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J Immunol 2003; 170:6133-6140; doi: 10.4049/jimmunol.170.12.6133

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Salmonella typhimurium Strains Carrying Independent Mutations Display Similar Virulence Phenotypes Yet Are Controlled by Distinct Host Defense Mechanisms

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The outcome of Salmonella infection in the mammalian host favors whoever succeeds best in disturbing the equilibrium between coordinate expression of bacterial (virulence) genes and host defense mechanisms. Intracellular persistence in host cells is critical for pathogenesis and disease, because Salmonella typhimurium strains defective in this property are avirulent. We examined whether similar host defense mechanisms are required for growth control of two S. typhimurium mutant strains. Salmonella pathogenicity island 2 (SPI2) and virulence plasmid-cured Salmonella mutants display similar virulence phenotypes in immunocompetent mice, yet their gene loci participate in independent virulence strategies. We determined the role of TNF-α and IFN-γ as well as different T cell populations in these Salmonella strains. After systemic infection, IFN-γ was essential for growth restriction of plasmid-cured S. typhimurium, while SPI2 mutant infections were controlled in the absence of IFN-γ. TNFRp55-deficiency restored systemic virulence to both Salmonella mutants. After oral inoculation, control of plasmid-cured bacteria substantially relied on both IFN-γ and TNF-α signaling while control of SPI2 mutants did not. However, for both mutants, ultimate clearance of bacteria from infected mice depended on αβ T cells. The Journal of Immunology, 2003, 170: 6133–6140.

The genus Salmonella consists of facultative intracellular enteropathogens responsible for diverse diseases in a wide range of hosts including humans. Although nontyphoidal Salmonella serovars commonly cause self-limiting gastrointestinal infections, they can also lead to life-threatening systemic infections in humans (1). Infection of mice by Salmonella typhimurium produces a systemic disease similar to human typhoid (2). In natural infections, the bacteria enter the host by the oral route, invade specialized Ag-transporting membrane cells (M cells) within the follicle-associated epithelium, colonize the Peyer’s patches of the small intestine, gain access to the gut-associated lymphoid tissue, migrate to the mesenteric lymph nodes (MLN) and disseminate to the liver and spleen (reviewed in Ref. 3). Within the lymphoid organs, S. typhimurium resides in intracellular compartments (4, 5). Intracellular survival and replication in host cells, including macrophages, is critical for bacterial pathogenesis and the development of serious systemic disease, since mutants that fail to replicate intracellularly are avirulent (6).

Attenuation due to defective intramacrophage replication can be due to two types of mutations, i.e., either mutations that alter the metabolic or structural integrity of the bacteria (6–8) or mutations that affect the expression of specific virulence traits mediating host-pathogen interactions. For example, intracellular survival of S. typhimurium depends on the two-component regulatory system PhoP/PhoQ (9–11), which regulates genes involved in resistance to antimicrobial peptides, nutrient scavenging, and LPS modification (12–14). In addition, the type III secretion system encoded by Salmonella pathogenicity island 2 (SPI2) is required for bacterial proliferation in macrophages and systemic growth in mice (15–17). SPI2-secreted effector proteins interfere with vesicular trafficking within the macrophage to avoid NADPH-oxidase dependent killing (18, 19). Moreover, many Salmonella serovars carry plasmids (20), which are crucial for establishing systemic disease by promoting bacterial growth inside phagocytes (21). Although these virulence plasmids considerably vary in size (50–100 kb), a highly conserved gene cluster designated spvRABCD (Salmonella plasmid virulence) can restore the virulence defect of plasmid-cured strains (22, 23). SpvR is a member of the LysR/MetR family of prokaryotic transcriptional activators (24) and participates in regulating the expression of the spv operon together with the starvation-associated σ factor RpoS (25). In recent reports, SpvB was shown to contain a C-terminal mono(ADP-ribosyl)transferase domain (26), which mediates modification of actin in epithelial cells and macrophage-like cells, thereby preventing actin polymerization (27).

However, in addition to their failure to grow inside phagocytes and to cause systemic infection (15, 20, 28–30), plasmid-cured and SPI2 mutant Salmonella strains share various other phenotypic similarities. Both classes of mutants cannot be complemented in trans by coinfection with virulent microorganisms (15) and both SPI2 and spv genes are induced inside host cells (31–33). Using a nonreplicating, temperature-sensitive plasmid to measure the relative rates of bacterial growth and killing during infection, both gene loci were shown to support systemic infection by increasing the bacterial growth rate within host cells of immunocompetent mice (34, 35). Although SPI2 and spv Salmonella mutants show all these phenotypic similarities, competitive index analyses with
single and double mutants did not provide any evidence that these gene loci cooperate as parts of the same virulence mechanism (35).

Despite their mechanistic differences in virulence strategies, both SPI2 and plasmid-cured *Salmonella* mutants grow with similar kinetics in vivo and similar bacterial loads are maintained in systemic organs of immunocompetent mice before infection is cleared 4–6 wk post inoculation (35, 36). This suggests that similar immune mechanisms are required for control of these *Salmonella* strains. However, attenuated *Salmonella* mutant strains have been shown to elicit differential host immune responses (37).

Therefore, we investigated the susceptibility of *S. typhimurium* SPI2 and plasmid-cured mutants to distinct host defense mechanisms. Since host resistance to *Salmonella* depends on Th1 cells and the proinflammatory cytokines TNF-α and IFN-γ (38–41), we focused on the role of these cytokines and of T cell populations in control of infection with SPI2 and plasmid-cured *Salmonella* strains. We used mutant mouse strains, in which the genes for IFN-γ, TNFRp55, and various molecules involved in T cell maturation had been deleted, to learn more about how clearance of attenuated *Salmonella* strains is achieved and how mutants with similar virulence phenotypes differ. In this study, we provide experimental evidence that, despite phenotypic similarities, early control of *S. typhimurium* SPI2 and plasmid-cured mutants displays differential requirements for proinflammatory cytokines. However, in both cases, ultimate clearance of bacteria from infected mice depended on αβ T cells. These data not only provide insight into the molecular basis of host-pathogen interactions, but also reveal guidelines toward the development of *Salmonella* vaccine carriers tolerable by immunocompromised hosts.

Materials and Methods

**Bacterial strains and growth conditions**

The attenuated *S. typhimurium* strains used in this study were derived from wild-type strain SL1344 (7). The plasmid-cured, and *phoP*–strains were a gift from Dr. B. Stocker (Stanford University, Stanford, CA). The SPI2 strain contains araA::mTn5::Km2 mobilized through F22H transduction from the *S. typhimurium* mutant P3F4, on the 12023 background (15). Unless noted otherwise, bacteria were grown statically in Luria-Bertani broth containing 0.3 M NaCl at 37°C to stationary phase with an OD600 of ~0.5. Antibiotics were included as appropriate at the following concentrations: streptomycin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (15 μg/ml).

**Mouse strains**

Mice were bred in our animal facilities at the Bundesamt für gesetzlichen Verbraucherschutz und Veterinärmedizin in Berlin, Germany, under specific pathogen-free conditions. For all experiments, sex- and age-matched animals were used under conventional housing conditions. Breeding pairs of homozygous IFN-γ−/−, β2 microglobulin (β2m−/−), AB−/−, TCR β−/−, and H2-KK−/−/H2-Dd−/− mice were kindly provided by T. Stewart (Genentech, South San Francisco, CA), R. Jaenisch (Massachusetts Institute of Technology, Cambridge, MA), D. Mathis (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France), S. Tonegawa (Massachusetts Institute of Technology, and B. Petna and F. Lemonnier (Institut Pasteur, Paris, France), respectively (42–46). TNFRp55-deficient animals were backcrossed six times onto the C57BL/6 background (47). Mice were tested by PCR for the presence of the wild-type or mutant Nramp1 allele using tail tissue (40). All breeding pairs proved to be homozygous for the susceptible allele.

**Animal treatments and infections**

Mice were infected by i.p. injection or oral gavage of the bacteria suspended in 0.2 ml of PBS. For oral infections, mice were starved overnight before inoculation. At the desired time points postinoculation, infected organs were homogenized in PBS and plated on Luria-Bertani agar plates to enumerate CFU. Routinely, the 5 caecum-proximal Peyer’s patches of the ileum were selected. Routinely, the following sublethal doses were chosen for infection: systemic challenge with SPI2 *S. typhimurium* mutants was performed with doses up to 5 × 10^9 CFU, since the LD₉₀ in BALB/c mice had been determined as >10⁶ CFU (35). For oral infections with plasmid-cured *S. typhimurium* mutants a dose of 10⁸ CFU was applied, since curing *S. typhimurium* wild-type strain SL1344 had been reported to raise the peroral LD₉₀ in BALB/c mice from 6 × 10⁶ CFU to around 6 × 10⁸ (36). Survival of mice was recorded daily and is given as percentage of live animals per time point.

**Histological analysis of infected tissues**

For histological analysis, a portion of infected mouse organs were embedded in Tissue-Tec OCT medium (OCT) and snap frozen in liquid nitrogen, while the remainder was homogenized and plated as described above. Tissues were sectioned in 5-μm sections, fixed and stored at ~70°C. FC activity was blocked with 1% FCS. The following primary rat mAb were used for staining: RB6-8C5 for neutrophils (48) or F4/80 for red pulp macrophages (ATCC HB-198). Primary rat mAb were detected by a sequential incubation with goat anti-rat Ig (Callag Labortories, Burlingame, CA) and alkaline phosphatase-conjugated donkey anti-goat Ig (The Lasson Laboratory, West Grove, PA). Detection Abs were diluted in PBS containing 5% normal mouse serum. Alkaline phosphatase complexes were developed with naphthol AS-BI phosphate (Sigma-Aldrich, St. Louis, MO) and New Fuchsin (Merck, Darmstadt) as substrate. Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma-Aldrich). Slides were counterstained with hematoxylin.

**Statistics**

The Mann-Whitney U test was used to determine statistical significance of differences in bacterial load between control and experimental groups. Survival curves were compared using the logrank test.

**Results**

TNFRp55 deficiency restores virulence of different *S. typhimurium* mutants defective in intracellular growth.

Susceptibility to TNF of a virulence plasmid-cured *S. typhimurium* mutant was compared with that of strain P3F4 (srrf::mTn5), which is a regulatory mutant controlling effectors both within and outside of SPI2. In immunocompetent mice, these two mutants show similar virulence phenotypes. *Salmonella* mutants deficient in *aroA* and *phoP* were included, since these attenuated strains stimulate different host responses (37). TNFRp55-deficient mice were infected i.p. and the bacterial loads in livers and spleens were determined. As previously shown (41), TNFRp55-deficient mice were highly susceptible to infection with wild-type *S. typhimurium*, but capable of controlling challenge with the metabolically attenuated *S. typhimurium aroA* vaccine strain (Fig. 1). In contrast to the *aroA*-deficient *Salmonella* strain, bacterial loads of all three virulence-attenuated mutants were markedly increased in TNFRp55-deficient animals. Bacterial numbers were at least 100-fold higher than in C57BL/6 control mice, indicating that TNF-α signaling is essential for growth restriction of *S. typhimurium* wild-type and virulence-attenuated strains, but not for control of metabolically attenuated strains.

Exacerbated *S. typhimurium* SPI2 infection in TNFRp55-deficient animals is in accordance with a recent report demonstrating defective localization of the NADPH phagocyte oxidase to *Salmonella*-containing phagosomes in TNFRp55-deficient macrophages (49) and suggests a role for SPI2 effectors in impairing TNF-α-induced vesicular trafficking. In contrast, increased susceptibility of TNFRp55-deficient mice to plasmid-cured *S. typhimurium* organisms was unexpected in view of a report claiming that infection of mice by *Spv*− *S. typhimurium* is not exacerbated by neutralization of TNF-α as well as IFN-γ (50).

Compatible results were revealed by histological examination of organs from normal and TNFRp55-deficient mice infected with *S. typhimurium* wild-type and mutants. Fig. 2 depicts the immunohistochemical analysis of spleen sections of these mice. Bacterial growth of SPI2 mutants was restricted in C57BL/6 mice after i.p. challenge (35) (Fig. 2C) with the splenic architecture being indistinguishable from that of naive animals (Fig. 2, A and D). The strongest morphological alterations were detected in the spleens of...
TNFRp55-deficient mice infected with wild-type *S. typhimurium* (Fig. 2E). The characteristic splenic architecture was disrupted and large areas of inflammatory cells predominantly consisting of polymorphonuclear neutrophils and mononuclear cells were seen. Control mice infected with wild-type *S. typhimurium* showed similar, although smaller and less abundant, areas of accumulating polymorphonuclear neutrophils and monocytes (Fig. 2B). The degree of tissue destruction after a *S. typhimurium* wild-type infection in control mice was comparable to that observed in TNFRp55-deficient animals infected with *S. typhimurium* SPI2 mutants. In the absence of TNF-α signaling, extended areas of inflammation were visible (Fig. 2F) paralleled by unrestricted bacterial replication of attenuated SPI2 mutants. Histopathological disorganization was comparable in spleens of mouse strains, which succumbed to uncontrolled *S. typhimurium* infection after early development of neutrophil-rich microabscesses and extensive necrotic lesions without progressing to mononuclear cell-rich granulomas (51). Thus, TNF-α may play a role in the focal development of granulomatous structures.

**Growth restriction of plasmid-cured but not SPI2 mutant *S. typhimurium* is abrogated in IFN-γ-deficient mice**

Survival experiments in which IFN-γ and TNFRp55-deficient and control mice were challenged with different *S. typhimurium* mutants were performed to clarify whether control of infection with plasmid-cured *S. typhimurium* relies on these proinflammatory cytokines. Although all *S. typhimurium* mutants were attenuated in C57BL/6 control animals (Fig. 3A), TNFRp55-deficient mice (Fig. 3B) rapidly succumbed to i.p. challenge with normally sublethal doses of all three virulence-attenuated strains, but not of the metabolically attenuated *aroA* strain. Intriguingly, plasmid-cured *S. typhimurium* mutants replicated unrestricted not only in TNFRp55-deficient mice, but also in animals lacking IFN-γ, while control of the *S. typhimurium* SPI2 defective strain occurred independent of IFN-γ (Fig. 3C, Refs. 19 and 52). Control of all other attenuated mutants required IFN-γ, though to different degrees as reflected by differential survival of IFN-γ-deficient mice postinfection (Fig. 3C). Thus, growth restriction of systemic infections with *S. typhimurium* SPI2, *aroA*-deficient, and plasmid-cured mutants differed in their requirement for TNF-α and IFN-γ.

**The proinflammatory cytokines TNF-α and IFN-γ are critical for clearance of plasmid-cured *S. typhimurium* strains after oral infection**

*S. typhimurium* is normally acquired via the oral route, and differential virulence of wild-type and plasmid-cured *Salmonella* strains is displayed more distinctly after oral rather than i.p. infection (36). We compared the growth kinetics of wild-type *S. typhimurium* and plasmid-cured *Salmonella* mutants after intragastric inoculation of $10^5$ CFU to TNFRp55- and IFN-γ-deficient and control mice. While this inoculation dose is sublethal to immunocompetent animals infected with plasmid-cured *S. typhimurium*, animals inoculated with wild-type microorganisms succumb to infection. At different time points postinfection, MLN, spleens, and livers were removed, and the bacterial loads in organ homogenates were determined. Bacterial persistence was monitored over a period of 6–14 days for animals infected with wild-type and plasmid-cured bacteria, respectively (Fig. 4). As to be expected, IFN-γ and TNF-α were essential for control of infections with wild-type *S. typhimurium* after oral challenge. Bacterial counts in infected organs of cytokine-deficient mice were significantly elevated compared with immunocompetent animals (Fig. 4, A and C). Susceptibility to infection was most prominent in IFN-γ-deficient mice, followed by TNFRp55-deficient animals and immunocompetent C57BL/6 controls.

As predicted for wild-type mice, the plasmid-cured *S. typhimurium* strain reached levels in the order of $10^3$–$10^4$ CFU in spleens 4–5 days postinfection (Fig. 4, B and D) and remained constant at this level over the time period examined (36). CFU in infected organs of IFN-γ- and TNFRp55-deficient animals did not significantly differ up to day 4 postinfection. However, subsequent to this time point, susceptibility of the plasmid-cured *Salmonella* strain to IFN-γ- and TNF-α-mediated defense mechanisms differed markedly. In both IFN-γ- and TNFRp55-deficient animals, bacterial growth was unrestricted (Fig. 4, B and D). While IFN-γ-deficient mice succumbed to overwhelming bacterial infection by day 8 postinoculation, TNFRp55-deficient animals were still alive two weeks postinfection, but displayed significantly elevated organ loads. In contrast to immunocompetent animals, which resolved infection with plasmid-cured *S. typhimurium* mutants ~4–6 wk postinfection, TNFRp55-deficient mice failed to restrict bacterial multiplication and ultimately succumbed by day 20 (data not shown). These data demonstrate that both IFN-γ and TNF-α are indispensable for control of infections with plasmid-cured *S. typhimurium* mutants following oral challenge, although IFN-γ displays its relevance in restricting bacterial growth earlier.

**Control of oral challenge with SPI2 mutant strains occurs independent of IFN-γ and TNFRp55 signaling**

To compare the requirements of proinflammatory cytokines for the control of *S. typhimurium* mutants following inoculation via the natural route, TNFRp55-deficient, IFN-γ-deficient, and wild-type control mice were infected orally with different *S. typhimurium* strains, including the wild-type strain SL1344, and the plasmid-cured, SPI2, *phoP* and *aroA* mutants. Survival was monitored for 40 days. As previously described, cytokine-deficient mice were significantly more susceptible to oral challenge with wild-type *S. typhimurium* SL1344 than immunocompetent controls (Refs. 41 and 53 and data not shown). *S. typhimurium* *aroA* mutants were virulent in IFN-γ-deficient animals after oral inoculation with $5 \times$
10^9 bacteria (Ref. 37, Fig. 5D), while this inoculum was controlled by TNFRp55-deficient and wild-type control mice (Ref. 41, Fig. 5D). The avirulent phoP and SPI2 Salmonella mutant strains were severely attenuated even in the absence of either IFN-γ or TNF-α signaling (Fig. 5, A and C). In contrast, both IFN-γ- and TNFRp55-deficient mice were highly susceptible to infection with plasmid-cured S. typhimurium (Fig. 5B) as reflected by the significantly reduced survival times as compared with control mice (20 days; p < 0.0001). The median survival time of TNFRp55-deficient mice (10 days) was greater than that of animals lacking IFN-γ (6 days). To substantiate the role of TNF-α and IFN-γ in control of infection with plasmid-cured S. typhimurium mutants after oral inoculation, a similar challenge experiment was performed using a sublethal inoculum. A dose of 10^8 CFU was chosen, since curing S. typhimurium wild-type strain SL1344 had been reported to raise the peroral LD_{50} in BALB/c mice from 6 × 10^4 CFU to around 6 × 10^8 (36). While C57BL/6 control animals survived oral challenge with 10^8 plasmid-cured S. typhimurium mutant, IFN-γ- and TNFRp55-deficient mice succumbed to infection with a median survival time of 10.5 days (p = 0.0015) and 16 days (p = 0.0056), respectively. Hence, IFN-γ and TNF-α are essential for growth restriction of plasmid-cured S. typhimurium in the early phase after oral infection.

Clearance of both plasmid-cured S. typhimurium and SPI2 mutant strains from infected mice depends on αβ T cells

Chronic stages of Salmonella infection are controlled by T cell mediated immunity (39, 54). Mouse strains lacking T cells fail to resolve Salmonella infections and succumb to challenge despite initial control of bacterial growth (39, 55). To define the contribution of αβ T cells to clearance of infections with S. typhimurium SPI2 and plasmid-cured mutants, TCR-β−/− mice were challenged orally with normally sublethal doses of bacteria (Fig. 6A). TCR-β−/− mice rapidly succumbed to challenge with plasmid-cured S. typhimurium. All mice died by day 20 postinfection, compared with TCR-β−/− animals infected with aroA-deficient S. typhimurium, which had a median survival time of 75 days. Clearance of the S. typhimurium SPI2 mutant strain also required the presence of αβ T cells. Mice lacking αβ T cells could control infection with both plasmid-cured and aroA-deficient S. typhimurium until a distinct time point at which rapid exacerbation of disease and subsequent death occurred. In contrast, TCR-β−/− mice infected with S. typhimurium SPI2 mutant died gradually over a period of 50 days.

To define the T cell populations required for control of infections with SPI2 and plasmid-cured Salmonella strains and to determine the relative contribution of MHC-class I- and class II-dependent immune mechanisms to clearance, we used H-2I-Aβ−/− mice (lacking MHC class II molecules and thus devoid of functional CD4^+ αβ T cells), H2-Kb−/−/H2-D^b−/− mice (lacking classical MHC class Ia molecules and thus devoid of conventional CD8^+ αβ T cells), and β_m-deficient mice (lacking all β_m-dependent surface molecules including classical MHC class Ia and nonclassical MHC class Ib and thus devoid of conventional and nonconventional CD8^+ and NK T cells). The median survival time of Aβ−/− mice (28 days) as well as of β_m-deficient and H2-Kb−/−/H2-D^b−/− mice (33 days) infected with plasmid-cured S. typhimurium (Fig. 6B) was significantly prolonged compared with TCR-β−/− animals (24 days). Hence, both CD4^+ and CD8^+ T cell-dependent immune mechanisms could not compensate for each other, suggesting either distinct or interactive functions in bacterial clearance. The T cell subsets required for control of oral infection with S. typhimurium SPI2 mutant could not be dissected in survival studies (Fig. 6C), since from all the different T cell-deficient mouse strains tested only the TCR-β−/− animals succumbed to infection. To analyze whether survivors were still carrying sublethal amounts of bacteria in their organs or had completely cleared the infection, the animals were sacrificed at day 100 postinfection and the bacterial load in the liver and spleen was determined. No residual S. typhimurium SPI2 mutant organisms could be recovered from organ homogenates (data not shown). In addition, when TCR-β−/− animals started to die, all other mouse strains were analyzed for shedding of S. typhimurium SPI2 mutant cells. This inoculum was controlled by TNFRp55-deficient and wild-type control mice (Ref. 41, Fig. 5D). The avirulent phoP and SPI2 Salmonella mutant strains were severely attenuated even in the absence of either IFN-γ or TNF-α signaling (Fig. 5, A and C). In contrast, both IFN-γ- and TNFRp55-deficient mice were highly susceptible to infection with plasmid-cured S. typhimurium (Fig. 5B) as reflected by the significantly reduced survival times as compared with control mice (20 days; p < 0.0001). The median survival time of TNFRp55-deficient mice (10 days) was greater than that of animals lacking IFN-γ (6 days). To substantiate the role of TNF-α and IFN-γ in control of infection with plasmid-cured S. typhimurium mutants after oral inoculation, a similar challenge experiment was performed using a sublethal inoculum. A dose of 10^8 CFU was chosen, since curing S. typhimurium wild-type strain SL1344 had been reported to raise the peroral LD_{50} in BALB/c mice from 6 × 10^4 CFU to around 6 × 10^8 (36). While C57BL/6 control animals survived oral challenge with 10^8 plasmid-cured S. typhimurium mutant, IFN-γ- and TNFRp55-deficient mice succumbed to infection with a median survival time of 10.5 days (p = 0.0015) and 16 days (p = 0.0056), respectively. Hence, IFN-γ and TNF-α are essential for growth restriction of plasmid-cured S. typhimurium in the early phase after oral infection.

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organisms in their feces. Neither Aβ−/− mice nor β2-m-deficient and H2-Kb−/−/H2-D b−/− animals still excreted *S. typhimurium* at this time point. Thus, the results indicate that individual T cell subsets are sufficient to control oral infections with SPI2-deficient *S. typhimurium* and that CD4+ and CD8+ T cells can compensate for each other.

**Discussion**

In this study we show that a distinct repertoire of host defense is required for effective growth control of different attenuated *S. typhimurium* strains. We have compared *S. typhimurium* mutant strains in which attenuation is based on either a metabolic defect, as is the case for the *aroA*-deficient vaccine strain, or the inability to survive or replicate inside phagocytes, such as the virulence attenuated strains defective in *phoP*, SPI2, or the virulence plasmid. The observed differences are complex, since the requirements of cytokines for the control of attenuated *Salmonella* strains also vary depending on the route of infection (Table I). We demonstrate differential impacts of TNF-α and IFN-γ on the control of these mutants in vivo. Early control of plasmid-cured *S. typhimurium* organisms was mediated by both IFN-γ- and TNF-α-dependent effector mechanisms. In contrast, SPI2-deficient *Salmonella* mutants, which in immunocompetent mice display an apparently comparable attenuation phenotype to plasmid-cured strains despite carrying an independent mutation, were eliminated by IFN-γ-independent mechanisms.

Furthermore, our results emphasize that the relative importance of individual control mechanisms varies with the route of infection. Absence of TNF-α signaling led to loss of control of all virulence-attenuated *S. typhimurium* strains following systemic challenge, while infections with the metabolically attenuated *aroA* vaccine strain were controlled efficiently. In contrast, following oral infection, TNF-α was only required for growth restriction in infections with the plasmid-cured *S. typhimurium* mutant strain. TNFRp55-deficiency affected the LD50 of plasmid-cured *S. typhimurium* following oral challenge and SPI2 *Salmonella* mutants following systemic challenge by three orders of magnitude (data not shown, Refs. 35, 36, and 49). In contrast, TNFRp55-deficient mice infected orally with as many as 2 × 1010 CFU SPI2 mutant did not succumb to challenge and transient infection was successfully resolved (data not shown). However, it remains unclear whether TNF-α-independent elimination of *S. typhimurium* SPI2 mutants following oral challenge directly translates into TNF-α-independent delivery of ROI to the *Salmonella*-containing phagosome, because this result could also reflect limitations of the test system with the precise determination of the peroral LD50 of *S. typhimurium* SPI2 mutants being technically impossible. Alternatively, TNF-α-independent components of the mucosal immune system may suffice to prevent transition of *S. typhimurium* SPI2 mutants from their intestinal location to systemic sites. As described recently, specific intestinal mucosal IgA responses can develop in the absence of both TNF-α signaling and T cells (56). It is tempting to assume that mucosal immunity represents the key effector for control of *S. typhimurium* SPI2 mutants after oral infection, while the TNF-α-independent delivery of toxic oxygen species mediates clearance after systemic challenge.

IFN-γ and TNF-α are critical components of the host response to microbial pathogens. However, experimental analyses using neutralizing cytokine-specific Abs, genetically deficient mice or mice treated with recombinant cytokines, have created a complex and sometimes contradictory picture regarding the role of TNF-α and IFN-γ in murine salmonellosis. Technical details, including inoculation route, host susceptibility, immune status of the animals and virulence of the *Salmonella* strains, mainly account for the differences described (57–59). In addition, some confusion must be attributed to the experimental limitations of the test systems applied. Depletion of cytokines using neutralizing Abs to determine their contribution to infection with different *Salmonella* strains is particularly critical, given that neutralization tends to be relatively short-lived, yet differences between Ab-treated animals and untreated controls do not manifest until after day 4 postinfection (53). Hence, we decided to use mice with gene deletions in IFN-γ and TNFRp55 to elucidate the role of TNF-α and IFN-γ in control of distinct virulence strategies of *S. typhimurium*. Our data not only prove the essential role of TNF-α and IFN-γ in control of infections with wild-type *S. typhimurium*, but also identify both cytokines as critical effectors for clearance of *S. typhimurium* strains lacking the virulence-plasmid. The latter result provides an example for how methodological differences in experimental design can lead to apparently contradictory conclusions despite providing overlapping data sets. An earlier report using mAbs to deplete cytokines (50) claimed that infection of mice by Spv−/− *S. typhimurium* is not exacerbated by neutralization of TNF-α as well as
IFN-γ. This statement provides a valid conclusion from the data obtained with cytokine-depleted mice until day 4 post infection, which is the time period during which Ab depletion is effective. Using gene-deletion mouse mutants, however, significant differences in bacterial load of immunocompetent and cytokine-deficient animals did not become apparent until day 5 postinfection with plasmid-cured *S. typhimurium*. Since the role of IFN-γ and TNF-α in control of infections with plasmid-cured *S. typhimurium* is only detectable in long-term analyses, the relevance of these cytokines had to be overlooked in previous studies based on Ab neutralization.

While our experiments reveal distinct differences in the immune mediators required for early control, it is tempting to speculate that both mutants share a comparable virulence strategy. Elegant studies by Vazquez-Torres et al. (49) suggest that SPI2 effectors and both mutants share a comparable virulence strategy. It is possible that TNF-α and IFN-γ are required for early control, but it is also possible that these cytokines had to be overlooked in previous studies based on Ab neutralization.

In marked contrast, resolution of oral infections with *S. typhimurium* SPI2 mutant was achieved by either CD4+ T cells or CD8+ T cells. Increased susceptibility to infection was only detected in animals deficient in all CD8+ T cell populations. Although this observation may result from the inoculum size used (bearing in mind that the highest technically possible dose of *S. typhimurium* SPI2 mutant (2 × 10^10 CFU) was used for oral challenge and the LD₅₀ for the bacterial strain cannot be determined), compensatory T cell functions should be considered.

In conclusion, our data demonstrate a defined hierarchy for the specific T cell subsets that contribute to control of plasmid-cured *S. typhimurium* with MHC class II-dependent CD4+ T cells being the dominating effectors. Yet, MHC class Ia restricted CD8+ T cells were also required and could not be fully compensated for by CD4+ T cells. Intracellular iron availability is a critical component of the host-*Salmonella* relationship. Uptake of transferrin bound iron into cells is regulated by Hfe (60). Surface expression of Hfe depends on β₂m as does MHC class I expression. Hence, in β₂m-deficient mice iron availability is altered. However, differences in intracellular iron availability did not affect bacterial clearance at this stage of infection, since both H₂-Kᵇ⁻/⁻/H₂-Dᵇ⁻/⁻ and β₂m-deficient mice orally challenged with 8 × 10^7 CFU plasmid-cured *S. typhimurium* strains (inverted triangle) and β₂m-deficient mice orally challenged with 8 × 10^8 CFU plasmid-cured *S. typhimurium* mutants (square) were monitored.
bactericidal effector mechanisms for clearance of the respective mutants. Although TNF-α and IFN-γ act synergistically to enhance the bactericidal activity of macrophages, our results show that triggering the respective signaling cascade of either cytokine can independently lead to the activation of different effector systems. Both qualitative and kinetic differences in control of S. typhimurium infections suggest that distinct cytokine combinations are directed at specific virulence gene products of a single pathogen. Effective control of infections can only occur when the host delivers the appropriate repertoire of effectors in a timely fashion.

Implications for vaccine design are obvious. Attenuated Salmonella strains have proven their efficacy in numerous preclinical trials, in which they were used not only as vaccines against typhoid, but also as heterologous carriers for various viral, bacterial and protozoal Ags (38). However, viable attenuated vaccines bear the intrinsic risk of causing disease in immunocompromised hosts. As shown in this study and elsewhere, different attenuated vaccine candidates vary with respect to the immune mechanisms responsible for host defense (37, 61). Better understanding of the molecular defense strategies required for control of different attenuated strains will facilitate selection of appropriate vaccine candidates, which as the ultimate goal, will be both effective and tolerable even in individuals with distinct immunodeficiencies.

Acknowledgments

We are grateful to Uwe Klemm, Karin Bordsch, and Manuela Prinke for assistance in establishing and maintaining the mouse colonies. We thank Helen Collins and David Weiss for carefully reading the manuscript and helpful comments.

References


Table 1. Differential requirements of cytokines for the control of attenuated Salmonella strains

<table>
<thead>
<tr>
<th>Salmonella Strain</th>
<th>IFN-γ</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δaro</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔphoP</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ΔSPI2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Δspv</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The table summarizes results obtained for the requirement of cytokines for control of infections with attenuated Salmonella strains. The qualitative overview combines data obtained in this study and/or previously published results.

† See also Refs. 37, 39, and 41.

‡ See also Ref. 37.

§ See also Refs. 19, 49, and 52.