by EliSpot assay. Approximately 0.1% of cells from immunized mice produced $\text{IFN}\gamma$ in response to LVS *in vitro* (Figure 4). Mice maintained at 28°C had 1.6-fold or 1.9-fold more circulating *Ft*-responsive T cells than mice at 22°C as assessed by the number of cells per 10^5 lymphocytes ($P < 0.021$; 95% confidence interval, 8 to 84) or per volume blood ($P < 0.008$; 95% confidence interval, 44 to 258), respectively. The nonimmunized control mice had a mean of 1.7 ± 0.9 spot-forming cells per 10^5 total cells as compared with 104 ± 43 in the immunized mice. In the phase

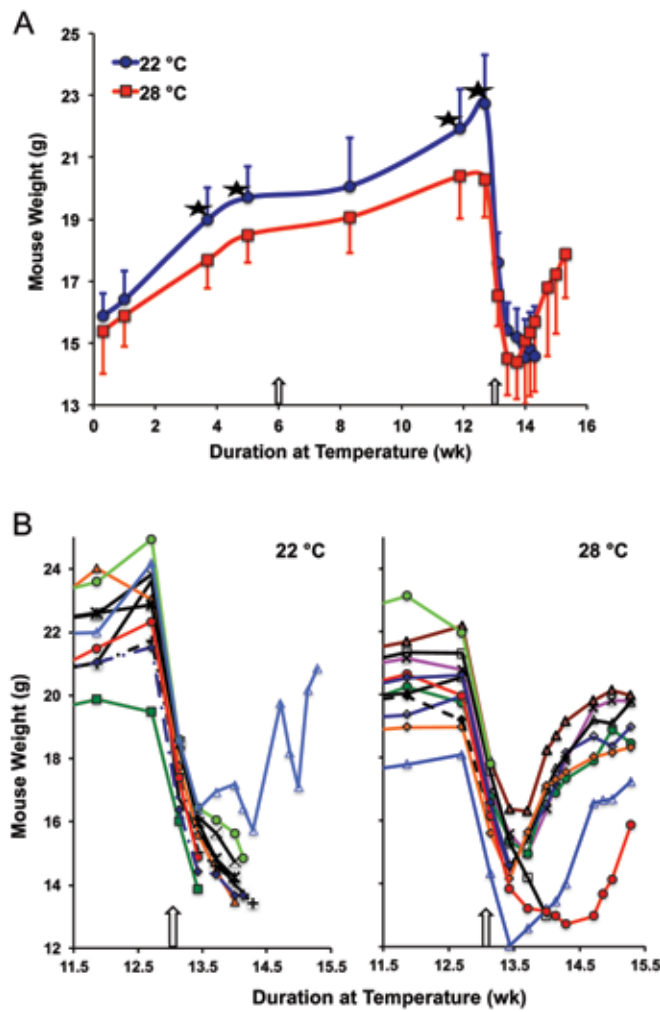


Figure 2. Effect of ambient temperature on mouse weight. (A) Body weight (mean + 1 SD [for 22 °C]; mean - 1 SD [for 28 °C]) of mice maintained at the 2 indicated temperatures. *, Significant ($P < 0.02$) difference between values at the indicated time point; the arrow at 6 wk depicts the time when mice were vaccinated with *Ft* LVS, and that at 12.9 wk shows when mice were challenged with a potentially lethal dose of *Ft* LVS. (B) Weights of individual mice just before challenge and for the subsequent 2 wk; curtailed data correspond to mice that died.

In 1 study, mice at 28 °C had 2.8-fold more LVS-specific T cells than mice at 23 °C ($P < 0.03$). The number of IL2-secreting T cells in response to *Ft* was 116 ± 41 from mice at 28 °C compared with 75 ± 56 from mice at 22 °C ($P < 0.062$; data not shown), which, although below statistical significance, included a higher background of IL2-secreting cells (5 ± 2) in the absence of antigen.

IgG antiLVS antibodies were highly elevated in *Ft* LVS-immunized mice but did not differ between mice maintained at the 2 temperatures (Figure 5). In the phase 1 protocol mice at 28 °C had a mean of 3.35 ± 1.12 OD₄₅₀ anti*Ft* compared with 1.80 ± 0.94 OD₄₅₀ from mice at 23 °C ($P < 0.004$). In addition, IgA anti*Ft* was detected in 200-fold diluted plasma only from immunized mice, but mean antibody levels did not differ between the mice maintained at the 2 temperatures (0.44 ± 0.48 OD₄₅₀ compared with 0.39 ± 0.22 OD₄₅₀; data not shown).

Antigen-naïve Balb/c mice survive intranasal challenge with 10^2 cfu *Ft* LVS but succumb within 7 d to intranasal inoculation with 10^3 cfu *Ft* LVS.¹⁰ In the current study, mice sensitized with 200 cfu *Ft* LVS were challenged 7 wk later with 2.6×10^7 cfu *Ft* LVS and monitored daily for morbidity and mortality. Two days

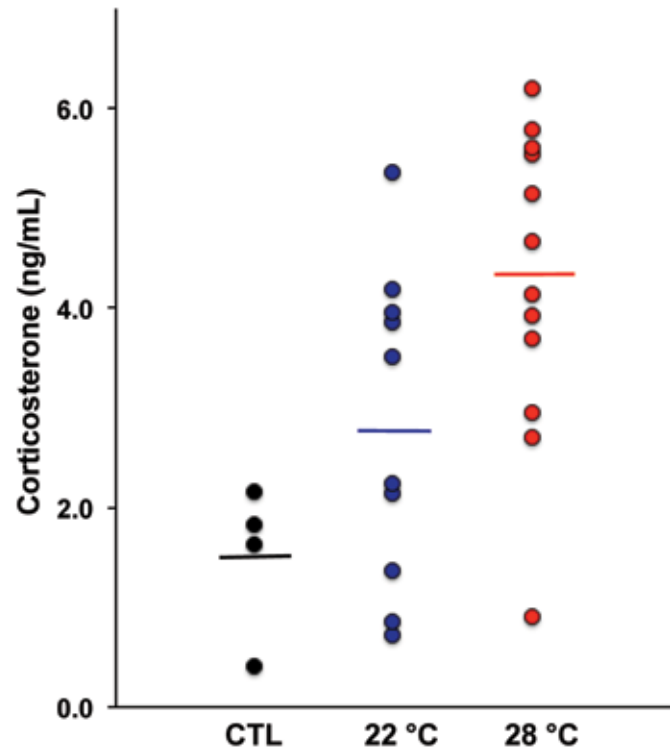


Figure 3. Plasma corticosterone levels. Each point represents an individual untreated control (CTL) mouse (at 22 °C) or mice in the immunization protocol maintained at the indicated temperature for 9 wk. Inoculated mice at 28 °C had higher corticosterone levels than inoculated mice at 22 °C ($P < 0.04$) and the CTL mice ($P < 0.004$). Lines indicate the mean plasma corticosterone concentration in each group.

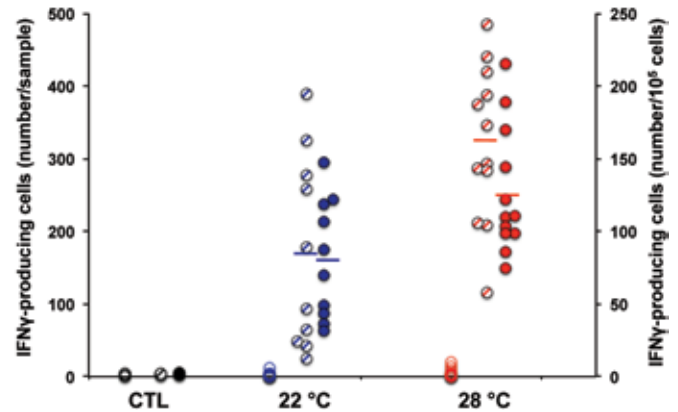


Figure 4. *Ft*-specific T cell enumeration. T cell response to LVS was measured in vitro at 3.4 wk after immunization of mice with *Ft* LVS. Values are expressed as the number of IFN γ -secreting spots per volume blood (hatched circles, left axis) or per 10^5 viable WBC recovered (filled circles, right axis). Open circles are IFN γ -secreting spots in the absence of *Ft* antigen addition in vitro. CTL mice were unimmunized. Lines indicate the mean number of responding T cells in each group. Mice maintained at 28 °C had more antigen-specific T cells than mice maintained at 22 °C ($P < 0.02$ per 10^5 cells and $P < 0.008$ per sample).

after challenge, mice at 22 °C had a 23% weight loss compared with an 18% loss in mice at 28 °C. Mouse weight continued to drop, with maximal losses of 36% and 26%, respectively, 8 d after challenge, when surviving mice that had been housed at 28 °C weighed 15.1 ± 2.0 g compared with 14.5 ± 1.2 g for mice at 22 °C (Figure 2). The lower morbidity of mice at 28 °C was reflected in their prolonged survival (Figure 6). By 12 d after

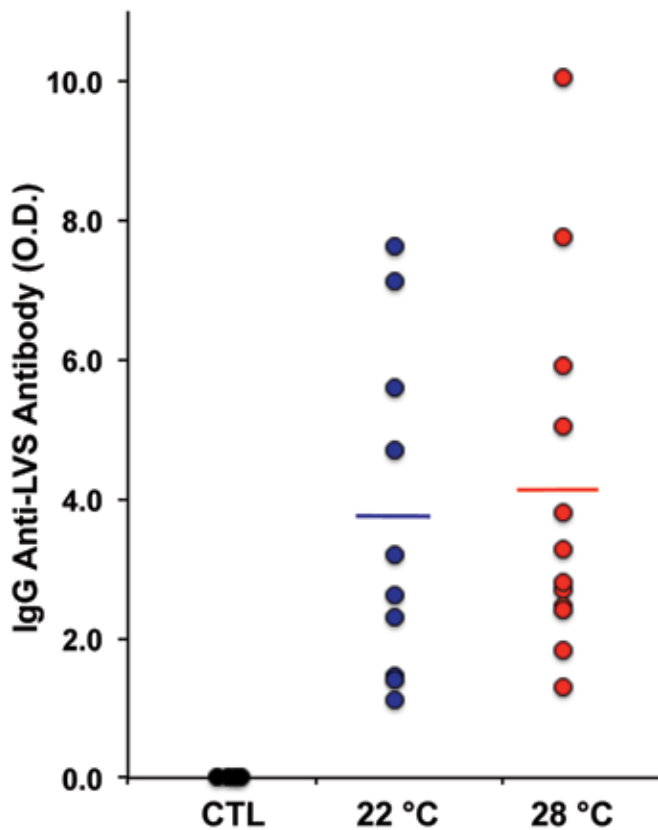


Figure 5. IgG antibody production in response to *F. tularensis* LVS. Anti*Ft* antibody activity was measured in 1500-fold diluted plasma collected 3.4 wk after immunization of mice with *Ft* LVS. Each point represents an individual untreated control (CTL) mouse (at 22 °C) or mice in the immunization protocol maintained at the indicated temperature for 9 wk. Lines show the mean antibody activity in each immunized group.

challenge, only 1 of the 10 mice maintained at 22 °C was alive, compared with 8 of the 12 mice at 28 °C ($P < 0.041$).

Discussion

This study indicates that mice housed at temperatures below their TNZ had a muted T-cell response and compromised survival in a bacterial infection animal model. *F. tularensis* is a facultative intracellular pathogen, which is sterilely cleared through cellular immune mechanisms dependent on IFN γ ,⁵ although antibody to *Ft* contributes substantially to immune protection.^{9,24} With decreased *Ft*-specific T cells, mice maintained at 22 °C presumably failed to mount adequate CD4⁺ T-cell help for macrophage-mediated killing or direct killing of *Ft*-infected cells by CD8⁺ T cells. In addition, mice housed at 28 °C gained less weight over a 13-wk period and lost less relative weight in response to a potentially lethal dose of *Ft* LVS, presumably reflecting the improved immune status and accelerated pathogen clearance of mice housed at a temperature within their TNZ.

In light of the almost 2-fold increase in *Ft*-specific T cells, it was surprising that neither IgG nor IgA antibody to *Ft* was consistently elevated in mice maintained at 28 °C, given isotype-switched antibody responses are strictly dependent on CD4⁺ T cells. Perhaps immunization of mice with *Ft* LVS at 22 °C elicited a T-cell response that was in excess with regard to help for antibody production, consistent with the remarkably high IgG anti*Ft* LVS activity elicited after just a single immunization.

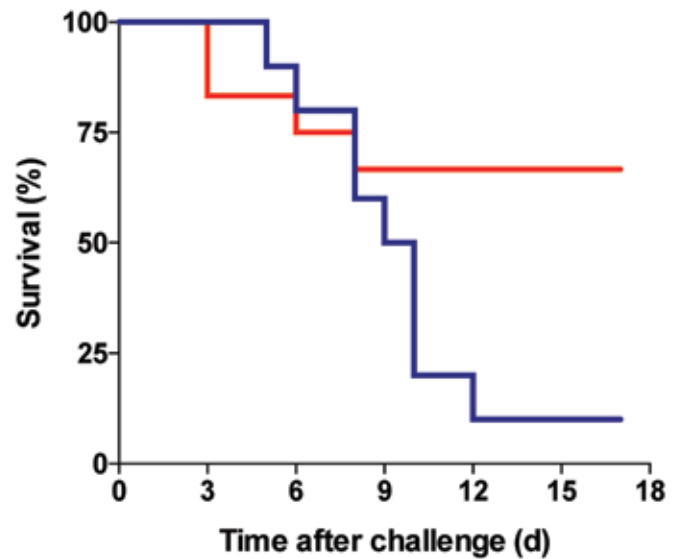


Figure 6. Kaplan–Meier plot of survival of mice after high-dose challenge with *Ft* LVS. Mice maintained at 28 °C showed greater survival than mice maintained at 22 °C ($P < 0.041$ by log-rank (Mantel–Cox) χ^2 analysis and $P < 0.012$ by 2-tailed, Fisher exact test at termination).

Alternatively, B-cell activity might be relatively unaffected by corticosteroid-mediated immune suppression. Nevertheless, the greater mortality of mice from *Ft* LVS at 22 °C compared with at 28 °C suggests that, although specific antibodies may contribute to survival,^{22,24} even a modest 2-fold increase in *Ft*-specific circulating T cells at 28 °C is more important in this regard. This conclusion is consistent with the finding that depletion of all CD4⁺ or CD8⁺ T cells from LVS-vaccinated Balb/c mice abrogated protection from a lethal dose of *Ft*.³³ An additional consideration is that interstitial T cells in the airway parenchyma or particular T-cell subsets, such as Th17 cells,^{29,32} may be more relevant to protection from primary infection with *Ft* LVS and perhaps are more profoundly affected by subTNZ temperatures than are circulating T cells.

Compared with mice housed at 28 °C, mice maintained at 22 °C for 4 wk were significantly heavier, and they continued to gain more weight over the subsequent 9 wk. This result may seem counterintuitive to the expectation that lower ambient temperatures should result in higher metabolic rate² and enhanced conversion by thermogenic brown fat of ingested calories to heat rather than to fat to compensate for heat loss to maintain core temperature at approximately 37 °C.³ However, a seminal study³⁴ revealed that both male and female ICR mice were significantly heavier when kept at 22 °C compared with 28 °C, beginning from just a few weeks after birth and maintained continuously for 9 wk, and this difference correlated with significantly greater food intake. Other investigators had similar observations, in that C57BL/6 mice housed at 22 °C gained more weight than mice housed at 30 °C,³¹ and the intradermal fat of Balb/c mice at 23 °C was 5-fold thicker than that of mice maintained at 31 °C, a cold stress response dependent on the lipoprotein uptake receptor syndecan 1.²³

The finding that the mean corticosterone level in mice at 28 °C was the same as or actually higher than that of mice at 22 °C was surprising, given that corticosterone production by the hypothalamus is enhanced in cold-stressed mice.^{2,19} However, because the glucocorticoid receptor is highly expressed in adipocytes,⁶ which are increased in cold-stressed mice,²⁰ the steady-state plasma corticosterone might have been unaffected

by chronic cold stress at 22 °C as a result of receptor-mediated uptake by adipocytes or nonspecific diffusion into adipose tissue due to its lipophilicity,²⁶ while negatively regulated through inhibition of ACTH release from the pituitary.⁷

In the earlier study in this facility,³³ Balb/c mice vaccinated intranasally with 200 cfu *Ft* LVS were protected against a respiratory challenge dose of 50,000 cfu LVS that is lethal in unvaccinated mice and against a 2000-cfu dose of *Ft* biovar A, an antigenically similar but more virulent strain of *Ft*; however, vaccinated mice succumbed to a 10-fold increased respiratory dose of *Ft* biovar A. Furthermore, the same vaccination protocol was not protective in C57BL/6 mice.^{5,33} Balb/c mice were unprotected from intranasal challenge with LVS, unless they were intradermally vaccinated with LVS twice over a 4-wk interval.¹⁷ Limited or no protective immunity after vaccination against other respiratory infectious agents has similarly been reported for *Chlamydia* spp. in C57BL/6 mice¹⁴ and for *B. anthracis* in DBA/2 mice.²⁷ Taken together, these findings are consistent with the view that mice maintained at typical (that is, subTNZ) vivarium temperatures display poor protective immunity in bacterial infection models.

This study has several limitations. Cage temperatures that are increased by using floor-embedded heating elements are not uniform because the static airflow environment results in a gradient of decreasing temperature from the bottom to top of cage, although this pattern also occurs (albeit to a lesser extent) in nonheated cages due to the floor-level heat generated by group-housed mice. Environmental chambers or ventilated microisolation cages, in which delivered air is heated above typical ambient temperatures, would provide more uniform temperatures within the mouse TNZ. In addition, the effect of temperature on protective immunity may be dependent on the nature, amount, or frequency of the immunogen as well as on the sex- and strain-dependent sensitivity of mouse immune systems to suboptimal ambient temperature, as suggested previously.^{5,33} The current study was essentially a pilot study using a minimal number of animals in 2 protocols, thus leading to uncertainty regarding the reproducibility and magnitude of the observations. Furthermore, the huddling effect of multiple animals in a single cage (as noted in the present study) as well as the type of bedding may elevate the effective internal cage temperature. Finally, mice may gradually adapt to temperatures below their TNZ, as suggested by the increased weight gain in the current study; consequently, possible effects of housing temperature on immune function may be dependent on the duration of exposure to subTNZ temperatures. To test the possibility that insufficient acclimation periods further compromise immune responses, ongoing studies are addressing the B and T cell responses to immunogenic virus-like particles in mice acclimated to subTNZ temperature for only 2 wk.

Because of the mixed success of mice as an animal model for vaccine development, other animal species, particularly rats and rabbits, have been used in vaccine research despite their numerous disadvantages. Although several real or imagined explanations for the difficulties encountered in mouse models of infectious disease have been offered, much of the understanding of cellular and molecular immunology is based on observations and experiments with mouse systems. Discrepancies between *in vitro* and *in vivo* studies and between studies performed in different vivaria may be due to the failure to control cold stress. As shown in the current study, simply raising the ambient temperature to the mouse TNZ may substantially expand the successful application of mouse models of infectious disease and vaccine development and thus reduce the number of sentient

experimental animals by improving the reproducibility of the experimental conditions. Performing studies at thermoneutral-ity may enhance the overall translational relevance of murine models to humans, for whom cold stress is generally not a factor.

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