UNDERSTANDING THE ROLE OF DENDRITIC CELL SUBSETS IN THE GENERATION OF A $CD8^+$ T CELL RESPONSE FOLLOWING PULMONARY VACCINIA VIRAL INFECTION

BY

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2'-5' OAS……2'-5' Oligoadenylate synthase

APC……Antigen presenting cells

BMDC……Bone marrow-derived dendritic cells

CCR……C-C chemokine receptor, i.e. CCR7

CD……"Cluster of differentiation" molecules, i.e. CD8

cDC……Common dendritic cells

CTL……Cytotoxic lymphocytes

CTO……Cell tracker orange

d……..day

DC……...Dendritic cells

E3L……Vaccinia virus protein

eGFP……..Enhanced green fluorescent protein

ER……...Endoplasmic reticulum

IFN……..Interferon, i.e. IFNγ

IL……Interleukin, i.e. IL-12

JNK……Jun N-terminal kinase

K3L……Vaccinia viral protein

LN……...Lymph node

LPS……Lipopolysaccharide

MCP……Monocyte chemotactic protein (AKA CCL2)

MHC……...Major histocompatibility complex

MIP……Macrophage inflammatory protein, i.e. MIP1α

MLN……Mediastinal lymph node

MMP……...Matrix metalopeptidase, i.e. MMP-9

NK cell……....Natural killer cell

NP……...Nucleoprotein (viral protein)

PAMP……Pathogen associated molecular pattern

pDC……Plasmacytoid dendric cell

PGE……Prostiglandin E

PolyI:C……Polyinosine polycytidylic acid

PFU……....Plaque forming unit

PMN……Polymorphonuclear cell.

PKR……Protein kinase R

RANTES……C-C motif ligand 5, i.e. CCL5

RSV……Respiratory syncytial virus

STAT……..Signal transduction and activator of transcription

TAP……Transporters associated with antigen-processing

TGFβ……..Transforming growth factor beta

TLR……Toll-like receptor

TNF……Tumor necrosis factor

VV……Vaccinia virus

ABSTRACT

Unlike many other tissues, the lung is constantly assaulted with foreign antigens, both environmental and infectious. This includes a large number of viruses which spread via aerosolized droplets. In order for the body to mount an adaptive immune response to a pathogen, T cells circulating through lymph nodes (LN) must be alerted to the presence of infection in the periphery. This occurs as a result of presentation of pathogen derived epitopes on professional antigen presenting cells (APC), primarily dendritic cells (DC). While an important role for dendritic cells (DC) as the activators of naive T cells is clear, the contribution of distinct DC subsets in this process is less understood. Multiple DC subsets are present within the lung tissue ($CD103^+$ DC and $CD11b^+$ DC) and draining lymph nodes (MLN) ($CD8\alpha^+$), and as such, all are potential regulators of T cell activation (for review see[1,2]). These studies sought to understand how DC subsets contribute to the generation of virus-specific $CD8^+$ T cells following pulmonary viral infection.

We have developed a model of pulmonary vaccinia (VV) infection in order to address the role of DC subsets in activating naïve $CD8^+$ T cells. The use of a recombinant virus expressing eGFP allowed us to identify DC that had access to viral antigen. Following intratracheal instillation of the cell permeable dye cell tracker orange (CTO) we were able to delineate DC in the MLN that had trafficked from the lung. These methods, along with cell sorting, have allowed us to determine which DC subsets were capable of priming naïve $CD8^+$ T cells *ex*

vivo. While $CD103^+$ DC and $CD11b^+$ DC in the lung showed similar expression of eGFP, the $eGFP^+CD11b^+$ DC failed to migrate to the MLN. The $eGFP^-$ $CD11b^+$ DC that did migrate were poor inducers of $CD8^+$ T cell activation, as were LN resident $CD8\alpha^+$ DC. Our data identified $CD103^+$ DC as the most potent activators of naïve $CD8^+$ T cells in response to pulmonary VV infection.

During the course of these studies, we identified $CD8\alpha^+CD103^+$ DC subset present in the MLN, but absent in the lung. While this DC subset has been noted in the past, this is the first set of studies to extensively characterize this population. We found that these $CD8\alpha^+CD103^+$ DC resemble the $CD8\alpha^-CD103^+$ DC in expression of surface markers CD205 and CD24. CTO labeling studies suggested $CD8\alpha^+CD103^+$ DC migrate to the MLN from the lung, although with delayed migration kinetics compared to $CD8\alpha^-CD103^+$ DC. Finally, we noted that while the $CD8\alpha^+CD103^+$ DC have enhanced expression of co-stimulatory molecules in response to toll-like receptor (TLR) stimulation, incubation with naïve $CD8^+$ T cells resulted in less T cell division than was seen with $CD8\alpha^-$ $CD103^+$ DC. While the role of the $CD8\alpha^+CD103^+$ DC in $CD8^+$ T cells activation has yet to be fully elucidated, it appears that these DC are a population with distinct properties, separate from airway $CD8\alpha^-{}^+CD103^+$ DC and LN resident $CD8\alpha^+CD103^-$ DC.

INTRODUCTION

Given that the lungs are a vital organ, it is necessary to tightly control immune responses at this site. This tissue is constantly exposed to foreign antigens, both environmental and infectious, including aerosolized virus. It is therefore important to understand how the immune system detects these infections and mounts subsequent $CD8^+$ T cell response. Recently, the dominant role of DC in the development of $CD8^+$ T cells has been established (for reviews[3,4]). There are multiple DC subsets are present in the lung and draining lymph nodes that have the potential to regulate T cell activation[5, 6]. It was our goal to determine the role of these DC subsets in establishing an adaptive $CD8^+$ T cell response following pulmonary infection with a pox virus.

Dendritic Cells and Activation of $CD8^+$ T cells.

Dendritic cells (DC) are considered the most potent antigen presenting cell (APC) with regard to the generation of an adaptive T cell response[7,8]. As naïve T cells are activated in lymph nodes (LN) and infection most often occurs in non-lymphoid tissue, it is necessary for the antigen in the periphery to enter the LN. DC in the periphery act as conduits, bringing antigen from the periphery to the LN, where an adaptive T cell response can be initiated.

DC initiate both a $CD4^+$ and $CD8^+$ T cell response. Antigen-specific $CD4^+$ T cells become stimulated when they encounter DC presenting cognate antigen in the context of major histocompatibility complex class-II molecules (MHCII). These

antigens (12-25 amino acids) are derived from proteins that the DC has obtained from an exogenous source, such as the phagocytosis of apoptotic cells or bacteria. Although the $CD4^+$ T cell response is an important aspect of adaptive $CD8^+$ T cell memory has proven protective against secondary VV challenge[9] and thus the focus of these experiments.

Antigen-specific T cell receptors (TCR) on the $CD8^+$ T cell recognize antigen bound to MHC class-I (MHCI) on the surface of DC. The peptides bound to MHCI are between 8-10 amino acids in length and are derived from proteins present in the cytoplasm of the DC. Following proteasome degradation of cytosolic proteins, peptides are shuttled into the endoplasmic reticulum (ER) and loaded onto MHCI molecules. Under non-infectious conditions, the peptides bound to the MHCI molecules represent an array of endogenous proteins being translated by the cell. However, should an intracellular pathogen infect a DC, the pathogen's proteins are then available for processing and presentation by MHCI through the same mechanism as the host's proteins.

The caveat of MHCI binding only endogenous peptides would be the lack of a sufficient $CD8^+$ T cell response to any extracellular pathogen. We know, however, that proteins from extracellular sources are able to elicit a $CD8^+$ T cell response. In the mid-1970 Bevan *et. al*. showed that mice injected with congenic cells could establish a $CD8^+$ T cell response specific for the donor cells[10]. This phenomenon was termed cross-presentation.

$CD8^+$ T cells require three individual signals from the DC in order for optimal activation to occur[11,12]:

1) MHCI/peptide

2) co-stimulatory molecules

3) cytokines

The first signal, MHCI/peptide binding to the TCR on the $CD8^+$ T cell, confers specificity to the $CD8^+$ T cell response. The binding of MHC/peptide to the TCR provides an initial mode of regulation for the T cell response. If binding of TCR to the MHCI/peptide complex occurs in the absence of the second and third signal, the $CD8^+$ T cell becomes tolerized to the antigen leading to anergy[13].

Co-stimulatory molecules expressed by the DC binding to their corresponding ligands on the $CD8^+$ T cells is the second required signal for optimal $CD8^+$ T cell stimulation[14], resulting in production of IL-2 and proliferation of $CD8^+$ T cells[15]. Among the most studied co-stimulatory molecules capable of providing signal two are CD80 and CD86. CD80 and CD86 are both members of the B7 family of molecules which bind CD28 on the $CD8^+$ T cells. Although CD80 and CD86 share a 25% sequence homology[16], their expression on DC does not appear to be redundant. In support of the non-redundant roles of these molecules, CD80 has been shown to be important for the up-regulation of CD25 on $CD8^+$ T cells following conjugation with DC infected with SV5 in vitro. In this model, SV5 matured DC have decreased CD80 expression, resulting in decreased $CD8^+$ T

cell proliferation and function[17]. Additionally, in the context of a pulmonary influenza infection, blocking CD80 binding to CD28, while leaving CD86 binding intact results in fewer virus specific $CD8^+$ T cells in the lung as well as a defect in $CD8^+$ T cell IFNγ production[18].

Production of cytokines by DC provides the third signal required by $CD8^+$ T cells. This signal is thought to play a critical role in the acquisition of effector function. IL-12 and IFNα/β are two of the most highly investigated cytokines capable of providing this third signal. Bioactive IL-12p70 is composed of a heterodimer of IL-12p40 and IL-12p35. Production of IL-12p70 requires two individual stimuli: an inflammatory signal for IL-12p40 production in addition to either CD40 ligation[19] or multiple signals through toll-like receptors (TLR)[20,21] for production of IL-12p35. IL-12 is essential for $CD8^+$ T cells to produce INFγ[22,23], while IFNα/β signaling modulates $CD8^+$ T cell survival and acquisition of effector function[24-28].

Effector functions associated with signal three include the production of IFNγ, TNFα and lytic components such as granzyme. INFγ acts in a paracrine capacity to increase antigen processing and presentation on APC[29,30] and to maintain a T_h1 cytokine environment[31,32]. TNFα acts as a feedback mechanism to stimulate DC maturation[33,34] as well as inducing cytolysis on airway epithelial cells in a perforin-independent manner[35]. Finally, granzyme release can induce apoptosis in target cells[36] through caspase-3[37] and cytochrome-c release[38,39].

In a naïve animal the DC exist in an immature state and lack the necessary signals needed to initiate $CD8^+$ T cells. However, the DCs express high levels of adhesion molecules and are highly phagocytic. DC must undergo a process called maturation wherein they up-regulate expression of co-stimulatory molecules and cytokines, resulting in their enhanced capability to effectively prime T cells. DC maturation can be initiated by a number of stimuli. Pathogen-associated molecular patterns (PAMPS) are conserved motifs associated with bacteria and viruses. These PAMPS are recognized by toll-like receptors (TLR) and other pattern recognition receptors (PRRs) expressed by the DC, initiating DC maturation. DC can also undergo maturation following exposure to inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interluken-1 (IL-1), interluken-6 (IL-6) and type one interferon (IFNα/β). Additionally, ligation of CD40 on the DC surface with CD40L can stimulate DC maturation.

Upon receiving a maturation signal the DC undergoes morphological changes, whereby they increase their surface area through the formation of dendrites as well as decrease adhesion molecule expression, while up-regulating CCR7 expression – leading to an increased motility and increasing their expression of co-stimulatory molecules: CD40, CD80 and CD86. Following maturation, the DC become less phagocytic, while at the same time increasing its rate of antigen processing and the expression of MHCII on its surface. With these changes the mature DC now has all of the necessary signals to optimally prime naïve T cells.

Dendritic Cell Subsets.

It has recently been demonstrated that DCs are not a homogenous population. A large body of work within the DC field has been dedicated to determining which markers delineate subsets with differential functions (Table 1) or lineages. Our studies will focus on the role of lung derived $CD103^+$ DC and $CD11b^+$ DC and LN resident $CD8\alpha^+$ DC in the generation of virus specific $CD8^+$ T cells following pulmonary VV infection. We will also characterize a new $CD8\alpha^+CD103^+$ DC subset and examine their potential role in the generation of adaptive immunity.

Subset	Location	Markers	Function
$CD103^+$	Lung epithelia	$CD11c^+$ $CD103^+$ $CD11b^-$ $CD8\alpha^{-/+}$ $Langerin^+$	IL-12 production, CD8 & CD4 T cell stimulation, cross-presentation
$CD11b^+$	Lung parenchyma	$CD11c^+$ $CD11b^+$ $CD103^-$ $CD8\alpha^-$ $Langerin^-$	CD8 & CD4 T cell stimulation, leukocyte recruitment to lung
$CD8\alpha^+$	LN	$CD11c^+$ $CD11b^-$ $CD103^-$ $CD8\alpha^+$ $Langerin^+$	IL-12 production, CD8 T cell stimulation, cross-presentation
pDC	Lung & LN	$CD11c^{lo}$ $B220^+$ $SiglecH^+$ $PDCA1^+$	IFNα/β production
tipDC	Lung	$CD11c^+$ $CD11b^+$ $Ly6C^+$	TNFα & inducible nitric oxide production

Table 1 – Characterization of Lung-relevant DC subsets

The $CD103^+$ DC were first described in 2006[40], making them one of the more recent DC subsets to be identified. CD103, a α_E-β_7 integrin, binds E-cadherin which is present on the basal surface of the lung epithelium and vascular endothelial cells[40]. Expression of tight junction proteins such as Claudin-1 and Claudin-7[40] allow the $CD103^+$ DC to intercalate between the epithelial cells of the airway and directly sample the airspace. $CD103^+$ DC have been shown to be able to cross-present intratracheally instilled Ova[41] and express Clec9A, which

has been shown to be necessary for the cross presentation of necrotic cell-associated antigens[42]. In response to TLR3, $CD103^+$ DC have been shown to respond with high IL-12 production[40]. Expression of IL-6 and TNFα are modest when stimulated with the TLR4 agonist, LPS, although expression increased following stimulation with CpG (TLR9)[43].

DC expressing CD103 have also been identified in the intestine and colon of mice. Under steady state conditions, gut $CD103^+$ DC induce FoxP3 expression in $CD4^+$ T cells[44,45] in a transforming growth factor β (TGFβ) and retinoic acid dependent fashion[44]. However, during periods of intestinal inflammation (e.g. colitis), the $CD103^+$ DC induce less FoxP3 expression within $CD4^+$ T cells[45] and are able to generate $CD8^+$ T cells to orally administered soluble antigens[46]. Importantly, the $CD8^+$ T cells stimulated by the $CD103^+$ DC in the intestine draining lymph node, express both CCR9 and $\alpha_4\beta_7$ integrins[47], which are necessary for effector $CD8^+$ T cells in homing back to the gut. Unlike the $CD103^+$ DC in the intestines, the lung $CD103^+$ DC have not been shown to exhibit any tolerogenic properties.

$CD11b^+$ DC are located in the parenchyma of the lung and as such do not have direct contact with the airway[40]. Microarray analysis has shown increased expression of scavenger receptor RNA in $CD11b^+$ DC compared to $CD103^+$ DC[48], leading to the hypothesis that $CD11b^+$ DC are superior at phagocytosis. Indeed, it has been shown that $CD11b^+$ DC have a higher rate of pinocytosis[40],

despite the $CD103^+$ DC ability to cross-present. $CD11b^+$ DC secrete IL-6 and TNFα in response to TLR4 and TLR7 stimulation, and, to a lesser extent with TLR9 stimulation[49]. In addition to their ability to stimulate naïve T cells, $CD11b^+$ DC are thought to play an important role in the recruitment of leukocytes into the lung during infection, as they secrete significantly more chemokines (MIP-1, MIP-1α MIP-1β, MIP-1γ and RANTES) than $CD103^+$ DC[50].

$CD11b^+$ and $CD103^+$ DC, with their close proximity to pulmonary viral antigens, are not the only DC subsets with the potential to stimulate a virus-specific CD8 T cell response following respiratory infection. $CD8\alpha^+$ DC are thought to enter the LN from the blood and are not regularly found within the tissue. Therefore in order for $CD8\alpha^+$ DC to present antigen, the antigen must access the LN. This subset was first characterized in the spleen and was shown to lack CD8β and CD3 expression, while expressing the mRNA for CD8α[51]. Early on, these DC were termed lymphoid-derived DC because of their expression of CD8α. However, this nomenclature has subsequently been abandoned and they are now characterized as conventional DC, along with $CD103^+$ DC and $CD11b^+$ DC. The $CD8\alpha^+$ DC subset are efficient at cross presentation of both soluble[52,53] and cell associated antigens[54,55]. Stimulated $CD8\alpha^+$ DC are known to produce high levels of IL-12p70, particularly in the spleen, but also in the LN[56].

This thesis also explores a $CD8\alpha^+CD103^+$ DC subset present in the lung draining LN. This is not the first documentation of such a subset. CD8α co-expression

with CD103 has been noted on DC of the skin[57,58], LN[59,60] and spleen[61]. While little is know about this population, a recent study revealed that among splenic DC $CD8\alpha^+CD103^+$ DC in the marginal zone are unique in their ability to phagocytose apoptotic cells[61]. To date, Qiu et. al is the only group to explore the function of $CD8\alpha^+CD103^+$ DC as most studies group them together with the $CD8\alpha^+$ DC or the $CD103^+$ DC.

While the plasmacytoid DC (pDC) and the TNF-α/inducible nitric oxide synthase (iNOS)-producing DCs (tipDCs) are not thought to play a major role in the generation of adaptive immunity through presentation of antigen to T cells in the draining LN, they may present antigen at the site of infection[62,63]. In addition these DC play an important role in innate immunity. PDC produce the greatest amount of IFNα/β in response to viral infection[64,65] compared to other DC. TipDC, as their name suggests, secrete TNFα and NO in response to stimuli. Together these DC help to enhance innate immune responses.

DC and Respiratory Virus Infection Models.

The most commonly studied experimental models of respiratory viral infections are influenza virus and the paramyxoviruses respiratory syncytial virus (RSV) and Sendai virus (SeV). Influenza and RSV are highly contagious and represent a health concern for the young and elderly. SeV, while not a human pathogen, provides a useful model for studying paramyxovirus immunity within a natural host (the mouse).

DC are known to be important to the clearance of paramyxoviruses[66,67,68]. In SeV models, active infection of lung resident DC led to their maturation and rapid migration into the mediastinal lymph node (MLN)[66]. Viral RNA was detected in both the $CD11b^+$ DC and $CD103^+$ DC in the MLN and both DC subsets could present viral antigen to CD8 and CD4 T cells[68].

Lung migratory DC also play a critical role in the response to influenza virus infection. The first study describing the ability of DC from the lung to prime $CD8^+$ T cells in the influenza model utilized CFSE to track DC[69]. It has since been shown that these DC are most likely the airway resident $CD103^+$ DC. $CD103^+$ DC play a large role in generating the $CD8^+$ T cell response to influenza. $CD103^+$ DC are more susceptible to influenza infection compared to the $CD11b^+$ DC and they produce the majority of IL-12 following infection[70]. The important role of $CD103^+$ DC in generating an adaptive response to influenza is further exemplified by the fact that if they are knocked down, either by clodronate treatment or in mice whose $langerin^+$ cells are susceptible to diphtheria toxin, mice show increased weight loss, decreased numbers of virus specific $CD8^+$ T cells in the lungs and increased time required to clear the virus[5,60].

The role of $CD11b^+$ DC priming a CD8 T cell response to influenza is less clear. Some studies suggest they play no role in the generation of the CD8 T cell response[70,69], while others contend that although they activate $CD8^+$ T cells, the

resulting $CD8^+$ T cells are decreased in effector function[60]. *In vivo*, $CD11b^+$ DC appear unable to prime $CD8^+$ T cells following exposure to soluble antigen[60] suggesting they are unable to cross present antigen and rely on direct infection in order to present antigen in the context of MHCI.

Vaccinia Virus.

Vaccinia virus (VV) is a member of the orthopoxvirus family and closely related to variola virus, the causative agent of smallpox. The large, ~190 kbp genome of vaccinia virus encodes approximately 250 genes. Many of these genes attenuate the immune response or help the virus avoid detection. Among these genes are receptor homologs for TNFα, IL-1, IL-6 and IFNγ[71].

The virus employs both extracellular and intracellular mechanisms to counteract the effects of type 1 IFN (reviewed[72,73]). B18R is an IFNα/β binding protein that can be both secreted or bind to the surface of cells in order to compete with IFN receptors for soluble IFNα/β in the environment. When IFNα/β binds to its receptor the resulting signaling cascade culminates in the production of proteins such as protein kinase R (PKR) and 2'-5' Oligoadenylate Synthetase (2'5'OAS). These proteins down regulate translation in response to dsRNA produced during VV infection. To combat this and ensure that viral protein continues to be translated, the virus encodes for a protein that binds dsRNA (E3L) and one that is a homologue for the target of PKR (K3L). While the IFNα/β binding protein

B18R helps to prevent initiation of the IFNα/β signal, E3L and K3L, act to dampen the effects of the IFN induced cellular proteins.

It has recently been demonstrated that toll-like receptor 2 (TLR2) is important in the innate recognition of VV[74] and that TLR9 is vital to survival following a lethal poxvirus infection[75]. VV encodes two proteins that block signaling through TLR. A52R binds to IRAK2 and TRAF6[76], while A46R binds MyD88, TRIF and TRAM[77] inhibit the downstream activation of NFκB that occurs following TLR stimulation. Despite all of these evasion methods, the immune system is still able to respond to and clear VV infection from mice.

An effective immune response to an initial VV infection includes $CD4^+$ and $CD8^+$ T cells along with B cells. Memory $CD8^+$ T cells are protective against secondary challenge[9]. IFNγ production by both $CD4^+$ and $CD8^+$ T cells is of particular importance, as mice lacking the IFNγR had a 60-fold increase in viral titers in their spleen, liver, lung and ovaries at day 22 post infection[78].

Because of its significant homology to variola virus (greater than 90%) and its attenuated nature, VV was used in the vaccine that eradicated smallpox in the 1970s. Variola spreads through an aerosolized transmission route[79,80]. Variola virus delivered through aerosolized droplets first infects the lung mucosa at the site of initial infection. This is followed by primary viremia, spread of the virus to

other tissue. Finally, an external rash indicates the secondary viremia stage of infection[81].

Our studies utilize a pulmonary route of VV infection. Although the dosage of the virus used was sublethal and mice were sacrificed soon after infection (within 1-4 days), respiratory infection of mice with high doses of cowpox virus has been shown to lead to meningitis and pneumonia[82]. However, differing lung pathology in mice infected with either cowpox or rabbit pox has made generalization about poxvirus induced lung pathology difficult[83]. Although systemic infection following VV is possible, given the length of infection in our studies, it is unlikely that VV was able to establish a systemic infection. These studies use VV as a model to understand how DC subsets contribute to the generation of $CD8^+$ T cells following a pulmonary viral infection.

MATERIALS AND METHODS

Mice

C57BL/6 mice (Frederick Cancer Research Facility, National Cancer Institute, Fredrick, MD) were used throughout this study. OT-I mice were from a colony established with breeding pairs obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in the Wake Forest University School of Medicine animal facilities, under specific pathogen free conditions and in accordance with approved ACUC protocols. Mice for these studies were between 6 and10 weeks of age.

Virus and Infection

The recombinant VV.NP-S-eGFP virus was the kind gift of Jack Bennink (NIH). This virus expresses a fusion protein, under the early viral promoter, containing the NP protein from influenza virus, the SIINFEKL epitope from ovalbumin and enhanced green fluorescent protein (eGFP) [84]. The recombinant VV.M and VV.P viruses express the M and P proteins from SV5, respectively, and were constructed on site, as previously described [85]. For infection, mice were anesthetized by i.p. injection of avertin, followed by intranasal administration of $1x10^7$ PFU of virus in a volume of 50µL. Mock infected mice received equivalent volumes of PBS. Intratracheal infections were performed following anesthetization with isofluorane by delivery of 10^7 PFU of virus in 30 µL PBS. Mice recover from infection with this dose of VV.NP-S-eGFP and generate a $CD8^+$ T cell response (our unpublished data).

Intratracheal Instillation of Cell Tracker Orange

Five hours following i.t. infection with vaccinia virus, mice were anesthetized with isoflourane and 50 µL of 1mM Cell Tracker Orange (Molecular Probes) was administered intratracheally. When the DC from the MLN were analyzed on day 2 post infection, this pulse with CTO resulted in 9.7±1.7% of the $eGFP^+$ DC co-staining for CTO.

For migration time lines with CTO (Figure 7), mice were infected on day zero. Twenty-four hours prior to MLN harvest, mice were treated with 1 mM CTO i.t.

DC isolation from the mediastinal LN

At the indicated day post infection, MLN were isolated and pooled within each experimental condition. The tissue was mechanically disrupted and allowed to incubate in complete media supplemented with 1 mg/mL collagenase D (Roche) for 45 minutes at 37º. Cells were then passed through a 70 µm nylon cell strainer (BD Falcon). RBC were removed by treatment with ACK lysis buffer (Lonza).

Analysis of DC maturation

Cells obtained from the MLN following collagenase digestion were incubated for 5h in the presence of GolgiPlug (BD BioSciences). Following the incubation, cells were stained with a combination of CD11c-APC (HL3) or PE/Cy7 (HL3), CD103-PE (M290), CD11b-PE/Cy7 (M1/70) CD86-Pacific Blue(GL-1), CD80-PE (16-10A1) and CD90.2-biotin(53-2.1). Streptavidin 525 Qdots (Molecular Probes)

were used to detect biotinylated antibodies. Expression of these fluorophores, along with eGFP expression from the virus was assessed using the BD FACSCanto II. Data were analyzed using FacsDiva software (BD Biosciences).

Naïve T cell activation

Prior to sorting, CD11c expressing cells were enriched by positive selection using the Miltenyi column system. Enriched populations were routinely 45-65% $CD11c^+$. The enriched population was stained with CD11c-APC and a combination of the following: CD8α-PerCP-Cy5.5, CD8α-V450, CD103-PE, CD103-PerCP-Cy5.5, CD11b-PE/Cy7 along with biotinylated CD19, CD90.2 and CD49b antibodies (all from BD BioSciences). Streptavidin 525 Qdots (Molecular Probes) were used to detect biotinylated antibodies. Cells positive for the 525 Qdots were gated out of the analysis prior to sorting. This approach was shown in preliminary studies to increase purity in the isolated DC subsets. Thus all sorted cells met the criteria of $CD11c^+$ $CD90.2^-$ $CD49b^-$ $CD19^-$. For the analysis of lung derived cells in the lymph node, DC were sorted into four populations based on the presence of the cell tracker orange and the expression of CD103 and CD11b. For the analysis of $CD8\alpha^+$ $CD103^+$ vs. $CD8\alpha^-$ $CD103^+$ DC cells were sorted based on CD8α and CD103 expression. All sorts utilized the BD FACsAria cell sorter and all sorted cells were $CD11c^+$ $CD90.2^-$ $CD49b^-$ $CD19^-$. Sorted populations were routinely 94-99% pure. To assess the ability of the DC subsets to induce naive T cell activation, CFSE-labeled OT-I T cells were co-cultured with sorted DC populations at a ratio of 1:4 (DC:OT-I) in a V-bottomed,

96-well plate. Cells were incubated for 60h at 37ºC. Following incubation, cells were stained with anti-CD8α-PerCP-Cy5.5 and anti-CD90.2-APC antibodies. Samples were acquired using a BD FACsCalibur. FlowJo softare (Treestar, Inc.) was used for analysis of cell division.

Surface Marker Staining

MLN were harvested from 5 B6 mice and prepared as described. Following incubation with CD16/32 (to bind Fc receptors on the DC), cells were stained with CD11c APC (N418), CD90.2 biotin (53.2.1), CD103 PE (M290), CD8α PerCP-Cy5.5 (53-6.7), CD205 FITC (MG38), CD24 Pacific Blue (M1/69) and CD36 PE (HM36). Data was acquired using a BD FACSCalibur. MFI and percentage of each DC subset expressing each marker was analyzed using FacsDiva software from BD.

Treatment with TLR agonists

Twenty-four hours prior to MLN harvest, B6 mice were treated with 10 μg of a TLR agonist: PolyI:C (TLR3), LPS (TLR4), CL097 (TLR7) or CpG (TLR9) in 50 μL volume i.t. MLN were then harvested and a single cell suspension was obtained as described. Following incubation with CD16/32, cells were stained with CD11c APC (N418), CD90.2 biotin (53-2.1), CD103 PE (M290), CD8α PerCP-Cy5.5 (53-6.7), CD80 FITC (16-10A1) and CD86 Pacific Blue (GL-1). Data was acquired on the BD FACSCalibur and analyzed using FacsDiva.

CHAPTER 1

Functional Divergence among CD103$^+$ Dendritic Cell Subpopulations following Pulmonary Poxvirus Infection

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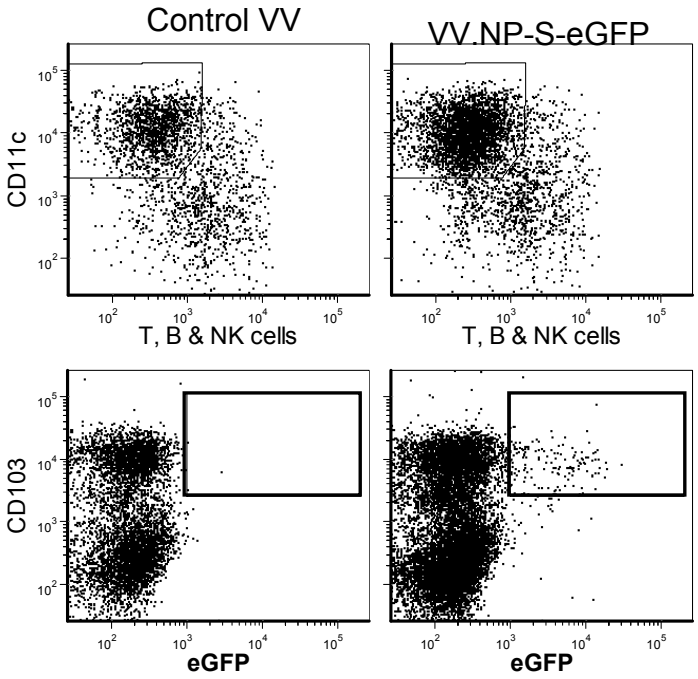
Summary

A large number of DC subsets have now been identified based on the expression of a distinct array of surface markers as well as differences in functional capabilities. More recently the concept of unique subsets has been extended to the lung, although the functional capabilities of these subsets are only beginning to be explored. Of particular interest are respiratory DC that express CD103. These cells line the airway and act as sentinels for pathogens that enter the lung, migrating to the draining lymph node where they add to the already complex array of DC subsets present at this site. Here we assessed the contribution that these individual populations make to the generation of a $CD8\alpha^+$ T cell response following respiratory infection with poxvirus. We found that $CD103^+$ DC were the most effective APC for naive $CD8\alpha^+$ T cell activation. Surprisingly, we found no evidence that lymph node resident or parenchymal DC could prime virus-specific T cells. The increased efficacy of $CD103^+$ DC was associated with the increased presence of viral antigen as well as high levels of maturation markers. Within the $CD103^+$ DC, we observed a population that bore CD8α on their surface. Interestingly, cells bearing CD8α were less competent for T cell activation compared to their $CD8\alpha^-$ counterpart. These data show that lung migrating $CD103^+$ DC are the major contributors to $CD8^+$ T cell activation following poxvirus infection. However the functional capabilities of cells within this population differ with the expression of CD8, suggesting $CD103^+$ cells may be further divided into distinct subsets.

RESULTS

eGFP$^+$ DC are specific to infection with VV.NP-S-eGFP. Early on in these investigations it became clear that, given the small numbers of events we would be analyzing, it was necessary to verify that the eGFP signal we were detecting in the MLN DC subsets was specific to the VV.NP-S-eGFP infection. We originally had some concern that infection with VV might alter DC autofluorescence, thereby leading to false positive results. EGFP expression was analyzed in DC from mice infected with either VV.NP-S-eGFP or a non-eGFP expressing control VV (Figure 1) and found to be specific to the DC from mice infected with VV.NP-S-eGFP.

Respiratory infection with vaccinia virus results in a generalized increase in DC in the MLN. Poxviruses are known to express an array of immunoregulatory molecules[86]. These include numerous cytokine receptor homologs, inhibitors of complement, and chemokine binding proteins[86]. As such, we first examined whether respiratory infection with the poxvirus vaccinia virus resulted in an influx of DC into the MLN, as has been reported for influenza virus infection[87]. Mice were intranasally infected with a recombinant vaccinia virus construct (VV.NP-S-eGFP) expressing a fusion protein containing the influenza virus nucleoprotein, the $Ova_{257-264}$ immunodominant ovalbumin epitope (SIINFEKL), and eGFP[84]. MLN were harvested on



Supplementary Figure 1. eGFP signal is only present following infection with VV.NP-S-eGFP. In order to verify that the eGFP expression we detected was a result of eGFP and not an autofluorescent artifact from VV infection, we infected mice with either VV.NP-S-eGFP or a non-eGFP expressing control VV. Two days post infection, MLN were harvested, pooled and enriched for $CD11c^+$ cells. The DC were determined by $CD11c^+$ $CD90.2^-$ $CD19^-$ $CD49b^-$ cells (top). The eGFP signal on $CD103^+$ DC was then analyzed (bottom).

days 1 to 4 post infection (p.i.) and DC recovered following enzymatic digestion in the presence of collagenase D. The number of $CD11c^+$ cells was calculated using flow cytometric data and the total number of cells recovered from the tissue (Figure 2A). $CD90.2^+$, $CD19^+$, and $CD49b^+$ cells were excluded by gating. As expected, by day 1 p.i., there was a significant increase in the number of $CD11c^+$ cells in the MLN (Figure 2A). The number of DC was similar at day 2 p.i., with a detectable, although not significant, transient decrease on day 3. MLN from animals at day 4 p.i. contained the largest number of $CD11c^+$ cells (a >19-fold increase compared to the level for mock-infected mice) (Figure 2A). Thus, infection with vaccinia virus resulted in a significant recruitment of DC to the draining lymph node that was detected as early as day 1 post infection.

We next evaluated the presence of defined DC populations. We used a panel of markers that included CD11c, CD103, CD8α, and CD11b to distinguish individual subsets. Lung airway-derived DC were identified as $CD11c^+$ $CD103^+$ $CD11b^-$ (here referred to as $CD103^+$ DC)[40]. In addition to this airway-derived population, a $CD11c^+$ $CD103^-$ $CD11b^+$ subset (here referred to as $CD11b^+$ DC) has been reported to reside in the lung parenchyma[40]. Of note, $CD11b^+$ cells in this analysis also contain LN-resident, conventional DC or monocyte-derived DC. Finally, $CD11c^+$ $CD8\alpha^+$ $CD11b^-$ lymph node-resident DC (here referred to as $CD8\alpha^+$ DC) were assessed. In addition to DC, we determined the number of macrophages in the draining lymph node. While these cells appear to play a limited role in the activation of vaccinia virus-specific T cells[84], they have the

potential to transport antigen to the MLN. This analysis revealed an early increase in $CD11b^+$ DC as well as macrophages (Figure 2B). No significant increase in $CD8\alpha^+$ or $CD103^+$ cells was detected, although this was challenging given the small sizes of these populations.

$CD103^+$ DC in the MLN are enriched for $eGFP^+$ cells. The vaccinia virus construct utilized for these studies allowed us to monitor the presence of viral protein in the various populations via assessment of eGFP. We began by quantifying cells within the lung as an indicator of antigen-bearing cells with the potential to traffic to the MLN. In the lung, both the $CD103^+$ and $CD11b^+$ DC populations contained a significant percentage of cells that were $eGFP^+$ on day 1 p.i. (Figure 2C). $eGFP^+$ cells were also detected within the macrophage population (Figure 2C). The percentage of $CD11b^+$ DC that was $eGFP^+$ was increased at day 2, while the percentage of $CD103^+$ DC that was $eGFP^+$ was similar to that at day 1 p.i. Macrophages exhibited a continuous increase in the percentage of cells that were $eGFP^+$ over all 4 days analyzed. As expected, there were few, if any, events that fell within the $eGFP^+$ gate when cells from the mock-infected mice (or mice infected with a recombinant vaccinia virus that did not express eGFP) were analyzed.

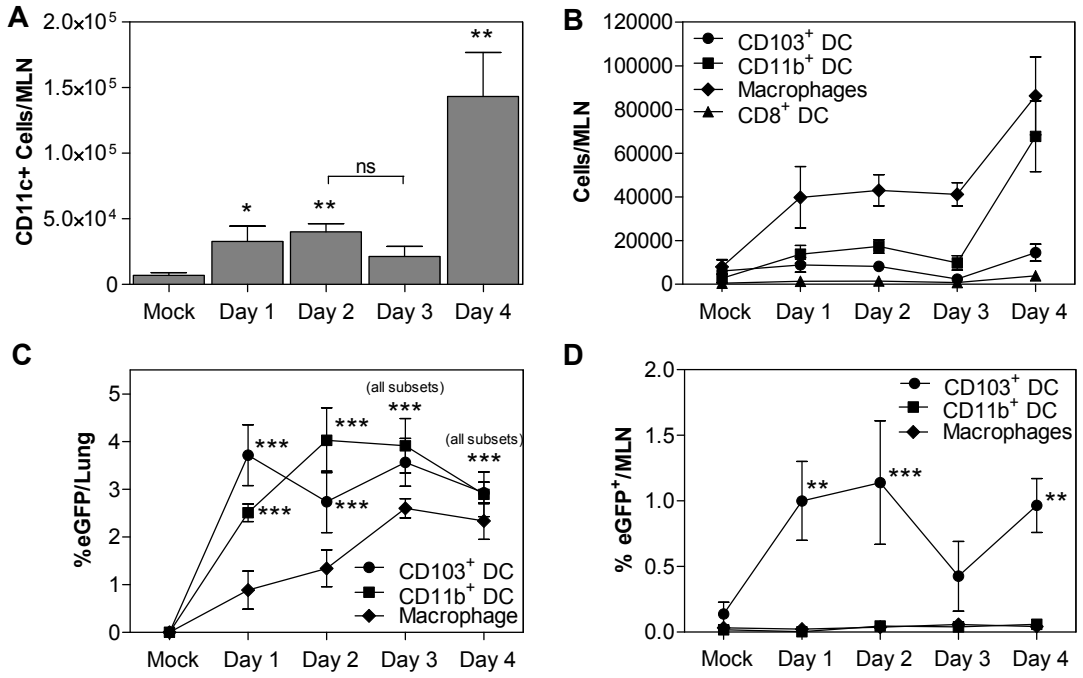


Figure 2. Dendritic cells increase in the lung draining MLN following VV infection. C57BL/6 mice were intranasally infected with 10^7 PFU of VV.NP-S-eGFP. On days 1-4 post infection, MLN were isolated and $CD11c^+$,$CD90.2^-$, $CD49b^-$, $CD19^-$ analyzed for expression of CD103, CD11b, CD8 and F4/80. The total number of $CD11c^+$ cells (A) and the number present within each DC subset as well as the number of macrophages (B) were calculated based on the total cells recovered. EGFP expression in the populations was analyzed in both the lung (C) and the MLN (D) and graphed as a percent of each APC type expressing eGFP. Data reflect the average of 4 independent experiments. In these experiments, to be considered valid for analysis the number of $eGFP^+$ events in each population had to be greater than five-fold that observed in mock infected mice. For day 1 significant $eGFP^+$ events among the different populations in the lung for individual mice ranged from 19-205, for day 2 from 17-588, on day 3 from 10-598, and on day 4 from 14-747. The variation in cell number was the result of differences in the size of the different APC populations. For the MLN significant $eGFP^+$ events were only observed for $CD103^+$ cells. For individual mice these ranged from 9-29 on day 1, from 14-32 for day 2, from 16-24 on day 3, and from13-39 on day 4. Significance was determined by a 2-way ANOVA with a Bonferoni post test comparing subsets to mock values. *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$, ns $p \geq 0.05$

eGFP$^+$ CD103$^+$ DC were also found in the MLN (Figure 2D). Interestingly, the percentage of eGFP$^+$ cells detectable in the CD11b$^+$ DC and macrophage populations was never significantly above the background for mock-infected animals. Analysis of B and NK cells in the MLN showed that there were no detectable eGFP$^+$ cells in these populations. Together, these data suggested that airway CD103$^+$ DC are infected or acquire viral antigen in the lung and subsequently traffic to the draining LN, where they have the potential to serve as activators of naive T cells. In contrast, while eGFP$^+$ parenchymal CD11b$^+$ DC were detected in the lung, they were not present above background in the draining LN.

Migrating CD11b$^+$ DC do not express eGFP. One caveat to this result is the presence of a large number of LN-resident DC that bare this marker. Thus, it remained possible that eGFP$^+$ lung-resident parenchymal DC were migrating to the MLN but were difficult to detect as a result of dilution within the LN-resident CD11b$^+$ DC population. To address this question, we labeled lung DC by intratracheal administration of Cell Tracker Orange (CTO). This approach was chosen to allow concurrent detection of lung-derived cells and eGFP positivity. Mice received virus by i.t. instillation and, 5 h later, received CTO by i.t. delivery. MLN were isolated and the percentages of eGFP$^+$ cells within the CTO$^+$ CD11b$^+$ and CTO$^+$ CD103$^+$ populations determined.

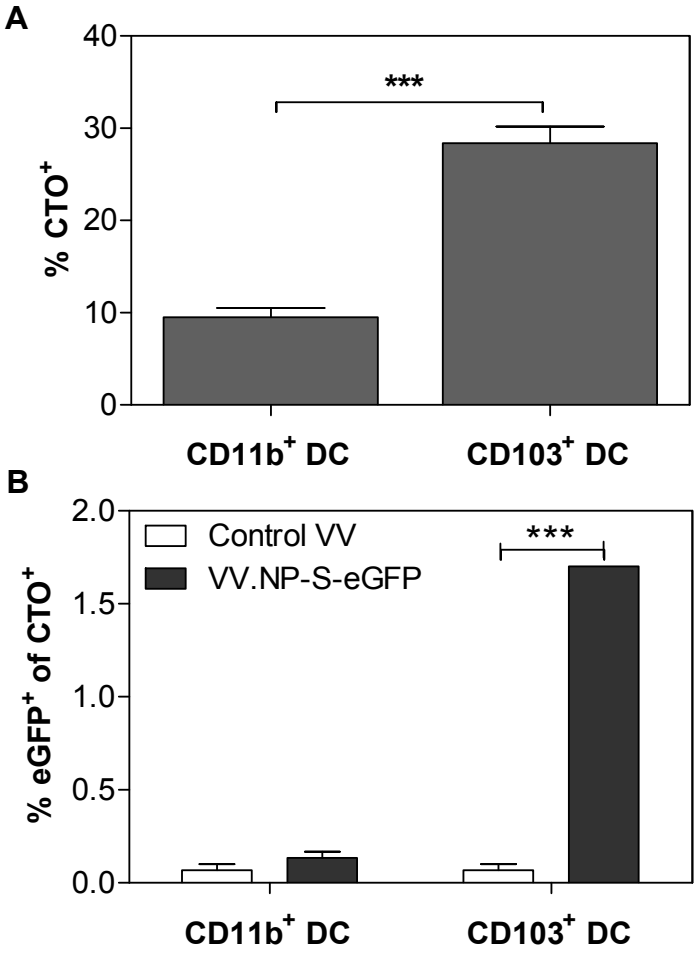


Figure 3. Migrating CD11b$^+$ DC are eGFP$^-$. Mice were infected and 5 hours later CTO was administered intratracheally. Cells were pre-gated by CD11c$^+$, CD90.2$^-$, CD49b$^-$, CD19$^-$ and subsequently CTO$^+$ CD11b$^+$ or CD103$^+$ DC were analyzed for CTO signal (A) and eGFP$^+$ cells (B) on day 2 post infection. The data reflect 3 independent experiments each utilizing between 23 and 25 pooled MLN for each condition. A students T-test was used to compare the percent CTO$^+$ between the DC subsets (A) and eGFP expression between control and day 2 within each subset (B). *** $p \leq 0.005$.

Of the analyzed CTO^+ cells from the MLN, approximately 41% were $CD11c^+$ DC; the remaining 59% were likely macrophages, as determined by their forward and side scatter profiles. Of the total $CD103^+$ DC and $CD11b^+$ DC present in the MLN, approximately 23.0% ± 4.3% and 9.7% ± 1.8%, respectively, were labeled with CTO (Figure 3A). The increase in CTO labeling of the $CD103^+$ DC compared to that of the $CD11b^+$ DC was likely due to $CD103^+$ DC proximity to the airway. These studies showed that only a minimal percentage of the CTO^+ $CD11b^+$ cells were positive for eGFP (0.13% ± 0.03%, not significantly different than background) (Figure 3B). In contrast, 1.7% ± 0.0% of CTO^+ $CD103^+$ cells were $eGFP^+$, a percentage similar to that seen in the total $CD103^+$ DC population of the MLN (Figure 2D). These data suggest that while parenchymal $CD11b^+$ DC in the lung showed evidence of infection, these $eGFP^+$ cells did not appear to migrate to the draining LN.

$CD103^+$ lung-resident DC are the most efficient activators of naive $CD8^+$ T cells. The above-described studies supported a potential role for lung-migrating DC in the activation of naive T cells. In order to determine the ability of these DC to activate naive $CD8^+$ T cells following pulmonary infection with vaccinia virus, we isolated CTO^+ $CD11b^+$ and CTO^+ $CD103^+$ DC from the MLN of mice infected with VV.NP-S-eGFP. Although there were limited $eGFP^+$ cells found in the CTO^+ $CD11b^+$ population, it remained formally possible that these cells contained viral antigen that had been processed for presentation, e.g., as a result of abortive infection or cross-presentation, that would allow them to activate naive T cells.

For these studies, mice were infected either with a recombinant vaccinia virus expressing the P protein from SV5 (VV.P), as a control for nonspecific stimulation by DC isolated from a virus-infected environment, or with VV.NP-S-eGFP. DC were isolated into subsets based on their CTO signal and the expression of CD103 or CD11b (CTO^+ $CD103^+$ and CTO^+ $CD11b^+$) (Figure 4) and subsequently co-cultured with CFSE-labeled OT-I cells for 3 days. Following the co-culture, proliferation and gamma interferon (IFN-γ) production in OT-I cells were assessed (Figure 4B and D). The $CD103^+$ DC from the lung were the only subset that was able to induce significant proliferation in the naive OT-I T cells, with an approximately 4-fold increase over that for OT-I cells incubated with $CD103^+$ DC infected with the control virus (Figure 4C). The CTO^+ $CD11b^+$ DC from the lungs of mice on day 2 showed no ability above those from the control mice to stimulate proliferation in naive OT-I T cells. Additionally, $CD103^-$ DC that were not labeled with CTO failed to induce proliferation in the OT-I T cells above the level seen with mock infection (Figure 4B to D).

The percentage of the OT-I T cells producing IFN-γ following culture with the sorted DC populations was also assessed to determine the ability of lung-migrating DC to stimulate function in $CD8^+$ T cells. Similarly to the proliferation data, the CTO^+ $CD103^+$ DC were the only DC capable of inducing acquisition of IFN-γ production in OT-I naive T cells, with a >10-fold increase in the percentage of cells producing IFN-γ in OT-I cells cultured with the $CD103^+$ DC compared to that of the $CD11b^+$ or CTO^- DC (Figure 4D). Together, the data in figure 4 show

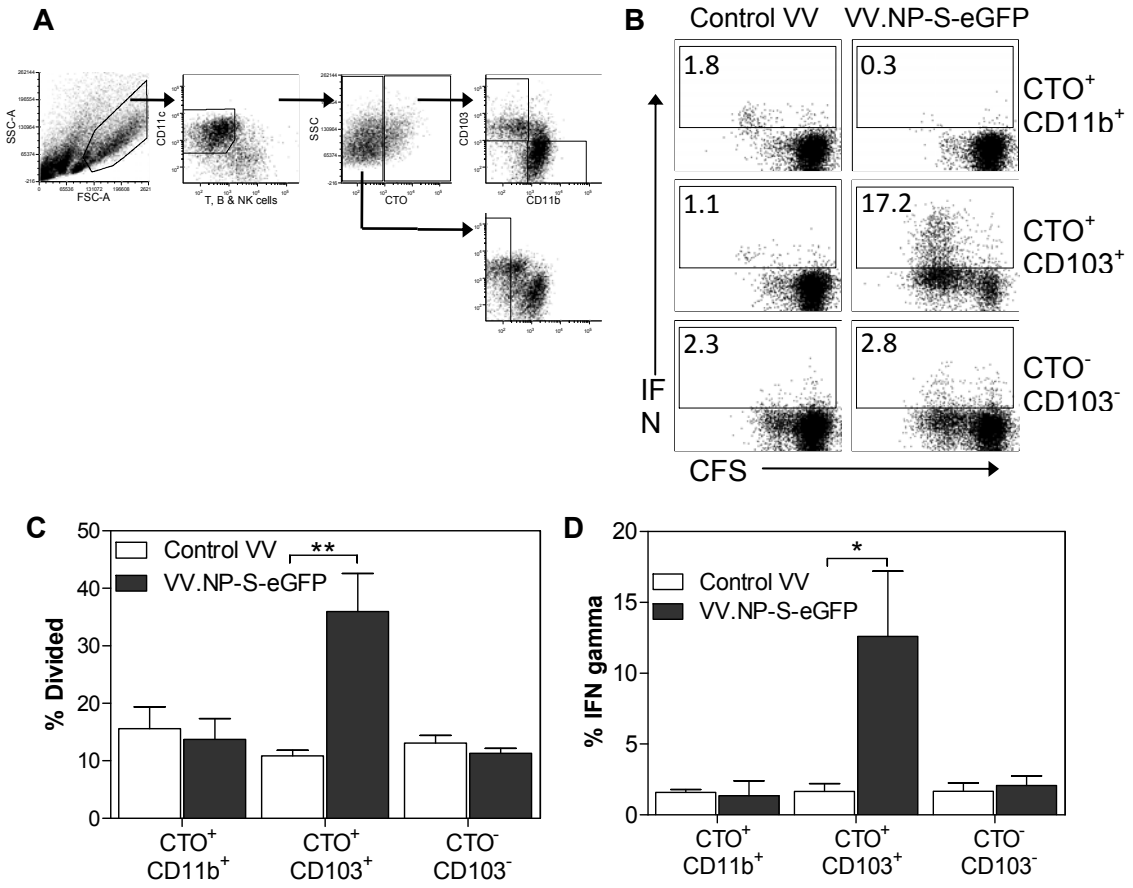


Figure 4. Airway derived CD103$^+$ DC are superior to parenchymal DC for priming naïve CD8$^+$ T cells ex vivo. Mice were intranasally infected with 10^7 PFU of either VV.NP-S-eGFP or the control virus VV.P. Five hours following infection mice were given 1 mM Cell Tracker Orange i.t. Two days post infection, mice were sacrificed and MLN harvested. Recovered cells were gated based on CD11c$^+$, CD90.2$^-$, CD49b$^-$, CD19$^-$ and were sorted based on their expression of CTO, CD103 and CD11b as shown in A. Sorted cells were then incubated with CFSE labeled naïve OT-I T cells for 3 days at a ratio of 1 DC:5 OT-I. OT-I cells were restimulated for 5 hours with 10^{-6} M Ova peptide. Cells were analyzed to determine proliferation and IFNγ production (representative data in B and averaged data in C and D). The percent divided was calculated using FlowJo software. MLN from 23-25 animals were pooled for each sort. Error bars represent the SEM of 2 individual experiments. Significance was determined using a student's T-test to compare mock and day 2. *p ≤ 0.05, ** p ≤ 0.01

that among CTO-labeled cells, only $CD103^+$ DC were capable of activating OT-I cells for division and acquisition of effector function. These data suggest a model wherein airway-derived DC are the predominant migrating DC population capable of activating naive $CD8^+$ T cells following a respiratory vaccinia virus infection.

$eGFP^+$ $CD103^+$ DC are enriched for mature cells. Optimal activation of naive T cells requires accessory signals provided in part by CD28 engagement of CD80/CD86 [88]. Thus, we assessed the expression of co-stimulatory molecules on the $CD103^+$ DC present in the MLN. The data in figure 5 show the results from the analysis of CD80 and CD86 expression within the $eGFP^-$ and $eGFP^+$ $CD103^+$ populations. Overall, we found that nearly all $eGFP^+$ cells expressed CD80 and CD86 at day 2 and beyond, demonstrating that these cells had undergone maturation (Figure 5A, B, and D). $eGFP^-$ cells also exhibited significant expression of CD80 (Figure 5B), but a much smaller percentage of cells expressed CD86 (Figure 5D), suggesting that these cells may have been exposed to a distinct maturation signal in the lung. When the levels of CD80 and CD86 on a per-cell basis were examined, we found no significant difference between $eGFP^+$ and $eGFP^-$ cells (Figure 5C and E). Together, these data show that the presence of detectable eGFP in DC correlated with a program of maturation that included up-regulation of both CD80 and CD86.

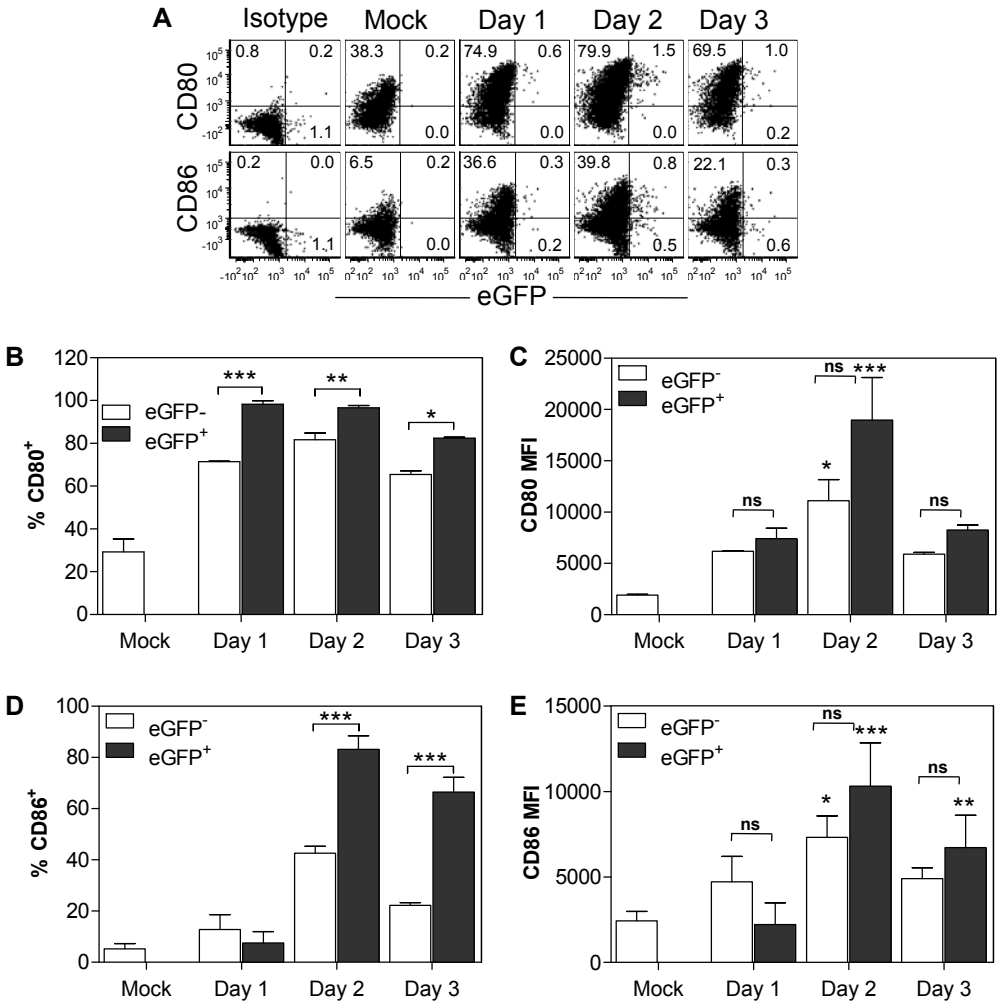


Figure 5. EGFP$^+$ CD103$^+$ DC are highly enriched for mature cells. Mice were intranasally infected with 10^7 PFU of VV.NP-S-eGFP or PBS as a control. On days 1-3 post infection, MLN from animals were assessed for the maturation of CD103$^+$ DC. EGFP$^+$ and eGFP$^-$ cells within the CD11c$^+$, CD103$^+$, CD90.2$^-$, CD49b$^-$, CD19$^-$ population were analyzed for CD86 and CD80 expression. Representative data are shown in A. The percent of cells that were positive for CD80 (B) or CD86 (D) as well as the intensity of staining for CD80 (C) or CD86 (E) within the positive population are shown. Error bars represent the SEM from 4-5 independent experiments each containing 2-5 animals per time point. For each graph, significance was determined using a 2-way ANOVA with Bonferoni post test. In B and D the eGFP$^+$ vs. eGFP$^-$ cells for each time point were compared. In C and E, significance determination was performed by comparing each time point to the mock value as well as comparing eGFP$^+$ and eGFP$^-$ as indicated by the brackets. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$, ns $p \geq 0.05$. For all data points the following minimum numbers of eGFP$^+$ events were analyzed: day 1: 18-41, day 2: 239-382, day 3:64-189. In addition to be considered valid for analysis the number of eGFP$^+$ events had to be a minimum of 5 fold above the mock samples which ranged from 1-5.

A portion of the CD103$^+$ DC in the MLN expresses CD8α. While examining the various populations of DC in the MLN, we noted that a portion of CD103$^+$ DC (approximately 20%) co-stained with anti-CD8α antibody (Figure 6A). Although the number of CD103$^+$ DC in the MLN increased over time, the percentage of those that co-expressed CD8α$^+$ remained relatively constant. This population was not dependent on infection with vaccinia virus, as it was present in the MLN at a similar frequency in mock-infected animals. This subset, while present in the MLN, was notably absent in the lungs (Figure 6B), in agreement with previous reports analyzing CD103$^+$ cells in the lung[40].

CD8α$^-$CD103$^+$ DC are superior stimulators of naive CD8$^+$ T cells compared to CD8α$^+$CD103$^+$ DC in their ability to stimulate naïve CD8$^+$ T cells following viral infection. As was demonstrated in figure 5, CD103$^+$ migrating DC are superior to CD11b$^+$ migrating DC with regard to the capacity to activate naive T cells. Given the presence of CD8α$^+$ and CD8α$^-$ subsets within this population, it was next determined whether there were differences in the abilities of these populations to promote activation of naive T cells. MLN were harvested from mice infected intranasally with VV.NP-S-eGFP or a control vaccinia virus (VV.M), and CD11c$^+$ cells were enriched by column purification. The cells were stained and sorted based on their expression of CD8α and CD103. These sorted DC were then incubated with CFSE-labeled, naive OT-I T cells for 3 days, after which the CFSE signal was assessed to determine proliferation.

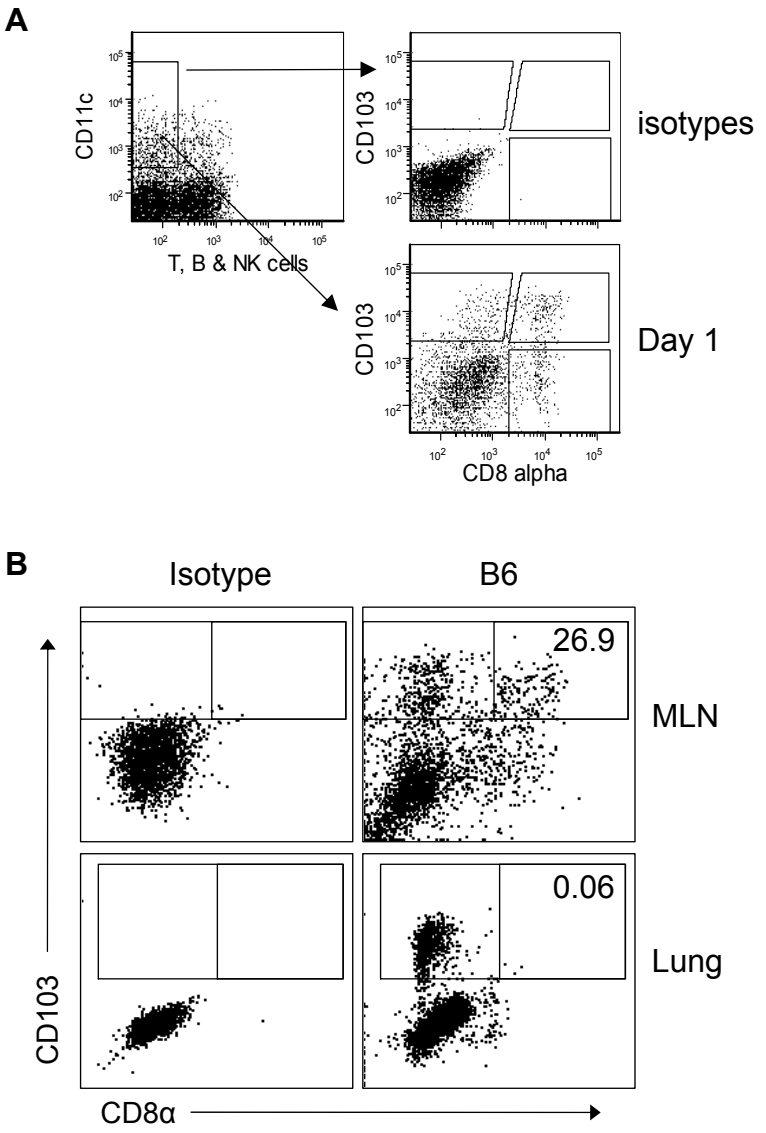


Figure 6. A subset of CD103$^+$ expressing CD8α$^+$ is present in the MLN. MLN from mock treated or infected (10^7 PFU of VV.NP-S-eGFP) animals were isolated on the indicated days. CD11c$^+$, CD90.2$^-$, CD49b$^-$, CD19$^-$ MLN cells were analyzed for the expression of CD8α and CD103$^+$. Representative data showing the gating strategy (A) and expression of CD103 and CD8α in the lung and MLN (B).

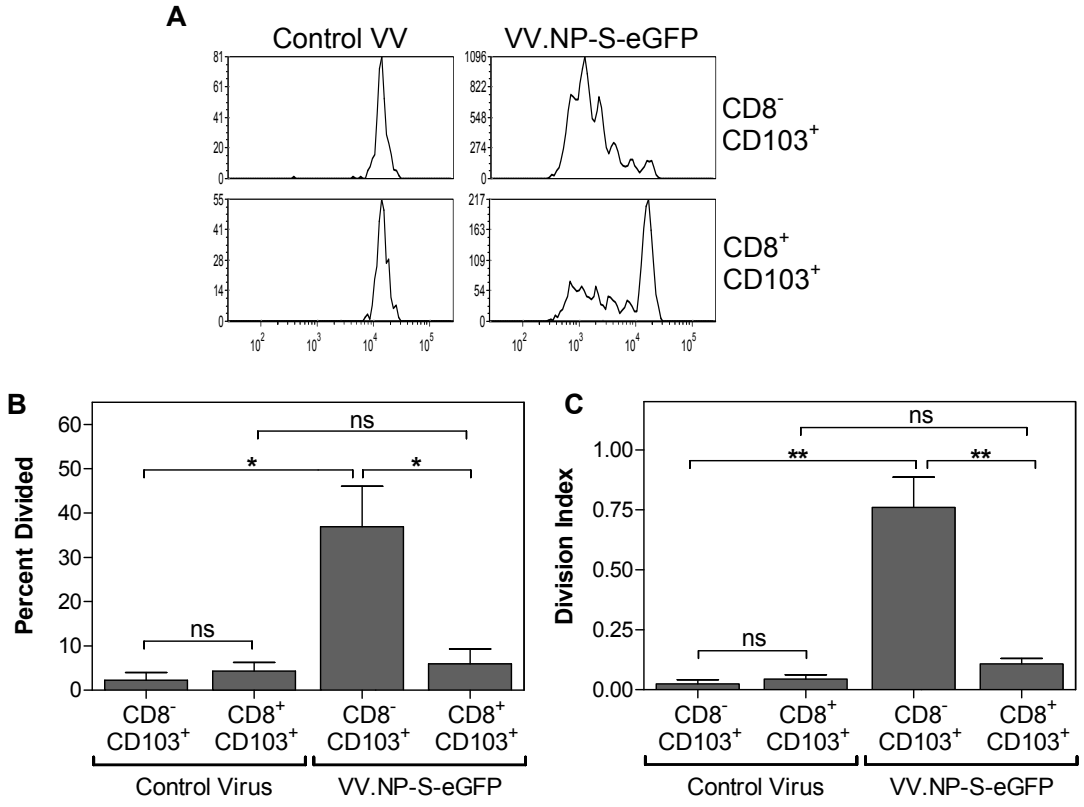


Figure 7. Functional divergence between CD8α$^+$CD103$^+$ and CD8α$^-$ CD103$^+$ DC in their ability to stimulate naïve CD8$^+$ T cells following viral infection. Mice were infected intranasally with either VV.NP-S-eGFP or VV.M (10^7 PFU). On day 2 post infection, MLN cells were isolated, pooled and CD11c$^+$ cells enriched by column purification. The enriched population was sorted into subsets based on CD11c$^+$,CD90.2$^-$, CD49b$^-$, CD19$^-$ staining together with expression of CD8α and CD103. Sorted cells were incubated for 3 days with CFSE labeled naïve OT-I T cells at a ratio of 1 DC:4 OT-I. Following culture, OT-I cells were identified by staining with CD90.2 and analyzed for CFSE expression. A representative experiment is shown in (A) and average data from three independent experiments in (B). Between 22 and 25 mice were used for each group for each experiment. Error bars represent the SEM. Significance was determined using the student's T-test. * $p \leq 0.05$ ** $p \leq 0.01$, ns $p \geq 0.05$.

We found that $CD8\alpha^-$ $CD103^+$ DC were the more potent stimulators of naive OT-I T-cell proliferation, as demonstrated by the significant increase in the percentage of OT-I cells that entered division as well as in the calculated division index following incubation with $CD8\alpha^-CD103^+$ DC compared to results following incubation with $CD8\alpha^+CD103^+$ DC (Figure 7B and C). $CD8\alpha^+CD103^+$ DC did not induce significant proliferation in the OT-I T cells above that observed with DC from animals infected with the control virus. In the absence of antigen (i.e., OT-I cells cultured with DC from control vaccinia virus-infected animals), naive T cells did not undergo division and exhibited poor survival during the 3-day culture period (Figure 7).

In the course of these studies, we also isolated lymph node-resident $CD8\alpha^+CD103^-$ DC, as this population has been implicated in the activation of virus-specific $CD8^+$ T cells[89]. These DC did not induce proliferation of OT-I cells that was above that detected with the corresponding DC population isolated from mice infected with the control virus.

$CD103^+$ DC subsets display a similar percentage of $eGFP^+$ DC.

The functional divergence in the ability of $CD8\alpha^-CD103^+$ DC and $CD8\alpha^+CD103^+$ DC to stimulate naïve $CD8^+$ T cells could have been explained if the $CD8\alpha^+CD103^+$ DC had lower access to viral antigen than the $CD8\alpha^-CD103^+$ DC. When eGFP signal was analyzed within both of these subsets, it was noted that there was not a statistically significant difference in the percent of $CD8\alpha^-CD103^+$

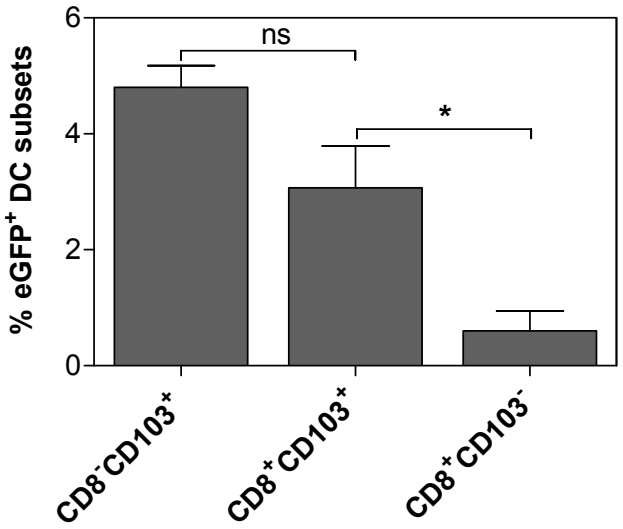


Figure 8. A similar proportion of CD8α$^+$CD103$^+$ DC and CD8α$^-$CD103$^+$ DC are positive for eGFP. MLN DC were harvested at day 2 post VV.NP-S-eGFP infection and analyzed for percent eGFP$^+$ (A) and the MFI of eGFP within the eGFP$^+$ DC (B). Bar graphs represent the mean of three independent experiments, with error bars graphing SEM. Statistical analysis performed by Student's T-test. * $p \leq 0.05$, ns $p \geq 0.05$

DC and $CD8\alpha^{+}CD103^{+}$ DC that were positive for eGFP (Figure 8). We therefore concluded, that antigen access alone could not explain the inability of the $CD8\alpha^{+}CD103^{+}$ DC to stimulate division of naïve $CD8^{+}$ T cells to levels seen with $CD8\alpha^{-}CD103^{+}$ DC stimulation.

CHAPTER 2

$CD8\alpha^{+}CD103^{+}$ DC Resemble Airway $CD8\alpha^{-}CD103^{+}$ DC in both Function and Origin

Parts of this chapter are being prepared for publication

We thank Jim Wood for and Beth Holbrook for helping sort DC populations

Summary

During the course of our studies of lung DC migration following pulmonary vaccinia virus infection, we noted that while the $CD103^+$ DC in the lung lack CD8α expression, there exist in the lung draining mediastinal lymph node (MLN) a subpopulation of $CD103^+$ DC that co-expressed CD8α. These $CD8\alpha^+CD103^+$ DC were inferior to their $CD8^-$ counterpart with regard to their ability to prime $CD8^+$ T cells. These results led us to examine the origin and function of $CD8\alpha^+CD103^+$ DC. In order to do this, we addressed the $CD8\alpha^+CD103^+$ DC migration from the lung at various times post infection, surface molecule expression of the $CD8\alpha^+CD103^+$ DC compared to both the $CD8\alpha^-CD103^+$ DC and the $CD8\alpha^+CD103^-$ DC subsets and the up-regulation of co-stimulatory molecules following TLR agonist stimulation for all three DC subsets. We found that $CD8\alpha^+CD103^+$ DC more closely resemble the airway resident $CD8\alpha^-CD103^+$ DC with regard to both cell surface marker expression and response to TLR agonists than LN resident $CD8\alpha^+CD103^-$ DC. The superior maturation response to TLR agonists in this subset suggests they have the capacity to play a key role in the control of an adaptive immunity.

RESULTS

CD8α$^+$CD103$^+$ DC do not express either CD8β or CD3 on their surface.

CD8α exists as a homodimer and a hetrodimer with CD8β on CD8$^+$ T cells. However, DC in the LN express only the CD8α homodimer. We first addressed the expression of CD8 isomers on the surface of the CD103$^+$ DC in the MLN. While 21% of the CD103$^+$ DC expressed CD8α, we found negligible expression of CD8β and CD3 on CD103$^+$ DC within the MLN (Figure 9A).

It has been postulated, although never formally presented by data in the literature, that the CD8α expression on the DC in the MLN is a result of membrane sharing with a CD8$^+$ T cell following a conjugation event; a processetermed trogocytosis. In order to address whether CD8α expression on CD103$^+$ DC in the MLN was a result of trogocytosis, we examined CD103$^+$ DC for CD8α expression in the MLN of mice lacking CD8$^+$ T cells. In this model, CD8α is unable to be acquired through trogocytosis. While there was a slight decrease in the percent of the CD103$^+$ DC that co-expressed CD8α, the CD8α$^+$CD103$^+$ DC were present in the MLN despite the lack of CD8$^+$ T cells (Figure 9B). This data, along with the lack of CD8β and CD3 on CD103$^+$ DC, supports a model where CD8α is actively expressed by the CD8α$^+$CD103$^+$ DC.

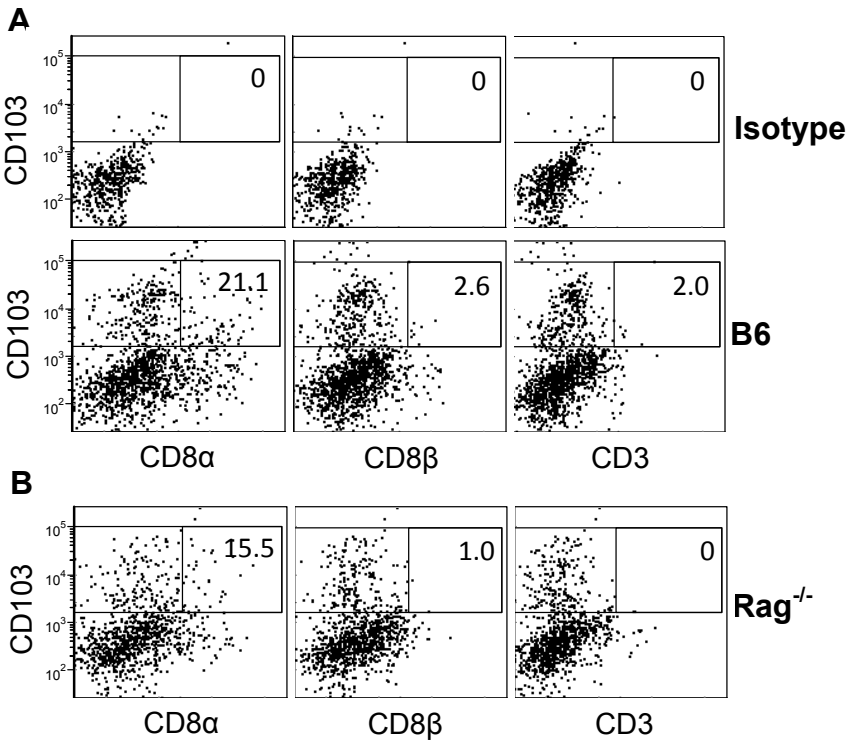


Figure 9. CD8α^{+}CD103^{+} DC do not co-express CD8β or CD3. Expression of CD8α, CD8β and CD3 were analyzed on the DC of the MLN of naïve B6 (A) and Rag$^{-/-}$ (B) mice. Plots are pre-gated on CD11c^{+} CD90.2^{-} cells. Data is representative of three individual animals.

Migration kinetics of DC from the lung to the MLN

The CD103 molecule is a marker of tissue resident DC, while CD8α has long been used to delineate a LN resident DC. As the DC population in question epresses both of these markers, we wanted to determine if the $CD8\alpha^{+}CD103^{+}$ DC had migrated through the lung prior to entering the MLN. To do this we monitored the daily migration kinetics of DC from the lung to the MLN following infection. We treated the mice with Cell Tracker Orange (CTO) 2, 24, 48 and 72 hours post infection. The mice were sacrificed and the MLN examined 24 hours post CTO treatment (figure 10A). This method allows for the monitoring of migration that occurs within the 24 hour period prior to analysis, as opposed to a cumulative migration of DC to the MLN over time, as is routinely done. The number of CTO^{+} DC in each subset was compared to uninfected mice treated with CTO as a reference to homeostatic migration. We chose to label the lung with CTO as, in our hands, it does not result in either lung inflammation or non-specific migration of lung DC to the MLN, as has been previously shown for CFSE labeling of the lung[90].

In these analyses we found, that within the first 24 hours of infection the number of CTO^{+} DC in the MLN doubles compared to homeostatic migration (figure 10B). This migration continues to increase between 24 and 48 hours post infection, when the migration of CTO^{+} DC is three times that of homeostatic migration. We see the peak of DC migration from the lung to the MLN in the 24-48 hours following infection as the number of CTO^{+} DC in the MLN decrease after 48

hours post infection and within 72 to 96 hours post infection, the levels of CTO^+ DC in the MLN are similar to homeostatic migration.

The number of DC migrating from the lung to the MLN is delayed in the $CD8\alpha^+CD103^+$ DC compared to the $CD8\alpha^-CD103^+$ DC (Figure 10C). The number of CTO^+ $CD8\alpha^-CD103^+$ DC in the MLN increases significantly within the first 24 hrs post infection, while the number of $CD8\alpha^+CD103^+$ DC does not reach significant levels until 48 hrs post infection, although there is the trend of an increase at 24-48 hrs, but large variance in cell numbers at 24-48 hrs negates the significance. At 72-96 hours post infection, the number of $CTO^+CD8\alpha^-$ $CD103^+$ DC, but not $CTO^+CD8\alpha^+CD103^+$ DC, have returned to homeostatic migration levels.

When we analyze the percentage of $CTO^+CD8\alpha^-CD103^+$ DC and $CTO^+CD8\alpha^+CD103^+$ DC within the total CTO^+ DC we see that within the first 48 hours of infection $CD103^+$ DC make up at least 50% of the CTO^+ DC, with $CD8\alpha^-$ $CD103^+$ DC making up a majority of the migrating $CD103^+$ DC. However, as the infection progresses the percent of migratory $CD103^+$ that express CD8α has increased (Figure 10D). As the infection progresses into 72 hours, fewer of the migrating DC are $CD103^+$. At this time point a majority of the migrating DC are $CD11b^+$.

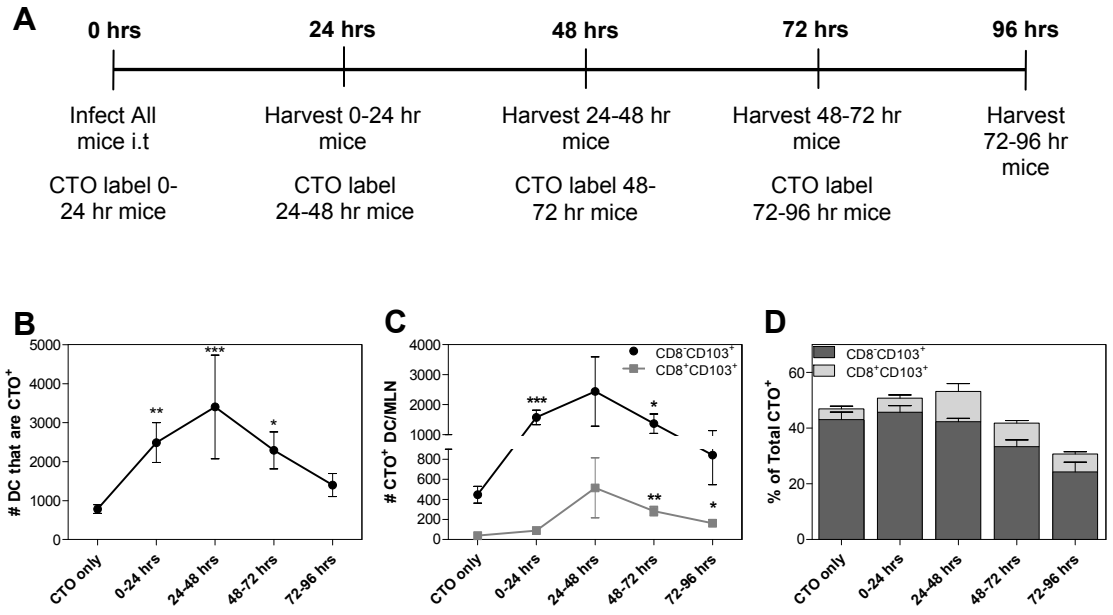


Figure 10. Migration Kinetics of the DC subsets from the lung to the MLN. Mice were treated with 1 mM CTO i.t. 24 hrs prior to sacrifice and MLN were harvested 1 – 4 days post infection with VV (A). The $CD11c^+$ $CD90.2^-$ cells were analyzed for CTO signal (B). Numbers of CTO^+ DC in each subset were calculated (C). All CTO^+ DC were then analyzed for the subset markers (D). The data is graphed as the mean of six animals, collected from two individual experiments, with error bars representing the SEM. Students T-test was used in B and C to compare each time point to the CTO only value. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$, ns = no significance.

While these data do not conclusively prove the origin of the $CD8\alpha^{+}CD103^{+}$ DC, they do strongly suggest that the $CD8\alpha^{+}CD103^{+}$ DC are likely to have migrated to the MLN from the lungs, rather than from the blood, as occurred for LN resident $CD8\alpha^{+}CD103^{-}$ DC.

Expression of CD24, CD205 and CD36 is similar on $CD8\alpha^{+}$ and $CD8\alpha^{-}$ $CD103^{+}$ DC. As these $CD8\alpha^{+}CD103^{+}$ DC have functional capabilities unlike $CD8\alpha^{-}CD103^{+}$ DC or $CD8\alpha^{+}CD103^{-}$ DC in the context of a VV infection, we looked to see if they had phenotypic characteristics similar to either the $CD103^{+}$ airway DC or the CD8α LN resident DC. We examined the expression levels of CD205, CD24 and CD36 on $CD8\alpha^{-}CD103^{+}$ DC, $CD8\alpha^{+}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{-}$ DC found in the MLN of naïve mice (figure 11A).

CD8α is the surface marker most often used to identify lymph node resident DC in the mouse. However there are other surface markers that have been identified on the surface of LN resident DC.

These DC also express CD205 (Dec205), a mannose receptor important in endocytosis and subsequent antigen presentation. CD205 is highly co-expressed with CD8α[91,92,93,94] in the spleen and on $CD103^{+}$ DC in the LN[41], spleen[51,95] and dermis[96].

CD205 was similarly expressed on $CD8\alpha^-$ and $CD8\alpha^+$ $CD103^+$ DC 57.6% ± 0.15 and 63.3% ± 0.9, respectively. This is in contrast to $CD8\alpha^+CD103^-$ DC where only 10.8% ± 1.7 were positive for this marker. The $CD8\alpha^-CD103^+$ DC and $CD8\alpha^+CD103^+$ DC expressed four-fold more CD205 on their surface than the $CD8\alpha^+CD103^-$ DC (figure 11B), but there was no significant difference in expression level of CD205 on $CD8\alpha^-CD103^+$ DC vs. $CD8\alpha^+CD103^+$ DC.

CD24 (heat stable antigen) is a variably glycosolated membrane protein. While it has some co-stimulatory properties, it is also extensively studied as a marker of precursors that give rise to $CD8\alpha^+$ DC. In the spleen, $CD24^+CD8\alpha^-$ DC give rise to the $CD8\alpha^+$ DC. In support of this, BMDC generated in the presence of Flt3L include a $CD24^{hi}$ DC subset which gives rise to $CD8\alpha^+$ DC following transfer *in vivo*. Recently, in a microarray analysis, $CD103^+$ DC from the lung were found to express CD24 RNA[97]. To the best of our knowledge, data presented here are the first to examine the surface expression of CD24 on $CD103^+$ DC in the LN.

Both $CD103^+$ DC subsets expressed CD24 on nearly 100% of their cells, while a significantly lower percent of $CD8\alpha^+CD103^-$ DC (LN resident) expressed CD24 (70.1% ± 4.8). The more striking difference, however, was observed in the level of expression on these various DC subsets. While there was a modest increase in the level of expression of CD24 between the $CD8\alpha^-CD103^+$ DC and the $CD8\alpha^+CD103^+$ DC, $CD8\alpha^+CD103^-$ DC had an almost three-fold decrease in the CD24 MFI compared to the $CD103^+$ DC subsets (figure 11C).

CD36 is a scavenger molecule that binds to a variety of ligands, including thrombospondin, collagen (types 1 and IV) and long fatty-acid chains. CD36 is preferentially expressed by the $CD8\alpha^+$ DC in the spleen[98]. This is the first study to address the expression of CD36 on the $CD103^+$ DC in the LN.

With regard to CD36, there was no significant difference in the percent of DC expressing this marker: 7.2% ± 2.1, 15.6% ± 4.5, 4.4% ± 1.7 for the $CD8\alpha^-$ $CD103^+$ DC, $CD8\alpha^+CD103^+$ DC and $CD8\alpha^+CD103^-$ DC, respectively. The pattern of expression in populations was similar to that of CD24, in that there was a modest increase in expression between $CD8\alpha^+CD103^+$ DC compared to the $CD8\alpha^-CD103^+$ DC (figure 11D).

The expression levels of CD205, CD24 and CD36 on MLN DC indicate that the $CD8\alpha^+CD103^+$ DC more phenotypically resemble the $CD8\alpha^-CD103^+$ DC of the airway than the $CD8\alpha^+CD103^-$ DC LN resident DC population.

$CD8\alpha^+CD103^+$ DC up-regulate CD86 and CD80 to higher levels than $CD8\alpha^-$ $CD103^+$ DC or $CD8\alpha^+CD103^-$ DC in response to TLR agonist stimulation

Although $CD8\alpha^+CD103^+$ DC have been reported, there is little information available with regard to their functional capabilities in vivo. To address this question, we wanted to determine if there was similarity in their response to individual TLR agonists.

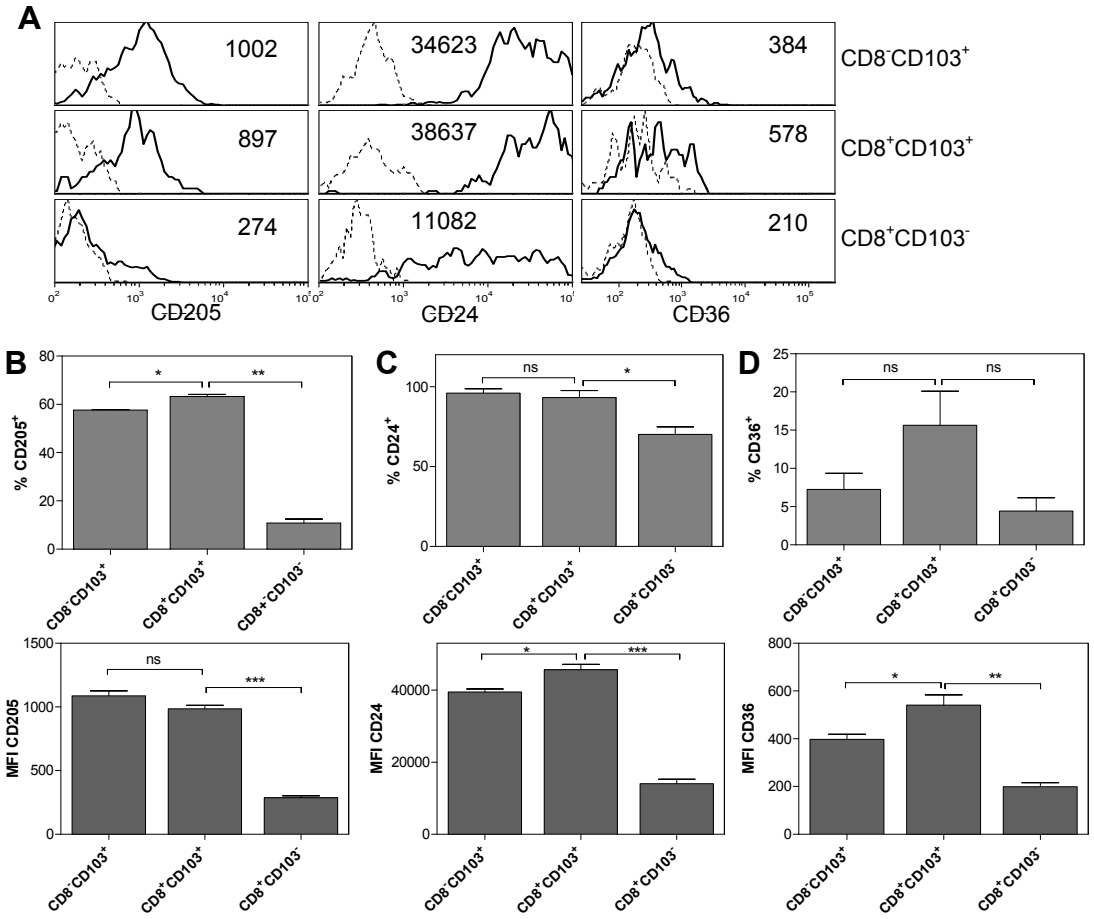


Figure 11. Expression of CD205 and CD24 are similar between CD8α⁻ CD103⁺ DC and CD8α⁺CD103⁺ DC. MLN 5 from naïve C57BL/6 mice were harvested and pooled. CD8α⁻CD103⁺ DC, CD8α⁺CD103⁺ DC and CD8α⁺CD103⁻ DC were analyzed for the expression of CD205, CD24 and CD36. In the histograms (A) the solid black lines represent the stain for the corresponding surface marker while the isotype controls are represented by a dotted black lines. The DC subsets were analyzed for MFI and percent positive for CD205 (B), CD24 (C) and CD36 (D). Data in A is representative of three individual experiments and the error bars on the graphs represent standard error. Statistical analysis performed Student's T test. * $p \leq 0.05$, ** $p \leq 0.01$, ns $p \geq 0.05$

PolyI:C (TLR3), LPS (TLR4), CL097 (TLR7) or CpG (TLR9) was administered i.t. Twenty-four hours post treatment, DC in the MLN were analyzed for expression of CD86 and CD80. Compared to PBS treated mice, all DC subsets from mice treated with PolyI:C, LPS or CpG demonstrated a significant up-regulation of their expression of both CD80 and CD86 (Figure 12A).

On a percent basis there was no significant difference in the percent of DC expressing CD86 in the $CD8\alpha^{-}CD103^{+}$ DC versus $CD8\alpha^{+}CD103^{+}$ DC following stimulation with PolyI:C, LPS or CpG, with upwards of 94% of each subset expressing this molecule. In contrast to the $CD103^{+}$ DC subsets, $CD8\alpha^{+}CD103^{-}$ DC had a smaller percent of cells that had undergone maturation, with a statistically significant difference in the percent of $CD8\alpha^{+}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{-}$ DC expressing CD86 with LPS (94.2% ± 1.5 and 53.6% ± 6.6, respectively) and CpG treatments (95.2% ± 1.8 and 74.8% ± 0.8, respectively). With regard to the level of CD86 expression, the $CD8\alpha^{+}CD103^{+}$ DC displayed significantly higher levels of expression than the $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{-}$ DC (Figure 12B).

Unlike CD86, the percentage of $CD8\alpha^{+}CD103^{+}$ DC expressing CD80 is significantly higher than $CD8\alpha^{-}CD103^{+}$ DC following treatment of PolyI:C (92.2% ± 1.0 and 71.4% ± 3.1, respectively) and CpG (88.5% ± 3.2 and 61.2% ± 7.8, respectively). The $CD8\alpha^{+}CD103^{+}$ DC had a higher percentage of CD80 expression when compared to the $CD8\alpha^{+}CD103^{-}$ DC for PolyI:C (92.2% ± 1.0

and 70.4% ± 4.1, respectively), LPS (92.8% ± 0.7 and 49.1% ± 4.5, respectively) and CpG (88.5% ± 3.2 and 67.7% ± 3.0, respectively). The trend of CD80 expression is similar to that of CD86, in that the $CD8\alpha^{+}CD103^{+}$ DC expressed significantly higher levels of CD80 than $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{-}$ DC (Figure 12C). As was seen with CD86 expression, the CD80 expression on the $CD8\alpha^{+}CD103^{+}$ DC was between two and four fold higher than the $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{-}$ DC.

It has previously been reported that $CD8\alpha^{+}$ DC in the spleen do not express TLR7. However, the expression of TLR7 on $CD103^{+}$ DC has not been previously addressed. Not only did the $CD8\alpha^{+}CD103^{-}$ DC not show any increase in the expression of the maturation markers in response to the TLR7 agonist CL097, the $CD8\alpha^{+}CD103^{+}$ DC and the $CD8\alpha^{-}CD103^{+}$ DC also showed a lack of up regulation of CD80 and CD86 expression in response to CL097.

Thus we have shown that while the $CD8\alpha^{+}CD103^{+}$ DC show a significantly higher level of CD86 and CD80 expression than both of the $CD8\alpha^{-}CD103^{+}$ DC and the $CD8\alpha^{+}CD103^{-}$ DC in response to PolyI:C, LPS and CpG treatment, the $CD8\alpha^{+}CD103^{+}$ DC population, as a whole, responds similar to the airway $CD8\alpha^{+}CD103^{+}$ DC.

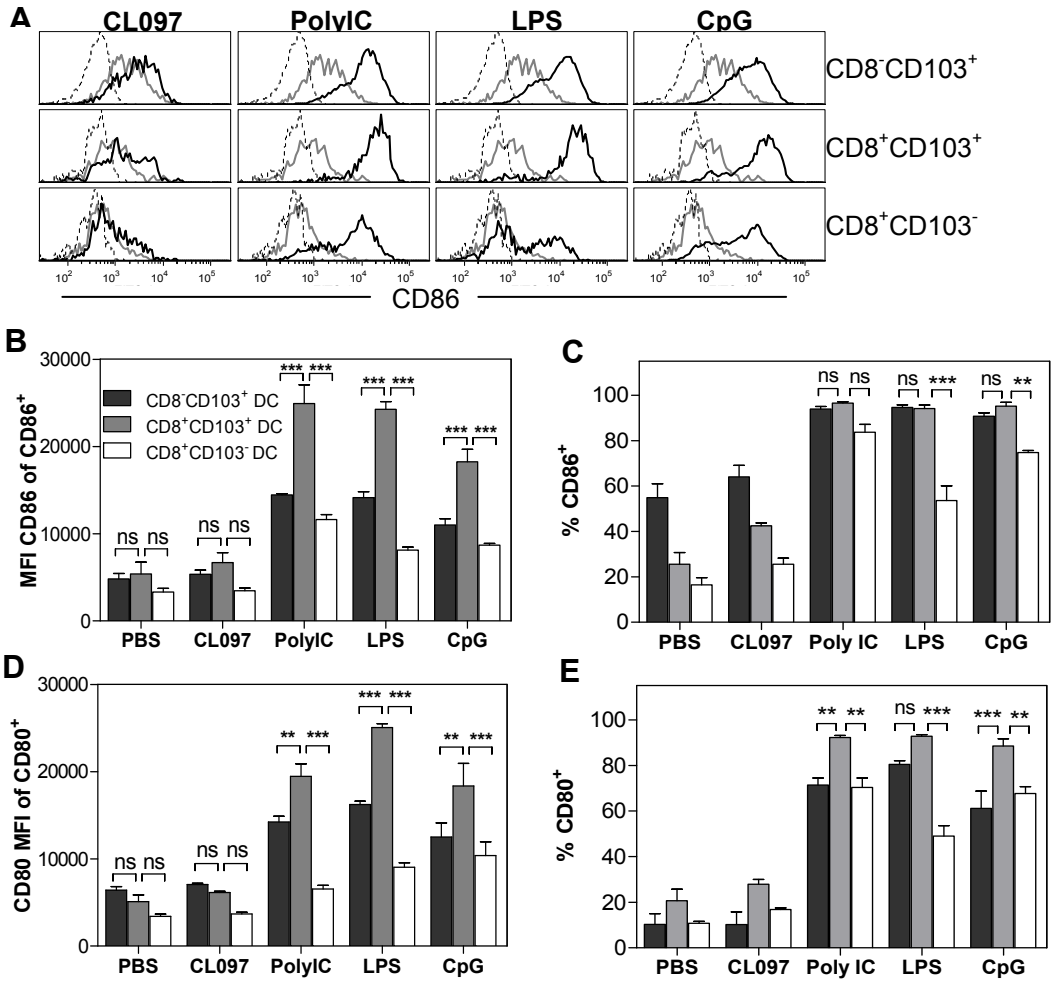


Figure 12 - CD8α⁺CD103⁺ DC have an enhanced response to TLR agonists. TLR agonists were delivered i.t. 24 hours prior to sacrifice. The DC subsets in the MLN were analyzed for expression of co-stimulatory molecules with flow cytometry (A) Dotted black likes represent the isotype control, gray lines represent PBS treatment and solid black lines represent the CD86 staining. The response to each TLR agonist was analyzed for level and percent of CD86 (B & C) and CD80 (D & E) for each DC subset in the MLN. Data in A is representative of CD86 expression for 3 independent experiments. Statistical analysis performed using a 2-way ANOVA with Bonferoni post-test. ** $p \leq 0.01$, *** $p \leq 0.001$, ns $p \geq 0.05$

DISCUSSION

In these studies, a mouse model of pulmonary VV infection was used to determine the contribution of various DC subsets in the generation of a virus-specific $CD8^+$ T cell response. We found that airway resident, $CD103^+$ DC have the greatest potential to prime naïve $CD8^+$ T cells. These studies further not only the understanding of how VV, specifically, is recognized by the immune system, but also, together with other models in the literature, how a $CD8^+$ T cell response is mounted in response to pulmonary viruses. As vaccination campaigns strive to employ more effective vaccination strategies, it has become increasingly necessary to understand how pathogens are recognized and adaptive immunity is generated following infection.

Lung resident $CD103^+$ DC are able to prime virus specific $CD8^+$ T cells following pulmonary VV infection.

Following a respiratory infection with VV, we noted an increase in the number of $CD11c^+$ cells in the MLN. Specifically, the number of $CD11b^+$ DC, $CD103^+$ DC increased following infection, as did macrophage. This influx of DC into the MLN was consistent with DC migration from the lung following respiratory infections with influenza[99,69,100,60] RSV[68] and SeV[66]. Legge *et al* noted that the DC migration from the lung to the MLN following respiratory infection occurred rapidly, peaking 18 hours post infection and decreasing sharply by 24 hours post infection[99]. However, more recent work out of this lab with HINI influenza (as opposed to H2N2 in previous reports), has reported a slower, more sustained

migration of lung-derived DC to the MLN, with the total number of $CD103^+$ DC peaking at day 3 post infection while the $CD11b^+$ DC peaked later at day 6 post infection [60,70,101]. So while it is clear that different viruses may lead to distinct migration kinetics, pulmonary viral infection provided the necessary stimuli for migration of DC from the lung to the MLN and these migrating DC appeared to play a role in T cell priming.

Although we saw a general increase in the number of DC in the MLN following pulmonary VV infection, it was important to determine how many of those DC had access to viral antigen and therefore, had the potential to stimulate $CD8^+$ T cells. Our use of a VV construct encoding for the eGFP protein allowed us to track the presence of viral antigen within cells of the lung and MLN. While both DCs and macrophages contained $eGFP^+$ populations, macrophages had significantly fewer $eGFP^+$ cells. Within the DC of the lung, eGFP was detectable in 2.5%–3.5% of the DC at day 1 post infection. This continued to be the case through day 2, indicating that regardless of whether they were located at the airway ($CD103^+$ DC) or in the parenchyma ($CD11b^+$ DC), the lung DC show a similar susceptibility to infection early following the infection. This is in contrast to influenza infection, where $CD11b^+$ DC exhibited a marked decrease in the percent of infected cells when compared to $CD103^+$ DC[70]. It is possible that this divergence is a result of greater destruction of the lung architecture by VV, allowing the infection to spread deeper into the parenchyma and infect a greater percentage of $CD11b^+$ DC.

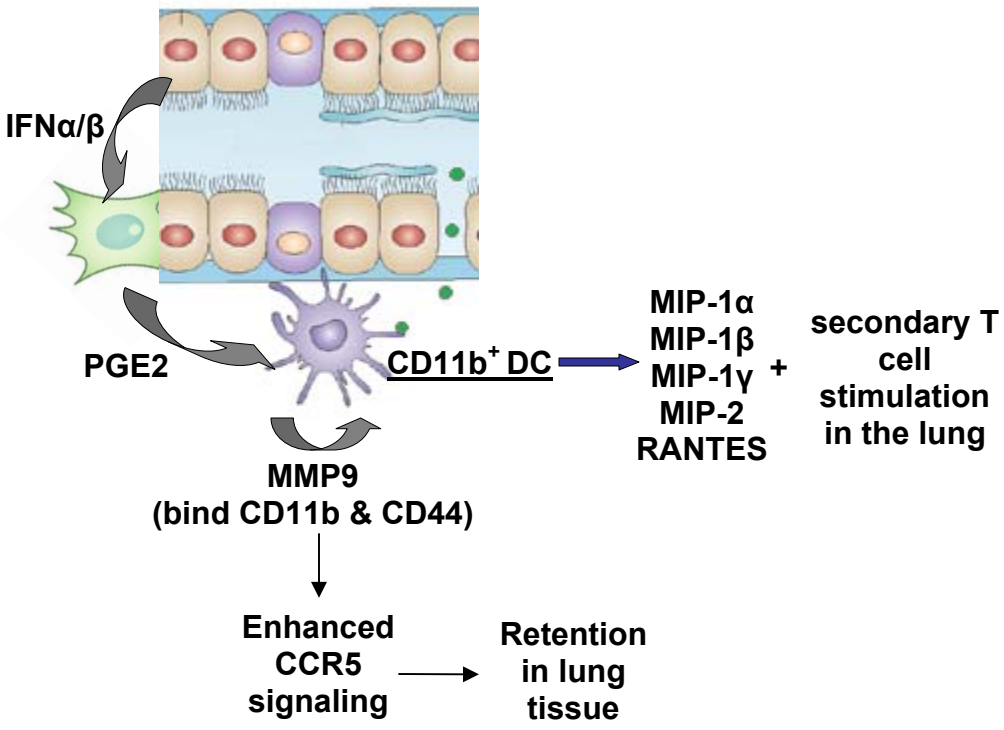
When we analyzed the lung migratory DC in the MLN following infection, we found eGFP expression only in $CD103^+$ DC, indicating that there was a failure of the $eGFP^+$ $CD11b^+$ DC to migrate to the MLN. It was possible that the $CD11b^+$ DC were more susceptible to VV induced apoptosis or that they failed to up-regulate CCR7, CCR8[102,6,103] or sphingosine-1-phosphate receptor[104], leading to an inability to migrate to the MLN. Normally, the up-regulation of CCR7 corresponds to a down-regulation in the expression of CCR5, the receptor necessary for migration into tissue. It was possible that the $eGFP^+$ $CD11b^+$ DC failed to down-regulate CCR5, effectively enhancing their response to lung chemokines and thus retention in the tissue. However, in preliminary studies, we saw no difference in the levels of CCR5 or CCR7 between $CD103^+$ DC and $CD11b^+$ DC or between the $eGFP^-$ $CD11b^+$ DC and the $eGFP^+$ $CD11b^+$ DC in the lung.

Given the similar expression of chemokine receptors on the DC subsets of the lung we devised an alternative hypothesis (Figure 13). Following influenza infection, NP protein expression is not detected in the $CD11b^+$ DC subset in the MLN[60], similar to what we have seen for the expression of eGFP following VV infection; however, this phenomenon is not universal and does not occur following either RSV infection[68] or FITC-Ova instillation into the lung[60]. Since the divergence in the ability of $CD11b^+$ DC to migrate is not based on viral infection, but rather the specific virus, it is informative to identify potential factors that differ between RSV versus influenza and VV infection. Infection with both VV and

influenza result in robust IFNα/β production from both DC and infected epithelial lung cells, a process absent in RSV infection, due to RSV's ability to degrade STAT2 within the IFNα/β signaling cascade[105,106,107], and soluble antigen treatment. IFNα/β produced during VV infection stimulates lung fibroblasts to secrete prostaglandin E2 (PGE2)[108]. PGE2 can then act on DC in the lung leading to the secretion of MMP-9 (matrix metallopeptidase-9)[109]. MMP-9 is known to facilitate migration by degrading the extracellular matrix[110] and to be important for DC migration into the airway following allergy sensitization[111]. Binding of MMP-9 to CD11b has been reported to co-stimulate CCR5-mediated signaling through enhanced JNK activation[112]. The MMP-9/CD11b$^+$ interaction could condition the CD11b$^+$ DC to be more responsive to CCR5 signaling, causing them to remain in the lung. The eGFP$^+$ CD11b$^+$ DC could be more susceptible to the effects of MMP9, if they up-regulate CD44, an additional receptor for MMP9, as a maturation response[113] to viral infection[114]. It is also possible that the CD11b$^+$ DC have inherent differences in migration compared to CD103$^+$ DC following influenza virus and VV infection.

Given that the infected CD11b$^+$ DC appeared to be pre-disposed to remaining in the lung following both VV and influenza infections, we propose that these infected CD11b$^+$ DC are retained in the lung in order to promote/sustain the immune response. For example they may recruit additional leukocytes to the infected lung. In an analysis of chemokines produced by lung DC subsets, it was found, using both microarray analysis and RT-PCR, that CD11b$^+$ DC secrete

greater amounts of MCP-1, MIP-1α, MIP-1β, MIP-1γ, MIP-2 and RANTES compared to $CD103^+$ DC[50]. These chemokines would recruit polymorphic nuclear cells (PMN), macrophages, natural killer (NK) cells and activated T cells to the sight of infection. Additionally, McGill *et. al*. have proposed a model where effector $CD8^+$ T cells in the lung require a second encounter with antigen presenting DC in the lung in order to maximize division and retain effector function[100]. Following intratracheal administration of clodronate liposomes to deplete airway DC, McGill *et. al*. established that the resulting $CD8^+$ T cell response in the lung was impaired. Reconstitution of the lung with $CD11b^+$ DC restored the number and function of the pulmonary $CD8^+$ T cells. Indeed, $CD11b^+$ DC infected with influenza virus *in vitro*[70] have the ability to activate naïve $CD8^+$ T cells, suggesting they could perform this function in the lung. Additionally, our preliminary experiments show an up-regulation of CD86 on lung $CD11b^+$ DC (data not shown) following VV infection, suggesting they may be capable of stimulating T cells. By remaining in the lung following the pulmonary infections with VV (and influenza) the $CD11b^+$ DC could act to enhance the innate immune response as well as maintaining the adaptive immune response (Figure 13).



Graphics adapted from Förster *et al*. 2008. Nature Reviews Immunology 8, 362-371

Figure 13. eGFP$^+$ CD11b$^+$ DC are retained within the lung following VV infection. Following VV infection, IFNα/β is produced by pDC and epithelial cells in the lung. IFNα/β stimulates lung fibroblasts to secrete PGE2. The PGE2 signals DC to produce MMP9, which feeds back and binds to CD11b and CD44 expressed on the surface of the DC. This binding of PGE2 to CD11b enhances the signaling of CCR5 through JNK stimulation. The CD11b$^+$ DC therefore receive signals to remain in the lung and do not respond to chemokines signaling emigration from the lung to the MLN. These retained CD11b$^+$ DC secrete chemokines that allow for the trafficking of additional innate cells (NK cells, macrophages and eosinophils) into the lung and potentially to provide a source of secondary antigen stimulation for the effector CD8$^+$ T cells as they enter the lung.

As the CD11b$^+$ DC with access to viral antigen did not migrate to the MLN, it is not surprising that the lung derived CD11b$^+$ DC found in the MLN at day two post infection were unable to stimulate either division or IFNγ production in naïve CD8$^+$ T cells (Fig 3). The ex vivo priming of naïve CD8$^+$ T cells was limited to the lung-derived CD103$^+$ DC. These DC exhibit both access to viral antigen (as determined by presence of eGFP) and up-regulation of co-stimulatory molecule expression (Figure 4), two of the three signals required for optimal T cell activation. Other studies have shown CD103$^+$ DC to be capable of antigen presentation following RSV[68] and influenza[60,70] infection, suggesting that in general, airway derived CD103$^+$ DC play a critical role in establishing the virus-specific CD8 T cell response following a pulmonary virus infection.

Given that eGFP can potentially be obtained through uptake of apoptotic cells, we note that there is a strong correlation between eGFP expression and the percentage of CD103$^+$ DC expressing CD80 and CD86. While technical limitations preclude us from concluding that VV infection directly induces maturation, VV has been shown to induce DC maturation through a TLR2 dependent mechanism[74]. Intravenous infection with VV supports a correlation between eGFP positivity and the expression of co-stimulatory molecules[115]. However, it also appears that the CD103$^+$ DC population were able to undergo by-stander maturation. It is possible that pro-inflammatory cytokines present during the infection (IFNα/β, TNFα) lead to an increase in the percentage of eGFP$^-$ CD103$^+$ DC expressing CD86 and particularly CD80. Of interest is the

observation that the percentage of eGFP$^-$CD103$^+$ expressing CD80 was about two-fold greater than those expressing CD86. In general, CD80 was expressed at higher levels and at a higher percentage on the CD103$^+$ DC. This could reflect the reported importance of CD80 as a co-stimulatory molecule specifically vital to lung infections[18].

Unexpectedly, we also found that LN resident CD8α$^+$ DC were unable to stimulate naïve CD8$^+$ T cells *ex vivo*. While CD8α$^+$ DC appear to have a role in the generation of a CD8$^+$ T cell response following subcutaneous [89,116] or intravenous infection[115], the growing body of literature assessing pulmonary infections provide limited evidence for their participation in generating the CD8$^+$ T cell response. We note that we cannot fully rule out a role for CD8α$^+$ DC in priming naïve T cells as it is possible that their contribution to CD8$^+$ T cell priming is below the limit of detection or that they play a supportive role, such as secretion of additional IL-12. The latter is an attractive model given the finding that splenic CD8α$^+$ DC produce more IL-12 than CD8α$^-$ DC[56].

CD8α$^+$ DC have been the focus of many studies because of their well established ability to cross-present antigen to CD8$^+$ T cells. However, CD8α$^+$ DC are not the only DC subset known for their ability to cross-present antigen; the CD103$^+$ DC have also exhibited this trait[41,117]. While it is tempting to conclude that cross-presentation by CD103$^+$ DC plays a role in priming CD8$^+$ T cells following pulmonary viral infection, the complexity of the system and an inability to

specifically block either the direct or cross-presentation pathways in an *in vivo* viral infection model makes such conclusions speculative at best. We did find that approximately 1.5 percent of the airway resident $CD103^+$ DC in the lung were $eGFP^+$. The level of eGFP signal in these DC and the rapid kinetics by which protein are degraded/denatured once entering the endocytic pathway[118,119], lead us to conclude that these $CD103^+$ DC are most likely infected and thus presenting antigen through direct presentation. It is possible however, that mature $eGFP^-CD103^+$ DC (Figure 4) have acquired antigen through phagocytosis and that the amount of eGFP phagocytosed falls below the limit of detection or the eGFP has been degraded. These DC would then be able to cross present the Ova peptide to $CD8^+$ T cells. Unfortunately, the number of cells recovered from the MLN was limiting and does not allow us to separate the $eGFP^+$ and $eGFP^-$ $CD103^+$ DC for direct comparison *ex vivo,* by incubation with naïve $CD8^+$ T cells. While such an experiment could provide further evidence for the role of cross-presentation of antigen in the development of the resulting $CD8^+$ T cell response, we would still need to prove that the $eGFP^-$ cells were, in fact, uninfected. Thus the role of direct versus cross-presentation in the generation of a $CD8^+$ T cell response to pulmonary vaccinia viral infections remains to be defined.

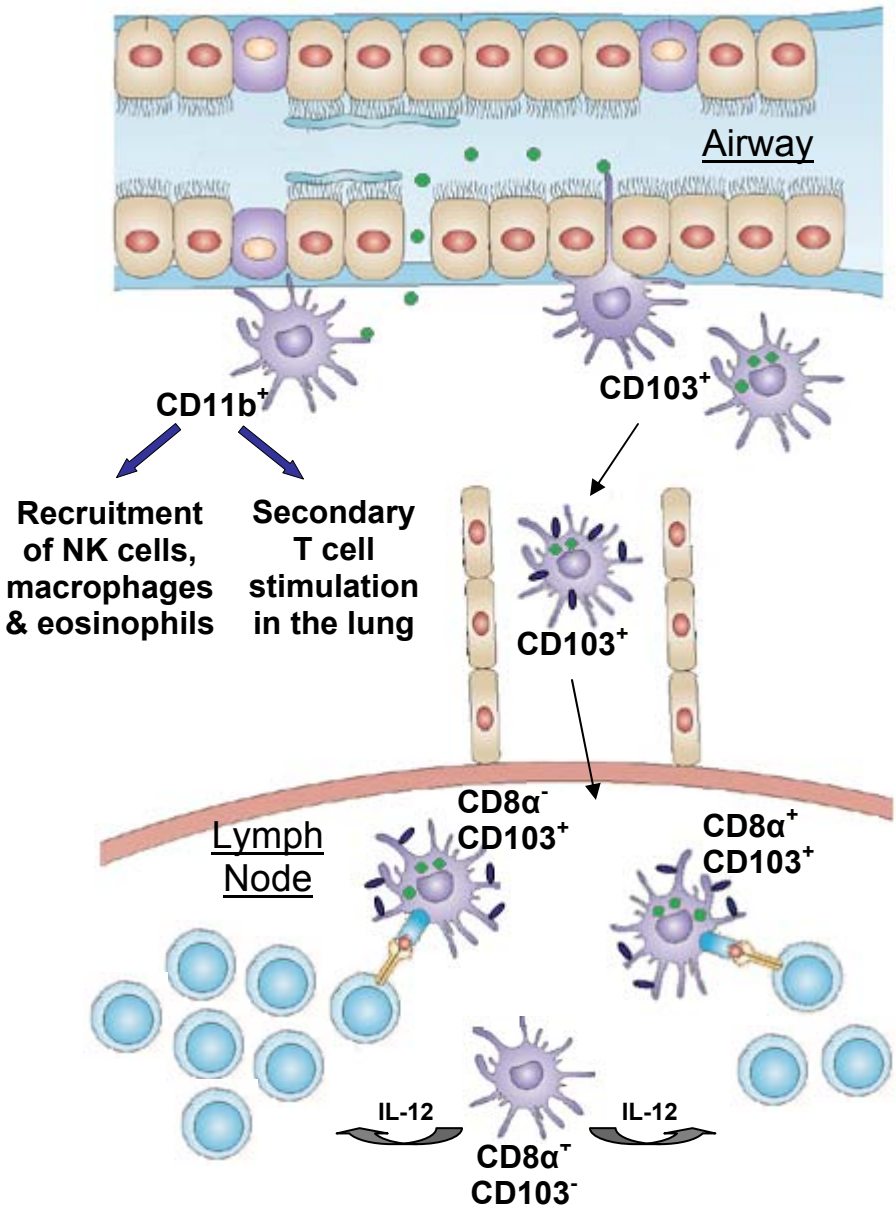
While analyzing DC from the MLN, we noted that a portion of the $CD103^+$ DC co-expressed CD8α (Figure 5) even in the absence of infection. There is evidence of this population in the literature[57,58,59,60,69,101] although this population is

relatively unexplored. CD8α expression on DC is noticeably absent from the lung tissue, though some studies suggest that CD8α$^+$ DC migrate into the lung at later time points post infection[59,100]. Vermaelon has noted co-expression of CD8α and CD103 on DC in the skin[58]; while Anjuere showed that Langerhan cells could be induced *in vitro* to express CD8α following CD40L stimulation[57]. Acute infection with *Bordetella pertussis* infection resulted in as many as 40% of the CD103$^+$ DC in the cervical LN co-expressing CD8α[59]. Following influenza infection the presence of a CD8α$^+$CD103$^+$ DC subset in the draining LN has been noted [60,101,69]. Given the limited information available regarding the function of these DC, we assessed the ability of the CD8α$^+$CD103$^+$ DC isolated from the lung draining MLN to serve as activators of naïve CD8$^+$ T cells.

Following VV infection, we found that while the CD8α$^+$CD103$^+$ DC could induce division in naïve CD8$^+$ T cells, they stimulated far fewer naïve CD8$^+$ T cells than did CD8α$^-$CD103$^+$ DC (Figure 7). This dichotomy existed despite a similar percentage of the CD8α$^+$CD103$^+$ DC and CD8α$^-$CD103$^+$ DC expressing eGFP (Figure 8). It is possible that the CD8α$^+$CD103$^+$ DC have acquired eGFP through uptake of apoptotic infected cells[61], explaining their positive eGFP signal but lack of antigen presentation. Alternatively, CD8α$^+$CD103$^+$ DC may be as susceptible to infection as the CD8α$^-$CD103$^+$ DC, but may have a defect in their ability to present antigen following infection. Perhaps these CD8α$^+$CD103$^+$ DC contribute to the generation of the CD8$^+$ T cell response to pulmonary VV, though production of cytokines such as IL-12, rather than antigen presentation.

Based on our data we have devised the following model for $CD8^+$ T cell activation following pulmonary infection with VV. Following virus administration, $CD103^+$ DC and $CD11b^+$ DC resident in the lung become infected. The $CD103^+$ DC mature and migrate from the lung to the MLN. In the MLN the mature $CD8\alpha^-$ $CD103^+$ DC are able to prime naïve virus-specific $CD8^+$ T cells, aided by the $CD8\alpha^+CD103^+$ DC. The LN resident DC do not appear to stimulate $CD8^+$ T cells directly, but may be a source of additional IL-12. Meanwhile, the $eGFP^+$ $CD11b^+$ DC are retained in the lung, secreting chemokines that will attract NK cells, macrophages and eosinophils, along with the activated T cells, to the sight of infection. Additionally, the $CD11b^+$ DC are present in the lung to provide additional antigen stimulation for the effector $CD8^+$ T cells (Figure 14).

Potential implications for this model exist in the design of vaccine vectors. In the case of a therapeutic vaccine against cancer, where a strong innate and adaptive immune response would be beneficial, a recombinant vaccinia virus might work particularly well[120]. The $CD11b^+$ DC retained within the tissue near the tumor could help to recruit innate immune cells to enhance innate anti-tumor immunity as well as support the anti-cancer $CD8^+$ T cell response with additional antigen presentation at the site of the tumor. It is unknown whether this retention of $CD11b^+$ at the site of infection is limited to the lung or extends to other mucosal sites. Vaccine strategies aside, these studies have provided greater insight as to how the immune system is able to recognize and respond to pulmonary viruses.



Modified from Förster *et al*. 2008. Nature Reviews Immunology 8, 362-371

Figure 14. The Generation of virus-specific CD8$^+$ T cells following pulmonary VV infection. Following infection, the CD103$^+$ DC mature and migrate to the MLN where they are able to stimulate naïve CD8$^+$ T cells. The LN resident CD8α$^+$ DC do not directly prime CD8$^+$ T cells, but may secrete IL-12 to enhance the activation of the CD8$^+$ T cells primed by the CD103$^+$ DC. The CD11b$^+$ DC are retained in the lung, secreting chemokines which attract both innate and adaptive immune cells to the site of infection. Also, infected CD11b$^+$ DC in the lung are able to interact with effector CD8$^+$ T cells and provide a secondary antigen encounter to enhance effector function and division.

$CD8\alpha^+CD103^+$ DC Represent a Distinct Subset of DC, Functionally Different from both $CD8\alpha^-CD103^+$ DC and $CD8\alpha^+CD103^-$ DC.

The reduced stimulatory ability of the $CD8\alpha^+CD103^+$ DC for $CD8^+$ T cells led us to investigate the origin and function of this subset. In the only report that addresses a specific function of these DC, it was demonstrated that only the splenic marginal zone DC co-expressing CD8α and CD103 were able to cross-present apoptotic cells[61]. The co-expression of CD8α and CD103 on DC in the MLN could result from either lung derived $CD103^+$ DC up-regulating the expression of CD8α upon entry into the MLN, or from the up-regulation of CD103 on LN resident $CD8\alpha^+$ DC. In the latter model, CD8α would upregulate expression of CD103, an integrin whose ligand, E-cadherin, is expressed by lung epithelia, in order to faicilitate homing of $CD8\alpha^+$ DC to the lung. At later time points of *Bordetella pertussis*[59] infection and some influenza infections[100,121] the presence of a $CD8\alpha^+$ DC population in the lung has been described. In both models of infection depletion of the $CD8\alpha^+$ DC in the lung impairs the clearance of the infection. While we have not addressed the presence of $CD8\alpha^+$ DC in the lung at later times post VV infection, we did not find $CD8\alpha^+CD103^+$ DC in the lung within the first three days post infection. It also remains a possibility that $CD103^+$ DC in the lung up-regulate CD8α when exposed to the proper inflammatory environment.

Our data are most consistent with a model where the lung-derived $CD103^+$ DC up-regulate expression of CD8α following a LN-specific stimulus. The presence

of the $CD8\alpha^{+}CD103^{+}$ DC in the MLN under steady-state conditions argues that the up-regulation of CD8α is MLN dependent and not infection dependent. When lung resident DC were labeled with CTO following viral infection, there was an increase in the number of $CTO^{+}CD8\alpha^{+}CD103^{+}$ DC in the MLN, suggesting that they had trafficked through the lung. The number of $CTO^{+}CD8\alpha^{-}CD103^{+}$ DC present in the MLN rose significantly 24 hours post infection while the number of $CTO^{+}CD8\alpha^{+}CD103^{+}$ DC was not significantly above steady-state until day 3 post infection. There are also more $CTO^{+}CD8\alpha^{-}CD103^{+}$ DC than $CTO^{+}CD8\alpha^{+}CD103^{+}$ DC in the MLN, reflective of the larger overall number of $CD8\alpha^{-}CD103^{+}$ DC in the MLN.

When the $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{+}$ DC subsets were analyzed as a percent of the migratory CTO^{+} DC, we found that $CD103^{+}$ DC accounted for at least half of all migrating DC within the first 48 hours following infection (Figure 10D). Beyond this point, the $CD11b^{+}$ DC became the predominant DC migrating from the lung. Additionally, there is an increase in the percentage of CTO^{+} DC that are $CD8\alpha^{+}CD103^{+}$ DC. This might indicate that DC recruited into the inflamed lung prior to the 24 hour time point are more likely to up-regulate CD8α upon migration to the MLN. It is possible that while infection is not required for the appearance of $CD8\alpha^{+}CD103^{+}$ DC in the MLN, it does enhance the conversion of $CD8\alpha^{-}CD103^{+}$ DC to $CD8\alpha^{+}CD103^{+}$ DC.

Since the kinetics of the $CD8\alpha^+CD103^+$ DC migration to the MLN are slightly delayed, it is possible that they might play a role in the generation of $CD8^+$ DC later than day 2 post infection. If this is the case, we would expect to see a greater division in the OT-I T cell cultured with $CD8\alpha^+CD103^+$ DC taken from the MLN of mice at days three or four post infection.

Surprisingly, there was a low, though detectable, level of $CTO^+CD8\alpha^+CD103^-$ DC in the MLN (less than 3% of trafficking DC). It is most likely that the CTO signal in the $CD8\alpha^+CD103^-$ DC was acquired through phagocytosis of apoptotic CTO^+ cells from the lung. And while the $CD103^+$ DC are also known for their phagocytic abilities, the significantly larger proportion of $CD8\alpha^+CD103^+$ DC positive for CTO, would indicate that either the $CD8\alpha^+CD103^+$ DC are far superior at phagocytosis than the $CD8\alpha^+CD103^-$ DC or, more likely, that the $CD8\alpha^+CD103^+$ DC have trafficked through the lung prior to entry into the MLN.

Given the likelihood that the $CD8\alpha^+CD103^+$ DC have trafficked through the lung and therefore have originated from the $CD8\alpha^-CD103^+$ DC, we wanted to examine the expression of surface markers on these DC subsets to determine if there were other phenotypic distinctions between the populations.

CD205 is a type 1 C-type lectin-like protein of the mannose-receptor family[122], whose ligands remain unknown. However, experiments with vaccinations of fusion proteins consisting of ovalbumin and an antibody for CD205 have shown

that the addition of α-CD205 enhances the $CD8^+$ T cell response to ovalbumin[123]. CD205 has also been implicated in binding and phagocytosis of necrotic and apoptotic cells[124]. Not surprising, given its potential as a receptor for cross presentation, CD205 expression has been shown on $CD8\alpha^+$ DC in the spleen[91,92,93,94]. CD205 has expression has also been reported for $CD103^+$ DC in the MLN[41], spleen[51,95] and dermis[96].

In the MLN of B6 mice, the expression of CD205 correlated to the $CD103^+$ DC populations. Both $CD8\alpha^-CD103^+$ and $CD8\alpha^+CD103^+$ DC expressed CD205 on over 50% of their cells. While there was a slightly higher percentage of $CD8\alpha^+CD103^+$ DC expressing CD205 compared to the $CD8\alpha^-CD103^+$ DC, the overall expression level of CD205 was not statistically different. The $CD8\alpha^+CD103^-$ DC, on the other hand, showed a significant decrease in both the percentage of $CD205^+$ DC as well as expression level of CD205.

Since both $CD103^+$ DC and $CD8\alpha^+$ DC are known to be highly efficient at cross presentation[41,52], it is interesting that there was such a dichotomy in their expression of CD205. It may be that the $CD103^+$ DC are more dependent on CD205 binding for uptake of apoptotic cells, while LN $CD8\alpha^+$ DC express alternative receptors. Additionally, as this is the first study to examine co-expression of CD8α, CD103 and CD205, it is possible that previous studies reporting expression of CD205 on $CD8\alpha^+$ DC in the spleen could actually be detecting $CD8\alpha^+CD103^+$ DC which are known to be present in the spleen[61].

Regardless, expression of CD205 suggests that the $CD8\alpha^{+}CD103^{+}$ DC are phenotypically similar to the $CD8\alpha^{-}CD103^{+}$ DC.

CD24 or heat stable antigen has been implicated as a co-stimulatory molecule important in the priming of $CD8^{+}$ T cells[125,126] and is expressed by $CD8\alpha^{+}$ DC in the spleen[93,127,94]. Additionally, CD24 is often used as a marker for DC in the blood and spleen that are committed to becoming $CD8\alpha^{+}$ DC[128,129] as well as a marker of a $CD8\alpha^{+}$ equivalent population of DC that is generated from the bone marrow following differentiation in the presence of Flt3L[130]. Although cell surface expression of CD24 has not been evaluated in lung derived $CD103^{+}$ DC, recently mRNA for CD24 has been reported in $CD103^{+}$ DC from the lung[97]. In our analysis we found that $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{+}$ DC express CD24 on almost 100% of their cells, while a significantly smaller proportion of $CD8\alpha^{+}CD103^{-}$ DC are $CD24^{+}$. Further the level of expression of CD24 is reduced more than 2.5 fold on the $CD8\alpha^{+}CD103^{-}$ DC compared to the $CD8\alpha^{-}$ $CD103^{+}$ DC or $CD8\alpha^{+}CD103^{+}$ DC.

In the mouse, CD24 has been reported to bind P-selectin[131]. P-selectin is expressed by endothelial cells during inflammation and plays a part in leukocyte recruitment into inflamed tissue[132-135]. While these data were obtained from analysis of naïve mice, it is possible that the high expression of CD24 by the $CD103^{+}$ DC might play a role in their migration from the blood into the lung under conditions of inflammation. Although the role of CD24 on DC remains unclear,

the expression profile of CD24, like that of CD205, suggests a relationship between the $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{+}$ DC.

CD36 is a B class scavenger receptor. While it has been implicated in the uptake of apoptotic cells[136], Belz et. al. has demonstrated that it is not required for cross-presentation on DC, although they did show that CD36 was preferentially expressed on the $CD8\alpha^{+}$ DC of the spleen[98]. We found that CD36 expression was low to moderate on all of the DC subsets analyzed from the MLN. There was no significant difference between the percentage of DC expressing CD36 on any of the subsets. While the $CD8\alpha^{+}CD103^{+}$ DC did show a significant increase in the expression level of CD36 when compared to both the $CD8\alpha^{-}CD103^{+}$ DC or $CD8\alpha^{+}CD103^{-}$ DC, the expression of CD36 does not show the strong correlation to CD103 expression that we have seen with CD205 or CD24.

Had the $CD8\alpha^{+}$ DC in the MLN up-regulated CD103 to result in the $CD8\alpha^{+}CD103^{+}$ DC population, we would expect to see phenotypic similarities in the expression of CD205, CD24 and CD36 between the $CD8\alpha^{+}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{-}$ DC. These data again point to the likelihood that the $CD8\alpha^{+}CD103^{+}$ DC are a result of up-regulation of CD8α by the $CD103^{+}$ DC upon emigration into the MLN.

Although we have shown that the CD8α^{+}CD103^{+} DC have a phenotypic similarity to the CD8α^{-}CD103^{+} DC, expression of surface markers does not address the functional differences we have seen between these two DC subsets. We treated the mice with various TLR agonists i.t. in order to determine if the CD8α^{+}CD103^{+} DC displayed inherent defects in their ability to respond to inflammatory stimuli.

Following treatment with PolyI:C (TLR3), LPS (TLR4) and CpG (TLR9), all three DC subsets had an increase in the percentage of DC that were positive for both CD80 and CD86. In fact the level of CD80 and CD86 on the CD8α^{+}CD103^{+} DC significantly exceeded the expression levels on both CD8α^{-}CD103^{+} DC and CD8α^{+}CD103^{-} DC, following stimulation with PolyI:C, LPS or CpG. These data show CD8α^{+}CD103^{+} DC appear to have enhanced maturation in response to TLR agonists.

VV stimulates IL-6 and IL-1 production in DC as well as induces up-regulation of CD86 through a TLR2 dependent mechanism[137]. Additionally mice lacking TLR9 are more susceptible to infection with another member of the orthopoxvirus family, ectromelia virus infection[75]. Clearly the deficiency of CD8α^{+}CD103^{+} DC to prime CD8^{+} T cells *ex vivo*, is not due to an inherent inability to up-regulate expression of co-stimulatory molecules. However, as VV infection is far more complex than TLR stimulation, it is still possible that the VV infection could modulate the ability of the CD8α^{+}CD103^{+} DC to up-regulate co-stimulatory molecules, thereby decreasing their ability to prime naïve CD8^{+} T cells. Indeed,

in a preliminary experiment where DC from MLN of VV infected mice were pulsed with Ova peptide prior to incubation with naïve OT-I T cells, we found that the OT-I T cells incubated with $CD8\alpha^+CD103^+$ DC still underwent less division than those incubated with $CD8\alpha^-CD103^+$ DC (data not shown).

While the $CD8\alpha^+CD103^+$ DC show a significant increase in the level of co-stimulatory molecule expression, on a population level, the $CD8\alpha^+CD103^+$ DC respond more similarly to the airway $CD8\alpha^-CD103^+$ DC than the LN resident $CD8\alpha^+CD103^-$ DC. It could be argued that TLR agonist, inserted into the lungs, are not draining to the LN, resulting in lower expression levels and lower percentages of $CD80^+$ and $CD86^+$ $CD8\alpha^+CD103^-$ DC. However, if this is the case then the greater expression of co-stimulatory molecules on the $CD8\alpha^+CD103^+$ DC suggests that they have come into contact with the TLR agonists in the lung, adding to the evidence that the $CD8\alpha^+CD103^+$ DC are related to the $CD8\alpha^-CD103^+$ DC.

Previous reports have demonstrated that $CD8\alpha^+$ DC have a higher expression of TLR3 than their $CD8\alpha^-$ DC in the spleen[138] and recently, dermal $CD103^+$ DC have been shown to express high levels of TLR3[96]. Indeed, TLR3 stimulation resulted in greater than 80% of the DC in all three subsets expressing high levels of CD86. One of the TLR agonists that was tested was CL097, an agonist for TLR7. While $CD8\alpha^+$ DC have been reported to lack TLR7 expression[138], $CD103^+$ DC have not been examined for TLR7 expression. We have shown that, like

CD8α$^+$ DC, the CD103$^+$ DC do not respond to TLR7 agonists. The enhanced response to TLR3, as well as the lack of response to TLR7, may suggest a common precursor between the CD8α$^-$CD103$^+$ DC, CD8α$^+$CD103$^+$ DC and CD8α$^+$CD103$^-$ DC.

The development of DC into their respective subsets is a topic currently under much investigation. One model is that DC develop through a common pluripotent progenitor, whose development increasingly restricts the types of DC that can arise[139] (Figure 15). In this model, the CD8α$^+$ DC and CD103$^+$ DC can arise from the pre-DC population[139,140]. There is, however, also evidence to suggest that the tissue CD103$^+$ DC arise from a monocyte population[141,142].

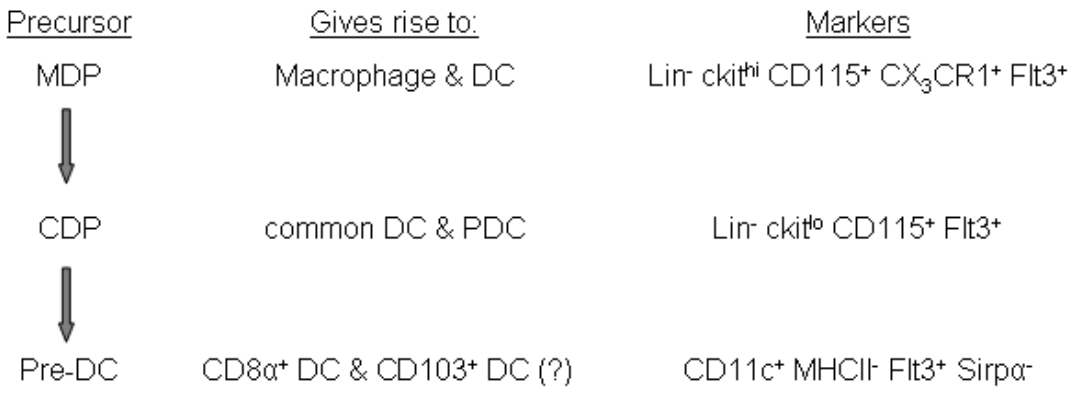


Figure 15. DC Precursor Development

There is mounting evidence that the CD8α$^+$ DC and CD103$^+$ DC have a common precursor, possibly at the later stages of DC development. Several transcription factors that have been shown to be vital for the development of CD8α$^+$ DC are also important to the CD103$^+$ DC compartment. Mice lacking either Batf3 or Irf8 do not develop tissue resident CD103$^+$ DC or CD8α$^+$ DC[97,143]. It is interesting

that Langerhan cells have been reported to up-regulate CD8α expression following *in vitro* stimulation with CD40L in mice[57]. In humans, DC generated from peripheral blood monocytes, stimulation with CD40L resulted in a 3-fold increase in the expression of Batf3, measured by microarray, 40 hours post stimulation[144]. It is possible that an interaction with $CD40L^+$ T cells in the microenvironment of the MLN, allows the $CD103^+$ DC to up-regulate Batf3 leading to CD8α expression. As attractive as this hypothesis may be, preliminary data examining the DC subsets in $CD40L^{-/-}$ mice revealed the $CD8\alpha^+CD103^+$ DC to still be present, indicating that this population does not depend on the presence of CD40L.

Most of the previous studies addressing the ability of $CD8\alpha^+$ DC in the MLN to stimulate naïve $CD8^+$ T cells have not assessed the expression of CD103 and assumed that $CD8\alpha^+$ DC in the lymph node are resident APC and, therefore obtain antigen through phagocytosis of cells migrating into the MLN from the lung. Here we provide data supporting the model that a portion of the $CD8\alpha^+$ DC in the MLN are not lymph node resident, but instead reflect a population of DC that acquired the expression of CD8 following emigration from the lung. These data suggest that the previously identified role of $CD8^+$ DC in the LN may merit re-examination. Additionally, there is evidence that there exists a potential plasticity within the DC pool, which may be able to be manipulated in the future.

We have shown that the airway derived $CD103^+$ DC become infected, undergo maturation and migrate to the draining LN following pulmonary VV infection and thus are capable of stimulating naive $CD8^+$ T cells. While the lung parenchymal $CD11b^+$ DC become infected, the infected DC fail to migrate to the MLN, resulting in poor stimulation of naïve $CD8^+$ T cells by $CD11b^+$ DC. Finally, it appears that a portion of the $CD103^+$ DC up-regulate expression of CD8α upon entering the MLN. These $CD8\alpha^+CD103^+$ DC appear to enter the MLN from the lung and be phenotypically related to the $CD8\alpha^-CD103^+$ DC. While the $CD8\alpha^+CD103^+$ DC have increased expression of CD80 and CD86 compared to the $CD8\alpha^-CD103^+$ DC following stimulation with TLR agonists, they are poor stimulators of naïve $CD8^+$ T cells following a pulmonary VV infection.

Future Directions

1. Determine why the $eGFP^+CD11b^+$ DC fail to migrate to the MLN following pulmonary VV infection.

We have already explored the expression of CCR5 and CCR7 on the $eGFP^-$ vs. $eGFP^+$ DC in both $CD11b^+$ and $CD103^+$ DC subsets and they do not appear to account for the differential migration. To test the proposed model and to see if the expression of IFNα/β alters the migration of $CD11b^+$ DC, the first experiment would be to infect IFNα/β receptor knock-out mice or mice treated with IFNα/β neutralizing antibody. Interfering with IFNα/β signaling most likely leads to enhanced viral spread, but given the short duration of infection (two days), it is possible that the animals will not succumb to illness in that time period. If by

blocking IFNα/β, there is detectible migration of the $CD11b^+$ DC, the involvement of PGE_2 and MMP-9 could then also be explored using mice deficient in PGE_2 and MMP-9.

2. Determine the cytokine production in $CD8\alpha^-CD103^+$ DC, $CD8\alpha^+CD103^+$ DC, and $CD8\alpha^+CD103^-$ DC in the MLN.

While attempts to analyze IL-12p40 expression via flow cytometry proved unsuccessful (the staining of the IL-12p40 was not above that of the isotype control), we could use either ELISA or ELISPOT analysis to determine the cytokine production (IL-12p70, IL-6, IL-10, IFNα/β) within these DC subsets. The DC subsets would have to be sorted prior to analysis. This does pose a technical problem as the recovery for the $CD8\alpha^+CD103^+$ DC and $CD8\alpha^+CD103^-$ DC are particularly low (~5000 – 7000 $CD8\alpha^+CD103^+$ DC for 25 pooled MLN). Since ELISA and ELISPOT can only analyze one cytokine at a time, the number of mice needed for these experiments could be prohibitive. However, given enough mice, these experiments would be highly informative.

3. Determine if $CD8\alpha^+CD103^+$ DC have a greater ability to stimulate naïve $CD8^+$ T cells at days three or four post infection.

Since there appears to be a delay in the migration of the $CD8\alpha^+CD103^+$ DC to the MLN, it is possible that by analyzing this population at day 2 post infection, we are simply looking too early to fully appreciate their role in naïve $CD8^+$ T cell priming. Sorting the DC from the MLN at days three and four post infection rather than day 2, might reveal a greater ability of the $CD8\alpha^+CD103^+$ DC in priming naïve $CD8^+$ T cells.

4. Determine if $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{+}$ DC prime $CD8^{+}$ T cells with differing avidity.

Using DC from the MLN of mice day 2 post infection to address this question is difficult as there is minimal stimulation of the OT-I T cells by the $CD8\alpha^{+}CD103^{+}$ DC at this time point. If however, the experiments in point 3 prove that the $CD8\alpha^{+}CD103^{+}$ DC have enhanced ablity to prime naïve $CD8^{+}$ T cells at later time points, this question could be addressed. The OT-I T cells primed off of $CD8\alpha^{-}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{+}$ DC would have to be re-stimulated with various concentration of Ova peptide following the three day incubation with DC, in order to determine the functional avidity of the OT-I T cells. This experiment, again, has some technical considerations regarding the DC recovery. Multiple wells of OT-I and DC would have to be set up for each DC subset and the number of mice required to yield enough $CD8\alpha^{+}CD103^{+}$ DC to do that could be prohibitive.

5. Determine if the $CD8\alpha^{+}CD103^{+}$ DC and $CD8\alpha^{+}CD103^{+}$ DC are able to stimulate naïve $CD4^{+}$ T cells and if either has the ability to prime tolerogenic $CD4^{+}$ T cells.

Throughout these studies we have only addressed the $CD8^{+}$ T cell priming ability of these $CD103^{+}$ DC subsets. It is possible that either or both might also have the ability prime $CD4^{+}$ T cells (OT-II). This would require the use of an alternative virus as the VV.NP-S-eGFP virus is specific for the Ova epitope able to stimulate $CD8^{+}$ T cells. As the $CD103^{+}$ DC in the gut are tolerogenic, it would be interesting to determine if either or both of these $CD103^{+}$ DC subsets found in the lung draining lymph node have a similar ability.

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Education

May 2011 PhD Molecular Medicine – concentration in Immunology
Wake Forest University School of Medicine, Winston-Salem, NC
Advisor: Dr. Martha Alexander-Miller
Disscertation: Understanding the Role of Dendritic Cell Subsets in the Generation of a $CD8^+$ T cell Response Following Pulmonary Vaccinia Viral Infection

May 2006 MS Biology
New Mexico Institute of Mining and Technology, Socorro, NM
Advisor: Dr. Scott Shors

May 2003 BS Chemistry
New Mexico Institute of Mining and Technology, Socorro, NM

Graduate Research

2006-present "The role of lung dendritic cell subsets in eliciting a $CD8^+$ T cell response following respiratory viral infection"
Dr. Martha Alexander-Miller
Wake Forest University School of Medicine

2003-2005 "The role of PKR-like ER Kinase (PERK) in redox and viral stress"
Dr. Scott Shors
New Mexico Institute of Mining and Technology

Undergraduate Research

2000 "The use of salicylic acid as a chelating agent in phytoremediation"
Dr. Christa Hockensmith
New Mexico Institute of Mining and Technology

Teaching experience

2004	Teaching Assistant: General Chemistry Lab I & II, Genetics Lab
2003	Teaching Assistant: General Biology Lab, Genetics Lab, Molecular Biology Lab
2002	Teaching Assistant: General Chemistry Lab I & II
2001	Teaching Assistant: General Chemistry Lab I

Awards and Honors

2009	National Institute of Allergy and Infectious Diseases – Travel Scholarship Keystone Symposia on Dendritic Cells, Banff, Canada
2007-2009	Ruth L. Kirschstein National Research Service Award Training Program in Molecular Medicine T32 GM063485 NIH/NIGMS

Laboratory Skills

Animal Models	Mouse Virus Infection Model: intranasal, intratracheal, intraperitoneal Vaccinia Virus SV5 Tissue isolation: lung, spleen, lymph nodes, bone marrow Transgenic mouse models Mouse colony breeding and maintenance Mouse genotyping
Flow Cytometry	Intracellular & Extracellular antibody staining Multicolor cell analysis Instruments: FACS Canto II, FACS Calibur, FACS Aria Analysis programs: BD DIVA, FlowJo, Cell Quest Pro, FCS express
Cell Culture	Sterile and aseptic technique Passaging of immortalized cell lines Generation of dendritic cells from mouse bone marrow Isolation and passage of primary CD8 T cells MACS column cell separation and enrichment Virus growth & recovery Plaque assays
Molecular Biology	PCR Gel electrophoresis SDS-PAGE electrophoresis Western Blotting ELISA

Research Presentations

2009 **Keystone Symposia on Dendritic Cells** - Banff, Canada
Nicole Beauchamp & Martha Alexander-Miller
"Lung derived dendritic cells are necessary and sufficient to prime CD8 T cells following pulmonary vaccinia virus infection"
Poster Presentation

2008 **American Association of Immunologists Annual Conference** – San Diego, CA
Nicole Beauchamp & Martha Alexander-Miller
"Analysis of dendritic cell maturation following respiratory infection with vaccinia virus"
Poster Presentation

2007 **American Association of Immunologists Annual Conference** – Miami, FL
Nicole Beauchamp & Martha Alexander-Miller
"Analysis of dendritic cell maturation following respiratory infection with vaccinia virus"
Poster Presentation

Publications

Beauchamp NM, Busick RY, Alexander-Miller MA. 2010. Functional divergence among $CD103^+$ dendritic cell subpopulations following pulmonary poxvirus infection. *Journal of Virology* 84(19):10191-9. Epub 2010 Jul 21. PMID: 20660207

Beauchamp NM, Holbrook BC, Alexander-Miller MA. 2010. Origin of CD8α expression on $CD103^+$ DC of the MLN.
Manuscript in preparation

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