Salmonella enterica Serovar Typhimurium-Induced Maturation of Bone Marrow-Derived Dendritic Cells

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Murine bone marrow-derived dendritic cells (DC) can phagocytose and process Salmonella enterica serovar Typhimurium for peptide presentation on major histocompatibility complex class I (MHC-I) and MHC-II molecules. To investigate if a serovar Typhimurium encounter with DC induces maturation and downregulates their ability to present antigens from subsequently encountered bacteria, DC were pulsed with serovar Typhimurium 24 h prior to coincubation with Escherichia coli expressing the model antigen Crl-OVA. Quantitating presentation of OVA epitopes contained within Crl-OVA showed that Salmonella-pulsed DC had a reduced capacity to present Crl-OVA-expressing E. coli for OVA(257-264)/Kb and OVA(265-277)/I-Ak presentation. In addition, time course studies of DC pulsed with Crl-OVA-expressing serovar Typhimurium showed that OVA(257-264)/Kb complexes could stimulate CD80/CD40 T-hybridoma cells for <24 h following a bacterial pulse, while OVA(265-277)/I-Ak complexes could stimulate OT4H T-hybridoma cells for >24 but <48 h. The phoP-phoQ virulence locus of serovar Typhimurium also influenced the ability of DC to process Crl-OVA-expressing serovar Typhimurium for OVA(265-277)/I-Ak presentation but not for OVA(257-264)/Kb presentation. Furthermore, pulsing of DC with serovar Typhimurium followed by incubation for 24 or 48 h altered surface expression of MHC-I, MHC-II, CD40, CD54, CD80, and CD86, generating a DC population with a uniform, high expression level of these molecules. Finally, neither the serovar Typhimurium phoP-phoQ locus nor lipopolysaccharides (LPS) containing lipid A modifications purified from phoP mutant strains had a different effect on DC maturation from that of wild-type serovar Typhimurium or purified wild-type LPS. Thus, these data show that Salmonella or Salmonella LPS induces maturation of DC and that this process is not altered by the Salmonella phoP virulence locus. However, phoP did influence OVA(265-277)/I-Ak presentation by DC infected with Crl-OVA-expressing serovar Typhimurium when quantitated after 2 h of bacterial infection.

Initiating a specific immune response to bacterial pathogens requires that bacterial antigens be captured, processed, and presented by antigen-presenting cells (APC) that activate naive T cells. Dendritic cells (DC) are the most potent APC for stimulating naive T cells (reviewed in reference 3) and thus are critical in initiating an immune response to a previously unencountered antigen. Immature DC can internalize and process bacteria for antigen presentation on both major histocompatibility complex class I (MHC-I) and MHC-II molecules (13, 41, 47, 48). This capacity of immature DC combined with their ability to migrate to lymphoid tissues after antigen capture (reviewed in reference 3) suggests that DC play a key role in initiating an immune response to bacterial infections. During migration, DC that have encountered inflammatory stimuli undergo a process of maturation in which they develop into fully competent APC. DC maturation involves downregulating their ability to capture and present antigens (44, 45, 56), upregulating MHC molecule synthesis (9, 41), altering MHC-II trafficking (9, 40), increasing the stability and surface expression of MHC molecules (9, 40, 41), increasing costimulatory molecule surface expression (13, 22, 41, 44, 45, 56), and enhancing cytokine secretion (10, 13, 22, 41, 56).

In order to survive the hostile environment encountered during the course of infection, bacterial pathogens coordinately regulate their gene expression (21, 31, 33). One such regulon, phoP-phoQ (34), promotes Salmonella enterica serovar Typhimurium virulence (15, 34, 35). The phoP-phoQ virulence regulon is a bacterial two-component regulatory system consisting of a membrane-associated sensor kinase (PhoQ) and a cytoplasmic transcriptional regulator (PhoP) (34). PhoP and PhoQ both positively and negatively regulate more than 40 gene products (4, 5, 37). Activation of the phoP-phoQ regulatory system is induced by Mg2+ limitation (16) and the low pH of the phagosomal environment within macrophages (MΦ) (2). PhoP-PhoQ regulates modifications of the lipid A moiety of lipopolysaccharide (LPS) (18), affects tumor necrosis factor alpha (TNF-α) expression by monocytes (18), regulates antimicrobial peptide resistance (15, 17), represses invasion genes (37), influences formation of spurious phagosomes (1) and bacterial survival within MΦ (35) after antibody-mediated opsonic uptake, and alters the efficiency of phagocytic processing of serovar Typhimurium by activated MΦ for peptide presentation on MHC-II molecules (54).

The previous observation that murine bone marrow-derived DC can process virulent serovar Typhimurium for peptide presentation on MHC-I and MHC-II molecules (47) led us to further investigate the properties of these Salmonella-pulsed DC. Here we examine the ability of serovar Typhimurium to induce maturation in murine bone marrow-derived DC and the effects of Salmonella phoP-regulated genes on DC maturation. This was assessed by analyzing the ability of DC pulsed with wild-type, phoP null (phoP−), or phoP constitutive (phoP+) serovar Typhimurium or of LPS purified from phoP mutant serovar Typhimurium strains containing lipid A modifications to present antigens from subsequently encountered bacteria. In addition, the influence of Salmonella infection of DC or of DC interaction with wild-type or mutant LPS on interleukin-12 (IL-12) production and surface expression of MHC and co-stimulatory molecules was analyzed. Finally, the influence of...
the phoP-phoQ locus, which controls numerous aspects of the pathogenesis of this bacterium, on antigen presentation by serovar Typhimurium-pulsed DC was investigated.

MATERIALS AND METHODS

Mice. C57BL/6 mice were bred in animal facilities at Lund University or purchased from Charles River Laboratories (Sulzfeld, Germany) and were used at 6 to 10 weeks of age. Bacterial strains, plasmids, and culture conditions. Bacterial strains used in this study are the wild-type serovar Typhimurium strain A1307 (35), the phoP

strains ATCC 15215 (35), the avirulent strain CSO22 (35), and the CTX whole-cell lysate. The presence of the avirulent phoP::phoQ insertion was confirmed by PCR using primers JLP-28f and JLP-28r (28) on genomic DNA from the T-bacterium cells, which secrete IL-2 upon specific recognition of the OVA(265-277) epitope. CD11c-expressing DC were incubated for 4 h at 37°C. The cultures were then washed thoroughly, and antigen-processing and presentation assays.

Antigen-processing and presentation assays. Antigen-processing assays were performed as described previously (47). Briefly, DC were resuspended in IMDM (without antibiotics) containing 5% fetal calf serum (FCS) and 5% GM-CSF and were seeded at 10^6 cells per well in 24-well plates. DC were stimulated with viable serovar Typhimurium, heat-killed bacteria on LB agar plates. DC were stimulated with viable serovar Typhimurium, heat-killed bacte-

Bacterial survival assays. DC were pulsed with the bacterial strains and were washed and treated with gentamicin as described for DC activation assays. After confluency reached of DC with Salmonella strains, two milliliters of a bacterial suspension at 10^9/ml was centrifuged at 1,700 g for 5 min, and the pellet was resuspended in 0.4 ml of PBS (pH 7.4) and lysed by sonication on ice using a Vibra Cell sonicator (Sonics and Material, Danbury, Conn.). The sonicated samples were centrifuged to remove cell debris, and the total protein content of the cleared lysate was determined using the bicinchoninic acid protein determination system (Sigma). Samples were normalized for protein content, serially diluted in PBS, and then seeded on 450 nm using a microplate reader (Molecular Devices Corp., Sunnyvale, Calif.). IL-12 p70 was measured as described for the IL-12 p40 subunit, except that 9A5 (Pharmingen) and biotinylated anti-phoP antibody (Sigma) were used as the capture and detection antibodies, respectively. Recombinant IL-12 was used as the standard and the concentration of IL-12 in test samples was calculated using the linear part of a standard curve run in parallel with the samples. The sensitivity of the assay was 45 pg/ml.

Detection of Crl-OVA expression by bacteria. An OVA-specific ELISA was used to quantify the level of Crl-OVA expression by the different Salmonella strains. Two milliliters of a bacterial suspension at 10^9/ml was centrifuged at 1,700 x g for 5 min, and the pellet was resuspended in 0.4 ml of PBS (pH 7.4) and lysed by sonication on ice using a Vibra Cell sonicator (Sonics and Material, Danbury, Conn.). The sonicated samples were centrifuged to remove cell debris, and the total protein content of the cleared lysate was determined using the bicinchoninic acid protein determination system (Sigma). Samples were normalized for protein content, serially diluted in PBS, and then seeded on 450 nm using a microplate reader (Molecular Devices Corp., Sunnyvale, Calif.). IL-12 p70 was measured as described for the IL-12 p40 subunit, except that 9A5 (Pharmingen) and biotinylated anti-phoP antibody (Sigma) were used as the capture and detection antibodies, respectively. Recombinant IL-12 was used as the standard and the concentration of IL-12 in test samples was calculated using the linear part of a standard curve run in parallel with the samples. The sensitivity of the assay was 45 pg/ml.

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RESULTS

DC exposed to Salmonella have a reduced capacity for MHC-I and MHC-II presentation of antigens derived from subsequently encountered bacteria. To investigate if exposure to live serovar Typhimurium down modulates DC's ability to

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process subsequently encountered bacteria for peptide presentation on MHC-I and MHC-II molecules, murine bone marrow-derived DC were pulsed for 2 h with wild-type serovar Typhimurium and cultured for 24 h before pulsing for 2 h with *Escherichia coli* expressing the model antigen Crl-OVA. Preexposure of DC to serovar Typhimurium reduced the cells’ ability to process subsequently added Crl-OVA-expressing *E. coli* for presentation of OVA(257-264) on K^b^ as well as presentation of OVA(265-277) on I-A^b^ (Fig. 1A and B). In addition, we investigated if exposure to phoP or phoP^-^-serovar Typhimurium strains also down modulates DC’s ability to process subsequently encountered bacteria for peptide presentation on MHC-I and MHC-II molecules. These results revealed that pulsing of DC with phoP or phoP^-^-serovar Typhimurium showed an ability similar to that of pulsing with wild-type serovar Typhimurium to decrease the capacity of DC to present OVA(257-264)/K^b^ (Fig. 1A) or OVA(265-277)/I-A^b^ (Fig. 1B) processed from subsequently encountered Crl-OVA-expressing *E. coli*. The reduced presentation capacity of DC exposed to bacteria is not due to loss of ability to stimulate T-hybridoma cells, since presentation of exogenously added OVA(257-264) or OVA(265-280) peptide is not affected by bacterial preincubation (Fig. 1C and D). Furthermore, coinoculation with *Salmonella* with DC for 24 h had no effect on DC viability, as determined by trypsin blue staining.

LPS purified from serovar Typhimurium also down modulated the ability of DC to process subsequently encountered Crl-OVA-expressing *E. coli* for OVA(257-264)/K^b^ and OVA(265-277)/I-A^b^ peptide presentation (Fig. 1E and F). Since phoP regulates structural modifications of serovar Typhimurium lipid A and alters the biological effects of the LPS, including TNF-α production by infected monocytes (18), we also tested whether this altered LPS induced DC maturation similar to that observed with purified wild-type *Salmonella* LPS. Purified LPS containing lipid A modifications controlled by PhoP resulted in a reduced capacity of DC to process subsequently encountered Crl-OVA-expressing *E. coli* for OVA(257-264)/K^b^ and OVA(265-277)/I-A^b^ presentation similar to that seen with LPS purified from wild-type serovar Typhimurium (Fig. 1E and F). The reduced presentation capacity of DC exposed to LPS is not due to loss of the ability to stimulate the T-hybridoma cells, since DC stimulated with any of the LPS preparations presented exogenously added OVA(265-280) or OVA(257-264) peptide equally well (data not shown). In addition, trypan blue staining showed that no reduction in viability of LPS-treated DC occurred during the 24-h incubation.

**DC encounter with *Salmonella* alters surface expression of MHC and costimulatory molecules.** In addition to reduced antigen-capture capacity, another hallmark of murine DC maturation is increased surface expression of MHC and costimulatory molecules (40, 41, 44, 45, 56). To investigate if *Salmonella* PhoP-regulated genes alter the DC surface expression of molecules important in stimulating T cells, DC were pulsed with viable wild-type, phoP^-^, or phoP^-^-serovar Typhimurium for 2 h and surface expression of MHC-I, MHC-II, CD80 (B7-1), CD86 (B7-2), CD40, and CD54 (ICAM-1) was analyzed following further incubation up to 48 h. At 24 h of total incubation time, the DC population shifted from one with heterogeneous levels of surface MHC-I, MHC-II, CD86, CD40, and CD54 to a more uniform population expressing a similar, high level of these molecules (Fig. 2A). In addition, the phoP mutants influenced surface expression of MHC-I, MHC-II, CD86, CD40, and CD54 in a fashion similar to that observed for wild-type bacteria (Fig. 2A). This altered surface molecule expression was also apparent when DC were infected with heat-killed wild-type bacteria or with wild-type bacteria in the presence of CCD (Fig. 2A), demonstrating that neither bacterial viability nor internalization was required for the observed effects. The expression pattern of the surface molecules shown in Fig. 2A on *Salmonella*-infected DC was similar at 24 h (Fig. 2A) and 48 h (not shown) of total incubation time.

The surface expression of CD80 on DC was also influenced
by *Salmonella* infection (Fig. 2B). However, unlike the case for the other surface molecules studied here, almost no alteration in surface expression of CD80 was detected at 24 h, while at 48 h after infection, the DC population consisted of more cells with a higher level of CD80 expression; the alteration of CD80 expression was similar regardless of the PhoP phenotype of the bacteria (Fig. 2B). In addition, little if any alteration in surface expression of CD80 was detected at 24 or 48 h after DC infection with heat-killed wild-type bacteria or when the initial pulse with bacteria occurred in the presence of CCD (Fig. 2B). This is in marked contrast to the effect of either heat-killed bacteria or inhibition of phagocytosis on the other surface molecules examined (Fig. 2A) and shows that bacterial viability and internalization are required for *Salmonella*-induced effects on CD80 surface expression but not for the effects observed on CD86, CD54, CD40, and MHC molecule expression.

Furthermore, coincubation of DC with LPS purified from the three *Salmonella* strains resulted in effects on surface molecule expression similar to those observed with intact bacteria at 24 h (Fig. 2C), that is, a shift from a population with heterogeneous levels of expression of MHC-II (Fig. 2C) and MHC-I and CD86 and CD40 (data not shown) to a more uniform population expressing a similar, high level of these molecules. Finally, a phagocytic stimulus per se was not responsible for the observed alteration in surface molecule expression following DC coincubation with serovar Typhimurium, as DC coincubated with 1-μm polystyrene beads did not alter surface expression of MHC-II (Fig. 2D), CD80, CD86, CD54, or CD40 (data not shown).

**T-cell-stimulatory capacity of peptide-MHC complexes on the surface of Salmonella-pulsed DC.** We next investigated the ability of MHC-I and MHC-II containing bacterium-derived peptides formed during a bacterial pulse to stimulate T-hybridoma cells after removing external bacteria. Thus, DC were pulsed with viable wild-type, phoP, or phoP* serovar Typhimurium expressing Crl-OVA for 2 h and were then washed to remove noninternalized bacteria. After 22 or 46 h of additional

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**FIG. 2.** DC coincubation with serovar Typhimurium alters expression of surface molecules important in signaling the immune system. (A and B) DC were coincubated with either viable wild-type, phoP, or phoP* serovar Typhimurium; viable wild-type serovar Typhimurium in the presence of CCD; or heat-killed (HK) wild-type serovar Typhimurium, as indicated to the left of each row of histograms. After an initial 2-h pulse with bacteria, the cells were washed, treated with gentamicin, and incubated an additional 22 h (A and B) or 46 h (B) before flow cytometry was performed. (C) DC were coincubated with LPS purified from either wild-type, phoP, or phoP* serovar Typhimurium as indicated. The surface expression of MHC-II molecules on DC after 24 h of stimulation with LPS (thick line) compared to that on DC incubated in medium only (thin line) is shown. (D) The surface expression of MHC-II molecules on DC 48 h after the addition of latex beads (dotted line) compared to that on DC incubated in medium only (thin line) or DC incubated with wild-type serovar Typhimurium (thick line) is shown. The upregulation of the different surface markers was not due to unspecific binding of Ig used for the fluorescence-activated cell sorter analysis, as appropriate Ig isotype subclass controls showed no difference in expression levels for infected and uninfected cells (not shown). Similar results were obtained in at least four independent experiments.
incubation, DC were fixed and OVA peptide presentation on MHC-I and MHC-II molecules was measured. Significant OVA(265-277)/I-A\(^{b}\) presentation by DC was detectable at 24 h (Fig. 3A), while no OVA(257-264)/K\(^{b}\) presentation above background was detectable at this time point (Fig. 3B). Furthermore, OVA(265-277)/I-A\(^{b}\) complexes generated from the \(phoP\) mutants stimulated OT4H T-hybridoma cells to a level similar to that observed for wild-type bacteria, suggesting that PhoP does not affect the level of surface peptide-MHC complexes remaining at this time point. After 48 h of total coinoculation time following the pulse of DC with bacteria, neither OVA(257-264)/I-A\(^{b}\) nor OVA(257-264)/K\(^{b}\) presentation by DC could be detected (data not shown). Thus, OVA(265-277)/I-A\(^{b}\) complexes are present on the DC cell surface for OT4H T-hybridoma cell stimulation for at least 24 h, while OVA(257-264)/K\(^{b}\) complexes are available on the cell surface at levels sufficient to stimulate CD8OVA T-hybridoma cells for less than 24 h following exposure to bacteria expressing Crl-OVA. Coincubation of \(Salmonella\) with DC for 48 h had no effect on DC viability, as determined by trypan blue staining.

Although PhoP did not influence the T-cell-stimulatory capacity of DC exposed to Crl-OVA-expressing serovar Typhimurium 24 h prior to quantitation of OVA(265-277)/I-A\(^{b}\) and OVA(257-264)/K\(^{b}\) presentation, it did, however, affect OVA(265-277)/I-A\(^{b}\) presentation by DC when quantitated after 2 h of infection with serovar Typhimurium (Fig. 4). These data show that \(phoP\) serovar Typhimurium was processed with greater efficiency for OVA(265-277)/I-A\(^{b}\) presentation than were \(phoP\)– bacteria expressing the same antigen (Fig. 4A). In contrast, both the \(phoP\) and \(phoP\)– bacteria were processed by DC with equal efficiency for OVA(257-264)/K\(^{b}\) presentation (Fig. 4B). The observed difference in processing efficiency for MHC-II presentation of the two bacterial strains was not due to lower antigen expression in the \(phoP\) strain, as \(phoP\) and \(phoP\)– bacteria showed relatively equal amounts of reactivity in an OVA-specific ELISA (Fig. 4A). Furthermore, it is likely that the difference in antigen-processing efficiency for MHC-II presentation following \(Salmonella\) internalization by DC is due to the \(phoP\) locus, since heat killing \(phoP\)– bacteria restore the level of OVA(265-277)/I-A\(^{b}\) presentation to that observed for viable \(phoP\)– bacteria (Fig. 4C). In contrast, heat killing the \(phoP\) strain did not alter the observed level of OVA(257-264)/K\(^{b}\) presentation (Fig. 4D). Finally, the observed difference in phagocytic processing efficiency for MHC-II presentation is not due to differences in internalization of the two bacterial strains, as equal numbers of \(phoP\) and \(phoP\)– \(Salmonella\) were recovered after a 2-h coincubation of bacteria with DC (Table 1).

### Intragranular survival of serovar Typhimurium within DC

Wild-type serovar Typhimurium can survive and replicate inside phagosomal compartments of infected M\(^{d}\) cells, whereas \(phoP\) and \(phoP\)– mutants are impaired in their ability to survive inside M\(^{d}\) following antibody-mediated opsonic uptake (35). However, little is known about the replication capacity of these strains in DC. To address the ability of infected DC to control intragranular replication of wild-type, \(phoP\), and \(phoP\)– serovar Typhimurium, DC were pulsed with bacteria for 2 h. After washing and gentamicin treatment to kill extracellular bacteria, intragranular survival was followed for 48 h. These data showed that the number of viable \(Salmonella\) recovered from infected DC did not increase over the 48-h time period examined (Fig. 6A). In addition, the number of bacteria recovered was similar regardless of the \(phoP\) phenotype. Coincubation of \(Salmonella\) with DC for 48 h had no effect on DC viability, while incubations of DC with or without bacteria for 72 h resulted in decreased DC viability. This latter result prevented obtaining meaningful data on bacterial survival in DC for more than 48 h of incubation.

To investigate if production of reactive nitrogen intermediates such as nitric oxide (NO) was a mechanism contributing to the ability of immature DC to restrict intragranular replication of serovar Typhimurium, the activity of iNOS was quantitated. Supernatants from DC infected with any of the three \(Salmonella\) strains did not increase iNOS activity, as assessed by quantifying NO\(^{−}\) accumulation (Fig. 6B), even though these cells were capable of iNOS induction, as demonstrated by NO\(^{−}\) accumulation after stimulation with gamma interferon (IFN-\(γ\)) and LPS (Fig. 6B). Thus, although DC can control serovar Typhimurium replication, at least within the 48-h time frame studied here, significant levels of NO do not appear to be induced by \(Salmonella\) infection of DC.

### IL-12 is produced by \(Salmonella\)-infected DC

IL-12 promotes the development of T-helper cell type 1 (Th1) responses and is a powerful inducer of IFN-\(γ\) production by T cells and NK cells (reviewed in reference 49). Since both IL-12 and IFN-\(γ\) are essential for resistance to \(Salmonella\)
infection in mice (23, 30), we tested the effect of Salmonella PhoP-regulated genes on production of IL-12 by DC. Thus, DC were pulsed with wild-type, \(\text{phoP} \) or \(\text{phoP}^{-}\) serovar Typhimurium, and supernatant samples were tested for the presence of the p40 subunit or the biologically active form of IL-12, the p70 heterodimer, following a total incubation time of 24 or 48 h. Although little IL-12 p40 was detected in culture supernatants from DC pulsed with bacteria and incubated for a total of 24 h, p40 was detected after 48 h of total incubation time (Fig. 7A). Neither bacterial internalization nor viability was required for production of IL-12 p40, as evident from the moderate levels of p40 detected when DC were coincubated with heat-killed wild-type bacteria or when the initial bacterial pulse was performed in the presence of CCD (Fig. 7A). However, although bacterial viability or internalization was not required to elicit IL-12 p40, the level of p40 produced was consistently higher when viable bacteria were used and when bacterial internalization occurred. Similar to the effects on DC maturation presented above, purified LPS from the bacteria was sufficient to elicit p40 production (Fig. 7B). Furthermore, all forms of LPS tested (LPS from wild-type serovar Typhimurium as well as LPS containing lipid A modifications purified from the \(\text{phoP}^{-}\) and \(\text{phoP}^{+}\) strains [18]) elicited IL-12 p40 secretion. Finally, eliciting p40 production by DC required stimulation with bacteria or LPS, as a phagocytic stimulus per se (1-μm polystyrene beads) was not sufficient to elicit p40 production by DC (data not shown).

Despite IL-12 p40 production by Salmonella-pulsed DC, only a modest increase in IL-12 p70 production was detected in culture supernatants after 48 h of DC coincubation (details in Fig. 7 legend) with wild-type, \(\text{phoP}^{+}\), or \(\text{phoP}^{-}\) serovar Typhimurium (Table 2). Furthermore, pulsing of DC with purified

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**TABLE 1.** Similar quantities of \(\text{phoP}^{+}\) and \(\text{phoP}^{-}\) bacteria are recovered following coincubation with serovar Typhimurium for 2 h

<table>
<thead>
<tr>
<th>Bacterium/DC ratio</th>
<th>(\text{phoP}^{+}) (No. of recovered bacteria)</th>
<th>(\text{phoP}^{-}) (No. of recovered bacteria)</th>
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<tbody>
<tr>
<td>15:1</td>
<td>(2.5 \times 10^{6})</td>
<td>(2.1 \times 10^{6})</td>
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<tr>
<td>50:1</td>
<td>(8.8 \times 10^{5})</td>
<td>(12.0 \times 10^{5})</td>
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<td>50:1 (+CCD)</td>
<td>(2.5 \times 10^{4})</td>
<td>(4.0 \times 10^{4})</td>
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</table>

*We coincubated \(10^{6}\) DC with \(\text{phoP}^{+}\) (MS7953) or \(\text{phoP}^{-}\) (CSO22) bacteria with rough LPS at the indicated bacterium/DC ratio for 2 h before lysing the cells and plating the bacteria onto Luria agar plates. The data are representative of at least three independent experiments.*
LPS from wild-type, \textit{phoP}, or \textit{phoP}c strains elicited levels of IL-12 p70 only slightly above those detected in culture supernatants of DC incubated in medium alone. Thus, although exposure of DC to serovar Typhimurium or purified LPS resulted in production of the p40 subunit, only a slight increase in secretion of the biologically active p70 heterodimer was induced, particularly when 1\,\mu g of purified LPS/ml was the stimulus.

**FIG. 5.** The CD11c\(^{+}\)MHC-II\(^{low}\) DC subpopulation is most active in phagocytosing serovar Typhimurium. DC were coincubated with \textit{Salmonella phoP}c bacteria expressing GFP at a bacterium-to-cell ratio of 15:1. After 2 h, extracellular bacteria were washed away. The DC were subsequently labeled with monoclonal antibodies, cells were fixed in 1\% paraformaldehyde and flow cytometry was performed. The dot plot shows the MHC-II and CD11c expression on infected DC and the regions R1 and R2, which were used to analyze the uptake of \textit{Salmonella phoP}c bacteria by CD11c\(^{+}\) cells. Histograms of flow cytometry analysis of CD11c\(^{+}\)MHC-II\(^{+}\) (R1 + R2), CD11c\(^{+}\)MHC-II\(^{low}\) (R1), and CD11c\(^{+}\)MHC-II\(^{high}\) (R2) cells are shown. The y axis represents the number of DC, and the x axis represents log fluorescence intensity. Infections were done in the absence (thick line) or presence (thin line) of CCD. The numbers shown represent the percentage of DC infected with bacteria in the absence of CCD (i.e., actively internalized bacteria). In this gate 0.5\% of the cells was infected when CCD was present. Similar results were obtained in at least four independent experiments.

**FIG. 6.** DC control intracellular replication of serovar Typhimurium within a 48-h period. (A) Following a 2-h pulse with either wild-type, \textit{phoP}, or \textit{phoP}c serovar Typhimurium, DC were washed and were either lysed to determine the number of bacteria recovered at the 2-h time point or treated with gentamicin before continuing the incubation for a total of 24 or 48 h. At these time points, DC were lysed and the number of viable bacteria remaining was determined by plating on LB agar plates. The actual initial bacterium-to-DC infection ratios were 15:1, 13:1, and 17:1 for wild-type, \textit{phoP}, and \textit{phoP}c bacteria, respectively, as determined by viable counts. (B) \textit{NO}_2\textsuperscript{-} was quantitated in the supernatants of DC that were incubated in medium alone or in medium containing IFN-\gamma (300 U/ml) and LPS (10\,\mu g/ml); or infected with wild-type, \textit{phoP}, or \textit{phoP}c serovar Typhimurium or with wild-type serovar Typhimurium in the presence of CCD or with heat-killed (HK) wild-type serovar Typhimurium; or incubated with 1-\mu m polystyrene beads, as indicated. DC were pulsed with bacteria for 2 h. The cells were washed and treated with gentamicin, and incubation was continued for an additional 46 h. At this time point the level of \textit{NO}_2\textsuperscript{-} in the culture supernatant was quantitated by using the method of Greiss with NaNO\textsubscript{2} as the standard. Data are presented as the means of triplicate samples \pm 1 standard deviation. Similar results were obtained in at least three independent experiments.
In the present study, we also show that the maturation of murine bone marrow-derived DC. For example, LPS purified from this bacterium induces maturation of CD11c+ DC that have been stimulated with viable wild-type, phoP, or phoPc serovar Typhimurium; viable wild-type serovar Typhimurium or were incubated in medium alone, as indicated. After an initial 2-h pulse with the bacteria, the cells were washed, treated with gentamicin, and incubated an additional 46 h. At this time point, the relative level of the IL-12 p40 subunit in the culture supernatant was quantitated by sandwich ELISA. (B) DC were either incubated in medium alone, as indicated. After an initial 2-h pulse with the bacteria, the cells were washed, treated with gentamicin, and incubated an additional 46 h. At this time point, the relative level of the IL-12 p40 subunit in the culture supernatant was quantitated by sandwich ELISA. Data are presented as the means of triplicate samples ± 1 standard deviation. Similar results were obtained in at least three independent experiments.

**DISCUSSION**

Immature DC are specialized in capturing and processing antigens into peptides for MHC presentation. Upon maturation, which can be triggered by microbial products or inflammatory cytokines, these cells function as initiators and modulators of the immune system (reviewed in reference 3). The DC activation process that results in a mature phenotype appears to be a crucial step in generating a specific immune response. It is therefore important to understand how DC are affected when they encounter pathogens. For example, it has been shown that the pathogen *Plasmodium falciparum* (51), *Trypanosoma cruzi* (53), and herpes simplex virus (43) prevent DC maturation and that the bacteria *Mycobacterium tuberculosis* (22), *Staphylococcus aureus* (54), *Salmonella enterica* (43), and *Chlamydia psittaci* (36) induce activation and maturation of DC. In the present study, we also show that the facultative intracellular bacterium *S. enterica* serovar Typhimurium or LPS purified from this bacterium induces maturation of murine bone marrow-derived DC. For example, *Salmonella*-stimulated DC showed a reduced capacity to process and present antigens into peptides for MHC presentation. This finding along with the observed *Salmonella*-induced alteration in surface molecule expression suggests that an encounter with serovar Typhimurium or purified *Salmonella* LPS indeed triggers maturation of murine bone marrow-derived DC.

Increased surface expression of MHC and costimulatory molecules following a pulsing of DC with *Salmonella* is consistent with that observed using other stimuli to induce DC maturation, such as exposure to TNF-α (44, 45, 56), gram-positive bacteria (56), or *M. tuberculosis* (22). Alteration in surface expression of MHC-I, MHC-II, CD86, and CD54 was maximal by 24 h of total incubation time. Neither viable serovar Typhimurium nor bacterial internalization was required to influence surface expression of these molecules. These results suggest that the observed increase in expression is due to surface interaction of bacteria with DC or soluble mediators released by bacterium-DC contact rather than phagocytic uptake of bacteria. This is consistent with the finding that only a small percentage of immature DC internalizes bacteria during a 2-h time frame (Fig. 5), while the whole population of immature DC undergoes alteration in MHC-I, MHC-II, CD86, and CD54 surface expression when analyzed at 24 h (Fig. 2).

In contrast, alteration in surface expression of CD80 had requirements different from those for the other surface molecules examined here. For example, alteration in surface expression of CD80 required that the DC be coincubated with viable serovar Typhimurium and also required active internalization of the bacteria. Furthermore, upregulation of CD80 expression was not significant until 48 h of total incubation time, with only a slight alteration in CD80 expression being apparent after 24 h. The mechanism for upregulation of surface molecules on DC following a pulse with serovar Typhimurium or *Salmonella* LPS is presently not known. However, TNF-α, a stimulus that induces DC maturation (44, 45, 56), was detected in culture supernatants of DC stimulated with bacteria or LPS (data not shown). It is possible that TNF-α produced by DC following a serovar Typhimurium infection may be involved in the maturation process. Clearly the regulation of surface molecule expression, and in particular the apparently differential regulation of CD80 expression, on DC following a serovar Typhimurium infection deserves further attention.

We and others (29) have found that bioactive IL-12 is produced following DC exposure to *Salmonella*. Although the quantity of IL-12 p70 detected was low and only detectable in supernatants collected after 48 h of incubation, both viable bacteria and purified LPS induced IL-12 p70 secretion by DC. IL-12 production by DC that encountered serovar Typhimurium would be favorable to the defense against this bacterium, as IL-12 production favors development of Th1 CD4+ T cells (reviewed in reference 49). *Salmonella*-specific T cells developing under the influence of IL-12 would produce cytokines such as IFN-γ that in turn enhance the microbicidal effects of phagocytic cells and facilitate bacterial elimination. It has also been demonstrated that DC maturation induces increased synthesis and stability of MHC-I and MHC-II (9, 40, 41). These observations, combined with previous data showing

**TABLE 2. IL-12 p70 production by DC 48 h after a bacterial pulse**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IL-12 p70 production (pg/ml)</th>
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<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Bacteria</td>
<td>154 ± 6</td>
</tr>
<tr>
<td>LPS</td>
<td>115 ± 7</td>
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See the legend to Fig. 7 for details of the incubation conditions. IL-12 p70 production in medium alone was 90 ± 5 pg/ml. The values shown are determined from a standard curve run in parallel and represent the means ± 1 standard deviation of triplicate supernatant aliquots from a single well infected with the indicated bacterial strain or stimulated with purified LPS from the indicated bacterial strain. The data are representative of three independent experiments.
that DC display OVA(257-264)/K b complexes for at least 4 h following a 2-h pulse with Ctrl-OVA-expressing serovar Typhimurium (47), led us to investigate the duration that peptide-MHC complexes are present on the DC surface following phagocytic processing of the bacteria. T-cell stimulation assays revealed detectable levels of OVA(265-277)/I-A b but not OVA(257-264)/K b complexes on the DC surface after at least 24 h following an initial pulse of DC with bacteria expressing Ctrl-OVA. OVA(265-277)/I-A b complexes were not, however, present at sufficient levels to stimulate the T-hybridomas cells 48 h after an initial bacterial encounter. Although several features of peptide-MHC–T-cell receptor interaction, such as the sensitivity of the two hybridomas, the quantity of specific peptide-MHC complexes formed, and/or the affinity of the T-cell receptor for the ligands, could influence the relative level of T-hybridoma cell stimulation, our data are consistent with biochemical data that measured an increase in the stability of total MHC-I and MHC-II molecules from 3 h to 9 h (41) and from 12 h to 36 h to 40 h (40), respectively, following the maturation of murine DC. DC exposure to maturation stimuli, including gram-positive (41) and gram-negative (Fig. 1 through 3) bacteria, results in DC expressing surface peptide-MHC complexes and costimulatory molecules enabling T-cell stimulation for a fairly short period following the antigen encounter. This suggests that to be able to activate both CD4 + and CD8 + Salmonella–specific T cells, DC need to meet specific T cells within ~24 h after the bacterial encounter to produce productive triggering of a Salmonella–specific immune response.

Bacterial pathogens synthesize numerous proteins that contribute to the ability of the bacteria to escape immune surveillance and persist inside host cells. For example, serovar Typhimurium expresses several genes upon contact with eukaryotic host cells (2, 8, 26). Furthermore, expression of some of these loci is under the control of the transcription regulator PhoP (34), which is critical for the virulence of serovar Typhimurium in murine models (15, 34, 35). PhoP also regulates lipid A modifications on LPS (18), affects the level of TNF-α production by monocytes (18), influences bacterial survival within Mφ after antibody-mediated opsonic uptake (35), and alters the efficiency by which Mφ process serovar Typhimurium for peptide presentation on MHC-II molecules (54). In the present study, we also show that PhoP affects the ability of DC to process Ctrl-OVA-expressing serovar Typhimurium for OVA (265-277)/I-A b presentation when quantitated following a short (2-h) coinubcation of bacteria with DC. In contrast, no effect of PhoP on OVA(265-277)/I-A b presentation was apparent when peptide presentation was quantitated after 24 h of total Salmonella-DC coinubcation. Furthermore, despite its effect on numerous aspects of bacterium-APC interactions as mentioned above, PhoP does not appear to influence IL-12 production by DC or surface expression of molecules important in signaling the immune system by Salmonella-infected DC. Finally, our results with LPS purified from wild-type or phoP DC. Finally, our results with LPS purified from wild-type or

Such information is crucial to developing effective vaccines that use attenuated versions of bacteria, such as serovar Typhimurium, to induce specific immune responses to recombinant antigens.

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