RESPIRATORY INFECTION WITH THE PARAMYXOVIRUS SV5 RESULTS IN MATURATION AND CYTOKINE SECRETION IN CD103^{-} AND CD103^{+} DENDRITIC CELL SUBSETS.

BY

JINGYING ZHA

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Approved By:

Martha A. Alexander-Miller, Ph.D., Advisor _____

Examining Committee:

Kevin P. High, M.D., M.Sc., Chairman _____

Elizabeth M. Hiltbold, PhD. _____

Jason M. Grayson, PhD. _____

Griffith D. Parks, PhD. _____

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

APC……..……..……Antigen-presenting cells

BMDC……...……..….....Bone marrow-derived dendritic cell

CD……”Cluster of differentiation” molecules

CTL……..…….Cytotoxic T lymphocytes

DC…….Dendritic cell

mDC…….…….myeloid dendritic cell

pDC……plasmacytoid dendritic cell

FBS……Fetal bovine serum

GM-CSF……Granulocyte macrophage-colony stimulating factor

HN protein…….……Hemagglutinin-neuraminidase protein (viral protein)

ICAM…….……Intercellular adhesion molecule

IFN…….Interferon

IL……..……..Interleukin

M protein……Matrix protein (viral protein)

MHC……..……Major histocompatibility complex

MoDC……Monocytic DC

MOI……...….Multiplicity of infection

PRR……..……..Pattern recognition receptors

PAMP…….Pathogen associated molecular patterns

PFU……..……..Plaque forming units

P.I. …….Post Infection

RDC……Respiratory dendritic cell

RSV……Respiratory syncytial virus

rSV5……..…….Recombinant Simian virus 5

TLR……...…….Toll-like receptor

Jingying Zha

ABSTRACT

RESPIRATORY INFECTION WITH THE PARAMYXOVIRUS SV5 RESULTS IN MATURATION AND CYTOKINE SECRETION IN CD103$^-$ AND CD103$^+$ DENDRITIC CELL SUBSETS.

Thesis under the direction of Martha A. Alexander-Miller, Ph.D.,

Associate Professor of Microbiology and Immunology.

Dendritic cells (DC) bridge innate and adaptive immunity as the effective activators of naïve T cells. Three signals from DC contribute to the CD8$^+$ T cell activation and acquisition of effector function: 1. peptide/MHC, 2. costimulatory molecules (eg. CD80, CD86), and 3. secreted cytokines (e.g. IL-12, type I IFN). The first two signals require direct DC and T cell contact. In contrast, the third signal could be obtained from the local environment.

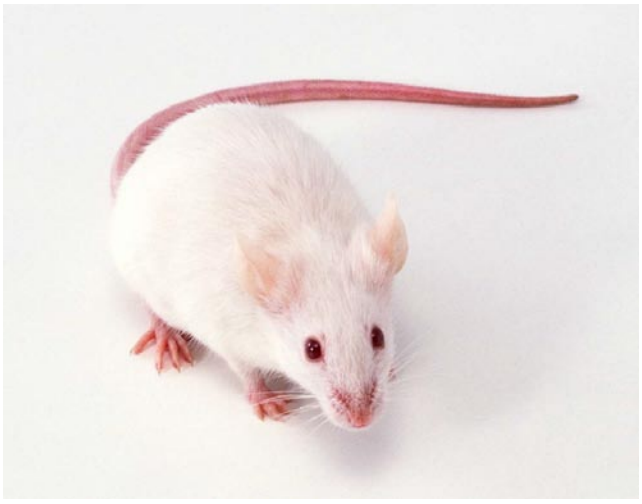
In this study, the DC in the MLN are divided into four subsets based on the expression of CD8 and CD103 molecules. Among the four subsets, the two CD103$^+$ DC subsets are from the airway while the CD8$^+$ CD103$^-$ DC are lymph node resident.

The $CD8^-$ $CD103^-$ DC subset is a mixed population, including $CD103^-$ $CD11b^+$ lung parenchyma DC, $CD8^-$ lymph node resident DC, pDC and monocytic $Gr\text{-}1^+$ DC. This division allows us to study the subsets important for the naïve $CD8^+$ T cell priming: $CD103^+$ DC and $CD8^+$ lymph node resident DC.

We utilized intranasal infection of BALB/c mice with paramyxovirus recombinant Simian Virus 5(rSV5-eGFP-p60) as a model to explore the behavior of these four DC subsets in vivo with regard to regulation of costimulatory molecules and cytokine secretion. We found that the number of mature DC, defined as CD80 and CD86 double positive DC, increased over time following infection, reached the maximum at day 2 post infection (p.i.) and rapidly decreased in both $CD103^+$ DC subsets while that of the two $CD103^-$ DC subsets continued to increase from day 1 to day 4 p.i. Specifically, the mature $CD103^-$ DC increased significantly from day 3 to day 4, following the peak of $CD103^+$ DC cell number. The number of IL-12p40 producing DC increased in a similar pattern in each DC subset following infection.

Interestingly, we found that all the DC subsets were able to undergo full maturation, which means the upregulation of both CD80 and CD86 expression. This result was reported in BMDC in response to productive rSV5 infection at M.O.I of 50 in vitro. We proposed that the complex vivo inflammatory environment, which includes other cell populations and inflammatory cytokines, were responsible for the full maturation of both $CD103^+$ DC and $CD103^-$ DC. Specifically, we proposed a model which described the direct or indirect interaction between lung migrant DC and lymph node resident DC contributed to the induction of the maturation of the latter population.

These data provided new insights into the control of the innate immune response in response to viral infection and might reveal novel understanding of the initiation of anti-viral adaptive immune response.



INTRODUCTION

Jingying Zha

Dendritic Cells

Dendritic cells (DC) are considered the superior professional APC and form a central component of the immune system. They participate in the important process of immune surveillance by patrolling the peripheral tissues, searching for pathogens or non-pathogenic particles. DC bridge innate immunity with adaptive immunity by obtaining, processing and presenting foreign antigens to the adaptive immune cells. They are adept at priming naïve and memory $CD8^+$ T cells. (Bhardwaj et al., 1994; Bohm et al., 1995; Lanzavecchia, 2001; Young et al., 1990)

DC progenitors from the bone marrow circulate in the blood. Most of them home to peripheral tissues but some of them home directly to lymphoid organs, for example, the draining lymph nodes. In both the peripheral tissues and the draining lymph nodes, those newly arrived DC reside as immature DC. They have high phagocytic capacity and are efficient at antigen uptake. But they express low levels of MHC-I and MHC-II molecules, costimulatory molecules, and adhesion molecules. Those immature DC are poor stimulators for naïve T cell priming (Banchereau et al., 2000).

In the peripheral tissues, once upon being directly infected by pathogens or have taken up foreign antigens, those immature DC will undergo maturation (Banchereau et al., 2000). Basically, some “danger” signals, including pro-inflammatory cytokines (e.g. TNF-α, IL-1β) and pathogen associated molecular patterns (PAMP), provide strong stimulus for DC maturation (Gallucci and Matzinger, 2001). As a result of maturation, the DC in the peripheral tissue migrate into the draining lymph node. Their chemokine receptors which help them home to peripheral tissues, including CCR1, 5 and 6, are down regulated. Facilitated by the upregulated chemokine receptor CCR7, those DC migrate

towards the CCL19/CCL21 gradient (Saeki et al., 1999) and enter the T cell zone of the draining lymph node, where the naïve T cell priming occurs.

At the mean time, these DC switch from antigen-acquiring cells into antigen-presenting cells with several phenotypic changes. They present antigens on the MHC class I and II molecules, whose expression has been rapidly upregulated on the cell surface due to the increased synthesis along with the decreased internalization and degradation (Inaba et al., 1998). And they decrease the expression of antigen capture receptors, upregulate their costimulatory molecules such as CD80 and CD86 (Sallusto et al., 1998) and adhesion molecules, for example, LFA-1, ICAM-I, etc. Besides the upregulation of those molecules, DC also change their shape from round to a typical "dendritic" look (Grayson and Holtzman, 2007). Those long extensions provide DC with a larger surface area for antigen-presentation. After entering the lymph node, DC can be further activated by T helper cells via CD40 and CD40L engagement. This activation process is presented by the upregulation of MHC, costimulatory molecules, adhesion molecules (Caux et al., 1994) as well as IL-12 secretion (Cella et al., 1996).

The life span of mature DC is typically short after they enter the lymph nodes (Banchereau et al., 2000). They will undergo an apoptotic death when an appropriate stimulation of T cells has been accomplished. Presumably, this apoptotic death helps down regulate the immune response (Grayson and Holtzman, 2007). It was reported by Legge et al. that the influx of respiratory dendritic cells (RDC) into the lung draining lymph node, the peribronchial lymph node (PBLN), in response to the influenza virus intranasal infection was followed by a rapid decrease of RDC cell number (Legge and Braciale, 2003), suggesting that the mature RDC might have a limited life span. This

phenomenon is still waiting for further experimental verification to identify the mechanism.

Similar to the DC in the peripheral tissues, the immature dendritic cells which reside in the lymph node remain immature when there is no infection or stimulating signals. Compared to the DC in the peripheral tissues, these lymph node resident DC have less access to the foreign antigens when infection occurs. Although these DC do not have the chance to interact with pathogens in the peripheral infection sites, the interaction with the lymph-borne pathogenic molecules as well as the cytokines secreted by cells in the surroundings will trigger the maturation of lymph node resident DC (Borderia et al, 2008; Dejnirattisai et al., 2008; Nightingale et al, 2008; Pollara et al., 2004). They will undergo the same maturation process as the DC from peripheral tissues. More details about the antigen-capture and naïve T cell priming in the draining lymph node will be discussed along with the structure of the draining lymph node.

Different subsets of DC

Based on the recent studies, DC are a heterogenous population, which means they have many different subsets. As we mentioned above, DC can be divided by their location: tissue DC and DC residing in lymphoid organs. Specifically, lung DC and the DC in the lung draining lymph nodes are the two corresponding groups in the respiratory tract.

Several distinct subsets of lung DC have been identified: $CD11c^+$ $CD103^+$ DC airway-derived DC, $CD11c^+$ $CD11b^+$ DC parenchyma DC and the $CD11c^+$ $B220^+$ plasmacytoid DC (pDC) (Hao et al., 2008). CD103, the α chain of αE-β7 integrin, has

been used as a new surface marker to describe DC subsets. It is expressed on DC in the lung (Sung et al., 2006), in the mesenteric lymph nodes (Kilshaw, 1993) and in the small intestine lamina propria of rats (Brenan and Rees, 2000; Turnbull, 2005). Lung $CD103^+$ DC usually reside in the airway mucosa and pulmonary vessels (Sung et al., 2006). Their surface expression of tight junction proteins, including Claudin-1, Claudin-2 and ZO-2, help them open up tight junctions between epithelial cells. As a consequence, $CD103^+$ DC can send their long extensions or even migrate across the barrier of epithelia into the airway mucosa to capture antigens (Sung et al., 2006). This ability facilitates $CD103^+$ DC to sense microbial invasion, gain antigens and be ready to present them to adaptive immune cells. Therefore, $CD103^+$ DC are potent for immune surveillance and initiation of adaptive immunity. Among the three lung DC subsets, $CD11c^+$ $CD103^+$ DC, $CD11c^+$ $CD11b^{hi}$ DC and $CD11c^+$ $B220^+$ pDC, $CD11c^+$ $CD103^+$ DC subset was found to be the most permissive subset to influenza infection in vitro (Hao et al., 2008). An in vivo study also demonstrated the susceptibility of this subset: The $CD103^+$ DC found in the lung draining MLN after day 3 post influenza infection were positive for NP, the viral protein of influenza virus (Kim and Braciale, 2009). Besides the permissivity to direct infection, many studies explored the cross-presentation ability of $CD103^+$ DC subset. Dr. Braciale and his colleagues showed that $CD103^+$ DC were capable of cross-presenting peptides derived from non-infectious intact influenza virions to cognate naïve $CD8^+$ T cells and triggering their proliferation in vivo (Kim and Braciale, 2009). Another study done by del Rio et al. showed that $CD103^+$ DC could cross-present inhaled innocuous antigens to $CD8^+$ T cells in the lung draining bronchial lymph node in vivo (del Rio et al., 2007). Those studies indicated that $CD103^+$ DC could do cross-priming. The permissivity to

viral infection and the ability to cross-present, as well as the proximity to foreign antigens in the airway, along with their expression of MHC molecules, costimulatory molecules and IL-12 (Sung et al., 2006), make $CD103^+$ DC potential candidates for antigen-presentation to naïve T cells. Moreover, the expression of CCR7 on $CD103^+$ DC (Kim and Braciale, 2009) facilitates them to migrate from the airway to the T cell zone of the draining lymph node, where naïve T cell priming occurs. In short, this DC subset is important for the initiation of adaptive immunity in the respiratory tract.

$CD11c^+$ $CD11b^+$ parenchyma DC locate in the submucosa and the lung parenchyma (Kim and Braciale, 2009). Among the three lung DC subsets, the parenchyma DC show the immediate permissivity to influenza viral infection in vitro, less susceptible than the $CD103^+$ DC (Hao et al., 2008). This DC subset and the $CD103^+$ DC subset are the two primary antigen-presenting cells in the lung (Kim and Braciale, 2009). Like the $CD103^+$ DC, the $CD11b^+$ parenchyma DC can take up viral antigens, migrate to the draining lymph nodes, and accumulated in the MLN following i.n. influenza infection. However, an ex vivo study following intranasal influenza infection showed that, different from the $CD103^+$ DC, which can prime both naïve $CD4^+$ T cells and naïve $CD8^+$ T cells, $CD11b^+$ parenchyma DC can only stimulate the activation of $CD4^+$ T cells (Kim and Braciale, 2009). This study also showed that the parenchyma DC subset did not have the ability to cross-present antigens.

Compared to the above two cDC subsets, the plasmacytoid DC (pDC) are less efficient in antigen presentation (Colonna et al., 2004). Dr. Lambrecht and his colleagues reported that the depletion of pDC in the lung and the draining lymph node did not alter the course of influenza viral infection albeit pDC was shown to carry viral NP protein in

this infection (GeurtsvanKessel et al., 2008). Although the pDC do not have efficient antigen-presentation ability, they contribute to antiviral responses by producing a high level of type I IFN and a relatively lower level of IL-12 (Colonna et al., 2004). In mouse models of RSV infection, the depletion of pDC caused a more severe RSV infection: airway inflammation, the increased viral titers in the lung and airway hyperresponsiveness (Smit et al, 2006; Wang et al, 2006). This phenomenon is in line with the need for effective type I IFN production to control RSV viral infection and the fact that pDC serve as a major source of type I IFN following infection. The pDC can migrate to the draining lymph node through high-endothelial venules (HEVs) and create an inflammatory environment by secreting type I IFN and IL-12. A subset of pDC, which express CD19 in addition to other normal pDC surface markers, however, are shown to have suppressive regulatory effect for T cell proliferation (Munn et al., 2004). Besides the above functions, pDC can also skew Th2 response towards a Th1 response and as a consequence, reduce the development of allergic airway disease (De Heer et al., 2004; De Heer et al., 2005).

The DC subsets residing in the draining lymph nodes are derived directly from the precursors in the blood. They can be divided by CD8 expression: $CD8^+$ lymph node resident DC and $CD8^-$ DC which include $CD4^+$ $CD8^-$ $CD11b^+$ DC and $CD4^-$ $CD8^-$ $CD11b^+$ DC.

$CD8^+$ lymph node resident DC ($CD8^+$ DC) do not express CD11b and CD4 but express high level of CD205 on their cell surface (Lopez-Bravo and Ardavin, 2008) . They are very efficient at antigen-presentation through the MHC-I pathway by both direct presentation and cross-presentation in vivo (del Hann et al., 2000; Schnorrer et al., 2006).

Upon a microbial infection, this DC subset can prime both naive $CD4^+$ and $CD8^+$ T cells, by direct presentation to $CD4^+$ and $CD8^+$ T cells or cross-presentation to $CD8^+$ T cells (Allan et al., 2003; Allan et al., 2006; Belz et al., 2004a; Belz et al., 2004b; Mount et al., 2008) . Their cross-priming ability is correlated with their capability to phagocytose dying cells and the potential associated antigens (Schulz and Reis e Sousa, 2002) as well as their high expression level of CD205, which is a C-type lectin antigen receptor that facilitates viral antigen uptake (Dudziak et al., 2007). Also, $CD8^+$ lymph node resident DC express high level of IL-12 in vivo following microbial infection (Maldonado-Lopez et al., 2001). It is believed that $CD8^+$ lymph node resident DC are the superior DC subset for the induction of $CD8^+$ T cell responses in vivo, especially in the case of viral infection (Allan et al., 2003; Allan et al., 2006; Belz et al., 2004a; Belz et al., 2004b).

In the two $CD8^-$ DC subsets, $CD4^+$ $CD8^-$ $CD11b^+$ DC do not express CD205 or CD8 on their surface. This DC subset only represents a small portion of the total lymph node resident DC. The other $CD8^-$ DC subsets, $CD4^-$ $CD8^-$ $CD11b^+$ DC express none or moderate level of CD205. These DC are usually refered as double negative lymph node resident DC and represent a relatively large portion of the lymph node resident DC population (Shortman and Liu, 2002). Both $CD8^-$ DC subsets can capture antigens by phagocytosis and endocytosis, and present them to $CD4^+$ T cells via MHC-II pathway (Villadangos and Schnorrer, 2007). Compared to $CD8^+$ DC, however, $CD8^-$ DC are much less efficient in cross-presentation in vivo (den Haan et al., 2000). This is probably due to their lack of cross-presentation machinery and the relatively low expression of C-type lectin receptors on their surface (Lopez-Bravo and Ardavin, 2008).

Upon the inflammatory situation, there is a large influx of migrant DC into the local draining lymph nodes. In the case of respiratory infection, many airway-derived DC migrate into the draining MLN following infection. Besides those lung-origin DC, large number of Gr-1^+ monocytic DC (MoDC) derived from circulating Gr-1^+ monocytes are present in the lymph node. Those MoDC are $CD11c^{lo}$ $CD8^-$ $F4/80^+$ $CCR7^-MHC^{lo}$ and have been observed migrating into the MLN following influenza intranasal infection in a CCR7-independent way (Kim and Braciale, 2009). In their study, these MoDC were shown to present low level of viral antigens and their antigen-presentation was restricted to $CD4^+$ T cells albeit other studies utilizing dermal infection model suggested that this MoDC population work as important antigen-presenting cells (Le Borgne et al., 2006; Leon et al., 2007; Randolph et al., 1999). This mobilization of blood-borne monocytes only occurs in response to inflammatory stimuli but not in the steady state (Geissmann et al., 2008; Kim and Braciale, 2009; Shortman and Naik, 2007). In parallel with the large influx of MoDC, an accelerated migration of pDC is also induced in response to the infection (Diacovo et al., 2005; Yoneyama et al., 2005).

Activation of naïve $CD8^+$ T cells

Mature DC are competent for the naïve $CD8^+$ T cell priming, which is an important part of the initiating of potent adaptive immunity. The activation of naïve T cells needs three signals from activated antigen presentation cells 1) the cognate antigen presented by MHC molecules 2) costimulatory molecules and 3) secreted cytokines (Curtsinger, Lins and Mescher, 2003). The three signals will be discussed separately.

1) Signal one: the cognate antigen presented by MHC molecules on DC to $CD8^+$ T cell

The first signal involves the antigen presentation by dendritic cells to T cells. DC can present antigenic peptide of 8-10 amino acids by MHC-I to the T cell receptor (TCR) on $CD8^+$ T cells (Engelhard, 1994). Those short peptides are normally from processed foreign pathogens or non-pathogenic particles. There are two ways for the peptides to be presented to $CD8^+$ T cells by the MHC-I molecules: direct presentation and cross-presentation. The direct presentation involves the presentation of peptides from the proteins degraded by the proteasome in the cytosol. In the case of viral infection, those proteins are viral proteins which have been synthesized in the infected DC. The processed peptides are transported into the endoplasmic reticulum (ER) by TAP, a heterodimeric ATP- binding protein, for binding with MHC-I and are presented on the surface of DC. On the contrary, cross-presentation allows the viruses which are not able to infect DC to stimulate $CD8^+$ T cell responses (Heath and Carbone, 2001). This process involves exogenous antigen uptake, antigen degradation and the entry into the major MHC-I pathway (Lin et al., 2008). Among the different models for presentation of exogenous antigens through MHC-I pathway, the model of cytosolic pathway is considered the most relevant one in vivo (Delamarre et al., 2003; Rodriguez et al., 1999). This model proposes that the internalized antigens can be translocated into the cytosol of DC from the phagosome. By utilizing the proteasome of the conventional MHC-I pathway, the exogenous antigens are degraded into peptides. And another component of the MHC-I pathway, TAP (transporters associated with antigen processing), will transport the resultant peptides into the ER to be loaded onto MHC-I molecules. As mentioned above,

$CD8^+$ DC and $CD103^+$ DC are the types of DC which can cross-present efficiently in vivo (del Rio et al., 2007; den Haan et al., 2000; Kim and Braciale, 2009). This peptide-loaded MHC-I complex is recognized by the TCR/CD3 complex present on the cognate $CD8^+$ T cells. This signal gives a great stimulation to the naïve T cell, resulting in T cell survival and cytokine secretion (Gett et al., 2003). But a naïve T cell will be tolerated to the specific antigen if they only see the cognate peptide-MHC at physiologic dose but without the other two signals. This situation is called anergy (Gett et al., 2003). By contrast, for the effector T lymphocytes, the first signal will provide enough stimulation for their activation.

2) ***Signal two: The binding of costimulatory molecules on DC with the corresponding receptors on $CD8^+$ T cells.***

The second signal is normally provided by mature DC. These mature DC express high level of surface molecules which facilitate the interaction between DC and T cell. Among them, the costimulatory molecules (CD80, CD86, 41BBL, etc) are reported as essential factors for successful activation of naïve T cells (Boise et al., 1995; Song et al., 2005). This thesis focuses on the expression of CD80 and CD86 and uses the term "mature DC" to define the CD80 and CD86 double positive DC.

CD80 and CD86 belong to the B7 family. They are also named B7-1 and B7-2, respectively. These two costimulatory molecules can bind to the same receptors: CD28 and CTLA-4 (Bhatia et al., 2005). Their engagement with CD28 will pass anti-apoptosis signals to T cells to promote their survival and activation (Noel et al., 1996). On the contrary, the interaction between CD80 or CD86 and CTLA-4 will deliver the inhibitory signal to the T cells (Carreno et al., 2000). This pro-apoptosis signal will limit the number

of T cells in order to prevent any excess immune response that would cause tissue damage. Both of the molecules can bind with CD28 and CTLA-4, but previous work done by Pejawar-Gaddy and Alexander-Miller demonstrated that compared to CD86, CD80 is essential for promoting CD25 expression on $CD8^+$ T cells (Pejawar-Gaddy and Alexander-Miller, 2006). Other studies also suggested that CD80 can provide superior signal compared to CD86 (Bhatia el al, 2005; Jasson et al., 2005). Those studies suggested that CD80 and CD86 are not redundant.

3) Signal Three: the cytokines secreted by DC are required for $CD8^+$ T cell activation.

The cytokines secreted by mature DC are considered the third signal for naïve T cell priming. Among the array of cytokines produced by DC (IL-12, TNFα, IL-15 and IL-18, type I IFN, etc), IL-12 and type I IFN are the ones that have been demonstrated to play an important role for naïve T cells to acquire effector function, especially the IFN-γ secretion, and proliferation (Trinchieri, 1998). It has been reported that bystander DC are responsible for enhancing effector T cell expansion, inducing their cytolytic activity by providing IL-12 in the process of naïve CD8 T cell priming (Cui et al., 2009). This study suggested that as a soluble factor, IL-12 can play important role in the naïve T cell priming but does not have to come from the antigen-presenting cell.

The bioactive form of IL-12 is the heterodimeric IL-12p70. It consists of two subunits, IL-12p40 and IL-12p35. The expression of the two subunits is controlled by different signals, respectively (Schulz et al., 2000). Normally, IL-12p40 subunit is produced at a much higher concentration than IL-12p70 heterodimer. IL-12p40 production could be induced by a single TLR agonist. Stimulation from one of the following ligands: the TLR4 agonist LPS, the TLR7 agonist R-848 and the TLR9 agonist

CpG can induce the high level expression of IL-12p40 in BMDC (Theiner et al., 2008; Wagner et al., 1999). Compare to the above agonists, Poly (I:C), a TLR3 agonist, induces lower level of IL-12p40 secretion in BMDC (Gautier et al., 2005). In contrast, the expression of IL-12p70 is controlled in a more restricted way. Single TLR agonist stimulation would induce very low level of IL-12p70 expression. However, a much higer level of IL-12p70 expression could be obtained by the combination of R-848 with LPS or Poly (I:C) (Gautier et al., 2005). A synergistic effect induced by CpG pretreatment with followed LPS stimulation was observed in the manner of elevated expression of both IL-12p40 and IL-12p70 in murine DC (Theiner et al., 2008). In contrast, this study showed that these ligands could only induce very low levels of IL-12p70 expression when they were used alone. This suggested that the expression of IL-12p35 is controlled in a more restricted way. Besides the agonists of TLR, other signals are also reported to be critical for IL-12 secretion. It was reported that the engagement of CD40-CD40L between $CD4^+$ T cell and DC can facilitate the expression of IL-12p35 of DC (Cella et al., 1996; Koch et al., 1996). STAT1 is reported to be required for optimal IL-12p70 production (Gautier et al., 2005).

Type I IFN is another cytokine important in the naïve T cell priming. It is produced by most cells during viral infection as a key anti-viral cytokine. Described as a third signal for T cell activation, type I IFN has been shown to be able to promote T cell survival and development of effector function via a STAT4 dependant pathway (Curtsinger et al., 2005). In addition to T cell activation, type I IFN can act on DC maturation. Many studies have shown the maturation of DC is related to the production of type I IFN (Honda et al., 2003; Montoya et al., 2002; Santini et al., 2000) or the

activation of type I IFN pathway (Lopez et al., 2003). The previous research done in our lab showed that the binding of secreted type I IFN to its receptor is required for the upregulation of costimulatory molecules, CD40, CD80 and CD86 of BMDC in SV5 infection, but an additional signal present during productive SV5 infection is needed for an optimal CD80 upregulation (Pejawar, Parks and Alexander-Miller, 2005). Type I IFN has also been reported to induce the maturation of bystander uninfected DC as a paracrine activator (Borderia et al., 2008; Dejnirattisai et al., 2008; Nightingale et al., 2008; Pollara et al., 2004).

The naïve T cell priming occurs in the draining lymph node

In the naïve repertoire, T cells specific for a particular antigen are sparse (Blattman et al., 2002). It is difficult for the limited number of antigen-specific naïve T cells to meet with their cognate antigens by randomly travelling in the peripheral tissues all over the body. However, by patrolling the regional lymph nodes, where the antigens and antigen-presenting cells from the peripheral tissues are largely concentrated, the antigen specific naïve T cells obtain a better chance to meet with the cognate antigens, gain their effector function and protect the host against a potential infection in a short period of time (Willard-Mack et al., 2006; Young, 1999). Two main parameters, the cognate antigens and the antigen-presentation process led by antigen-presenting cells, are essential for the efficient priming of naïve T cells.

1) Two ways for antigens to enter the draining lymph nodes

Basically, upon microbial invasion, the antigens from the peripheral infection site have two ways to reach the regional draining lymph nodes. In the first way, the antigens

take advantage of the tissue DC which migrate from the peripheral site to the lymph node, whereas in the second way, the antigens were drained to the lymph node, independent of the migration of tissue DC.

The first way is well characterized by many studies. As we already mentioned, tissue DC fulfill the task of immune surveillance by watching the surroundings for foreign antigens. Once they sense any microbial antigens, they phagocytose, process and present the antigenic peptides on the cell surfaces (Grayson and Holtzman, 2007). By migrating to the draining lymph node via afferent lymph along a chemokine gradient, those DC work as antigen messengers to provide the cognate antigen-specific T cells with the dedicated antigens (Cavanagh and Andrian, 2002; Manickasingham and Reis e Sousa, 2001; Randolph, 2001; Sallusto and Lanzavecchia, 2000).

The second way for antigens to reach regional lymph nodes is independent of the travel of antigen-presenting cells from the peripheral tissue. Antigens from the infection site can be carried by the afferent lymph to the subcapsular sinus of the draining lymph node (Grayson and Holtzman, 2007). A three dimensional network known as the conduit system emerges from the subcapsular sinus and spreads throughout the T cell zone into the medulla (Sixt et al., 2005). Basically, this conduit system is an extracellular matrix specialized for transporting low molecular weight contents, such as chemokines, antigens and cytokines. This meshwork consists of basement membrane, microfibrillar zone (the inner space) and collagen core (Sixt et al., 2005). It is covered with fibroblast reticular cells (FRC) and DC (Katakai et al., 2004). Facilitated by this conduit system, the antigen molecules which weigh less than 70-80 KDa can move directly from the subcapsular sinus to the area around HEVs in the T cell zone (Gretz et al., 2000), the location where

most naïve T cell priming occurs. Those molecules are transported through the enclosed part of the conduit system which is an extracellular matrix formed by different types of collagens. The molecules that weigh more than 70-80 KDa circumvent the lymphocyte zone and subsequently, drain into the efferent lymph vessel through the sinuses (Gretz et al., 2000). They have very limited access to the T cell zone.

2) The interaction between naïve T cell and migrant DC or LN resident DC

Besides the accessibility of the cognate antigens to the naïve T cell priming site, the draining lymph nodes, the initiation of an antigen-specific T cell response also needs the help from antigen-presenting cells. The migrating DC from the peripheral tissue which have loaded the antigen on their surfaces gather around the HEVs, the entrances of lymphocytes from the arteriole into the lymph node parenchyma. The naïve T cells with CCR7 expressed on their surface are attracted by the ligands CCL19 or CCL21, secreted by those mature DC (Sallusto et al., 1999). Clusters of antigen-specific T cells are found centered around DC with the cognate antigen in the proximity to HEVs (Bajenoff et al., 2003). This finding is consistent with the model that the naïve T cell priming events are not equally distributed all over the T cell zone but are restricted to the areas near HEVs (Bajenoff et al., 2007). The presence of the concentrated DC population around the entrances maximizes the chance for a naïve T cell to quickly meet with the antigen it is specific for.

The immature lymph node resident DC are concentrated around the network of conduit system. They adhere to the conduit basement membrane, which works as a specific anchoring structure for lymph node resident DC, to screen the afferent lymph for potential antigens (Bajenoff et al., 2007). Once they have obtained antigens, they will

undergo maturation followed by the inactivity of the surface integrin (Sixt et al., 2005). As a consequence, they leave the conduit and have increased mobility for free movement around the T cell zone to carry out naïve T cell priming.

If one naive T cell is not able to find the right antigen after spending one day in a particular lymph node, it will go back into the bloodstream and travel to other secondary lymphoid tissues to continue the search (Cyster et al., 1999). If the naïve T cell is successfully activated by DC, it will upregulate the expression of the activation marker CD69 on its surface (Shiow et al., 2006). As a consequence, the naïve T cell will retain in the lymph node, proliferate and acquire the effector functions. After fully activated, the effector T lymphocytes will egress from the lymph node and travel to peripheral tissue to search for target cells that bear the cognate antigen (Weninger et al., 2001).

Taken together, the information showed above indicates that T cell-DC interaction is not a random process. Instead, this process includes a series of highly organized events, including but not limited to the migration of tissue DC into the draining lymph node, the antigens drained into the lymph nodes through the conduit system and achieved by immature lymph node resident DC, the recruitment of naïve T cells into the lymph node via chemotaxis and their subsequent interactions with DC present in the same area. The occurance and combination of those events facilitate the process of T cell priming.

Interaction between DC and viruses

Among the options for DC to obtain viral antigens to process and present, direct infection by viruses is the most straight forward way. Some viruses, such as measles virus

and paramyxovirus simian virus 5 (SV5), have been shown to be able to productively infect DC in vitro (Fugier-Vivier et al., 1997; Pejawar, Parks, and Alexander-Miller, 2005). However, in vivo, most kinds of viruses either do not easily infect DC or can hardly cause a productive infection in DC (Grayson and Holtzman, 2007; Tortorella et al., 2000). For example, paramyxovirus respiratory syncytial virus (RSV) has been reported to replicate in cDC in vitro, but in vivo, RSV appears to replicate predominantly in airway epithelial cells (Guerreo-Plata et al., 2006). This suggested that abortive infection and phagocytosis of exogenous antigens are likely to be the major ways for DC to obtain the viral antigens in vivo.

It has been reported that many kinds of viruses can induce the maturation of DC, which is important for the naïve T cell priming ability of these DC. Studies that focused on interaction between viruses and DC have demonstrated that the infection of influenza virus, dengue virus, Sendai virus, murine cytomegalovirus and measles virus can result in the upregulation of costimulatory molecules (Libraty et al., 2001; Lopez et al., 2003; Raftery et al., 2002).

The previous in vitro study done in our lab has shown that paramyxovirus SV5 infection can induce the upregulation of CD40 and CD86 of bone marrow dendritic cells (BMDC) at M.O.I. 10. But the optimal CD80 upregulation correlates with the permissivity of viral infection at a relatively high M.O.I., M.O.I. 50 (Pejawar, Parks, and Alexander-Miller, 2005). This suggests that the expression of CD80 might be controlled by different signals compared to CD40 and CD86 in vitro. Also, the amount of viruses encountered by DC or the permissivity would have impact on the maturation phenotype of different DC subsets.

However, some viruses have been reported to inhibit the maturation of DC. Ebola virus, Marburg virus and Lassa virus can induce productive infection and their infection will inhibit the maturation of DC (Baize et al., 2004; Bosio et al., 1995; Bosio et al., 2003; Pollara et al., 2003). It has been shown that the vaccinia virus will abortively infect human DC and induce apoptotic cell death in vitro (Engelmayer et al., 1999). For the murine cells, the studies done in our lab have shown that vaccinia viral infection were not able to induce the maturation of BMDC in vitro (Yates and Alexander-Miller, 2007) but were capable of upregulating expression of the costimulatory markers CD80 and CD86 in vivo (Yammani et al., 2008). These results suggested that the mechanism of DC maturation in vitro could be distinct from that in vivo.

Respiratory viral infection and DC migration

In the case of respiratory viral infection, DC located in the respiratory tract are essential for the induction of adaptive immune responses against the infection. Upon viral infection, respiratory DC transport viral antigens from the infected respiratory site to the draining lymph nodes as a consequence of maturation (Grayson and Holtzman, 2007). Many studies have reported the migration of DC following respiratory viral infection. Legge et al. reported that intranasal infection of influenza virus could cause the accelerated migration of RDC to the draining peribronchial lymph nodes during the first 24 hours p.i. But those RDC did not undergo further migration in spite of the viral infection were still not cleared in the lung (Legge and Braciale, 2003). Another influenza infection study done by the same group showed that the number of migrant $CD103^+$ DC, peaked on day 3 in the MLN, which was followed by a decrease in that DC population.

This relatively rapid decrease was in contrast with a slower decrease of Gr-1^{+} monocytic DC in the MLN that occured on day 4 p.i. (Kim and Braciale, 2009). A study utilizing intranasal infection model of paramyxoviruses human metapneumovirus (hMPV) and RSV reported that both kinds of paramyxovirus infection could cause a rapid recruitment of lung DC into the MLN (Guerrero-Plata et al., 2009). In this study, the total cDC in the MLN, including the migrating RDC and lymph node resident DC, peaked at day 6 p.i. and decreased afterwards. This peak was identical in hMPV and RSV infection. Another study focused on paramyxovirus Sendai virus infection showed that cDC began to migrate from the lung to the draining lymph nodes within 2 hours after viral infection whereas pDC were recruited into the lung and became the predominant DC population (Grayson et al., 2007). In short, the respiratory infection would cause a rapid but less persistent migration of RDC to the draining lymph node for initiation of the adaptive immune response, while the number of total cDC in the draining lymph node, which includes both the lung migrating DC and lymph node resident DC, could maintain at a high level for a longer period of time.

The recombinant Simian Virus 5 model

In order to obtain a better understanding of infection in the respiratory tract, our lab has developed an intranasal infection model with paramyxovirus Simian Virus 5 in BALB/c mice. Simian virus 5, also called parainfluenza virus 5, belongs to the paramyxovirus family, which includes viruses that cause acute respiratory infection in clinic, for example, human parainfluenza virus, RSV, measles virus and mumps virus, etc.

This close relationship to clinical relevant viruses makes SV5 a valid model virus in the research of respiratory infection.

SV5 is an enveloped negative stranded RNA virus. Its genome is approximately 15kb long, which encodes nucleocapsid protein (NP), phosphoprotein P, V protein, matrix protein M, fusion protein F, small hydrophobic protein SH, hemagglutinin-neuraminidase HN and large protein L (Fig.1.).

SV5 infects several mammalian species. In human, SV5 evades IFN signaling by targeting STAT1 for proteasomal degradation in a STAT2 dependent reaction (Kraus, Garza, and Horvath, 2008). But in mice, SV5 is not able to block the IFN signaling pathway due to the inability of SV5 V protein to bind murine STAT2, which is indispensable for the proteasomal degradation of STAT1 (Kraus, Garza, and Horvath, 2008) .

In this project, we used 10^7 PFU/mouse for SV5 intranasal infection. The recombinant SV5 we used has enhanced green fluorescent protein (eGFP) inserted after HN protein for detection of virus-infected cells. An 18 amino acid long peptide from p60, the protein from Listeria, is inserted after eGFP and expressed as a fusion protein with it. Due to some unknown reasons, the flow cytometry data did not show any eGFP positive cells in the MLN and in the lung following rSV5-eGFP-p60 infection. But through V5 staining of lung sections as well as lymph node sections done in our lab, there did exist some cells positive for the V protein in the lung and the MLN. So, it is possible that the eGFP did not work in vivo due to some unknown reason.

Fig.1. Schematic diagram of recombinant SV5-eGFP-p60 viruse.
The negative single stranded RNA genome of recombinant Simian Virus 5 is depicted as a rectangle with blocks indicating the single genes.18 amino acid long peptide p60 (from *Listeria*) is expressed as fusion protein with enhanced green fluorescent protein (eGFP). This fusion protein is inserted between HN and L. le, leader; tr, trailer.

Fig.1.

MATERIALS AND METHODS

Jingying Zha

Mice and infections

BALB/c mice at the age of 6-8 weeks were purchased from the Charles River research center. The virus we have utilized in the research project is the recombinant SV5 eGFP p60, which is provided by Dr. Parks in our department. This rSV5 has enhanced green fluorescent protein (eGFP) inserted after HN protein for the detection of virus-infected cells (Fig1). An 18 amino acid long peptide from p60, the protein from Listeria, is inserted after eGFP and expressed as fusion protein with eGFP. It works as an epitope for L9.6 p60 TCR transgenic T cells to recognize.

Mice were anesthetized with Avertin (2, 2, 2-tribromoethanol) by intraperitoneal injection. Then, we deliver 10^7 pfu rSV5 per mouse in 50ul of PBS intranasally. We bring the volume up to 50μl to facilitate the virus are delivery into the lung. The mock mice were infected with 50μl PBS.

Tissue sampling

Mediastinal lymph nodes (MLN) were harvested from mock mice and infected mice at different time points post infection: day 1, day 2, day 3 and day 4. We had four mice for the mock group and day 1 p.i. group, and three mice for day 2 to day 4 p.i. groups. The harvested MLNs were pooled in a small petri dish with 5ml digestion mixture. The digestion mixture consisted of RPMI 1640 (Invitrogen Life Technologies), 10% FCS (Sigma), 1mg/ml collagenase D (Roche). After 45 min incubation in 37^{o}C, the MLNs were pushed through a nylon cell strainer to make a single cell suspension. All the red blood cells are removed by treatment with ACK lysis buffer (Sigma).

Cell staining and flow cytometry

The samples were separated into two parts. Surface staining was performed with the following antibodies: allophycocyanine-conjugated anti-CD11c, phycoerythrin-conjugated anti-CD103, and PerCpCy5.5 conjugated anti-CD80, Fluorescein isothiocyanate-conjugated anti-CD8α (all from BD, San Diego, CA) and Pacific Blue conjugated anti-CD86 antibodies (BioLegend, San Diego, CA). For intracellular cytokine staining cells were cultured for 5 hours in the presence of Golgi Plug in RPMI 1640 medium (Invitrogen Life Technologies) with 10% FCS (Sigma), HEPES, gentamicin sulfate (BioWhittaker) and $5x10^{-5}$M 2-ME. After the culture, the cells were stained with allophycocyanine-conjugated anti-CD11c, phycoerythrin-conjugated anti-CD103, fluorescein isothiocyanate-conjugated anti-CD8α and Pacific Blue conjugated anti-CD86 antibodies. Cells were then washed, permeabilized by cytofix/cytoperm and stained with anti-IL-12p40-PerCPCy5.5.

Samples were acquired on a BD Canto II and the data analyzed with BD FACS Diva Software (both from BD, San Diego, CA). The percent of DC expressing high levels of costimulatory molecules or IL-12p40 secretion was determined by gating on the cells that were positive vs the isotype control.

Statistical analysis

A general Student's t-test (two-tailed distribution) was used to compare significance of individual time points post infection. $P \leq 0.05$ was considered statistically significant. Single asterisk * was used to denote a $P \leq 0.05$ and double asterisks ** were used to denote a $P \leq 0.01$.

RESULTS

Jingying Zha

rSV5-eGFP-p60 intranasal infection resulted in the increase of total numbers of DC in the MLN

The mediastinal lymph nodes (MLN) contain distinct populations of DC. In the steady state or upon the respiratory tract infection, pulmonary DC migrate from the lung to the draining lymph node. It was reported by Dr. Braciale and his colleagues that the $CD103^+$ DC accumulated in the MLN and reached the maximum number at day 3 post p.i. following influenza viral infection (Kim and Braciale, 2009). However, the accumulation of lung migrant DC and the lymph node resident DC following paramyxovirus SV5 infection has not been clearly defined yet. We hypothesize that the lung migrant DC would have a considerable influx into the MLN during the early stage: the first two to three days of viral infection, while the number of lymph node resident DC would have a longer and continued increase following infection.

To understand the immigration kinetics of DC subsets in the MLN following rSV5-eGFP-p60 infection, BALB/c mice were infected with viruses or instilled with PBS (mock) intranasally. The MLNs were harvested from mock mice and viral infected mice on day 1, day 2, day 3 and day 4 p.i. Each sample was counted and stained with antibodies to CD11c, CD8 and CD103. CD11c staining helps us identify the dendritic cells, which are CD11c positive. Then we divided the DC into four subsets based on the expression of CD8 and CD103 molecules. Among the four subsets, the two $CD103^+$ DC subsets: $CD8^-$ $CD103^+$ DC and $CD8^+$ $CD103^+$ DC are from the airway while the $CD8^+$ $CD103^-$ DC are lymph node resident. The $CD8^-$ $CD103^-$ DC subset is a mixed population, including $CD103^-$ $CD11b^+$ lung parenchyma DC, $CD8^-$ lymph node resident DC, pDC and monocytic $Gr\text{-}1^+$ DC. This division allows us to study the subsets important for the

naïve $CD8^+$ T cell priming: $CD103^+$ DC (del Rio et al., 2007; Kim and Braciale, 2009) and $CD8^+$ lymph node resident DC (Allan et al., 2003; Allan et al., 2006; Belz et al., 2004a; Belz et al., 2004b). The cell numbers of those four subsets were calculated based on the percentage of each subset and the total cell number from MLN on each day p.i.

The data in Fig. 2., compiled with four experiments, showed the total number of DC from the four subsets of interest present in the MLN following infection. Compared to the mock infected mice, the total cell number of both $CD103^-$ DC subsets and $CD103^+$ DC subsets increased significantly over the course of rSV5-eGFP–p60 infection. Presumably, the increase of $CD103^+$ DC was due to their migration from the peripheral infection site of the respiratory tract to the draining lymph node. This migration process contributed to the significant increase of both $CD103^+$ DC subsets before day 2 p.i. However, after day 2 p.i., the two $CD103^+$ DC subsets differed in the changing patterns of cell numbers. The cell number of $CD8^-$ $CD103^+$ DC subset had a significant decrease from day 2 to day 3 and then remained at a comparable level on day 4. In contrast, the cell number of $CD8^+$ $CD103^+$ DC subset showed an elevation at day 4 compared to day 3. One possible explanation for this observation was that these $CD8^+$ $CD103^+$ DC were newly generated from $CD8^-$ $CD103^+$ DC. It was possible that part of the $CD8^-$ $CD103^+$ DC were triggered by one or more unknown activation signals to express CD8, resulting in a second wave of $CD8^+$ $CD103^+$ DC cell number increasing.

Regardless of the distinct changing trend of $CD8^+$ $CD103^+$ DC and $CD8^-$ $CD103^+$ DC, the total $CD103^+$ DC number decreased from 71720±7922 cells on day 2 p.i. to 53610±3724 cells on day 4 p.i. The decreased cell number could be explained by the death of a group of cells following their maturation. Or alternatively, these DC, which

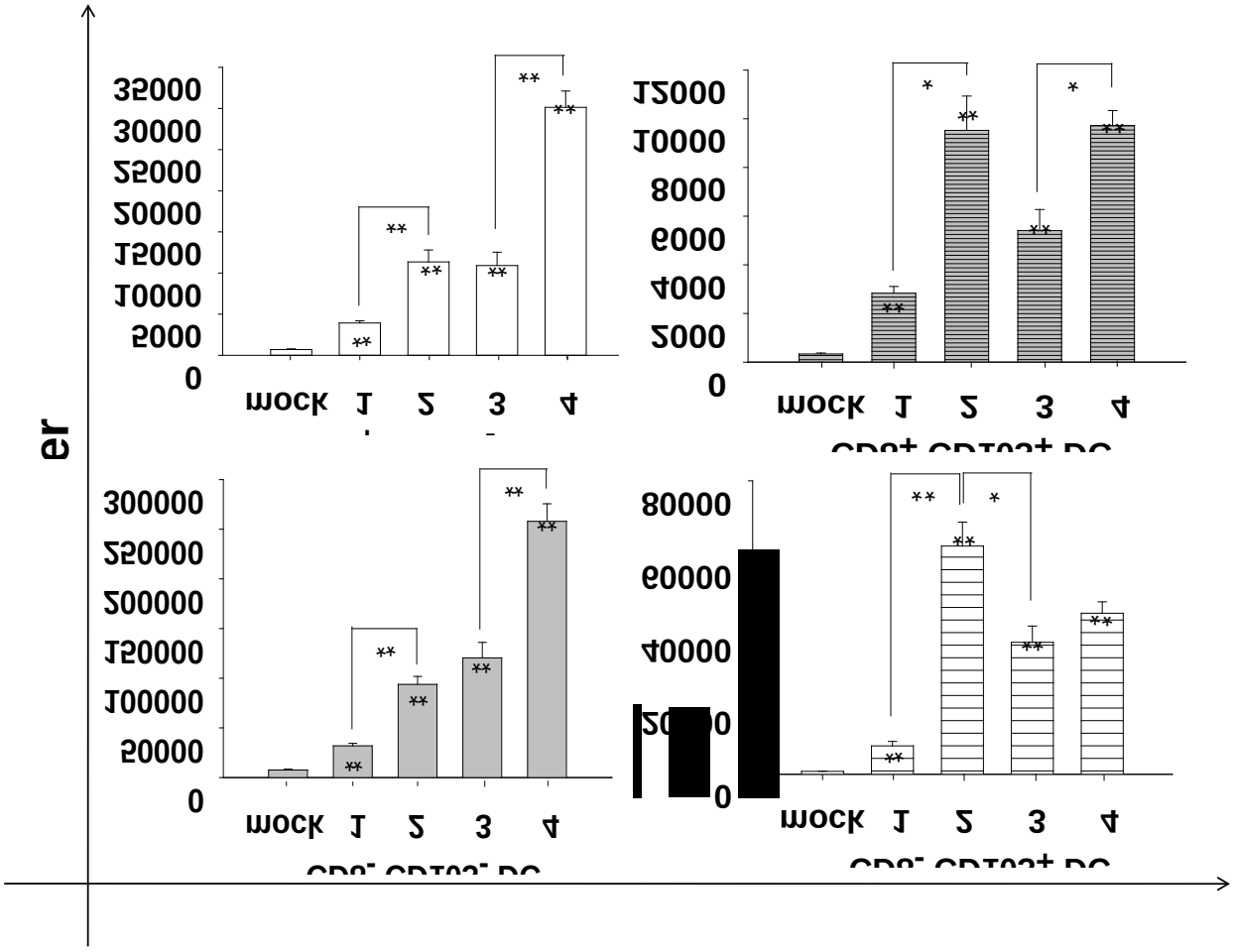
were likely to be antigen-bearing, could be killed by the cognate cytotoxic T lymphocytes (CTL), whose function was observed on day 3 p.i.(Gray, Parks and Alexander-Miller, 2003). Also, the migration from the lung into the lymph node may be suppressed at later time points p.i., resulting in the decreased cell number. The study done by Dr. Braciale and his colleagues reported a similar phenomenon: the respiratory DC were suppressed for further migration after 24 hours in the lung draining PBLN following intranasal influenza infection (Legge and Braciale, 2003).

In contrast to the decreased number of $CD103^+$ DC following day 2 p.i., the cell numbers of both $CD103^-$ DC subsets continued to increase in the days p.i. and were significantly different compared to mock. These $CD103^-$ DC were not from the airway. As shown in the figure, the cell number of both $CD103^-$ DC subsets showed two waves of significant increase ($P \leq 0.01$) following infection. This pattern could be explained by a robust mobilization of DC precursors and monocytes in the bone marrow or the blood, which differentiated into lymph node cDC, pDC and monocytic $Gr\text{-}1^+$ DC. Also, these newly generated DC would proliferate rapidly in response to the inflammatory environment. These two combined factors contributed to the accumulating of $CD103^-$ DC in the lymph nodes.

In short, rSV5-eGFP-p60 intranasal infection caused significant accumulation of both $CD103^+$ DC and $CD103^-$ DC population in the MLN. However, the immigration kinetics of the two populations differed due to distinct origins of the two DC populations.

Fig. 2. The maximum numbers were reached at day 2 p.i. in CD103^{+} DC population while the numbers of CD103^{-} DC continued to increase.
BALB/c mice were instilled with PBS (mock) or with rSV5-eGFP-p60 at the dose of 10^{7} pfu/mouse intranasally. At different days p.i., mice were sacrificed and MLN were harvested. Cells were stained with antibodies to CD8, CD11c and CD103 or the corresponding isotype antibodies (as negative control for flow cytometry). Based on the above surface staining, the cells were classified into four different subsets. The cell numbers of the four subsets were calculated based on the percentage of each subset and the total cell number from MLN on each day post infection. These data are the average of four experiments (±SEM). (All the asterisks represent significant differences compared to mock except those indicated by brackets which compare alternative time points.)
* $P \leq 0.05$, ** $P \leq 0.01$

Fig.2.



The mature DC percentage in each DC population peaked at day 2 p.i., but the mature cell number of CD103$^+$ DC and CD103$^-$ DC showed distinct changing trends

As stated earlier, DC with a relatively high level of costimulatory molecule expression and cytokine secretion are potent naïve T cell primers. To understand the naïve T cell priming ability of different DC subsets, we first evaluated the expression of costimulatory molecules CD80 and CD86. Here we defined CD80 and CD86 double positive DC as "mature DC". We proposed that the CD103$^+$ DC which migrate from the respiratory infection site would have a higher percentage of mature cell than the CD103$^-$ DC.

To evaluate the expression of CD80 and CD86 on DC, we stained all the DC populations with antibodies to CD80 and CD86 and ran them on the flow cytometer Canto. For analysis, we drew quadrants based on the isotype staining of the individual population and divided the whole population into four parts: negative, CD80 single positive, CD86 single positive, as well as CD80 and CD86 double positive. Fig. 3a. showed representative dot plots for the four experiments. The averaged percentages of mature cells were calculated for individual DC subsets (Fig. 3b.). Based on the averaged percentages of mature cells and the total number of each DC subset, the mature cell numbers for individual subset were calculated on each day p.i. (Fig. 3c.).The data was compiled with four experiments.

As shown in Fig. 3a., each DC population were able to upregulate both CD80 and CD86 expression following rSV5-eGFP-p60 infection. Compared to mock, the percentage of the double positive DC population in each subset showed significant increase (Fig. 3b.). In contrast, in each DC subset, neither the CD80 single positive

population nor the CD86 single positive population showed significant increase in percentage following rSV5-eGFP-p60 infection (data not show). This full maturation pattern as upregulating both CD80 and CD86 was also observed on the BMDC of BALB/c mice productively infected by rSV5 infection at M.O.I. 50 in vitro (Pejawar, Parks and Alexander-Miller, 2005). However, this does not necessarily suggest that DC in the MLN were productively infected in vivo. Instead, it is possible that the signaling pathway that triggers the upregulation of costimulatory molecule expression in the complex vivo environment is different from that in vitro.

As denoted in Fig. 3a., both $CD103^+$ DC subsets had much lower percentages of immature cells, which were double negative for CD80 and CD86 expression, compared to the $CD103^-$ DC subsets, following viral infection. This phenomenon is consistent with the different origins of the $CD103^+$ DC and $CD103^-$ DC. $CD103^+$ DC migrate from the lung to the draining MLN in response to respiratory infection (Kim and Braciale, 2009).These migrant $CD103^+$ DC are stimulated by rSV5-eGFP-p60 infection and undergo maturation during migration. In contrast, the lymph node resident DC, including both $CD8^+$ and $CD8^-$ lymph node resident DC, are differentiated from the DC precursors from the circulation (Geissmann et al., 2008). In the inflammatory conditions, the mobilization of these DC precursors and the division of newly generated DC in the lymph node are accelerated. As a result, these “new-born” lymph node resident DC quickly accumulate in the lymph node. Before or shortly after they are exposed to the draining viral antigens or the inflammatory cytokines, they will maintain immature. Therefore, in our experiments, more immature DC were observed in lymph node resident DC subsets

compared to the migrant DC subsets. In short, the different maturation status of $CD103^-$ DC and $CD103^+$ DC reflected their different origins.

As shown in Fig. 3b., the averaged percentages of mature population increased significantly following infection, compared to mock. The percentages of mature DC peaked on day 2 p.i. in all subsets. Among them, the $CD8^-$ $CD103^+$ DC subset had the highest percentage, 67% ± 2.67%, of mature cells on day 2 p.i. Then, this DC subset showed two constituted significant decrease until day 4. This decreasing trend correlated with the data of mature cell number of the same DC subset albeit there was no significant decrease following day 2 p.i., which probably caused by the relatively large error bar on day 2 p.i.(Fig.3c.). This suggested that the mature $CD8^-$ $CD103^+$ DC population might undergo cell death due to post maturation apoptosis.

The other $CD103^+$ population, the $CD8^+$ $CD103^+$ DC also had a relatively high percentage of the mature population, 52% ± 4.27%, on day 2 p.i. Then, there was a significant decrease percentagewise from day 3 to day 4 in the mature population of this subset. However, the mature cell numbers on day 3 (1665±242 cells) and day 4 (1712±345 cells) did not show a decrease (Fig. 3c.). This suggested that there were more immature or partially mature cells appeared in this population on day 4. This could be caused by the CD8 expression of a small group of partially mature or immature $CD103^+$ DC.

The mature population of $CD8^-$ $CD103^-$ DC composed 36% ±2.31% of the whole $CD8^-$ $CD103^-$ DC population on day 2. There was no significant decrease following day 2 and the percentage of mature population kept the same level until day 4. Also, the mature cell number increased continually from day 1 (5670±548 cells) to day 4 (61275±4910

cells) following infection (Fig. 3c.). This kinetics of cell increasing in $CD8^-$ $CD103^-$ DC was very different from any $CD103^+$ DC subsets. It is possible that many immature $CD8^-$ $CD103^-$ DC continued to undergo maturation during the course of infection to replenish the loss of mature cells due to death. These $CD8^-$ $CD103^-$ DC could benefit from other DC populations and underwent maturation continuously as a consequence. The maturation of $CD8^-$ $CD103^-$ DC could be triggered by the cytokines secreted by other DC subsets or viral proteins and viral nuclear acids from the phagocytosed apoptotic DC or the exosomes secreted by other DC (Bosnjak et al., 2005; Fleeton et al., 2004; Maranon et al., 2004; Motta et al., 2001; Popescu et al, 2003; Ronchetti et al.,1999; Thery et al., 2002).

As shown in Fig 3b, $CD8^+$ $CD103^-$ DC had 42% ±3.38% of mature DC on day 2 p.i. and the percentage underwent significant decrease from day 3 to day 4. This decrease in percentage of mature cells from day 3 to day 4, in contrast with the fact that the mature cell number of this population increased in the same time period, from 2,867 ±461cells to 4,997±527 cells (Fig. 3c.), suggested that there was a great influx of immature cells newly differentiated from DC precursors joined this DC subset. As a consequence, the percentage of mature DC decreased.

The number of IL-12p40 secreting $CD103^-$ DC kept increasing while that of $CD103^+$ DC peaked at day 2 following SV5 infection

Besides the costimulatory molecules expressed on the surface of DC, IL-12 is also important for the strong proliferation, development of cytolytic functions and IFN-γ secretion of cytotoxic T cells. It is secreted by mature DC and considered the third signal

Fig. 3. The percentage of mature DC in each population peaked at day 2 post infection while the mature cell number of $CD103^+$ DC and $CD103^-$ DC showed distinct changing trends.

BALB/c mice were inoculated with PBS (mock) or infected with rSV5-eGFP-p60 at the dose of 10^7 pfu/mouse. At different days post infection, mice were sacrificed and MLN were harvested. The MLN cells were stained with antibodies to CD8, CD11c, CD103, CD80 and CD86 or the corresponding isotype antibodies.

Fig. 3a. showed the raw data representative of four experiments. The quadrants were drawn based on the corresponding isotype staining. The numbers on the quadrants denote the percentage of mature DC.

Fig. 3b. showed the averaged percentage of mature DC in each population. The percentages of double positive populations from the four DC subsets are averaged based on four experiments. The two hatched bars denote the two $CD103^+$ DC subsets while the solid bars demonstrate the two $CD103^-$ DC subsets. The data was compiled of four experiments (±SEM). (All the asterisks represent significant differences compared to mock except those indicated by brackets which compare alternative time points.)
* $P \leq 0.05$, ** $P \leq 0.01$.

Fig. 3c. showed the mature cell number in each population. The numbers of mature DC were calculated based on the percentage of mature DC and the total cell number from MLN on each day p.i. The data was compiled of four experiments (±SEM). (All the asterisks represent significant differences compared to mock except those indicated by brackets which compare alternative time points.)* $P \leq 0.05$, ** $P \leq 0.01$.

Fig. 3a.

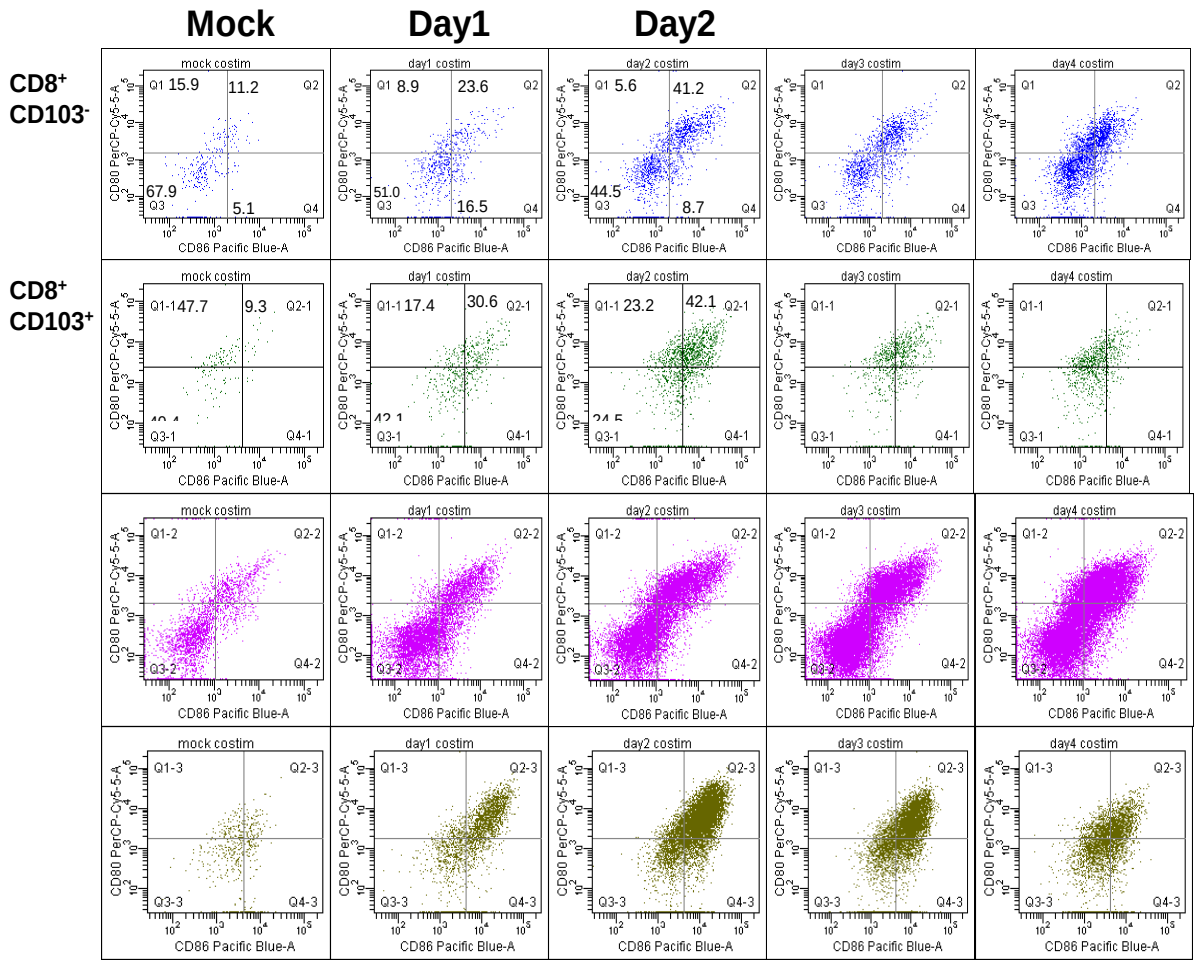

Mock
Day1
Day2
CD8+ CD103-
CD8+ CD103+
mock costim
day1 costim
day2 costim
day3 costim
day4 costim
CD80 PerCP-Cy5-5-A
CD86 Pacific Blue-A
Q1 15.9
11.2
67.9
5.1
Q1 8.9
23.6
51.0
16.5
Q1 5.6
41.2
44.5
8.7
Q1-1 47.7
9.3
Q1-1 17.4
30.6
Q1-1 23.2
42.1

Fig. 3b.

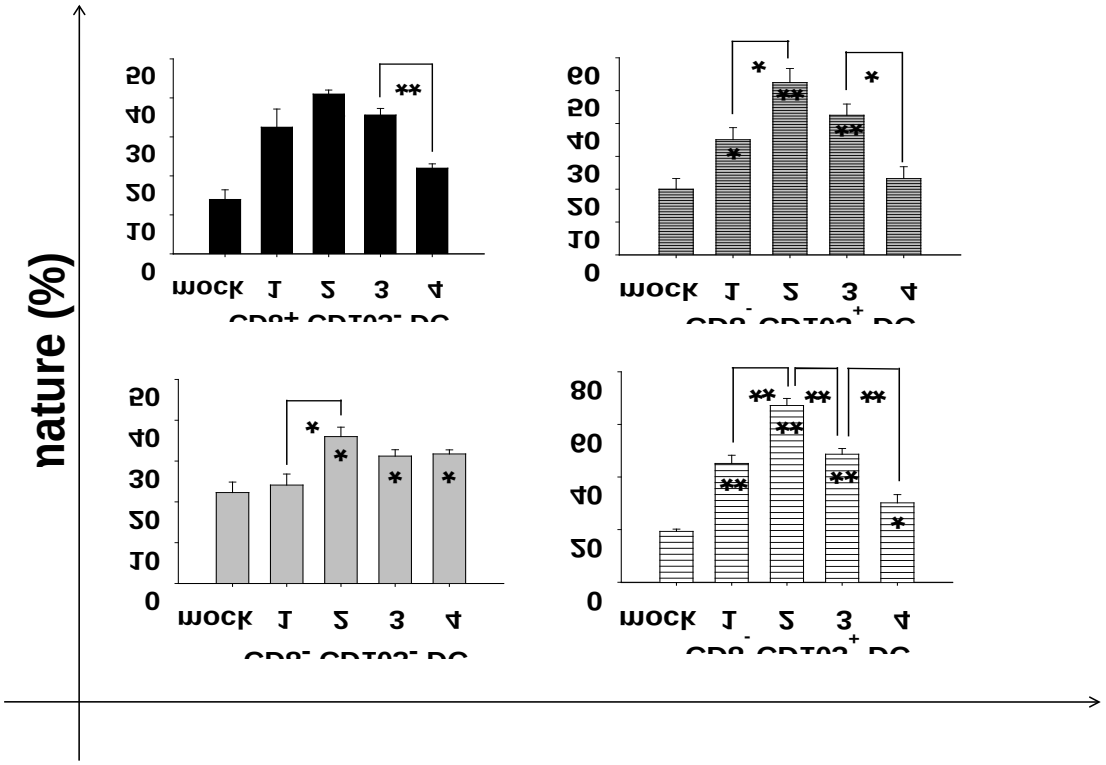
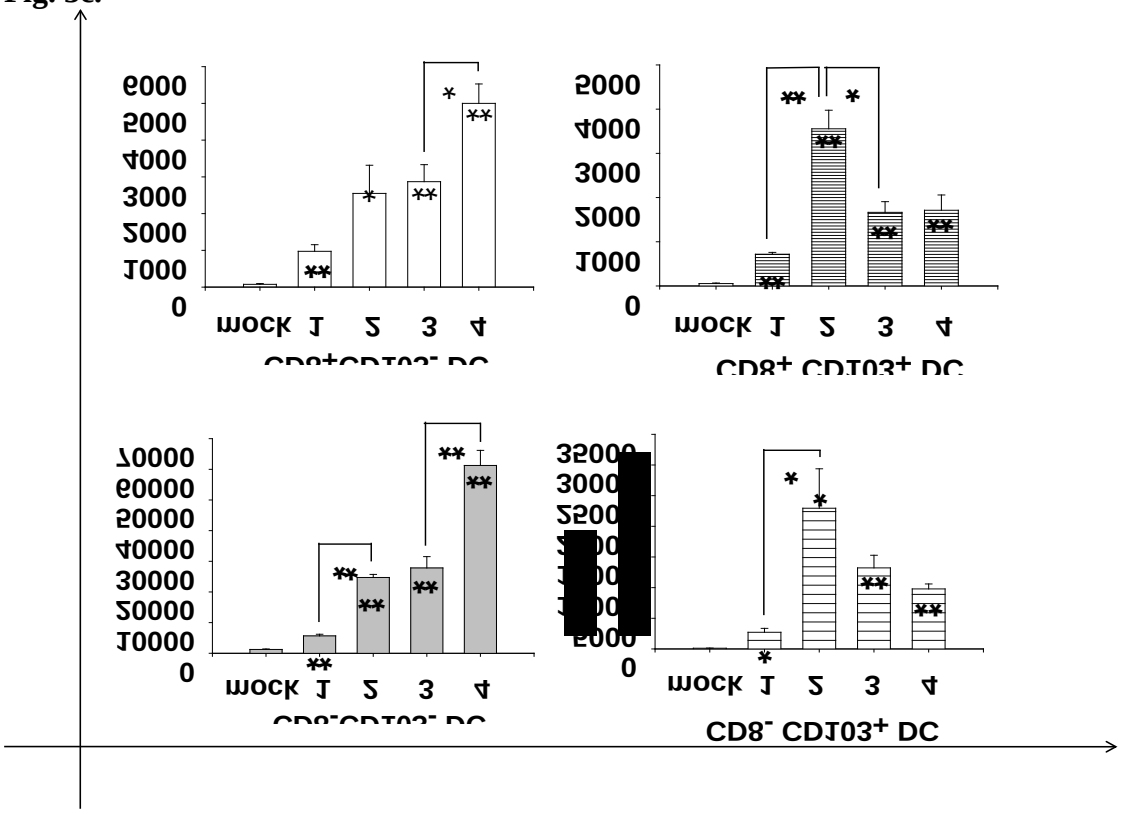

nature (%)
mock
1
2
3
4
CD8+ CD103- DC
CD8- CD103+ DC
CD8- CD103- DC

Fig. 3c.


mock
1
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3
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CD8+ CD103+ DC
CD8- CD103+ DC
CD8- CD103- DC

for naïve T cell priming. The bioactive form of IL-12 is IL-12p70, consisting of two subunits: IL-12p40 and IL-12p35. As aforementioned, the control mechanisms of the expression of the two subunits are different (Schulz et al., 2000) and as a consequence, IL-12p40 is expressed at a relatively higher level compared to IL-12p35. We are interested in studying the numbers of IL-12p40 secreting DC in different DC populations and their changing trends following rSV5-eGFP-p60 infection. Our hypothesis is that following rSV5-eGFP-p60 infection, the changing trends of cell numbers of IL-12p40 secreting DC from migrant DC and lymph node resident DC are consistent with that of mature DC in migrant DC subsets and lymph node resident DC subsets, respectively, and the IL-12p40 secretion at a per cell basis maintains a similar level at mock and each day p.i.

To evaluate the number of IL-12p40 secreting DC in each population, we stained the MLN cells from PBS inoculated mock mice and rSV5-eGFP-p60 infected mice of different days p.i. with antibodies to IL-12p40 via intracellular cytokine staining and ran them on the flow cytometer Canto. For analysis, we drew gates to divide the whole population into two parts: IL-12p40 negative and IL-12p40 positive. All the gates were drawn based on the respective isotype control of each DC subset. Fig.4. showed the cell number of IL-12p40 secreting DC in mock (the negative control), and different days p.i. The data was compiled with four experiments.

As shown in Fig.4., $CD103^+$ DC subsets and $CD103^-$ DC subsets showed different trends of IL-12p40 secreting DC over the course of infection. IL-12p40 secreting $CD103^+$ DC peaked at day 2 p.i. with both $CD103^+$ DC subsets increased significantly from day 1 to day 2. This peak correlated with that of the cell number of mature DC population,

which was consistent with our understanding that IL-12p40 secretion is one of the hallmarks of DC maturation. The amount of IL-12p40 secreted by individual DC is similar among different days p.i. and mock in all the DC subsets. It will be interesting to compare the amount IL-12p40 secreted by individual DC in response to different stimulation, for example, other microbial infection or TLR agonists. It is possible that other stimulation could give rise to a higher amount of IL-12p40 secreted by each DC. Or alternatively, the amount of IL-12p40 secreted in our experiment following rSV5-eGFP p60 infection is the optimal IL-12p40 amount an individual DC can secrete. This would suggest that certain signals available to uninfected BALB/c mice provided stimulation and thus, trigger the optimal IL-12p40 secretion of a small group of DC. We are not sure whether this phenomenon is caused by fungi, mycoplasma or other microbes usually seen in the environment BALB/c mice stay. But based on the isotype control corresponding to IL-12p40 secretion, we do notice that this small group of DC secrete IL-12p40.

In the $CD103^+$ DC population, the cell numbers of IL-12p40 secreting $CD8^-$ $CD103^+$ DC increased approximately 6 fold from day 1 to day 2 p.i., while that of $CD8^+$ $CD103^+$ DC increased only 2 fold at the same time. In comparison with the distinct fold increases in IL-12p40 secretion of the two $CD103^+$ DC subsets, the mature cell numbers of these two populations both increased about 4 fold from day 1 to day 2, thus suggesting that a larger portion of mature $CD8^-$ $CD103^+$ DC were induced to secrete IL-12p40 than the $CD8^+$ $CD103^+$ DC.

Compared to the cell numbers of IL-12p40 secreting $CD103^+$ DC, the cell numbers of $CD103^-$ DC continued to increase from day 1 to day 4 p.i. This trend was also consistent with the increase shown in the mature cell population of $CD103^-$ DC subsets.

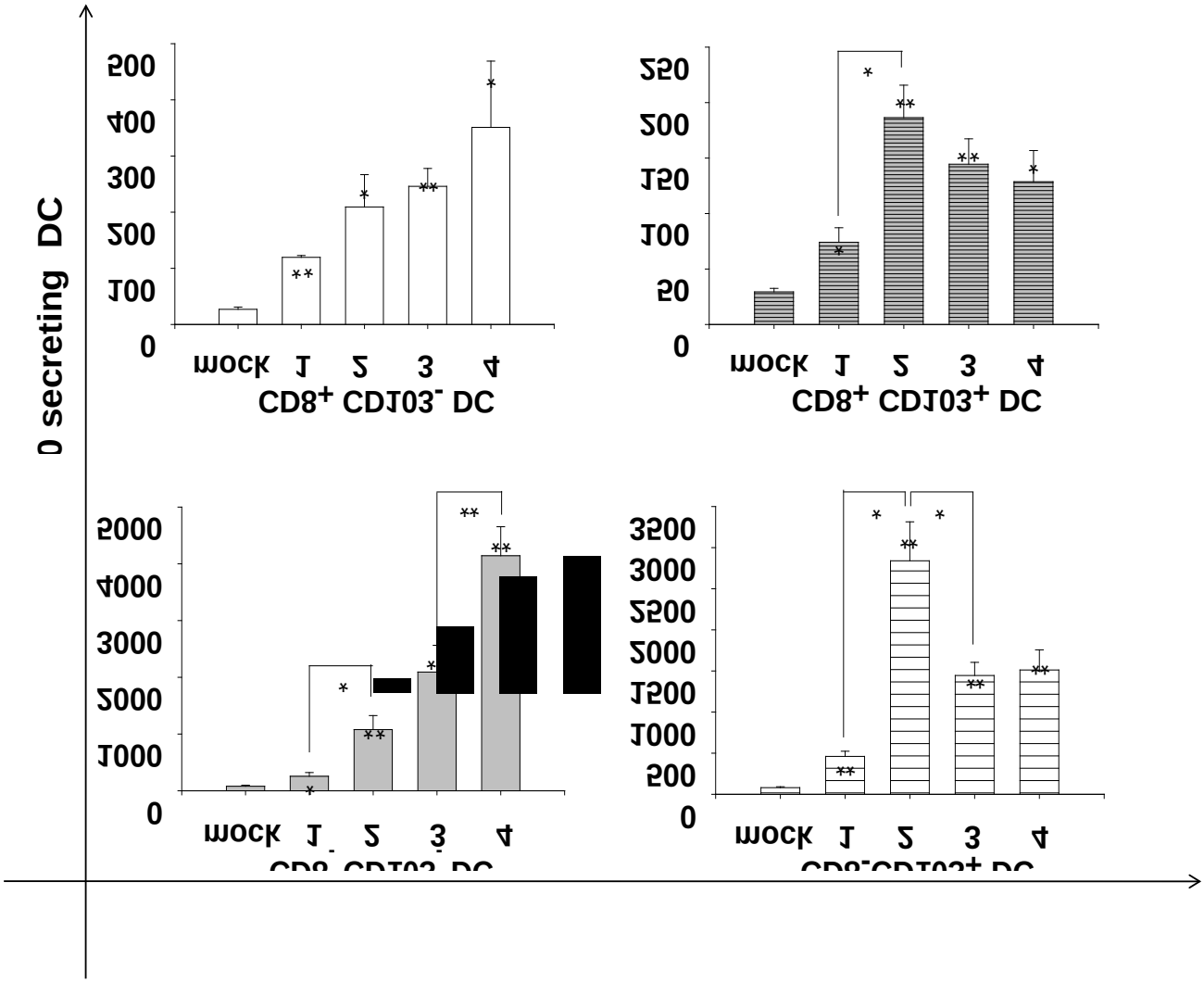
While the $CD8^-$ $CD103^-$ DC exhibited a trend of continual and significant increase ($P<0.01$) from day 1 to day 2 and from day 3 to day 4, the $CD8^+$ $CD103^-$ DC exhibited a trend of increase but without any statistical significance. This lack of significant increase could be explained by the relatively large variance (showed as larger error bars) caused by the limited number of IL-12p40 secreting $CD8^+$ $CD103^-$ DC.

The cell numbers of IL-12p40 secreting DC exhibited significant increase at different days p.i. compared to mock. This increased number of IL-12p40 secreting DC was also reported in DC from spleen following Listeria infection in vivo (Cui et al., 2009). The increased cell number of IL-12p40 secreting DC, along with the similar amount of IL-12p40 secreted by individual DC p.i., would result in an increased concentration of IL-12p40. This potential result is consistent with the increased concentration of IL-12p40 secreted by lung DC following in vitro influenza virus infection (Hao et al., 2008), in vivo herpes simplex virus infection (Elsawa and Bost, 2004), in vivo paramyxovirus human metapneumovirus infection and in vivo respiratory syncytial virus infection (Guerrero-Plata, 2009). But since those studies used ELISA instead of intracellular cytokine staining to measure IL-12p40, neither the IL-12p40 secretion level of individual DC nor the changing trend of IL-12p40 secreting DC number following infection was reported.

Based on the above discussion, the potential model of the regulated IL-12p40 secretion could be that in response to rSV5-eGFP-p60 intranasal infection, the immune system of the BALB/c mice increases the concentration of IL-12p40 in the naïve T cell priming site by upregulating the number of IL-12p40 secreting DC significantly rather than changing the amount of IL-12p40 secretion on a per cell basis.

Fig. 4. The number of IL-12p40 secreting CD103$^-$ DC kept increasing while that of CD103$^+$ DC peaked at day 2 following SV5 infection.
BALB/c mice were instilled with PBS (mock) or infected with SV5 at the dose of 10^7 pfu/ mouse. MLN were harvested from mock infected mice and viral infected mice at day 1, day 2, day 3 and day 4 p.i. The cells from the MLN were cultured in the presence of GolgiPlug for 5 hours. And then, these cells were stained with antibodies to CD8, CD11c, CD103 and IL-12p40 or the corresponding isotype antibodies by intracellular cytokine staining. For analysis, the gates of IL-12p40 positive DC were drawn based on the isotype control of each DC subset. The numbers of IL-12p40 positive DC were calculated based on the percentage of IL-12p40 secreting DC and the total cell number from MLN on each day p.i. The data was compiled by using four experiments (±SEM). (All the asterisks represent significant differences compared to mock except those indicated by brackets which compare alternative time points.) * $P \leq 0.05$, ** $P \leq 0.01$.

Fig.4.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Mock
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CD8+ CD103- DC
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CD8+ CD103+ DC
0
1000
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Mock
1
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4

DISCUSSION AND CONCLUSIONS

Jingying Zha

The immigration kinetics of different DC subsets in the MLN following respiratory rSV5-eGFP-p60 infection

In this study, we divide the DC in the MLN into four subsets based on the expression of CD8 and CD103 molecules. Among the four subsets, the two $CD103^+$ DC subsets are from the airway while the $CD8^+$ $CD103^-$ DC are lymph node resident. The $CD8^-$ $CD103^-$ DC subset is a mixed population including $CD103^-$ $CD11b^+$ lung parenchyma DC, $CD8^-$ lymph node resident DC, pDC and monocytic $Gr\text{-}1^+$ DC. This division allows us to study the subsets important for the naïve $CD8^+$ T cell priming: $CD103^+$ DC (del Rio et al., 2007; Kim and Braciale, 2009) and $CD8^+$ lymph node resident DC (Allan et al., 2003; Allan et al., 2006; Belz et al., 2004a; Belz et al., 2004b).

$CD103^+$ DC, which are from the airway, bring viral antigens to the draining lymph node and are capable of priming naïve $CD8^+$ T cell via direct presentation and cross-presentation (del Rio et al., 2007; Kim and Braciale, 2009). In our study, the $CD103^+$ DC showed a robust increase in total number and mature cell number at day 1 and day 2 following infection. However, following day 2 p.i., the cell numbers of mature $CD103^+$ DC decreased significantly. This rapid loss of mature respiratory DC was also seen by Dr. Braciale and his colleagues in the lung draining peribronchial lymph nodes (PBLN) at 24 hours following influenza viral infection (Legge and Braciale, 2003). Another influenza infection study from the same group showed that the number of $CD103^+$ DC peaked on day 3 p.i. in the MLN, after which a decrease in this DC population occurred. But the immigration kinetics of mature DC into the MLN was not clearly identified in this study (Kim and Braciale, 2009).

The absence of continuous accumulation of mature $CD103^+$ DC could be explained by a suppressed immigration of airway-derived cells into the MLN following the initial respiratory DC influx, and the cell death of mature DC. The suppressed immigration of tissue DC has also been reported in the gut mesenteric lymph nodes following TLR agonist exposure and could be reversed by blocking IL-10 function (Monteleone et al., 2008). This suggested that the suppressed migration of tissue DC to the draining lymph node could be caused by anti-inflammatory cytokines in the peripheral tissue following microbial stimulation to keep a homeostasis of DC.

The idea of a limited life span of mature DC in the lymph node is consistent with the current information of activated mature DC (Banchereau et al., 2000) but lacks experimental verification. It is possible that the migrant DC undergo apoptotic death shortly after their arrival in the draining lymph node. These dying DC could provide a source of viral antigens when they are phagocytosed by immature lymph node resident DC (Fleeton et al., 2004; Maranon et al., 2004; Popescu et al., 2003; Ronchetti et al., 1999).

Contrasting with the relatively early peak of mature cell number in $CD103^+$ DC subsets, the mature $CD103^-$ DC continued to increase from day 1 p.i to day 4 p.i. (Fig.3a.). Among the $CD103^-$ DC, the $CD8^+$ $CD103^-$ DC are the DC subset superior at naïve T cell priming (Allan et al., 2003; Allan et al., 2006; Belz et al., 2004a; Belz et al., 2004b). So the significant increase of the mature $CD8^+$ lymph node resident DC is of great importance to the naïve T cell priming. As shown in Fig. 3b. and Fig. 3c., the mature $CD8^+$ $CD103^-$ DC percentage decreased from day 3 to day 4 while the mature cell number of that subset increased significantly. This suggested that there were more

immature $CD8^+$ $CD103^-$ DC present in the MLN, resulting in the decreased mature $CD8^+$ $CD103^-$ DC percentage. The kinetics of the $CD8^-$ $CD103^-$ DC number was very similar. It was likely that this great increase of immature $CD103^-$ cells were caused by an accelerated recruitment of DC precursors from the circulation. This mobilization and accumulation of DC from the blood was also observed in the lung draining PBLN as well as the MLN following influenza viral infection (Legge and Braciale, 2003; Kim and Braciale, 2009). It was noteworthy that the robust increase of $CD103^-$ DC on day 3 p.i. correlated with a significant increase of the mature $CD103^-$ DC on that same date, following the peak of the $CD103^+$ DC on day 2 p.i. (Fig 3.c.). This phenomenon suggested that it is possible that the mature lung migrant DC could release inflammatory factors, along with the viral products available via the conduit system, to induce the maturation of the newly derived DC from the precursors. Although the migrant DC stopped to accumulate in the MLN and their number decreased rapidly, their presence in the MLN has induced a large wave of lymph node resident DC increase as well as their subsequent maturation. Therefore, the stop of accumulation of migrant DC in the MLN will not be the end of naïve T cell priming. These DC will continue the initiation of adaptive immunity until the anti-viral specific response is well-founded. Like other steps in the immune response, this elegant mechanism of maturation induction between DC subsets is well-developed in the inflammatory environment.

The expression of CD8 molecules on CD103 DC

From our experiment, we observed the CD8 expression on some of the CD103 DC. This CD8 expression was also observed by Dr. Braciale and his colleagues in the

MLN following influenza viral infection. They, as well as other groups in the field, tended to interpret this phenomenon as the trogocytosis of CD8 molecules of CD103 DC from T cells in the MLN (Joly and Hudrisier, 2003; Kim and Braciale, 2009; Moron et al., 2002). However, Anjuere et al. reported that the CD8 expression of langerhans cells could be induced by CD40 ligand stimulation (Anjuere et al., 1999; Anjuere et al., 2000). Another study focused on lung DC population reported the CD8 expression of lung DC after their arrival in the draining lymph node (Vermaelen et al., 2001). We also consider that the CD8 expression of CD103 DC is likely to be a real phenomenon resulted from the interaction with the cell populations in the lymph node. As shown in our experiment, the total number of $CD8^+$ $CD103^+$ DC increased from day 3 p.i. to day 4 p.i.(Fig. 2.) but the number of mature $CD8^+$ $CD103^+$ as well as the number of IL-12p40 secreting DC maintained at the same level (Fig. 3c., Fig.4.). These data suggested that in the case of rSV5-eGFP-p60 intranasal infection, the CD8 expression in CD103 DC was not correlated with either the upregulation of both CD80 and CD86 or the IL-12p40 secretion. It could be that the CD8 expression of partially mature $CD103^+$ DC, which did not contribute to the mature cell number or IL-12p40 secreting cell number, was upregulated in response to certain stimulation during or after their migrating to the MLN. But at this point, we still do not fully understand what the CD8 expression in $CD103^+$ DC means and how $CD8^+$ $CD103^+$ DC are different from their $CD8^-$ $CD103^+$ counterparts.

Mobilization of DC precursors and the subsequent DC division are the major cause of the robust increase of lymph node resident DC

As shown in our experiment, $CD103^-$ DC had a robust increase following rSV5-eGFP-p60 infection. Except for the $CD11b^+$ $CD103^-$ $CD8^-$ DC, most DC in $CD103^-$ DC population are the lymph node resident DC derived from the precursor in the blood. The robust increase of $CD103^-$ DC population is predominantly due to the mobilization of DC precursors and the subsequent DC division in the lymph node.

Conventional DC subsets, including $CD8^+$ lymph node resident DC and $CD8^-$ $CD11b^+$ lymph node resident DC, are derived from their precursor pre-cDC, which arise and expand in the bone marrow (Geissmann et al., 2008; Liu et al., 2009). They differentiate into cDC in lymphoid tissues and undergo cell division in both steady state and the inflammatory situations (Diao et al., 2006; Liu et al., 2009). The increased cell number of $CD8^+$ lymph node resident DC observed in our experiment suggested that the cell division of cDC could be accelerated in response to the respiratory viral infection, possibly by enhancing the Flt3 receptor signaling (Waskow et al., 2008). In our experiment, it was likely that this accelerated division of cDC contributed to the increase of $CD8^-$ $CD103^-$ DC subset by $CD8^-$ $CD11b^+$ lymph node resident DC. Plasmacytoid DC in the lymph node are derived from CDP (Geissmann et al., 2008), which also serve as progenitors for pre-cDC. Therefore, pDC and pre-cDC share the same progenitor CDP. Monocytic $Gr\text{-}1^+$ DC in the lymph node are derived from the blood monocytes migrate from the bone marrow to the circulation. This process usually occurs in response to microbial invasion(Geissmann et al., 2008). The accumulation of monocytic $Gr\text{-}1^+$ DC

was reported by Dr. Braciale and his colleagues in the MLN following influenza intranasal infection (Kim and Braciale, 2009).

In conclusion, monocytic DC, pDC and cDC are derived from different corresponding precursors although they share the same early progenitors: myeloid progenitors (MP). In response to viral infection, the mobilization of the progenitors of these three kinds of DC in the bone-marrow or the blood stream, along with the accelerated cell division of these DC in the lymph node, is the major factor that contributes to the robust increasing of non-migrating DC in the draining lymph node.

Comparison between in vitro and in vivo viral infection models of DC maturation

The maturation of dendritic cells is a milestone in the process of microbial infection, which indicates the activation of innate immunity and the subsequent initiation of adaptive immune response. With the importance of this event being fully recognized, many studies have been focused on the regulation of DC maturation. There are a large number of in vitro reports but a relatively limited number of in vivo studies regarding the maturation of DC in response to viral infection. It has been widely accepted that in vivo study could provide very different answer compared to the in vitro study regarding the same kind of viral infection. For example, the study done by Dr. Alexander-Miller and her colleagues that showed DC underwent maturation following vaccinia virus infection in vivo(Yammani et al., 2008) was strikingly different from the observation that BMDC were not able to induce upregulation of costimulatory molecules following vaccinia virus infection in vitro (Yates and Alexander-Miller, 2007). The distinct results from the two

types of research models indicate that these two models are different in several aspects, as discussed below.

First of all, the in vitro model could not provide the complex biological environment, which includes various cell populations and the soluble factors secreted by them, for example, IFN-γ from nature killer cells, which can help trigger DC maturation (Della Chiesa et al., 2005). We can address the difference using the example of viral infection models. In the culture dish, the DC solely interact with viruses and as a consequence, their maturation is the result of the stimulation from viral products as the PRR agonists or from viral replication which can trigger maturation via certain signaling pathways (Pejawar, Parks and Alexander-Miller, 2005). On the contrary, the DC maturation in vivo following viral infection could be a combined result from multiple factors, which, in addition to the stimulation from viral products and viral replication, include the stimulation from the contact with other cell populations, such as the contact with antigen-specific $CD4^+$ T cells via CD40-CD40L binding (Sporri and Reis e Sousa, 2003), and the paracrine cytokines, e.g. type I IFN, from those cells in the surroundings (Borderia et al., 2008; Piqueras et al., 2006; Pollara et al., 2004).

Secondly, the in vivo study allows the analysis of different DC subsets while some in vitro culture can only provide us with the information from certain DC subsets, for example, the BMDC generated from GM-CSF differentiation culture. In some in vitro researches, this shortage is overcome by using the cell sorting technique. For example, Dr. Braciale and his colleagues sorted the lung DC into different subsets and studied their characteristics in vitro following influenza virus infection (Legge and Braciale, 2003).

Additionally, in vivo models can provide us with the immigration kinetics of

individual DC subset following viral infection. It is important to know the presence of DC in the local draining lymph node, where the naïve T cell priming occurs, following infection. This information will help us understand the role played by each DC subset in this priming process and further, the potential interaction between different DC subsets. In contrast, the in vitro studies are stationary and cannot provide us with the above information.

Addressing the maturation of lung migrant DC and lymph node resident DC in MLN following intranasal infection of paramyxovirus rSV5-eGFP-p60 in BALB/c mice, we observed a pattern of full maturation, defined as the upregulation of both CD80 and CD86, in both the migrant DC subsets and lymph node resident DC subsets (Fig.3a.). The in vitro study done in our lab reported that the BMDC of BALB/c mice were induced full maturation when productively infected at a relatively high M.O.I: M.O.I 50 (Pejawar, Parks and Alexander-Miller, 2005). This study also showed that BMDC of BALB/c mice were partially matured, defined as the upregulation of CD40 and CD86 but not CD80, when abortively infected at a lower M.O.I.: M.O.I. 10. This suggested that the expression of viral proteins and/or the production of viral progeny within the cell were required for BMDC to upregulate CD80 expression in vitro. However, the correlation between CD80 upregulation and viral replication in DC does not necessarily exist or work as the predominant factor for DC maturation in vivo.

As we discussed in the introduction part, in contrast to the productive infection of DC caused by some viral infection in vitro (Fugier-Vivier et al., 1997; Pejawar, Parks, and Alexander-Miller, 2005), the abortive infection and phagocytosis of exogenous antigens are likely to be the major ways for DC to obtain the viral antigens in vivo

(Grayson and Holtzman, 2007; Guerreo-Plata et al., 2006; Tortorella et al., 2000). In our study, the full maturation was shown in both migrant DC from the lung and the lymph node resident DC. The former DC subsets from the peripheral infection site could have the chance to interact with viruses. The latter DC subsets have less accessibility to the rSV5-eGFP-p60 infection because the size of rSV5-eGFP-p60 prevents itself to go through the conduit system and infect the DC in the T cell zone. Also, the mild infection in BALB/c mice caused by rSV5-eGFP-p60 intranasal infection was highly unlikely to cause a bloodstream infection. So the rSV5-eGFP-p60 viral particles were unlikely to enter in the MLN through HEVs and consequently, directly infect the lymph node resident DC. But it has been shown by Dr. Bennink and Dr. Yewdell's research that cells residing beneath the subcapsular sinus of the draining inguinal lymph node could be infected by vesicular stomatitis virus and vaccinia virus following subcutaneous infection (Hickman et al., 2008), thus suggesting that some lymph node resident DC could be infected during the infection. We know there are possibilities for both lung migrant DC and lymph node resident DC subsets to interact with rSV5-eGFP-p60, but we have no evidence to demonstrate either the M.O.I. was close to 50 in vivo or there were any progeny viruses produced in DC during the infection.

Due to the less possibility for DC in the MLN to be productively infected, it is likely that the full maturation pattern showed in our study could be resulted from reasons other than viral replication, which is the cause of the upregulation of both CD80 and CD86 expression of BMDC in vitro. The potential factors for triggering of DC full maturation in our study will be discussed in the following paragraphs.

Inflammatory cytokines from the viral infected DC induce maturation of the bystander DC

Upon viral infection, infected DC release a broad array of soluble factors, which play important roles in stimulating a potent immune response in the infection site as well as in the draining lymph node (Piqueras et al., 2006). Among these soluble factors, the pro-inflammatory cytokines contribute to the activation of not only naïve T cells but the bystander uninfected DC as well. The latter activation events have been described as bystander maturation of uninfected DC in many infection models (Borderia et al., 2008; Dejnirattisai et al., 2008; Nightingale et al., 2008; Pollara et al., 2004).

In our study, it was likely that some lymph node resident DC underwent bystander maturation. As aforementioned, the chances for lymph node resident DC to be infected by rSV5-eGFP-p60 were limited. Despite of that, the mature cells of lymph node resident DC subsets did increase significantly following infection. My hypothesis is that the maturation of the lymph node resident DC was closely related to the migrant DC, some of which were abortively infected by rSV5-eGFP-p60.

In a herpes simplex virus infection model, type I IFN secreted by infected DC was reported to induce the maturation of bystander uninfected DC as a paracrine activator (Pollara et al., 2004). Another study, using a type of paramyxovirus, Newcastle disease virus, indicated that bystander maturation could be triggered by type I IFN and other cytokines secreted by infected DC in a paracrine signaling pathway (Borderia et al., 2008). In this study, the matured bystander DC, described as “antiviral-activated DC,” were shown to express elevated level of antiviral genes, including RIG-I, PKR and OAS. This changed gene expression profile suggested that their resistance to viral infection was

largely enhanced compared to immature DC (Borderia et al., 2008). Researches using dengue virus infection showed that the paracrine TNF-α and type I IFN were responsible for the maturation of bystander DC and infected DC had distinct cytokine production compared to bystander uninfected DC (Dejnirattisai et al., 2008; Nightingale et al., 2008).

By sensing the inflammatory cytokines released by the infected DC, which serve as the danger signals, bystander DC undergo maturation and as a consequence, they become better naïve T cell primers and are more resistant to viral infection (Borderia et al., 2008; Salio et al., 1999; Yates and Alexander-Miller, 2007). Therefore, this bystander DC maturation phenomenon is an important anti-viral response of the host during the infection.

Antigen transfer from the migrant DC to the lymph node resident DC could potentially stimulate the maturation of the latter population

Different subsets of DC have been identified in the draining lymph nodes. Those DC could be divided into two groups: the lymph node resident DC and the migrant DC from the peripheral tissue. Upon infection, the migration of tissue DC is accelerated (Legge and Braciale, 2003). They undergo maturation and migrate to the lymph node in response to microbial invasion (Banchereau et al., 2000). Their presence in the draining lymph node provides antigens for the priming of antigen-specific adaptive immune cells, which is considered the initiation of adaptive immunity. However, compared to this classical model of antigen-presenting, the real situation in the draining lymph nodes is more complex.

Many studies reported the interaction between migrant DC and lymph node resident DC. Belz et al. showed that the airway-derived $CD8^-$ $CD11b^-$ DC could act as a

source of antigen for $CD8^+$ lymph node resident DC following the influenza infection. In that study, both airway-derived $CD8^-$ $CD11b^-$ DC subset and distinct $CD8^+$ lymph node resident DC were capable of presenting MHC-I restricted antigens following lung infection of the influenza virus and the Herpes Simplex Virus (HSV) (Belz et al., 2004b). This would suggest that the airway-derived DC provide the lymph node resident DC with antigens from the infection site through interaction between the two groups of DC. Allan et al., using the HSV cutaneous infection model, showed that blocking skin-DC migration could inhibit HSV-specific CTL priming in the skin draining lymph node (Allan et al., 2006). This study suggested the possibility that the lymph node resident DC relied on the migrant DC to gain their ability to prime naïve T cells if they were the population to do that. This is consistent with the study done by Dr. Braciale and his colleagues which showed that, in CCR7-deficient mice, the proliferation of the transferred CCR7-component $CD8^+$ CL-4 T cells specific for influenza HA in the MLN was largely reduced following the influenza infection (Kim and Braciale, 2009). The studies discussed above showed the interaction between different subsets of DC in the draining lymph node, suggesting that the migrant DC might play an indispensable role to assist lymph node resident DC to gain their capability for naïve T cell priming.

To explain the phenomenon mentioned above, an “inter-DC antigen transfer” model (Allan et al., 2006) was proposed indicating the possibility that the migrant DC serve as a source of antigens for the naïve T cell priming, albeit the mechanism for this model is still under debate. Thery et al. reported that DC-derived exosomes could act as a source of antigens for stimulating antigen-specific naïve T cells by other DC in vitro (Thery et al., 2002). Dr. Cunningham and colleagues reported that uninfected bystander

DC could phagocytose HSV-infected apoptotic DC and consequently, stimulate antigen-specific T cell activation via cross-presentation in vitro (Bosnjak et al., 2005). Similar results were reported by Dr. Kourilsky and his colleagues in a recombinant canarypox virus infection model (Motta et al., 2001). These studies are consistent with many other researches which demonstrated that DC are capable of deriving antigens from apoptosed cells both in vitro, ex vivo and in vivo (Fleeton et al., 2004; Maranon et al., 2004; Popescu et al., 2003; Ronchetti et al.,1999).

However, it is less likely that the passing of microbial molecules between different DC subsets would happen without the activation of the recipient DC. My hypothesis is that by phagocytosing the exosomes or the apoptosed infected DC, the lymph node resident DC could obtain the foreign antigens, which, in return, activate the DC via interacting with the PRR, such as toll-like receptors, RIG-I and MDA5. This activation process would trigger the maturation of the recipient DC by elevating the expression level of costimulatory molecules and MHC molecules as well as the secretion level of cytokines.

Besides the bystander maturation by paracrine inflammatory cytokines as discussed above, the maturation triggered by antigen exposure could also provide a reasonable explanation for the maturation of lymph node resident DC in our study. The rapid loss of mature migrant DC from day 2 to day 3 p.i. could be caused by cell apoptosis (Fig. 3c.). This decrease in $CD103^+$ DC was followed by the significant increase of the two $CD103^-$ DC populations from day 3 to day 4 (Fig. 3c.). This kinetics is consistent with the hypothesis that the phagocytosis of the apoptotic migrant DC could result in the exposure to viral antigens and the subsequent maturation.

While the secreted inflammatory cytokines can influence a relatively larger number of DC in the local environment, the maturation resulted from exposure to transferred antigens is limited to a small number of the recipient DC surrounding that donor DC. But the two stimuli for maturation induction stimulation are not mutually exclusive. Furthermore, the combination of the two stimuli would synergistically contribute to a much stronger activation of the uninfected DC, which leads to longer duration of maturation status (Bajenoff et al., 2007).

Model: The direct or indirect interaction with migrant DC contributes to the maturation of the lymph node resident DC

Like other steps of the immune response, the initiation of adaptive immunity is a well controlled process. One typical example is the co-localization of DC and naïve T cells in the draining lymph node, which is well regulated by the chemokines and cytokines in the inflammatory environment. Similarly, the initiation of the priming process is also delicately regulated. The maturation of dendritic cells, which determines whether these antigen-presenting cells are “licensed” to prime naïve T cells, is a response to combined signals. Many studies focused on DC maturation have revealed their stimulated maturation in response to the microbial invasion (Libraty et al., 2001; Lopez et al., 2003; Raftery et al., 2002). In addition to their sensing of the potential enemies, the cross-talk and collaboration with other cell populations, which build up the environment, are also important for this orchestrated campaign. These cross-talks include not only the interaction with other immunological cells (e.g. nature killer cells) or non-immunological populations (e.g. epithelial cells), but also the interaction between different DC subsets.

It has been reported by many groups that the migrant DC are essential for the initiation of adaptive immune response. As we mentioned above, the study done by Dr. Braciale and his colleagues utilized CCR7-deficient mice and observed a largely reduced naïve T cell proliferation following influenza infection (Kim and Braciale, 2009). They also demonstrated the absence of the proliferation of CL-4 $CD8^+$ T cell after the elimination of lung dendritic cells in DTR mice model following diphtheria toxin intranasal treatment. These results suggested that the absence of migrant DC in the MLN following respiratory infection would result in impaired naïve T cell proliferative expansion. The authors interpreted this phenomenon in the way that the migrant DC worked as a main source of antigens for the naïve T cell priming. However, it is also possible that the migrant DC can trigger the full maturation of lymph node resident DC, which is required for naïve T cell priming. Therefore, without the presence of migrant DC in the lymph node, very limited DC are fully matured in the MLN and could not initiate a naïve T cell priming in spite of the presence of the viral antigens draining through the lymph.

In our study, the cell numbers of mature $CD103^+$ DC of both subsets increased dramatically from day 1 to day 2 p.i. and peaked on day 2, but followed by a considerable decrease. As we mentioned above, other studies also reported the lost accumulation of DC from the respiratory tract in the draining lymph node. It has been reported that following i.n. influenza infection, migration of mature DC from the respiratory tract into the peribronchial lymph node only occurred in the first 24 hour p.i. The respiratory DC did not undergo further migration in spite of the viral infection in the lung (Legge and Braciale, 2003). Another influenza infection study from the same group showed that the

number of $CD103^+$ DC peaked on day 3 p.i. in the MLN, after which a decrease in this DC population occurred. But the immigration kinetics of mature DC into the MLN were not clearly identified in this study (Kim and Braciale, 2009). The absence of continuous accumulation of mature $CD103^+$ DC could be explained by a suppressed immigration of airway-derived cells into the MLN following the initial cell influx and the cell death of the mature DC. The idea of a limited life span of mature DC in the lymph node is consistent with current information of activated mature DC (Banchereau et al, 2000) but lacks experimental verification. Contrasting with the relatively early peak of mature cell number in $CD103^+$ DC subsets, the mature $CD103^-$ DC continued to increase until day 4 p.i. (Fig.3a.). Among the $CD103^-$ DC, the $CD8^+$ $CD103^-$ DC are the DC subset superior at naïve T cell priming (Allan et al., 2003; Allan et al., 2006; Belz et al., 2004a; Belz et al., 2004b). Therefore, the significant increase of the mature $CD8^+$ lymph node resident DC is of great importance to the naïve T cell priming. And it is possible that without the migrant DC and their stimulus, the maturation of $CD8^+$ lymph node resident DC would be very restricted, resulting in the impaired naïve $CD8^+$ T cell proliferative expansion.

Further, a full maturation pattern, the upregulation of both CD80 and CD86, was observed in all the DC subsets in the research following rSV5-eGFP-p60 infection. As mentioned earlier, it is not likely that this full maturation is caused by the replication of progeny viruses in the DC in vivo. While the migrant DC could be fully matured in response to the inflammatory conditions in the infection site, i.e. the high level of inflammatory cytokines, viral products, the surrounded apoptotic infected epithelia or the direct infection, it is less likely that the full maturation of the lymph node resident DC

could be solely attributed by their exposure to the viral products draining through the lymph in the MLN. There must be some other factors contributing to their full maturation.

It is very likely that the inflammatory environment induced by viral infection is responsible for the stimulation for full maturation of DC. In the respiratory infection site, the epithelia of the airway-mucosa are exposed to viruses and productively infected (Guerreo-Plata et al., 2006). Along with other permissively infected cells, they express considerable amount of inflammatory cytokines, such as type I IFN, in response to viral infection. Further, the potential cell death caused by viral infection and the released factors from these dying cells adds to the inflammatory environment of the airway. Thus, the inflammatory environment in the respiratory tract is presumably the major contributor to the full maturation of the migrant DC. Compared to the respiratory infection site, as we have discussed above, the lymph node resident DC in the draining MLN have less chances to be exposed to the virions. Except the cells which reside in the subcapsular sinus of the lymph node (Hickman et al., 2008), other cells are less likely to be directly infected.

Here we propose the model that the migrant DC from the peripheral infection site are the major contributor for the full maturation of lymph node resident DC following rSV5-eGFP-p60 infection (Fig.5). As we discussed above, the migrant $CD103^+$ DC induce the maturation of the lymph node resident DC in two ways: direct interaction, which includes passing antigens via exosomes to the recipient lymph node resident DC (Thery et al., 2002) or the apoptosed infected DC being phagocytosed by the recipient lymph node resident DC (Bosnjak et al., 2005; Motta et al., 2001) ; indirect interaction, which involves the stimulation from the array of inflammatory cytokines secreted by

migrant DC to the recipient DC (Borderia et al., 2008; Dejnirattisai et al., 2008; Nightingale et al., 2008; Pollara et al., 2004). These two ways of induction of maturation are not mutually exclusive. The situation that the indirect interaction works solely on the lymph node resident DC is called bystander maturation. But based on the information we know about the structure of the lymph node, it is more likely that the maturation of lymph node resident DC is the synergistic effect of the stimulation from the direct and indirect interaction with migrant DC as well as the interaction with the viral antigens draining through the conduit system, due to the possibility of the co-localization of the three events in the T cell zone.

Conclusions

In this study, by using the intranasal infection model of paramyxovirus rSV5-eGFP-p60 in BALB/c mice, we have obtained much information about the maturation pattern and the immigration kinetics of lung migrant DC and lymph node resident DC following paramyxovirus respiratory infection. We observed a pattern of full maturation, which indicated the upregulation of both CD80 and CD86, in both lung migrant DC and lymph node resident DC. Interestingly, this maturation pattern was also observed in the BMDC, in response to productive infection caused by rSV5 at M.O.I of 50 in vitro. In that model, it was proposed that the signaling pathway triggered by the replication of viral progenies was responsible for the induction of full maturation of BMDC.

However, based on the information of in vivo respiratory infection of paramyxovirus, it is not very likely that the productive infection should be responsible for the full maturation of DC upon respiratory tract infection. Instead, we considered that the

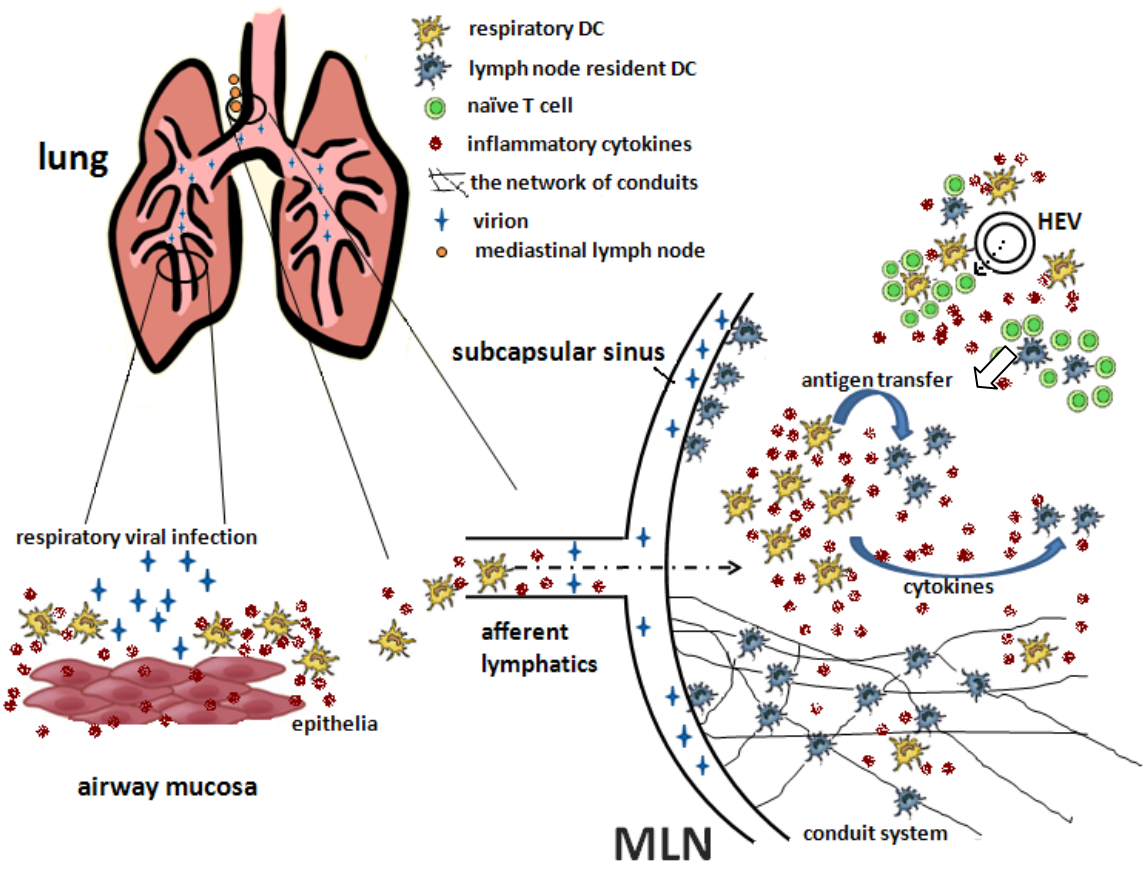
complex vivo environment which includes various cell population and soluble factors contributes to the full maturation of both lung migrant DC and lymph node resident DC.

Fig. 5. Model depicting the direct or indirect interaction with migrant DC contributes to the maturation of lymph node resident DC following respiratory viral infection.

Following respiratory viral infection, respiratory DC migrating from the airway mucosa to the draining MLN through afferent lymphatics. They can induce the maturation of the lymph node resident DC via direct interaction, which involves in antigen transfer and cytokine stimulation, or indirect interaction by their secreted cytokine in the environment. Also, the interaction with viruses restricted in the subcapsular sinus or sensing the viral products draining through the conduit system* could facilitate the maturation process of lymph node resident DC. In response to the combined stimulations, lymph node resident DC gain antigens and are capable to prime naïve T cells, which enter the draining lymph node via high endothelial venules (HEVs).

* The conduit system is a dense 3D network covering the whole T cell zone. But for the purpose of depicting other events in the draining lymph node, we display the network only in a part of the picture.

Fig. 5.


respiratory DC
lymph node resident DC
naïve T cell
inflammatory cytokines
the network of conduits
virion
mediastinal lymph node
lung
subcapsular sinus
HEV
antigen transfer
cytokines
respiratory viral infection
afferent
lymphatics
epithelia
airway mucosa
MLN
conduit system

The lung migrant DC, which are from the respiratory infection site, are possibly exposed to the complex inflammatory environment induced by rSV5-eGFP-p60 intranasal infection, including pro-inflammatory cytokines, viral products, and dying infected epithelia. And they themselves could be abortively infected by the virus. The lymph node resident DC in the MLN, although are less accessible to the virions, have many chances to interact with lung migrant DC, which serve as antigen-messengers to the draining lymph node. And the continued increase of lymph node resident DC in total cell numbers, mature cell numbers and IL-12p40 secreting cell numbers suggested that they were well triggered for maturation. Their robust increase from day 3 to day 4 p.i. followed the significant decrease of these DC from day 2 to day 3 p.i., which were presumably caused by the suppressed immigration from the lung and the considerable cell death. This suggested that the lymph node resident DC had the chance to interact with apoptotic lung migrant DC and derive antigens as TLR agonists.

Based on the above factors, we proposed that through direct or indirect interaction with live or apoptotic lung migrant DC, along with some stimulation from viral antigens draining into the MLN, lymph node resident DC get triggered for full maturation. These stimulation could be obtained by phagocytosing the released exosomes or the apoptosing lung migrant DC, which would possibly provide the lymph node resident DC with the viral molecules as TLR agonists, and by interacting with the secreted soluble factors, e.g. pro-inflammatory cytokines, from the lung migrant DC. This model gives us new insights into the control of anti-viral immunity: Besides the interaction between viruses and DC as well as the interaction between DC and naïve T cells, the direct or indirect contact between different DC population trigger the

maturation of the recipient DC and work as a “licensing” process to give them naïve T cell priming ability and thus, help induce an effective anti-viral adaptive immune response.

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CURRICULUM VITAE

Jingying Zha

1900 Queen Street, Apt B-1,

Winston-Salem, NC 27103

(336)782-3868

jingyingzha@yahoo.com, jzha@wfubmc.edu

EDUCATION

WAKE FOREST UNIVERSITY- SCHOOL OF MEDICINE Winston-Salem, NC

Master of Science, Microbiology and Immunology Sep 2006- May 2009 (expected)

Member: Wake Forest University Brain Awareness Council (BAC)

Activities: Poster section on Graduate School Research Day of Wake Forest Mar, 2008

FUDAN UNIVERSITY - SCHOOL OF LIFE SCIENCES Shanghai, China

Bachelor of Science, Life Science Sep 2002- Jun 2006

Honors: The People's Scholarship of Fudan University 2003, 2004, 2005

SKILLS

Cell Culture:

mammalian cell culture, foundation of cell lines with progenitor cells isolated from murine tissue, cell line expansion and purification

In Vitro Assays:

1. molecular biology assays:

western blot, immuno precipitation, PCR

cell transfection, DNA electrophoresis, plaque assay(virology)

2.immunology assays:

Elisa, Elispot, cell staining: surface staining & intracellular cytokine staining

Flow Cytometry Data Acquisition: Calibur (flow cytometer with 4 fluorescent parameters), Canto (complex flow cytometer with 8 fluorescent parameters)

Microbiology Techniques:

Aseptic techniques, molecular cloning methods

In Vivo Techniques:

1. anaesthesia of animal for surgical manipulation

2. drug delivery/viral infection via different route:

intravenous /intraperitoneal /intranasal/ intratracheal

Softwares:

Word, PowerPoint, Excel, Office One Note, Reference Manager, Adobe Reader, Photoshop, Sigma Plot, BD Diva software, Flow Jo, Cell Quest Pro

RESEARCH PROJECT

Respiratory infection with the paramyxovirus rSV5-eGFP-p60 results in maturation and cytokine secretion in CD103$^-$ and CD103$^+$ DC subsets in the MLN.

- Purpose: to understand the migration and maturation mechanisms of different DC subsets in the respiratory infection.
- Background: DC can prime naïve T cells through three signals: peptide/MHC, costimulatory molecules and secreted cytokines. DC maturation will upregulate the second and third signals, which contribute to better priming of naïve CD8$^+$ T cells. We focus on two DC populations: CD103$^+$ DC (airway-derived DC) and CD103$^-$ DC (lymph node resident DC), trying to understand their unique migration and maturation kinetics.
- Animal model: BALB/c mouse, intranasal infection model
- Conclusion: The total cell number, mature cell number and IL-12p40 secreting DC number of CD103$^+$ DC subsets peaked at day 2 post infection but decreased afterwards. In contrast, these numbers of CD103$^-$ DC subsets kept increasing over the course of infection. The different migrant kinetics of the two DC subsets provides us with the new insights of the DC migration and maturation mechanisms in the respiratory tract.