Salmonella efficiently enter and survive within cultured CD11c$^+$ dendritic cells initiating cytokine expression

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While *Salmonella* infects macrophages, this cell population may not be the only one important for disseminating intracellular bacteria from mucosal sites. Dendritic cells (DC) are present in the Peyer's patches and are mobilized following stimulation. Such characteristics would seem to be ideal for the dissemination of an intracellular, mucosal pathogen. However, it has been difficult to obtain sufficient numbers of DC to assess their ability to harbor *Salmonella* or to monitor DC *in vivo*. In the present study, this problem has been addressed by expanding DC *in vivo* using flt3 ligand, followed by the purification of CD11c$^+$ cells using antibody-coated magnetic beads or by fluorescence-activated cell sorting. *Salmonella dublin* were found to be efficiently internalized, and to survive and replicate within purified CD11c$^+$ DC, and also in CD11c$^+$, CD8α^+ or CD11c$^+$, CD11b$^+$ DC subpopulations. The ability of *Salmonella* to enter DC is of similar magnitude to that reported for macrophages, suggesting that this cell population could be an important host cell for dissemination of this pathogen from mucosal sites. Furthermore, infected DC responded to *Salmonella* by secretion of IL-1, IL-6 and IL-12. As such, these cells may be important sources of these cytokines during the host response against *Salmonella* infection.

Key words: Infectious immunity-bacteria / Dendritic cell / Cytokine / Rodent / Cellular activation

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1 Introduction

It is widely accepted that *Salmonella* species are intracellular pathogens of macrophages [1–3]. Survival and dissemination of pathogenic species of *Salmonella* into the peripheral tissues following invasion of the gut has been characterized as a host/pathogen interaction dictated by the ability of *Salmonella* to survive and replicate within infected macrophages. It is due to this intracellular sequestration and survival that *Salmonella* can avoid neutrophil- or antibody-mediated destruction which might be lethal for this pathogen.

A limited number of recent studies have suggested that dendritic cells (DC), located within mucosal lymphoid organs, may play an important role in the host/pathogen interaction involving intracellular bacterial infections [4–6]. These cells mobilize in response to infection [7, 8], and may have some phagocytic capability for certain microbes or particles [9–16]. The ability of DC to function as APC, and for their ability to serve as co-stimulators of T lymphocyte activation, are well documented [17, 18]. More recently, DC have been shown to be a significant source of cytokine production, in particular IL-12 [8, 10, 19–23]. This finding has added an important dimension to the proposed function of this cell population. Taken together, the properties of DC, coupled with their presence at mucosal sites such as the Peyer's patches [4, 6], suggest that invasion of mucosal surfaces by enteric pathogens such as *Salmonella* would result in interactions with cells of dendritic lineage.

Until recently, investigations into the role of DC in microbial pathogenesis have been limited by the availability of large numbers of purified DC [19], and compounded by the absence of unique markers that can identify this heterogeneous population [4, 6, 8]. Such limitations have been overcome by a more complete understanding of the maturation of these cells [17], and the specific observation that myeloid-derived DC precursors can be

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Abbreviations: DC: Dendritic cell(s) **RT:** Reverse transcription

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expanded from the peripheral blood or other tissues by *in vitro* culture in the presence of growth factors such as GM-CSF when used in conjunction with IL-4. In addition, soluble hematopoietic growth factors have been used to significantly expand populations of DC *in vivo*. For example, it has been recently shown that *in vivo* treatment with flt3 ligand expands multiple lineages of DC in humans or mice [24–28]. Such technologies have allowed investigations into the roles of DC that were previously considered technically challenging.

Here, we demonstrate that cultured DC can readily be infected by *Salmonella*, and that interaction with *Salmonella* results in the production of cytokines by these cells. These studies were made possible by methods employed to isolate large numbers of CD11c$^+$ DC following their generation *in vivo* by treatment of mice with flt3 ligand [24–28].

2 Results

2.1 Phenotype of flt3 ligand-induced DC

To isolate DC from flt3 ligand-treated mice [24–28], a magnetic separation system was employed using the mAb N418 [4, 6, 8, 25], coupled to magnetic microbeads. These purified CD11c$^+$ cells demonstrated the characteristic morphology of DC (*i.e.* abundant cytoplasm, veiled and dendritic processes, irregularly shaped nuclei, and multivesicular bodies) as revealed by phase contrast microscopy (results not shown), with less than 1 % of the cells being polymorphonuclear.

Furthermore, immunofluorescence analyses (Fig. 1) demonstrated that the isolated DC were CD11$^+$, MHC class II$^+$, and CD45R/B220$^-$, as expected. This phenotype is characteristic of DC and has been used to define this cell population as such [4, 6, 8, 25]. Approximately half of the DC expressed CD8α, and a smaller percentage (21 %) expressed CD11b. This result was also expected based on previously published work [25], and recently it has been suggested that DC expressing CD11c and CD8α represent lymphoid lineage DC and those expressing CD11c and CD11b represent myeloid lineage DC [26].

2.2 Entry of *Salmonella* into CD11c$^+$ DC

To determine whether these DC could serve as a host for *Salmonella*, purified CD11c$^+$ DC were cultured in the presence of varying numbers of wild type *Salmonella dublin* (strain SL1363). After eliminating extracellular bacteria, the number of viable, intracellular *Salmonella*

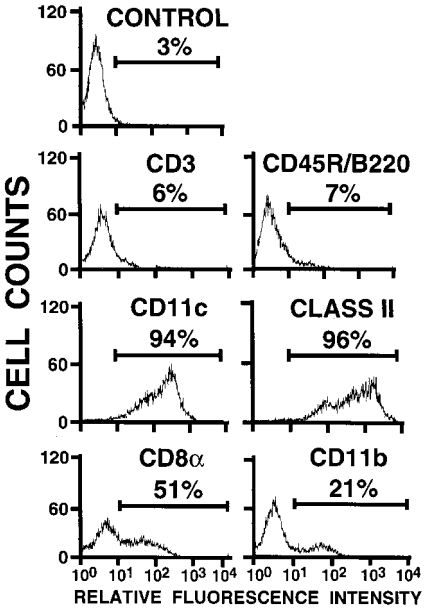

Figure 1. FCM analysis of cell surface markers or purified CD11c$^+$ DC. Immunofluorescence analyses were performed to define the surface markers present on CD11c$^+$ cells purified by magnetic separation. Purified DC were stained with the indicated FITC-conjugated antibody, and then analyzed for fluorescence using a FACSVantage flow cytometer. This analysis was performed three times with similar results.

were quantified by colony counts of lysed DC. As shown in Fig. 2, when varying numbers of *Salmonella* were co-cultured with DC for 90 min, these bacteria could enter and survive intracellularly in the presence of gentamycin-containing media. The ratio of intracellular *Salmonella* to total *Salmonella* added to each culture of 2×10^6 DC is of the order for that observed following invasion of cultured macrophages by this pathogen [29]. The ability of *Salmonella* to enter DC is particularly noteworthy when compared to the number of viable bacteria found in cultures of normal lymphocytes which were used to control for incomplete killing of bacteria by gentamycin or nominal uptake of bacteria by nonphagocytic cells. The dose-dependent nature of intracellular invasion by *Salmonella* is somewhat complicated by cell death when high numbers of wild-type organisms were used. Typically, cultures infected with 10^8 *Salmonella* had cell viabilities between 60 % and 75 %, whereas cultures exposed to 10^7 *Salmonella* or less had greater than 90 % viability at 1 h post infection.

To further demonstrate the intracellular nature of this infection, a non-invasive strain of *Escherichia coli* was unable to efficiently infect DC (< 0.001 %). In addition, DC which were infected with *Salmonella* and then permeabilized to allow gentamycin present in the culture

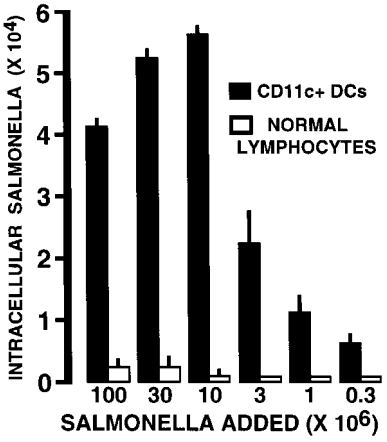

Figure 2. Quantification of viable, intracellular *Salmonella* within purified CD11c$^+$ DC. To quantify intracellular infection by *Salmonella*, 2×10^6 DC per well were exposed to varying numbers of viable *Salmonella* (ratios of 17:1 to 0.2:1, *Salmonella* to DC) which were added to the cultures for 90 min. After removal of extracellular bacteria, cells were incubated in gentamycin-containing medium to eliminate any remaining extracellular bacteria. Intracellular bacteria were quantified from cellular lysates using colony counts. Each dilution of *Salmonella* was performed in triplicate, and results are expressed as means ± SD. This experimental protocol was performed three times with similar results. Equal numbers of normal splenic lymphocytes were used to control for non-specific interactions with *Salmonella*.

media to access the intracellular space showed no viable intracellular bacteria.

Fig. 3A suggests that not only could *Salmonella* survive within CD, but that some replication of these bacteria could also occur intracellularly. This suggestion is supported by the fact that the numbers of viable intracellular *Salmonella* were increased at 90 min when compared to 1 h post infection. Furthermore, viable intracellular *Salmonella* were present even after 20 h of culture (Fig. 3A), demonstrating that DC can harbor bacteria for extended periods of time. The reduced number of intracellular *Salmonella* at 20 h compared to earlier time points might be explained, at least in part, by the observation that the viability of DC in these infected cultures was between 31 % and 48 %. Thus, as has been observed for cultured macrophages, *Salmonella* infection results in DC death.

To more fully define the CD11c$^+$ DC which were responsible for harboring *Salmonella*, fluorescence-activated cell sorting was used to isolate CD8α^- (negative) and CD8α^+ (high expressing) DC subpopulations. Surprisingly, each population had a similar ability to harbor *Salmonella* as determined 1 h post infection (Fig. 3B). This

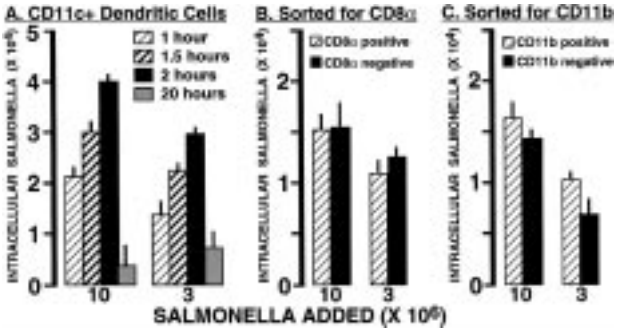

Figure 3. Kinetics of survival for viable, intracellular *Salmonella* within purified CD11c$^+$ DC. Identical methodologies were used to those described for Fig. 2 with the following exceptions. In (A), viable intracellular *Salmonella* were quantified at varying times post infection. In (B), CD11c$^+$ DC were sorted by FACS into CD8a negative or CD8a positive (high expressing) populations prior to quantifying viable intracellular *Salmonella* 1 h post infection. In (C), CD11c$^+$ DC were sorted by FACS into CD11b negative or CD11b positive (high expressing) populations prior to quantifying viable intracellular *Salmonella* 1 h post infection.

result was also obtained using CD11b$^-$ and CD11b$^+$ DC subpopulations (Fig. 3C). It has been suggested that DC expressing CD8α represent cells of lymphoid lineage, whereas those expressing CD11b represent myeloid lineage DC [26]. While these DC subpopulations may have different properties and/or functions, the ability to harbor *Salmonella in vitro* seems similar.

To further demonstrate the ability of *Salmonella* to infect DC, *in vitro* studies using total splenic leukocytes from flt3 ligand-treated mice were performed. Following treatment with flt3 ligand, splenic leukocytes were isolated and cultured with 30 or 100×10^6 *Salmonella* for 90 min. Extracellular *Salmonella* were eliminated by washing and incubation in media containing gentamycin. Fluorescence-activated cell sorting of CD11c$^+$ cell populations demonstrated the preferential localization of viable intracellular *Salmonella* in these cells as compared to the CD11c$^-$ and CD11b$^-$ population (Fig. 4). Clearly *Salmonella* could efficiently enter CD11c$^+$ DC, even in a mixed population of splenic leukocytes.

Finally, we wished to assess whether *Salmonella* were present within CD11c$^+$ DC following *in vivo* challenge with this pathogen. Flt3 ligand-treated mice were injected i. p. with *Salmonella*, and 24 h later splenic leukocytes were removed for fluorescence-activated cell sorting analysis. CD11c$^+$ populations were sorted from total splenic leukocytes, and the number of viable intracellular *Salmonella* determined by colony counting. As shown in Fig. 5, *Salmonella* were preferentially localized

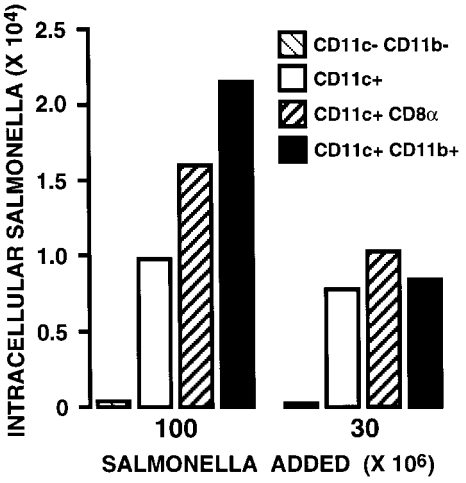

Figure 4. Quantification of viable intracellular *Salmonella* in splenic leukocyte subpopulations infected *in vitro* with *Salmonella*. Splenic leukocytes were acutely isolated from flt3 ligand-treated mice and exposed to 30 or 100×10^6 *Salmonella* for 90 min. Splenic leukocytes were then sorted by fluorescence-activated cell sorting into CD11c and CD11b negative; CD11c positive; CD11c and CD8α positive; and CD11c and CD11b positive populations. After sorting, cells were lysed and viable intracellular *Salmonella* were quantified using colony count and presented as number of bacteria/2×10^6 sorted cells. This experiment was performed three times with similar results.

within the CD11c$^+$ populations compared to the CD11c$^-$ and CD11b$^-$ population. These results demonstrate that CD11c$^+$ DC can harbor this pathogen *in vivo*.

2.3 Cytokine response to *Salmonella* infection by CD11c$^+$ DC

It was observed that the majority of DC would aggregate *in vitro* in response to ratios of *Salmonella* to DC that resulted in only a small percentage of DC being infected (results not shown). This observation suggested that the infected DC were expressing soluble factors or cell surface molecules which effect neighbouring, uninfected cells. To begin to understand this response, we investigated cytokine expression by cultures of purified CD11c$^+$ DC following interaction with varying numbers of *Salmonella*. As shown in Fig. 6, semi-quantitative reverse transcription (RT)-PCR was used to analyze cytokine gene expression in purified CD11c$^+$ DC exposed to culture media alone or to varying numbers of *Salmonella*. As shown in Fig. 6, significant increases in IL-1 (22.3-fold increase for 30:1 ratio, as determined by densitometric analysis), IL-6 (tenfold increase for 30:1 ratio) and IL-12p40 (3.1-fold increase for 30:1 ratio) were observed

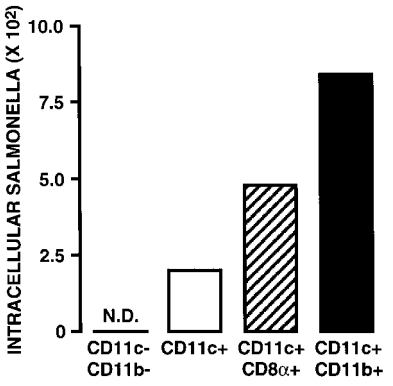

Figure 5. Quantification of viable intracellular *Salmonella* in splenic leukocyte subpopulations from *Salmonella*-infected mice. Mice treated with soluble human flt3 ligand (10 μg) for 9 days were injected i.p. with 1×10^5 *Salmonella*. After 24 h, splenic leukocytes were isolated and were sorted by FACS into CD11c and CD11b negative; CD11c positive; CD11c and CD8α positive; and CD11c and CD11b positive populations. After sorting, cells were lysed and viable intracellular *Salmonella* were quantified using colony count and presented as number of bacteria/2×10^6 sorted cells. N. D. signifies that bacteria were not detectable in this experimental group. This experiment was performed twice with similar results.

when compared to constitutive expression of control cultures. Conversely, nominal increases in IL-10 (1.5-fold increase for 30:1 ratio) or TNF-α (1.3-fold increase for 3:1 ratio) mRNA were observed following exposure to

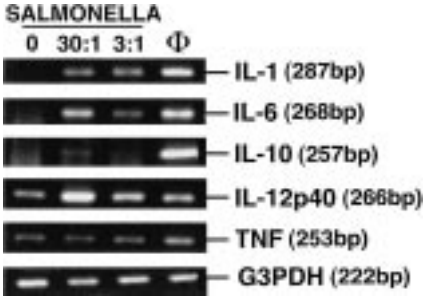

Figure 6. Semi-quantitative RT-PCR for cytokine mRNA expression in *Salmonella*-infected DC. RNA was isolated 9 h post infection from 2×10^6 DC infected with either 30:1 or 3:1 *Salmonella* to DC, and semi-quantitative RT-PCR performed. A positive control for each RT-PCR was also performed using *Salmonella*-stimulated macrophages (Φ). In addition, PCR amplification of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), was performed to ensure that similar amounts of input RNA and similar efficiencies of reverse transcription were being compared. These studies were performed four times with similar results.

Salmonella. Capture ELISA were then used to quantify secretion of these cytokines as shown in Fig. 7. These results largely parallel the mRNA findings in that significant increases in IL-1 (approximately 250 pg/ml), IL-6 (approximately 1200 pg/ml), and IL-12p40 (approximately 7000 pg/ml) were observed when DC interacted with *Salmonella*. Exposure of DC to *Salmonella* numbering between $30 \times 10^6 - 3 \times 10^6$ bacteria consistently produced the most significant cytokine secretion. Conversely, nominal amounts of IL-10 (approximately 60 pg/ml) and TNF-α (approximately 30 pg/ml) secretion were detected. For optimal cytokine secretion, exposure to viable organisms was required, since exposure to similar numbers of UV-killed *Salmonella* induced less than 5 % of the maximal IL-1, IL-6 or IL-12p40 secretion observed in Fig. 7.

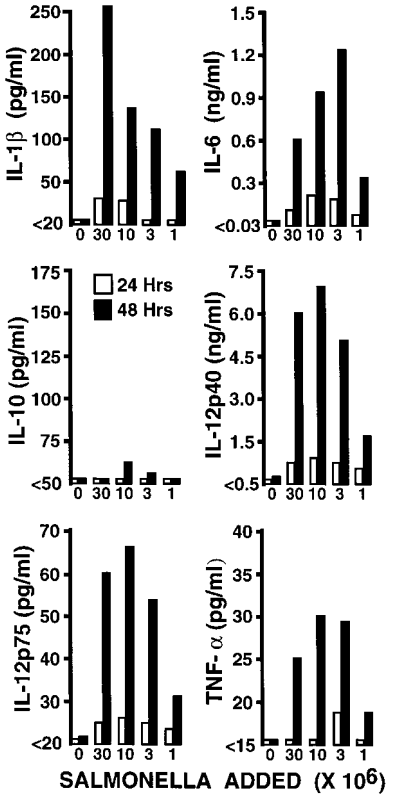

Figure 7. Quantification of cytokine secretion by *Salmonella*-infected DC. Secretion of IL-1β, IL-6, IL-10, IL-12p40, IL-12p75 and TNF-α by 2×10^6 DC infected with the indicated number of *Salmonella* was quantified using an appropriate capture ELISA for each. The sensitivities for each ELISA were determined using the appropriate recombinant cytokine diluted in culture medium and were found to be: 20 pg/ml for IL-1β, 30 pg/ml for IL-6, 50 pg/ml for IL-10, 50 pg/ml for IL-12p40, 20 pg/ml for IL-12p75 and 15 pg/ml for TNF-α. These studies were performed four times with similar results.

Surprisingly, most of the IL-12 secreted was in the form of IL-12p40 (approximately 7000 pg/ml), with significantly less heterodimer IL-12p75 being secreted (approximately 65 pg/ml). To further verify that only a limited amount of IL-12p75 was being secreted, a bioassay was also performed. This bioassay is based upon the ability of IL-12p75, but not IL-12p40, to induce the secretion IFN-γ in a dose-dependent manner. As shown in Fig. 8, the maximal amount of bioactive IL-12p75 induced by 10^6 *Salmonella* was 31 ($\pm$ 6) pg/ml. This value was similar to that observed using the ELISA for IL-12p75 (approximately 65 pg/ml). Thus, while exposure to wild-type *Salmonella* induces marked IL-12p40 secretion, the amount of IL-12p75 secreted is considerably less.

3 Discussion

The present work demonstrates that *Salmonella* can efficiently enter and survive within CD11c$^+$ DC. The ability of *Salmonella* to enter DC is of a magnitude similar to that reported for macrophages [29], suggesting that this cell population could be an important site for harboring intracellular bacteria *in vivo*. Populations of DC exposed to this intracellular pathogen respond by increased production of significant amounts of IL-1, IL-6 and IL-12p40.

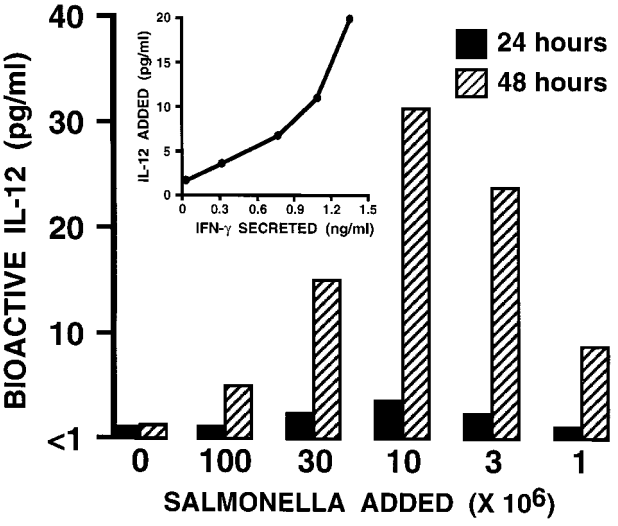

Figure 8. Quantification of bioactive IL-12p75 secretion by *Salmonella*-infected DC. A bioassay was performed to quantify the amount of IL-12p75 present in supernatants of 2×10^6 DC infected with the indicated number of *Salmonella*. Concentrations of IL-12 in experimental samples were determined by extrapolation of experimental values from a standard curve generated by adding known amounts of rIL-12 to splenic cultures, and measuring IFN-γ secretion. This experiment was repeated twice with similar results.

This cytokine secretion occurs within a population in which only a small percentage of cells contain viable intracellular organisms. Furthermore, interaction with viable organisms was required to elicit optimal cytokine secretion.

Several lines of evidence suggest that DC may be an important target for pathogenesis of wild-type *Salmonella in vivo*. These bacteria are known to be taken up by specialized epithelial cells, designated M cells, which overlie the Peyer's patches. Once inside the Peyer's patch, it has been suggested that *Salmonella* are disseminated as intracellular pathogens within macrophages. However, it has been noted that the Peyer's patches contain few macrophages, but relatively more DC in close proximity to M cells [4–6]. In addition, it has been noted that DC are rapidly recruited to mucosal sites of some microbial infections [7, 8]. Furthermore, DC are known for their mobility and efficiency with which they carry immunogens to draining lymph nodes [9, 10, 12–16]. Conversely, macrophages are not recognized for movement out of resident tissues, and are less efficient at presenting antigens to T lymphocytes. In the present study, we have shown that *Salmonella* can efficiently enter DC and that these bacteria can survive and replicate within these cells. Importantly, we demonstrate that *Salmonella* infection *in vivo* can result in DC harboring viable intracellular bacteria. Taken together, these studies suggest that DC are positioned, and possess characteristics, which would allow them to be important targets for intracellular survival and dissemination of *Salmonella*.

In addition, we demonstrate that cultures of infected DC secreted significant amounts of IL-1, IL-6 and IL-12p40. These results suggest that these cells may be important sources of these cytokines during the host response against *Salmonella* infection. It is important to note that the amount of IL-12p40 secreted was approximately 1000-fold higher than the amount of IL-12p75 secreted by cultured DC infected with *Salmonella*. Since a homodimer of IL-12p40 can be an antagonist of IL-12p75, it will be important to determine if co-stimuli are necessary to increase the percentage of IL-12p75 secreted.

Finally, this work combines several recent technical advances which allow the purification of large numbers of $CD11c^+$ DC. Using conventional techniques, DC are difficult to purify from normal tissues due to their small number and due to the loss of cell viability during involved isolation procedures. For this reason, studies using these cells have been limited by cell number and interpretation has been difficult due to contaminating cell populations. Here we describe technology which can be used to isolate very pur populations of $CD11c^+$ DC (approximately 95 %) at high numbers (5×10^7 per spleen) in a short period of time (approximately 4 h). Such technical advances should allow better assessment of the role played by these cells in immunity against infectious diseases.

Taken together, the present studies suggest that DC may be an important component of the host/pathogen interaction with *Salmonella*. The studies presented here, along with previous observations, suggest that DC are positioned, and posses characteristics, which would allow them to be important targets for intracellular survival and dissemination of *Salmonella*.

4 Materials and methods

4.1 Purification of $CD11c^+$ DC from flt3L-treated mice

To expand the population of DC present in peripheral lymphoid organs, BALB/c mice weighing 18 to 21 g (Charles Rivers, Wilmington, MA) were injected i. p. with 10 μg soluble human flt3 ligand (Immunex, Seattle, WA) for 9 consecutive days. This treatment reproducibly induced splenomegaly, lymphadenopathy and a dramatic increase in the numbers of $CD11c^+$ leukocytes, as has been extensively documented [24–28].

One day following cessation of treatment with flt3 ligand, mice were euthanized and spleens and mesenteric lymph nodes excised. Organs were injected with RPMI-1640 containing 15 mM Hepes and 1 mg/ml collagenase D (Boehringer Mannheim, Indianapolis, IN) using a 25 gauge needle, and then minced into small fragments. Tissue fragments were then incubated in the collagenase D solution at 37 °C for 20 min followed by manipulation to obtain single-cell suspensions. After washing, cells were suspended in RPMI-1640 containing 15 mM Hepes and 10 % FBS (Atlanta Biologicals, Norcross, GA), and incubated with magnetic microbeads conjugated to hamster anti-mouse CD11c mAb (clone N418, Miltenyi Biotec, Auburn, CA) for 20 min at 6 °C. $CD11c^+$ DC were then separated by passing the cell suspension over a magnetic-activated cell sorter (MACS) VS + column held in a VarioMACS magnetic separator (Miltenyi Biotec). The N418 mAb has been widely used as a marker for DC [4, 6, 8, 25], and was therefore selected for use in these studies. $CD11c^+$ DC adhering to the columns were then used for immunofluorescence or *in vitro* cultures.

4.2 Immunofluorescence and cell sorting

Immunofluorescence analyses (FACSvantage, Becton Dickinson, San Jose, CA) were performed to define the surface markers present on $CD11c^+$ cells purified by magnetic separation. FITC-conjugated antibodies for such analyses were purchased (PharMingen, San Diego, CA) and included anti-CD3 (clone 145-2C11), anti-CD8α (clone 53-6.7), anti-

CD11b (clone M1/70), anti-CD11c (clone HL3), anti-CD45R/B220 (clone RA3-6B2) and anti-class II MHC (clone 2G9). Control antibodies used for staining included FITC-conjugated rat IgG2a (R35-95) and FITC-conjugated hamster IgG (clone G235-2356).

Prior to staining, purified cells were incubated in siliconized tubes for 2 h at 37 °C in RPMI-1640 containing 15 mM Hepes and 10 % FCS to allow recovery of expression of receptors that may have been lost following collagenase treatment or following purification by magnetic beads. After incubation, cells were washed and stained with fluoresceinated antibodies.

To isolate subpopulations, purified $CD11c^+$ cells were stained with fluoresceinated anti-CD8α or anti-CD11b followed by cell sorting. Two populations (negative and high expressors) were isolated from each sorting experiment. In some experiments, acutely isolated splenocytes were stained with PE-labeled anti-CD11c and allophycocyanin-labeled anti-CD8α and anti-CD11b followed by cell sorting (FACSCalibur, Becton Dickinson). Four populations ($CD11c^+$; $CD11c^+$ and $CD11b^+$; $CD11c^+$ and $CD8\alpha^+$; $CD11c^-$ and $CD11b^-$) were isolated from each sorting experiment. Control antibodies used for staining included PE-conjugated hamster IgG and allophycocyanin-conjugated rat IgG2bκ.

4.3 Intracellular infection of magnetically purified DC by *Salmonella dublin*

To quantify intracellular infection by *Salmonella*, 2×10^6 DC per well were suspended in 0.5 ml RPMI-1640 containing 15 mM Hepes and 10 % FCS in 48-well culture plates. Varying numbers of viable *Salmonella dublin*, wild-type strain SL1363 (ratios of 50:1 to 0.2:1, *Salmonella* to DC) were added to the cultures for 90 min at 37 °C. Extracellular bacteria were washed off, and plates were then incubated in gentamycin-containing medium for 1 h to kill any remaining extracellular bacteria [30]. Gentamycin was selected as the antibiotic due to its limited uptake by eukaryotic cells [30], and so, the addition of gentamycin should have little effect on the viability of intracellular *Salmonella*. At the indicated times following 1 h incubation with gentamycin, the antibiotic-containing medium was removed. The DC were subsequently lysed with 0.5 % deoxycholate, and colony counts were performed of serial dilutions of cellular lysates on Luria agar to quantify the number of intracellular *Salmonella*. Each dilution of *Salmonella* was performed in triplicate, and results are expressed as mean colony counts ± SD. Viability of infected DC was assessed by trypan blue exclusion.

Equal numbers of normal splenic lymphocytes were used to control for nonspecific interactions with *Salmonella*. These lymphocytes were isolated by sequential passage over Sephadex G-10 columns to eliminate adherent cells followed by centrifugation on Hypaque-Ficoll gradients to remove red blood cells and polymorphonuclear leukocytes.

Additional controls to assure the intracellular localization of *Salmonella* were also performed. These included incubation of a non-invasive strain of *E. coli* (strain JM83) with DC for 90 min prior to the addition of gentamycin, and incubation of *Salmonella* with DC for 90 min prior to the addition of gentamycin with 0.01 deoxycholate to permeabilize the DC. Colony counts were then performed as described above.

4.4 Isolation and infection of total splenic leukocytes *in vitro*

Single-cell suspensions were prepared from acutely isolated spleens from mice injected i. p. with 10 μg soluble human flt3 ligand for 9 consecutive days. Red blood cells were removed by incubation in red blood cell lysing buffer (Sigma) for 10 min at room temperature. The resulting splenic leukocyte cell suspension was washed and 5×10^7 cells were exposed to 30 or 100×10^6 *Salmonella* for 90 min. Extracellular bacteria were washed off, and cells resuspended in gentamycin-containing medium to kill any remaining extracellular bacteria. Splenic leukocytes were then sorted into CD11c and CD11b negative; CD11c positive; CD11c and CD8α positive; and CD11c and CD11b positive populations. After sorting, cells were lysed and viable intracellular *Salmonella* were quantified by colony count.

4.5 Isolation and infection of total splenic leukocytes *in vivo*

Mice treated with soluble human flt3 ligand (10 μg) for 9 days were injected i. p. with 1×10^5 *Salmonella*. After 24 h, splenic leukocytes were acutely isolated from infected animals as described above, washed in gentamycin containing medium, and sorted into CD11c and CD11b negative; CD11c positive; CD11c and CD8α positive; and CD11c and CD11b positive populations. After sorting, cells were lysed and viable intracellular *Salmonella* were quantified by colony count.

4.6 Cytokine expression by DC following exposure to *Salmonella*

DC (10^7 per well) were placed in 6-well plates in RPMI-1640 containing 15 mM Hepes and 10 % FCS (without antibiotics). Varying numbers of *Salmonella dublin* (strain SL 1363) were added to the DC for 90 min at 37 °C. Extracellular bacteria were washed off, and infected cells were cultured for the indicated times in gentamycin-containing medium. Antibiotics were added to eliminate the possibility of contamination of cultures which were routinely maintained for 24–48 h. At the indicated times, cells were taken for RNA isolation and semi-quantitative RT-PCR [31–34], or culture superna-

tants were taken for ELISA, or bioassays to quantify cytokine secretion using techniques which have been previously described [29].

Densitometric analysis of the RT-PCR product bands was performed using NIH Image (obtained from the NIH Web site: http://rsb.info.nih.gov/nih-image). Each gel image was imported into NIH image by Adobe Photoshop (Adobe Systems, San Jose, CA), a gel-plotting macro was used to outline the bands, and the intensity was calculated on the Uncalibrated OD setting. Results are presented as the mean fold increase over those levels found in untreated cells.

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