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CD154 Is Essential for Protective Immunity in Experimental Salmonella Infection: Evidence for a Dual Role in Innate and Adaptive Immune Responses

Basel K. al-Ramadi,* Maria J. Fernandez-Cabezudo,† Azim Ullah,* Hussain El-Hasasna,* and Richard A. Flavell†

CD40-CD154 interactions are of central importance in the induction of humoral and cellular immune responses. In the present study, CD154-deficient (CD154−/−) mice were used to assess the role of CD40-CD154 interactions in regulating the immune response to a systemic Salmonella infection. Compared with C57BL/6 (CD154+/+) controls, CD154−/− mice were hypersusceptible to infection by an attenuated strain of Salmonella enterica serovar Typhimurium (S. typhimurium), as evidenced by decreased survival rate and mean time to death, which correlated with increased bacterial burden and persistence in target organs. CD154−/− mice exhibited a defect both in the production of IL-12, IFN-γ, and NO during the acute phase of the disease and in the generation of Salmonella-specific Ab responses and Ig isotype switching. Furthermore, when CD154−/− animals were administered a subdotal dose of attenuated S. typhimurium and subsequently challenged with a virulent homologous strain, all mice succumbed to an overwhelming infection. Similar treatment of CD154+/+ mice consistently resulted in ≥90% protection. The lack of protective immunity in CD154−/− mice correlated with a decreased T cell recall response to Salmonella Ags. Significant protection against virulent challenge was conferred to presensitized CD154−/− mice by transfer of serum or T cells from immunized CD154+/+ mice. For best protection, however, a combination of immune serum and T cells was required. We conclude that intercellular communications via the CD40-CD154 pathway play a critical role in the induction of type 1 cytokine responses, memory T cell generation, Ab formation, and protection against primary as well as secondary Salmonella infections. The Journal of Immunology, 2006, 176: 496–506.

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1 Address correspondence and reprint requests to Dr. Basel K. al-Ramadi, Department of Medical Microbiology and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates. E-mail address: ramadi.b@uae.ac.ae

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CD40-CD154-mediated intercellular collaboration would be critical for immunity against infection. In one published study, the administration of a CD40 agonist led to a small enhancement in resistance to virulent infection in normal BALB/c mice (16). Conversely, treatment with an antagonist Ab to CD154 resulted in ~25% reduction in mean survival after *Salmonella* infection. Moreover, the same study purported that CD154-deficient (CD154−/−) mice exhibited no increased susceptibility to *Salmonella* infection. Given the aforementioned role of the CD40-CD154 pathway in the control of humoral and cellular immune responses, the findings regarding the *Salmonella* model were surprising. We reasoned that these largely modest effects of the CD40-CD154 pathway on *Salmonella* resistance might have been a consequence of using a highly virulent strain of *Salmonella dublin* in that study, which may have precluded any demonstration of increased susceptibility by the CD154−/− mice. In the present study we have re-examined the role of CD40-CD154 interactions in *Salmonella* infections by comparing the immune responsiveness of CD154−/− and that of normal C57BL/6 (CD154+/+) mice to strains of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) of varying virulence. Our data clearly demonstrate that CD154−/− mice are hypersusceptible to both primary as well as secondary (challenge) *Salmonella* infections. The increased susceptibility correlates with the failure to mount an adequate Th1-type immune response as well as a protective neutralizing IgG humoral response. We conclude that intercellular communications via the CD40-CD154 pathway play a critical role in protection against experimental *Salmonella* infections.

**Materials and Methods**

**Mice**

CD154−/− mice on the C57BL/6 background were derived from a colony generated and described previously (17). They were bred at our animal facility and maintained in filter-topped isolator cages on Lagatrim-supplemented water (Lagap Pharmaceuticals). Control C57BL/6 mice were obtained from the same colony from which the CD154−/− mice were derived and are designated throughout as CD154+/+ mice. All animals were used at 8–12 wk of age. All studies involving animals were conducted in accordance with and after approval of the animal research ethics committee of the Faculty of Medicine and Health Sciences, United Arab Emirates University.

**Bacterial strains and growth conditions**

The characteristics of the bacterial strains have been previously described (18). BRD509 is an attenuated *aroA*/*aroD* mutant strain of *S. enterica* serovar Typhimurium, and SL1344 is the parental wild-type strain from which BRD509 was originally derived (19, 20). The LD50 in C57BL/6 mice of BRD509 given i.p. is >5–10^6 CFU; in contrast, the LD50 of SL1344 is <100 CFU (our unpublished observations). Log-phase bacterial suspensions were prepared in pyrogen-free saline and injected i.p. in a 0.5-ml volume. For all experiments, bacterial doses were confirmed by CFU plate counts.

**Enumeration of bacteria in liver and spleen homogenates**

To determine the bacterial load in liver and spleen, organs were removed aseptically and homogenized in 3–5 ml of cold sterile water, as previously described (18). A 100-μl aliquot of the homogenate or an appropriate dilution was plated on T-soy agar plates, and viable CFU were determined after overnight incubation. Duplicate plates were set up for each dilution or experimental group.

**Cell preparation**

Erythrocyte-depleted spleen single-cell suspensions were prepared as previously described (21) and suspended in RPMI 1640 supplemented with 5% FCS, l-glutamine, sodium pyruvate, essential amino acids, nonessential amino acids, penicillin/streptomycin, gentamicin, and 2-ME (all reagents from Invitrogen Life Technologies). Cells were cultured at a concentration of 2.5–10^6 cells/ml in 24-well plates with or without 50 μl of *Salmonella* sonicate, prepared as previously described (22), at the indicated final concentrations. Cells were incubated for 24–48 h at 37°C with 5% CO2. Culture supernatants were collected, spun free of any cells, and kept at −20°C until assayed for cytokines and nitrite content.

**Flow cytometric analysis**

Spleen cell suspensions were prepared from animals infected i.p. 90 days previously with a dose of 0.6 × 10^6 BRD509/mouse. Spreading of spleen cells for FACS analysis was conducted as described previously (18). Briefly, washed cells were resuspended in staining buffer (PBS/1% FCS/0.1% NaN3) to a concentration of 1 × 10^7/ml. Aliquots of 100 μl were dispensed into wells of a round-bottom, 96-well plate and incubated with a CD16/CD32-specific mAb (clone 2.4G2) for 30 min on ice to block FcγRIII/Iib sites. Cells were then double stained, according to the following combinations, with a pair of directly conjugated mAbs specific to TCR β-chain and B220, CD4 and CD8, and F4/80 and Gr-1 (all purchased from eBioscience). All Abs were pretitrated in preliminary experiments and used at saturating concentrations. Cell staining was performed for 30 min on ice, and washed cells were analyzed on a FACScan (BD Biosciences). Data collected on 20,000 cells were analyzed using CellQuest software (BD Biosciences).

**Cytokine analysis**

Production of IFN-γ was quantitated in culture medium after in vitro culture of spleen cells for 48 h. IFN-γ content was determined using a Duoset ELISA development kit from R&D Systems according to the manufacturer’s instructions. Serum IL-12p40 and TNF-α cytokines were also detected using monospecific Duoset ELISA development kits from R&D Systems. The limit of detection for all ELISAs was ~30 pg/ml.

**NO determination**

Accumulation of NO2− was used to determine the production of NO according to the Griess method, as described previously (18). Briefly, 100 μl of cell-free culture supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 10–15 min. The absorbance at 562 nm was measured in an automated microplate reader. The nitrite concentration was quantitated using NaN2 as standard and was expressed as micromolar concentrations of NO2− per 5 × 10^6 spleen cells.

**Measurement of Salmonella-specific Abs in serum**

Serum samples were obtained from *Salmonella*-infected or saline-treated mice at 7, 14, and 21 days after inoculation. The presence of Salmonella-specific Abs of various IgM and IgG3 isotypes was determined by ELISA. Maxisorb microplates (Nunc) were coated with a preparation of heat-killed BRD509 organisms overnight. The appropriate concentration (~1 × 10^7/ml) of heat-killed BRD509 was determined empirically in preliminary experiments. After coating, blocking buffer (PBS/1% BSA/5% sucrose/0.05% NaN3) was added to each well and incubated for 1 h at room temperature. Serum samples from individual animals were then serially diluted in the plate (range, 1/50 to 1/6400 dilution) and incubated for 2 h at room temperature. Titrations were detected using biotin-conjugated, mouse isotype-specific Abs (Serotec), followed by streptavidin-HRP. Wells were finally developed using 3,3′,5,5′-tetramethylbenzidine as a substrate and were read at 450 nm using a TECAN microplate reader.

**In vivo protection studies**

These were performed to assess the capacity of the attenuated BRD509 strain to protect CD154−/− and CD154+/+ mice against virulent challenge with SL1344. Mice (5–10 mice/group) were immunized i.p. with a subthal dose of BRD509 organisms (range, 0.2–0.5 × 10^6/mouse) and were challenged with 2–5 × 10^7 organisms of the virulent SL1344 strain 6–8 wk later. In some experiments the virulent challenge with SL1344 was conducted 16 wk after immunization. Survival of the animals was recorded for up to 60 days after challenge.

**In vitro recall immune responses**

Immune T lymphocytes were purified on enrichment columns (R&D Systems) from pooled spleen cells of mice (five mice per experimental group) immunized 60 days previously with a single i.p. injection of 0.5 × 10^6 BRD509 strain organisms/mouse. The resulting population consisted of >90% T cells by flow cytometric analysis (data not shown). For in vitro restimulation cultures, purified T cells (1.5 × 10^6 cells/ml) were added to 96-well plates (Nunc) and stimulated with varying concentrations of a sonicated *Salmonella* preparation, as indicated in the figures. Irradiated spleen cells (7.5 × 10^6/ml) were used as APCs. Cell-free culture supernatants...
were collected at 24 and 48 h for IL-2 and IFN-γ determinations, respectively. All cytokine analysis was conducted using specific ELISA kits from R&D Systems.

**Passive and adoptive transfer studies**

For these studies, purified splenic T cells were isolated, as described above, from CD154+/− mice immunized with BRD509 strains organisms 45 days previously. Pooled immune sera were obtained from CD154+/− mice immunized and boosted at three monthly intervals with BRD509 strain (5.0 × 10^6 CFU/mouse). Immune serum was collected within 7 days of the last booster immunization, spun down, and frozen at −40°C until use. As recipients of the transfer, we used CD154+/− mice inoculated with a sublethal dose of BRD509 (4.0 × 10^5/mouse) 2 mo previously. For the transfer, mice received serum alone (in a 0.5-ml volume i.p.), purified immune T cells alone (5.0 × 10^6/mouse in a 200-μl volume i.v.), or a combination of both. Control mice received an equivalent volume of saline. Twenty-four hours after the transfer, all mice were challenged i.p. with a lethal dose of virulent SL1344 strain (2.0 × 10^5 CFU/mouse). Survival was followed for up to 30 days.

**Statistical analysis**

Statistical significance was analyzed by Student’s *t* test, using the statistical program of PRISM software (GraphPad). Differences between experimental groups were considered significant at *p* < 0.05.

**Results**

**Increased morbidity and heightened susceptibility of CD154−/− mice to salmonella infection**

To analyze the role of CD154 in murine salmonellosis, we first studied the in vivo response of CD154−− mice to an attenuated, *aroA/aroD−* vaccine strain of *S. typhimurium* (designated strain BRD509). As shown in Fig. 1, CD154+/− mice inoculated with a moderate dose (1 × 10^6 CFU) of BRD509 organisms exhibited progressively increasing morbidity starting at ~3 days after infection. Infected mice showed gradual loss of activity, posture, and total body weight (Fig. 1A). By day 10, these mice began to succumb to infection, and by day 20, survival of the infected animals was only ~30% (Fig. 1B). In contrast, CD154+/− mice showed a transient weight loss initially (between days 3 and 7), followed by normal recovery and weight gain thereafter (Fig. 1A). No deaths were observed in this group of animals. The development of the symptoms in CD154−/− mice was dependent on the bacterial dose used (Fig. 1B). Thus, when the bacterial dose was 0.3 × 10^6/animal, both CD154−/− and CD154+/− mice showed 100% survival. The survival rate among CD154−/− mice fell to 30 and 0% in response to a dose of 1 × 10^6 or 3 × 10^6 CFU, respectively. In comparison, wild-type CD154+/− mice routinely exhibited 100% survival to inocula as high as 3 × 10^6 CFU (Fig. 1B). These data demonstrate that compared with normal mice, CD154−/− mice are more susceptible to infection with *S. typhimurium*.

**Heighened morbidity of infected CD154−/− mice correlates with increased bacterial burden**

We next determined whether the increased susceptibility of CD154−/− mice to *Salmonella* infection correlated with an increase in bacterial load in target organs. To determine bacterial CFU at different time points after infection, it was necessary to use a sublethal dose (~0.5 × 10^5/mouse) of the BRD509 strain for infection. As indicated in Fig. 2, at 3 days after infection, the numbers of *Salmonella* CFU recovered from the spleen (Fig. 2A) and liver (Fig. 2B) of CD154−/− and CD154+/+ mice were very similar. However, at subsequent time points, ranging from 7 to 21 days after infection, the bacterial burden in the organs of CD154−/− mice was consistently higher than that in normal CD154+/+ mice. The fold increase in CFU in CD154−/− mice ranged from 4.2- to 1850-fold that in the corresponding group in CD154+/+ mice. These data suggest that hypersusceptibility of CD154−/− mice to *Salmonella* infection was associated with a failure to control bacterial replication in target organs. As a consequence of the higher bacterial load, infection-induced splenomegaly was also more pronounced in CD154−/− mice (Fig. 3).

The degree of splenomegaly observed in CD154−/− mice was 4.6-, 6.0-, and 4.6-fold (compared with spleen weights of saline-treated mice) on days 7, 14, and 21, respectively, indicating that peak splenomegaly was reached at 14 days after infection (Fig. 3A). In contrast, the corresponding degrees of splenomegaly in CD154+/+ mice were 4.6-, 7.5-, and 9.5-fold of that in control mice on days 7, 14, and 21, respectively, demonstrating that infection-related splenic changes were both more pronounced and more prolonged in the deficient mice.

To assess these differences, groups of mice were analyzed 90 days after infection with a sublethal dose of BRD509 (6 × 10^7/mouse). As shown in Fig. 3B, splenomegaly was still evident in CD154−/− mice at 90 days after infection, with an average spleen weight 3-fold that of control mice. However, spleen weights of infected CD154+/+ mice had essentially returned to normal. Importantly, BRD509 organisms could not be detected in the spleen...
or liver of infected mice of either strain at this time point (data not shown). It is worth noting that in our assays, the minimum detectable level of bacterial CFU in target organs is ~40 CFU/spleen or ~40 CFU/200 mg of liver tissue. Flow cytometric analysis of spleen cells obtained from 90-day-infected mice revealed that the increased splenomegaly in the CD154−/− strain was associated with the presence of an inflammatory cell influx composed mainly of professional phagocytes (Figs. 4 and 5). Thus, within the gated (gate R4) cell population in Fig. 4 (A and D), which represents the cellular influx seen mostly in CD154−/− (20.6% of total spleocytes; Fig. 4D), but not CD154+/+ spleens (Fig. 4A), two CD11b-positive cell types (CD11b data not shown) predominated: neutrophils (Gr-1<sup>high</sup>F4/80<sup>−</sup>, 54.8%; M2 gate in Fig. 4E) and macrophages (Gr-1<sup>low</sup>F4/80<sup>−</sup>, 33.9%; M1 gate in Fig. 4E and M2 gate in Fig. 4F). The distribution of splenic cellular compartments in 90-day-infected CD154+/+ and CD154−/− mice is shown in Fig. 5. It is clear that delayed resolution of infection-associated splenomegaly in CD154−/− mice was predominantly due to increases in the ratio as well as the cell number of professional phagocytes (macrophages and neutrophils). Interestingly, the effect of long-term infection on the lymphocytic populations in the two mouse strains was quite different. In the case of B lymphocytes, although the ratio of B lymphocytes in the two mouse strains was not altered significantly, the absolute cell number was elevated (~1.7-fold) in CD154−/− mice, largely due to the higher cell yield. In contrast, despite the apparent decrease in the ratio of T lymphocytes in CD154−/− mice, the number of splenic T cells in the two mouse strains was virtually unchanged. Taken together, these data suggest that an accumulation of B lymphocytes occurs in the spleens of infected CD154−/− mice, which also contributes to the observed splenomegaly.

**CD154 is required for the proinflammatory cytokine response to Salmonella infection**

Resistance to *Salmonella* infections is thought to occur via Th1-mediated activation of host target cells, leading to bacterial clearance (23). Previous studies have demonstrated the requirement for CD40-CD154 interactions in pathways leading to the production of IL-12, IFN-γ, and NO (24–27). Therefore, the abilities of CD154+/+ and CD154−/− mice to produce these inflammatory mediators after inoculation by attenuated *S. typhimurium* organisms were compared. First, we evaluated the ex vivo production of IFN-γ and NO by spleen cells of infected mice. Compared with CD154+/+ mice, spleen cells from infected CD154−/− mice produced significantly less IFN-γ, which was evident early (4 days) as well as late (11 days) after infection (Fig. 6A). A similar trend emerged when splenocyte culture supernatants were tested for evidence of NO production. As shown in Fig. 6B, greater levels of

**FIGURE 3.** Protracted splenomegaly in *Salmonella*-infected CD154−/− mice at late time points of infection. A. The extent of splenomegaly was compared at different time points in mice injected with ~0.7 × 10<sup>8</sup> organisms of the BRD509 *Salmonella* strain. The data were compiled from three independent experiments and represent the mean weight ± SEM of eight to 10 spleens/group. The spleen weights of saline-injected control mice were typically 80–100 mg (data not shown). B. Spleen weights on day 90 of infection. The data are the mean ± SEM of six mice per group. The experiment shown is representative of two independent experiments. Asterisks denote statistically significant differences between CD154+/+ and CD154−/− mouse strains (**, p < 0.01; ***, p < 0.001).
No (range, 1.4–2.1-fold) were produced by infected splenocytes of CD154+/+ than CD154−/− mice, particularly early in the infection ($p$ values of 0.015 and 0.0009 on days 4 and 6 after inoculation, respectively). We also assessed the production of inflammatory mediators in vivo by determining the level of IL-12p40 in the sera of normal and infected CD154+/+ and CD154−/− mice. As indicated in Fig. 6C, the level of IL-12p40 in the serum of infected CD154−/− mice was significantly less (range, 1.8–4.4-fold) than that in infected CD154+/+ mice at all time points tested ($p$ values of 0.034, 0.003, and 0.017 on days 4, 6, and 11 after inoculation, respectively). Because it has been demonstrated that cells of the innate immune response, NK cells and macrophages, are the source of these cytokines in the early phase of infection, our data suggest that CD154 also plays a role in the regulation of these responses (21, 28).

**Impaired Ab production and Th1-dependent isotype switching in CD154−/− mice**

*Salmonella typhimurium* is a facultative intracellular pathogen; as a result, protection against this pathogen requires good humoral as well as cellular immune responses (14, 15). We assessed the serum levels of *Salmonella*-specific IgM and IgG3 Abs, the latter being an isotype indicative of a Th1 response, in infected CD154+/+ and CD154−/− mice. As shown in Fig. 7, *Salmonella*-specific IgG3, an isotype induced by IFN-γ, was not found in infected CD154−/− animals 3 wk after infection. By contrast, a small, but significant, titer of *Salmonella*-specific IgG3 was detected in wild-type mice. By 7 wk of infection, IgG3 Abs in CD154+/+ animals were increased several-fold, reaching a titer of 3200 (a titer is defined as the last dilution giving a response above background). Sera of infected CD154−/− mice were also checked for the presence of other IgG isotypes (IgG1, IgG2a, and IgG2b), all of which were negative (data not shown). *Salmonella*-specific IgM was also measured in both mouse strains, and at both time points, the IgM level in CD154+/+ mice was significantly higher than that in CD154−/− animals (Fig. 7). As expected, the IgM response predominated at 3 wk after infection, whereas at 7 wk the dominant Ab isotype was IgG3. At the latter time point, the *Salmonella* organisms were essentially cleared, with <0.02% of the infecting CFU being detectable in the liver and spleen of either mouse strain (data not shown).

These data demonstrate that CD154−/− mice are deficient in their ability to produce pathogen-specific IgM Abs. Furthermore, these mice fail to switch the *Salmonella*-specific Ab response to appropriate isotypes, such as IgG3, IgG1, or IgG2a (Fig. 7 and data not shown).

**CD154 is required for priming the immune system and for protection after secondary lethal *Salmonella* challenge**

In the next series of experiments, we assessed whether CD154−/− mice were able to mount an effective memory immune response after immunization with a sublethal dose of the attenuated BRD509 strain. Mice were inoculated i.p. with a sublethal dose (range, 0.2–0.5 × 10⁶ CFU/mouse) of BRD509, then challenged with a high dose (200- to 500-fold the LD₅₀ dose) of a virulent strain of *S. typhimurium* (strain SL1344; LD₅₀ <10/mouse i.p.) 45–60 days later. Survival of the animals was monitored daily for a total period of 60 days after challenge. As shown in Fig. 8A, in data combined from five independent experiments, nonimmunized CD154+/+ and CD154−/− mice succumbed to an overwhelming infection (mean time to death, 5.0 ± 0.3 and 5.0 ± 1.4 days, respectively). Immunized CD154+/+ mice showed an 86% survival rate with a mean time to death of 50.6 ± 3.9 days. By contrast, BRD509-pretreated CD154−/− mice still exhibited significant mortality after infection with SL1344 (mean time to death, 17.8 ± 3.0 days; $p < 0.0001$ compared with immunized CD154+/+ mice), with an overall survival rate of only 8.7%. It is interesting to note that pretreatment of CD154−/− mice with attenuated BRD509 organisms resulted in a significant delay in the onset of mortality (from 5.0 ± 1.4 to 17.8 ± 3.0 days; $p = 0.0002$), but this was still insufficient for the majority of mice to survive the lethal challenge. Postmortem analysis of organ CFU in nonsurviving, SL1344-challenged, CD154−/− mice confirmed that death was associated with high SL1344 CFU (>10⁵ organisms/organ) in the liver and spleen. No BRD509 organisms were detectable at this time (data not shown).

In the above protection studies, mice were challenged with the virulent SL1344 strain at 7–8 wk after immunization. At this time
We determined whether the failure of immunized CD154 mice to survive a virulent challenge correlated with defective memory T lymphocyte responses. Splenic T cells were purified from BRD509-immunized mice and tested ex vivo in recall cultures in the presence of antigen (Ag). The response of saline-injected mice is shown as a control. The results, shown in Fig. 9, demonstrate that the response of CD154 mice is significantly decreased compared with that in wild-type mice. The Salmonella-specific IL-2 and IFN-γ recall responses of CD154 mice were significantly reduced by ~33 and 60%, respectively (Fig. 9).

**Transfer of protection to secondary challenge is best achieved by a combination of immune serum and sensitized T cells**

The ability of serum or sensitized T cells from preimmunized immune T cells were reduced by a combination of immune serum and sensitized T cells. Transfer of protection to secondary challenge is best achieved by a combination of immune serum and sensitized T cells. The results, shown in Fig. 9, demonstrate that the response of CD154 mice is significantly decreased compared with that in wild-type mice. The Salmonella-specific IL-2 and IFN-γ recall responses of CD154 mice were reduced by ~33 and 60%, respectively (Fig. 9).
serum alone, sensitized T cells, or a combination of both serum and T cells. Control mice received saline. After challenge, survival of the mice was followed for 30 days. As shown in Fig. 10, all saline-treated control CD154−/− mice succumbed to SL1344 infection within 1 wk (mean time to death, 6.0 ± 0.2 days). Passive transfer of immune serum led to a significant degree of protection, resulting in a survival rate of 40% and a mean time to death of 21.2 ± 2.7 days (p < 0.0001 compared with control mice). Adoptive transfer of sensitized T cells alone led to 70% survival, with a mean time to death of 23.4 ± 3.4 days (p < 0.0001). Superior protection was observed in the mouse group that received both immune serum and T cells, resulting in 90% protection and a mean time to death of 28.6 ± 1.4 days (p < 0.0001). Differences in survival and mean time to death between mice receiving serum alone or serum plus immune T cells were statistically significant (p = 0.0253). Differences between other groups did not reach significance. We conclude that the failure to develop protective immunity against virulent secondary challenge in CD154−/− mice is due to a combination of defective humoral as well as T cell immune responses.

**Discussion**

In this study we evaluated the requirement for CD4-CD154 interactions in *Salmonella* infections by comparing the in vivo responses of CD154−/− and wild-type mice. Our findings demonstrate that CD154 protein is critical for 1) defense against a primary *Salmonella* infection and 2) development of a protective memory response in a secondary infection. This implies that in the *Salmonella* murine model, CD154 appears to be required for both innate and adaptive immune responses. The heightened susceptibility of CD154−/− mice to *Salmonella* infection correlates with a quantitative defect in the generation of an inflammatory immune response, particularly in the production of IL-12p40, NO, and IFN-γ, and the absence of an adequate Ab response to *Salmonella*. In our study the humoral anti-*Salmonella* response in CD154−/− mice was characterized by severely reduced IgM levels as well as defective isotype switching to IgG. These findings are consistent with previous data demonstrating that CD154−/− mice fail to develop IgM responses to T-dependent Ags despite having normal/ elevated levels of total serum IgM (17). Other investigators have reported similar defects in IgM and IgG syntheses in another experimental infection model (29). Intriguingly, CD154−/− mice were susceptible to even an attenuated *aroA*/*aroD* strain of *S. typhimurium*. *Salmonella* mutants carrying single or double mutations in *aro* genes have been used as vaccine vectors in numerous animal studies because of their excellent safety profile in mouse strains of different genetic backgrounds (30). This includes strains regarded as hypersusceptible to *Salmonella*, such as TLR-4-defective or *nramp*1-mutant mice (19, 31–34). The fact that CD154−/− mice succumbed to infection by an *aro* gene mutant *Salmonella* strain underscores the degree of susceptibility caused by the loss of CD154 expression.

In our study the majority of CD154−/− animals began to show signs of morbidity by day 9 and succumbed to infection 10–16 days after inoculation. This pattern of susceptibility in response to a primary infection suggests a defective development of innate immune responses in these animals. Being intracellular pathogens, *Salmonella* organisms invade host macrophages and establish a niche inside discrete vacuoles, known as *Salmonella*-containing vacuoles (35). The ability of the bacteria to establish and maintain their intracellular niche within macrophages is crucial for their survival and, consequently, pathogenicity (36, 37). During the innate phase of the immune response, phagocytic cells (primarily macrophages and neutrophils) function to limit the intracellular replication of the bacteria as well as their spread to surrounding tissue in the liver parenchyma (38, 39). The efficiency of this containment depends to a great extent on the production of TNF-α and IFN-γ, which are secreted primarily by macrophages and NK cells and augmented by IL-12, a product of macrophages and dendritic cells (40). CD154 has been previously implicated in the regulation of macrophage and NK cell activation during the early as well as late phases of infection (41, 42). Our findings demonstrate that CD154−/− mice are unable to produce normal levels of IL-12, NO, and IFN-γ in response to infection, thereby decreasing the effectiveness of their innate immune mechanisms. This would have been expected to produce a breakdown in the early containment of bacterial replication, leading to an overwhelming bacterial load and, ultimately, death of the animal. In this report, data have been
presented showing an increased bacterial burden in target organs in CD154/−/− mice. Moreover, postmortem analysis of CD154/−/− mice infected with a high dose of (1 × 10⁶/mouse) of the aroA/aroD BRD509 mutant has consistently shown high CFU (10⁷–10⁸/organ) in liver and spleen (data not shown). It is known that bacterial CFU of this magnitude induce death by toxic shock (43). These findings support our conclusion regarding the underlying defect in CD154/−/− mice.

The susceptibility of CD154/−/− mice to a primary infection by a vaccine strain of S. typhimurium is reminiscent of previous data obtained using mice in which the functional expression of proinflammatory cytokines, primarily IFN-γ, TNF-α, and IL-12, has been either genetically knocked out or inhibited by the use of neutralizing mAb (43–46). Mice genetically deficient in IFN-γ or its receptor succumb to infection by an aroA mutant of S. typhimurium with similar kinetics (the majority of animals die in wk 2 and 3 after infection) to what we have observed in CD154/−/− mice (46). By contrast, when functional TNF-α expression is deleted or neutralized, infection by virulent Salmonella was exacerbated, but...
replication of attenuated aroA mutant Salmonella was partially controlled, with no mortality reported (44). Furthermore, neutralization of IL-12 in susceptible mice was also found to exacerbate the severity of infection by attenuated Salmonella in an IFN-γ-dependent fashion, because that effect was reversed by the administration of exogenous IFN-γ (43). Taken together with these other studies, our findings strongly suggest that CD154 is required for the proper development of an innate inflammatory cytokine response to Salmonella infections that acts to control initial bacterial growth and replication in target organs.

The finding that aromatic-dependent strains of Salmonella can be virulent is surprising if one considers the nature of the attenuating auxotrophic mutations in these organisms. The safety of such mutants was thought to be linked to their strict requirement for essential aromatic compounds, such as p-aminobenzoic acid, which are not readily synthesized by eukaryotic hosts. However, we and others (Refs. 44 and 46–49 and this report) have demonstrated that these mutant Salmonella strains can be virulent or persist over long periods of time in severely immunocompromised hosts, including T cell-deficient nu/nu, H-2I-Aβ−/−, TCRβ−/−, IFN-γR−/−, TNF-op55R−/−, CD28, and CD154−/− mice. It has been suggested that the in vivo persistence and/or virulence of such mutants may be due to the exogenous supply of p-aminobenzoic acid in sufficient quantities in the animal feed (47).

The data presented in this report demonstrate that in addition to being important in the acute phase of the disease, CD154 is also critical for the development of adaptive immunity in response to secondary Salmonella infections. The important role of CD40-CD154 interactions in the regulation of T and B cell responses has been well documented (4, 50). CD40-CD154 interactions regulate the development of Th1-type responses through the induction of IL-12 synthesis by macrophages and dendritic cells and the up-regulation of costimulatory molecules on APCs, thereby enhancing and optimizing the conditions for T cell activation (26, 42, 51–54). Our findings demonstrate a significant reduction in the production of IL-12 and IFN-γ in response to inoculation of attenuated Salmonella organisms. This may well have an impact on the induction of an effective anti-Salmonella T cell response during the primary infection. A requirement for CD154 in the generation of a memory response in experimental viral infections was previously demonstrated (55). However, this requirement does not seem to be universal, because it has been reported that CD154−/− mice develop secondary protective immune responses to the intracellular fungal pathogen Histoplasma capsulatum (13). In this report, CD154−/− mice immunized with a sublethal dose of the attenuated Salmonella strain and challenged 7–8 wk later with a highly lethal dose (equivalent to 500-fold the LD50 dose) of a virulent strain of S. typhimurium had a survival rate of <10%, which is in sharp contrast to similarly treated CD154+/+ mice, which had a survival rate of ~90%. Furthermore, to rule out the possibility that the lack of survival in CD154−/− mice was due to a superimposed secondary infection, mice were challenged at 16 wk after immunization, a time at which the primary infection was demonstrably completely cleared. The findings demonstrate that even under these conditions, all BRD509-treated CD154−/− mice succumbed to the challenge infection, whereas similarly treated CD154+/+ mice were fully protected.

Investigation of the mechanism underlying the poor survival seen after secondary challenge revealed that two factors are involved. First, there is a defective production of Igs. In infected CD154−/− mice, the development of IgM Abs against Salmonella was delayed, and its magnitude was consistently lower than that in CD154+/+ mice. Furthermore, consistent with their inability to undergo Ig isotype switching, infected CD154−/− animals fail to develop any IgG response. A good Ab response is known to be required for protection against virulent, but not attenuated, Salmonella organisms (49, 56). Thus, failure to develop a protective Ab response would render CD154−/− mice susceptible to secondary lethal challenge with the SL1344 strain. Passive transfer studies demonstrated that up to 40% of CD154−/− mice could be protected by the transfer of serum alone. Second, there is evidence of inadequate generation of memory T cells in CD154−/− mice after immunization with a sublethal dose of attenuated BRD509 strain. This is supported by two observations. First, the absence of a protective memory response in CD154−/− mice correlated with a significant decrease in the production of type 1 cytokines by sensitized T cells in Salmonella-specific recall responses. Second, adoptive transfer of CD154+/+ sensitized T cells afforded good protection to CD154−/− mice, with 70% of the recipients surviving the lethal challenge. The demonstration that both Igs and T cells are needed for efficient immunity against virulent Salmonella is consistent with previous reports (14, 15, 57). We therefore conclude that CD154 is essential for the generation of protective, memory, humoral, and cell-mediated immune responses in Salmonella infections.

In experimental infection models, a deficiency in CD154 expression has been shown to result in increased susceptibility to a variety of intracellular pathogens, including Leishmania spp. (8–10), Toxoplasma gondii (11), C. parvum (58), P. carinii (59), Candida albicans (60), and S. typhimurium (this report). The susceptibility is mainly due to defects in the production of IL-12 and IFN-γ and activation of macrophage microbicidal functions. In X-linked hyperIgM syndrome patients, it was recently reported that the most prominent clinical infections were pneumonia, upper respiratory infections, recurrent/protracted diarrhea, CNS infections, sepsis, cellulitis, and hepatitis. These infections were caused by encapsulated bacteria and a group of opportunistic pathogens, such as P. carinii, C. parvum, Candida, Histoplasma, and members of the herpes virus family, including CMV (61). Importantly, CD40-CD154 interactions have also been shown to be critical for T cell-dependent macrophage activation in human patients (reviewed in Ref. 62). Susceptibility to Salmonella and Mycobacteria organisms has been reported in children born with a deficiency that affects major proteins in the type 1 cytokine (IL-12/IL-23-IFN-γ) axis (63), but, interestingly, not in CD40- or CD154-deficient individuals. The underlying reason for the differential pattern of susceptibilities observed in patients defective in the CD40-CD154 pathway or the IL-12/IL-23-IFN-γ cytokine activation pathway is not yet known.

In conclusion, using the murine systemic Salmonella model, evidence is provided supporting an essential role for CD154 in resistance to infection during the innate phase of disease as well as in the development of a protective memory response in secondary infections. The data suggest that CD154 may mediate its effects by influencing the release of proinflammatory cytokines by APCs, which are required for control of the infection during the early phase of disease, and by regulating the development of Ab and T cell responses, which are critical for protection in secondary infections.

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Disclosures

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References


