Regulation of host immune responses by modification of *Salmonella* virulence genes

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Modifying bacterial virulence genes to probe the nature of host immunity is mostly unexplored. Here we investigate whether host immune responses can be regulated by modification of bacterial virulence genes. In mice, attenuated *Salmonella* mutant strains with clinical relevance elicited differential host immune responses. Oral administration of a mutant strain with a PhoP-null phenotype promoted potent innate immune responses of macrophages that were sufficient for host defense. In contrast, administration of an Aro$^-$ mutant strain elicited stronger specific antibody and T-helper (Th)-cell responses, wherein Th1-type cells were required for clearance. Thus, genetic manipulation of bacteria may be used to broadly alter immune mechanisms that regulate attenuation within the host and to tailor host immunity to specific bacterial pathogens.

Modifying virulence genes in bacteria by genetic techniques has facilitated the development of mutant strains that have potential as vaccines and antigen carrier vehicles. In particular, mutant strains of *Salmonella* have shown promise as live oral vaccines in humans, capable of stimulating both mucosal and systemic immune responses[1]. However, the immune mechanisms involved in host pathology and the roles that bacterial virulence genes have in evading, suppressing and stimulating immune responses have not been fully elucidated[2].

Survival of *Salmonella* within the mammalian host is accomplished partly by coordinate expression of virulence genes[3]. In turn, host resistance to *Salmonella* depends on the action of Th1-type cells and interferon gamma[4–6] (IFN-γ). A variety of defined deletions in *Salmonella* virulence genes can substantially reduce survival in the host. For example, there has been considerable interest in strains with mutations in *phoP–phoQ* and *aro* genes. The PhoP–PhoQ virulence regulon is a bacterial two-component regulatory system that controls expression of genes necessary for survival within macrophages and resistance to antimicrobial peptides[3]. Disruption of these genes results in the PhoP-null phenotype[3] (PhoP$^-$). In contrast, *aro* genes regulate synthesis of aromatic acid metabolites that are normally unavailable in mammalian hosts[7]. *S. typhimurium* PhoP$^-$ and Aro$^-$ mutant strains are immunogenic and attenuated for virulence in animal models, as are PhoP$^-$ and Aro$^-$ derivatives of *S. typhi* in humans[8–12].

Here we investigate the effects of the modification of *Salmonella* virulence genes on immune responses that ensue after the oral inoculation of mice. We focused on *Salmonella* Aro$^-$ and PhoP$^-$ mutant strains in part because of their relevance to vaccine development. We used immunodeficient animals, mice deficient in IFN-γ (IFN-$\gamma^{-/-}$)(ref. 13) and TCR-β x $\delta^{-/-}$ mice[14,15], to clarify the relative importance of particular immune pathways involved in the immunogenicity and control of each strain. We show that *S. typhimurium* Aro$^-$ and PhoP$^-$ mutant strains promote different immune responses in the host and are not equally susceptible to a variety of host defenses. These results demonstrate that virulence genes in *Salmonella* can be genetically modified to regulate immune responses.

IFN-γ and T cells influence the virulence of *Salmonella* mutants

An Aro$^-$ mutant of *S. typhimurium* was highly virulent in IFN-$\gamma^{-/-}$ mice after oral inoculation with 5×10^9 colony-forming units (CFU), whereas normal mice tolerated this dose (Fig. 1*a* and *d*). Other avirulent mutant strains that were lethal in IFN-$\gamma^{-/-}$ mice included a PhoP-constitutive (PhoP^c) mutant, with a single amino-acid substitution in the *phoQ* gene[16], and mutants blocked in adenylate cyclase synthesis and the cyclic AMP receptor protein[17] (*cya*$^-$*crp*$^-$)and heat-shock proteins[10] (*htrA*$^-$)(data not shown). These results indicate a more general requirement for IFN-γ in host resistance to a wide array of attenuated *S. typhimurium* strains[4,5].

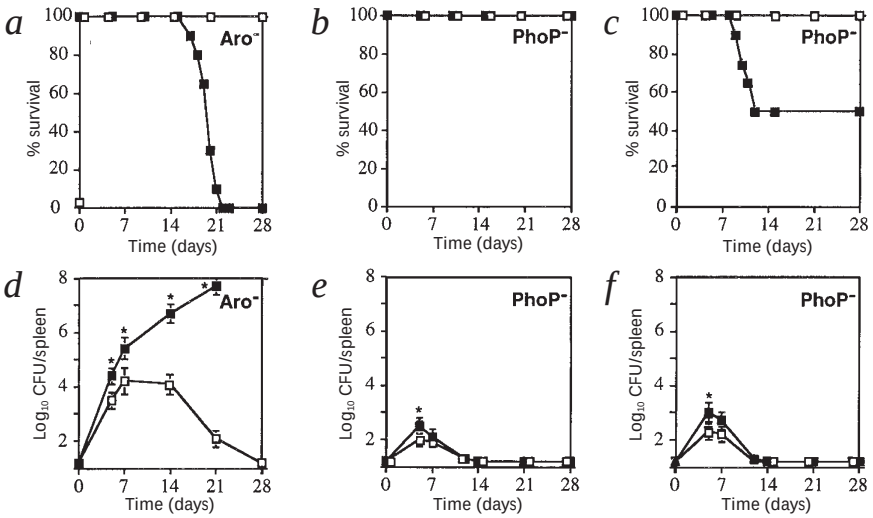

Fig. 1 Susceptibilities of normal and IFN-$\gamma^{-/-}$ mice to infection with Aro⁻and PhoP⁻ *S. typhimurium*. IFN-$\gamma^{+/+}$ (□) and IFN-$\gamma^{-/-}$ (■) C57BL/6 mice were orally inoculated (5×10^9 CFU per mouse) with the Aro⁻ strain (***a*** and ***d***) and the PhoP⁻ strain (***b*** and ***e***). Other IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ mice received 1.2×10^{10} CFU of the PhoP⁻ strain (***c*** and ***f***). The liver, Peyer's patches and spleen showed similar growth kinetics except that the PhoP⁻ strain was occasionally not recovered from spleens and livers of IFN-$\gamma^{+/+}$ mice at the detection level of 50 CFU; only results from the spleen are shown. Data are from twenty mice per group for survival curves (*a, b* and *c*) and each point in the growth curves (*d, e* and *f*) represents the mean ± s.d. of five mice. The Aro⁻ strain was consistently less virulent than wild-type bacteria; a dose of 5×10^9 CFU of wild-type strain was lethal in less than 7 days in IFN-$\gamma^{+/+}$ mice and less than 2 days in IFN-$\gamma^{-/-}$ mice. *, $P < 0.01$ compared with IFN-$\gamma^{+/+}$ or IFN-$\gamma^{-/-}$ mice.

In contrast, a PhoP⁻ mutant strain showed significant attenuation in IFN-$\gamma^{-/-}$ mice (Fig. 1*b* and *e*). In the absence of IFN-γ, bacterial burdens were increased by fivefold on day 5, but clearance was achieved by day 14 (Fig. 1*e*). Thus, IFN-γ influenced the *in vivo* growth kinetics of both the Aro⁻ and PhoP⁻ strains of *Salmonella*, but was only required for clearance of the Aro⁻ strain (Fig. 1*d* and *e*). When inoculating with a 50% lethal oral dose (LOD_{50}; 1.2×10^{10} CFU) of the PhoP⁻ strain, bacterial clearance was achieved before IFN-$\gamma^{-/-}$ mice died (Fig. 1*c* and *f*). Additionally, in TCR-β x $\delta^{-/-}$ mice, which lack functional αβ and γδ T cells, only the Aro⁻ strain was lethal. The *in vivo* growth kinetics and timing of the clearance of the PhoP⁻ strain were similar in normal and TCR-β x $\delta^{-/-}$ mice (data not shown), whereas the Aro⁻ strain was lethal in TCR-β x $\delta^{-/-}$ mice after 60 days, a result consistent with previous reports[5,6]. These results indicated that different immune mechanisms were mediating control of the growth and lethal effects of the two strains within the host.

Effects of Aro⁻ and PhoP⁻ mutant strains on specific immunity

To determine whether the PhoP⁻ and Aro⁻ mutant strains stimulated different host defense mechanisms, we initially characterized the Th phenotype that developed in normal and IFN-$\gamma^{-/-}$ mice. Five days after oral inoculation with 5×10^9 CFU of each strain, $CD4^+$ T cells were isolated from splenocyte and Peyer's patch populations for analysis of IFN-γ and IL-4 mRNA expression. The Aro⁻ strain stimulated more Th1-type IFN-γ expression than did the PhoP⁻ strain in normal mice (Fig. 2*a*), and only the Aro⁻ strain stimulated a shift to Th2-type IL-4 expression in IFN-$\gamma^{-/-}$ mice (Fig. 2*b*). Decreasing the dose of the Aro⁻ strain or increasing the dose of the PhoP⁻ strain to produce similar bacterial loads in either normal or IFN-$\gamma^{-/-}$ mice failed to significantly alter the differential abilities of the two strains to induce Th1/Th2 responses (data not shown).

To demonstrate the consequence of Th1- and Th2-cells on serum antibody responses, we measured *Salmonella*-specific IgE, IgG, and IgG subclass responses. In mice, IFN-γ and IL-4 provide essential signals for antibody isotype switching to IgG2a and IgG3 or to IgE and IgG1, respectively[18]. Total IgE levels (Fig. 2*c*), *Salmonella*-specific IgE titers (Fig. 2*d*) and IgG1-to-IgG2a ratios (Fig. 2*e*) were increased in the sera of IFN-$\gamma^{-/-}$ mice given the Aro⁻ strain, whereas no such increases were evident in response to the PhoP⁻ strain. Moreover, the Aro⁻ strain elicited higher *Salmonella*-specific IgG titers in normal and IFN-$\gamma^{-/-}$ mice than did the PhoP⁻ strain (Fig. 2*f*).

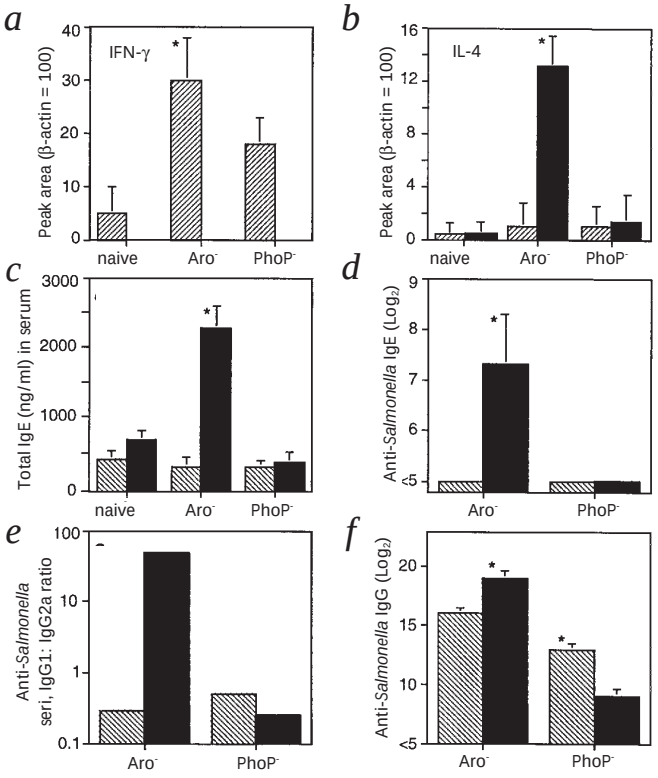

Fig. 2 Th1- and Th2-type cytokine profiles and antibody responses in normal and IFN-$\gamma^{-/-}$ mice inoculated with Aro⁻and PhoP⁻ strains of *S. typhimurium* or uninoculated (naive). IFN-γ (***a***) and IL-4 (***b***) mRNA expression in $CD4^+$ T cells from the spleens of IFN-$\gamma^{+/+}$ (□) and IFN-$\gamma^{-/-}$ (■) mice 5 days after oral inoculation with 5 x 10^9 CFU of bacteria. Total IgE levels (***c***), *Salmonella*-specific IgE titers (***d***), *Salmonella*-specific IgG1-to-IgG2a ratios (***e***) and *Salmonella*-specific IgG titers (***f***) in serum samples. Data are expressed as the mean peak area ± s.d. (*a* and *b*), $\log_2$ endpoint titer ± SD (*d* and *f*) and serum IgE concentration (ng/ml) ± s.d. (*c*); and the IgG1:IgG2a ratio of endpoint titers (*e*) from three experiments with five mice per group. *, $P < 0.01$ compared with IFN-$\gamma^{+/+}$ or IFN-$\gamma^{-/-}$ mice.

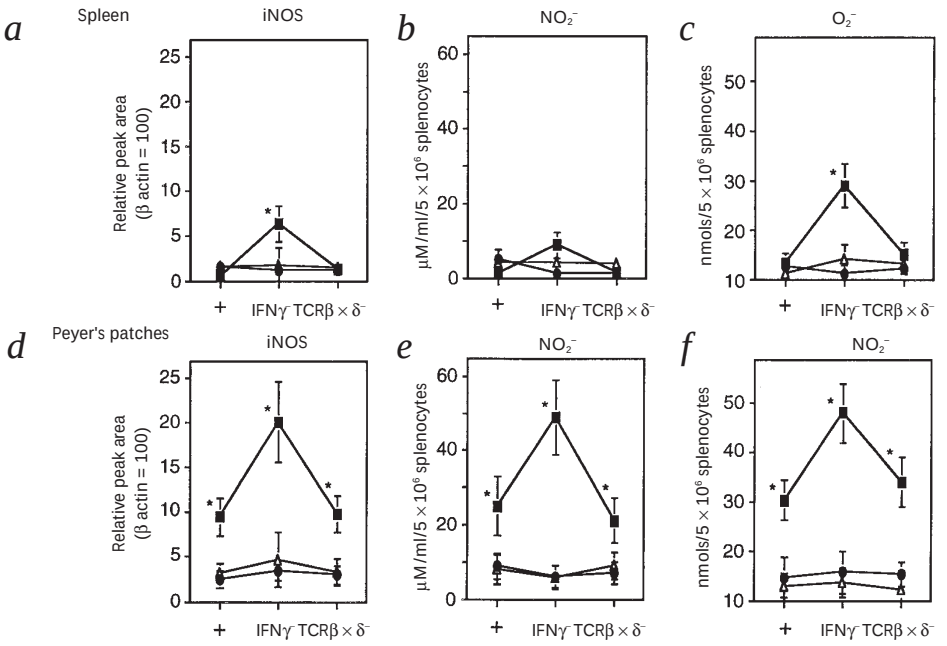

Fig. 3 Effect of *S. typhimurium* mutant strains and wild-type bacteria on antimicrobial production in IFN-$\gamma^{+/+}$, IFN-$\gamma^{-/-}$ and TCR-β x $\delta^{-/-}$ mice. Mice were orally inoculated with the Aro$^-$ (●), PhoP$^-$ (■) and wild-type (△) strains, and samples were collected and analyzed 5 days later. Inducible NOS mRNA expression in freshly isolated macrophages (Mac-1$^+$ CD4$^-$) from spleens (***a***) and Peyer's patches (***d***). NO_2^- and O_2^- release in supernatants from splenic (***b*** and ***c***) and Peyer's patch (***e*** and ***f***) cultures. A dose of 5×10^9 CFU was used for the Aro$^-$ and PhoP$^-$ strains, whereas a lower dose of wild-type bacteria (10^6 CFU) was used to produce similar bacterial loads in organs of mice infected with the Aro$^-$ strain and wild-type strain at day 5. The data represent five experiments with five mice per group (mean ± s.d.). *, $P < 0.01$ compared with IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ mouse groups.

Effects of the Aro$^-$ and PhoP$^-$ mutant strains on innate immunity

Macrophages contribute to the strong innate immune response during salmonellosis by producing antimicrobial agents such as reactive nitrogen and oxygen intermediates[19–21]. Therefore, we investigated whether the two mutant strains had different effects on innate immune responses of macrophages. Measurement of nitric oxide (NO_2^-) and O_2^- release and the inducible form of nitric oxide (NO) synthase (iNOS) mRNA, which catalyzes the release of NO_2^-, in macrophages from spleens and Peyer's patches showed that the PhoP$^-$ strain was a more effective inducer of each in comparison with the Aro$^-$ and wild-type *Salmonella* strains (Fig 3). The PhoP$^-$ strain had particularly profound effects on macrophages from Peyer's patches and stimulated high levels of iNOS/NO_2^- and O_2^- in the absence of either T cells or IFN-γ. In contrast, production of antimicrobial factors in splenic macrophages from normal and TCR-β x $\delta^{-/-}$ mice in response to the PhoP$^-$ strain was low, possibly because the PhoP$^-$ mutant organisms were marginally detectable in the spleens of these mice. Relatively low expression of iNOS occurred after inoculation of the Aro$^-$ and wild-type strains at both low and high doses, indicating that the high bacterial loads failed to impede this response (data not shown). Normalization of the data to bacterial burdens showed that the PhoP$^-$ strain was more than 300-fold more efficient in stimulating antimicrobial agents in spleens and Peyer's patches than was the Aro$^-$ strain. For example, the Aro$^-$ strain elicited 0.017 μM NO_2^-/ml and the PhoP$^-$ strain elicited 5 μM NO_2^-/ml of culture supernatant per 100 CFU in Peyer's patches from normal mice. Although the Aro$^-$ strain was a poor inducer of iNOS/O_2^-, it elicited higher levels relative to bacterial loads in IFN-$\gamma^{+/+}$ (normal) mice and TCR-β x $\delta^{-/-}$ mice, in comparison with IFN-$\gamma^{-/-}$ mice, indicating that IFN-γ has an important role in triggering optimal iNOS/O_2^- production early after inoculation with the Aro$^-$ strain.

Innate immunity to PhoP$^-$ LPS is CD14-dependent

Stimulation of macrophages through a CD14-dependent pathway initiates events leading to the release of antimicrobial agents and pro-inflammatory cytokines[22]. The CD14-dependent pathway may be an essential mechanism through which the PhoP$^-$ strain activated macrophages even in the absence of IFN-γ or T cells. To investigate this, we added live *S. typhimurium* strains to cultures of splenocytes from naive IFN-$\gamma^{+/+}$, IFN-$\gamma^{-/-}$ and TCR-β x $\delta^{-/-}$ mice in the presence or absence of anti-CD14 receptor antibody (5 μg/ml). Without anti-CD14 blockade, the Aro$^-$ and wild-type strains were weak inducers of NO_2^- (4–6 μM NO_2^-/ml per 5×10^6 splenocytes) in each mouse strain, whereas the PhoP$^-$ strain was a potent inducer in all cases (25–35 μM NO_2^-/ml per 5×10^6 splenocytes). NO_2^- release by splenocytes after stimulation with PhoP$^-$ bacteria was abrogated by blockade of the CD14 receptor.

The central role of the CD14 receptor in NO_2^- release indicated that PhoP$^-$ LPS, which contains an altered lipid A structure when compared with wild-type bacteria[23], may partially account for the increased NO_2^- response to the PhoP$^-$ strain. LPS was purified from wild-type bacteria and Aro$^-$ and PhoP$^-$ mutant strains and added (10 ng/ml) to IFN-$\gamma^{+/+}$ splenocyte cultures (5×10^6 cells/ml). After 24 hours, supernatants from splenocytes from PhoP$^-$ LPS-stimulated cultures had 42–65 μM NO_2^-/ml, whereas supernatants from Aro$^-$ LPS- and wild-type LPS-stimulated cultures had 5–10 μM NO_2^-/ml.

Different immune mechanisms control *Salmonella* mutants

The above results showed that early control and subsequent clearance of the Aro$^-$ strain required IFN-γ and Th1 cells. To investigate the *in vivo* mechanism responsible for control of the PhoP$^-$ strain, we treated IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ mice with inhibitors of iNOS (L-N^6-(1-Iminoethyl)-lysine (L-NIL)) and O_2^- (allopurinol) and with antibody against TNF-α. Inhibition of either iNOS or TNF-α had little effect on the *in vivo* growth of the PhoP$^-$ mutant strain, whereas its virulence was significantly enhanced by inhibition of O_2^- (Fig. 4*a* and *b*). Conversely, inhibition of iNOS and O_2^- had little effect on Aro$^-$ virulence, whereas recombinant TNF-α reduced and anti-TNF-α antibody increased Aro$^-$ virulence (Fig. 4*a* and *b*). Mouse strains with the Ity^r genotype have increased iNOS activity and increased resis-

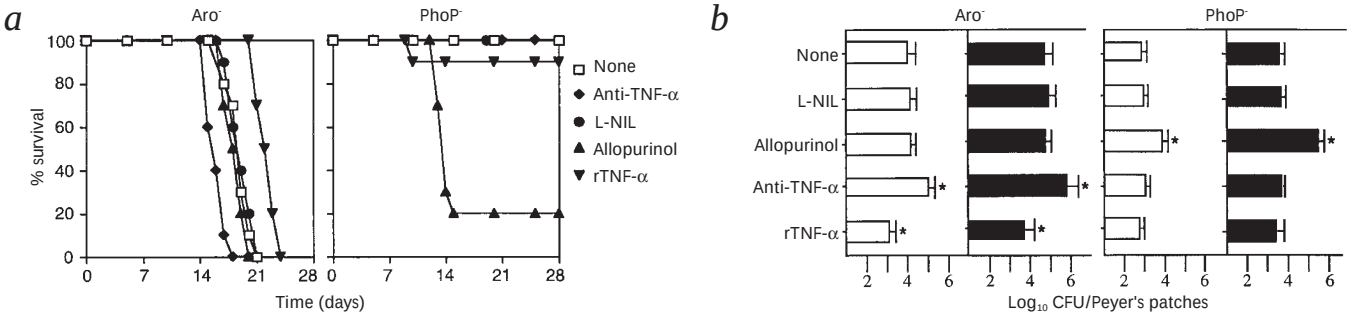

Fig. 4 Effects of L-NIL, allopurinol, TNF-α neutralization and recombinant TNF-α on the virulence of Aro⁻ and PhoP⁻ *S. typhimurium* in mice. Survival rate of IFN-$\gamma^{-/-}$ mice (***a***) and growth of the Aro⁻and PhoP⁻strains in Peyer's patches of IFN-$\gamma^{+/+}$ (□)and IFN-$\gamma^{-/-}$ (■)mice (***b***) after oral inoculation with 5×10^9 CFU. The survival rate of IFN-$\gamma^{+/+}$ mice was 100% for all conditions (data not shown in *a*). No mortality occurred in control mice receiving L-NIL, allopurinol, anti-TNF-α antibody or rTNF-α alone. The data represent the mean ± s.d. of five (*a*) and ten (*b*) mice per group. *, $P < 0.01$ compared with control group of mice (None), infected but without treatment.

tance to *Salmonella* infection[24,25], supporting the hypothesis that the relative impact of NO and possibly other antimicrobial factors on *Salmonella* virulence may differ substantially among mouse strains. To investigate this, we inoculated groups of 10 C3H/HeN mice (Ity^r) with 5 x 10^9 CFU of the Aro⁻ strain or the PhoP⁻ strain and provided L-NIL (4.5 mM) in their drinking water. All of the mice survived the infection. However, on day 14, bacterial loads in the organs of mice treated with L-NIL and infected with the Aro⁻ strain were elevated 10-fold compared with those in the untreated control C3H/HeN mice ($P < 0.01$). Bacterial loads in C3H/HeN mice infected with the PhoP⁻ strain and given L-NIL, however, were increased by less than twofold compared with untreated controls. Thus, inhibition of iNOS production in C3H/HeN mice by L-NIL treatment increased the virulence of the Aro⁻ strain, but had little effect on the growth of the PhoP⁻ strain.

The toxic and immunosuppressive effects of the PhoP⁻ strain

We next studied whether NO or inflammatory cytokines (endogenous effector molecules of LPS-induced endotoxic shock[26,27]) could account for the lethal effects of the PhoP⁻ strain in IFN-$\gamma^{-/-}$ mice (Fig. 1*c*). The PhoP⁻ strain stimulated more TNF-α, IL-1β and serum amyloid P (SAP) in sera from IFN-$\gamma^{-/-}$ mice than did the Aro⁻ strain (Fig. 5). Therefore, to determine whether these molecules had detrimental effects in PhoP⁻-infected mice, we treated IFN-$\gamma^{-/-}$ mice with neutralizing antibody to either IL-1β or TNF-α and inoculated them with the LOD_{50} of the PhoP⁻ strain. By day 14 after inoculation **[AUTHOR: OK?]**, 4 of 10 mice in the untreated group had died, whereas 0 of 10 and 1 of 10 mice treated with anti-IL-1β antibody and anti-TNF-α antibody, respectively, died; treatment with L-NIL failed to protect IFN-$\gamma^{-/-}$ mice from the lethal effects of the PhoP⁻ strain.

NO has been shown to impart immunosuppressive effects during murine salmonellosis[28,29]. To determine whether there was an inverse relationship between NO and specific immune responses, we measured *Salmonella*-specific IgG responses in normal and IFN-$\gamma^{-/-}$ mice treated with L-NIL. By day 12, serum IgG titers were increased by sixfold ($P < 0.01$) in PhoP-infected IFN-$\gamma^{-/-}$ mice, and by threefold in PhoP⁻-infected normal mice treated with L-NIL, whereas IgG titers were not increased further in mice inoculated with the Aro⁻ strain (data not shown). These results indicated that NO was partially responsible for the reduced *Salmonella*-specific immune responses early after inoculation with the PhoP⁻ strain.

Discussion

We report here that Aro⁻ and PhoP⁻ mutant strains of *Salmonella* invoked different immune responses in mice. We also found that IFN-γ and T cells were essential for distinguishing the immune mechanisms elicited to the two mutant strains *in vivo*. The PhoP⁻ strain stimulated the innate immune responses of macrophages with greater efficiency than did the Aro⁻ strain, whereas the Aro⁻ strain promoted the development of higher immune responses of B cells and Th cells. In the absence of IFN-γ, infection with the PhoP⁻ strain led to higher bacterial loads in systemic tissues and increased levels of toxic molecules in the circulation, such as NO_2^-, IL-1β and SAP. However, in the presence of IFN-γ, the PhoP⁻ strain was less able to reach systemic tissues in normal mice. Consequently, in the presence of IFN-γ, the PhoP⁻ strain had more profound effects on local macrophage activity, resulting in limited pro-inflammatory and immunosuppressive effects in the systemic compartment.

During infection with the Aro⁻ strain, IFN-γ was necessary to trigger antimicrobial activity of macrophages, essential for early bacterial control. In the absence of IFN-γ, the Aro⁻ strain stimulated strong Th2-type and IgG1/IgE responses but macrophage activity was negligible; the Aro⁻ strain also showed uncontrolled growth, demonstrating that Th2-type responses were inadequate to effect immunity to this strain. Although *in vivo* growth

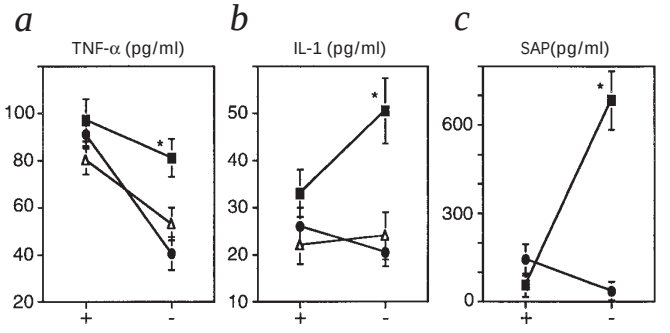

Fig. 5 Effect of *S. typhimurium* mutant strains and wild-type bacteria on inflammatory responses in normal and IFN-$\gamma^{-/-}$ mice. Mice were orally inoculated with the Aro⁻ (●), PhoP⁻ (■) and wild-type (△) strains, and samples were collected and analyzed 5 days later. Serum levels of TNF-α (***a***) IL-1β (***b***) and SAP (***c***). The data represent five experiments with five mice per group (mean ± s.d.). *, $P < 0.01$ compared with the other *Salmonella*-infected IFN-$\gamma^{-/-}$ mouse groups.

of the Aro⁻ strain was controlled by mechanisms dependent on IFN-γ in the absence of T cells for up to 60 days, Th1 cells were subsequently needed for clearance. In contrast, the PhoP⁻ strain stimulated potent antimicrobial macrophage activity whether or not T cells or IFN-γ were available. Moreover, in the absence of T cells or IFN-γ and combined with the extreme sensitivity of the PhoP⁻ mutant strain to the respiratory burst (O_2^-), innate immune mechanisms were sufficient for its control. Other host responses, such as defensins[30–32] or the host environmental milieu (for example, low magnesium concentrations[33]), may have also contributed to the control of the PhoP⁻ strain. Thus, defined mutations in virulence genes can have profound effects on *Salmonella* immunogenicity within the host and the resulting effector responses.

To determine whether the differences in the immune responses to the PhoP⁻ and Aro⁻ strains were attributable to differences in overall bacterial loads, we analyzed the ability of lower doses of the Aro⁻ strain or higher doses of the PhoP⁻ strain to elicit specific and innate immune responses. These experiments confirmed a greater ability of the PhoP⁻ strain to elicit NO_2^- and O_2^- in both normal and IFN-$\gamma^{-/-}$ mice and the Aro⁻ strain to stimulate Th and antibody responses. Moreover, analysis of similar amounts of LPS from PhoP⁻, Aro⁻ and wildtype bacteria showed that the PhoP⁻ LPS induced more NO_2^- *in vitro*. These results were consistent with a previous report showing that PhoP⁻ LPS stimulated more endothelial E-selectin expression and more monocyte TNF-α expression *in vitro* than wild-type LPS[23]. Similarly, LPS from spontaneous variants of *Francisella tularenis* induced different amounts of NO_2^- in rat macrophages[34]. Our observations show that mutant strains of bacteria may be generated that induce qualitatively distinct immune responses *in vivo*.

Intraperitoneal injection of *S. typhimurium* Aro⁻ mutant resulted in more NO production and immunosuppression in spleens from C3HeB/FeJ mice (Ity^r) than did the same dose of a PhoP⁻ mutant[35]. Therefore, the higher NO production in response to the Aro⁻ strain may have reflected the overwhelmingly higher number of Aro⁻ organisms (as many as 10^6 CFU) in comparison with the number of PhoP⁻ organisms (fewer than 250 CFU) in spleens of these mice[35]. Thus, when NO production was normalized to bacterial loads in the present study, we showed that the PhoP⁻ strain was 300-fold more efficient than the Aro⁻ strain in stimulating splenic NO_2^- release. Moreover, the PhoP⁻ strain stimulated more total NO_2^- in local Peyer's patches than did the Aro-strain.

Our observations demonstrate a delicate balance between *Salmonella* virulence/attenuation and inflammation within the host. Defined alterations of *phoP–phoQ* virulence genes and in genes involved in aromatic biosynthesis resulted in distinct effects on attenuation and on both specific and innate immune responses in the host. IFN-γ was not required for clearance of the PhoP⁻ strain, whereas alteration in the PhoP–PhoQ regulon, yielding the PhoP^c strain, resulted in IFN-γ-dependent clearance. The ability of the PhoP⁻ strain to elicit high levels of reactive nitrogen and oxygen intermediates is relevant to possible anti-neoplastic therapeutic application of salmonellae[36]. Thus, by manipulating virulence genes, bacterial mutants could be produced that specifically modulate immune mechanisms within the host (that is, pathways independent of T cells involving CD14 and iNOS/O_2^-, or Th1-dependent pathways). It remains important to continue to identify virulent genes in this manner to enable selection of new attenuation strategies for defined medical purposes.

Methods

Mice. IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice of a C57BL/6 background and C3H/HeN mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). TCR-β x $\gamma^{-/-}$ mice of a C57BL/6 background were supplied by S. Tonegawa and K. Fujihashi[14,15]. All mice were provided sterile food and water *ad libitum* and maintained in pathogen-free conditions in animal facilities of the UAB Immunobiology Vaccine Center. Animal care was in accordance with institutional guidelines.

Reagents and *in vivo* treatments. Anti-CD14 receptor antibody (rmC5-3; Zymed, San Diego, California) was added to splenic and Peyer's patch cultures (5×10^6 cells/ml) at 5 μg/ml. NO_2^- and O_2^- in 24-hour culture supernatants were assayed by the Griess method to measure nitrite generated from NO_2^- (ref. 37) and by reduction of ferricytochrome C to measure superoxide production[38]. L-NIL (Alexis, San Diego, California) was added to the drinking water (4.5 mM) over a 4-week period. Allopurinol (400 μg/mouse per day; Sigma) in 200 μl of 0.25 mM NaOH was administered by oral intubation on days 1–5 and 8–12; higher or additional doses were toxic (based on the appearance of ruffled fur, weight loss and lethargy in mice). An intraperitoneal dose of 2×10^4 U/mouse of rabbit anti-mouse TNF-α (IP-400; Genzyme, Cambridge, Massachusetts) in 200 μl of PBS was administered at the time of *Salmonella* administration and repeated 2 and 14 days later. Treatment with recombinant TNF-α (TNF-M; Genzyme, Cambridge, Massachusetts) was achieved by intraperitoneal injection with 1 μg in 100 μl of PBS at the time of inoculation and again 14 days later. Treatment with IL-1β was accomplished by intraperitoneal injection with 600 μg/mouse on day 0 and again on day 14 (1997-01, Genzyme, Cambridge, Massachusetts). LPS was purified from the Aro⁻ and PhoP⁻ strains and wild-type bacteria as described[39]. The number of bacteria present in spleens of mice were determined by viable count of organ homogenates on LB agar[40].

***Salmonella* strains**. The three attenuated *S. typhimurium* strains used in this work were derived from wild-type strain ATCC14028. The PhoP⁻ strain (LH430) has a 956-bp deletion in the contiguous *phoP-phoQ* genes; this in-frame deletion is identical to that in *S. typhi* Ty800 (ref. 12). The AroA⁻ strain (SL7731) has *aro*A Δ407 mobilized through P22HT from SL7728 using linkage of the transposon insertion *serC* 1121::Tn10 by transduction; this *aroA*⁻ deletion allele is identical to that in *S. typhimurium* SL3261, the strain most extensively evaluted in mice[7]. The PhoP^c strain (CS022) has *pho*-24 mobilized through P22HT from strain TA2367 (ref. 16). Other attenuated *S. typhimurium* strains studied included an *htrA*⁻ mutant (BRD726) derived from wild-type strain SL1344 (ref. 10) and a *cya*⁻ *crp*⁻ mutant (χ4062) derived from wild-type strain SR-11 (ref. 17).

Antibody analysis. Total IgE levels in sera were determined by ELISA. For this assay, Falcon MicroTest III plates (Becton Dickinson, San Jose, California) were coated with 2 μg/ml of rat monoclonal anti-mouse IgE antibody (R35-72; PharMingen, San Diego, California). Serial dilutions of standard mouse IgE (PharMingen, San Diego, California) were then added followed by addition of 100 μl of a biotinylated rat monoclonal anti-mouse IgE antibody (R35-92; PharMingen, San Diego, California). Peroxidase-labeled streptavidin was used for detection and plates were then developed with the chromogenic substrate, ABTS with H_2O_2 (Moss, Pasadena, Maryland). Antigen-specific IgE titers in sera were determined by endpoint ELISA using biotinylated monclonal antibodies specific for IgE (R35-92) and streptavidin-conjugated, polyadenylated peroxidase-80 (1:2000 dilution; Research Diagnostics, Flanders, New Jersey). The coating antigen was heat-killed preparations of *S. typhimurium*. *Salmonella*-specific IgG1 and IgG2a antibody titers in sera were determined by endpoint ELISA (ref. 40). Biotinylated monoclonal antibodies specific for IgG1 and IgG2a and streptavidin-conjugated peroxidase were employed (PharMingen, San Diego, California).

Th1/Th2 analysis. CD4⁺ Mac-1⁻ T cells and CD4⁻ Mac-1⁺ spleen and Peyer's patch cells were purified by flow cytometry using FITC-conjugated anti-L3T4 monclonal antibody (GK 1.5) and biotinylated anti-CD11b (Mac-1) antibodies and PE-conjugated streptavidin[40] (PharMingen, San Diego, California). Total RNA was isolated from CD4⁺ T cells and Mac-1⁺ cells with

Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) and reverse-transcribed with Superscript RT (Promega) according to standard protocols. The resulting cDNA was subjected to PCR amplification using IFN-γ, IL-4 and iNOS-specific primers[41,42]. Levels of amplified cDNA were determined by capillary electrophoresis[41] (P/ACE 5000 Beckman Instruments, Fullerton, California). All cytokine and iNOS values were normalized to the corresponding β-actin values[41].

Pro-inflammatory cytokines. Serum levels of TNF-α and IL-1β were determined by ELISA using Quantikine™ M kits from R&D systems (Minneapolis, Minnesota). SAP in serum was also assayed by ELISA[43].

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