B7-H1 (Programmed Cell Death Ligand 1) Is Required for the Development of Multifunctional Th1 Cells and Immunity to Primary, but Not Secondary, Salmonella Infection

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B7-H1 (Programmed Cell Death Ligand 1) Is Required for the Development of Multifunctional Th1 Cells and Immunity to Primary, but Not Secondary, Salmonella Infection


Robust Ab and CD4 T cell responses are required for the resolution of Salmonella infection in susceptible mice. In this study, we examined the role of B7-H1 (programmed cell death ligand 1) in resistance to primary Salmonella infection. Infected B7-H1−deficient mice had significantly higher bacterial burdens at day 21 and day 35 postinfection compared with wild-type mice, demonstrating that B7-H1 plays an important role in immunity to Salmonella. B7-H1−deficient and wild-type mice both generated Salmonella-specific IgM and IgG2c Ab responses to infection, and clonal expansion of endogenous and adoptively transferred Salmonella-specific CD4 T cells was similar in both groups. However, although Salmonella-specific IFN-γ–producing Th1 CD4 T cells were generated in Salmonella-infected B7-H1−deficient mice, these cells did not expand to the level observed in wild-type mice. Furthermore, fewer multifunctional Th1 cells that simultaneously secreted IFN-γ, TNF-α, and IL-2 were detected in Salmonella-infected B7-H1−deficient mice. Together, these data demonstrate that B7-H1 is required for the generation of multifunctional Th1 responses and optimal protective immunity to primary Salmonella infection. The Journal of Immunology, 2010, 185: 2442–2449.

Salmonella enterica serovar typhi is responsible for human typhoid, a disease that is endemic in the developing world and responsible for >200,000 deaths every year (1, 2). Although two typhoid vaccines are available (3, 4), neither of these has reduced the incidence of disease in developing nations, due to concerns about vaccine efficacy, safety, or financial cost (5). Thus, development of an effective typhoid vaccine that could lower the burden of typhoid in developing nations remains a global healthcare priority. For this objective to be achieved, greater knowledge of the adaptive immune response to Salmonella is required.

Immunity to Salmonella infection is often studied using inbred strains of mice infected with S. enterica serovar typhimurium (hereafter referred to as S. typhimurium) (6–8). Although S. typhimurium does not usually cause systemic disease in immune competent humans, this pathogen causes a fatal systemic infection, or nonfatal persistent infection in mice. Indeed, several important features of human typhoid are faithfully reproduced in murine S. typhimurium infection, making this the best available model to study systemic Salmonellosis (6). For example, susceptible mice can be infected orally (9), bacteria invade the host intestinal epithelium by targeting Peyer’s patch M cells (10), invasive bacteria replicate within infected macrophages (11), and the primary sites of systemic colonization are the spleen, liver, and bone marrow.

Infection of susceptible mice with virulent Salmonella rapidly causes fatal infection, thus protective immunity is often studied after infection with auxotrophic S. typhimurium strains (12, 13). These attenuated Salmonella replicate within the macrophages of the spleen, liver, and bone marrow but, in contrast to virulent Salmonella, are eventually cleared from systemic tissues (14). Importantly, the clearance of this primary infection requires the development of Salmonella-specific adaptive immunity (15, 16), and this model has therefore allowed precise dissection of the requirements for immune protection. For example, it has been demonstrated that clearance of primary attenuated Salmonella requires IFN-γ–producing CD4 Th1 cells, and thus mice with deficiencies in CD4 (17), MHC class-II (17), CD28 (18), IFN-γ (19), IFN-γR (17), or the Th1 transcription factor T-bet (20), succumb to fatal infection. Salmonella-specific B cells and CD8 T cells also play an additional protective role, although this is more commonly observed in resistance to secondary infection (21). Therefore, this particular infection model is ideally suited to understanding the development and role of Th1 cells in protective immunity to a natural human pathogen.

Given their important role in bacterial clearance, it is not surprising that Salmonella are capable of inhibiting the development and/or function of CD4 Th1 cells in vivo (22). Indeed, our laboratory recently reported that activated Salmonella-specific CD4 T cells are progressively depleted after Salmonella infection of mice (23). During the completion of these in vivo experiments, we noticed that activated Salmonella-specific CD4 T cells expressed higher levels of the inhibitory ligand B7-H1, also known as programmed cell death ligand 1 (PD-L1), suggesting that this molecule may be important for CD4 Th1 cell inhibition by Salmonella. Indeed, several reports using viral infection models have demonstrated that T cell function can be profoundly inhibited by signals delivered via B7-H1 or the receptor for this molecule (24, 25).

B7-H1 is constitutively expressed by several cell populations, including T cells, dendritic cells, and macrophages, and this expression is increased on activation (24, 26). PD-1 is the canonical receptor for B7-H1, but B7-H1 also displays affinity for CD80 (27). The role of PD-1 and B7-H1 has been extensively examined in models of autoimmunity and viral infection where signaling through these receptors is thought to deliver a negative signal to T cells and restrain
adaptive immune responses (24, 26). Inhibition of PD-1 binding with B7-H1 is therefore a promising therapeutic strategy for increasing T cell functionality during chronic viral infection or vaccination (28).

Based on this known inhibitory role for B7-H1, our prediction was that B7-H1–deficient mice would develop an enhanced Th1 response to Salmonella and display evidence of greater protective immunity to infection. However, in contrast, we present data showing that B7-H1 is required for optimal development of multifunctional Th1 cells and protective immunity in the mouse model of Salmonella infection.

Materials and Methods

Mouse and bacterial strains

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME) and were used at 6–12 wk of age. CD90.1 congenic, RAG-deficient SM1 TCR transgenic mice were originally generated on a C57BL/6 background and express a monoclonal TCR specific for Salmonella flagellin (29, 30). SM1 transgenic and RAG-, or B7-H1–deficient mice (31) were all maintained on a C57BL/6 background by intercrossing at the University of Minnesota. The initial breeding stock for our B7-H1–deficient colony was kindly provided by Dr. L. Chen (The Johns Hopkins University, Baltimore, MD). All mice were cared for in accordance with University of Minnesota Research Animal Resource guidelines. Salmonella BRD509 strain (32) was kindly provided by Dr. D. Xu, University of Glasgow, Glasgow, U.K.

Salmonella infection and bacterial counts

BRD509 (AroA−D−) and SL1344 were grown overnight in LB broth without shaking and diluted in PBS after estimation of bacterial concentration using a spectrophotometer. Mice were infected i.v. in the lateral tail vein with 5 × 10^3 BRD509, and monitored daily for signs of infection. Salmonella-infected mice were determined to be moribund if they were unresponsive to gentle prodding. Mice that resolved primary infection with BRD509 were administered 0.1 ml 5% sodium bicarbonate to neutralize stomach pH before oral challenge with 5 × 10^7 virulent Salmonella (SL1344) and daily monitoring to determine protection. In all experiments, the actual bacterial dose administered was confirmed by plating serial dilutions of the original culture onto MacConkey agar plates. To determine bacterial colonization in vivo, spleens and livers from infected mice were homogenized in PBS and serial dilutions were plated onto MacConkey agar plates. After overnight incubation at 37°C, bacterial plates were counted and bacterial burdens calculated for each individual organ.

Salmonella-specific Ab responses

Blood was collected retro-orbitally from mice infected with BRD509 and sera were prepared by centrifugation. High-protein binding plates (Costar) were coated overnight with heat-killed S. typhimurium diluted in 0.1 M NaH₂PO₄, pH 7.2. After incubation in 10% PBS/PBS for 1 h at 37°C, these plates were washed twice in PBS/0.05% Tween 20, and serum samples were added in serial dilutions in 10% FBS/PBS. After incubation for 2 h at 37°C, plates were washed four times before the addition of biotin-conjugated Ab specific for the desired Ab isotype (BD Bioscience and eBioscience, San Diego, CA). After incubation in 10% FBS/PBS/0.05% for 1 h at 37°C, these plates were washed twice in PBS/0.05% Tween 20, and serum samples were added in serial dilutions in 10% FBS/PBS. After incubation for 2 h at 37°C, plates were washed four times before the addition of biotin-conjugated Ab specific for the desired Ab isotype (BD Bioscience and eBioscience, San Diego, CA). After a further incubation for 1 h at 37°C, plates were washed six times and incubated for 1 h at 37°C with HRP-conjugated streptavidin (Sigma-Aldrich, St. Louis, MO) diluted in 10% FCS/PBS. Plates were then washed eight times and an HRP substrate (O-phenylenediamine dihydrochloride, Sigma-Aldrich) was used to develop the plates. After sufficient color-change was observed, the reaction was stopped by adding 50 µl 2 N H₂SO₄ and plates were analyzed using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

TCR transgenic adaptive transfers

Spleen and lymph node cells (inguinal, axillary, brachial, cervical, mesenteric, and periaortic) were harvested from SM1 mice and a single-cell suspension was generated. An aliquot of this sample was stained using Abs to CD4 and the relevant TCR Vβ to determine the percentage of TCR transgenic cells. SM1 T cells were labeled with 5 µM CFSE (Molecular Probes, Eugene, OR) and incubated at 37°C for 10 min. After several washes in cold HBSS, volumes were adjusted based on the percentage of SM1 cells and 1 × 10⁶–1 × 10⁷ SM1 were injected i.v. into recipient mice. Three days or 1 wk postinfection, spleens were harvested and a single-cell suspension generated in Eagle’s Ham’s amino acids (Intrivigen, San Diego, CA) medium containing 2% FBS. Samples were incubated on ice for 30 min in Fc

block containing Fcγ, PE-, PerCP-, or APC-conjugated Abs specific for CD4, CD11a, CD90.1, CD62L, CD69, or CD25 (eBioscience and BD Bioscience). After staining, cells were fixed using paraformaldehyde and examined by flow cytometry using a FACS Canto. All flow data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Examination of endogenous CD4 T cell responses using class-II tetramers

Endogenous Salmonella flagellin-specific CD4 T cell responses were monitored using a previously described methodology (33). Biotinylated I-A^d monomers with an attached peptide derived from Salmonella flagellin (34) (I-A^d-flag), were produced in S2 insect cells cultured using a Wave Bioreactor (GE Healthcare Biosciences, Pittsburgh, PA). Purified monomers were tetramerized using fluorochrome-conjugated streptavidin and batch-tested for optimal binding to SM1 T cells, as previously described (35). Spleen and lymph nodes were harvested from infected or uninfected mice and stained with I-A^d-flag and antifluorescence magnetic beads (Miltenyi Biotech, Auburn, CA) before tetramer-specific cells were enriched using magnetic columns. Column-bound and unbound fractions were stained with fluorochrome-conjugated Abs specific for CD3, CD4, CD8, CD11b, CD11c, CD44, B220, and F4/80 (eBioscience). Cells were then examined using an LSR II (BD Bioscience) and endogenous tetramer-specific T cells identified using a previously described gating strategy (33). All data were analyzed using FlowJo software.

Analysis of cytokine production and transcription factor expression by CD4 T cells

Salmonella-infected or naive mice were injected i.v. using a mixture of bacterial Ags to activate Salmonella-specific T cells (total of 10⁷ heat-killed S. typhimurium) (14). Four hours later, spleens were harvested in Eagle’s Ham’s amino acids containing 2% FBS and a single-cell suspension was generated. After rapid surface staining on ice, cells were fixed with formaldehyde, permeabilized using saponin (Sigma-Aldrich), and stained intracellularly using IFN-γ, IL-4, IL-2, IL-10, IL-17A, and/or TNF-α specific Abs (eBioscience and BD Bioscience). In some experiments, cells were surface stained and fixed before intracellular staining using Abs specific for T-bet, GATA-3, or RORγt (eBioscience and BD Bioscience). After intracellular staining, cells were examined by flow cytometry using a FACS Canto (BD Bioscience) and flow data were analyzed using FlowJo software (TreeStar). The percentage of CD4 T cells producing IFN-γ alone or IL-2, IFN-γ, and TNF-α was determined by sequential gating using individual samples stained with Abs specific for all three cytokines.

Statistical analysis

Statistical differences between groups of normally distributed data were examined using InStat (GraphPad Software, La Jolla, CA). For bacterial burdens, Log10 CFUs are normally distributed and were also compared using InStat. Data in each group were compared using an unpaired t test and were considered significantly different with a p value of <0.05.

Results

B7-H1 is required for optimal protective immunity to Salmonella infection

To examine the role of the inhibitory molecule B7-H1 in Salmonella infection, wild-type (Wt), RAG-deficient, or B7-H1–deficient mice were infected i.v. with attenuated Salmonella. Institutional animal guidelines do not allow the use of death as an end point, therefore Salmonella-infected mice were monitored twice daily and animals in a moribund state (unresponsive to gentle prodding) were sacrificed and considered to have succumbed to infection. As expected, RAG-deficient mice were unable to resolve primary infection with Salmonella, whereas Wt mice survived (Fig. 1). In marked contrast, ~30% of B7-H1–deficient mice failed to resolve primary Salmonella infection, with these animals succumbing to infection around the second or third week after challenge (Fig. 1).

These survival data suggested that B7-H1 played an unexpected but important protective role during the resolution phase of primary Salmonella infection. We examined this issue more carefully by monitoring bacterial loads in the spleen and liver of Wt and B7-H1–deficient mice at various time points postinfection. At 1 wk postinfection, no difference in bacterial loads was observed in the
spleen or liver of B7-H1–deficient or Wt mice (Fig. 2). Indeed, at this early time point, control of bacterial replication is mediated primarily by innate immune defense mechanisms (36), and no deficiency is observed in MHC class-II-, or T-bet–deficient mice (17, 20). In contrast, at both 3- and 5-wk postinfection, bacterial loads were significantly higher in the spleens of B7-H1–deficient mice compared with Wt mice (Fig. 2). Furthermore, at 5-wk postinfection, bacterial colonization of the liver was also significantly higher in B7-H1–deficient mice (Fig. 2). It should be emphasized that these data only show the bacterial burdens in surviving B7-H1–deficient mice, because around 30% had already succumbed to infection prior to these later time points (Fig. 1). Together, these data demonstrate an unexpected requirement for B7-H1 during the resolution phase of primary Salmonella infection.

Despite this deficiency in resolving primary Salmonella infection, the majority of B7-H1–deficient mice eventually cleared attenuated bacteria from the spleen and liver. Wt mice that have resolved primary infection with attenuated Salmonella develop robust protective immunity to secondary infection. Although B cells and Ab responses are not required to resolve primary Salmonella infection, they play a major role in protective immunity to secondary challenge (18). Therefore, to examine whether immunity to secondary infection was altered by the absence of B7-H1, we reinfected B7-H1–deficient mice with virulent Salmonella strain SL1344. Both Wt and B7-H1–deficient mice were able to fully resist secondary infection (Fig. 1B), indicating that B7-H1 is not required for acquired resistance to Salmonella.

Development of Salmonella-specific Ab responses in B7-H1–deficient mice

As noted previously, despite the fact that Salmonella is an intracellular pathogen, bacterial-specific Ab responses play an important protective role in murine and human Salmonellosis (18, 37–39).

We therefore examined Salmonella-specific IgM, IgG1, and IgG2c responses in Wt and B7-H1–deficient mice. Our laboratory has previously reported that these are the major Ab isotypes found in this infection model (18, 40). At 2-wk postinfection, similar Salmonella-specific IgM and IgG2c responses were detected in both Wt and B7-H1–deficient mice (Fig. 3, day 14). However, at 5-wk postinfection, B7-H1–deficient mice had a higher Salmonella-specific IgG2c response and similar or slightly higher IgG1 response (Fig. 3, day 35). Together, these data demonstrate that B7-H1 do not have a deficiency in generating Salmonella-specific Ab responses and in fact develop higher levels of Salmonella-specific IgG2c.

Clonal expansion of Salmonella-specific CD4 T cells in B7-H1–deficient mice

Despite a well-known inhibitory role for B7-H1 in autoimmune and viral infection models (24, 41), there are also reports suggesting that B7-H1 is required for optimal T cell priming and expansion to bacterial infection. In particular, Ab blocking of B7-H1 was shown to reduce T cell expansion to Listeria infection and delay bacterial clearance (42, 43). Therefore, we examined whether B7-H1 was required for optimal expansion of Salmonella-specific T cells after Salmonella infection. Wt and B7-H1–deficient mice were adoptively transferred with Salmonella flagellin–specific SM1 cells and infected the following day. SM1 cells expanded similarly and completed several rounds of CFSE-dye dilution, 3 d after infection of Wt mice or B7-H1–deficient mice (Fig. 4A). A slightly lower...
frequency of SM1 T cells was detected at day 7 postinfection of B7-H1–deficient mice compared with Wt mice, but the absolute number of SM1 T cells in the spleen of infected B7-H1–deficient mice was not statistically different to that detected in Wt mice (Fig. 4B). These data demonstrate that B7-H1 is not required for clonal expansion of Salmonella-specific CD4 T cells.

Next, we examined SM1 T cell activation in more detail by monitoring the expression of activation markers on SM1 T cells in the first week after Salmonella infection. As expected, naive SM1 T cells in both Wt and B7-H1–deficient mice expressed high levels of CD62L, but low surface levels of CD25 and CD69 (Fig. 5, day 0). One day postinfection, the majority of SM1 T cells in Wt and B7-H1–deficient mice decreased expression of CD62L and increased expression of CD25 and CD69 (Fig. 5, day 1), indicating activation had occurred. At later time points, SM1 T cells in both groups of mice reduced expression of CD25 and CD69 and regained expression of CD62L (Fig. 5, days 3 and 7). Thus, SM1 T cells displayed similar kinetics of activation and expansion in Wt mice and mice lacking expression of B7-H1.

To confirm our adoptive transfer data and also examine T cells that lacked expression of B7-H1, we used I-Ab–flagellin class-II tetramers and magnetic bead enrichment to examine the endogenous CD4 T cell response to Salmonella. This technique allowed direct tracking of the endogenous response without a requirement for TCR transgenic adoptive transfer. Six days postinfection, an expanded population of flagellin-specific CD4 T cells was clearly detected in infected Wt and B7-H1–deficient mice (Fig. 6). These Salmonella-specific CD4 T cells were uniformly CD44Hi in both groups of mice indicating that they had previously encountered Ag (Fig. 6A). It should be noted that endogenous flagellin-specific cells are still expanding at this time point, whereas the SM1 cells peak at day 3 and are contracting by day 7. This is because of differences in the initial precursor frequency of flagellin-specific T cells after adoptive transfer of SM1 T cells (23). However, as with our analysis of the SM1 response, no difference in peak clonal expansion of this population was detected between Wt versus B7-H1–deficient mice (Fig. 6).

Reduced number of Th1 cells in Salmonella-infected B7-H1–deficient mice

Development of Th1 cells with the capacity to produce IFN-γ is absolutely critical for the resolution of primary Salmonella infection (19, 20). We therefore examined whether the expanded Salmonella-specific CD4 T cells in B7-H1–deficient mice had acquired the ability to secrete IFN-γ. To examine polyclonal CD4 responses...
The percentage of cells positive for each surface marker. T cells and are representative of three mice per group. Numbers indicate shown). Furthermore, the production of IL-4, IL-10, and IL-17A was CD4 T cells from infected Wt and B7-H1–deficient mice (data not deficient mice could not be explained by enhanced Th2 or Th17 mice (Fig. 7a–d). In contrast, although IFN- production of CD4 T cells was clearly detected in Wt-infected B7-H1–deficient mice. Wt and B7-H1–deficient mice were adoptively transferred with 500,000 CFSE-labeled SM1 T cells and infected i.v. the following day with 5 × 10^5 attenuated Salmonella, BRD509. On day 6, endogenous Salmonella flagellin-specific CD4 T cells were stained with 1-A^+–flag tetramer, enriched using magnetic microbeads and stained and examined by flow cytometry. CD4 T cells from uninfected (Naive, left) and infected (right) mice, were gated from CD11c^+CD11b^+ F4/80^- B22^+CD3^+ cells and further analyzed for CD44 and I-A^+–flag tetramer positive cells. A. Contour plot show staining from a representative individual mouse and show the percentage of flagellin-specific tetramer positive CD4 T cells. B, Graph shows the mean number ± SEM of endogenous flagellin-specific CD4 T cells from Wt and B7-H1–deficient mice and three mice per group. Differences between these groups are not statistically significant.

In contrast, the proportion of these cells varied considerably at different time points. However, only a small proportion of CD4 clonal expansion appears to be unaltered by B7-H1 deficiency, the development or maintenance of a Th1 response to Salmonella is significantly reduced in the absence of B7-H1.

Reduced number of multifunctional Th1 cells in Salmonella-infected B7-H1–deficient mice

A recent report indicated that the development of multifunctional Th1 cells secreting IFN-γ, TNF-α, and IL-2, correlated with protective immunity against another intracellular infection pathogen, Leishmania major (45). Multifunctional Th1 cells have not previously been examined in Salmonella infection. Therefore, we examined the development of multifunctional Th1 cells in Salmonella-infected Wt and B7-H1–deficient mice. After Salmonella infection of Wt mice, up to 25% of IFN-γ–producing CD4 T cells simultaneously produced TNF-α (Fig. 8A, 8B, day 12), although the proportion of these cells varied considerably at different time points. However, only a small proportion of IFN-γ–producing CD4 T cells produced both IL-2 and TNF-α at any time point (Fig. 8C, 8D). These multifunctional Th1 cells were first detected at low frequency 6 d postinfection but became a larger proportion of the memory response around the time that bacterial clearance occurs, day 35 (Fig. 8C, 8D). Among the IFN-γ–producing CD4 T cell population in B7-H1–deficient mice, TNF-α–producers were detected at day 6, but the proportion of these cells did not increase as observed in Wt mice (Fig. 8A, 8B). Furthermore, a lower frequency

As expected, CD4 T cells from naive mice did not produce IFN-γ, whereas only Ag-experienced CD11a^HI CD4 T cells produced IFN-γ in infected Wt or B7-H1–deficient mice (Fig. 7A). In Wt-infected mice, IFN-γ–producing CD4 T cells were clearly detected 6 d postinfection, this response peaked at day 12, and then declined steadily thereafter (Fig. 7A, 7B). In contrast, although IFN-γ–producing CD4 T cells were detected postinfection of B7-H1–deficient mice, a lower percentage of these cells was observed after day 6 (Fig. 7A, 7B). In addition, we examined Th1 lineage commitment in infected mice by monitoring CD4 cell expression of T-bet, the major transcription factor associated with Th1 development. The percentage of T-bet^+ CD4 cells increased significantly in Wt-infected mice (Fig. 7C, 7D), indicating effective development of Th1 cells in response to Salmonella infection. In contrast, the percentage of T-bet^+ CD4 T cells was lower in B7-H1–deficient mice (Fig. 7C, 7D). The lower percentage of Th1 cells in B7-H1–deficient mice could not be explained by enhanced Th2 or Th17 development as both GATA-3 and RORgt staining was low in CD4 T cells from infected Wt and B7-H1–deficient mice (data not shown). Furthermore, the production of IL-4, IL-10, and IL-17A was
of IL-2/IFN-γ* multifunctional Th1 cells was detected in B7-H1–deficient mice at every time point after day 6 (Fig. 8C, 8D). Together, these data indicate that B7-H1 is required for the development of multifunctional Salmonella-specific Th1 cells.

**Discussion**

B7-H1 can bind PD-1 and CD80 and these interactions are thought to deliver a negative signal to T cells and thus inhibit immune responses (24, 26). Strategies to block PD-1–B7-H1 interactions are currently being explored as therapeutic strategies for increasing T cell functionality during chronic viral infection or after vaccination (28). Many fewer studies have examined the role of B7-H1 in bacterial infection, but the available data are markedly different from viral infection studies and actually suggest a protective role for B7-H1, at least in Listeria infection. For example, Ab blockade of B7-H1 inhibited T cell expansion and delayed the clearance of bacteria from infected tissues (42, 43). Similarly, our data using the Salmonella infection model suggest a protective role for B7-H1 against a Gram-negative bacterial infection and therefore point to an unexpected role for this inhibitory receptor in the development of protective immunity to pathogenic bacteria. It is not yet clear whether this protective role requires the interaction of B7-H1 with PD-1 or with CD80, or whether this requirement is typical of all bacterial infections. However, these findings would suggest that a cautious approach to B7-H1 blockade in the clinic would be appropriate.

Clearance of primary infection in the Salmonella mouse model requires the development of CD4 Th1 cells, although CD8 T cells are also required for an optimal response during secondary infection (21). B cells play a prominent role in clearance of Salmonella (18, 38), and recent studies suggest that thymus-independent Ab responses can confer significant protection (46). Our data demonstrate a modest increase in primary Ab responses and normal clonal expansion of Salmonella-specific CD4 T cells in the absence of B7-H1. Together, these data would indicate that B7-H1 is not required during initial T cell–APC or T cell -B cell interactions that drive the early adaptive response to Salmonella infection. This conclusion differs markedly from the protective role noted for B7-H1 during Listeria infection where a major effect on clonal expansion was detected (42, 43). It is not clear why these bacterial models should display a divergent role for B7-H1 in clonal expansion but this could be related to the different requirement of CD4 T cells in protective immunity to these pathogens. Indeed, Salmonella infection induces much greater clonal expansion of endogenous CD4 T cells than has been reported in viral or bacterial infection models that depend more heavily on CD8 T cells for clearance (14, 47). Thus, robust clonal expansion in the Salmonella model may simply be less dependent on B7-H1 signals.

Instead, our data point to a major role for B7-H1 in the maturation or survival of effector CD4 Th1 cells after initial clonal expansion has already occurred. The fact that Salmonella-infected B7-H1–deficient mice succumb to infection around 2–3 wk concurs with a known requirement for effector T cell responses at this time point. The uncoupling of clonal expansion and effector T cell maturation in this model suggests that Th1 cells take several weeks to develop full effector potential. Indeed, this conclusion is supported by recent data demonstrating that Th1 cell development required Ag persistence and was impeded by early antibiotic clearance of Salmonella (40). Sustained Ag presentation has also been noted as a requirement for CD4 T cell maturation in other model systems (48, 49). Thus, we propose that B7-H1 most likely plays a major role in maturing Th1 cell effector potential during this period of sustained Ag presentation, but is not required for early CD4 T cell activation and expansion. Given the relatively poor development of multifunctional CD4 T cells in B7-H1–deficient mice, it seems likely that these cells represent a more mature differentiation stage that requires
interaction with additional costimulatory molecules, including B7-H1 and perhaps others. Future experiments will examine the role of PD-1 and PD-L2 in the development of these multifunctional CD4 T cells.

Our analysis of endogenous flagellin-specific CD4 T cells using class-II tetramers suggests that these cells represent a small fraction of the overall memory response to *Salmonella*. This stands in contrast to previous studies where flagellin was described as a dominant Ag in the murine typhoid model (34, 50). However, it should be noted that most analysis of flagellin-specific CD4 T cells has focused on the very early responses to *Salmonella* infection (51), in contrast to previous studies where flagellin was described as a dominant Ag in the murine typhoid model (34, 50). However, it should be noted that most analysis of flagellin-specific CD4 T cells has focused on the very early responses to *Salmonella* infection (51), in part due to the failure of these cells to survive to seed the memory pool (14, 23). It should also be noted that the CD4 response to epitope 427–441 was previously described to be very low after primary infection with *Salmonella* (34), raising the possibility that other flagellin epitopes are more dominant in the memory response. Additional tools for examining CD4 responses to *Salmonella* epitopes are not currently available making it difficult to compare the flagellin-specific memory frequency to the wider *Salmonella*-specific CD4 repertoire.

Although B7-H1–deficient mice exhibited increased bacterial burdens in the spleen and liver, only around 30% of these B7-H1–deficient mice died after primary infection. Indeed, surviving mice were able to eliminate all remaining bacteria from tissues and fully resisted secondary challenge with virulent *Salmonella*. Thus, the inability of B7-H1–deficient mice to clear primary infection *Salmonella* is not as profound as that previously reported for class-II–deficient or T-bet–deficient mice. It is therefore likely that other costimulatory molecules participate in the maturation of Th1 cells during *Salmonella* infection and can contribute even in the absence of B7-H1. The ability of B7-H1–deficient mice to resist secondary infection is likely explained by the robust Ab response induced in these mice, because Ab is known to play an important role in secondary protection (18). Thus, in the presence of *Salmonella*-specific Ab response even the reduced Th1 response noted in B7-H1–deficient mice may be sufficient for pathogen clearance.

In conclusion, our data report a surprising requirement for B7-H1 in the maturation of multifunctional Th1 cells and protective immunity to primary *Salmonella* infection. Our data differ markedly from previous findings that B7-H1 functions as an inhibitory receptor in chronic viral infections and serve to emphasize the differing roles played by costimulatory molecules during infection with different pathogens. Understanding the basis of these differences may suggest future strategies for improving vaccination against intracellular bacterial infections.

**Disclosures**

The authors have no financial conflicts of interest.

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