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Virulent *Salmonella enterica* Serovar Typhimurium Evades Adaptive Immunity by Preventing Dendritic Cells from Activating T Cells

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Dendritic cells (DCs) constitute the link between innate and adaptive immunity by directly recognizing pathogen-associated molecular patterns (PAMPs) in bacteria and by presenting bacterial antigens to T cells. Recognition of PAMPs renders DCs as professional antigen-presenting cells able to prime naïve T cells and initiate adaptive immunity against bacteria. Therefore, interfering with DC function would promote bacterial survival and dissemination. Understanding the molecular mechanisms that have evolved in virulent bacteria to evade activation of adaptive immunity requires the characterization of virulence factors that interfere with DC function. *Salmonella enterica* serovar Typhimurium, the causative agent of typhoid-like disease in the mouse, can prevent antigen presentation to T cells by avoiding lysosomal degradation in DCs. Here, we show that this feature of virulent *Salmonella* applies in vivo to prevent activation of adaptive immunity. In addition, this attribute of virulent *Salmonella* requires functional expression of a type three secretion system (TTSS) and effector proteins encoded within the *Salmonella* pathogenicity island 2 (SPI-2). In contrast to wild-type virulent *Salmonella*, mutant strains carrying specific deletions of SPI-2 genes encoding TTSS components or effectors proteins are targeted to lysosomes and are no longer able to prevent DCs from activating T cells in vitro or in vivo. SPI-2 mutant strains are attenuated in vivo, showing reduced tissue colonization and enhanced T-cell activation, which confers protection against a challenge with wild-type virulent *Salmonella*. Our data suggest that impairment of DC function by the activity of SPI-2 gene products is crucial for *Salmonella* pathogenesis.

Dendritic cells (DCs) are key elements for the generation of an efficient adaptive immune response against bacterial pathogens (15, 52). They have the unique ability to initiate adaptive immunity by activating naïve T cells. Recognition of pathogen-associated molecular patterns from bacteria (43) leads to DC maturation and migration to secondary lymphoid organs (4, 24). As part of maturation, DCs show an increased density of major histocompatibility complex (MHC) and costimulatory molecules on their surface and become extremely efficient at activating bacterium-specific naïve T cells (27, 42). Therefore, interfering with DC activity would be advantageous for virulent bacteria, enabling them to survive and disseminate efficiently inside the host (1, 17).

Previously, we have shown that *Salmonella* enterica serovar Typhimurium, the causative agent of a typhoid-like disease in the mouse, can escape from antigen presentation by DCs (47). We observed that vacuoles containing virulent serovar Typhimurium inside DCs do not colocalize with lysosomal markers (47). These data suggested the avoidance of lysosomal degradation as a mechanism likely to be responsible for the escape of antigen presentation in DCs (47). This escape mechanism seems to be restricted to virulent strains, because attenuated *Salmonella* strains, such as mutants of the *phoP*/*Q* locus, fail to escape from DC processing and presentation (37). Considering that this locus encodes a two-component regulatory system that controls expression of virulence genes encoded within *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2, respectively) (16, 28), it is likely that these genetic elements could be responsible for interfering with DC function. SPI-1 and SPI-2 encode type three secretion systems (TTSS) that translocate effector proteins (also encoded by SPI-1 and -2) into the cytoplasm of host cells. These effector proteins subvert cellular metabolism to mediate bacterial entry and survival inside host cells. Translocation of effector proteins by the SPI-2 TTSS increases intracellular bacterial survival by preventing the fusion of *Salmonella*-containing vacuoles (SCV) with lysosomes (48).

Consistent with this notion, it has been recently shown that strains of *Salmonella* in which the entire SPI-2 region has been deleted are rendered unable to evade presentation of bystander antigens by DCs to MHC class II (MHC-II)-restricted T cells in vitro (6). Here we extend these findings by evaluating the mechanism by which SPI-2-encoded genes of serovar Typhimurium contribute to the escape from presentation of bacterium-expressed antigens by murine DCs to MHC-I- and MHC-II-restricted T cells, both in vitro and in vivo. To approach this question, we deleted the entire SPI-2, as well as specific genes contained within this genetic region that are needed for the assembly of TTSS and for the interference with vesicular trafficking in host cells. Our results show that each of the mutations on SPI-2 impaired the ability of serovar Typhimurium to escape from antigen processing and presentation by DCs. The mechanism responsible seems to be the loss of the ability to avoid intracellular degradation by lysosomes. Consistent with these findings, SPI-2 mutants showed decreased vir-

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ulence in vivo, as demonstrated by reduced tissue colonization and increased bacterium-specific T-cell and antibody responses. In addition, infection with each of the SPI-2 mutated strains led to protective immunity against challenge with the wild-type (WT) virulent *Salmonella* strain. Our data support the notion that the ability to interfere with DC function promotes serovar Typhimurium pathogenicity by avoiding bacterial degradation and activation of adaptive immunity. We have identified some of the virulence factors of serovar Typhimurium that mediate this strategy and which subvert DC function and could promote systemic disease in the host.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased and maintained at the animal facilities of P. Universidad Católica de Chile. OT-I (8) and OT-II (40) transgenic mice with a specific T-cell receptor (TCR) for H-2K^b/OVA$_{257-264}$ and I-A^b/OVA$_{323-339}$, respectively, were provided by R. Steinman. SM1 transgenic mice expressing a TCR specific for I-A^b/FliC$_{427-441}$ (33) were provided by S. McSorley. Animal work was performed according to institutional guidelines.

Bacterial strains. *Salmonella enterica* serovar Typhimurium 14028s (herein WT) was used as the parental strain (47). Mutants for *spiA*, *spiC*, and SPI-2 were generated by allelic exchange (9). Primers designed according to the serovar Typhimurium LT2 sequence (32) were as follows (5′ to 3′): for disruption of *spiA*, GGGGTATAAAATGGTAGTAAATAAACGTTTAATCTTAATTTGT AGGCTGGAGCTGCTTCG and ACTCTTTGGATTAACCATGAGATATG CCATTATTTACTACCATATGAATATCCTCCTTAG; for disruption of *spiC*, TTTGATAGAAACTCCCATTTATGTCTGAGGAGGGATTCATTGTAGG CTGGAGCTGCTTCG with GCCAGGTGTTTTTCTATCTCAATAGCAATA AGCTCAGAGCCATATGAATATCCTCCTTA; for disruption of SPI-2, GCG CATAATGTCAATCGAGCAACTTTTTGCCTTCCAGGTCTGTAGGCTGG AGCTGCTTCG and CCAAAAGCATTTATGGTGTTTCGGTAGAATGCGC ATAATCCATATGAATATCCTCCTTAG. Genomic deletions were confirmed by PCR using primers up- and downstream from the exchanged site.

To complement the *spiA* and *spiC* mutants, the complete coding sequences of these genes were amplified using the following primers (5′ to 3′): for SpiA, SpiA-F (GCCCATGGTTAACCATGAGATATGC) and SpiA-R (GGCCATG GTAGTAAATAAACGTTTA); for SpiC, SpiC-F (ATGTCTGAGGAGGGAT TCATGC) and SpiC-R (CGGAATTCTTATACCCCACCCGAAT). The products were inserted into the low-copy-number pACYC184 vector. Expression of both complemented genes was controlled by the endogenous promoter by cloning SpiC promoter with primers PSpiC-F (GCGAATTCAATGCTTCCCTCCA GTT) and PSpic-R (GCCCATGGAAATGGGAGTTTCTATC) as described elsewhere (7). Ovalbumin (OVA)-expressing and green fluorescent protein (GFP)-expressing *Salmonella* strains were obtained by transformation with pOVA and pGFP and evaluated by Western blotting, using an OVA-specific rabbit anti-antiserum (ICN, Biomedicals Inc.) and fluorescence microscopy, respectively (47).

Reverse transcription-PCRs (RT-PCRs) and Western blotting. cDNAs obtained from total RNA from bacterial cultures grown overnight in N salts medium at pH 5 (2, 18) were tested for amplification of *spiA* and *spiC* using primers SpiA-F with -R and SpiC-F with -R, respectively. Protein secretion by the SPI-2-encoded TTSS was evaluated by Western blot detection of SseB in supernatants of bacteria cultures, as previously described (20).

Antigen presentation assays, bacterial survival, and DC viability. As previously described (47), bone marrow-derived DCs (day 6 of culture) were pulsed for 4 h with OVA-expressing wild-type or SPI-2 mutant *Salmonella* strains at a multiplicity of infection (MOI) equal to 50 and treated with gentamicin (100 μg/ml; Sigma) to remove extracellular bacteria. As a control, DCs were loaded with various MOIs (5, 10, 25, 50, and 100) of live or heat-killed (65°C for 1 h [35]) *Salmonella* strains. After 12 h of culture, DCs were cocultured with either 1×10^5 B3Z or OT4H.2D5 (herein, OT4H) T-cell hybridomas, which are specific for H-2K^b/OVA$_{257-264}$ and I-A^b/OVA$_{265-280}$, respectively (29, 45). T-cell activation of transgenic T cells was evaluated by coculture of pulsed DCs with either OT-I or OT-II T cells (1×10^5) obtained from lymph nodes (LNs) of transgenic mice. Interleukin-2 (IL-2) release was measured after 20 h of DC–T-cell coculture (26). To evaluate bacterial infectivity and survival, 1×10^3 gentamicin-treated DCs were permeabilized for 30 min with 0.1% Triton X-100 in phosphate-buffered saline (PBS) and plated on LB agar 1 and 8 h postinfection. At the same time points, DC viability was determined by trypan blue exclusion.

Detection of bacterium-derived pMHC complexes. DCs were infected at an MOI of 50 for 4 h with either wild-type or SPI-2 mutant *Salmonella* strains that expressed OVA, treated with 100 μg/ml gentamicin to remove extracellular bacteria, washed at 4°C, and incubated for 16 h at 37°C in the presence of 100 μg/ml gentamicin. As a control for DC function upon bacterial pulse, DCs were copulsed with nonrecombinant WT Salmonella and 10 ng/ml of SIINFEKL peptide. Controls for background staining were unpulsed DCs and DCs pulsed with nonrecombinant WT *Salmonella*. DC surface H-2K^b/SIINFEKL complexes were detected by staining with anti-CD11c–phycoerythrin (PE) (clone HL3; Pharmingen) and supernatant from 25-D1.16 cells, a mouse-derived hybridoma which secretes a monoclonal antibody specific for pMHC H-2K^b/SIINFEKL (39). After washing, cells were stained with goat anti-mouse immunoglobulin G (IgG)–fluorescein isothiocyanate (Pharmingen), and the percentage of H-2K^b/SIINFEKL-positive cells in the CD11c$^+$ population was determined by fluorescence-activated cell sorter analysis.

Confocal and electron microscopy. DCs pulsed (MOI equal to 50) with wild-type or SPI-2 mutant *Salmonella* strains that expressed GFP were stained with anti-CD11c–PE, washed with gentamicin-supplemented PBS, fixed with 2% paraformaldehyde, and permeabilized with Triton X-100 (0.5% Triton in 5% fetal calf serum–PBS). The lysosomal marker LAMP-2 was detected by a rabbit antiserum (Zymed Laboratories, Inc.) and revealed with a rhodamine Red-X-labeled goat anti-rabbit IgG (Molecular Probes). DCs were examined on a Zeiss LSM 5 Pa confocal microscope. Semiquantitative analysis was performed by counting DCs showing *Salmonella*–LAMP-2 colocalization on several randomly selected fields, and fluorescence extension analyses were performed using the Carl Zeiss LSM 5 Examiner software. DCs pulsed for 4 h with either wild-type or SPI-2 mutant *Salmonella* strains were prepared as previously described (47) and analyzed on a Phillips Tecnai 21 electron microscope.

In vivo attenuation assays. Mice (6 to 8 weeks of age) were orally inoculated with 100 μl of PBS containing 10^6 CFU of either OVA-expressing wild-type or SPI-2 mutant *Salmonella* strains, grown at logarithmic phase, by intragastric gavage with a 20-mm feeding tip. This dose had been previously shown to induce an immune response by oral infection with attenuated *Salmonella* strains (35). Uninfected control mice received an equivalent volume of PBS. At day 5 postinfection, serum anti-OVA IgG and organ colonization were measured by enzyme-linked immunosorbent assay and plating on LB-ampicillin agar, respectively. Activation of naïve T cells in mice challenged with wild-type or SPI-2 mutant *Salmonella* strains was evaluated on single-cell suspensions from spleens and LNs by measuring gamma interferon (IFN-γ) and IL-2 release in response to stimulation for 72 h with peptides derived from OVA (OVA$_{257-264}$ and OVA$_{265-277}$) or flagellin (FliC$_{427-441}$), at a concentration equal to 10 ng/ml. This peptide concentration had been previously shown to be appropriate to expand peptide-specific T-cell populations (23, 34). For ex vivo antigen presentation assays, mice were challenged in the footpads with 10^5 CFU of OVA-expressing wild-type or SPI-2 mutant *Salmonella* strains. After 48 h, cell suspensions obtained from popliteal LNs were used to measure H-2K^b/SIINFEKL complexes on the CD11c$^+$ population as described above. To test the capacity of LN antigen-presenting cells (APCs) to activate T cells ex vivo, IL-2 release was measured on cocultures consisting of 10^5 LN-derived cells and either OT-I or OT-II T cells. For protection assays, mice were orally infected with 10^6 CFU OVA-expressing SPI-2 mutant strains or with PBS as a control. At day 14, mice were orally challenged with 10^8 CFU of wild-type *Salmonella* pOVA; this dose is 1,000-fold higher than the 50% lethal dose reported for this strain following oral inoculation (19). Mouse survival was evaluated within a period of 7 weeks.

In vivo T-cell proliferation and cytokine production. Single-cell suspensions obtained from spleens and LNs of either OT-I, OT-II, or SM1 transgenic mice were stained with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) and intravenously (i.v.) injected into syngeneic C57BL/6 recipient mice. Each splenocyte suspension was adjusted to a final volume of 500 μl containing 1×10^6 Vα2$^+$ CD8$^+$ (for OT-I), Vα2$^+$ CD4$^+$ (for OT-II), or Vβ2$^+$ CD4$^+$ (for SM1) CFSE$^+$ cells, as determined by flow cytometry (22, 41). Cells were injected into each recipient mouse, and 24 h later 1×10^5 CFU of OVA-expressing wild-type or SPI-2 mutant *Salmonella* strain cells were i.v. injected. Spleens were extracted from recipient mice 3 days after bacterial injection, and OT-I, OT-II, and SM1 T-cell proliferation was evaluated by dilution of CFSE labeling in the respective CD8$^+$ or CD4$^+$ population (22, 41). Detection of in vivo IFN-γ production by OT-I transgenic T cells was evaluated as previously described (46). Briefly, splenocytes obtained from mice previously transferred with OT-I T cells as described above and challenged with *Salmonella* were incubated with 10 nM OVA$_{257-264}$ in the presence of brefeldin A (2.5 μg/ml) for 6 h. Next, cells were washed and stained with anti-CD8–PE and fixed in 2% paraformaldehyde. After washing, cells were permeabilized with PBS–2% bovine serum albumin–0.5% Saponin, incubated with anti-IFN-γ–biotin (clone

XMG1.2), washed again, and stained with streptavidin-Tc. Staining for IFN-γ in the $CFSE^+$ population was determined by gating on $CD8^+$ cells.

RESULTS

Generation of *spiA*, *spiC*, and SPI-2 serovar Typhimurium mutants. To evaluate the role of the SPI-2 TTSS and SPI-2-encoded effectors on the capacity of serovar Typhimurium to interfere with antigen presentation by DCs, mutant strains of *Salmonella* carrying a deletion of either *spiA* or *spiC* were generated. The SpiA protein (SsaC) is a component of the outer membrane ring of the TTSS needle complex encoded by SPI-2. Deletion of the *spiA* gene impairs TTSS function, rendering bacteria unable to translocate effector molecules into the host cell cytoplasm (38). One of these effectors is SpiC (SsaB), an acidic protein also encoded by SPI-2 (21, 51). Although SpiC also participates in effector protein translocation by the TTSS (13), in addition it plays a role in serovar Typhimurium pathogenesis by inhibiting vesicular trafficking to prevent fusion of SCV with lysosomes. Accordingly, *spiC* mutant strains show impaired intracellular survival in macrophages (48) and colocalize with lysosomal markers (54).

Deletion of *spiA* and *spiC* from the chromosome was confirmed by PCR (not shown) and by RT-PCR of total RNA obtained from strains grown in N salt medium at pH 5.0, which induces expression and secretion of SPI-2-encoded effector proteins to the culture supernatant (2, 18). As shown in Fig. 1A, *spiA* or *spiC* transcripts could not be detected in the respective mutant strain. As a control for transcription of both coding sequences, an SPI-2 null mutant was also generated (Fig. 1A). In these mutants, translocation was evaluated by Western blot detection of SseB, an effector protein encoded by SPI-2 and secreted by the SPI-2 TTSS. Consistent with previous observations (13), a significant reduction on SseB secretion was observed for the Δ*spiA*, Δ*spiC*, and ΔSPI-2 *Salmonella* mutants (Fig. 1B). SseB secretion could be restored to wild-type levels upon complementation in *trans* with *spiA* and *spiC* genes for each respective null mutant (Fig. 1B).

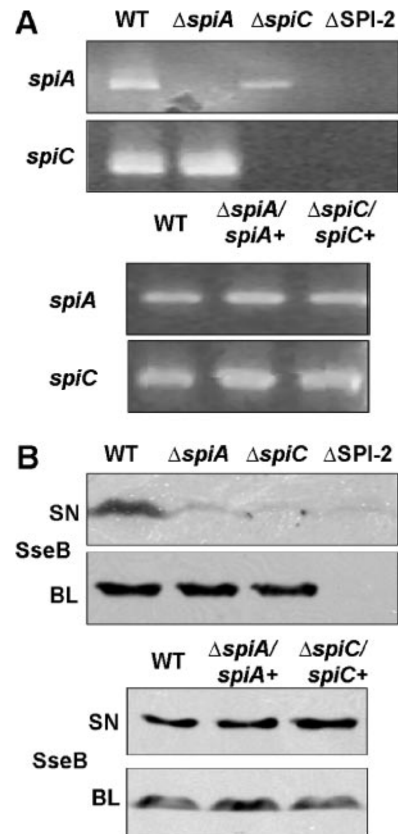

FIG. 1. Transcriptional and functional characterization of *S. enterica* serovar Typhimurium SPI-2 mutants. A. *spiA* and *spiC* RT-PCR amplification was absent on mutant strains (upper panels) and restored by complementation (lower panels). B. Western blot analyses show that secretion of SseB by SPI-2-encoded TTSS was significantly reduced in the supernatants (SN) of mutant *Salmonella* strains. Bacterial lysates (BL) are included as controls.

Antigens derived from serovar Typhimurium SPI-2 mutants are efficiently processed and presented by DCs. To evaluate whether SPI-2-encoded effectors and TTSS are important for the capacity of *Salmonella* to evade antigen presentation, DCs were infected with OVA-expressing wild-type or mutant strains of serovar Typhimurium. Bacterial load in infected DCs was measured by gentamicin protection assays (47, 53). One hour after infection, Δ*spiA*, Δ*spiC*, and ΔSPI-2 null mutant strains invaded DCs with equivalent efficiency as the wild-type strain (Fig. 2A, left panel), which is consistent with previous reports (38, 48). In contrast, 8 hours postinfection a significant reduction of intracellular bacterial load for the three SPI-2 mutants was observed (Fig. 2A, left panel). Evaluation of DC survival by trypan blue exclusion at each time point after *Salmonella* infection showed no significant decrease in DC viability compared to uninfected cells (Fig. 2A, right panel). These data suggest that, although DCs internalize equivalent amounts of wild-type and SPI-2 *Salmonella* mutants, a deficiency in SPI-2 function impairs bacterial survival inside DCs.

When the capacity of infected DCs to present bacterial antigens to T cells was tested, neither K^b/OVA- nor I-A^b/OVA-specific T-cell lines were activated in response to DCs infected by wild-type *Salmonella* expressing OVA (Fig. 2B, upper panels), which is consistent with our previous data (47). In contrast, DCs infected with SPI-2 mutants expressing OVA were able to efficiently activate both $CD8^+$ and $CD4^+$ OVA-specific T cells (Fig. 2B, upper panels). Similarly, when OT-I and OT-II transgenic T cells were used instead of T-cell hybridomas, only DCs pulsed with SPI-2 mutant strains of *Salmonella* expressing OVA were able to activate T cells (Fig. 2B, lower panels). The observation that mutations on SPI-2 impaired the ability of *Salmonella* to prevent activation of T cells by DCs was independent of the MOI, as shown by assays in which the number of bacteria used to infect DCs was titrated (Fig. 2C). Furthermore, interference with T-cell activation by DCs required live bacteria, because heat-killed wild-type *Salmonella* showed no differences with live or heat-killed SPI-2 mutants at each of the tested MOIs. No measurable IL-2 was released by T cells in response to DCs infected with *Salmonella* strains which do not express OVA (data not shown). The absence of T-cell activation by DCs infected with wild-type *Salmonella* was not due to nonspecific bacterium-mediated inhibition of T cells, as supported by the observation that *Salmonella*-infected DCs pulsed with exogenous SIINFEKL peptide were able to efficiently activate T cells (47) (data not shown).

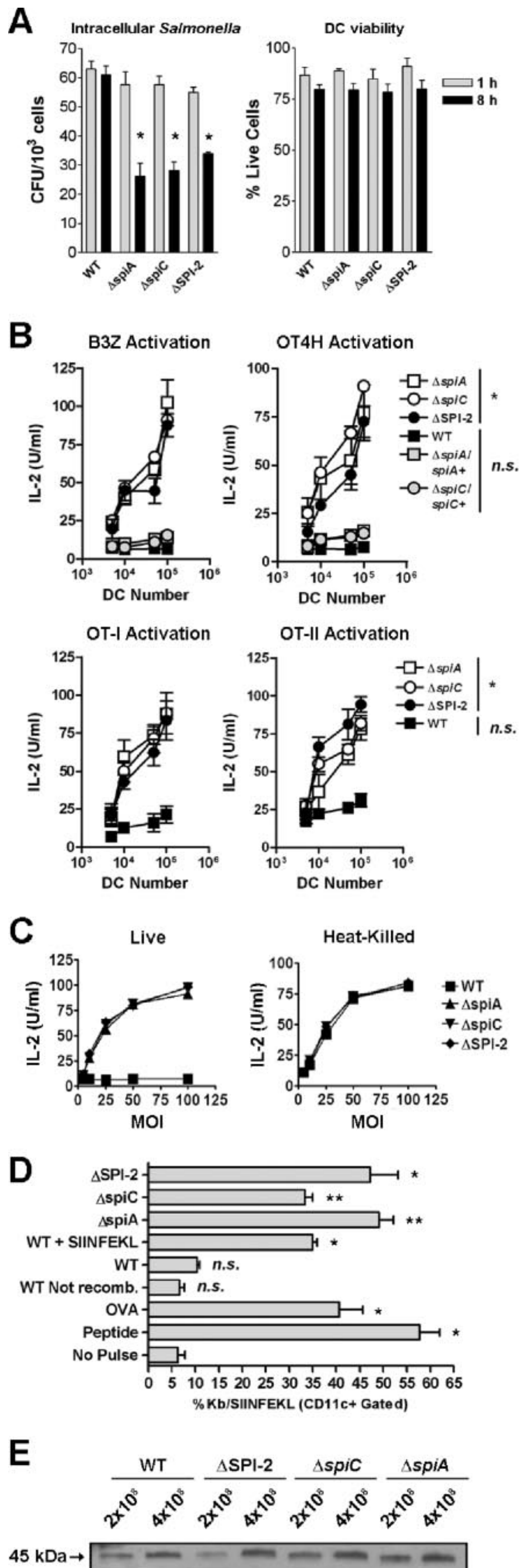

FIG. 2. SPI-2 mutant strains of *Salmonella* fail to evade antigen presentation by DCs. A. DCs were infected at an MOI of 50 for 4 h, washed, and incubated to evaluate *Salmonella* infection and survival capacity after 1 and 8 h by gentamicin protection assay (left panel). In addition, DC viability was determined at the same time points by trypan blue exclusion assay (right panel). *, $P < 0.05$, Student's *t* test, relative to WT *Salmonella*. B. Activation of T-cell hybridomas (B3Z and OT4H; upper panels) or transgenic T cells (OT-I and OT-II; lower panels) in response to DCs pulsed as above with OVA-expressing *Salmonella* and left in culture for 12 h. *, $P < 0.05$; n.s., nonsignificant (Student's *t* test relative to WT *Salmonella*). C. IL-2 release by OT-II T cells in response to DCs challenged with different MOIs of live (left panel) or heat-killed (right panel) WT or SPI-2 mutant *Salmonella* strains. D. Density of H-2K^b/SIINFEKL complexes on the surface of DCs treated as described above. OVA- and peptide-pulsed DCs were used as positive controls. Unpulsed DCs and DCs infected with the parental strain of *Salmonella* (14028s), which does not express OVA, were included as negative controls to show background staining. Data shown are the percentages of CD11c$^+$ cells positive for surface H-2K^b/SIINFEKL. *, $P < 0.05$; **, $P < 0.01$; n.s., nonsignificant (Student's *t* test relative to WT *Salmonella*). E. OVA expression on WT and SPI-2 mutant *Salmonella* strains was evaluated by Western blotting with two different amounts of bacterial protein extracts.

Complementation of ΔSpiA or ΔSpiC mutant strains with the respective wild-type gene restored the ability of *Salmonella* to evade antigen presentation to T cells (Fig. 2B, upper panels). These results suggest that the phenotypes shown by the serovar Typhimurium mutants are caused by the deletion of those specific genes and not due to nonspecific genetic modifications.

To evaluate the mechanism responsible for the absence of T-cell activation, generation of bacterium-derived pMHC complexes was evaluated using an H-2K^b/SIINFEKL-specific antibody. Consistent with the T-cell activation data, H-2K^b/SIINFEKL complexes were barely detected on the surfaces of DCs infected with wild-type *Salmonella* expressing OVA (Fig. 2D). The absence of H-2K^b/SIINFEKL complexes was not due to reduced expression of H-2K^b molecules on the surface of DCs, because a copulse with WT *Salmonella* and exogenous SIINFEKL peptide restored the assembly of H-2K^b/SIINFEKL complexes (Fig. 2D). In contrast to WT *Salmonella*, DCs infected with SPI-2 mutants expressing OVA showed a significant increase in the level of H-2K^b/SIINFEKL complexes on the surface (Fig. 2D). These data support the notion that the TTSS encoded by SPI-2 and the SpiC protein are critical for the evasion of antigen presentation in DCs. H-2K^b/SIINFEKL complexes were not detected in DCs infected with strains of *Salmonella* not expressing OVA (Fig. 2D). It is worth mentioning that differences in T-cell activation and generation of H-2K^b/SIINFEKL complexes were observed between wild-type *Salmonella* and each of the SPI-2 mutants despite equivalent OVA expression for each of these bacteria strains, as shown by Western blot analyses (Fig. 2E).

SPI-2 deficiency restores targeting of serovar Typhimurium to lysosomal degradation in DCs. The TTSS and effector proteins encoded by SPI-2 are important for bacterial survival and intracellular proliferation by arresting maturation of the SCV (51). While virulent strains of serovar Typhimurium reside inside DCs in vacuoles that lack lysosomal markers, efficient

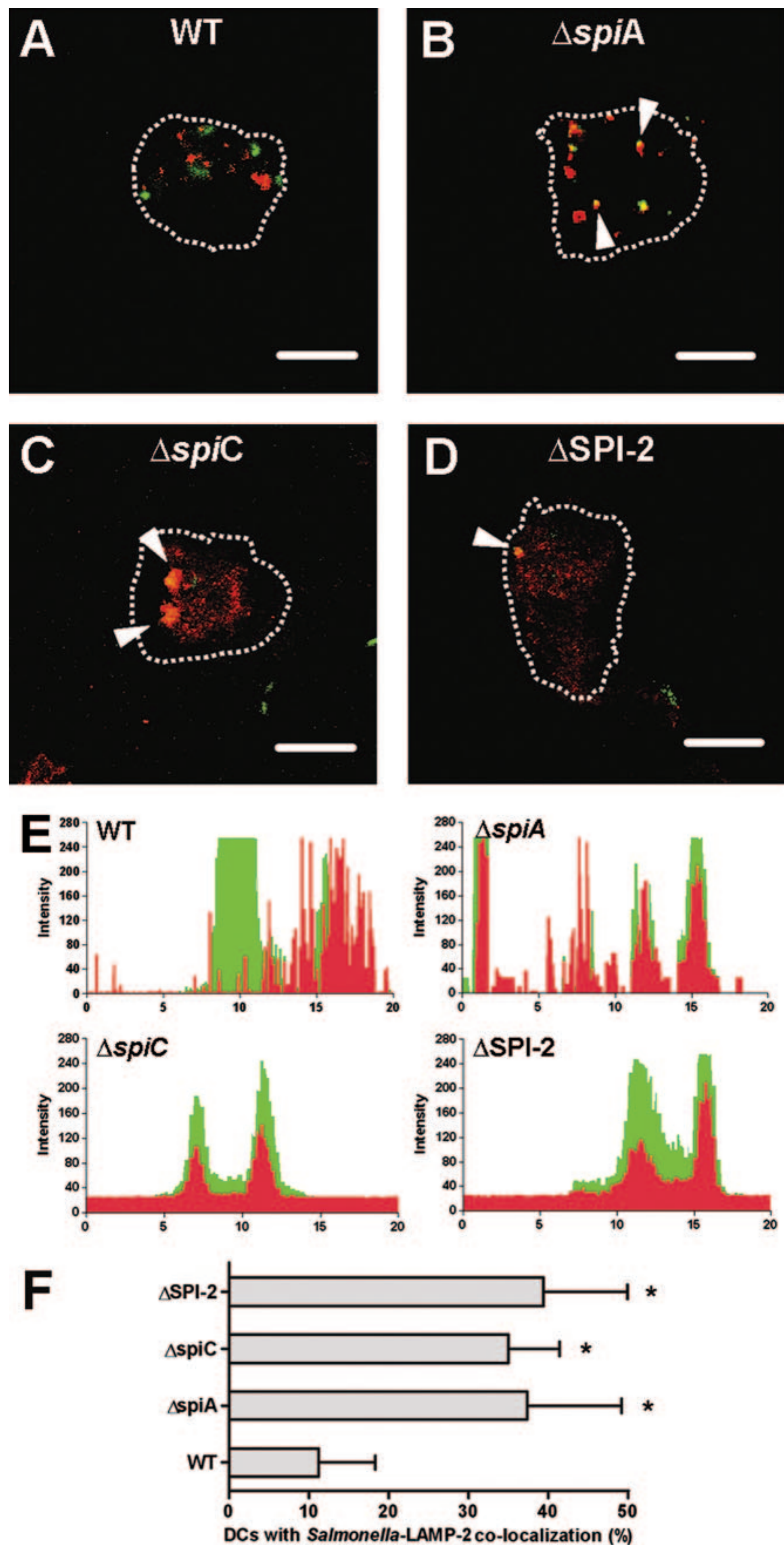

FIG. 3. SPI-2 mutant strains of *Salmonella* colocalize with lysosomal markers inside DCs. (A to D) Representative merged images show green (GFP) and red (LAMP-2) fluorescence in DCs infected either with GFP-expressing wild-type *Salmonella* (A), Δ*spiA* (B), Δ*spiC* (C), or ΔSPI-2 (D). Arrowheads show colocalization of GFP- and LAMP-2-derived fluorescence. Bar, 5 μm. (E) Fluorescence extension analyses for each *Salmonella* strain, showing the degree of overlay between green and red fluorescence. (F) Quantitative analysis of DCs showing *Salmonella*–GFP–LAMP-2 colocalization. *, $P < 0.05$ (Student's t test relative to WT *Salmonella*).

presentation of antigens derived from virulent *Salmonella* to T cells by DCs requires targeting bacteria for lysosomal degradation (25, 47).

Intracellular destination of *Salmonella* mutants in DCs was evaluated using wild-type and SPI-2 mutant *Salmonella* strains that express GFP. Quantitative confocal microscopy analyses showed that vacuoles containing GFP-expressing wild-type *Salmonella* did not colocalize with the lysosomal marker LAMP-2 in DCs (Fig. 3A, E, and F). In contrast, colocalization with LAMP-2 was observed for the three SPI-2 mutant *Salmonella* strains analyzed (Fig. 3B to D and F). As shown by fluorescence extension analysis, significant colocalization for fluorescence intensity derived from LAMP-2 and GFP inside DCs was observed only for ΔSpiA, ΔSpiC, and ΔSPI-2 serovar Typhimurium mutants but not for wild-type serovar Typhimurium (Fig. 3E).

Furthermore, electron micrographs showed an intact ultrastructure and no signs of degradation for wild-type serovar Typhimurium residing inside large vacuoles in DCs (Fig. 4A and B). In contrast, evidence for bacterial degradation was observed as electron light structures in the cytoplasm of ΔSpiA, ΔSpiC, and ΔSPI-2 mutant strains of serovar Typhimurium (Fig. 4C to H). Such structures have been previously associated with bacterial lysis (37).

The data presented above support the notion that functional SPI-2 expression is required for the capacity of *Salmonella* to survive inside DCs, avoiding lysosomal degradation and antigen presentation. Accordingly, lysosomal targeting and generation of bacterium-derived pMHC complexes were only observed when DCs were infected with SPI-2-deficient serovar Typhimurium.

SPI-2 deficiency leads to virulence attenuation and prevents serovar Typhimurium from evading T-cell activation in vivo. The capacity of *Salmonella* mutants to avoid activation of the immune system was measured in vivo. Mice were infected orally with 10^6 CFU of OVA-expressing wild-type or one of the SPI-2 mutant *Salmonella* strains. Bacterial colonization capacity as well as OVA-specific IgG titers and T cells were evaluated at day 5 postinfection. Mice infected with wild-type bacteria showed three to fourfold higher CFU in liver, spleen, and LNs compared to mice infected with any of the *Salmonella* SPI-2 mutants (Fig. 5A). These data are consistent with the notion that a deficient SPI-2 function attenuates virulence and reduces bacterial dissemination and organ colonization (6). However, whether attenuation due to SPI-2 mutations could lead to a reduced capacity of bacteria to evade adaptive immunity has not been evaluated. When humoral responses were measured in mice infected with OVA-expressing *Salmonella*, anti-OVA IgG titers elicited by each of the SPI-2 mutants were twofold higher than with wild-type virulent *Salmonella* (Fig. 5B). In addition, IFN-γ and IL-2 production by T cells specific for OVA- or flagellin-derived peptides could only be measured in mice challenged with ΔSPI-2, ΔSpiA, or ΔSpiC mutant *Salmonella* strains and not in mice infected with wild-type *Salmonella* (Fig. 5C). No measurable cytokine release was observed in the absence of antigenic peptide (not shown). Although T cells obtained from mice challenged with the ΔSpiA mutant showed lower cytokine release when stimulated with FliC peptide than with OVA peptides, the response of these cells to FliC was significantly higher than T cells obtained from mice challenged with WT *Salmonella* (Fig. 5C). The reduced FliC

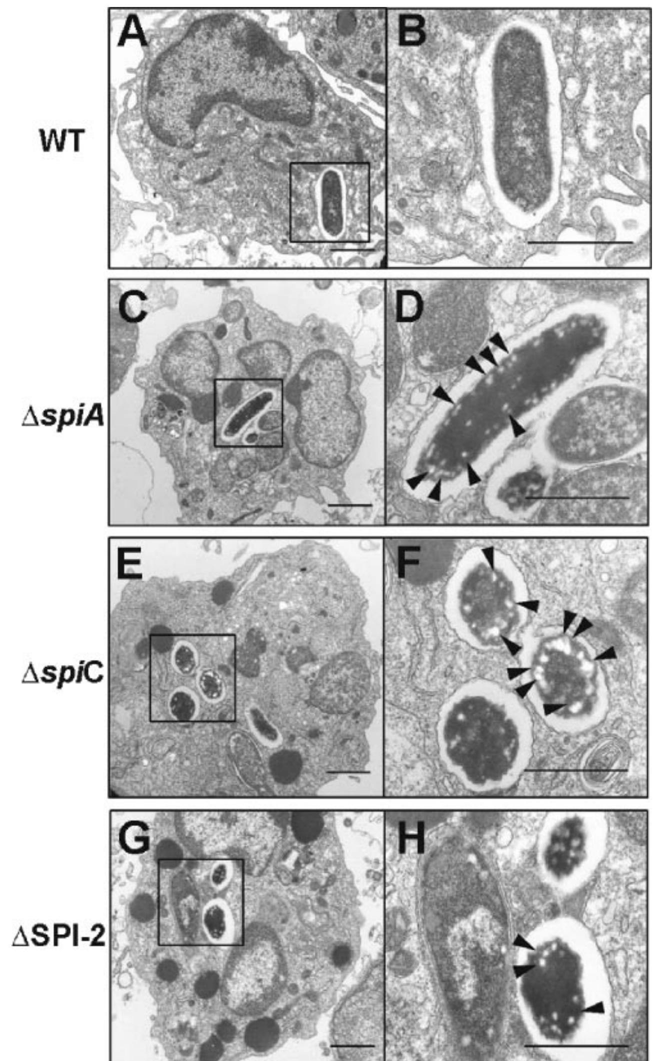

FIG. 4. Degradation signs are shown by SPI-2 mutant strains of *Salmonella* inside DCs. DCs were infected with wild-type or SPI-2 mutant *Salmonella* strains at an MOI of 50 and evaluated ultrastructurally by electron microscropy. Left panels show bacteria residing inside DCs. A zoom of the indicated area is shown in the right panels. Arrowheads show electron-light structures corresponding to degradation of bacteria. Bars, 500 nm.

response shown by mice challenged with the ΔSpiA mutant could be due to the expression of an immature form of flagellin by this mutant, which is likely to be less available for T-cell priming (31, 38).

Consistent with the reduced capacity shown in vitro by SPI-2 mutants to avoid antigen presentation to T cells (Fig. 2), APCs obtained from draining LNs were able to activate OVA-specific T cells only when mice were challenged with SPI-2 mutant *Salmonella* that expressed OVA (Fig. 5E). Activation of OVA-specific T cells was consistent with the presence of specific H-$2K^b$/SIINFEKL complexes on the surface of DCs residing in these LNs (Fig. 5D). These data support the notion that loss of virulence caused by SPI-2 deficiency could be due to an impaired ability of bacteria to avoid degradation by DCs, leading

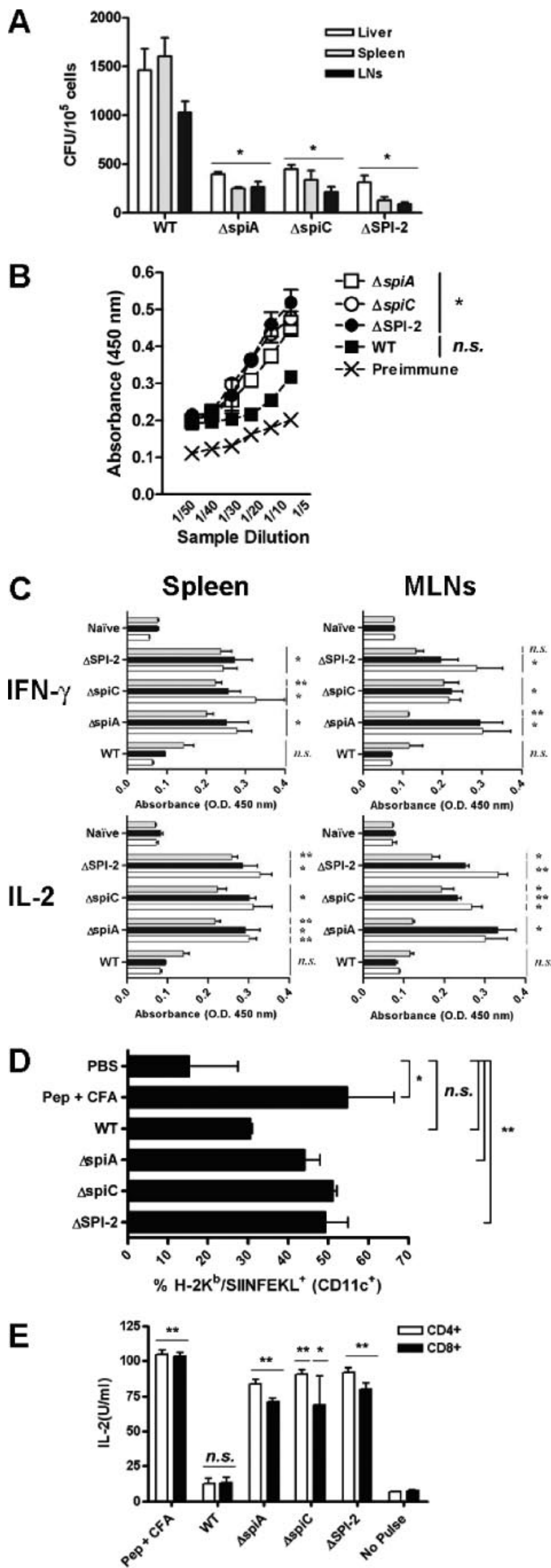

FIG. 5. SPI-2 mutant strains of *Salmonella* are attenuated in vivo and fail to evade T-cell activation. (A to C) Mice orally infected with OVA-expressing wild-type or SPI-2 mutant *Salmonella* strains were evaluated 5 days after infection for bacterial organ colonization (*, $P < 0.05$, Student's *t* test relative to WT *Salmonella*) (A), serum anti-OVA IgG (*, $P < 0.05$, Student's *t* test relative to preimmune serum) (B), and IFN-γ and IL-2 secretion by spleen and mesenteric LN cell suspensions after 72 h of stimulation with $OVA_{257-264}$ (white bars), $OVA_{265-280}$ (black bars), and $FliC_{427-441}$ (gray bars) peptides (*, $P < 0.05$; **, $P < 0.01$; n.s., nonsignificant, Student's *t* test relative to naïve mice) (C). (D) Frequency of H-$2K^b$/SIINFEKL$^+$ cells in the CD11c$^+$ DCs obtained from popliteal lymph nodes 48 h after a footpad challenge with OVA-expressing wild-type or SPI-2 mutant *Salmonella* strains. (E) Activation of CD8$^+$ and CD4$^+$ OVA-specific T cells by APCs obtained from popliteal lymph nodes 48 h after a footpad challenge with OVA-expressing wild-type or SPI-2 mutant *Salmonella* strains. *, $P < 0.05$; **, $P < 0.01$; n.s., nonsignificant (Student's *t* test relative to PBS-pulsed and unpulsed DCs in panels D and E, respectively).

to presentation of bacterium-derived antigens on MHC molecules and T-cell activation.

To further evaluate this notion, an in vivo T-cell proliferation assay was performed using OVA-expressing wild-type or SPI-2 mutant *Salmonella* strains. CFSE-labeled T cells obtained either from OT-I, OT-II, or SM1 transgenic mice were i.v. injected into syngeneic recipient mice (3). Twenty-four hours later, 10^5 CFU of the wild type or one of the SPI-2 mutant strains of *Salmonella* were administered by the same route (41). Three days after bacterial injection, proliferation of OT-I, OT-II, or SM1 T cells was evaluated in spleens of recipient mice by dilution of the CFSE signal. Proliferation of CD8$^+$ (OT-I) or CD4$^+$ (OT-II and SM1) was only observed in mice injected with SPI-2 mutant strains of *Salmonella* and not in mice challenged with wild-type virulent *Salmonella* (Fig. 6A). Differences in the efficiency of CFSE labeling and proliferation between OT-I, OT-II, and SM1 observed in these assays could be due to intrinsic features of each TCR transgenic system (5). To determine whether T cells that proliferated in response to bacterial challenge are functional, we measured IFN-γ secretion by OT-I transgenic T cells by intracellular cytokine staining. As shown in Fig. 6B, proliferating OT-I T cells were positive for intracellular IFN-γ. These data suggest that mutant bacteria were efficiently processed and presented to naïve transgenic T cells in vivo by endogenous APCs. Furthermore, the observation that equivalent results were obtained with OT-II and SM1 T cells suggests that the SPI-2-mediated bacterial evasion of antigen presentation to T cells is common to autologous (flagellin) and heterologous (OVA) antigens expressed by *Salmonella*.

Infection with SPI-2-deficient serovar Typhimurium promotes immunity against virulent wild-type serovar Typhimurium. The data presented above suggested that DCs that have captured SPI-2 mutants would be able to process and present bacterial antigens in vivo to prime *Salmonella*-specific T cells and confer immunity against a lethal infection with wild-type serovar Typhimurium. To evaluate this notion, mice were orally inoculated with each of the SPI-2 mutants or PBS as a control. Two weeks after immunization, mice were challenged with a lethal dose of wild-type serovar Typhimurium, and survival was measured over a period of 5 weeks. As shown

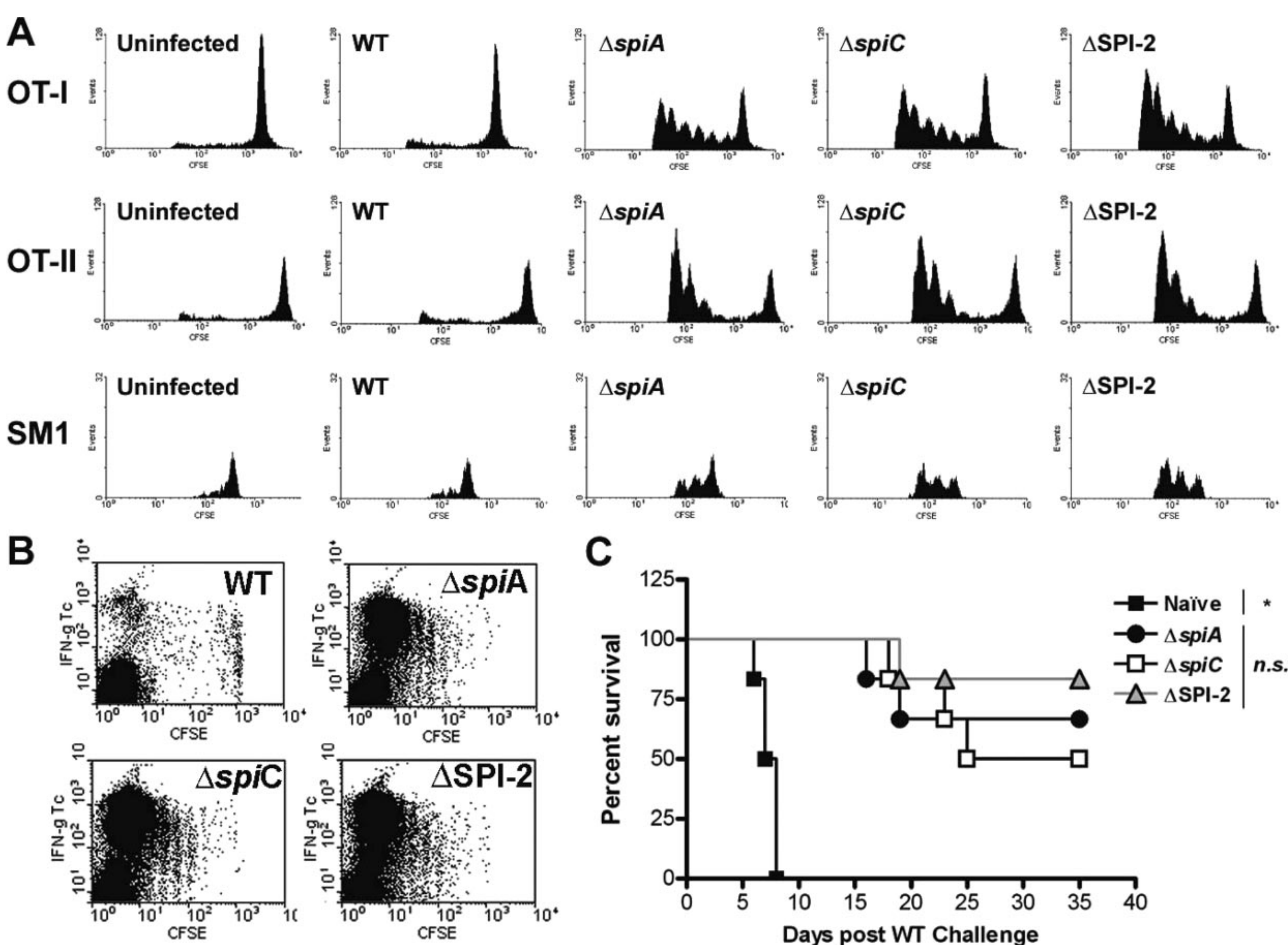

FIG. 6. Only infection with SPI-2 mutant strains of *Salmonella* leads to activation of naive T cells in vivo. C57BL/6 mice were adoptively transferred with CFSE-labeled OT-I, OT-II, or SM1 transgenic T cells and challenged 1 day later with virulent or SPI-2 mutant *Salmonella* strains. A. Mice were evaluated for in vivo activation of OT-I (upper panel), OT-II (middle panel), and SM1 (lower panel) transgenic T cells 3 days after adoptive transfer. Histograms show CFSE-derived fluorescence for $CD8^+$ T cells (OT-I transfer) or $CD4^+$ T cells (OT-II and SM1 transfer). B. Production of IFN-γ by OT-I T cells recovered from mice treated as described above based on intracellular staining. Dot plots show the fluorescence associated with CFSE and IFN-γ in the $CD8^+$ cell population. C. Mice were infected orally with PBS (naïve) or SPI-2 *Salmonella* mutant strains expressing OVA and challenged with a lethal dose of wild-type *Salmonella* 14 days later. Survival of infected mice was monitored over a 7-week period. Data shown are means of three independent experiments. *, $P = 0.05$; n.s., nonsignificant (log-rank test performed for each curve relative to the others).

in Fig. 6C, mice infected with the ΔSPI-2 mutant strain of serovar Typhimurium, and to lesser extent those infected with ΔSpiA and ΔSpiC mutant strains, were protected against a challenge with wild-type serovar Typhimurium. Infection with WT *Salmonella* led to death of naïve mice before the time of challenge (data not shown) (10).

DISCUSSION

Here we have provided evidence for the contribution of the TTSS and effector proteins encoded by the SPI-2 gene to the ability of virulent serovar Typhimurium to survive inside DCs and evade antigen processing and presentation to T cells. Our data support the capacity of virulent *Salmonella* to avoid fusion of SCV with DC lysosomes as the mechanism likely to be responsible for the evasive strategy of this pathogen. This feature is restricted only to virulent *Salmonella* strains, as suggested by the observation that attenuated strains fail to evade antigen presentation by DCs (47). The findings presented here are consistent with the notion that the ability of *Salmonella* to avoid bacterial degradation by DCs contributes to preventing the activation of naïve T cells, which could be crucial for the systemic infection caused by this pathogen.

To study the role of SPI-2 gene products in the interactions between *Salmonella* and DCs, we generated mutant strains by removing either the complete SPI-2 or two specific genes, *spiA* and *spiC*. A deficiency of SpiA prevents the assembly of the TTSS needle complex encoded by SPI-2 and impairs the translocation of bacterial effector proteins into the host cell cytoplasm (14, 38). One of these effectors corresponds to SpiC, which is required both to translocate effector proteins by the SPI-2-encoded TTSS (13) as well as to interfere with vesicular trafficking in host cells to prevent SCV-lysosome fusion (14, 38). Although wild-type *Salmonella* and each of the three SPI-2 mutants showed equivalent capacities to infect DCs, a significantly reduced capacity to survive inside DCs was demonstrated for all of the SPI-2 mutants (Fig. 2A). In a previous report, no differences in survival were observed for serovar Typhimurium and a $PhoP^c$ mutant after infecting DCs (36). Different mouse strains as DC sources (47) as well as the pleiotropic effect caused by the $PhoP^c$ mutation could account for this apparent discrepancy.

Consistent with previous reports (36, 44), under the experimental conditions applied during this study we did not observe significant DC death as result of infection with serovar Typhimurium (Fig. 2). However, evidence for induction of apoptosis as a strategy for interfering with DC function has been provided recently (50). The difference could be explained by the superior aggressiveness shown by strain SR-11 χ3041 used in reference 50, compared to strain 14028s used here (50% lethal dose, 2.4×10^4 and 10^5, respectively [determined in the same mouse strain and under equivalent experimental conditions]) (19, 30).

Taken together, our data suggest that SPI-2 *Salmonella* mutants fail to survive inside murine DCs. Accordingly, bacterial colocalization with lysosomal markers, generation of pMHC complexes loaded with bacterium-derived antigens, and activation of antigen-specific T cells were only observed for DCs infected with SPI-2 mutant strains of *Salmonella*. These results were consistent with the observations made in vivo where SPI-2 deficiency impaired organ colonization by bacteria and led to a significant increase in the adaptive immune response against *Salmonella*. Furthermore, infection with each of the SPI-2 mutants studied here led to significant protection against a lethal challenge with wild-type serovar Typhimurium (Fig. 6C). Although significant differences were only seen between naïve and each of the SPI-2-immunized mice, the observation that infection with SpiA and SpiC mutants led to different outcomes in mouse survival after challenge with virulent *Salmonella* (Fig. 6C) suggests that these mutations are not phenotypically redundant (38, 48).

The findings described here support the notion that interference with DC function is a mechanism of pathogenicity employed by virulent *Salmonella* to evade T-cell recognition. We showed that this feature of *Salmonella* virulence requires functional expression of SPI-2. Both the functional assembly of an SPI-2-encoded TTSS and the activity of effector proteins, such as SpiC, are critical for the capacity of *Salmonella* residing inside DCs to evade antigen presentation to T cells. Recently, evidence has been provided for the induction of inducible nitrogen oxide synthase in DCs as a result of the interaction with virulent *Salmonella* (6, 12). Considering that NO has been shown to inhibit T-cell function (11), it is conceivable that the avoidance of T-cell activation by virulent *Salmonella* infecting DCs could be at least in part the result of NO secretion. However, we think this scenario is unlikely, based on recent data showing no differences in the amount of NO secreted by DCs infected by wild-type and SPI-2 mutant strains of *Salmonella* (6). In addition, DCs infected with wild-type *Salmonella* were able to prime T cells when exogenously pulsed with MHC-I- and MHC-II-restricted peptides (data not shown) (47). Furthermore, the observation that virulent *Salmonella* keeps DCs from presenting bacterium-expressed antigens on MHC-I and -II, both in vitro and in vivo, would be sufficient to explain the absence of T-cell activation.

Our results are consistent with a recent study showing that virulent *Salmonella* can impair activation of MHC-II-restricted T cells by DCs in vitro (6). However, it is important to note that in that study OVA was not expressed by *Salmonella* and was used instead as an accompanying soluble antigen (6). Here we have further expanded on this notion by showing that virulent *Salmonella* is able to keep DCs from activating T cells that recognize antigens expressed by bacteria on class I and class II MHC molecules. In addition, we show that this mechanism of evasion has biological significance in vivo by adoptive transfer experiments using transgenic mice expressing TCRs that recognize antigens expressed by *Salmonella* on MHC class I and class II. Furthermore, we show that equivalent results are obtained in naïve (nontransgenic) mice challenged with wild-type virulent *Salmonella*, in which the pathogen suppresses cellular and humoral adaptive immune responses (Fig. 5). According to our data, this pathogenic feature of virulent *Salmonella* also depends on the functional expression of SPI-2. However, in addition to deleting the entire SPI-2 region, we have identified specific genes that seem responsible for the capacity of *Salmonella* to avoid presentation of bacterium-derived antigens by DCs, with the consequent impairment on T- and B-cell-mediated host immunity. In the absence of functional SPI-2- or SPI-2-specific genes encoding TTSS components or effector proteins, *Salmonella* is unable to prevent presentation of bac-

terium-expressed antigens to T cells by infected DCs. As a result of a deficiency in either SPI-2, *spiC*, or *spiA*, T cells are activated in vitro by infected DCs (Fig. 2). Furthermore, challenge of mice with SPI-2 mutant strains of *Salmonella* leads to activation of transgenic T cells in vivo and secretion of IFN-γ (Fig. 6). Accordingly, SPI-2-deficient *Salmonella* strains are unable to prevent antibody- and T cell-mediated immunity and fail to cause systemic infection and colonization of internal tissues (Fig. 5). In addition to the evasion of T-cell activation we report here, evidence has been provided recently for an additional mechanism displayed by *Salmonella* to interfere with T-cell function which seems to require bacterium–T-cell contact (49). Given that under our experimental conditions T cells interact with DCs that have previously captured *Salmonella* and extracellular *Salmonella* are removed by treatment with gentamicin, we think that in our assays direct *Salmonella*–T-cell interactions are unlikely. The observation that *Salmonella* can at least employ two distinct strategies to prevent T-cell activation underscores the molecular sophistication developed for this bacterial pathogen to evade adaptive immunity in the host.

Survival inside DCs in the absence of presentation of bacterial antigens to T cells is likely to be highly significant for the capacity of *Salmonella* to cause systemic disease in the host, because on one hand it prevents activation of adaptive immunity and on the other it could exploit DCs as reservoirs and means for bacterial dissemination from the site of infection to internal tissues. The involvement of DCs as key targets for *Salmonella* pathogenesis emphasizes the necessity to identify the virulence factors responsible for interfering with DC function, which would provide valuable insights for designing new strategies to prevent systemic infection caused by this pathogen.

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