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The effects of Bordetella pertussis on dendritic cell imprinting of CD4+ T cells

Sana Waheed
San Jose State University

THE EFFECTS OF *BORDETELLA PERTUSSIS* ON DENDRITIC CELL IMPRINTING

OF CD4+ T CELLS

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Sana Waheed

August 2010

The Designated Thesis Committee Approves the Thesis Titled

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OF CD4+ T CELLS

by

Sana Waheed

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

SAN JOSÉ STATE UNIVERSITY

August 2010

Dr. Tzvia Abramson   Department of Biological Sciences

Dr. John Boothby   Department of Biological Sciences

Dr. Kenneth Youngman   Department of Biological Sciences

ABSTRACT

THE EFFECTS OF *BORDETELLA PERTUSSIS* ON DENDRITIC CELL IMPRINTING
OF CD4+ T CELLS

by Sana Waheed

Bordetella pertussis is an aerobic gram-negative bacterial pathogen that causes

the human respiratory disease whooping cough. Despite widespread vaccination,

whooping cough is reemerging due to decreased vaccine efficacy. One of the hallmarks

of infection is lymphocytosis, which is induced by the pertussis toxin. Lymphocytes such

as CD4+ T cells navigate to infected tissues through surface-trafficking molecules, which

are imprinted during their interaction with tissue-associated dendritic cells. We

hypothesized that the pertussis toxin affects the imprinting process resulting in altered

expression of trafficking molecules on CD4+ T cells. We tested this hypothesis using a

mouse model of infection. Imprinting levels on CD4+ T cells were compared to

Bordetella parapertussis, a related strain that lacks pertussis toxin. Our results indicated

that 5 days post-infection, the percentage of lung dendritic cells increased and adopted a

mature phenotype (displaying an increased capability to migrate and present antigen to T

cells) in response to *B. pertussis* infection, and there was an overall downregulation of

trafficking molecules on CD4+ T cells. However, 25 days post-infection with *B.

pertussis*, dendritic cells continued to express elevated levels of MHC class II, and the

expression of trafficking markers on CD4+ T cells also increased compared to uninfected

controls. These results enable identification of molecules that are specific for

lymphocyte trafficking to the respiratory airways and contribute to knowledge useful in

the development of better vaccines.

ACKNOWLEDGEMENTS

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PREFACE

This thesis is comprised of three chapters and the appendices. Chapter I provides an overview of the research project and includes information regarding each part of the project. The second chapter is presented in journal format according to the guidelines set by the *Journal of Immunology*. The final chapter includes the conclusions from the research as well as a discussion of future directions. The appendices provide additional, supplementary data associated with this study.

CHAPTER I

INTRODUCTION

Overview of the Research Project

Immune cells such as lymphocytes circulate between the blood, lymph nodes, and organs due to chemotactic interactions between secreted and surface molecules in tissues and surface molecules on immune cells. This process is termed lymphocyte trafficking and is responsible for the recruitment of T lymphocytes to sites of infection (1). Lymphocyte trafficking (homing) to infected organs and tissues is crucial for the elimination of airborne pathogens and for restoration of a homeostatic environment such that unnecessary inflammation and tissue damage is prevented. T lymphocytes are able to home to specific organs due to a special combinatorial pattern of surface trafficking molecules that are "imprinted" on their surface during interaction with dendritic cells.

The trafficking molecules responsible for exclusive homing to the airways remain unknown. We report here our study of CD4+ T cell trafficking using two respiratory pathogens, *B. pertussis*, which produces pertussis toxin and, *B. parapertussis*, which does not. Pertussis toxin is responsible for lymphocytosis (extremely elevated levels of lymphocytes in the bloodstream) and is known to inhibit G proteins, which are responsible for cell signaling leading to chemotaxis or cell migration. Therefore we designed this study to observe the effects of pertussis toxin on dendritic cell imprinting of trafficking molecules on CD4+ T cells that could lead to preventing their exit from the bloodstream and entry into the lungs.

This project had two main objectives. The first objective was to analyze the presence of homing molecules on CD4+ T cells in three different compartments (lymph nodes, blood, and lungs), which was conducted primarily by Brian Kwong. Imprinting of

homing molecules occurs in the lymph nodes so it was important to include the CD4+ T cell trafficking levels in our analyses. Levels of these molecules were also measured for CD4+ T cells in the blood and lungs to observe their phenotype after imprinting. Together the information from these tissues allowed us to compare the level of each trafficking marker in various compartments during experimental infection.

The second objective of this project was to mimic the *in vivo* imprinting process that takes place in the lymph nodes, and observe the resulting interactions that occur between isolated lung dendritic cells and naïve allogeneic splenocytes in an *in vitro* co-culture system. The focus of this thesis was objective two of the overall research project, which should inform us about the effects of pertussis toxin on the ability of dendritic cells to imprint homing molecules on naïve T cells. To study this process, a co-culture system was developed using isolated lung dendritic cells from uninfected and *Bordetella*-infected mice and co-cultured overnight with naïve splenocytes to allow for imprinting. The expression levels of trafficking molecules on CD4+ T cells from uninfected and *Bordetella*-infected co-cultures were compared by flow cytometry. These experimental results will contribute to our understanding of the pathogenesis of *B. pertussis*, as well as the role of imprinting molecules in respiratory trafficking of CD4+ T cells. This knowledge may lead to improved vaccine development targeting molecules that participate in lung homing.

CHAPTER II

THE EFFECTS OF *BORDETELLA PERTUSSIS* ON DENDRITIC CELL IMPRINTING

OF CD4+ T CELLS

THE EFFECTS OF *BORDETELLA PERTUSSIS* ON DENDRITIC CELL IMPRINTING

OF CD4+ T CELLS [1]

Sana Waheed, Tzvia Abramson,*[2]**

Department of Biological Sciences,
San José State University,
San José, CA 95192

[2] Address correspondence and reprint requests to Tzvia Abramson, Department of Biological Sciences, One Washington Square, San José State University, San José, CA 95192

Abbreviations used in this paper: DCs, dendritic cells; *B. pertussis*, *Bordetella pertussis*; *B. parapertussis*, *Bordetella parapertussis*; PTx, Pertussis Toxin; MHC, major histocompatibility complex; CD, cluster of differentiation; PBS, phosphate buffered saline; FCS, fetal calf serum; FSC, Forward Scatter; SSC, Side Scatter; PSGL-1, P-selectin glycoprotein ligand-1; VLA-1, very late antigen-1; LFA-1, lymphocyte function-associated antigen-1; VLA-4, very late antigen-4; CCR5, chemokine receptor type 5; CCR6, chemokine receptor type 6; CXCR3, CXC chemokine receptor 3; VCAM-1, vascular cell adhesion molecule-1; CLA, cutaneous lymphocyte antigen.

Abstract

Bordetella pertussis is an aerobic gram-negative bacterial pathogen that causes the human respiratory disease whooping cough. Despite widespread vaccination, whooping cough is reemerging due to decreased vaccine efficacy. One of the hallmarks of infection is lymphocytosis, which is induced by the pertussis toxin. Lymphocytes such as CD4+ T cells navigate to infected tissues through surface-trafficking molecules, which are imprinted during their interaction with tissue-associated dendritic cells. We hypothesized that the pertussis toxin affects the imprinting process resulting in altered expression of trafficking molecules on CD4+ T cells. We tested this hypothesis using a mouse model of infection. Imprinting levels on CD4+ T cells were compared to *Bordetella parapertussis*, a related strain that lacks pertussis toxin. Our results indicated that 5 days post-infection, the percentage of lung dendritic cells increased and adopted a mature phenotype (displaying an increased capability to migrate and present antigen to T cells) in response to *B. pertussis* infection, and there was an overall downregulation of trafficking molecules on CD4+ T cells. However, 25 days post-infection with *B. pertussis*, dendritic cells continued to express elevated levels of MHC class II, and the expression of trafficking markers on CD4+ T cells also increased compared to uninfected controls. These results enable identification of molecules that are specific for lymphocyte trafficking to the respiratory airways and contribute to knowledge useful in the development of better vaccines.

Introduction

  Bordetella pertussis is an aerobic gram-negative bacterium that causes the

respiratory disease whooping cough in humans. Considerable mortality was attributed to

B. pertussis in the early twentieth century, which declined once a vaccine was introduced.

However, whooping cough is reemerging in both adults and children due to drop in

vaccine efficacy (2-3). Vaccinated adults can be carriers of pertussis and manifest a

prolonged, but relatively mild respiratory disease, which may be transmitted to

unvaccinated infants (4). In infants the manifestations can include vomiting, pneumonia,

hypoxia, seizures or apnea. Throughout the world there are 300,000 pertussis-related

deaths each year, mostly in children (5). Although these cases are mainly seen in

developing areas of the world, there have been incidents of pertussis outbreaks in the

United States and, according to the Centers for Disease Control and Prevention, the U.S.

had over 10,000 reported cases of pertussis in 2007 (6). The onset of the disease is

characterized by a persistent, forceful whoop-like cough that can last for several weeks to

months, even after the clearance of the bacteria. The hallmark of the disease is

leukocytosis with extreme lymphocytosis, which is attributed to one of the bacterial

virulence factors, pertussis toxin (PTx) (7-10).

  Bordetella pertussis is an extracellular pathogen that colonizes the upper

respiratory system and bronchial epithelial cells. The respiratory system is constantly

exposed to airborne antigens. The mucosal immunity maintains a steady state in which

harmless antigens do not trigger unnecessary immune responses (11-12). Tissue resident

DCs sample the airways and are key players in supporting immunological tolerance in

response to non-pathogenic antigens (11). *Bordetella pertussis* overcomes this suppressive mechanism and disrupts these homeostatic conditions in the airways. Several bacterial factors contribute to the pathogenesis of *B. pertussis* in the respiratory tract. PTx is a secreted AB subunit exotoxin that is associated with numerous pathogenic functions and immunomodulation. It is responsible for inhibiting chemotaxis in T cells, macrophages, neutrophils, and reduced B cell survival (13). The A subunit is the enzymatically active component that is responsible for ADP-ribosylation and inhibition of the alpha subunit of G proteins (Gα) on target host cells (14). G proteins are coupled to chemokine receptors and mediate cell signaling. PTx prevents activation of Gα and interferes with signaling that leads to chemotaxis and cell migration (8, 15). Administration of soluble PTx was reported to delay the initial recruitment of immune cells such as neutrophils and macrophages, which are responsible for engulfing and killing bacteria (16-20). Impaired neutrophil and macrophage function can lead to colonization of *B. pertussis* in the respiratory tract

During respiratory infection, DCs sample and pinocytose (internalize) antigen from the tracheal lumen and tissue-penetrating antigens. Immediately after antigen internalization, DCs develop a mature phenotype that is comprised of upregulation of surface maturation markers such as MHC class II, CD40, and CD86, which facilitate DC entry into the lymphatics and migration to the draining lymph nodes where antigen presentation to T cells occurs (21-23). The interaction of mature DCs with naïve T cells in the lymph nodes results in T cell activation, signaling, and upregulation of trafficking receptors, a mechanism called imprinting. Mature DCs derived from a specific tissue

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will then imprint T cells with trafficking receptors that direct them to the area of infection where the antigen was originally encountered by the DC (24).

Lymphocyte trafficking is a multi-step process that allows activated T cells to exit the lymph nodes, enter the bloodstream, extravasate, and home to infected tissue (1, 24-26). The first step of this process is a rolling/tethering action mediated by selectins that loosely bind the T cell to inflamed endothelium and slows its transit in the bloodstream. This is followed by attachment and activation of the T cell by chemokine receptors. The third step is mediated by integrins, which completely halt the T cell in the bloodstream and mediate extravasation.

The concept of trafficking receptor imprinting on lymphocytes is well documented in the gut and skin where the trafficking molecules and environmental factors influencing this interaction have been identified (26). For example, DCs from gut origin imprint T cells in the presence of retinoic acid (a dietary environmental factor) with high levels of CCR9 and α4β7, which are the surface molecules that direct T cells to the small intestine (1, 25, 27-30). Trafficking of T cells to the skin is mediated by DCs and activated Vitamin D3 derivatives that imprint CCR10, CLA, and CCR4, which leads T cells to the skin (1, 31-33). As of now, the imprinting factors that direct T cell trafficking exclusively to the respiratory system have not been described (34).

In this thesis, we studied the interactions between DCs and T cells during a respiratory infection with *B. pertussis*. We hypothesized that *B. pertussis* (via PTx) alters DC imprinting of trafficking molecules on T cells. This may lead to a high degree of lymphocytosis in the circulation with reduced levels of trafficking molecules that can

orient lymphocyte homing to the lungs. We tested our hypothesis using *B. pertussis* and *Bordetella parapertussis* experimental infections in mice. *Bordetella parapertussis* lacks PTx and was used as a control. This comparison elucidated the effects of PTx on G protein-coupled receptor signaling, as well as DC and T cell interactions leading to T cell homing (35-36). Since defective homing could lead to delayed clearance during *B. pertussis* infection, knowledge in this area should contribute to understanding the pathogenesis of this bacterium and aid in vaccine development based on lung associated trafficking molecules.

Materials and Methods

All studies using animals have been approved by the Institutional Animal Care and Use

Committee (IACUC) (Protocol #921) at San José State University, San José, CA.

Bacteria

Two *Bordetella* strains were used, *B. pertussis* 338, which is a nalidixic acid-resistant

derivative of the Tohama strain, and *B. parapertussis*, which was obtained from ATCC

(#9305). Both strains were grown on Bordet-Gengou blood plates, and single colonies

were used to inoculate Stainer-Scholte broth containing Heptakis (Sigma-Aldrich, St.

Louis, MO) prior to experimental infection.

Experimental infection

Six-week-old female Balb/c mice were inoculated intranasally with 20 μl of 5×10^{6} colony

forming units of *B. pertussis* or *B. parapertussis*. Uninfected control mice received 20 μl

of PBS (37-38). Two time-points of experimental infection were established, 5 days

post-infection (peak infection) and 25 days post-infection, which were determined by

performing a time-course experiment where bacterial load in the lungs was measured for

ten days and again on day 25 (see Appendix A). To confirm infection and determine the

bacterial load, lungs from experimentally infected mice were harvested, homogenized,

and plated on Bordet-Gengou plates prior to each experiment.

Preparation of single cell suspensions from lungs and spleen

Mice were exsanguinated followed by perfusion of lungs with 30-50 ml of PBS. The lungs and trachea were removed, minced and digested in RPMI-1640, 10% FCS, 75U/ml DNase I (Sigma-Aldrich) and 250 U/ml Type I Collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in a 37ºC orbital shaker for 45 to 60 minutes. The digested lungs were passed through a 40-micron nylon mesh strainer and cells were washed with RPMI-1640 to remove residual digest medium. Digested lung tissue was resuspended in 40% Percoll (GE Lifescience, Piscataway, NJ), slowly underlaid with 70% Percoll and centrifuged for 25 minutes to create a density gradient. Cells at the 40-70% interface (mononuclear cells) were collected and used for enrichment of DCs. The spleens from uninfected allogeneic mice were harvested and pressed through a nylon mesh to obtain a single cell suspension (39). Splenocytes were incubated with 3 ml red blood cell lysis buffer (Sigma-Aldrich) for 5 minutes and washed with RPMI-1640 with 10% FCS. Cell pellets from the lungs were resuspended in buffer (PBS, 0.5% FCS, 2 mM EDTA) suitable for MACS columns.

Enrichment of lung DCs

All cell enrichments were performed using magnetic microbeads (Miltenyi Biotech, Auburn, CA). For DC enrichment from lungs, CD11c microbeads (N418) were used and lung mononuclear cells were enriched for CD11c+ cells using positive selection with a MiniMACS separator. Briefly, lung mononuclear cells were incubated with 100 µl of CD11c microbeads for 15 minutes at 4°C. Cells were washed with MACS buffer (PBS,

0.5% FCS, 2 mM EDTA) and resuspended in 500 µl of buffer. One MS column was used to enrich for CD11c+ cells.

T cell activation and co-cultures

For co-culturing experiments, 24-well plates were coated with the purified monoclonal antibodies CD3 (1 µg/ml) and CD28 (1 µg/ml) in PBS, and incubated at 4ºC, as adapted from Sigmundsdottir (33) and Campanelli (39). Enriched lung DCs were cultured with splenocytes from uninfected allogeneic mice at a 1:25 ratio in RPMI-1640 supplemented with 10% FCS, penicillin, streptomycin, IL-2 (12.5 ng/ml) and IL-12 (2.5 ng/ml) (R&D Systems, Minneapolis, MN). Cells were co-cultured overnight at 37ºC with 5% CO_2 to allow for imprinting, followed by flow cytometry analysis. T cell activation was determined using intracellular antibody staining with Ki-67 (B56) and interferon γ (XMG1.2), which served as markers of T cell proliferation in an allogeneic system. Briefly, cells were stained for extracellular markers and resuspended in 250 µl Fixation/Permeabilization (BD Biosciences, Franklin Lakes, NJ) solution and incubated at 4°C for 20 minutes. Cells were washed twice with 1 ml of $1\times$ Perm/Wash Buffer (BD Biosciences) and then resuspended in 100 µl of Perm/Wash Buffer containing the antibody or negative control. Cells were incubated at room temperature in the dark for 30 minutes before being washed twice with 1 ml/wash of $1\times$ Perm/Wash Buffer and resuspended in staining buffer (0.1% Sodium Azide, 1% FCS, PBS).

Flow cytometry

Analysis of surface and intracellular markers was done by flow cytometry. Lung derived
DC phenotyping was measured by the expression of myeloid associated markers [CD11c
(N418), CD11b (M1/70)] and maturation markers [MHC class II (M5/114.15.2), CD40
(3/23), CD86 (GL-1)]. Additional trafficking markers were evaluated on DCs as detailed
for T cells. Expression of trafficking molecules on CD4+ T cells included CD4 (RM4-5,
BD Biosciences and Invitrogen, Carlsbad, California), CD38 (90) and the following
trafficking molecules: CD11a (M17/4), CD49f (GoH3), CD162 (2PH1, BD Biosciences),
CXCR3 (220803, R&D Systems), CD49a (HMalpha1, ABDSerotec, Raleigh, NC),
CD49d (9C10 MFR4.B), and CD103 (2E7). All antibodies were purchased from
BioLegend (San Diego, CA) unless otherwise indicated. The levels of these markers
were compared for *B. pertussis* and *B. parapertussis* infections and normalized to
uninfected controls as explained in the figure legends. Samples were acquired using the
FACS Calibur (BD Biosciences) at San José State University and the LSRII (BD
Biosciences) at Stanford University, and were analyzed using Flowjo.

Results

Experimental design

Balb/c mice were inoculated intranasally with PBS (uninfected controls), *B. pertussis* or *B. parapertussis* for a period of 5 and 25 days. At each time-point lungs were harvested, mononuclear cells were obtained and enriched for DCs (CD11c+ cells) using Miltenyi Microbeads (Fig. 1). DCs were enumerated and phenotyped for each treatment using multicolor flow cytometry (Fig. 2, 4). Imprinting of trafficking molecules was observed in co-cultures of lung DCs with naïve allogeneic splenocytes from Swiss Webster mice (Fig. 3, 5).

Peak infection: DCs matured in response to Bordetella infection

Five days post-infection we found 23% of CD11c+ cells in uninfected control lungs, whereas *B. parapertussis* had 33.1% and *B. pertussis* had 70.6% (Fig. 2A). The percent DCs was estimated by the overall analysis of CD11c+ cells isolated from the lungs. DC phenotype was characterized using CD11c+ and a second marker (Table I) using flow cytometry (Fig. 2B). Levels of maturation markers were assessed with MHC class II, CD40, and CD86 (Fig C-D). These molecules bind to the T cell receptor, CD40L, and CD28 respectively on CD4+ T cells, forming an immunological synapse that results in T cell activation and stimulation (11). Since *B. pertussis* is an extracellular bacterial pathogen, we analyzed MHC class II, which is the molecule responsible for presenting antigen to CD4+ T cells. Lung DCs from uninfected mice revealed low levels of MHC class II, CD40, and CD86 (7.72%, 10.64%, 6.11% respectively), whereas *B.*

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parapertussis-infected DCs showed increased expression (20.64%, 13.67%, 14.04%). Comparatively, lung DCs from *B. pertussis* infection displayed the highest levels of maturation (64.92%, 36.64%, 60.26% respectively).

The phenotypic marker CD11b is present on myeloid DCs of lung origin (40). Relatively low expression of CD11b (10.21%) was observed in lung DCs from uninfected control mice while this percentage increased in *B. parapertussis* infection (20.5%), but even more so in *B. pertussis* infection (61.04%). Additionally, CCR5 was analyzed to observe DC migratory abilities to inflamed tissues. There was low expression of CCR5 in uninfected DCs (7.76%) compared to *B. parapertussis* (13.95%,) and *B. pertussis* (55.84%). Together, this data indicate that DCs mature during *B. pertussis* infection and do not appear to be inhibited by PTx.
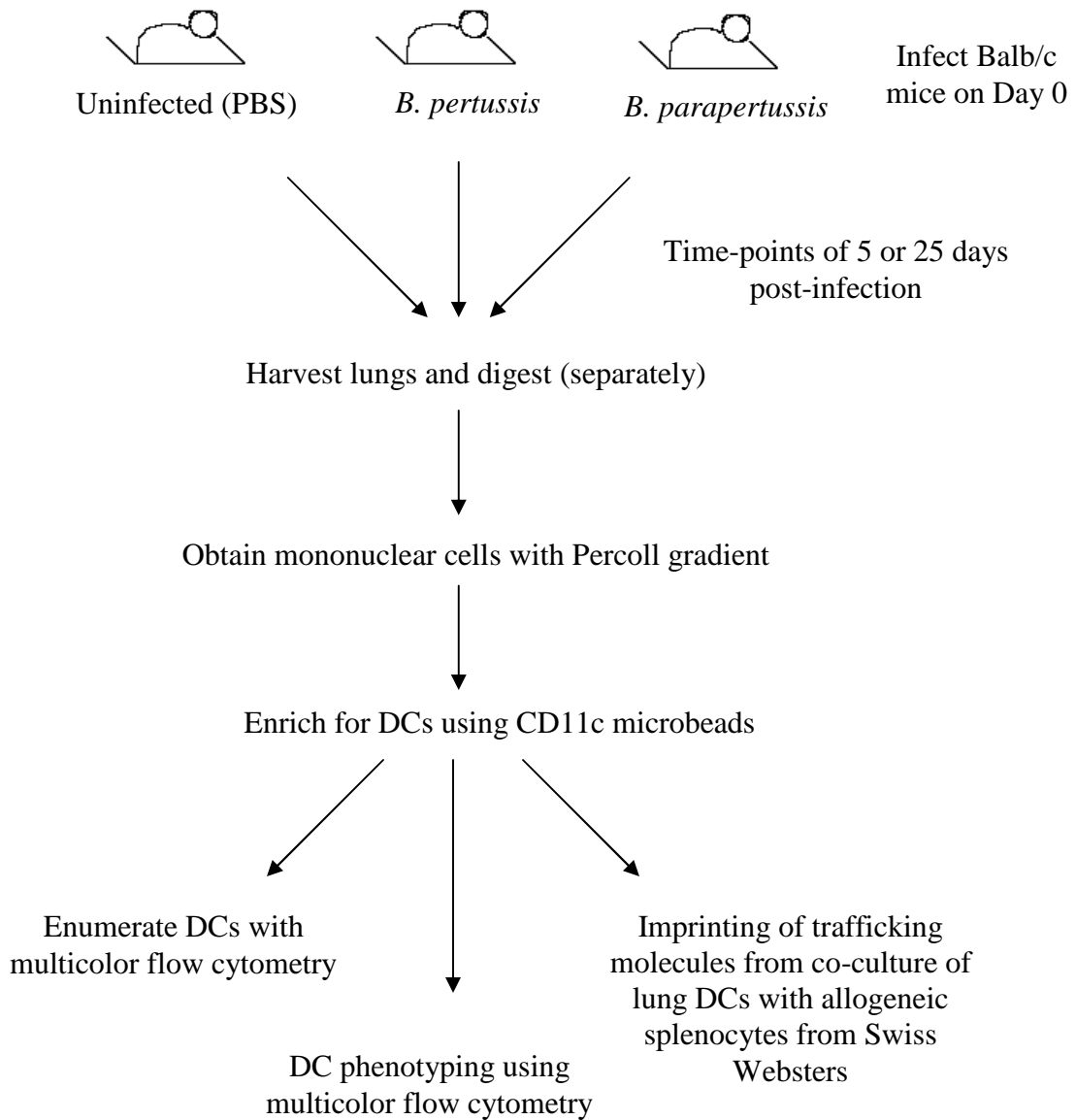
FIGURE 1. Experimental design. Balb/c mice were infected and lungs were harvested 5 and 25 days post-infection. Lungs were processed and mononuclear cells were obtained to further enrich for CD11c+ cells. DCs were enumerated, phenotyped and analyzed for imprinting abilities in co-cultures with allogeneic splenocytes from Swiss Webster mice.

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TABLE I. DC phenotypic markers. The following markers were used to phenotype DCs and determine maturation levels for uninfected, *B. pertussis* and *B. parapertussis* infected lungs.

Marker	Function
CD11c	Expressed primarily on DCs
CD40	Co-stimulatory molecule
CD86	Co-stimulatory molecule
MHC class II	Extracellular antigen presentation molecule
CCR5	Allows DC migration to inflamed tissue (24)
CD11b	Integrin expressed on DCs and monocytes

2A   **Percent CD11c+ Cells per Treatment**
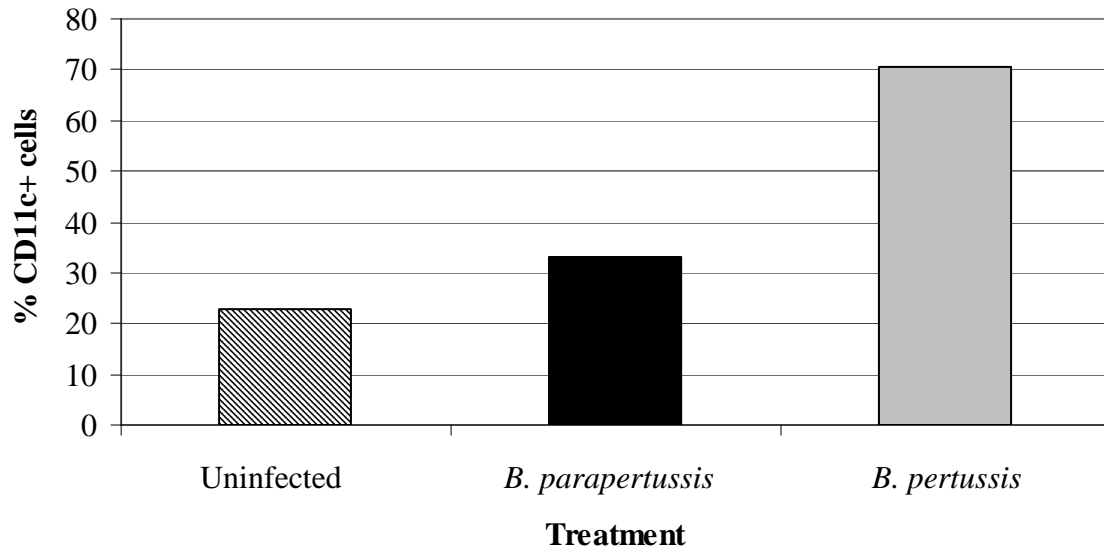
2B
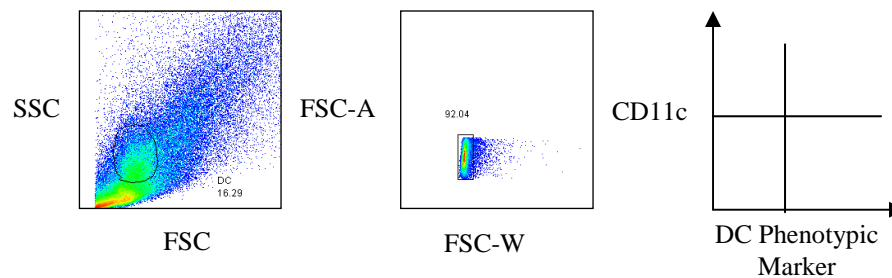
FIGURE 2. DC maturation 5 days post-infection with *B. pertussis* and *B. parapertussis*. Mononuclear cells were isolated from lungs and enriched for DCs. Percent of CD11c+ cells was estimated using quadrants that were placed according to positive controls (*A*). Mononuclear cells were gated on monocytes, and doublets (cell clumping) were removed (*B*). CD11c+ cells (DCs) were analyzed for a second phenotypic marker as detailed in *C*. (See Table I for a description of the markers). For DC maturation, we measured levels of CD40, CD86, MHC class II; myeloid associated marker CD11b, as well as ability to migrate to lymph nodes with CCR5 (*C-D*). Data are representative of at least 4 independent experiments.

2C

Uninfected   *B. parapertussis*   *B. pertussis*

CD11c

CD40

CD86

CCR5

MHC II

CD11b

2D

DC Phenotype 5 Days Post-Infection

Bar chart. Y-axis: % Expression on CD11c+ Cells (0 to 70). X-axis categories: MHC II, CD40, CD86, CD11b, CCR5. Legend: Uninfected, *B. parapertussis*, *B. pertussis*.
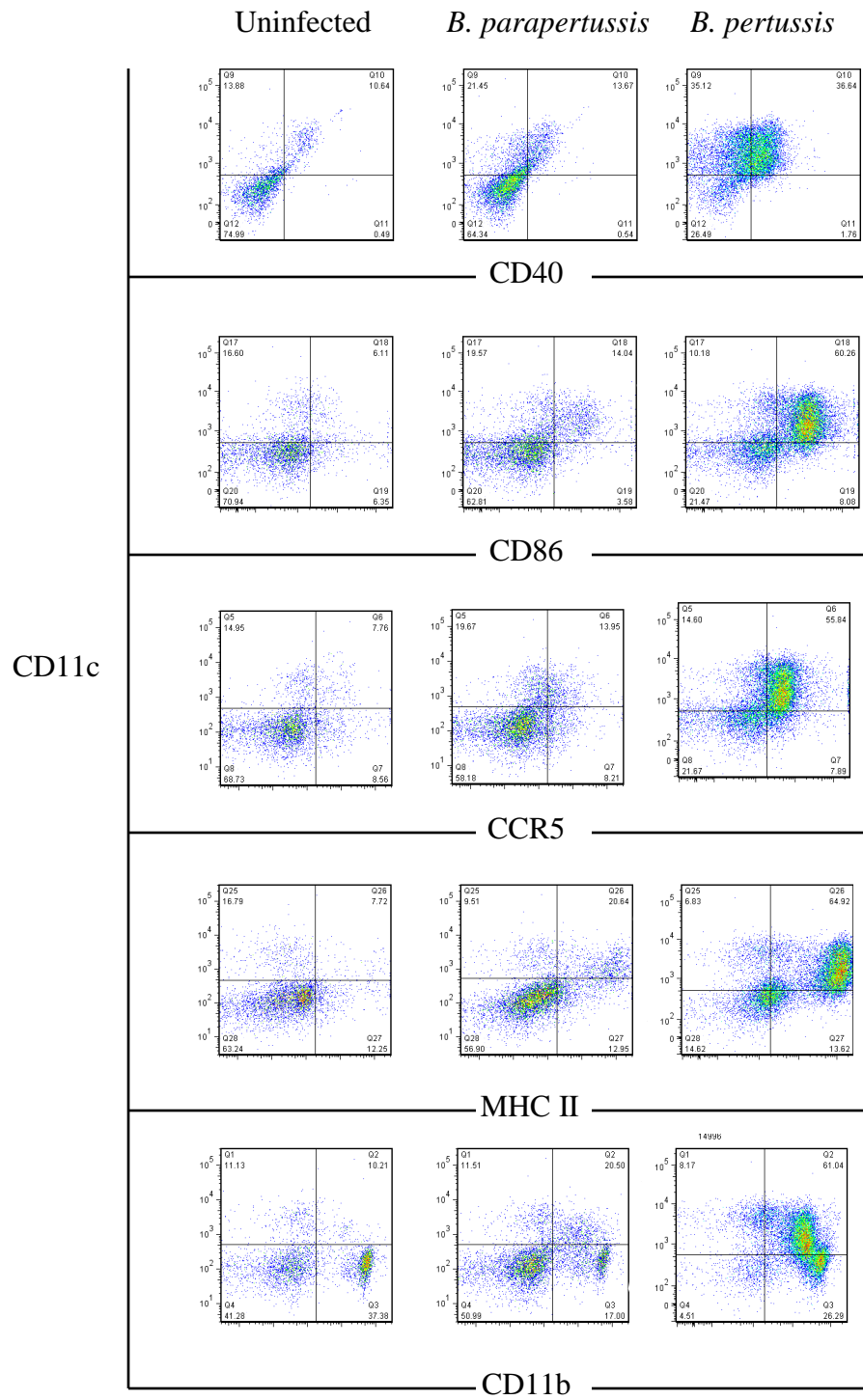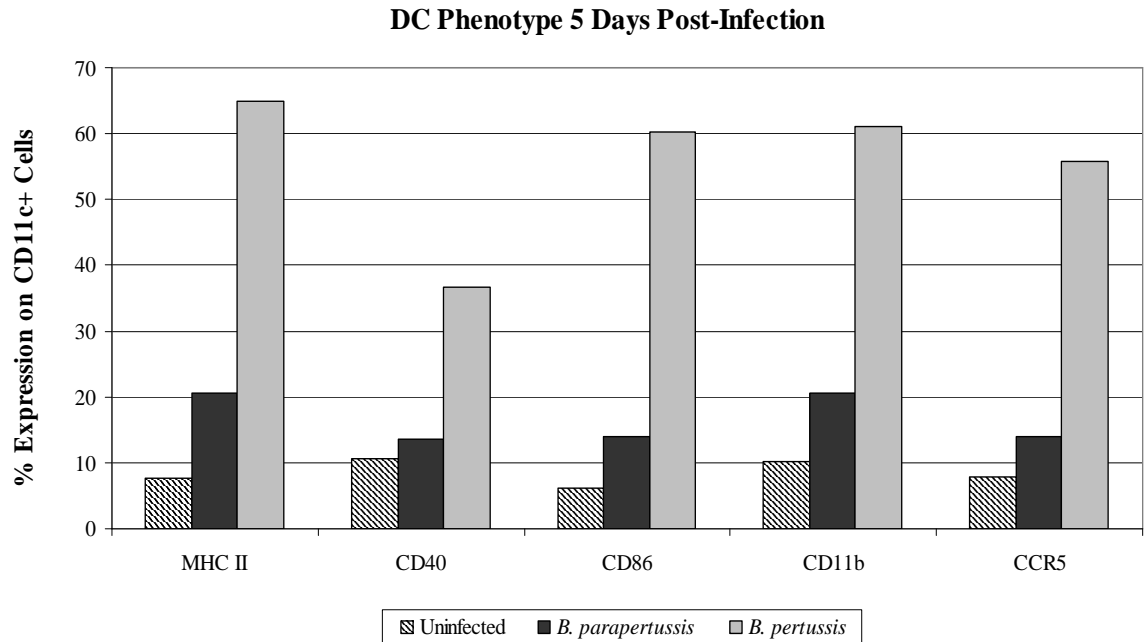
Infection with Bordetella alters trafficking receptor levels on naïve CD4+ T cells

In order to verify the imprinting ability of lung DCs derived from experimentally infected mice, we co-cultured lung DCs with naïve splenocytes from allogeneic mice. Establishing an allogeneic system ensures that imprinting occurs on proliferating T cells, where DCs interact through MHC class II with the T cell receptor and trigger non-specific T cell proliferation. This leads to transcription of certain patterns of molecules that guide the T cell to the site of infection (33). These homing molecules are a combination of chemokine receptors and integrins that interact with inflamed endothelium and allow transmigration of the T cell from the bloodstream into the tissue.

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The proliferative nature of the allogeneic system was confirmed by intracellular staining for interferon γ (secreted during T cell activation) and Ki-67 (a nuclear factor of proliferation) in activated CD38+ cells (Fig. 3A). A proliferating population was observed in each treatment; 0.5-1.8% of cells were positive for interferon γ and roughly 2% were positive for Ki-67 indicating that we had a proliferating T cell system. DC ability to imprint trafficking markers on allogeneic T cells was then analyzed. Since little is known about specific lung homing markers, we analyzed a panel of trafficking molecules to determine whether the levels of imprinted homing molecules were altered in infected co-cultures compared to uninfected controls (Table 2). Our results indicate an overall downregulation of homing molecules on CD4+ T cells during experimental *B. pertussis* infection compared to uninfected controls at 5 days post-infection. These results were normalized to uninfected control values set at 100%.

Recent studies indicate that CD11a, CD162, and CD103 are important markers for trafficking and cell retention in the lungs (28, 41-45). On day 5 of infection, CD4+ T cells from *B. parapertussis* co-cultures showed upregulation of CD11a (125.9%) and CD162 (114.9%) but downregulation of CD103 (81.5%) compared to uninfected controls (100%) (Fig. 3C). In contrast *B. pertussis* co-cultures showed downregulation of CD11a (94.4%) and CD162 (76.1%) with upregulation of CD103 (130.3%). Additionally, the integrins CD49a and CD49d can dimerize with CD29 and have been reported to migrate to the respiratory system (21, 46-47). However, downregulation was observed in both *B. parapertussis* and *B. pertussis* infections for CD49a (53.6%, 75.6% respectively) and CD49d (63.9%, 81.9% respectively) compared to uninfected controls (100%). Another

adhesion molecule, CD49f, may play a role in lung homing and was downregulated more

in *B. pertussis* co-cultures (69.9%) than in *B. parapertussis* (89.6%) (27). There is also

evidence in the literature that CXCR3 is involved in T cell homing to the lungs (11, 48-

50). Five days post-infection with *Bordetella*, CXCR3 was downregulated in both *B.*

parapertussis (49.6%) and *B. pertussis* (58.7%,) compared to uninfected controls (100%).

TABLE II. Trafficking markers and their functions. A variety of markers that are
implicated in the homing process were analyzed, such as molecules involved in tethering
or rolling and other integrins that mediate attachment to the endothelium and lead to
extravasation.

Marker	Function	Receptor Family
CD162 (PSGL-1)	Adhesion (43)	Selectin
CXCR3	Chemotaxis of T cells (49)	Chemokine receptor
CD11a (LFA-1)	Cell adhesion, costimulation (45)	Integrin
CD49a (α1)	Involved in T cell adhesion (46)	Integrin
CD49d (α4)	Cell migration, adhesion, homing (47)	Integrin
CD49f (α6)	Cell adhesion, migration (27)	Integrin
CD103 (α_ε)	Tissue retention of lymphocytes (42)	Integrin
CD38	Activation marker	---

3A

Uninfected   *B. parapertussis*   *B. pertussis*

CD38

Interferon γ

Ki-67
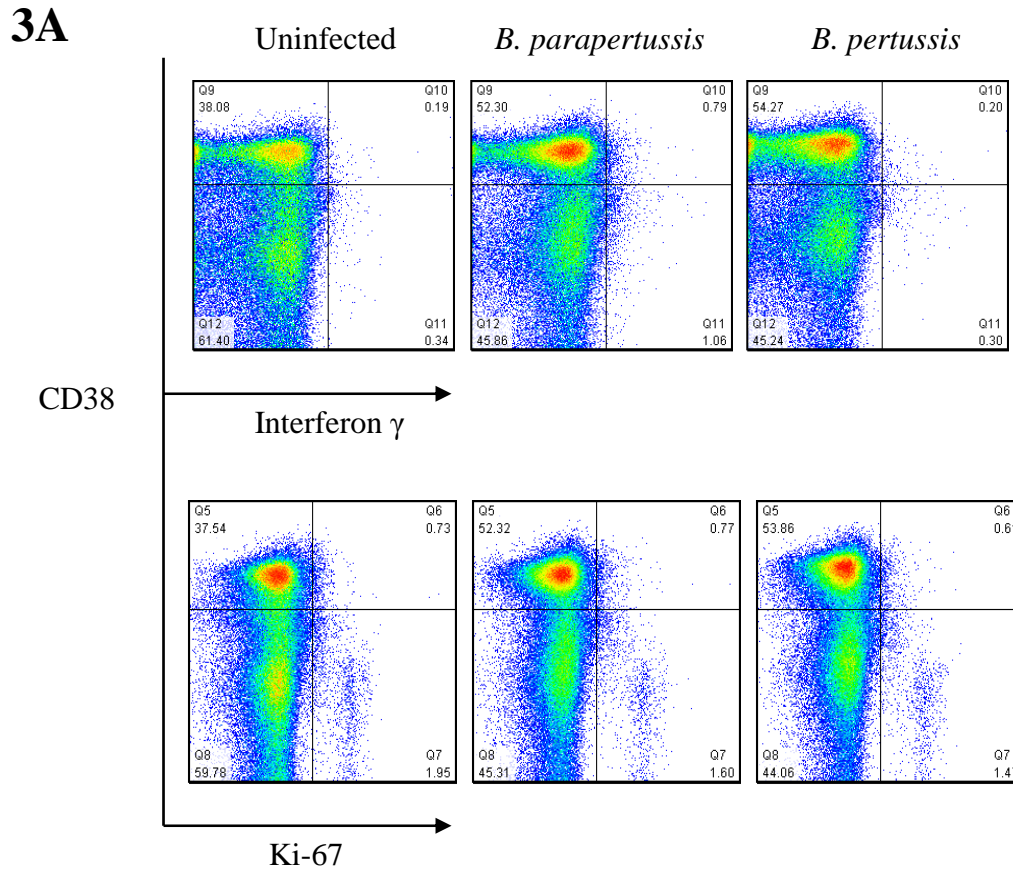
FIGURE 3. Imprinting of trafficking molecules, 5 days post-infection, occurs when DCs interact with CD4+ T cells. Lung DCs were cultured with naïve allogeneic splenocytes to allow for imprinting. Intracellular flow cytometry was used to confirm that co-cultures were an allogeneic system using CD38 with Ki-67 and interferon γ (*A*). For co-culture experiments flow cytometry was used to gate on the lymphocytes, doublets (cell clumping) were removed and CD4+ cells were identified to determine the pattern of trafficking receptors (*B*). In order to observe whether imprinting occurred, levels of the chemokine receptor CXCR3 and various integrins (CD11a, CD162, CD49f, CD49a, CD49d, and CD103) were measured (*C*). (See Table II for a description of the markers). Data was normalized to uninfected controls (100%) and are representative of at least 4 independent experiments.

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3B
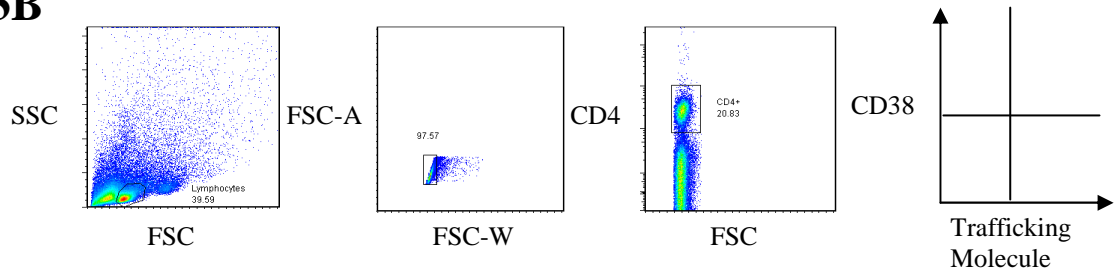
3C

Trafficking Molecule Levels on CD4+ T cells 5 Days Post-Infection
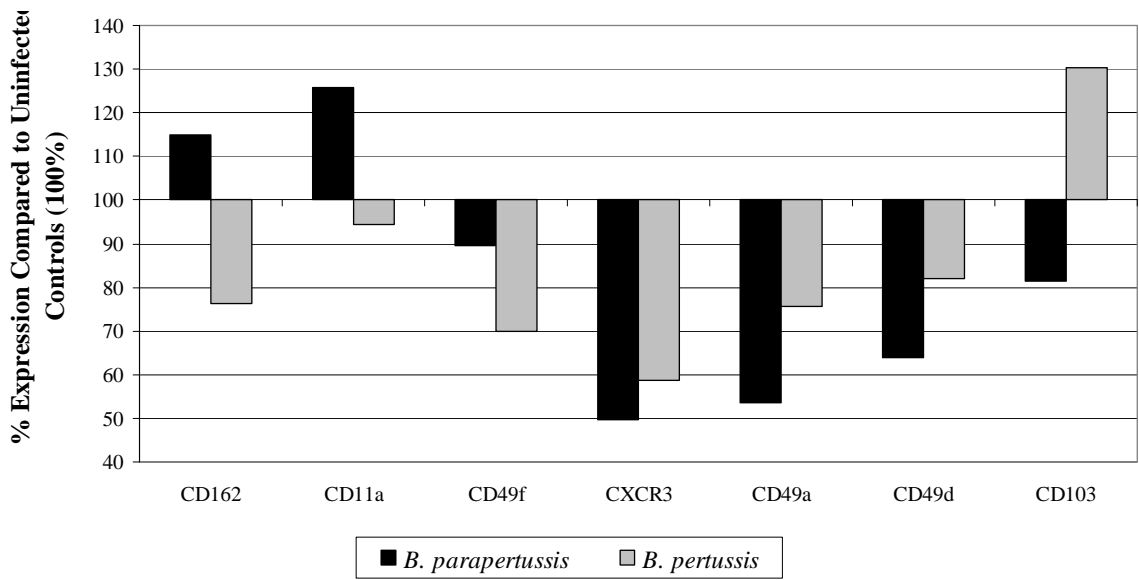
Twenty-five days post-infection: DC maturation levels decrease except for some markers in B. pertussis infection

In our previous experiment for 5 days post-infection, we observed an increase in both percent CD11c+ cells and DC maturation levels during experimental *Bordetella* infection. In order to establish the phenotypic state of DCs 25 days post-infection, lungs were harvested and lung mononuclear DCs were isolated. In the lungs, the percent gated CD11c+ cells decreased for *B. pertussis* (20.7%) and *B. parapertussis* (23.1%) infections to levels similar to uninfected controls (20.4%) (Fig. 4A). DC phenotypic levels were then analyzed where lung DCs from *B. parapertussis* infection had reduced expression of the maturation marker CD86 (4.14%) compared to *B. pertussis* (8.04%) (Fig. 4B). In contrast, MHC class II remained upregulated in *B. pertussis* (18.3%) whereas the levels for uninfected controls and *B. parapertussis* decreased to 6.74% and 7.57% respectively.

The lung-associated marker CD11b was 5.24% for lung DCs from uninfected controls, whereas DCs from *B. pertussis* infection had elevated levels (13.1%) while DCs from *B. parapertussis* were downregulated (6.56%). The DC migratory marker CCR5 was upregulated on DCs from *B. pertussis* (7.24%) compared to *B. parapertussis* (3.59%) and uninfected controls (3.75%). Together, this indicates that the effect of *Bordetella* on DC phenotype diminished 25 days post-infection with the exception of *B. pertussis* infection where MHC class II, CD86, CD11b, and CCR5 remained elevated.

4A

Percent CD11c+ Cells per Treatment

4B

DC Phenotype 25 Days Post-Infection
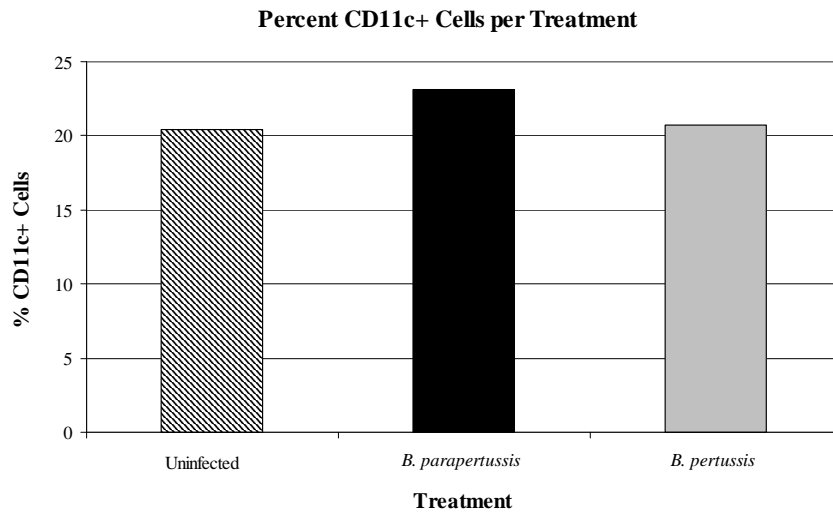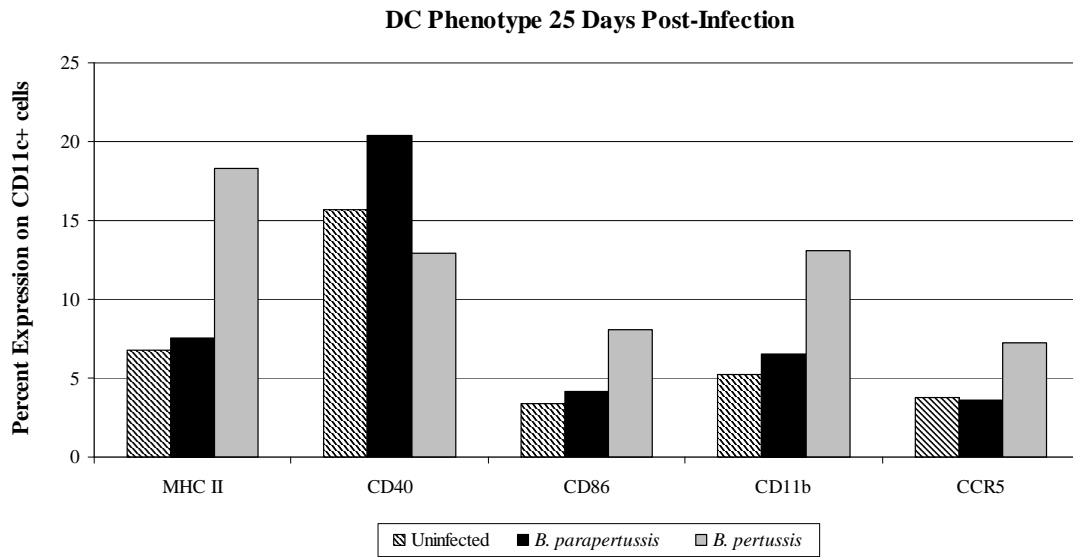
FIGURE 4. DC maturation 25 days post-infection with *B. pertussis* and *B. parapertussis*. After 25 days of infection lung mononuclear cells were obtained, enriched for DCs and gated similarly to that shown in Fig. 2B. We assessed the percent CD11c+ cells in each treatment (as determined using quadrants from flow cytometry dot plots) (*A*) and evaluated DC maturation with MHC class II, CD40, CD86; myeloid and lung associated markers CD11b, CD11c, and migratory capability with CCR5 (*B*). Data are representative of at least 3 independent experiments.

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Trafficking receptor levels decreased in B. parapertussis co-cultures but increased in B. pertussis co-cultures 25 days after infection

Intracellular flow cytometry results of interferon γ and Ki-67 confirmed the proliferative ability of each co-culture, as illustrated by the interferon γ positive and Ki-67 positive populations (Fig. 5A). The level of trafficking receptors on CD4+ T cells was measured to determine whether DCs continued to actively imprint 25 days post-infection. In *B. pertussis*-infected co-cultures, the adhesion and lung retention marker CD11a was upregulated to 201.1% whereas it was downregulated in *B. parapertussis*-infected co-cultures (113.5%) when compared to uninfected controls (100%) (Fig. 5B).

The integrins CD49d and CD49f are associated with migration and had reduced expression in *B. parapertussis* co-cultures (96.7% and 95.1% respectively) when compared to *B. pertussis* co-cultures, which displayed elevated levels of CD49d (164.6%) but maintained downregulation of CD49f (50.5%). For *B. pertussis* co-cultures, CXCR3 remained downregulated (21.4%) compared to uninfected controls (100%). This suggests that 25 days post-infection, *B. pertussis*-infected DCs can imprint some trafficking molecules on CD4+ T cells, while this is not the case for *B. parapertussis*-infected co-cultures, where these molecules are expressed at low levels.

5A
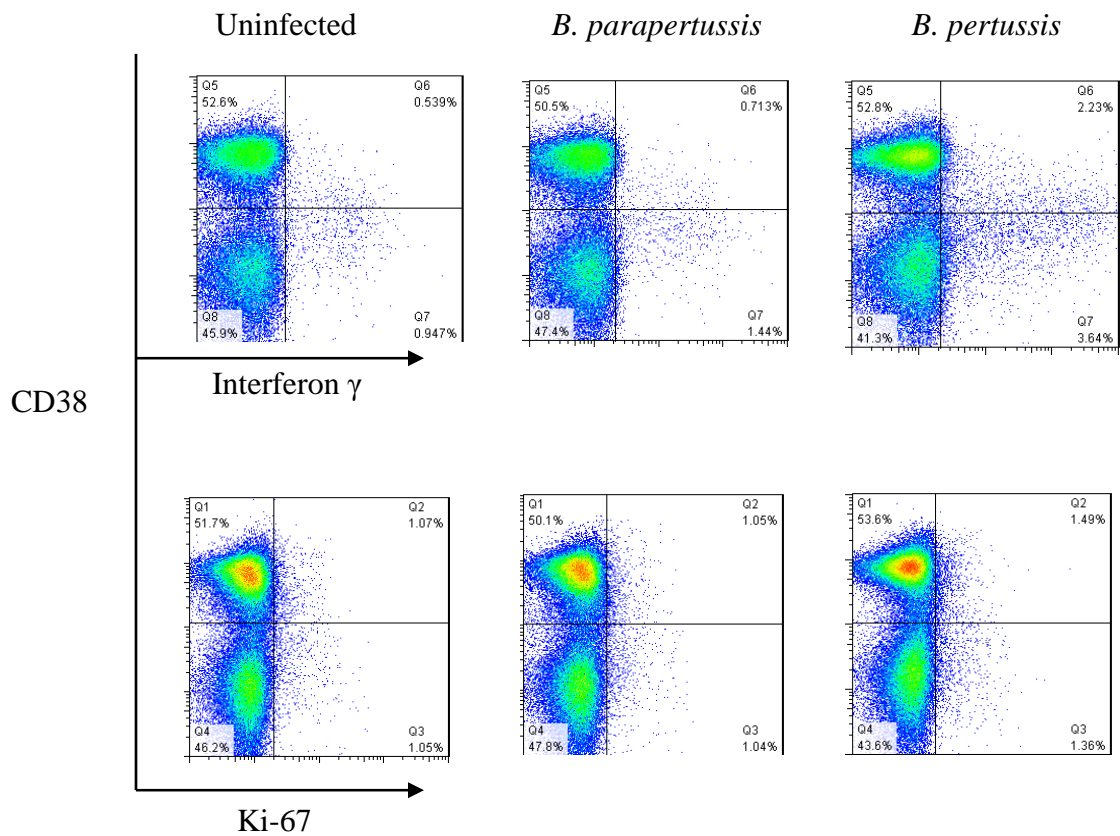
FIGURE 5. The levels of trafficking molecules increase 25 days post-infection in *B. pertussis* infection. Lung DCs were cultured with naïve splenocytes overnight to allow for imprinting. Intracellular staining with interferon γ and Ki-67 was included to confirm that imprinting occurred in the allogeneic co-cultures (*A*). To determine the levels of imprinted markers, we gated on the lymphocytes, removed doublets (cell clumping), and identified trafficking markers on CD4+ cells (gating similar to that shown in Fig. 3B). On day 25 of infection we measured levels of lung retention molecules (CD11a, CD162, CD103), integrins (CD49a, CD49d, CD49f), and the chemokine receptor CXCR3 (*B*). All values were normalized to uninfected controls (100%). Data are representative of at least 3 independent experiments.

5B

Trafficking Molecule Levels on CD4+ T cells 25 Days Post-Infection
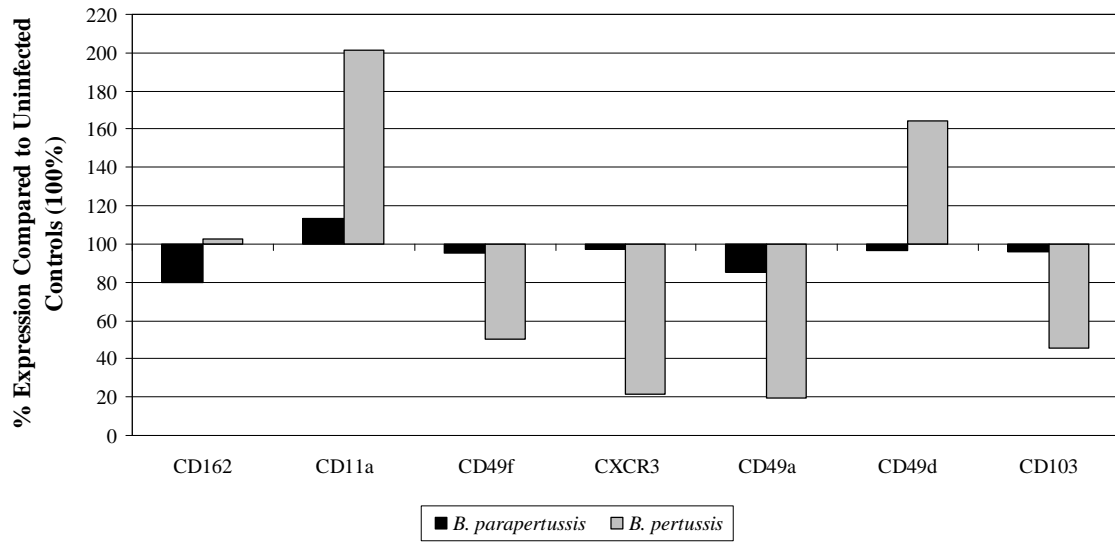
Discussion

Unlike other areas of the body such as the skin and gut where specific homing molecules that govern lymphocyte trafficking have been established (26), a unique trafficking molecule pattern to the respiratory airways remains to be defined. We were interested in elucidating the role PTx has on DC imprinting of trafficking molecules on CD4+ T cells that guides them to the respiratory system. PTx inhibits G protein activation and hinders chemotaxis of cells (8, 13, 15). Therefore, we established an experimental respiratory infection model using two bacteria from the *Bordetella* species: *B. pertussis* (produces PTx) and *B. parapertussis* (lacks PTx).

DC origin and their level of maturation are critical components for imprinting. Therefore, we utilized flow cytometry to determine DC phenotype for uninfected controls and *Bordetella*-infected DCs. At 5 days post-infection, CD11c+ cells increased 1.4 times in *B. parapertussis* infection, whereas there was a 3-fold increase in *B. pertussis* infection compared to uninfected controls. This increase in CD11c+ cells may either be due to recruitment of DCs in the lungs during a state of infection or inability to leave the lungs and migrate to the lymph nodes, a phenomenon that could be attributed to PTx inhibition of chemokine receptors such as CCR7. However, by 25 days post-infection, the percent CD11c+ cells in the lungs decreased for both infections, possibly due to a halt in recruitment of these cells to the lungs.

In contrast to those who report partial DC maturation during *B. pertussis* infection (51), our results indicated that 5 days post-infection, lung DCs from *B. pertussis* infection had upregulated MHC class II, CD40 and CD86 and had generally higher levels of

maturation compared to that in *B. parapertussis* infection or uninfected controls. This

discrepancy between our results and those of others may be attributed to the timing of

post-infection sampling or differences in bacterial strains. The phenotype of DCs

changed 25 days post-infection for *B. parapertussis* so that MHC class II, CD86, CD11b,

and CCR5 were downregulated compared to *B. pertussis*. However MHC class II

remained upregulated in *B. pertussis*, which may be due to continued antigen presentation

to CD4+ T cells in the regional lymph nodes even at late stages of infection. Together

this information indicated that 5 days post-infection, *B. pertussis* does not prevent DCs

from acquiring a mature phenotype that is required for imprinting CD4+ T cells.

However, by 25 days after infection, *B. parapertussis*-infected lungs appeared to revert to

steady-state conditions, which may not be the case for *B. pertussis* infection. An

important chemokine receptor, CCR7, is required for entry into lymph nodes (24). In

order to further characterize the migratory ability of *B. pertussis*-infected DCs, CCR7

assessment will be included in future experiments.

Once the DC phenotype was established, isolated lung DCs were co-cultured

with naïve splenocytes harvested from allogeneic mice, and imprinting of trafficking

molecules on CD4+ T cells was analyzed. CD11a and CD162 have been reported to be

important for adhesion and retention of cells in the lungs (43, 45). Interestingly, we

found that on day 5 post-infection, both CD11a and CD162 were downregulated in *B.*

pertussis infection but upregulated in *B. parapertussis*, which suggests that PTx has a

role in suppressing these molecules during peak infection. However, on day 25 post-

infection, CD11a levels were elevated, suggesting that this marker is upregulated only at later stages of infection, perhaps after the effects of PTx have diminished.

Bordetella pertussis also showed elevated levels of CD103 on day 5 post-infection, while it was downregulated in *B. parapertussis* co-cultures. CD103 has been shown to be involved in tissue retention of lymphocytes (42), and perhaps CD4+ T cells are retained in the tissue instead of migrating during *B. pertussis* infection. There is also evidence that CXCR3 is important for CD4+ T cell recruitment to the airways (11) and both *Bordetella* infections showed decreased levels of CXCR3 on day 5 of infection, while *B. pertussis* infection continued to show downregulation on day 25. This indicates that perhaps both species prevent initial T cell recruitment to the lung through this chemokine receptor.

The results from this research project suggest that *B. pertussis* downregulates certain trafficking molecules during peak infection; however, at later stages of infection these molecules are upregulated. Since *B. pertussis* is a prolonged respiratory disease, it is possible that imprinting of trafficking molecules on CD4+ T cells persists during later stages of infection, perhaps due to the decreased effects of PTx. Our experiments indicate that the lung environment for *B. parapertussis* returned to a state of homeostasis 25 days after infection, whereas this is not the case for *B. pertussis* infection.

Further studies can also include additional trafficking molecules such as CLA, CCR9 and α4β7 due to their presence on T cells that traffic exclusively to the skin and gut respectively. Therefore, low levels of these molecules may be observed on T cells that home to the respiratory system (30, 48, 52). Additional experiments may include *in*

vitro infection of uninfected lung DCs pulsed with PTx, to assess the antigen specific

response and imprinting of trafficking molecules that are PTx-dependent. Antigen pulsed

DCs could be co-cultured with naïve splenocytes to allow for imprinting, and results can

be compared to *in vivo* experimental *Bordetella* infection. In order to further confirm the

role of PTx, an experimental infection with *B. parapertussis* complemented with soluble

PTx could be included and compared to *B. pertussis* (18). This information will increase

our understanding of the pathogenesis of *B. pertussis*, and the role of PTx in preventing

lymphocyte recruitment to the respiratory system through trafficking molecule

modulation. This line of research may identify the molecules responsible for the

exclusive recruitment of CD4+ T cells to the airways, leading to a more efficacious

vaccine design that targets trafficking molecules important in *B. pertussis* infection.

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CHAPTER III

CONCLUSION

Conclusions

These experiments were designed to study the interactions between DCs and CD4+ T cells that lead to the imprinting of trafficking markers, which are especially important for respiratory illnesses such as *B. pertussis*. The discovery of lung specific trafficking markers will aid in the development of improved vaccines that target the upregulation of such molecules and will enhance the clearance of respiratory pathogens. Our results from this research project indicated that DCs are activated and adopt a mature phenotype from *B. pertussis* infection on day 5, and that perhaps PTx interferes with the imprinting process, as observed by the downregulation of CD11a and CD162 on CD4+ T cells, which are important molecules for T cell retention in the lungs. However, 25 days post-infection with *B. pertussis* there is continued elevated expression of MHC class II, CCR5, and CD11b in addition to upregulation of CD11a and CD49d, which could be due to the fading effects of PTx. This suggests that *B. pertussis* may initially alter the DC imprinting process to avoid T cell recruitment to the lungs.

Future directions

In order to further investigate the effects *B. pertussis* has on the respiratory system, we plan to perform immunohistochemical analysis of lung sections from uninfected and *Bordetella*-infected mice. These lung sections will illustrate the degree of inflammation and tissue damage inflicted by the bacteria compared to uninfected lungs. We will also include fluorescence tagged antibodies specific to T cells to determine whether these cells are recruited to the lungs. In order to broaden our analysis of

lymphocyte trafficking we will also incorporate CD8+ T cells to observe whether PTx

differentially imprints homing molecules on this cell type during infection.

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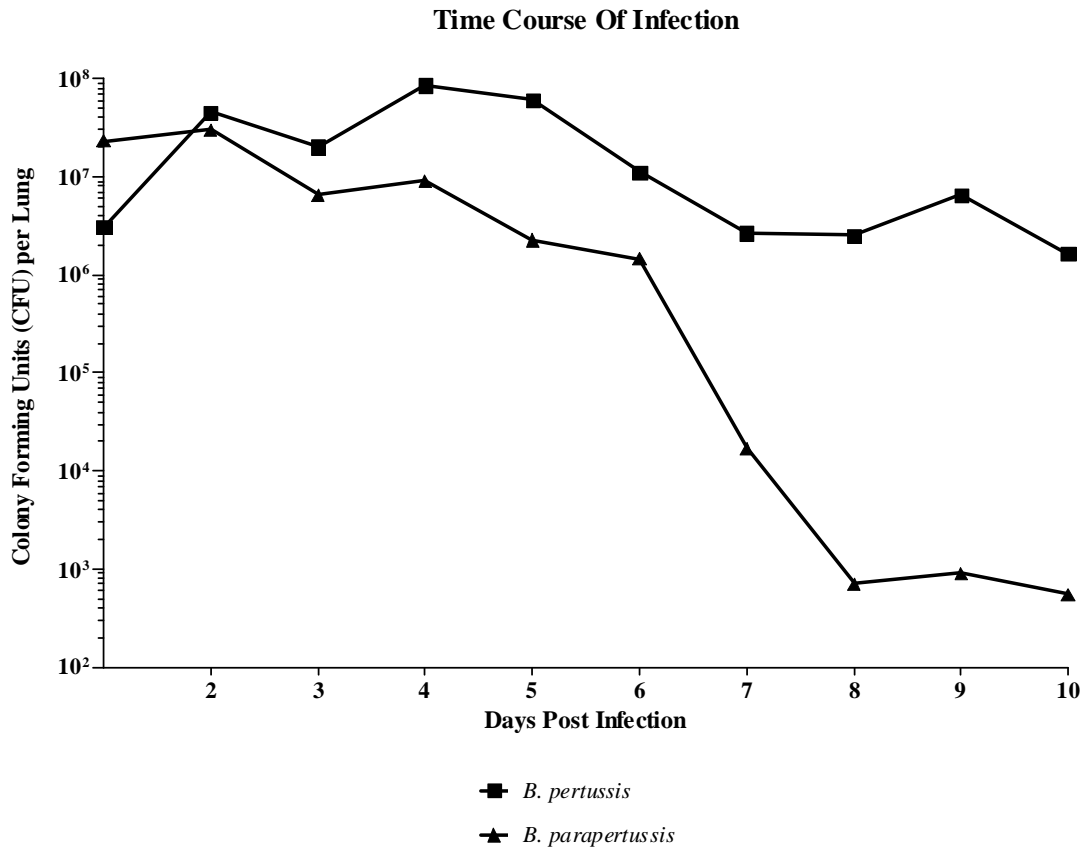
Time Course Of Infection

FIGURE 6. A time course of infection shows the peak infection days for *B. pertussis* and *B. parapertussis*. In order to determine the optimal day for peak infection, we administered 5×10^6 CFU per 20 μl by intranasal administration and performed a time course experiment with two Balb/c mice per time-point. Lungs were harvested at each time-point, homogenized and plated on Bordet-Gengou blood plates. By day 25 post-infection, zero colonies of *B. pertussis* and *B. parapertussis* were recovered from lungs (data not shown). Experiment and analysis was done by Nicole Tarlton.

7A

Percent CD11c+ Cells per Treatment

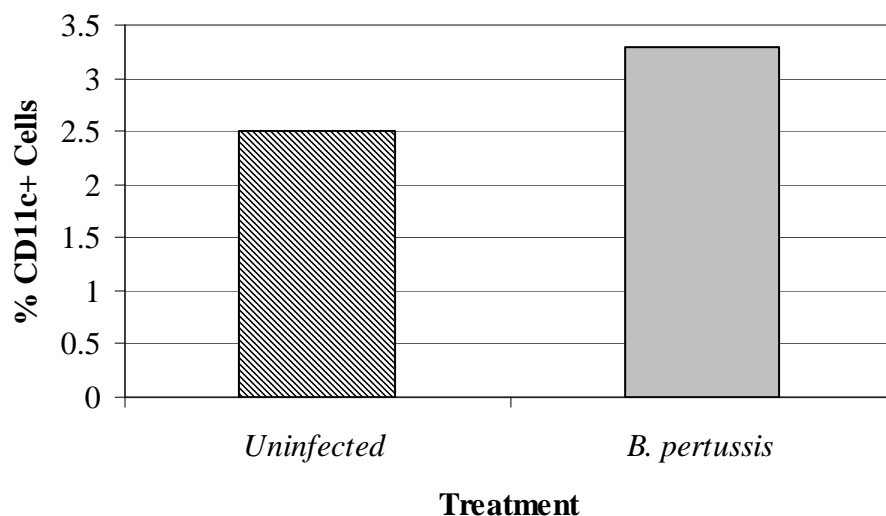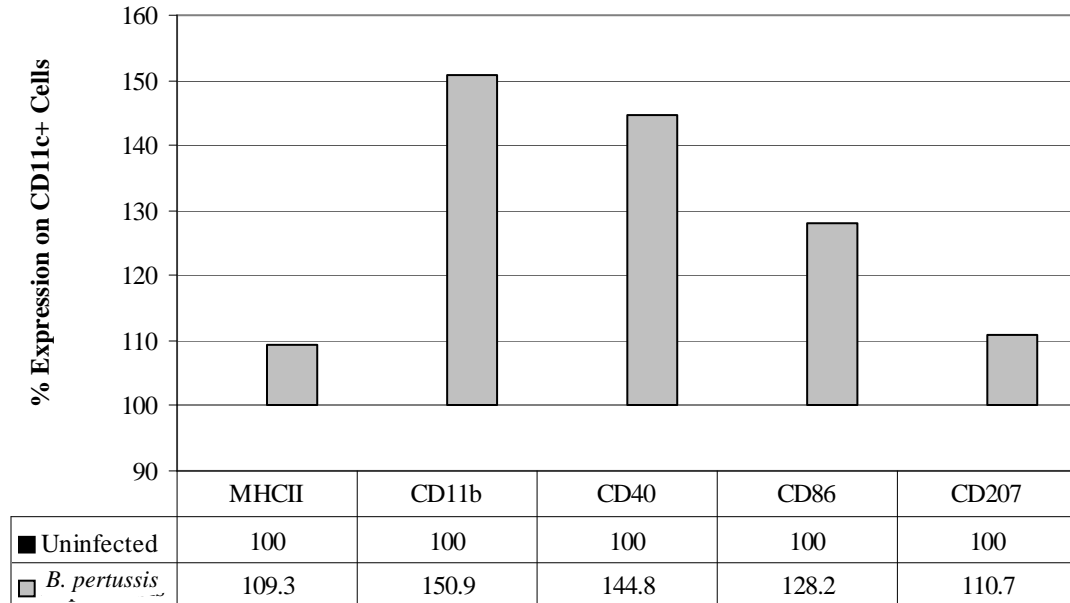**FIGURE 7.** DCs mature in response to *B. pertussis* infection even with smaller doses of inoculum. BALB/c mice were infected intranasally with 5×10^5 CFU in 20 μl and percent DCs were measured in each treatment at a peak infection time of 10 days (*A*). Maturation markers (MHC class II, CD40, CD86) and lung-associated markers (CD11b, CD207) were also measured (*B*). *Bordetella pertussis*-infected DCs were co-cultured with naïve splenocytes and levels of CCR5, CCR6, CD103, CD106, CD11a, CD162, CD49d, and CD49f were assessed (*C*). All values for the co-cultures were normalized to uninfected controls (100%).
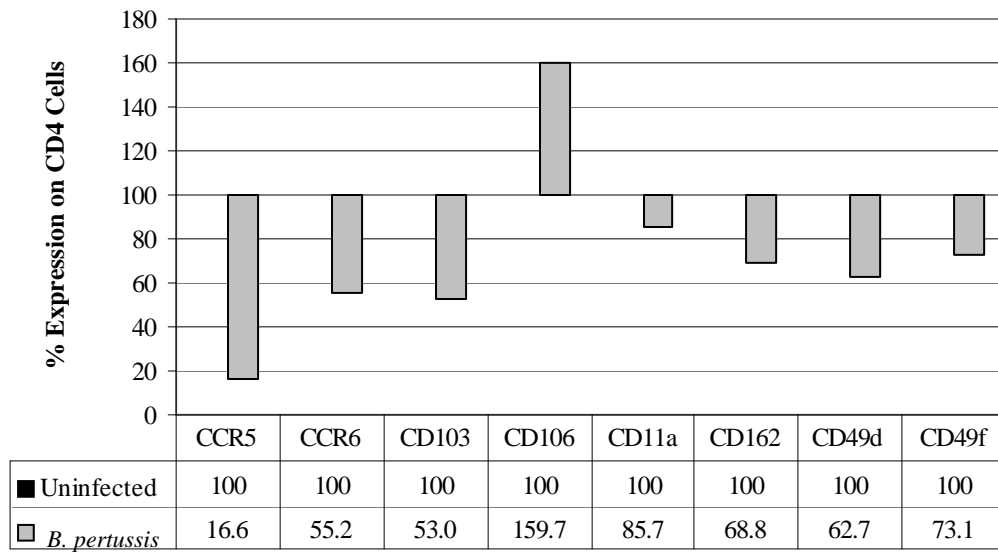
7B

DC Maturation During Peak Infection

	MHCII	CD11b	CD40	CD86	CD207
■ Uninfected	100	100	100	100	100
□ *B. pertussis*	109.3	150.9	144.8	128.2	110.7

y-axis: % Expression on CD11c+ Cells

7C

Trafficking Molecule Expression During Peak Infection

	CCR5	CCR6	CD103	CD106	CD11a	CD162	CD49d	CD49f
■ Uninfected	100	100	100	100	100	100	100	100
□ *B. pertussis*	16.6	55.2	53.0	159.7	85.7	68.8	62.7	73.1

y-axis: % Expression on CD4 Cells

8A
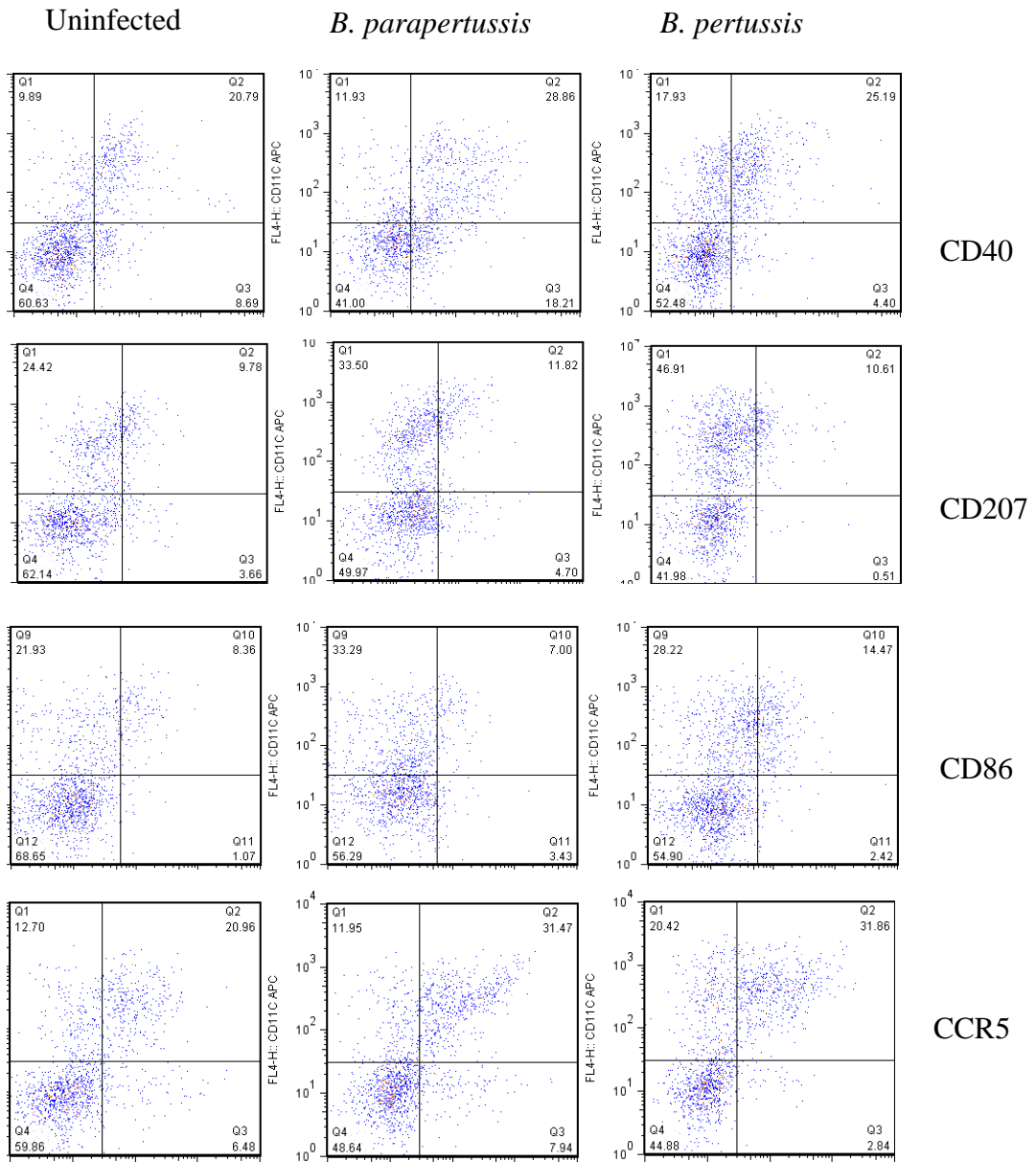

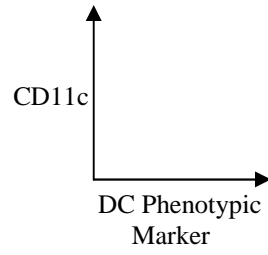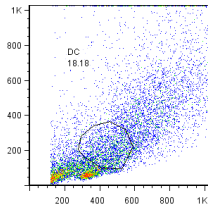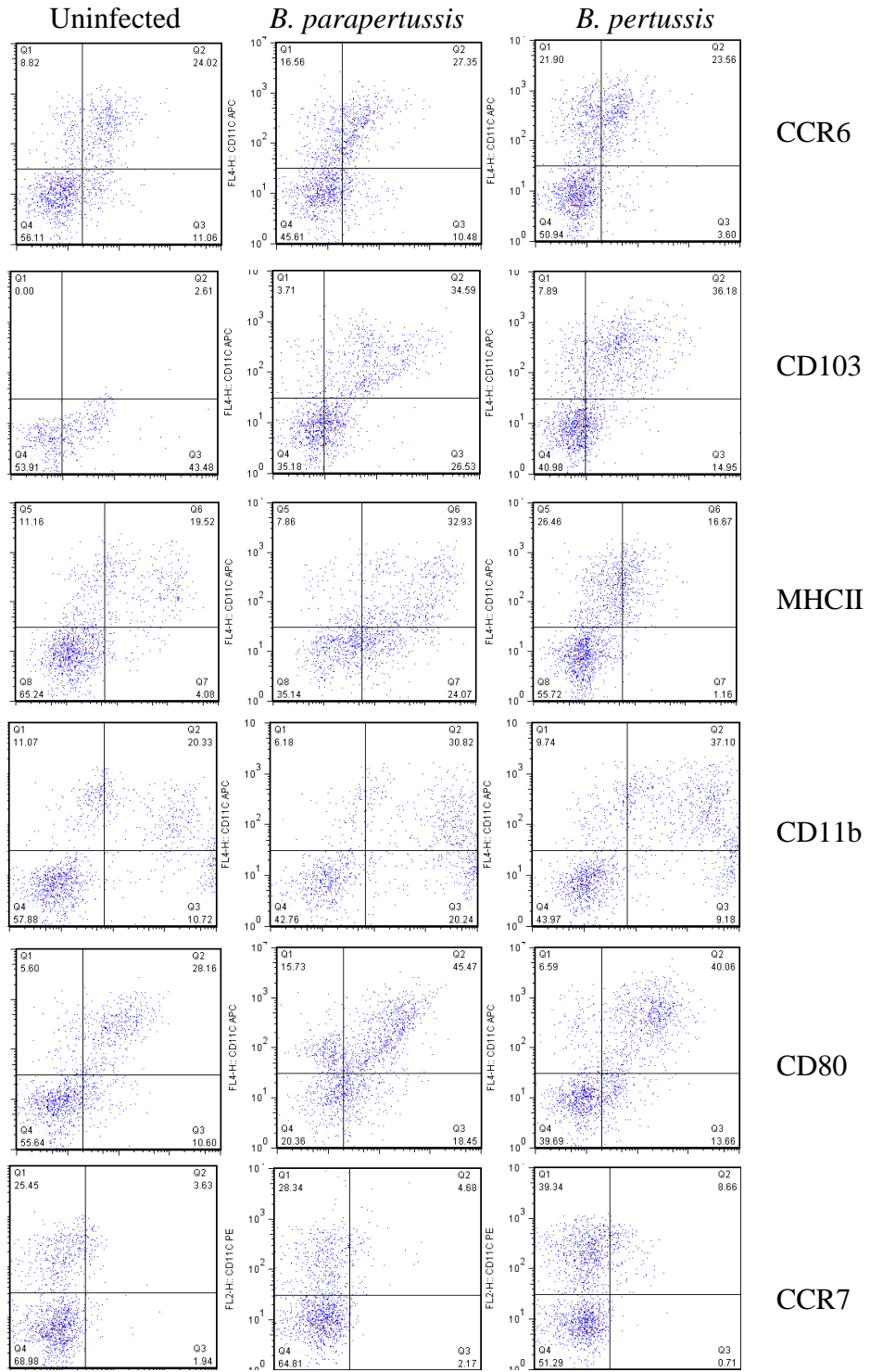**Percent CD11c+ Cells per Treatment**
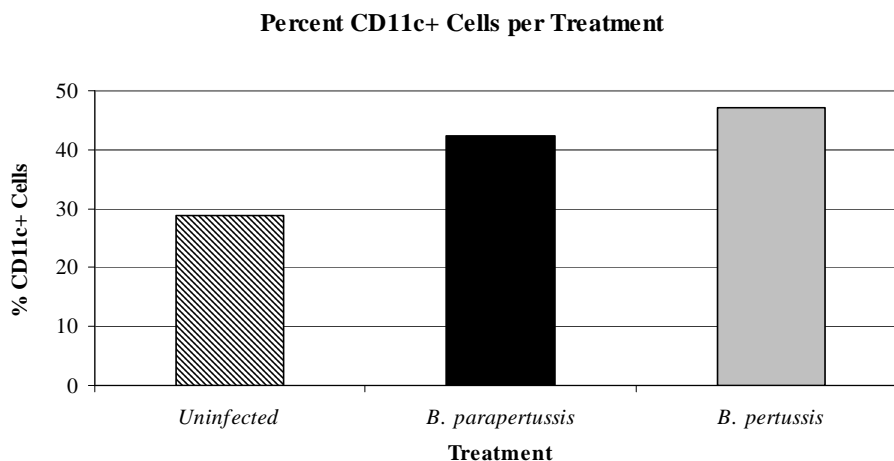
FIGURE 8. DCs mature in response to *Bordetella* and downregulation of homing molecules on CD4+ T cells was observed in *B. pertussis* co-cultures. We assessed the percentage of CD11c+ cells in each treatment using quadrants that were placed according to positive controls (*A*). DC maturation markers observed included MHC class II, CD40, CD80, CD86; myeloid and lung associated markers, CD11b, CD207 and migratory makers CD103, CCR5, CCR6, CCR7 (*B*). After co-culturing, levels of the chemokine receptors CCR5, CCR6, CXCR3, and adhesion markers CD11a, CD29, CD49a, CD49d, CD106, and CD162 were measured (*C*). All values were normalized to uninfected controls (100%).
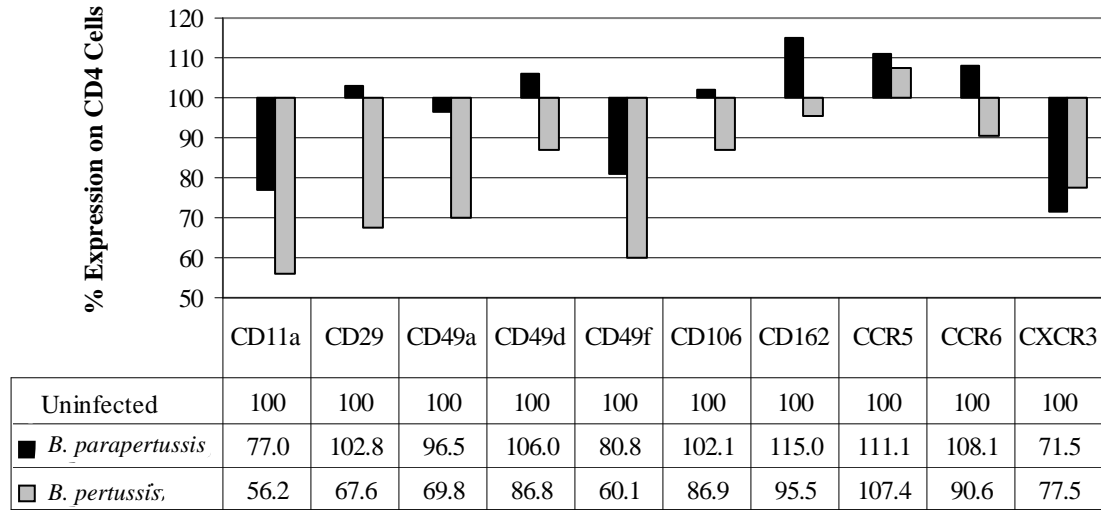
APPENDIX B: SUPPLEMENTARY DATA

8B

CD11c

DC Phenotypic
Marker

Uninfected   *B. parapertussis*   *B. pertussis*

CD40

CD207

CD86

CCR5

APPENDIX B: SUPPLEMENTARY DATA

56

8C

Trafficking Molecules Expression During Peak Infection

	CD11a	CD29	CD49a	CD49d	CD49f	CD106	CD162	CCR5	CCR6	CXCR3
Uninfected	100	100	100	100	100	100	100	100	100	100
■ *B. parapertussis*	77.0	102.8	96.5	106.0	80.8	102.1	115.0	111.1	108.1	71.5
□ *B. pertussis*	56.2	67.6	69.8	86.8	60.1	86.9	95.5	107.4	90.6	77.5

Trafficking Molecule Expression After Recovery

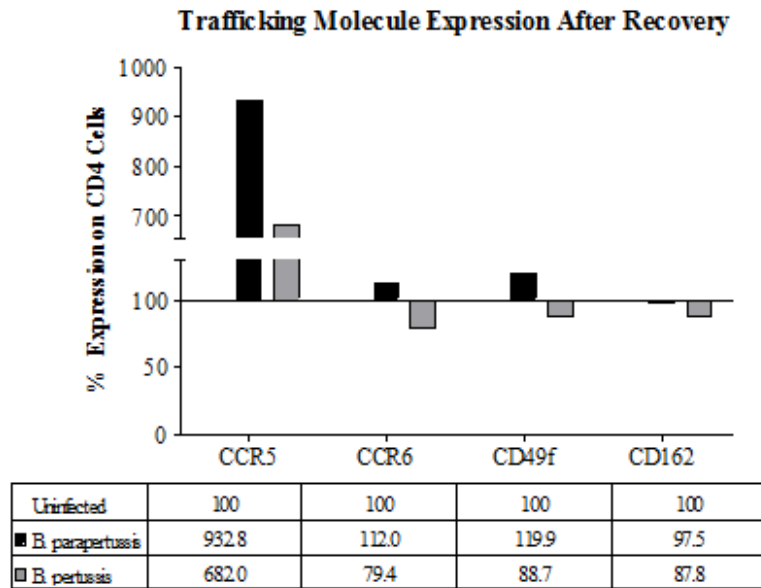|	CCR5	CCR6	CD49f	CD162
Uninfected	100	100	100	100
■ B. parapertussis	932.8	112.0	119.9	97.5
▨ B. pertussis	682.0	79.4	88.7	87.8

FIGURE 9. 25 days post-infection, the pattern of trafficking markers changes compared to peak infection. 25 days after infection with *Bordetella*, levels of CCR5, CCR6, CD49f, and CD162 were measured on co-cultured CD4+ T cell. All values were normalized to uninfected controls (100%).