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Susceptibility to *Salmonella typhimurium* Infection and Effectiveness of Vaccination in Mice Deficient in the Tumor Necrosis Factor Alpha p55 Receptor

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Mice defective in the ability to produce the tumor necrosis factor alpha p55 receptor (TNFαp55R) were orally challenged with a number of *Salmonella typhimurium* HWSH derivatives that differ in virulence. In comparison to TNFαp55R$^{+/+}$ mice, TNFαp55R$^{-/-}$ mice succumbed earlier to challenge with wild-type *S. typhimurium* HWSH and *S. typhimurium* HWSH *purE*. In contrast, TNFαp55R$^{-/-}$ mice were able to control an *S. typhimurium* HWSH *aroA* challenge, although greater numbers of *Salmonella* organisms were present in the tissues for a longer time period than was observed with TNFαp55R$^{+/+}$ mice. Vaccination of normal and TNFαp55R knockout animals with *S. typhimurium* HWSH *aroA* showed that TNFαp55R$^{-/-}$ mice, unlike TNFαp55R$^{+/+}$ mice, were not protected against a virulent *S. typhimurium* HWSH challenge. Splenocytes from TNFαp55R$^{-/-}$ mice exhibited a reduced ability to proliferate in the presence of *S. typhimurium* antigen compared to TNFαp55R$^{+/+}$ mice. Thus, TNFαp55R is essential for controlling *Salmonella* growth in tissues and for recall of immunity in murine salmonellosis.

Salmonella enterica causes a wide variety of disease syndromes in humans, many of which are associated with significant levels of fever (15). Perhaps the most dramatic example of this is typhoid, or enteric fever, caused predominantly by *Salmonella typhi* (15, 18, 19). Typhoid is an invasive enteric infection in which viable bacteria can often be isolated from the blood of infected individuals. Since *S. typhi* does not cause significant disease in animals other than higher primates, murine typhoid, caused by certain *S. enterica* serotypes including *Salmonella typhimurium*, has been used extensively as a model for systemic salmonellosis (4). Following oral infection of naive mice with virulent *S. typhimurium*, bacteria quickly spread from the gut, probably through the Peyer's patches of the gut-associated lymphoid tissue to organs of the reticuloendothelial system (RES) including the liver and spleen. With a combination of virulent bacteria and a susceptible mouse strain, the rate of bacterial growth is rapid and the mice die within several days, showing signs of endotoxic shock and harboring high levels of bacteria (up to 10^8) in liver and spleen (3–5, 7, 25, 35). However, the relative contribution of endotoxin to the mortality associated with systemic *Salmonella* infection remains to be fully defined. In sublethal infections or in immunized animals, bacterial growth is suppressed by the host response, leading to a plateau phase after the early rapid growth and to eventual clearance. The mechanisms controlling bacterial growth are also not fully defined, although T cells are not thought to play a major role early in infection and production of both tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) is required (17, 23, 25, 26, 29, 38). The plateau phase is followed by T-cell-dependent clearance of bacteria from the RES. Both CD4$^+$ and CD8$^+$ T cells contribute to clearance, although CD4$^+$ cells apparently play the predominant role (22, 23, 26, 34).

TNF-α plays a key role in the control of infections caused by a number of different pathogens (1, 10, 11, 36). This cytokine can act synergistically with IFN-γ to enhance the bactericidal activity of macrophages and is associated in mice with the induction of nitric oxide (10, 11, 36, 38). Although TNF-α is essential for the expression of immunity, it can also contribute significantly to pathology and mortality, mediating cachexia and endotoxin-associated septic shock. The balance between the beneficial aspects of immunity and pathology vary in different host-pathogen interactions. A number of studies have investigated the role of TNF-α in immunity to *Salmonella* infection by using TNF-α-neutralizing antibodies (22, 23). Mice treated with anti-TNF-α were more susceptible to infection and failed to display immunity when neutralizing antibody was administered simultaneously with challenge. Mice can express at least two independent receptors for TNF-α (28). Different functions have been assigned to the receptors, although some redundancy is present. One of the receptors, with an extracellular domain of 55 kDa (TNFαp55R), is implicated in the majority of known TNF-associated effects, including cytotoxicity and synergistic interaction with IFN-γ (9). Mice that lack TNFαp55R due to gene-targeted homozygous deletion have been developed (9). These mice show increased resistance to shock with lipopolysaccharide (LPS) and vary in susceptibility to infection with different pathogens (21). These mice provide an approach to defining the role of TNFαp55R in controlling salmonellosis in the mouse. In this paper we describe the use of TNFαp55R knockout mice and their abilities to control *Salmonella* infections of differing virulence, along with attempts to prevent infection by vaccination with live *S. typhimurium* HWSH *aroA* mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type *S. typhimurium* HWSH was isolated from a calf dying of salmonellosis and is highly virulent in mice and

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calves. *S. typhimurium* HWSH *purE* and *S. typhimurium* HWSH *aroA* are auxotrophic mutants of the wild-type strain (6, 30, 31). *S. typhimurium* HWSH *purE* is partially attenuated in mice but is able to induce pathology, including abscess formation, in the internal organs of infected mice and to cause sporadic deaths. *S. typhimurium* HWSH *aroA* is a highly attenuated strain that is unable to kill wild-type mice by the oral route of infection. *S. typhimurium* HWSH *aroA* can enter the RES but is efficiently cleared by normal mice after several weeks and is an effective single-dose oral vaccine against homologous *Salmonella* challenge. Bacteria were routinely cultured on Luria agar or in Luria broth. Minimal medium was supplemented with nutrients or antibiotics at the appropriate concentrations. Solid media contained 1.6% Noble agar (wt/vol) (Difco, Surrey, United Kingdom).

Infection of mice and enumeration of bacteria in murine organs. TNFαp55R knockout mice (−/−) along with normal (+/+) controls were bred at Imperial College. They were maintained on a mixed 129Sv × C57BL/6 genetic background and have been described previously (37). Mice were tested in-house by PCR for the presence of the receptor gene by using tail tissue. Bacteria were grown static in L broth, and bacterial numbers were determined by the optical density at 650 nm and then by surface viable counting. For oral inoculation, bacteria were administered in 0.2-ml volumes to lightly halothane-anesthetized mice by gavage as described previously (30). Livers and spleens were removed and homogenized as previously described (30). Viable counts were performed on these whole organ homogenates with L agar as the growth medium and are expressed in the figures as geometric means ± 2 standard errors of the means for four mice per time point.

Isolation of spleen cells. Spleen cells from infected animals, three mice per time point, were isolated by teasing out the cellular contents with a sterile needle and washing them twice in Dulbecco's modified Eagle's medium (Sigma, Poole, Dorset, United Kingdom) with 10% heat-inactivated fetal calf serum, L-glutamine, and penicillin-streptomycin (Sigma). Erythrocytes were lysed with ammonium chloride-potassium lysis buffer, and the cells were again washed twice and counted in a hemocytometer to give a final suspension of 5×10^6 cells/ml for splenocyte proliferation assays. Cells not excluding trypan blue were not included in the final count. Experiments were repeated at least six times.

Cell proliferation assays. Isolated spleen cells were plated in 96-well round-bottomed plates (Costar) in 100-μl volumes containing 5×10^6 cells/ml. The antigen used was detoxified *Salmonella* lysate (41) to give final concentrations of antigen of 1 to 10 μg/ml per well. Detoxified *Salmonella* lysate had been treated with sodium hydroxide in order to eliminate the mitogenic and cell toxicity effects of LPS of the gram-negative envelope. Thus, cell proliferation is a response to protein antigens of the bacteria and not to LPS. Ten microliters of the diluted antigen was applied to appropriate wells. The positive control concanavalin A was used at a final concentration of 5 μg/ml. The cells were incubated for 72 h at 37°C in 5% carbon dioxide. Supernatants for cytokine assays were removed to a 96-well plate, covered with parafilm, and frozen at −70°C. The cells were pulsed with 10 μl of a solution of 100 μCi of [^{3}H]thymidine per ml in RPMI to give a final concentration of 1 μCi/well. The cells were incubated for a further 6 h and then harvested with a cell harvester (Tomtec). The filters were placed in scintillation fluid and counted in a beta-plate counter (Wallac, Milton Keynes, United Kingdom).

Antibody subclass enzyme-linked immunosorbent assay (ELISA) protocol. Costar 96-well plates were coated overnight with formalin-killed and washed *S. typhimurium* at a concentration of 10 μg of protein per ml at 4°C. Wells were washed three times in phosphate-buffered saline (PBS)-Tween (PBST) and blocked with 200 μl of 1% bovine serum albumin (BSA). Mouse serum was diluted in PBS in the dilution range 1/50 to 1/50,000. Fifty microliters of diluted serum was added to all wells, and the plate was incubated for 2 h at 37°C. After this time the plates were washed in PBST and a rabbit anti-mouse horseradish peroxidase conjugate (Sigma) was added at a 1/1,000 dilution for each subclass, 50 μl/well for 2 h at 37°C. The plates were washed three times with PBST, and 50 μl of substrate was added. The color was developed at 37°C or at room temperature (rt). After the reaction was judged complete, it was stopped by the addition of 50 μl of 12.5% (3 M) sulfuric acid. The absorbance was read at 492 nm in a plate reader.

Cytokine ELISA protocol. A purified anticytokine capture monoclonal antibody was diluted to 2 μg/ml in 0.1 M sodium hydrogen carbonate, pH 8.2. Fifty microliters of this was added to a 96-well EIA/RIA plate (Costar), and the plate was left at 4°C overnight. The plates were washed twice with PBST and blocked with 3% BSA in PBS at 200 μl/well. The plates were left at rt for 2 hours and again washed with PBST. Standards and samples were diluted in PBS–3% BSA and added at 100 μl/well, and the plates were left at rt for 4 h or overnight at 4°C. The plates were washed four times with PBST, and diluted biotinylated anticytokine antibodies in PBS–3% BSA were added at 100 μl/well. Plates were left at rt for 45 min and then washed six times in PBST. Avidin-peroxidase was diluted 1:400 in PBS–3% BSA and added at 100 μl to each well. Plates were left at rt for 30 min, and then were washed eight times with PBST. One hundred microliters of substrate and hydrogen peroxide was added per well, and the color reaction was allowed to develop at rt. The color reaction was stopped, and the plates were read at 490 nm. The antibodies used were rat anti-mouse interleukin 4 (IL-4) clone BVD6-24G2, IFN-γ clones XMG1.2 and R4-6A2, IL-10 clone SXC-1, IL-5 clone TRFK4, and TNF-α clones G281-2626 and MP6-XT3 (Pharmingen, San Diego, Calif.). The sensitivity of detection for the cytokine ELISA was 15 pg/ml.

Histological sections. Five-micrometer-thick sections stained by hematoxylin and eosin were prepared from tissues fixed in 10% (vol/vol) formal saline and embedded in paraffin wax. Sections were also cut by cryostat from livers and spleens frozen at −70°C for immunohistochemistry.

Vaccination and challenge experiments. Normal and TNFαp55R knockout mice were vaccinated orally with 10^9 cells of *S. typhimurium* HWSH *aroA* vaccine strain. Counts of the vaccine strain in internal organs were performed at days 7, 14, and 21 postvaccination. After 28 days, both groups of animals and an uninfected control group were orally challenged with 10^8 cells of the *S. typhimurium* HWSH wild-type strain.

Nitric oxide determination. Both groups of knockout mice were challenged with the *S. typhimurium* HWSH wild type, and serum was taken at day 2 postinfection. Serum was assayed for nitric oxide by the Griess reaction, which detects nitric oxide by determining nitrate-nitrite in the medium.

RESULTS

Susceptibility of TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice to *Salmonella* infection. TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice were subjected to oral challenge with a number of derivatives of *S. typhimurium* HWSH. TNFαp55R$^{-/-}$ mice were significantly more susceptible than TNFαp55R$^{+/+}$ to virulent *S. typhimurium* HWSH following oral challenge. Death occurred 3 to 5 days (average, 4 days) after oral inoculation of TNFαp55R$^{-/-}$ mice with a challenge of 10^8 *S. typhimurium* HWSH bacteria. In contrast, TNFαp55R$^{+/+}$ mice did not start to die from infection until after 5 days, but most were dead at day 8 (average, day 7). Both TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice succumbed to overwhelming bacterial infection, although the actual cause of mortality is unknown. Interestingly, TNFαp55R$^{-/-}$ mice displayed symptoms typically associated with lethal *Salmonella* infection of normal mice, including ruffled fur, sweating, and immobilization. The TNFαp55R$^{-/-}$ mice had small spleens compared to TNFαp55R$^{+/+}$ mice at the time of death, and no Peyer's patches were detectable macroscopically. Indeed, no Peyer's patches were detected macroscopically in uninfected TNFαp55R$^{-/-}$ mice at the age they were used (6 to 8 weeks).

TNFαp55R$^{-/-}$ mice were also more susceptible to oral challenge with the partially attenuated *S. typhimurium* HWSH *purE* strain. All TNFαp55R$^{-/-}$ mice succumbed to oral challenge with *S. typhimurium* HWSH *purE* within 3 to 5 days. TNFαp55R$^{+/+}$ mice partially controlled an *S. typhimurium* HWSH *purE* challenge, with 70% of animals surviving infection and clearing the challenge bacteria. The remaining 30% of TNFαp55R$^{+/+}$ mice developed pathology, including abscess formation in the livers and spleens, and died. This is in agreement with previously published data (30). In contrast, *S. typhimurium* HWSH *aroA* was well controlled by these mice. TNFαp55R$^{-/-}$ mice orally challenged with *S. typhimurium* HWSH *aroA* exhibited a slight increase in spleen size and, interestingly, developed gut-associated tissue resembling abnormal looking Peyer's patches.

Enumeration of *S. typhimurium* HWSH derivatives in the internal organs of orally challenged TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice. The numbers of bacteria present in selected organs of mice were determined at various times after oral challenge with different *S. typhimurium* HWSH derivatives. Counts of *S. typhimurium* HWSH in the livers and spleens of TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice increased at about 1 log unit per day until death (Fig. 1). The same growth rate was observed in TNFαp55R$^{-/-}$ mice infected with *S. typhimurium* HWSH *purE* (Fig. 1). In contrast, TNFαp55R$^{+/+}$ mice either controlled the infection or succumbed to death associated with abscess formation (30). TNFαp55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice orally challenged with *S. typhimurium* HWSH *aroA* all cleared the vaccine strain from internal organs, but TNFαp55R$^{-/-}$ mice had higher levels of the vaccine strain persisting,

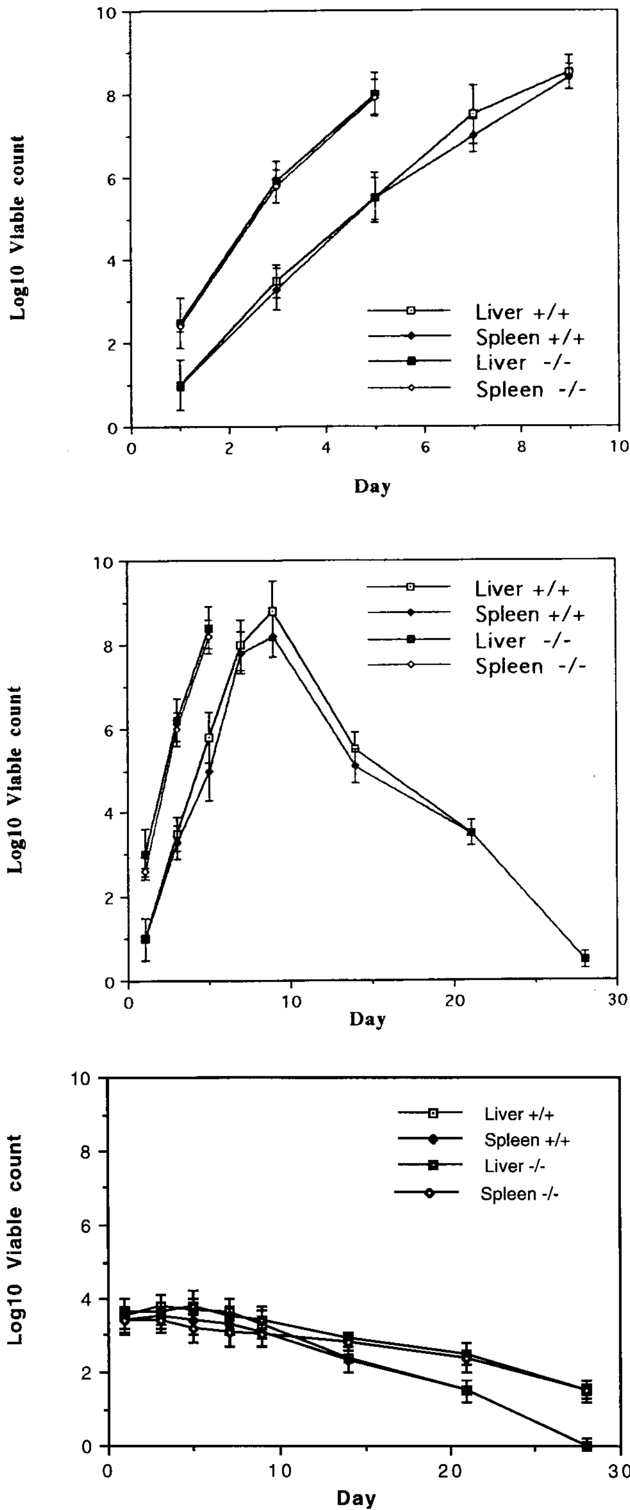


FIG. 1. Viable counts of the *S. typhimurium* HWSH wild type and *purE* and *aroA* strains in the spleens and livers of mice following oral challenge. (Top) *S. typhimurium* HWSH wild-type bacteria (10^8) administered orally to TNFα p55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice. (Middle) *S. typhimurium* HWSH *purE* bacteria (10^8) administered orally to TNFαp55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice. (Bottom) *S. typhimurium* HWSH *aroA* bacteria (10^8) administered orally to TNFα p55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice. Counts were performed on four animals per time point, with the experiment repeated three times.

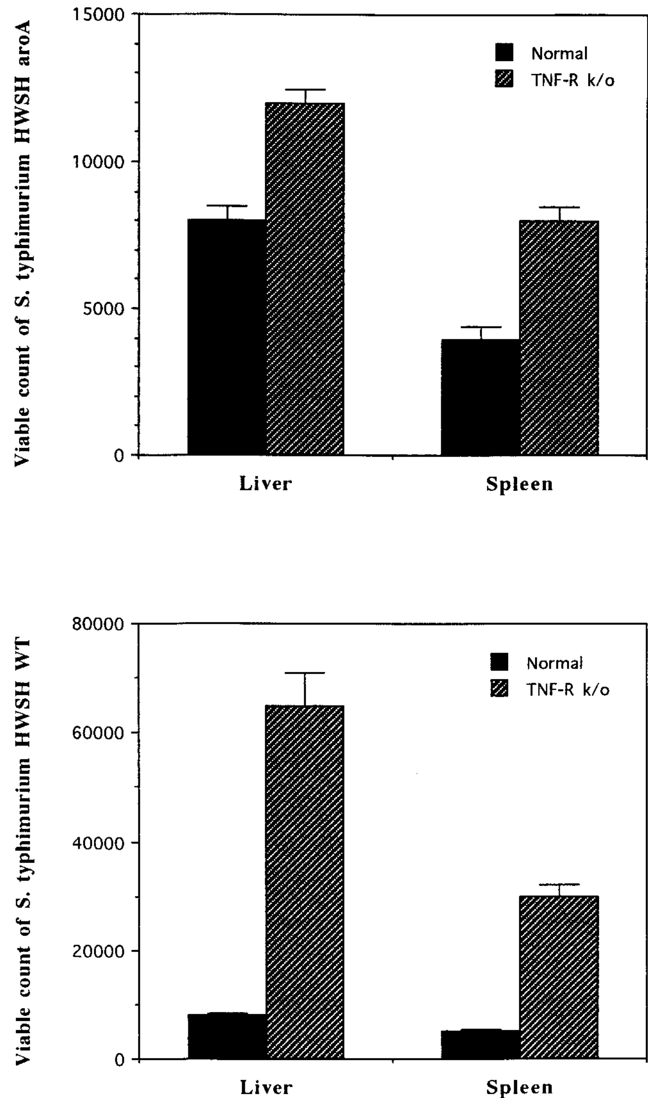


FIG. 2. Viable counts of *S. typhimurium* HWSH *aroA* bacteria at 28 days postvaccination in the livers and spleens of TNFαp55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice. (Bottom) Viable counts of the *S. typhimurium* HWSH wild-type strain at 2 days postchallenge in the livers and spleens of TNFαp55R$^{+/+}$ and TNFα p55R$^{-/-}$ mice.

indicating that they did not clear the vaccine strain as efficiently as normal mice (Fig. 1).

Vaccination studies. Groups of TNFαp55R$^{-/-}$ and TNFα p55R$^{+/+}$ mice were orally vaccinated with 5 × 10^8 or 10^9 *S. typhimurium* HWSH *aroA* bacteria. At day 28 postvaccination, both groups were orally challenged with virulent *S. typhimurium* at a dose of 10^8 bacteria. All TNFαp55R$^{+/+}$ mice survived challenge and were protected, but all of the TNFα p55R$^{-/-}$ group were dead by day 5. The experiment was performed independently three times with identical results. TNFα p55R$^{-/-}$ mice had larger numbers of *S. typhimurium* HWSH *aroA* organisms in their tissues at day 28 after immunization and 2 days after virulent *S. typhimurium* HWSH challenge (Fig. 2).

Splenocyte proliferation in response to *Salmonella* antigen. Splenocyte proliferation experiments were performed with spleens from TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice following vaccination with *S. typhimurium* HWSH *aroA* and

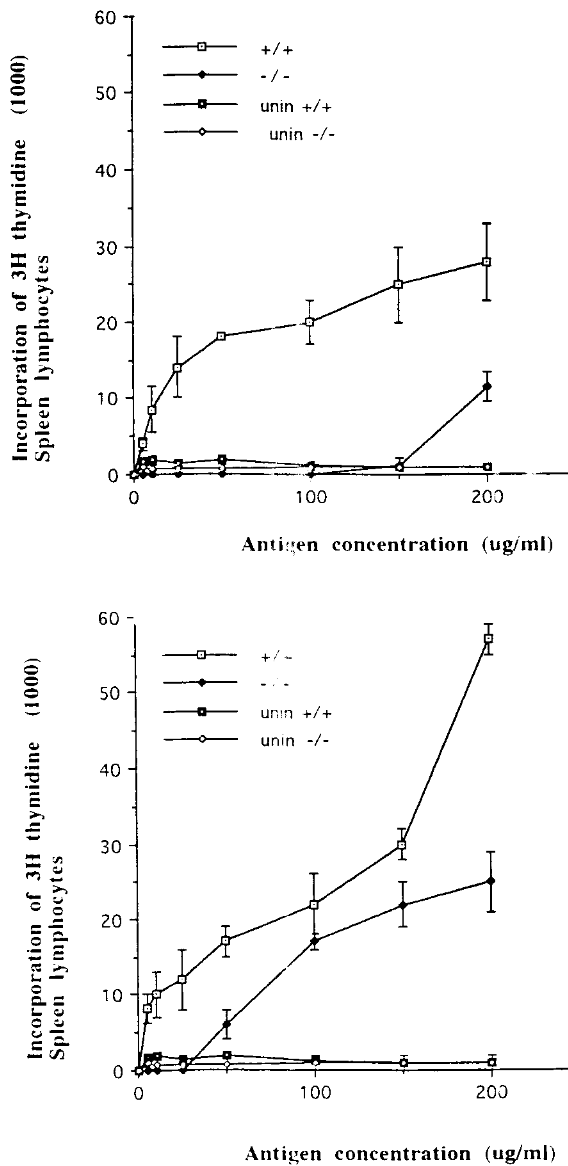


FIG. 3. Proliferation of spleen cells isolated from *Salmonella*-infected mice. (Top) Spleen cells isolated from TNFαp55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice at 28 days after vaccination with *S. typhimurium* HWSH *aroA* proliferate after stimulation with detoxified *Salmonella* lysate. (Bottom) Spleen cells isolated from TNFαp55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice at 2 days after challenge with the *S. typhimurium* HWSH wild-type strain. The results of both experiments are averages of spleen cells from six mice per time point. unin, uninfected mice.

subsequent challenge with virulent *S. typhimurium* HWSH. Splenocytes prepared from TNFαp55R$^{+/+}$ mice at 28 days postvaccination with *S. typhimurium* HWSH *aroA* proliferated in response to detoxified *Salmonella* lysate (detoxified LPS) (Fig. 3). Splenocytes prepared from TNFαp55R$^{-/-}$ mice at 28 days postvaccination proliferated only in response to high concentrations of *Salmonella* antigen and at lower levels than equivalent splenocytes prepared from the TNFαp55R$^{+/+}$ mice (Fig. 3). Two days after *S. typhimurium* HWSH wild-type challenge, previously vaccinated mice were sacrificed and the ability of their splenocytes to proliferate in the presence of *Salmonella* antigen was assessed. As expected, splenocytes prepared from *S. typhimurium* HWSH-challenged TNFαp55R$^{+/+}$ mice demonstrated a stronger proliferative response than did similar mice immunized with *S. typhimurium* HWSH *aroA*. Interestingly, splenocytes prepared from TNFαp55R$^{-/-}$ mice previously vaccinated with *S. typhimurium* HWSH *aroA* and challenged with the *S. typhimurium* HWSH wild type proliferated well in response to *Salmonella* antigen. However, the proliferative response was not as vigorous as in the TNFαp55R$^{+/+}$ mice. This response was ineffective in controlling infection, as all TNFαp55R$^{-/-}$ mice died.

Measurement of cytokines in supernatants of splenocytes prepared from TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice and stimulated with *Salmonella* antigen. High levels of IFN-γ were detected in supernatants from *Salmonella* antigen-stimulated splenocytes prepared from *S. typhimurium* HWSH *aroA*-challenged TNFαp55R$^{-/-}$ and TNFαp55R$^{+/+}$ mice. IFN-γ was also detected in supernatants of splenocytes prepared from TNFαp55R$^{-/-}$ mice 2 days after challenge with wild-type *S. typhimurium* HWSH (Fig. 4). IFN-γ production was decreased upon secondary challenge in knockout mice compared to normal animals. At the time points assayed, TNF-α, IL-4, IL-5, and IL-10 were not detected in supernatants from either TNFα p55R$^{-/-}$ or TNFαp55R$^{+/+}$ mice (days 7 and 21). Splenocytes from control, uninfected animals did not proliferate in the presence of *Salmonella* antigens, whereas concanavalin A-stimulated cells produced all of the above cytokines.

Serum nitric oxide and antibody subclass postvaccination and postchallenge. The levels of nitric oxide in the sera of TNFαp55R$^{+/+}$ mice orally challenged with the *S. typhimurium* HWSH wild type were more than double those present in similarly infected TNFαp55R$^{-/-}$ mice (Fig. 5). Uninfected control animals had very low levels of nitric oxide in their sera. TNFα p55R$^{+/+}$ mice orally challenged with *S. typhimurium* HWSH *aroA* mounted an anti-*Salmonella* immunoglobulin G (IgG) antibody response that was predominantly IgG2a, indicative of a strong Th1 response in these animals. IgG1 was also detected in normal mice. However, similarly challenged TNFαp55R$^{-/-}$ mice were also able to mount smaller IgG2a and IgG1 responses. Sixty percent of these mice demonstrated an IgG2a response, with only 12% producing anti-*Salmonella* IgG3. Postchallenge, all animals exhibited boosted responses in both groups, with the TNFαp55R$^{-/-}$ mice exhibiting lower titers than the TNFαp55R$^{+/+}$ mice (Fig. 6). The TNFαp55R$^{-/-}$

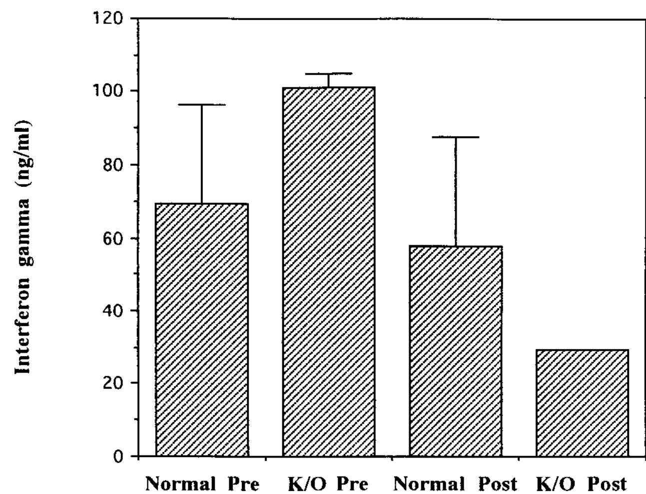


FIG. 4. Splenocytes from both TNFαp55R$^{+/+}$ (Normal) and TNFαp55R$^{-/-}$ (K/O) mice vaccinated with *S. typhimurium* HWSH *aroA* (Pre) taken at day 28 postvaccination and then challenged with the *S. typhimurium* HWSH wild-type strain (Post) produce IFN-γ in the presence of *Salmonella* antigen.

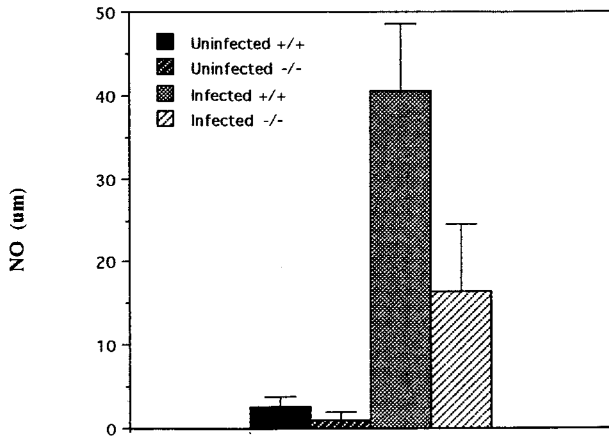


FIG. 5. Serum nitric oxide (NO) levels in TNFαp55R$^{+/+}$ and TNFαp55R$^{-/-}$ mice infected with wild-type *S. typhimurium* HWSH (day 2 after infection).

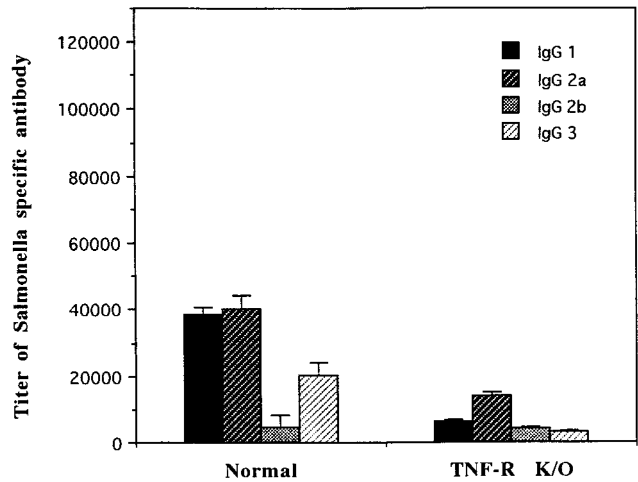
mice had lower titers of antibody to *Salmonella* than did normal mice postvaccination.

Histopathology of infected mice. Histological examination of livers and spleens from infected normal and knockout animals was undertaken 4 days after oral inoculation of the *S. typhimurium* HWSH wild type. In TNFαp55R$^{-/-}$ mice, the livers showed focal areas of inflammatory cells consisting of predominantly neutrophils (polymorphonuclear leukocytes [PMNs]), with some mononuclear cells (Fig. 7). These were scattered throughout the entire section and were numerous and extensive. The spleens of TNFαp55R$^{-/-}$ mice showed large areas of inflammatory cells, with the typical tissue architecture of the spleen disrupted by predominantly PMNs and mononuclear cells. In contrast, normal mice showed similar areas but they were smaller and less numerous in both livers and spleens (Fig. 7).

DISCUSSION

In this study we demonstrate, by using gene knockout mice combined with *Salmonella* HWSH derivatives of differing virulence, the importance of TNFαp55R to both innate and acquired immunity to salmonellosis. Several interesting observations have emerged from these studies. We have shown that TNFαp55R is essential for controlling infections by fully virulent *S. typhimurium* HWSH as well as partially attenuated strains such as *S. typhimurium* HWSH *purE*. TNFαp55R$^{-/-}$ mice die of overwhelming infection before any appreciable immune cell recruitment occurs in the spleen, as judged by the small spleen size at the time of death and the disorganized tissue architecture. Mice differ in the number of organisms present on day 1 of infection, with the knockout mice having higher counts in the livers and spleens. It is not known why this difference in bacterial numbers between the two groups of mice is distinct so early in infection. TNF-α may be needed for PMN recruitment to control *Salmonella* at this early time point. TNF-α is required to efficiently clear the *S. typhimurium* HWSH *aroA* vaccine strain from internal organs. However, the *S. typhimurium* HWSH *aroA* strain did not kill orally challenged TNFαp55R$^{-/-}$ mice, and the bacterial growth was partially controlled. Others have shown that IFN-γ gene knockout mice are hypersusceptible to *S. typhimurium aroA* challenge (14, 40). We have confirmed that IFN-γ gene knockout mice can be killed by oral challenge with *S. typhimurium* HWSH *aroA* (our results not shown), and thus gene knockout mice exhibit significant differences in their abilities to control the growth even of highly attenuated *Salmonella* strains in normal mice. From these observations, it is evident that the expression of TNF-α in IFN-γ gene knockout mice is not sufficient alone to protect mice from overwhelming infection by *Salmonella* strains. TNFαp55R$^{-/-}$ mice produce IFN-γ in response to challenge and vaccination. However, the IFN-γ production is not sufficient to protect the TNFαp55R$^{-/-}$ mice from wild-type *S. typhimurium* HWSH challenge, demonstrating the importance of TNF-α alone or more probably the synergistic effects of IFN-γ and TNF-α in recall of immunity and bacterial killing (9, 24, 32). Antibody does not protect knockout animals from lethal infection. This observation comes from the boosted antibody levels seen in knockout animals by prevaccination and then challenge. Boosted animals still died from wild-type challenge.

Postmortem, the TNFαp55R$^{-/-}$ mice orally challenged with the virulent *S. typhimurium* HWSH wild type or unchallenged



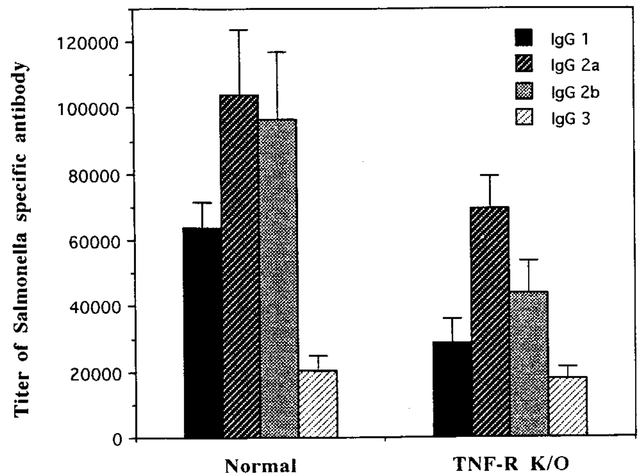


FIG. 6. Subclass-specific antibodies in murine serum. (Top) Subclass-specific titers of antibody against *S. typhimurium* HWSH at 28 days after vaccination with *S. typhimurium* HWSH *aroA*. (Bottom) Subclass-specific titers of antibody against *S. typhimurium* HWSH at 2 days after wild-type challenge of vaccinated mice. K/O, knockout.

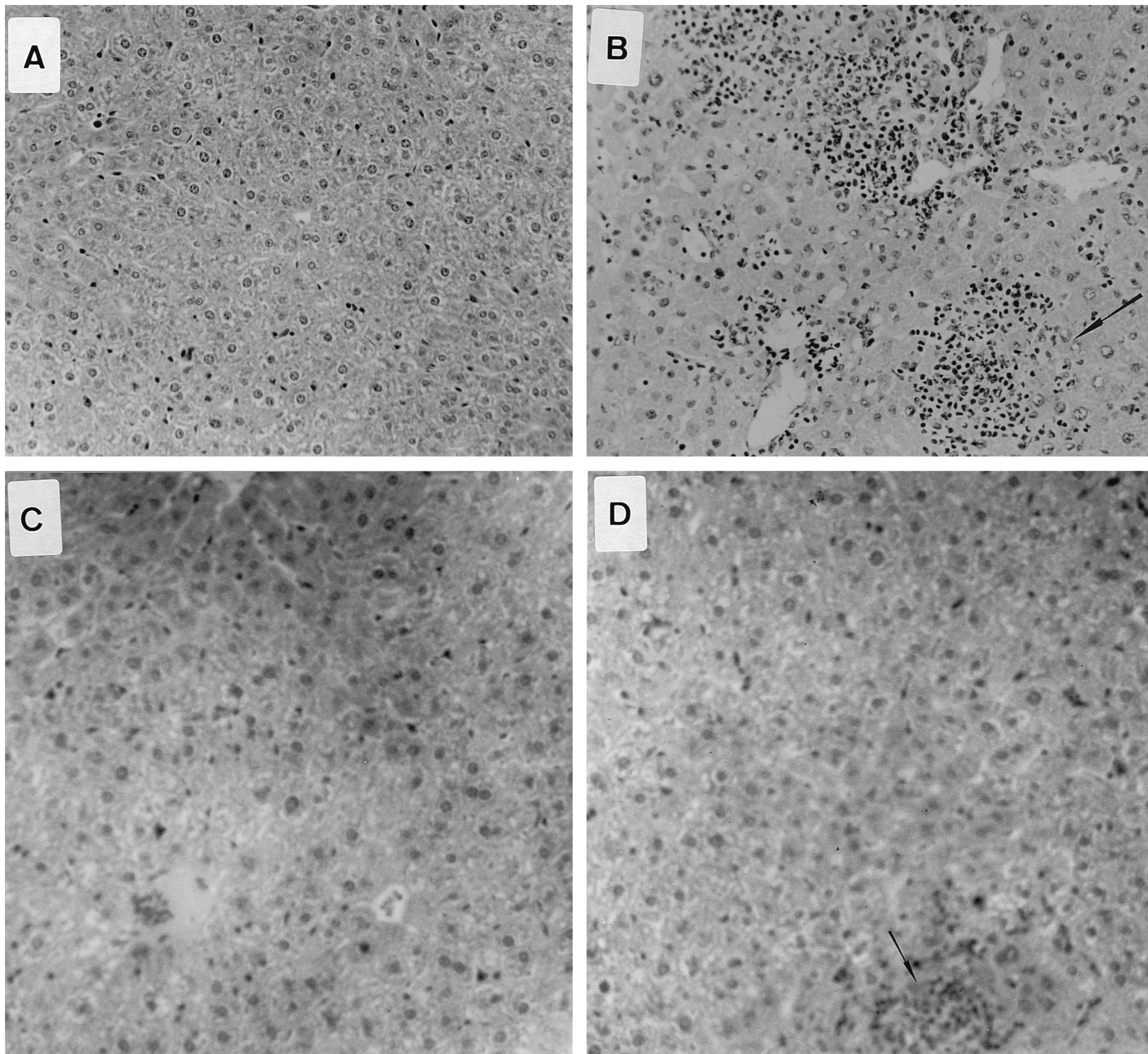
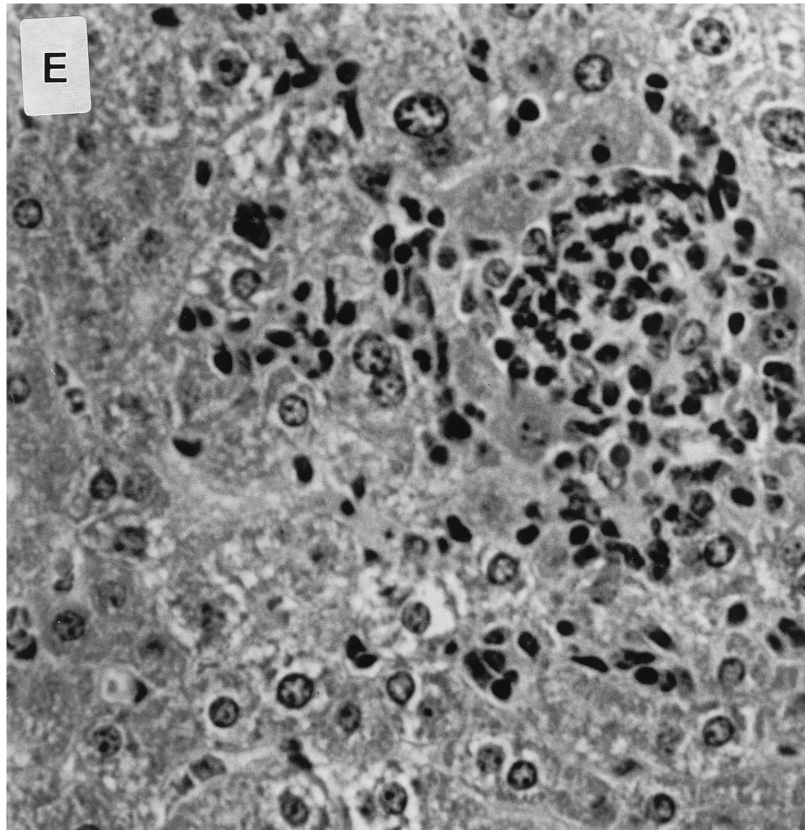


FIG. 7. Pathology in livers and spleens of mice infected with *S. typhimurium* HWSH derivatives and of uninfected controls. (A) Uninfected liver of a TNFαp55R$^{-/-}$ mouse (magnification, ×13.6 [low power]). (B) Liver of a TNFαp55R$^{-/-}$ mouse infected with wild-type *S. typhimurium* HWSH. Livers of these mice have microabscess formations with many neutrophils and macrophages scattered widely within the tissue (arrow) (magnification, ×13.6 [low power]). (C) Uninfected liver of a TNFα p55R$^{+/+}$ mouse (magnification, ×13.6 [low power]). (D) Liver of a TNFαp55R$^{+/+}$ mouse infected with wild-type *S. typhimurium* HWSH. Focal granulomas consisting mainly of macrophages and some PMNs were smaller than in TNFαp55R$^{-/-}$ animals and less numerous throughout the tissues (arrow) (magnification, ×13.6 [low power]). (E) High-power magnification (×40) of focal inflammation in the liver of a TNFαp55R$^{+/+}$ mouse infected with wild-type *S. typhimurium* HWSH. (F) High-power magnification (×40) of a large inflammatory lesion within the liver of a TNFαp55R$^{-/-}$ mouse showing many inflammatory cells, consisting mainly of PMNs and macrophages. (G) Spleen of a TNFαp55R$^{+/+}$ mouse infected with wild-type *S. typhimurium* HWSH, showing normal tissue organization within an infected spleen (magnification, ×16 [low power]). (H) Spleen of a TNFαp55R$^{-/-}$ mouse infected with wild-type *S. typhimurium* HWSH, showing large necrotic areas of poorly organized aggregates of neutrophils and macrophages (magnification, ×16 [low power]).

naive mice had no obvious Peyer's patch structures upon macroscopic observation. Others have reported the lack of Peyer's patch-like structures in TNFαp55R$^{-/-}$ mice (27). Significantly, tissues resembling Peyer's patches were observed associated with the guts of TNFαp55R$^{-/-}$ mice orally challenged with the highly attenuated *S. typhimurium* HWSH *aroA* vaccine strain. Interestingly, however, the Peyer's patches look macroscopically different from the Peyer's patches of TNFαp55R$^{+/+}$ mice (our unpublished observations). Pasparakis et al. (33) observed the formation of Peyer's patches in TNFαp55R$^{-/-}$ mice. In their animals, which are from the same source as ours, they observed small, flat Peyer's patches, the numbers being reduced to two to four per mouse. They also observed defective formation of B-cell follicles. The ages of the mice they used for their experiments are not stated, and for our experiments we used only mice between 6 and 8 weeks old. It seems probable that our uninfected mice would have developed Peyer's patches such as those observed by Pasparakis et al., but the influence of infection with a *Salmonella aroA* mutant upon morphology is not known.

The bacterial product most frequently implicated in TNF-α induction in vivo is LPS. TNF-α induction is involved in the



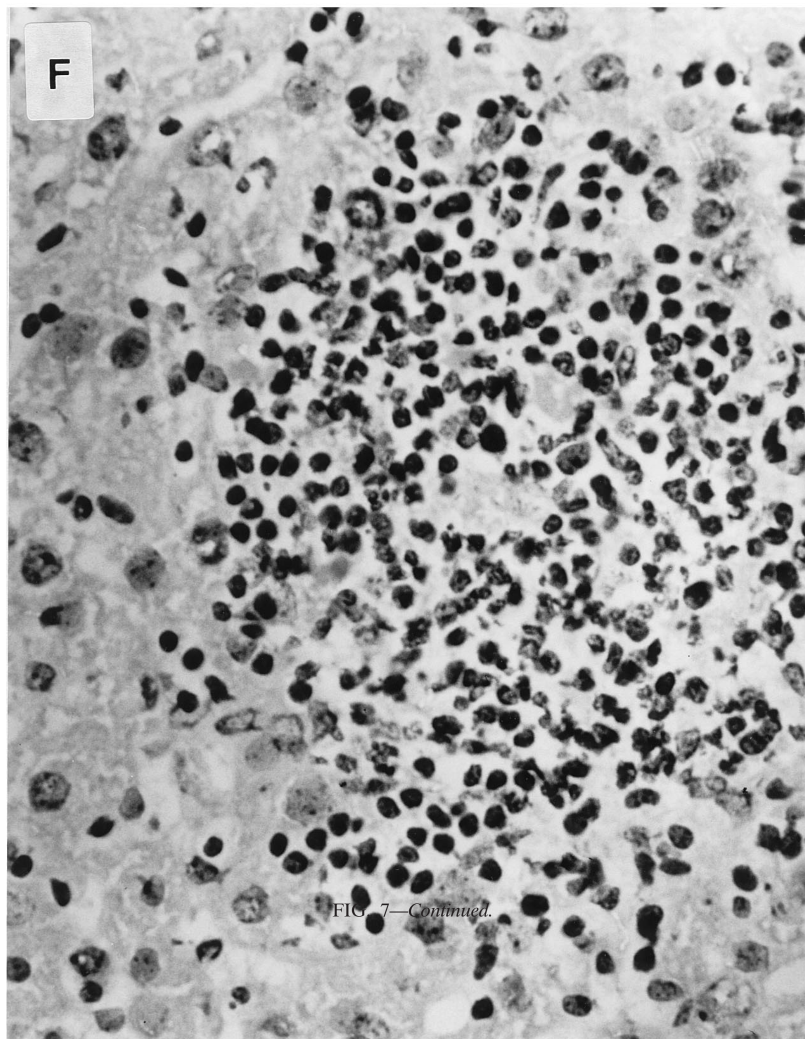
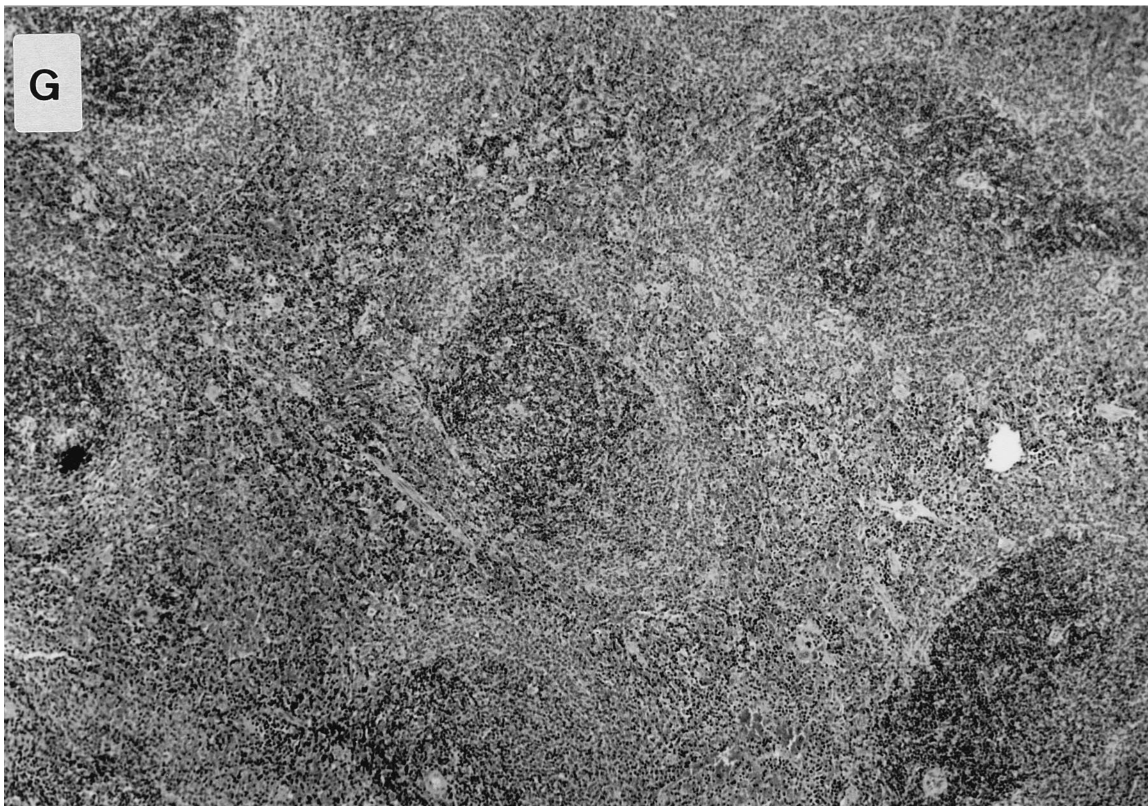


FIG. 7—*Continued.*



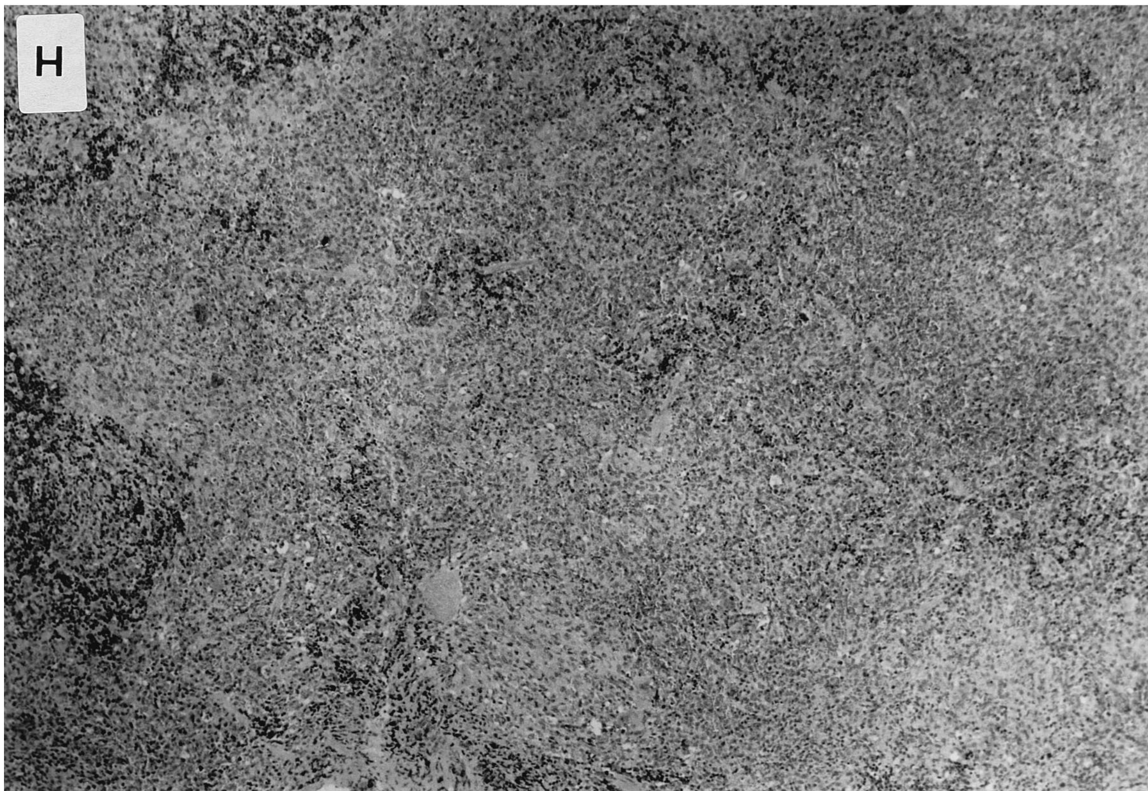


FIG. 7—*Continued.*

generation of reactive nitrogen intermediates in mice that can lead to bacterial killing (12–14). This pathway involves LPS-mediated induction of TNF-α and IFN-γ in macrophages linked to the induction of inducible nitric oxide synthase, which generates nitric oxide. In TNFαp55R$^{-/-}$ infected mice, there were significantly reduced detectable serum nitric oxide levels compared to TNFαp55R$^{+/+}$ infected mice, where double the amount of nitric oxide was detected during infection. The defect in bacterial killing exhibited in TNFαp55R$^{-/-}$ mice, and thus the inability of these animals to control infection, may be attributable in part to their inability to kill via the nitric oxide pathway. Flynn et al. (9) found that macrophages from TNFα p55R$^{-/-}$ mice were unable to synthesize large amounts of reactive nitrogen intermediates following mycobacterial infection. It was postulated that this effect was exerted either directly on macrophages or through a blockade of TNF-α-mediated activation of IFN-γ.

Our data, taken together with that of Mastroeni et al. (22, 23), who used anti-TNF-α antibody to assess the contribution of TNF-α to the control of *Salmonella* infection, suggests that neutralization of TNF-α or deletion of TNFαp55R increases murine susceptibility to salmonellosis. For example, a normally sublethal infection is lethal after anti-TNF-α administration, and treatment with antibodies to TNF-α or IFN-γ abolishes the plateau phase in *ityr* mice, allowing bacteria to grow unchecked.

In summary, we have demonstrated a central role of TNFα p55R in controlling infection by *S. typhimurium*. It would be of interest to see if TNF-α plays a role in humans in controlling *S. typhi* growth associated with typhoid fever. We are currently undertaking a study to investigate the nature of TNF-α production in this disease and to screen for potential genetic polymorphisms associated with the human TNF-α and TNFp55R genes.

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