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Francisella tularensis LVS-induced Interleukin-12 p40 cytokine production mediates dendritic cell migration through IL-12 Receptor β1

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ABSTRACT

Three cytokines use the IL-12p40 cytokine subunit namely: IL-12p70 (IL-12-comprised of IL-12p40 and IL-12p35), IL-23 (comprised of the IL-12p40 and IL-23p19 subunits) and homodimeric IL-12p40 (IL-12$(p40)_2$). Following activation, immature dendritic cells (DCs) upregulate the chemokine receptor Chemokine-C-Receptor 7 (CCR7), and migrate in response to homeostatic chemokines such as chemokine (C–C motif) ligand 19 (CCL19). Induction of the cytokine IL-12p40 in response to pathogen-exposure, likely in its homodimeric form, is one of the primary events that mediates migration of DCs in response to CCL19. Here we show that following exposure to *Francisella tularensis* Live Vaccine Strain (LVS), DCs produce IL-12p40 and promote the migration of DCs to the chemokine CCL19 in an IL-12Rβ1- and IL-12p$(40)_2$-dependent manner. Induction of IL-12p40 and resulting chemokine responsiveness in DCs is TLR2-dependent and coincides with the uptake of *F. tularensis* LVS and activation of DCs. Importantly, we show that IL-12Rβ1 signaling is required for DC migration from the lung to the draining lymph node following *F. tularensis* LVS exposure and coincides with accumulation of IL-12p40 expressing DCs in the draining lymph nodes. Together, these findings illustrate that IL-12p40 is induced rapidly in response to *F. tularensis* LVS and is required for DC migration through an IL-12Rβ1-IL-12$(p40)_2$ dependent mechanism.

1. Introduction

Francisella tularensis, a gram-negative facultative intracellular bacteria, is a highly infectious pathogen known to be the causative agent of the zoonotic disease, tularemia. The routes of *Francisella* infection include contact, ingestion or inhalation. However, inhalation with even low doses of airborne bacteria (<10 colony forming units, CFU) causes serious illness and therefore projects its use as a possible bioterrorism tool [1]. An *F. tularensis* Live Vaccine Strain (LVS) has been developed from the *F. tularensis* SCHU S4 strain, but is currently not licensed for use in humans [1]. Due to the absence of licensed vaccines against tularemia, much effort is directed towards understanding immune regulation in response to *F. tularensis*.

Three cytokines use the Interleukin (IL)-12p40 cytokine subunit namely IL-12p70 (IL-12-comprised of IL-12p40 and IL-12p35), IL-23 (comprised of the IL-12p40 and IL-23p19 subunits) and homodimeric IL-12p40 (IL-12$(p40)_2$). The IL-12p40 subunit cytokines play critical and distinct functions in the generation of adaptive T cell responses to pathogens. For example, IL-12$(p40)_2$ is required for DC migration and initiation of adaptive immune responses [2,3], while IL-12 and IL-23 are critical for generation of distinct T helper cell (Th) responses. The initiation of the host immune response to pathogen exposure is the activation of antigen presenting cells (APCs), mainly dendritic cells (DCs) by pathogen associated receptors and production of cytokines. Activated DCs upregulate chemokine receptors, specifically Chemokine-C-Receptor 7 (CCR7) and migrate to secondary lymphoid organs in response to the homeostatic chemokine such as chemokine (C–C motif) ligand 19 (CCL19) [4,5]. The migration of pathogen-activated DCs in response to CCL19 is critical for the generation of an effective adaptive immune response [6,7]. Recently, we and others have shown that following bacterial stimulation, DCs deficient in IL-12p40 fail to migrate toward CCL19 [2,3,8] and do not generate effective adaptive T cell responses [2,8]. Furthermore, migration of pathogen-exposed IL-12p40 gene-deficient DCs can be rescued by addition of IL-12$(p40)_2$ [2] and is mediated by its receptor IL-12 Receptor β1 (IL-12Rβ1) [9]. These data suggest that production of IL-12$(p40)_2$ by pathogen-activated DCs is a crucial first step in initiation of the host immune response [2]. Downstream of DC activation and migration to the secondary lymphoid organs, production of DC-derived IL-12p40 dependent cytokines is crucial for generation of T cell responses, specifically IL-12 is critical for the induction of IFNγ

Abbreviations: Th, T helper; BMDCs, bone marrow dendritic cells; IFNγ, interferon gamma; IL-17, Interleukin-17; LVS, Live Vaccine Strain; DLNs, draining lymph nodes.

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responses [10], while the production of DC-derived IL-23 is required for generation and maintenance of Th17 cells [11]. Accordingly, cellular immunity against *F. tularensis* requires the induction of both T helper 1 (Th1) [12,13] and T helper 17 (Th17) cells [14]. Infection of mouse bone marrow derived DCs (BMDCs) with *F. tularensis* LVS induces the production of IL-12p40 and IL-12 [15], while infection of macrophages with *F. tularensis* LVS induces IL-12p40 and IL-12p35 mRNA [16] and IL-12p40 protein in human monocyte derived macrophages [17]. *In vitro* infection of human monocytes with the virulent strain of *F. tularensis* SCHU S4 induces IL-23 [18]. These studies suggest that all three IL-12p40 subunit cytokines are induced in response to *Francisella* infection. However, it is not known whether IL-12p40 induced in response to *F. tularensis* LVS exposure has a role to play in chemokine responsiveness to CCL19 and in DC migration from the lung to the draining lymph nodes (DLN). In this study, using *in vitro* chemotaxis assays we show that *Francisella*-activated DCs induce TLR2-dependent IL-12p40 production and migrate in response to the homeostatic chemokine CCL19 in an IL-12p$(40)_2$ and IL-12Rβ1-dependent manner. Furthermore, we show that migration of DCs from the *Francisella*-exposed lung *in vivo* is also mediated by IL-12Rβ1 signaling and this coincides with rapid accumulation of IL-12p40-expressing DCs in the DLNs. Our data therefore suggest that IL-12p40 expression by DCs is the first crucial step in initiation of downstream immune responses in pulmonary tularemia.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) were purchased from Taconic Laboratory (Hudson, NY). IL-12p40-IRES-GFP reporter (*yet40*), IL-12p40$^{-/-}$ mice and TLR2$^{-/-}$ mice originated from The Jackson Laboratory (Bar Harbor, ME). IL-12Rβ1$^{-/-}$ mice were used as previously described [19] and were a generous gift from Dr. Michael J Walter, Washington University School of Medicine. Experimental mice were used between the ages of six to eight weeks. All mice were treated in accordance to University of Pittsburgh IACUC guidelines.

2.2. Bacteria

The LVS strain of *F. tularensis* (BEI) was grown in Mueller–Hinton (MH) broth or cultured on MH agar, supplemented with ferric pyrophosphate and isovitalex [12]. Bacteria were grown to mid-log phase at 37 °C and the CFU was determined by plating the bacterial stocks on MH agar plates. Bacterial colony formation was counted after 3 days of incubation at 37 °C. *F. tularensis LVS* stocks were then heat inactivated by incubating bacterial stocks grown to mid-log phase at 60 °C for 1 h. Bacteria were frozen in 1 ml aliquots without glycerol at −70 °C until needed. Total bacterial protein was determined with the Pierce BCA protein Assay Kit (Thermo Scientific) following the manufacturer's protocol. Irradiated *Mycobacterium tuberculosis* H37RV whole cells was obtained under National Institutes of Health (NIH) contract AI-75320.

2.3. Generation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated from the bone marrow cells of mice as previously described [14]. Briefly, cells were extracted from mouse femurs and 1×10^7 cells were plated with 10 ml of DMEM supplemented with 10% FBS (complete DMEM (cDMEM) containing 20 ng/ml recombinant murine GM-CSF (rmGM-CSF; Peprotech). Cells were cultured for 3 days at 37 °C in 5% CO_2, after which an additional 10 ml of cDMEM containing 20 ng/ml rmGM-CSF was added. On day 7, the non-adherent cells were collected by centrifugation, counted and used as BMDCs.

2.4. Exposure of BMDCs to F. tularensis LVS

BMDCs from 7-day cultures were placed in 24-well plates at a concentration of 1×10^6 cells/well. Cells were treated with heat-inactivated (100 μg/ml) or live *F. tularensis* LVS grown at 37 °C at a multiplicity of infection (MOI) of 100 bacteria per cell in 1 ml of cDMEM. Irradiated *M. tuberculosis* (100 μg/ml) was used as a positive control [2]. Cells were incubated for 24 h at 37 °C in 5% CO_2 followed by centrifugation and removal of the supernatants for protein analysis. In some wells, B6 DCs were treated with either Goat IgG isotype control or IL-12p40 neutralizing antibody (both from R and D Biosystems-100 ng/ml), while IL-12p40$^{-/-}$ DCs were treated with IL-12$(p40)_2$ (500 ng/ml) for 24 h. Untreated and treated BMDCs were washed extensively and used in assays described below.

2.5. BMDC uptake of F. tularensis LVS

F. tularensis LVS was conjugated to Alexa Fluor 488 following the manufacturer's protocol (Invitrogen). Air-dried heat inactivated *F. tularensis* LVS were suspended in 0.1% sodium bicarbonate buffer containing 200 μg/ml Alexa Fluor dye and labeled at 37 °C, 500 rpm for 1 h. Conjugated *F. tularensis* LVS was then washed twice in PBS and resuspended in cDMEM. BMDCs were treated with labeled *F. tularensis* (100 μg/ml). Cells were incubated for 24 h at 37 °C in 5% CO_2 and the percentage of CD11c$^+$ cells that phagocytized labeled *F. tularensis* was determined using flow cytometry.

2.6. In vivo tracking of BMDCs

Day 7 *in vitro*-generated BMDCs were used for *in vivo* tracking as described previously by us [2]. In brief, BMDCS were activated with *F. tularensis* LVS for 3 h or left untreated. Cells were washed and stained with 6 μg/ml TAMRA orange (Invitrogen) for 3 min at 37 °C, where upon they were washed and resuspended in PBS. 5×10^6 cells were delivered intratracheally into B6 mice and after 18 h post instillation, the lungs and DLN were harvested, processed into single cell suspensions, and the number of CD11c$^+$ TAMRA$^+$ cells determined by flow cytometry.

2.7. In vivo tracking of lung CD11c+ DCs

B6 or IL-12Rβ1$^{-/-}$ mice each received a suspension of 10 μg of heat inactivated LVS in a 5 mM CFSE (Invitrogen) solution delivered intratracheally. Control mice received CFSE alone. In some experiments, IL-12p40 reporter mice received PBS or 10 μg of heat inactivated LVS delivered in PBS. 18–24 h after instillation, lungs and DLNs were harvested and single cell suspensions prepared. Flow cytometry was used to determine the frequency of CFSE-labeled CD11c$^+$ cells or IL-12p40$^+$ expressing CD11c$^+$ cells within the DLNs.

2.8. Chemotaxis assay

The responsiveness of treated and untreated BMDCs to CCL19 was determined as previously described [2]. Treated and untreated BMDCs were resuspended at a concentration of 1×10^6 cells/ml in 1× Hanks Balanced Salt Solution (HBSS) without calcium and magnesium (Mediatech-Cellgro) plus 1% heat inactivated Fetal Bovine Serum (FBS) (Sigma–Aldrich). 100 μl of the cell suspension was added to the upper chamber of a transwell in a 24 well plate (Fisher Scientific) while the lower chamber contained 25 ng/ml CCL19 (R and D Biosystems) in 600 μl of HBSS. Transwell plates were incubated at 37 °C for 90 min following which the transmigrated cells from the lower chamber were fixed by addition of 1%