CONTRIBUTION OF LUNG MIGRATORY
DENDRITIC CELLS TO THE GENERATION OF
INFLUENZA IMMUNITY

NG SEE LIANG

SCHOOL OF BIOLOGICAL SCIENCES

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CONTRIBUTION OF LUNG MIGRATORY DENDRITIC CELLS TO THE GENERATION OF INFLUENZA IMMUNITY

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School of Biological Sciences

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1.2.3.3 Migration of virus-antigen harboring DCs from lung to MLN 23
1.2.3.4 Virus antigen presentation by lung migratory cDCs in the MLN 23
1.2.3.5 Contribution of DCs to the maintenance of memory T cell population 25
1.2.4 Adaptive immunity to influenza virus infection 26
  1.2.4.1 Anti-viral CD8 T cell response 26
  1.2.4.2 Induction of CD8 T cell response 26
  1.2.4.3 Contraction of effector CD8 T cell response and generation of memory CD8 T cell pool 28
1.2.5 Anti-viral CD4 T cell response 29
  1.2.5.1 Induction of CD4 T cell response 29
1.2.6 Humoral Immunity 31
1.2.7 Immunopathology 33
1.3 In vivo analysis of Dendritic cell function 37
  1.3.1 Strategies to study the function of Dendritic cell 37
  1.3.2 DT/DTR systems for Dendritic cell ablation 39
    1.3.2.1 CD11c-DTR and zDC-DTR 39
    1.3.2.2 Clec9a-DTR and Clec4a4-DTR 41
    1.3.2.3 Clec9a lineage marker and Clec9a-DTR 41
    1.3.2.4 Clec4a4 lineage marker and Clec4a4-DTR 42
1.4 Aims and Objectives 43

2. Chapter 2: Materials and Methods

2.1 Materials
  2.1.1 Mice 44
  2.1.2 Influenza A virus 44
  2.1.3 Chemicals, Reagents and Kits 45
  2.1.4 Media, Buffers and Solutions 45
  2.1.5 Commercial antibodies and Live/dead cell dye 46
  2.1.6 Computer software 46

2.2 Methods
  2.2.1 Influenza virus infection and weight loss monitoring 47
  2.2.2 Tissue collection, processing and isolate of cells 47
2.2.3 Isolation of PBMCs
2.2.4 Cells labeling for flow cytometry
2.2.5 Serum passive immunization
2.2.6 Cytokines ELISA
2.2.7 DT-mediated ablation
2.2.8 Preparation of BMDCs
2.2.9 Preparation of cells for intracellular cytokine staining

2.3 Statistical Analysis

3. Chapter 3: Results I
Characterization of Clec9a-DTR and Clec4a4-DTR transgenic mice

3.1 Gating strategies for the analysis of 2 distinct DC populations in the lung and 4 distinct DC populations in the mLN

3.2 In lung, DT efficiently ablates lung migratory CD103+ DCs and CD24+CD11b+ DCs in the Clec9a-DTR and Clec4a4-DTR mice respectively

3.3 In mLN, DT efficiently ablates lung migratory CD103+ DCs and mLN-resident CD8+ DCs in Clec9a-DTR mouse while lung migratory CD24+CD11b+ DCs and mLN-resident CD11b+ DCs (33D1+) are both ablated in Clec4a4-DTR mouse

3.4 Non-targeted cells are not affected by DT

3.5 In lung, CD103+ cDCs’ turnover is substantially slower than CD24+CD11b+ cDCs’

3.6 Discussion

4 Chapter 4: Results II
Impact of myeloid DCs to influenza virus immunity

4.1 Influenza infection induces massive recruitment of inflammatory innate cells to the lung

4.2 Ablations of lung migratory cDCs increase susceptibility of the DTR transgenic mice to influenza infection

4.3 Loss of lung migratory cDCs reduces total and influenza virus-specific CD8 T cell population in the lung

4.3.1 Loss of lung migratory cDCs reduces total CD8 T cell population
4.3.2 Loss of lung migratory cDCs reduces influenza-specific CD8 T cell population

4.4 Ablation of lung migratory CD103+ cDCs, but not CD24+CD11b+ cDCs, impairs antigen presentation in the mLN

4.5 Ablation of lung migratory CD103+ cDCs, but not CD24+CD11b+ cDCs, leads to inefficient NP<sub>366-374</sub>-specific CD8 T cell migration from the mLN

4.6 Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced viability of NP<sub>366-374</sub>–specific CD8 T cells in the lung

4.7 Reduced frequency of proliferating NP<sub>366-374</sub>-specific CD8 T cells in Clec9a-DTR and Clec4a4-DTR mice during CD8 T cells contraction phase

4.8 Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced number of IFN-γ-producing and IL-10-producing cells

4.9 CD8 T cells surface marker expression profiles
   4.9.1 NP<sub>366-374</sub>-specific CD8 T cells in Clec9a-DTR mice display T<sub>CM</sub>-like property
   4.9.2 NP<sub>366-374</sub>-specific CD8 T cells in Clec4a4-DTR mice lack KLRG1 expression
   4.9.3 Significantly higher frequency of CD62L+ CD8 T cell population in Clec9a-DTR while significantly lower frequencies of CD69+ CD8 T cell populations are observed in Clec9a-DTR and Clec4a4-DTR mice

4.10 Higher levels of pro-inflammatory cytokines in Clec9a-DTR and Clec4a4-DTR mice

4.11 Lung migratory cDCs are dispensable for the generation of homosubtypic immunity

4.12 Lung migratory cDCs regulate heterosubtypic immunity

4.13 Discussion

5. Chapter 5: General Conclusion and Implication

6. References

7. Appendix
8. Supplementary Figure 1  152
9. Author's Publications  153
10. Posters  154
LIST OF FIGURES

Chapter 1: Introduction
Figure 1: Ontogeny of DC subsets in lymphoid (spleen) and non-lymphoid (lung, small intestine, kidney) tissues.
Figure 2: Pre-cDC gives rise to CD8+ cDCs and CD4+ cDCs (equivalent to CD8- cDCs) within tissues.
Figure 3: Lung anatomy, antigen uptake and migration patterns for immune induction in the lung.
Figure 4: The structure of influenza A virus.
Figure 5: Innate and adaptive immune responses during primary influenza virus infection.
Figure 6: Experimental design for DT/DTR system-mediated inducible cell specific ablation approach.

Chapter 2: Materials and Methods
Supplementary Figure 1: Targeting construct for the generation of Clec4a4-DTR knock-in mouse.

Chapter 3: Result I
Characterization of Clec9a-DTR and Clec4a4-DTR transgenic mice
Figure 3.1: Gating strategy for lung migratory CD103+ DC and CD24+CD11b+ DC subsets in lung and mLN.
Figure 3.2: In lung, DT efficiently ablates lung migratory CD103+ and CD24+CD11b+ DCs in Clec9a-DTR and Clec4a4-DTR mice respectively.
Figure 3.3: In mLN, DT efficiently ablates lung migratory CD103+ DCs and mLN-resident CD8+ DCs in Clec9a-DTR mouse while lung migratory CD24+CD11b+ DCs and mLN-resident CD11b+ DCs (33D1+) are both ablated in Clec4a4-DTR mouse.
Figure 3.4: Non-targeted cells are not affected by DT.
Figure 3.5: In lung, CD103+ cDCs’ turnover is substantially slower than CD24+CD11b+ cDCs’
Chapter 4: Results II

Impact of myeloid DCs to influenza virus immunity

Figure 4.1: Influenza infection induces massive recruitment of inflammatory innate cells (neutrophils, monocytes and NK cells). Ablations of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs are maintained during the course of infection

Figure 4.2: Ablations of lung migratory cDCs increase susceptibility of the DTR transgenic mice to influenza infection

Figure 4.3.1: Loss of lung migratory cDCs reduces total CD8 T cell population

Figure 4.3.2: Loss of lung migratory cDCs reduces influenza-specific CD8 T cell population

Figure 4.4: Ablation of lung migratory CD103+ cDCs impairs antigen presentation in the mLN

Figure 4.5: Ablation of lung migratory CD103+ cDCs, but not CD24+CD11b+ cDCs, leads to inefficient NP366-374-specific CD8 T cell migration from the mLN

Figure 4.6: Loss of either lung migratory CD103+ cDCs or CD24+CD11b+cDCs results in reduced viability of NP366-374-specific CD8 T cells in the lung.

Figure 4.7: Reduced frequency of proliferating NP366-374-specific CD8 T cells in Clec9a-DTR and Clec4a4-DTR mice during CD8 T cells contraction phase

Figure 4.8: Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced number of IL-10-producing cells in the lung. Ablation of lung migratory CD103+ cDCs leads to reduced number of IFN-γ producing cells in the mLN

Figure 4.9.1: NP366-374-specific CD8 T cells in Clec9a-DTR mice display TCM-like property

Figure 4.9.2: NP366-374-specific CD8 T cells in Clec4a4-DTR mice lack KLRG1 expression

Figure 4.9.3: Significantly higher frequency of CD62L+ CD8 T cell population in Clec9a- DTR while significantly lower frequencies of CD69+ CD8 T cell populations are observed in Clec9a-DTR and Clec4a4-DTR mice
**Figure 4.10:** Higher levels of pro-inflammatory cytokines in Clec9a-DTR and Clec4a4-DTR mice

**Figure 4.11:** Lung migratory cDCs are dispensable for the generation of homosubtypic immunity

**Figure 4.12:** Lung migratory cDCs regulate heterosubtypic immunity

**LIST of TABLES**

**Chapter 3: Result I**

**Table 3.1:** Summary of fluorescent antibody combinations used for FACS analysis of DC subsets in the lung and MLN
SUMMARY

DCs are important for the induction of CTL response and elicitation of protective immunity against influenza virus infection. Owing to the functional heterogeneity of DCs in the lung, there is a need to unravel the in vivo contribution of various DC subpopulations to the generation of influenza immunity. Using our Clec9a-DTR and Clec4a4-DTR transgenic mouse models which allow us to specifically deplete CD103+ cDCs and CD24+CD11b+ cDCs respectively in the lung, we aimed to study the biology of these DC populations using mouse-adapted influenza A virus strain H1N1/PR8. We have shown that Clec9a-DTR and Clec4a4-DTR mice were highly susceptible to influenza virus infection compared to the wild type. Both the infected transgenic mouse models displayed suboptimal number of influenza-specific CD8 T cells in the lung indicating that lung-derived CD103+ cDCs and CD24+CD11b+ cDCs are required to achieve high number of pulmonary antiviral CD8 T cells for the elicitation of protective immunity against primary influenza virus infection. We have also shown that lung-derived CD103+ cDCs and CD24+CD11b+ cDCs differentially regulated induction of influenza-specific CD8 T cell response in the mLN with the former contributed significantly to the cross-presentation of virus antigens to the naive influenza-specific CD8 T cells after influenza virus infection. When compared to wild type and Clec4a4-DTR mice, Clec9a-DTR mice had less trafficking of influenza-specific CD8 T cells from the MLN indicating defective mobilization of differentiated CD8 T cells. The survival of pulmonary influenza-specific CD8 T cells, as our data indicate, was dependent on both lung-derived CD103+ cDCs and CD24+CD11b+ cDCs in which absence of either DC population led to reduced viability of these T cells in the lung. Collectively, we concluded from these analyses that the mechanisms contributing to the diminished accumulation of influenza-specific CD8 T cells in the lung of Clec9a-DTR and Clec4a4-DTR mice were different.

In examining the impact of lung-derived DC on CD8 T cell property and functional ability, we observed that the numbers of cytokine IFN-γ and immunosuppressive IL-10 secreting CD8 T cells were severely blunted in both
Clec9a-DTR and Clec4a4-DTR mice. Moreover we have also observed that CD8 T cells differentiated in the absence of CD103+ cDCs exhibited CD8 T_{CM} phenotype whereas CD24+CD11b+ cDCs uniquely controlled the expression of KLRG1 on CD8 T cells. In establishing lung-derived DCs involvement in immunity against secondary infection, we assessed the susceptibility of immunized Clec9a-DTR and Clec4a4-DTR mice to re-infection with identical virus (homosubtypic challenge) and to secondary challenge with serotypically distinct influenza virus (heterosubtypic challenge). Our data show that CD103+ cDCs and CD24+CD11b+ cDCs were dispensable for homosubtypic challenge whereas they were important for the efficacy of heterosubtypic immunity. Ablation of CD103+ cDCs led to complete loss of cross-reactive immunity in which x-31-Clec9a-DTR mice and naive mice show no difference in weight loss kinetics and susceptibility following challenge with lethal PR8 infection. Our findings in this study have expanded the current understanding of lung DC biology and concurrently revealed the in vivo role of distinct DC subpopulations to the generation of T cell immunity and memory protection. This knowledge may contribute to the development of more efficient flu vaccine and the improvement of DC immunotherapy.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>alveolar macrophage</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin (fluorophore)</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BAL</td>
<td>broncho-alveolar lavage</td>
</tr>
<tr>
<td>Batf3</td>
<td>basic leucine zipper transcription factor, ATF-like 3</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow derived dendritic cells</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of distribution</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CX3CR</td>
<td>Chemokine (C-X3-C motif) receptor</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DT</td>
<td>diphtheria toxin</td>
</tr>
<tr>
<td>DTR</td>
<td>diphtheria toxin receptor</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced GFP</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescent-activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin (influenza virus protein)</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>i.p</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v</td>
<td>intravenous</td>
</tr>
<tr>
<td>iIDC</td>
<td>inflammatory monocyte-derived DCs</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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</tbody>
</table>
IMDM  Iscove’s modified Dulbecco’s medium
IRES  internal ribosome entry site
ISG   IFN-stimulated genes
MHC  major histocompatibility complex
MLN  mediastinal lymph node
Mφ   macrophage
NA   neuraminidase
NK   natural killer
NO   nitric oxide
NP   nucleoprotein
P.I  post-infection
PAMPs  pathogen associated molecular patterns
PBS  phosphate buffered saline
pDC  plasmacytoid dendritic cell
PE   phycoerythrin
PFA  paraformaldehyde
PFU  plaque forming unit
PRRs  pattern recognition receptors
RBC  red blood cell
SAP  SLAM-associated protein
SPF  specific pathogen-free
SSC  side scatter
TCR  T cell recognition
TLR  Toll-like receptor
TNF  tumor necrosis factor
TRAIL tumor necrosis factor-related apoptosis-inducing ligand
VSV  vesicular stomatitis virus
WT   wild type
Chapter 1: Introduction

1.1 Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells (APC) that initiate and shape adaptive responses according to the peripheral cues. They are equipped to recognize pathogens, vaccines and self-antigens and covert these antigens into MHC-peptide complexes that can be recognized by T lymphocytes. While DCs constitute a unique hematopoietic lineage distinct from other leukocytes, they display remarkable functional and developmental heterogeneity.

Figure 1: Ontogeny of DC subsets in lymphoid (spleen) and non-lymphoid (lung, small intestine, kidney) tissues. CDP, a DC restricted progenitor, is derived from MDP in the bone marrow after which CDP gives rise to pDC and Pre-cDC. Pre-cDC migrates from the bone marrow to seed lymphoid and non-lymphoid tissues. Pre-cDC gives rise to CD8+ and CD8- cDCs in lymphoid tissues and CD103+ cDCs and CD11b+ cDCs in non-lymphoid tissues. A cDC subpopulation expressing both CD103 and CD11b markers (CD103+CD11b+cDCs) can be found in small intestine (adapted from [1]).
1.1.1 Dendritic Cell lineage and tissue distribution

Developmentally, tissue DCs can be broadly classified into two major subsets, classical dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs). Conventionally defined cDCs, originally discovered by Ralph Steinman in 1970s, can be further classified based on surface expression of CD8 molecule, CD8+ cDCs and CD8- cDCs. DC progenitors are generated in bone marrow (BM) in which the first dedicated DC progenitor in the BM are known as common dendritic cell progenitors (CDP) (Figure 1). CDPs give rise to pDCs and pre-cDCs in the BM shortly after which both pDCs and pre-cDCs move from the BM into the blood circulation and traffic to various tissues (Figure 1 and 2). Pre-cDCs are direct precursors of CD8+ and CD8- cDCs (Figure 1). BM cell commitment to CDP requires fms-like tyrosine kinase 3 (Flt3). Expression of Flt3 can be found on DC precursors, CDP as well as pre-cDCs, and is maintained on all cDCs and pDCs [2]. Most cDCs, with the exception of Langerhan cells, are relatively short-lived and are constantly replenished by their precursors [2].

Figure 2: Pre-cDC gives rise to CD8+ cDCs and CD4+ cDCs (equivalent to CD8- cDCs) within tissues. CD8+ cDCs and CD4+ cDCs mediate cross presentation and MHC class II antigen presentation respectively. CDP gives rise to pDC in the BM and pDC can be found in circulation and in tissues. pDCs are major source of type I IFN (adapted from [3]).
1.1.1.1 Lymphoid tissue (LT) Classical DC

Conventional cDCs, CD8+ cDCs and CD8- cDCs represent two major DC subsets in the spleen and all peripheral lymph nodes (LNs) which are collectively referred to as lymphoid tissue (LT) cDCs. After seeding in LT from the BM, Pre-cDCs give rise to both CD8+ and CD8- cDCs [4]. CD8+ cDCs and CD8- cDCs are commonly described phenotypically as MHC class II+ CD11c+CD8+CD205+ and MHC class II+ CD11c+CD8-33D1+ respectively. During uninfected steady state, they are functionally and phenotypically immature [5]. Due to the intrinsically different antigen processing mechanisms, CD8+ cDCs and CD8- cDCs are specialized in cross-presentation and in MHC class II-mediated presentation respectively (Figure 2). The superior cross-presenting ability of CD8+ cDCs can be partially attributed to the expression of CD36 and C-type lectin receptors Clec9a in which both are involved in the uptake of apoptotic cells [6, 7]. While both CD8+ and CD8- cDCs express TLR9, TLR3 is expressed on CD8+ cDCs, whereas TLR7 is restricted to CD8- cDCs [5]. This differential pattern of TLR expression suggests unique pattern of response against pathogens [5].

1.1.1.2 Non-lymphoid tissue (NLT) Classical DC

Conventional cDCs residing in the peripheral tissues for instance lung, intestine, kidney, epidermis etc are referred to as non-lymphoid tissue (NLT) cDCs. While two major cDC subsets in LT are distinguished by CD8 marker, the two major cDC subsets in NLT can be distinguished by the expression of CD103 and integrin CD11b (Figure 1). Phenotypically, NLT CD103+ cDCs and CD11b+ cDCs are commonly identified as MHC class II+ CD11c+ CD103+ and MHC class II+ CD11c+ CD11b+ respectively. [8] In most NLT, expression of CD103 and CD11b are mutually exclusive on CD103+ cDCs and CD11b+ cDCs. An additional subset expressing both CD103 and CD11b can be found in gut lamina propria (Figure 1) [8]. Like cDCs in LT, cDCs in NLT are derived from Pre-cDCs that arrive in NLT from BM.
The development of these phenotypically distinct DC subsets is differentially regulated [8]. CD103+ cDCs are dependent on Flt3L, Id2, BATF3 and IRF8; on the other hand, CD11b+ cDCs are dependent on Flt3L and MCSF-R (also known as Csf-1R) but not Id2 and IRF8 [9]. As briefly mentioned above, Flt3L is key to DC commitment and its receptor, Flt3, is expressed on all pre-cDC precursors (MDP), pre-cDCs and tissue DCs but not in other non-DC lineage [9, 10]. MCSF-R on the other hand is expressed at very low amount on pre-cDCs and is not expressed on tissue DCs [11]. Instead, MCSF-R is expressed abundantly on MDPs, monocytes and macrophages [11]. MDPs differentiate in the presence of CSF-1 into macrophages. The dependence of CD11b+ cDCs on both Flt3L and MCSF-R means CD11b+ cDC subset contains a mixture of cDCs and monocytes/macrophages. Due to lack of specific markers to distinguish between the cDCs and contaminating monocytes/macrophages in CD11b+ cDC subset, contribution of CD11b+ cDCs to tissue immunity has been lacking. Until very recently, Schlitzer et al indicated cDCs and contaminating monocytes/macrophages in CD11b+ cDC subset can be distinguished based on surface expression of CD24 and CD64. The cDCs in CD11b+ cDC subset can be identified as MHC class II+ CD11c+ CD11b+ CD24+, hereafter referred to as CD24+ CD11b+ cDCs. The contaminating monocytes/macrophages in CD11b+ cDC subset can be identified as MHC class II+ CD11c+ CD11b+ CD64+, hereafter referred to as CD64+ CD11b+ Macs. The term CD11b+ cDCs will be reserved to indicate population including both CD24+ CD11b+ cDCs and CD64+ CD11b+ Macs. Unlike CD11b+ cDCs, CD103+ cDC subset contains a homogeneous population of cDCs.

NLT CD103+ cDCs share their function and origin with LT CD8+ cDCs [12]. Aside from sharing the same origin (pre-cDC-derived) and dependency on transcription factor (TF) Id2, BATF3 and IRF8, phenotypically they share similar expression profile of TLR, CLR and chemokine receptors and functionally they are equally endowed with superior ability in cross-presenting cell-associated antigen [12]. Comparatively, relationships between LT CD8-cDCs and NLT CD11b+ cDCs remain elusive due to their heterogeneity, uncertainty in origin and less defined TF dependency of the NLT CD11b+ cDCs. Several previous studies indicate the capability of NLT CD11b+ cDCs to
cross-present viral antigen to CD8+ T cells in ex vivo assays [13-15]. Similarly in ex vivo assays using HSV-1 and influenza virus infection model, studies indicated the presentation capacity of NLT CD11b+ cDCs in presenting viral antigen to CD4+ T cells [10, 14, 16]. It should be noted that functional roles of NLT CD11b+ cDCs should be interpreted with care considering the presence of contaminating CD64+ CD11b+ Mφ.

1.1.1.3 Tissue migratory DC

Tissue migratory DCs are found in the peripheral LNs. [2] They are essentially the DC populations that migrate from NLT to the respective draining LNs. Hence, depending on where the LNs drain from, the nature of tissue migratory DCs differs. Tissue migratory DCs express chemokine receptor CCR7. The 2 known CCR7 ligands CCL19 and CCL21 are produced by lymphoid organs stromal cells. CCR7-CCL19/CCL21 axis is critical for tissue migratory DCs trafficking from NLTs to the respective draining LNs. In CCR7-deficient mice, the DC number is substantially reduced in the draining lymph node LN. The migration from tissue to its respective draining LN (via afferent lymphatics) is a constant process in which this process magnifies tremendously during inflammation.[17] Tissue migratory DCs undergo a maturation process during the migration in both steady state and inflammation context. In contrast to steady state DCs, DCs migrating during an inflammatory event secrete pro-inflammatory cytokines and up-regulate a panel of co-stimulatory molecules.

1.1.2 Dendritic cells and lung

The lung can be divided into two functionally distinct compartments, conducting airways and lung parenchyma (Figure 3) [18]. Conducting airway comprises mucosal tissue and respiratory epithelia, on the other hand, lung parenchyma comprises extensive network of bronchi and bronchioles that further branch out to form alveolar ducts and alveolar sacs [18]. Both compartments are populated with various immune cells like Mφ, T cells, B cells, DCs and plasma cells. There are two types of macrophages in the lung,
alveolar macrophages (AM) and interstitial macrophages (IM). AMs, which are exposed to the external environment, reside within the alveolar space whereas IMs can be found in the region underlying the alveolar epithelial layer [19]. Various different subsets of lung resident DCs have been identified in recent years in which they are shown to regulate important functions during lung homeostasis and infection [18].

1.1.2.1 DC subpopulations in lung

Three phenotypically and functionally distinct DC subpopulations can be found in the lung: CD103+ cDCs, CD11b+ cDCs and pDCs. In addition, a very minor DC population termed alveolar DC resides in the alveolar space. CD103+ cDCs and CD11b+ cDCs constantly migrate from the lung to mediastinal lymph node (mLN) in a CCR7-dependent manner and are referred as tissue migratory DCs (or lung migratory DCs). Hence, four DC subsets can be found in mLN, two lung-derived migratory DC subsets (CD103+ cDCs and CD11b+ cDCs) and two mLN resident DC subsets (CD8+ cDCs and CD8- cDCs).

In the lung, CD103+ cDCs and CD11b+ cDCs are positioned adjacent to the respiratory epithelial cells and underlying lamina propria respectively. Some studies suggest CD103+ cDCs are able to extend protrusions between epithelial cells perhaps due to the expression of tight junction proteins. CD103+ cDCs and CD11b+ cDCs express different TLRs, chemokine receptors and bridging molecules [20]. For instance, CD103+ cDCs and CD11b+ cDCs preferentially express TLR3 and TLR7 respectively. Another receptor which was reported to be expressed on CD103+ cDCs but not on CD11b+ cDCs is Clec9a (DNGR-1), a key receptor for apoptotic cell recognition. In particular, CD103+ cDCs have been implicated as the major DC population that induces tolerance against self-antigen (MOG-model) or the major cross-presenting DC subset that induces CD8 T cell response during influenza virus infection and malaria [21, 22].
Figure 3: Lung anatomy, antigen uptake and migration pattern for immune induction in the lung. There are 2 functionally distinct lung compartments, conducting airways and lung parenchyma. Each compartment is populated with DC, macrophages, lymphocytes and plasma cells. Lung migratory DCs migrate to the draining LNs via afferent lymphatics to interact with naïve T cells for either tolerance or immunity elicitation. Activated T cells traffic back to conducting airways and lung parenchyma via postcapillary venules and pulmonary capillaries respectively (adapted from [18]).

1.1.2.2 Role of DCs in lung immune response

The general consensus is that lung migratory DCs (CD103+ cDCs and CD11b+ cDCs) maintain the tolerance to self-antigen and potently induce adaptive immune responses upon infection [23]. Lung migratory cDCs, that are exposed to inhaled antigens, traffic to the MLN and induce T cell unresponsiveness when the treatment is devoid of strong TLR ligands [24]. In the presence of inhaled pathogen (respiratory virus e.g. influenza virus) or
harmful antigen (antigen coupled to TLR ligands), migratory cDCs are fully activated and express co-stimulatory molecules while trafficking to mLN to induce CD4 and cD8 T cell responses [24].

### 1.1.2.2.1 Role of DCs in lung tissue homeostasis and infection

Mucosal surfaces in the respiratory tract are constantly exposed to environmental innocuous antigens [18]. To prevent immune system from mounting responses against innocuous antigen, local immune response takes the forms of either immune ignorance or tolerance [25]. Immune ignorance can be induced after repeated inhalation of antigen (e.g. OVA). This antigen presenting cell-mediated suppression of responsiveness correlates with the presence of antigen-specific IgE subtypes, suggesting a ‘default and non-pathological’ local Th2 response against harmless antigens [25]. In addition, pDC and CD103+ cDCs are heavily involved in the induction of tolerance against airway antigens by generating adaptive, antigen-specific T_{reg} [26]. The presence of antigen-specific T_{reg}, and to a lesser extent non-specific T_{reg}, promotes immune tolerance and prevents the development of massive often pathological inflammatory response [26].

AMs represent another important immune-regulatory population that contributes significantly to maintain tolerance in the lung [27]. Though present in the alveoli space, AMs adhere on the alveolus wall and therefore are as close as 0.2-0.5um from the DCs and T cells lying in the interstitium [27]. These interstitial DCs and T cells are kept in quiescent by AMs in which AMs have been shown to suppress DC immunogenicity and to compromise T cell expansion [28, 29].

An arsenal of pattern recognition receptors (PRR) are found on lung migratory DCs [30]. PRRs recognize pathogen associated molecular patterns and upon activation, trigger a cascade of signals that leads to release of pro-inflammatory cytokines and chemokines [30]. More importantly, pro-
inflammatory cytokines and engagement of PRRs coordinately initiate the process of lung migratory cDCs maturation and their trafficking to mLN for the induction of adaptive immunity. Essentially, PRR activation on lung migratory cDCs bypasses default Th2 responses and tolerance and efficiently initiates protective immunity [18].
1.2: Immunity to Influenza Virus Infection

1.2.1 Influenza Virus Infection

1.2.1.1 Virus physiology

Influenza viruses are single-stranded RNA viruses of the Orthomyxoviridae family. Phylogenetically there are three distinct types of virus, A, B and C (Influenza A, B and C virus). Influenza A virus genome (total length of 13kb) comprises 8 separate RNA segments, PB2, PB1, PA, HA, NP, NA, M and NS. HA and NA segments encode surface glycoproteins hemagglutinin and neuraminidase respectively. The various different Influenza A virus subtypes (serotypes) are classified based on hemagglutinin (HA) and neuraminidase (NA). Currently 16 antigenically distinct forms of HA (H1-H16) and 9 antigenically distinct forms NA (N1-N9) have been identified in influenza A virus. All 16HA and 9NA subtypes can be found in its natural host, the aquatic birds of which they are the source of all the influenza A viruses that become adapted in other animals. There are currently limited to 3 HA (H1-H3) and 2 NA (N1-N2) influenza A virus subtypes circulating in human population (Hereafter the term Influenza virus refers to influenza virus type A).

Figure 4: The structure of influenza A virus. Virus genome is composed of 8 separate RNA segments. Surface proteins include HA, NA and M2. M2 proteins form ion channels that traverse HA and NA (adapted from [31]).
The 3 largest RNA segments encode for 3 polymerase proteins (PB2, PB1 and PA) which are involved in synthesizing the virus gene segments to be incorporated into the progeny virus [32]. HA encodes for the surface protein hemagglutinin that binds the virus to the host cell surface receptors specifically the sialic acid residues [32]. Following virus attachment to the host cell surface and endocytosis-mediated entry, acidic environment in the endosome induces a conformational change in HA that triggers fusion of virus envelope and endosome membrane after which the virus genetic contents are released into host cells [32]. NP encodes for a nucleoprotein which is a structural protein that binds to all 8 separate RNA segments. NP-bound RNA segment together with 3 subunits polymerase form a complex called ribonucleoprotein particles (RNP) [32, 33]. RNP is the site where virus RNA transcription and replication occurs in which NP plays a critical role in the switch from transcription to replication [32]. Neuraminidase (NA) cleaves the sialic acid at the end of virus life cycle before the release and dissemination of new progeny virus. M segment encodes for 2 proteins, M1 (most abundant viral proteins) and M2 of which are responsible for virus assembly and disassembly respectively [32]. Similarly, NS segment encodes for 2 proteins, NS1 and NS2 in which NS1 mediates multiple functions including its extensively studied role in antagonizing host Type I IFN anti-viral response and NS2 functions as nuclear export machinery in particular facilitate transport of RNP from host nucleus to the cytosol (Figure 4) [34].

Due to the selection pressure from the immune response and low fidelity of viral polymerase, HA glycoprotein undergoes constant genetic mutations to evade host antibodies recognition. Attempts to find a neutralizing antibody capable of targeting all 16 subtypes failed for years. Antigenic shift and antigenic drift are two important processes by which mutant influenza viruses are generated. Antigenic drift is a process by which circulating influenza viruses undergo frequent yet subtle genetic changes involving point mutations predominantly within HA and NA surface proteins and occasionally in some other viral internal proteins PB1 and PB2. Most of the mutations are silent mutations, however some mutations cause changes to HA and NA especially
in the binding regions targeted by host neutralizing antibodies. As a consequence of the altered antigenicity, these “drifted” influenza strains can no longer be targeted effectively by the host preformed neutralizing antibodies specific to the earlier circulating strains. Antigenic shift is a process by which circulating human influenza viruses exchange/reassort their RNA segments with non-human influenza viruses. As a consequence, the new human influenza virus acquires a non-human HA (with or without accompanying non-human NA subtype). 1 of the 16 antigenically distinct HA subtypes in the avian (aquatic birds) influenza virus gets introduced into the human influenza virus every time this type of major genetic crossover occurs. Essentially this process of antigenic shifting results in the emergence of new human influenza virus strain with the potential to cause outbreaks in a pandemic scale in which human population has no prior immunity against this novel HA of avian origin.

1.2.1.2 Vaccine against influenza virus

An antibody response is induced following influenza virus infection. Though antibodies generated against other virus proteins (for instance NA or M1) may provide varying levels of protection in vivo, antibodies specific for HA are capable of neutralizing influenza infection and has been shown to confer protection against viral infection in humans [35]. HA proteins are involved in virus binding and attachment to host cell receptors, a key step for virus entry to host cells before initiating infection. When HA specific antibodies bind to virus HA, they abrogate the interaction between virus HA and host receptor thereby preventing host cells from being infected. As such, the protections which are conferred by HA specific antibodies are known as sterilizing immunity, a state indicating that all infectious virus particles being neutralized by anti-HA antibodies and which inhibits a productive infection [36].

Currently licensed flu vaccines, which include inactivated or cold adapted influenza virus strains, strongly elicit neutralizing anti-HA antibodies in the vaccinated healthy hosts [37]. Neutralizing antibodies mediated protection is strain-specific and therefore are non-protective against HA mismatched
circulating influenza virus strains. Therefore, flu vaccines are re-formulated annually to include the best predictions of the influenza virus strains which will be circulating in the upcoming year. Such efforts aim to maximize the protection conferred by the neutralizing antibodies elicited by the vaccine inactivated viruses.

*Homosubtypic and Heterosubtypic Immunity*

Current flu vaccines or infection-induced neutralizing HA specific antibodies protect the hosts from being re-infected by identical virus strains, a protection referred to as homosubtypic immunity. However, antibody-mediated selection pressure drives frequent changes (antigenic drift) in the HA. In addition, there is occasional emergence of new pandemic strain (antigenic shift) from non-human reservoir. Therefore, humans are very likely to encounter serologically distinct influenza virus strains from the one they previously experienced. As such, neutralizing antibodies elicited by vaccines or previous infection are rendered ineffective and do not protect against these HA mismatched variants [37]. In recent years, several scientific advances have been attempted to develop a broadly protective vaccine capable of inducing heterosubtypic immunity that cross-reacts between virus strains of distinct subtypes [36]. The strategies for this universal vaccine have been focusing on regions of the virus proteins which are highly conserved across different virus subtypes for instance extracellular domain of virus M2 proteins and stalk domain of virus HA proteins [38]. Besides neutralizing antibodies, effector CD8 T cells that recognize relatively conserved virus proteins such as NP, PA, PB1 and M1 have been shown to contribute to heterosubtypic immunity against influenza A virus of different subtypes [39]. It would therefore be useful to include CD8 T cell activating component in the antibodies based-vaccine.
1.2.1.3 Influenza virus-induced lung immune responses

Respiratory virus such as influenza virus establishes acute infection mainly restricted to the respiratory tract and lung parenchyma [30]. The use of mouse adapted strains of influenza virus to investigate immunity to influenza virus infection has generated a wealth of information regarding the dynamics of immune response in the lung as well as insights on a myriad of individual components of the response necessary for the successful resolution of the infection.

A common feature of respiratory virus is the initial infection in the respiratory tract epithelial cells, which are productively infected by the virus and as such represent the major source of new virions production and spread, followed by a release of pro-inflammatory cytokines and chemokines that recruit innate immune cells (neutrophils, macrophages/monocytes and natural killer cells) to the infected lung [30]. Respiratory epithelial cells and recruited inflammatory infiltrates in the early phase of infection may not be the primary source of massive pro-inflammatory cytokines and chemokines release, called cytokines storm [40]. Endothelial cells in the lung instead have been reported by Teijaro et al to be the central regulators of early inflammatory infiltrates and cytokine storm initiation [41]. In their study, they showed that endothelial cells orchestrate two important events during the early phase of infection, firstly a direct recruitment of massive numbers of innate immune cells and secondly an induction of type I IFNs, predominantly IFN-α, release in which IFN-α is directly responsible for the initiation of cytokine storm.

The early influx of innate immune cells and the presence of cytokines limit virus replication prior to the adaptive response (Figure 5). However, an overly aggressive innate response which is characterized by the excessive early recruitment of inflammatory infiltrates was a key contributor to the morbidity observed in 1918 H1N1 pandemic human influenza virus infection [42]. Similarly excessive early cytokines events were associated with severe clinical
symptoms observed in 1997 H5N1 epidemic avian influenza virus infection [43].

Figure 5: Innate and adaptive immune responses during primary influenza virus infection. Virus detection in the lung via PRRs initiates cascade of cytokines/chemokines production that recruit neutrophils, NK cells, monocytes/macrophages from the circulation to the lung. Concurrently, activated, viral antigen bearing lung migratory DCs traffick to the mLN where they activate virus-specific naïve T cells to generate an expanded population of differentiated effector T cells. CD4 T cells provide help for B cell activation and GC formation in the mLN. Effector CD4 and CD8 T cells migrate to and accumulate in the infected lung in massive numbers. Cytotoxic activities and pro-inflammatory cytokine secretion from the effector T cells and virus-specific class switched antibodies constitute effective machineries to clear the virus (adapted from [30]).

Amidst the robust innate immune events occurring in the lung upon influenza virus infection, lung migratory DCs upon acquisition of virus-infected epithelial cells, migrate from the lung to draining mLN (Figure5) [44]. Trafficking of lung migratory DCs from infected lung to mLN is a key step in the initiation of
influenza-specific adaptive immunity [44]. Viral-antigen harboring lung migratory DCs while travelling through the lymphatics, mature en route and process viral antigens for T cell priming in the mLN [44]. Activated and differentiated virus-specific CD4 and CD8 T cells first move from mLN into the blood, migrate and exit to the infected lung to clear the infection.

**1.2.2 Innate Immunity to Influenza virus infection**

When influenza virus is inhaled via nasal cavity into the lung compartments, it first encounters mucus covering the respiratory epithelia before attaching to the epithelial lining and infecting immune and non-immune cells in the vicinity. The innate system detects influenza virus infections through recognition of PAMPs that are present on the virus or that are generated during infection. Influenza virus PAMPs include single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), 5′-triphosphate viral ssRNA (a product resulted from viral replication) and the proton pump activity of viral protein M2 inside the infected host cells [45].

**1.2.2.1 PRR-mediated recognition of influenza virus**

Influenza virus infection is detected by three distinct classes of innate sensors (PRRs), Toll-like receptors (TLR), RIG-I like receptors (RLR) and Nod-like receptors (NLR) [45]. TLR and RLR detect viral pathogen associated molecular pattern (PAMP), in contrast, NLR senses cellular damage or stress as a result of viral infection. The major difference between TLR and RLR is that TLR-mediated virus recognition occurs in the endosomal compartment whereas RLR-mediated virus recognition occurs in the cytosol compartment. The two extensively studied TLRs in influenza virus recognition are TLR3 and TLR7 which recognize dsRNA and ssRNA respectively [45].

Host cells do not generate dsRNA, therefore presence of dsRNA signals presence of intruders. Recognition of dsRNA by TLR3 in the endosome is triggered when phagocytes engulf dying influenza virus infected cells. Another
endosomal TLR, TLR7 is directly activated by virus genomic ssRNA when virus membrane and capsid is degraded in the acidic endosome, a context in which the host cell itself is infected. TLR7-ssRNA recognition process does not require virus replication. Unlike TLR7 which recognizes non-replicating RNA, RLR detects replicating viral RNA specifically the 5`-triphosphate region of viral ssRNA. NLRP3, which belongs to NLR family, is activated by the activity of virus M2 protein [46]. Viral M2 ion channel activity that acidifies host cell cytosol is deemed as distress signal that triggers NLRP3 [47]. IL-1β, product of NLRP3 activation, has diverse effect on the host cellular and humoral immunity against influenza virus infection [48, 49].

1.2.2.2 Immune cells that sense influenza virus via PRRs

TLR3-expressing cells include macrophages, splenic CD8+ cDCs and lung migratory CD103+ DCs [20, 50]. TLR3-mediated virus dsRNA recognition occurs in the endosome where virus dsRNA is released from the phagocytosed dying virus-infected cells. Therefore, expressions of TLR3 are mostly found on the immune cells which are able to recognize and uptake apoptotic cells for instance CD8+ cDCs and lung CD103+ cDCs.

TLR7-expressing cells include predominantly pDCs, macrophages, LT CD8-cDCs and NLT CD11b+ cDCs [20, 47, 51, 52]. In most circumstances, cells that are activated by TLR7-mediated-ssRNA recognition are themselves infected though in some cases uptake of virus-infected cells can equally trigger TLR7 activation.

RIG-I, which is crucial for the detection of replicating virus in cytosol, is an important innate sensor for lung epithelial cells to detect influenza virus infection. Other immune cells that express RIG-I include LT cDCs and alveolar macrophage [53]. High NLRP3-expressing cells include LT cDCs and monocytes, while macrophages show modest expression and pDCs practically
do not express NLRP3. RIG-I and NLRP3 expression have not been studied in lung resident CD103+ cDCs and CD11b+ cDCs [45].

1.2.2.3 Roles of various innate immune cells

Natural Killer (NK) cells are important in the defense against influenza virus infection in which in vivo NK cells depletion using anti-asialo GM1 or NK1.1 antibodies increases susceptibility and mortality of the infected mice [54]. NK cells have been shown to utilize cytotoxicity machineries and produce pro-inflammatory cytokines/chemokines to contain influenza virus at the early stage of infection [55]. Recent studies provided evidence on the direct involvement of NK cell activation receptor NCR1 in recognizing influenza virus HA protein [56]. Loss of NCR1 resulted in enhanced susceptibility of mice to influenza virus infection suggesting the activity of NCR1 against influenza virus is crucial and thus NK cells have a critical role in the in vivo eradication of influenza virus [56].

AMs are indispensable for the containment of influenza virus in the early stage of infection absence of which resulted in uncontrolled virus replication, elevated level of pro-inflammatory cytokines, aggravated lung lesion and higher mortality [57-60]. AMs actively suppress lung-migratory DC immunogenicity and T cells during homeostatic condition to avoid elicitation of immunity against innocuous antigen. Concurrently, AMs are suppressed by a critical repressor pathway mediated by CD200-CD200R [61]. CD200R is expressed almost exclusively on myeloid cells including AMs while its ligand CD200 is expressed by the alveolar epithelial cells and as such CD200 inhibitory signaling is delivered continuously to the adjacently positioned alveolar macrophages to keep their inflammatory function in check during homeostasis [61]. CD200-deficient mice display more macrophage activity, delayed resolution of inflammation and are highly susceptible to influenza virus infection strongly suggesting CD200R signaling is critical for AM to restrict the amplitude and duration of inflammatory events during influenza virus infection [61].
Neutrophils are massively recruited to the infected lung during the early stage of mild and highly virulent influenza virus infection [62, 63]. In vivo depletion of neutrophils using Ly6G-specific mAb 1A8 leads to uncontrolled virus growth and spread, more severe disease condition and higher mortality suggesting protective role of neutrophils against influenza virus infection [57, 64]. In addition, absence of neutrophils has been shown to reduce the overall magnitude of CD8 T cell response and impair cytokine production and cytotoxic activities [65]. Collectively, these observations indicate neutrophils restrict virus replication and participate in the direct anti-viral mechanism by promoting influenza virus-specific CD8 T cell responses [65].

1.2.3 Roles of Dendritic cells in the innate and adaptive immunity against influenza virus infection

In the steady state condition, lung migratory cDCs are immature, phagocytic and non-immunogenic [66]. Influenza virus infection triggers antigen acquisition and activation of these migratory cDCs, resulting in their mobilization and migration out of the infected lung, followed by their trafficking to the draining mLN [67]. In mid 1980s and early 1990s, Holt et al showed the ability of lung DCs to acquire inhaled antigen and to prime T cell response in rats after intranasal challenge with bacterial stimulus. Subsequent studies demonstrated DCs in lung are also capable of acquiring HEL antigen and FITC-labeled beads. In early 2000s, Braciale et al demonstrated antigen acquisition, migration and T cell priming aspects of DCs in the lung during influenza virus infection. Following the discovery of multiple DC subpopulations in the lung, investigations in recent years focus on the role of these individual DC subsets in T cell response induction in the draining mLN. Intense studies on this aspect revealed these lung migratory DC subpopulations migrate to the draining mLN at different kinetics during influenza virus infection. In addition these multiple DC subsets do not possess the same capacity to prime T cell response in the mLN. On top of this, some studies suggest the involvement of mLN-resident DCs in T cell priming apart from the lung migratory DCs. In short, consensus is that there is a division of
labor among various DC subsets in the lung and mLN and each subset appears to take control of non-redundant roles during primary as well as secondary infection.

1.2.3.1 DCs acquisition of virus in the lung

Lung DCs’ ability to acquire antigen in the lung and migrate to mLN was first explored by Vermaelen et al.[68] In this study, they delivered FITC-labeled dextran/OVA intranasally and monitored the presence of FITC-positive DCs in the mLN. They observed lung derived DCs are FITC-positive in the mLN but those mLN resident CD8+ cDCs and CD8- cDCs do not acquire the FITC-labeled molecules. These FITC-positive lung migratory cDCs that arrive in the mLN are phenotypically mature and are able to cross-present to T cells [68]. The ability to acquire viral antigen was later demonstrated in the study conducted by Manicassamy et al in which they used a recombinant PR8 influenza virus carrying a GFP-reporter gene in the NS segment (NS1-GFP) to infect the mice [69]. In GFP-PR8 infected mice, they observed 20% of CD103+ cDCs and 20% of CD11b+ cDCs in the lung were GFP+ at 48 hours P.I [70]. As in the case for mLN, they observed 8.5% of CD103+ cDCs and 0.5% of CD11b+ cDCs were labeled GFP+ [70]. This observation indicates that even though similar percentage of both CD103+ cDCs and CD11b+ cDCs were initially labeled GFP+ in the lung, frequency of GFP+ CD11b+ cDCs in the mLN was significantly lower than the frequency of GFP+ CD103+ cDCs suggesting that CD103+ cDCs possess greater migration potential.
1.2.3.2 DCs activation during early phase of Influenza Virus Infection

Differential patterns of innate sensors PRRs (TLRs, RLRs and NLRs) expression on various immune cells (refer to section) suggest the unique response undertaken by each cell type [45]. For instance, using TLR7- and TLR3-deficient mice, study showed TLR7 (and its adaptor protein, MyD88) signaling contributes significantly to the magnitude of virus-specific antibody response and isotype switching, whereas TLR3 (and its adaptor protein, Trif) signaling does not regulate the anti-viral humoral immunity [71]. The use of knockout mice that lack specific PRRs (e.g. TLR3 or TLR7) in specific immune cell subsets will reveal the cell type-specific requirements for these innate sensors in instructing the multiple aspects of adaptive immunity. Specific anatomical location of lung migratory DCs may determine the activation (in situ) and migration kinetics. For instance, lung migratory CD103+ cDCs which are positioned adjacent to respiratory epithelium are the first APC to be activated and arrive in mLN earlier than its counterpart CD11b+ cDCs which are positioned in the underlying lamina propria [67].

A recent study by Iwasaki group suggests there is a hierarchy of those viral signatures recognized by PRRs in the instruction of adaptive immunity to influenza virus [45]. In this study, they hypothesized that each of these viral signatures (ssRNA, dsRNA, replicating ssRNA, and viral M2 activity) represents varying degree of threats and therefore each correlates with qualitatively different immunogenicity. The signal emitted from virus-induced damage (viral M2 activity) via NLRP3 outweighs those signals derived from TLR7 or RIG-I for CD8 T cell induction [46]. CD8 T cell responses, which remain unaffected in the absence of TLR7 or RIG-I signaling, are sub-optimal in the absence of NLRP3 activation during influenza virus infection [48].

Robust DC activation is the key step towards generating optimal adaptive immune response against influenza virus infection. Factors that undermine DC activation correspondingly impair effector T cells and humoral immunity.
Recent studies demonstrated that IL-1β, an important cytokine potently activates DCs during infection, is constitutively generated in the form of pro-IL-1β (inactive form) in lung during the steady uninfected state. This tonic/basal level of pro-IL-1β is the instant source for IL-1β which can then act directly on DCs and initiate DC activation and migration upon infection [71]. Diminished pro-IL-1β pool, reduced IL-1β secretion, and loss of IL-1R signaling severely compromise DC activation and migration, leading to fewer lung migratory cDCs in the mLN and ultimately result in sub-optimal CD8 T cell response and enhanced susceptibility to infection [48, 71].
1.2.3.3 Migration of virus-antigen harboring DCs from lung to mLN

In as early as 12 hours post infection (P.I), DCs residing in the lung migrate in a CCR7- and sphingosine-1-phosphate (S1P) receptor dependent manner to mLN [14, 72-74]. Indeed, in CCR7-deficient mice and mice deficient for CCR7 ligands (also known as plt/plt mutant mice, lacking ccl19 and ccl21), lung DCs do not migrate to the MLN. [73] On the other hand, presence of S1P receptor antagonist, a sphingosine-1-analog, severely blunts T cell responses in LCMV infection models suggesting the significance of S1P receptor-mediated DC migration in the generation of optimal T cell response [72]. Relatively recent studies reported the indirect involvement of CCR5-CCL5 axis in the DC emigration from the lung to mLN using Sendai virus infection model. The mechanism of CCR5-CCL5 mediated DC trafficking appears to be associated with an increase in CCR7 expression in the presence of CCL5. In both CCR5- and CCL5-deficient mice, there are very few lung-derived DCs that travel to the MLN upon infection [75, 76]. It is noteworthy to point out that when influenza virus is administered intranasally, T cells in the mLN require lung migratory cDCs are required to carry viral antigens from lung to mLN in order to stimulate mLN T cells. Alternatively if the virus is delivered intravenously, the virus is able to gain direct access to mLN resident cDCs (CD8+ cDCs and CD8- cDCs) and hence these blood borne viruses induce T cell response via mLN resident cDCs obviating the need for lung migratory cDCs [14].

1.2.3.4 Virus antigen presentation by lung migratory cDCs in the MLN

CD4 and CD8 T cell priming and expansion in mLN requires viral-antigen-bearing lung migratory cDCs. Interestingly, GFP signal is undetectable in MLN of GFP-PR8 infected CCR7-deficient mice [77] indicating that influenza virus is highly unlikely to have migrated from lung to mLN independently of lung migratory cDCs [70]. Besides, there is no GFP signal in sub-capsular CD169+...
macrophage in which this macrophage population would be positive for GFP if virus migrates to the MLN independently of lung migratory cDCs [78]. Therefore, lung migratory cDCs, CD103+ cDCs and CD11b+ cDCs are the main APCs for the activation of naïve T cells in the MLN. It has been proposed that mLN-resident CD8+ cDCs also contribute to the cross-presentation and activation of naïve CD8 T cells in the MLN after influenza virus infection [79]. Investigations from some other studies however argued against the participation of mLN-resident CD8+ cDCs in the cross-priming of influenza-specific CD8 T cells in the MLN [70]. Though the relative contribution of mLN resident cDCs towards naïve T cell activation remain to be fully explored during influenza virus infection, several lines of evidence suggest that lung migratory cDCs represent the most dominant APCs that efficiently present viral antigens to naïve T cells in mLN [70].

In 2012, Helft et al demonstrated CD103+ cDCs as the predominant DC subpopulation in the lung carrying viral antigen from the lung to the mLN at the early phase of infection (12-72hours) [70]. In this time window, only 1 out of 10 lung migratory cDCs arriving in mLN are CD11b+ cDCs. Their results also showed that lung CD103+ cDCs and CD11b+ cDCs arriving in the mLN do not carry infectious viral particles and are not infected by virus. Instead, the viral antigens which are carried by lung migratory cDCs are derived from the dying virus-infected cells that are previously phagocytosed by these cDCs in the infected lung [70]. Using CD103+ cDCs-deficient mouse, lung CD103+ cDCs have been shown to be required for cross presenting viral antigens to mLN CD8 T cells and subsequent generation of effector CD8 T cell response. However, contribution of CD11b+ cDCs to the generation of CD8 T cell response during influenza virus infection is inconclusive due to lack of CD11b+ cDCs-deficient mouse model and the presence of contaminating monocytes/macrophages. Functional studies regarding the role of CD11b+ cDCs in the generation of protective CD8 T cell response are mainly derived from in vitro assay [13, 80, 81].

It is important to note that CD103+ cDCs and CD11b+ cDCs migrate at different speed and reach peak numbers in mLN at day 3 and day 5
respectively [13]. Therefore, contribution of CD103+ cDCs and CD11b+ cDCs in cross priming may dominate at different stages of infection [70, 79, 82]. In line with this argument, mLN CD103+ cDCs and CD11b+ cDCs have been shown to induce CD8 T cell proliferation at different stages of infection (early stage 2-4 days P.I versus intermediate stage 5-7 days P.I respectively) [13, 70].

1.2.3.5 Contribution of DCs to the maintenance of memory T cell population

The outcome of the interaction between virus infection and host immune response is either the clearance of virus or the development of chronic infection. Viruses such as LCMV, RSV and Sendai virus are known to produce chronic persistent infections because these viruses are able to evade or suppress the host immune response. Therefore viral antigens can be detected months after the infection. Recent data suggest that influenza virus persists in the form of mRNA and virus protein which is detectable in the lung long after the clearance of infectious virus [83-86]. The reservoir for viral antigen is believed to reside in both non-heamatopoietic (CD45-) and heamatopoietic (CD45+) cells in the lung. Using DC-deficient mouse, lung migratory DCs were shown to be responsible for the maintenance of memory T cells by carrying residual viral antigen depot in the lung to the mLN long after acute infection and virus clearance [87]. This means that virus-specific memory T cells are selectively enriched in the draining mLN and lung migratory DCs may influence the quality of the memory T cell response to secondary infection [87, 88].
1.2.4 Adaptive immunity to Influenza virus infection

1.2.4.1 Anti-viral CD8 T cell response

CD8 T cell response is indispensable for the efficient clearance of influenza virus infection in the lung. CD8 T cell response-deficient mice (MHC class I-deficient β2-microglobulin, β2m-/-) unequivocally display enhanced susceptibility to mild infectious dose [89]. CD8 T cell response is also strictly required during secondary influenza virus challenge for the efficient clearance of virus even when memory CD4 T cell response is intact [89].

1.2.4.2 Induction of CD8 T cell response

The generation of CD8 T cell protection is an intricate process. The induction phase requires naïve CD8 T cells to recognize cognate MHC-virus peptide complex displayed on the surface of lung migratory cDCs in mLN. Cognate recognition leads to naïve CD8 T cells activation, proliferation and differentiation to become effector CD8 T cells and eventually survive by a small pool of memory CD8 T cell population representing 5-10% of the expanded CD8 T cells. Interaction between lung migratory cDCs and naïve CD8 T cells and the subsequent CD8 T cell activation events are exclusively restricted to mLN during the first 3 days P.I [90]. During this period, activated CD8 T cells undergo phenotypic changes including up-regulation of activation marker CD69 and down-regulation of adhesion molecule CD62L [90]. In the process of differentiating from naïve to effector cells, these cells expand a few rounds concomitantly [90]. Proliferating CD8 T cells appear to be in contact with the virus antigen only for the first 2 divisions after which they proliferate in an antigen-independent manner [91, 92]. Effector CD8 T cells are equipped to secrete cytokines and release cytotoxic granules in the mLN even before the migration to the infected lung. After multiple divisions in the mLN, CD8 T cells exit from MLN and travel to the lung approximately 4-5 days P.I. Pioneer cohort of virus-specific CD8 T cells arriving in the lung proliferate extensively in situ [93]. Though influenza virus infection is a localized infection, effector CD8 T cells exiting from the MLN were seen disseminated to the spleen and
peripheral LNs as well. Nonetheless, these effector CD8 T cells arriving in lung and spleen are dissimilar in the expression of phenotypic marker CD69, a marker which is upregulated by the engagement between TCR and MHC-virus peptide complex [90].

**Effector mechanisms of CD8 T cells**

Effector CD8 T cells eliminate influenza virus infected cells by Perforin-/Granzyme B-, FasL- and TRAIL-mediated cytotoxic mechanisms (Effector CD8 T cells are also known as Cytotoxic T lymphocytes, CTL) [67]. Perforin and Granzyme B act synergistically to induce apoptosis. They are packaged in CTL granules in which the granules release is triggered when TCR comes in contact with virus infected cells displaying cognate MHC class I-virus peptide. Due to the highly toxic nature of Perforin and Granzyme B, granules release is activated in a short burst and directed at the contact point between CTL and targeted cells [94]. Fas ligand (FasL) - Fas interaction induces apoptosis in Fas expressing virus infected cells. Unlike granules release which occurs in a short burst, FasL expressions on CTLs persist for longer periods and are less tightly regulated [95, 96]. TRAIL expression augments CTL cytotoxicity in which loss of TRAIL leads to elevated virus titers and increased disease severity. Up-regulation of DR5 (TRAIL receptor) on virus infected cells render these cells susceptible to TRAIL-induced apoptosis [97].

Apart from cytotoxic effector function, effector CD8 T cells produce cytokines IFN-γ, TNF-α and IL-10. Though IFN-γ has been to shown to influence cellular infiltrate in the lung and antibody isotypes, impact of IFN-γ in the course of primary infection is modest and generally does not affect virus clearance, CD8 T cells recruitment and CTL cytotoxic activities [67, 98, 99]. However, adoptive transfer study showed that wild type CD8 T cells but not IFN-γ-deficient CD8 T cells ameliorated disease severity and lung damage [100]. While some studies observed decisive role of IFN-γ in conferring protection during secondary virus challenge, observations from other studies suggest a dispensable role of IFN-γ in mounting effective response during the re-infection challenge [98, 99, 101, 102]. TNF-α by effector CD8 T cells have been noted for its role in inducing lung damage and severe pathology [40, 103]. Recognition of MHC-I-virus
antigen complex which is displayed on virus infected epithelial cells by effector CD8 T cells triggers TNF-α secretion [103]. TNF-α in situ promotes cytolysis in infected epithelial cells and induces these dying epithelial cells to produce chemoattractants for various inflammatory infiltrates hence greatly sustaining and augmenting the inflammation process in the lung [103]. Therefore respiratory epithelial cells actively participate in inflammation and lung injury in the presence of TNF-α sufficient CD8 T cells. Effector CD8 T cells are major producers of regulatory cytokine IL-10 during the peak of response in lung followed by rapid disappearance of IL-10- secreting-CD8 T cells in the resolution phase of infection [104]. Neutralization of IL-10 leads to severe lung pathology, increased morbidity and susceptibility [104].

1.2.4.3 Contraction of effector CD8 T cell response and generation of memory CD8 T cell pool

IFN-γ signaling and IL-7 are both important during the contraction phase of the generated CD8 T cell response though IL-7 effect is apparent only when IFN-γ signaling is abrogated. IFN-γ is required for the contraction of effector CD8 T cell population. IFN-γ-deficiency leads to up-regulation of IL-7R expression whereby IL-7 signaling is known to induce anti-apoptotic mediators. Therefore, in the absence of IFN-γ, IL-7 signaling protects and increases resistance of differentiated CD8 T cells from cell death and subsequently this abrogation of contraction phase eventually results in larger percentage of memory cells. It is important to note that role of IFN-γ in CD8 T cell contraction is specific to influenza virus infection. While some studies using LCMV (systemic virus infection) and Listeria (bacterial infection) infection models reported possible role of IFN-γ in CD8 T cell response contraction, [105, 106] others studies using CMV and VSV infection models reported irrelevant role of IFN-γ signaling in this aspect [107, 108]. On the other hand, CD40 signaling is required to maintain robust CD8 T cell response, preventing premature contraction [109]. Specifically, CD40-CD40L (also known as CD154) interaction between CD40-expressing DC and CD40L-expressing CD4T cells
is required to counteract Treg cells-mediated suppression of CD8 T cell response [109].

TRAIL, chemokine receptor CCR5 and CXCR3 have also been reported to regulate the contraction of anti-viral effector CD8 T responses [110, 111]. Influenza-specific CD8 T cells in Trail -/- mice were less proliferative and more viable in which this resulted in a larger pool of influenza-specific CD8 T cells in the lung [110]. CCR5 and CXCR3 double deficiency led to increased viability of influenza-specific CD8 T cells and followed by a greater population of CD8 memory T cells [111].

1.2.5 Anti-viral CD4 T cell response

Naïve CD4 T cells are not required for virus clearance [112, 113]. CD4-T cell-deficient mice, aside from displaying only slightly delayed virus clearance, show similar lung immunopathology compared to wild type mice [112, 113]. However, adoptive transfer of in vitro- or in vivo- generated HA-specific effector CD4 T cells has been shown to confer protection against lethal influenza infection suggesting that during primary infection, activation of naïve CD4 T cells into effector CD4 T cells is not sufficiently fast to combat the exponential increase of replicating virus [114, 115].

1.2.5.1 Induction of anti-viral CD4 T cell response

The process of effector CD4 T cell generation closely parallels effector CD8 T cell generation in terms of the tempo of activation and tissue distribution [116]. Unlike effector CD8 T cell response that primarily originates in the mLN, generation of effector CD4 T cell response occurs in the spleen as well as the mLN [90]. At the end of the induction phase in mLN, the effector CD4 T cells (and also effector CD8 T cells) are heterogeneous in terms of their differentiation and division status [117]. In fact, while some of the primed CD8 or CD4 T cells have undergone sub-optimal differentiation, some other T cells have undergone complete differentiation after the encounter between naïve CD4 or CD8 T cells and lung migratory cDCs in the mLN. Consequently, those sub-optimally primed T cells for instance CD8 T cells can only produce IFN-γ
or IL-2 but do not acquire cytolytic whereas those primed CD8 T cells which have undergone complete differentiation program acquire cytolytic function [117]. The division status of these primed T cells are asynchronous and the activation markers are differentially expressed the time they leave mLN for lung. Nonetheless, among this broad spectrum of phenotypically and functionally distinctive T cells, the most activated and differentiated ones are likely end up in the lung [90, 116].

**Effector mechanisms of CD4 T cells**

One of the most extensively studied CD4 T cell effector functions is the helper function (provided by specialized subset of CD4 T cells known as follicular-helper T cell (Tfh)) for B cells activation, germinal centre (GC) formation, isotype switching and affinity maturation of virus-specific antibodies. Pioneer study by Crotty et al described that Tfh communicate with B cells through signaling lymphocytic activation molecule-associated protein, or SLAM-associated protein (SAP) without which the mice showed loss of virus-specific plasma cells and memory B cells [118]. Subsequent study by Kampschroer et al reported Tfh are indispensable for the optimal expansion of antigen-specific B cells in the early stage of primary infection [119]. Provision of CD40L and ICOS signals by Tfh is equally critical for the optimal generation of humoral response to influenza infection [119]. Unlike in the context of innocuous antigen or HSV infection model, helper function of effector CD4 T cells is not required for the generation of primary CD8 T cell response during primary influenza virus infection [120, 121]. In addition, neither CD8 cytotoxic activities nor recruitment of effector CD8 T cells to lung requires presence of effector CD4 T cells.[120] However, there is a clear role for CD4 T cell-mediated help for the generation of effective memory CD8 T cell protection [121]. CD4-deficient mice exhibited diminished in size and recall response of memory CD8 T cells regardless whether homologous or heterologous virus strains are used for secondary virus challenge [121].

Effector CD4 T cells are not only lymphocyte helpers, they migrate and accumulate in lung as efficiently as CD8 effector T cells [116, 122]. Effector CD4 T cells utilize perforin-mediated apoptosis-inducing pathway to eliminate
virus-infected cells. This specific cytotoxic CD4 Th1 subset is considered a distinct functional T cell population on its own as it does not depend on transcription factor T-bet, which is a CD4 T-helper-1 specific transcription factor [123]. Unlike CTL granules release that is triggered upon recognizing MHC class I-virus peptide complex displayed on virtually all infected cells, cytotoxic CD4 T cell perforin release requires recognition of MHC class II-virus peptide complex. Therefore during influenza virus infection, there are limited number of targets susceptible to cytotoxic CD4 T cell killing since majority of the cells do not express MHC class II. However, some cells other than APCs are capable of up-regulating MHC class II expression. The most notable ones include respiratory epithelial cells which upon infection or IFN-γ influence, up-regulate expression of MHC class II [124, 125].

1.2.6 Humoral Immunity

Influenza virus infection initiates robust B cell response that is characterized by multiple B cell subsets working coordinately to provide protective antibodies during and after infection [126]. Before infection, naturally occurring IgMs of which a portion is influenza-specific constitute the first line of defense against the virus [127-129]. Of the 10 influenza proteins, antibody epitopes have been identified in 5 (HA, NA, M2, M1 and NP) of them. Majority of the investigations focus on the study of surface proteins HA, NA and M2 [130].

In the early phase of influenza virus infection (Day 1 to 5 P.I), antibody response against the virus is dominated by steady state IgMs (natural IgM) and infection-induced neutralizing IgMs [131]. Both are secreted by B-1 cells. However, these steady state IgMs (natural IgMs) are able to recognize influenza virus [128, 132]. B-1 cells are the only cell source for natural IgMs with little to no contribution from follicular B cells (also known as B-2 cells) to this natural IgM pool [128]. Unlike follicular B cells which robustly secrete tremendous amount of influenza specific IgMs and IgGs following infection, B-1 cells do not up-regulate antibody production in response to influenza virus infection [128].
In the later phase of influenza virus infection, T-helper cells induce T-dependent B cell response (refer to follicular B cell subset) and formation of germinal centre (GC). CD40 signaling is absolutely required for B cell activation, expansion, somatic hypermutation, isotype class-switching and GC formation during influenza virus infection. Influenza virus-specific long lived antibody forming cells (AFCs) and memory B cells, which are hallmarks of T dependent B cell activation, have been demonstrated in both human and mouse. They are important source of virus-specific antibodies that provide long term protection from secondary infection. Among the class-switched anti-viral antibodies, IgG2 (IgG2a and IgG2b in balb/c, IgG2b and IgG2c in C57Bl/6) represents the largest faction within GC in the MLN and spleen. Despite strong GC reaction in spleen, splenectomized mice are able to survive lethal influenza virus challenge. Anti-viral IgAs are scarce in these 3 sites throughout the infection.

While provision of CD40 signaling by Tfh cells alone is both sufficient and necessary for antibody isotype class switching, innate signal IFN-α is able to fine tune influenza specific IgG antibodies specifically it has been shown to increase IgG2c class switching at the expense of IgG1 class switching [133]. Factors affecting IgG subtypes are vital given the importance of IgG subtypes (in particular IgG2c in influenza infected C57BL/6 mice) [134] in both prophylactic and therapeutic benefits in passive serum transfer experiment [135]. IgG2c antibodies represent the more effective subtypes than IgG1 in terms of virus clearance and protection against lethal influenza challenge. [136] On the other hand, IgM and IgA do not cure the host when given after infection starts, though these subtypes confer protection against infection when given prophylactically [135].
**1.2.7 Immunopathology**

Virus burden or its intrinsic cytopathic effect does not necessarily contribute to mortality [40]. In fact, higher virus load in influenza-infected lung does not always lead to higher mortality. Indication of dysregulated immune response-mediated, rather than influenza-virus cytopathic-triggered, pathology came from the study of patients infected with highly pathogenic viruses showing multiple organ failures although influenza virus replicates primarily in infected lung tissue only. More direct evidence was derived from a recent study on mice infected with mutant virus expressing 1918 influenza virus HA and NA in which investigators observed exaggerated production of pro-inflammatory cytokines and chemokines. Excessive presence of cytokines and chemokines, termed cytokine storm, is believed to be the mastermind underlying the cytokine-driven clinical feature observed in severely infected patient called reactive haemophagocytosis, a disorder associated with multiple organ failure. In general, severity of the infection correlates strongly with cytokines and chemokines level with reported cases on elevated serum levels of IL-6, TNF-α, IFN-α and IFN-γ. Detailed studies using mouse models infected with experimental influenza virus strains have shed important insights on the dynamics of the virus-host interactions, in particular how the hosts mount and orchestrate effective anti-viral response. Identification of the cellular infiltrates that are responsible for the production of excessive pro-inflammatory cytokines and chemokines, reactive-oxygen species and nitric oxide radicals would provide potential targets for clinical interventions [137, 138].

Respiratory epithelial cells, being the first to get infected, are the main replicating site for virus and subsequent spreading to alveolar macrophages [40, 139]. Infected respiratory epithelial cells release the first wave of cytokines and chemokines in particular chemokine CCL2 (also known as MCP-1) has been shown to recruit significant amount of circulating monocytes which later become inflammatory macrophages (also termed exudate macrophage) [140]. Exudate macrophages induce massive cell death in alveolar epithelial cells via TRAIL-inducing pathways as a result of which causing severe lung leakage.
and increased morbidity [141]. Exudate macrophages are the major producers of TNF-α and Nitric Oxide (NO) both of which aggravate lung injury with no apparent effect on reducing virus load [142, 143]. Another early inflammatory infiltrate, NK cells, have been associated with increased lung pathology in high dose influenza virus challenged mice but confer protection against low dose challenged mice. The mechanism behind NK cell-driven lung pathology is uncertain [144, 145].

Similarly, whether CD8 T cells cytotoxic activities are protective or detrimental to the lung physiology are virus dose dependent [146]. It is believed that timing of CTLs appearance in the infected lung is a key factor to determine the infection outcome. Early appearance and intervention by CTLs followed by rapid contraction positively impact the disease outcome [146]. Dosage effects have also been reported in the comparison of CTL-mediated effect for young and old mice in which CTLs were associated with protective effect in young mice infected with low dose influenza virus, on the contrary CTLs aggravated lung pathology and produced higher mortality in young mice infected with a more virulent strain [147, 148]. So far there is no general consensus regarding the effect of TNF-α produced by virus-specific CD8 T cells during influenza virus infection. While some studies suggest TNF-α production by CD8 T cells is associated with increased lung pathology, investigations from other studies demonstrated that TNF-α regulates CD8 T cell response and limits lung pathology. This discrepancy is likely due to different experimental settings but the less disputed proposition is that high levels of TNF-α usually contribute to severe lung pathology especially if mice were infected with more virulent virus strains that induce overt pro-inflammatory cytokine signatures [146, 149-152].

**Immunotherapies for influenza-induced lung acute injury**

Current immunotherapeutic strategies to mitigate acute lung injury (ALI) can be classified into those intended to enhance anti-viral resistance, increase host tolerance and reduce tissue damage that is mediated either by virus intrinsic virulence (e.g. replication capacity and virus NS1 protein) or host aberrant immune response. Agents to boost anti-viral resistance should be administered early in the infection and should be without inflammatory side
effect. Recombinant Type I IFN is a good candidate but it induces flu like symptoms. Recent study reported that protectin D1, a polyunsaturated fatty acid related molecule, effectively blunt influenza virus replication by blocking the export of virus RNA from the nucleus into the cytosol [153]. It has been shown to confer protection and reduce virus titers in mice infected with lethal dose of influenza virus [153].

The importance to increase host tolerance is due to the fact that certain cells, for instance vascular endothelial and respiratory epithelial cells, are very vulnerable to oxidized species that are generated during infection. The well-being of these cells is important for the proper functioning of vasculature and respiratory system. Therefore, protecting endothelial and respiratory epithelial cells from the reactive oxidized species, for instance oxidized phospholipids which are generated in situ have been shown to bind TLR4 and trigger inflammation and damage in the lung, would be beneficial to the hosts. Recombinant human catalase and apocynin, both of which reduce the amount of reactive oxygen species have been shown to confer partial protection [154, 155].

Exceedingly high level of pro-inflammatory cytokines is an overt signature of the infection caused by highly pathogenic influenza virus strains. Generally, severity of lung lesions correlates with cytokine levels. Reducing cytokine-induced inflammation is a straightforward approach to ameliorate lung damage. However patients, who have been given neutralizing antibodies against these inflammatory mediators, were consistently rendered incapable of clearing virus effectively and therefore led to elevated level of virus titers [40]. These inflammatory mediators thus represent double-edge swords; on one hand they are positively correlated with the disease severity on the other hand they are required for effective virus clearance. TNF-α is an interesting exception in which anti-TNF-α neutralizing antibody treatment reduce lung pathology and mortality without compromising virus clearance [40]. TLR4 dependent inflammation in lung during influenza virus infection leads to severe lung pathology. Eritoran, a TLR4 antagonist, blocks the binding of oxidized
phospholipids to TLR4 and has been shown to protect mice from lethal infection [156].
1.3 In vivo analysis of DC function

1.3.1 Strategies to study the function of Dendritic cell

Several different approaches have been proposed to investigate the functions of DCs in vivo. In vivo targeting of antigen via lectin receptors, DCs transfers and constitutive cell ablation are valuable tools to disentangle the contribution of DCs in innate and adaptive immune response. Also gene targeting of transcription factors is a powerful strategy to selectively deplete targeted cell population. To achieve selective DC ablation, mice have been engineered for deficiency in crucial transcription factor that regulates DC development. Batf3 is one such transcription factor that is highly expressed in cDCs with little or no expression in other immune cells and deficiency in batf3 (batf3 -/- mice) leads to selective loss of CD8+ cDCs. Studies on batf3 -/- mice showed that these transgenic mice displayed defect in cross presentation, lacked virus specific CD8 T cell responses and were unable to reject syngeneic tumors indicating that CD8+ cDCs are important in cytotoxic T cell immunity in responses to viruses and tumor rejection [157-159].

Unfortunately, constitutive loss of specific cell population may trigger compensatory mechanisms in the host of missing targeted population and there is a possibility that the physiology of the deficient host is no longer the same especially when the targeted population is developmentally important. To circumvent this problem, an inducible cell specific ablation system therefore represents a more favorable strategy to analyze in vivo functions of cells. In 2001, Saito et al introduced a conditional and cell-specific ablation approach utilizing DT-DTR system (Figure 6) [160]. DT is a bacterial exotoxin secreted by Corynebacterium diptheriae. Its receptor (DTR) has been identified as a membrane anchored form of the heparin binding epidermal growth factor (EGF) like growth factor (hbEGF precursor). DT mediated cytotoxicity is strictly dependent on DTR-mediated endocytosis. Upon binding to DT subunit B of the heterodimeric toxin, DTR triggers DT endocytosis and incorporation of DT into the endosomal compartment. Subsequently, DT subunit A translocates from the endosome into the cytosol where it induces cell apoptosis as described.
above [160]. DT is an extremely potent toxin in human and primates yet rodents are $10^5$ more resistant to DT [161]. Such drastic difference is due to the three amino acids changes in the binding region of DTR to DT that significantly reduce the affinity of rodent DTR towards DT. Therefore, inducible ablation of specific cells can be achieved by the expression of human DTR in the target cells of transgenic mice and the delivery of DT. The advantage of DT-DTR system is that ablation should be specific and collateral damage should be minimal in the transgenic DTR mouse models considering the non-targeted cells express highly-resistant rodent DTR. Another advantage in this system is that targeted population should be ablated with high efficiency given one molecule of cytosolic DT-A is sufficient to kill the cell [162].

Figure 6: Experimental design for DT/DTR system-mediated inducible cell specific ablation approach. The transgene construct contains human DTR (hbEGF) cDNA and tissue or cell specific promoter. This transgene is subsequently microinjected into fertilized egg for the generation of DTR transgenic mouse (adapted from [160]).
1.3.2 DT/DTR systems for Dendritic cell ablation

1.3.2.1 CD11c-DTR and zDC-DTR

In 2002, Jung et al described a CD11c-DTR mouse model for the functional study of DCs in vivo. In CD11c-DTR transgenic mice, human DTR gene is expressed under the control of a cloned Itgax promoter (a minimal promoter region to drive expression of CD11c gene, hence referred to as CD11c promoter).[163, 164] As such, human DTR would be expressed on all CD11c-expressing cells and render these cells susceptible to DT toxicity [163]. Jung et al showed CD11c-expressing cells were efficiently ablated in spleen, bone marrow, colon, lymph nodes and lung one day after a single dose DT injection. Their study showed reduced CTL response against Listeria monocytogenes bacteria and plasmodium yoelii parasite in absence of CD11c-expressing cells which indicates their importance in cross presenting and induction of protective CTL response. However, CD11c promoter is active not only in DCs, but also in some non-DC lineage, for instance splenic marginal zone and metallophilic macrophages, pulmonary alveolar macrophages, activated CD8+ T cells and plasmablast, making these cells also targets in CD11c-DTR transgenic mice [165-168].

CD11c-DTR mice created by S. Jung are vulnerable to repeated DT injection probably due to the aberrant expression of human DTR on non-immune cells such as epithelial cells of the gut. CD11c-DTR mice do not survive a second DT infection within less than 7 days and depletion is sustained only for 2-3 days after a single dose of DT.[169] Experiments involving prolonged DC depletion therefore require radiation chimera in which irradiated wild type mice are reconstituted with CD11c-DTR mice bone marrow. In these CD11c-DTR chimeras, non-immune cells are wild type origin and therefore cannot express DTR, obviating the deleterious effect of DT in the chimeric mice. However, there are radiation resistant DCs for instance langerhan cells and a subset of dermal DCs; as such they may complicate the interpretation of DC depletion experiments using CD11c-DTR chimeric mice [169]. In 2008, Hochweller et al described a novel CD11c-DTR transgenic mouse called CD11c-DOG. Both CD11c-DTR and CD11c-DOG mice share identical cell ablation profile but
CD11c-DOG mice allow effective depletion over prolonged periods without non-specific cytotoxicity. In 2012, Tittel et al described another CD11c-DTR transgenic mouse called CD11c-LuciDTR in which it displayed similar cell ablation profile as the CD11c-DTR mice. Tittel et al reported neutrophilia in all the CD11c-DTR transgenic mice but the kinetics of onset and recovery is different. They observed that CD11c-LuciDTR mice displayed a more delayed onset of neutrophilia than CD11c-DTR and CD11c-DOG. CD11c-LuciDTR mouse is therefore more suited for short term analysis in particular those experimental outcomes that may be affected by neutrophilia.

Although CD11c-DTR transgenic mice have proven useful for the in vivo functional study of DC biology, the presence of CD11c expression on non-DC lineage have greatly limited its usage in the study addressing DC-specific functions [169]. Functional roles assigned to DCs using CD11c-DTR transgenic mice should therefore be carefully examined. Nonetheless, DT-DTR system presents a conditional and efficient cell-specific ablation strategy that is extremely useful in the functional study of dendritic cells in vivo. To achieve DC-restricted ablation, Meredith et al described a zDC-DTR mouse model in which they inserted human DTR into the 3’ untranslated region of the zinc finger transcription factor (zbtb46 aka zDC) gene [170]. Zbtb46 expression is restricted to cDCs and some minor population of activated monocytes but is absent on pDCs, macrophages and other immune cells making it a DTR model suited for cDCs study [170]. Though zDC-DTR mouse is a useful model for the function study of DC-restricted lineage, in view of the numerous subsets of DCs in various LT and NLT (refer to DC lineage and distribution) compartment and the wide-ranging yet non-overlapping functions of these subsets, there is a need to generate DC-DTR mouse to inducibly deplete these specific DC subsets rather than all DCs.
1.3.2.2 Clec9a-DTR and Clec4a4-DTR

Genetic strategy in cell-ablation hinges on the uniqueness of the cell-specific promoter as it determines the specificity of the targeted ablation. Identification of the specific markers to define various different DC subsets represents the main limitation. Recent studies have shed some light on a number of DC-lineage specific marker and these markers have since received special attention due to their unique expression patterns on various specific DC subsets. Two of these markers Clec9a (also known as DNGR-1) and Clec4a4 (also known as DCIR-2) are of outstanding interest due to their restricted expressions on certain DC subsets.

1.3.2.3 Clec9a DC-lineage marker and Clec9a-DTR

Clec9a belongs to a family of C-type lectin-like molecules which are encoded by genes positioned on mouse chromosome 6. Many of the C-type lectin family members are expressed on the surface of DCs. Clec9a seems to be more tightly restricted to both mouse and human DCs and shows no expression on monocytes, macrophages and T cells. Detailed study shows that Clec9a expression is DC-lineage restricted including lymphoid tissue CD8+ cDCs, some pDCs and non-lymphoid tissue CD103+ DCs. In contrast, lymphoid tissue CD8- cDCs, non-lymphoid tissue CD11b+ DCs and monocyte-derived inflammatory DCs do not express Clec9a.

Therefore clec9a can be potentially used to distinguish different DC subtypes in the mouse. Its restricted expression profile makes it a useful target for antigen delivery to Clec9a-expressing DC subset. After a single injection of antigen-Clec9a conjugates, the antigen was effectively targeted to Clec9a-expressing DC. Consequently there is striking increase in antibody response and enhancement in CD4 and CD8 response in these treated mice. Functionally, clec9a is a dead-cell recognizing receptor. It recognizes normal cell component which is exposed when the cell membrane is ruptured. In particular, splenic CD8+ cDCs have been shown to take up dead cell
remnants and cross-present to CD8+ T cells against cell-associate antigens in clec9a-dependent manner.

Clec9a is an extremely useful marker for two reasons, firstly its expression is highly DC-lineage restricted and secondly it is expressed by certain DC subsets. Our lab has since generated a Clec9a-DTR transgenic mouse in which we have shown specific ablation of CD8+ cDC subset and partial pDC population in spleen and CD103+ DCs in the gut (unpublished data) [22].

**1.3.2.4 Clec4a4- DC-lineage marker and Clec4a4-DTR**

Clec4a4 belongs to a C-type lectin superfamily [171]. Cytoplasmic tail of Clec4a4 harbors an ITIM motif, suggesting inhibitory functions for this receptor molecule [171]. 33D1 antibody recognizes Clec4a4 and is used to identify Clec4a4 expression. Clec4a4 expression is DC-lineage restricted and is specifically found on CD8- cDCs in spleen, lymph nodes and Peyer’s patches (PP) but is absent on the CD8+ cDC subset in these tissues. Clec4a4-expressing splenic CD8- cDCs (33D1+ CD8- cDCs) are splenic DC population specialized for antigen presentation on MHC class II contrasting with the splenic CD8+ cDCs which are specialized for antigen cross-presentation on MHC class I [172]. The differential antigen presentation displayed by CD8+ cDCs and CD8- cDCs is related to the corresponding increased expression of proteins involved in MHC class I and MHC class II processing respectively [172]. Clec4a4 restricted expression pattern allows it to distinguish CD8- cDCs from CD8+ cDCs in the lymphoid tissue compartment. Using clec4a4 promoter to drive human DTR expression, our Clec4a4-DTR mouse displayed specific ablation of CD8- cDCs in the lymphoid tissue and a specific DC subset in the gut (CD103+ CD11b+ DCs, unpublished data) [22].
1.4 Aims and objectives

While the importance of lung cDCs in the generation of optimal CD8 T cell response is well established, the relative contribution of distinct DC subpopulations to this process remains poorly addressed owing to the lack of mouse models to specifically ablate these distinct DC subsets. In this study, we sought to understand the roles of two major cDC subpopulations in the lung, CD103+ cDCs and CD24+CD11b+ cDCs in influenza immunity. Clec9a-DTR and Clec4a4-DTR transgenic mice allow specific ablation of CD103+ cDCs and CD24+CD11b+ cDCs respectively hence providing a valuable tool to address their in vivo contribution to the immune protection and disease pathogenesis during influenza virus infection. Our specific aims were:

1. To assess the impact of distinct cDCs on the immune protection against influenza virus infection
2. To investigate relative contribution of distinct cDCs to the generation of anti-viral CD8 T cell response in lung, MLN and BALs
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Mice

Wild type C57BL/6 and BALB/c mice were purchased from the Centre for Animal Resources of National University of Singapore (NUS) or obtained from animal facility in Nanyang Technological University (NTU). Clec9a-DTR and Clec4a4-DTR transgenic mouse models were generated in BALB/c genetic background. Influenza virus infection was carried out in F1 mice, generated by crossing Clec9a-DTR or Clec4a4-DTR BALB/c with C57BL/6. Transgenic mice were bred and maintained under specific pathogen-free (SPF) conditions in NTU animal facility. All experiments were approved by the Institutional Animal Care and Use Committee.

Clec9a-DTR mouse strain was generated in our laboratory via BAC recombineering approach and was described in a published study [22]. Clec4a4-DTR mouse strain was obtained via gene targeting approach. Briefly, the targeting construct containing IRES-DTR cassette followed by removable selection marker (PGK-NeoR) was inserted after the stop codon of Clec4a4 gene (Supplementary Figure 1). After electroporation of this targeting construct, several BALB/c ES colonies carrying desired DTR insertion after the stop codon of Clec4a4 were obtained. These ES colonies were selected and used for blastocyst microinjection which led to the generation of chimeric animals and ultimately germ line transmission of the modified allele. Obtained Clec4a4-DTR knock-in mice are viable, fertile and phenotypically indistinguishable from the WT mice.

2.1.2 Influenza A virus

Influenza virus strain A/PR/8/34 (H1N1) and recombinant OTI-PR8 were gifts from Dr. Sivasankar Balasubramanian (Singapore Institute for Clinical Sciences/SICS, Singapore). Influenza virus strain A/X-31 (H3N2) was a gift from Prof. David Michael Kemeny (NUS Graduate School for Integrative
Sciences and Engineering). PR8 were used in all influenza experiments. X-31 were used to immunize mice prior to secondary lethal PR8 challenge in the heterosubtypic immunity experiment.

2.1.3 Chemicals, Reagents and kits

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2.1.4 Media, buffers and solutions

Please refer to Appendix.
# 2.1.5 Commercial Antibodies and live/dead cell dye

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2.1.6 Computer software

Flow cytometry data analyses were done using Flowjo 7.6.1 software (TreeStar Inc, Ashland, OR). Graphs and statistical analyses were generated using Graphpad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA).

2.2 Methods

2.2.1 Influenza virus infection and weight loss monitoring

Each mouse was given 10mg ketamine/kg body weight mixed with 2mg xylazine/kg body weight intraperitoneally to induce anesthesia before intranasal delivery with the designated dose (plaque forming unit, PFU) PR8/X-31 in 25ul of PBS. Female F1 mice of 6-8 weeks of age were routinely used for influenza infections. Weights were measured daily throughout the course of infection.

2.2.2 Tissue collection, processing and isolation of cells

Mice were culled by cervical dislocation. Broncho-alveolar lavage (BAL) fluid was extracted by performing lung lavage 3 times with 0.5 ml PBS. BAL contained cells residing in the alveolar space.

After BAL extraction, lung tissues were perfused with 10ml PBS before excision. Excised lung tissues were cut into small pieces and incubated with 2mg/ml Collagenase D for 60 mins with agitation. Digested lung tissues were passed through 70 μm cell strainer to obtain single cell suspension. The cell suspension was spun down and resuspended with 3 ml 70% Percoll™ followed by overlay of 3ml 40% Percoll™. The gradient was centrifuged at 2800 rpm for 15 mins at room temperature without brake after which a ring of cells formed at the interface between the two solutions. The ring of cells was transferred to clean tube containing 7 ml of PBS 2%. The lung cell suspension was centrifuged at 450 g for 10 mins followed by resuspension in PBS 2%.
The MLN were harvested, cut into small pieces and incubated with 2 mg/ml Collagenase D for 60 mins. Digested MLN were resuspended a few times to obtain single cell suspension. BAL fluid, lung and MLN single cell suspensions were spun down at 300 g for 5 mins, followed by resuspension with RBC lysis buffer to lyse red blood cells. After 10 mins incubation at room temperature, these cell suspensions were centrifuged and resuspended with PBS 2%. BAL, lung and MLN cellularity was enumerated by Trypan blue exclusion.

2.2.3 Isolation of peripheral blood mononuclear cells (PBMCs)

Mice were anesthetized with isofluorane while blood was collected via retro-orbital route. Blood was collected into tube containing 3% Sodium Citrate solution to prevent coagulation. This mixture was subsequently layered above Ficoll-Paque™, a solution that is commonly used to separate PBMCs from erythrocytes. The gradient was centrifuged at 900 g for 30 mins with no brake. After centrifugation, a layer of cells consisting of PBMCs would be formed and this layer of cells was transferred to clean tube containing 5 ml PBS 2%.

2.2.4 Cells labelling for flow cytometry

Single cell suspensions of BALs, lung, MLN and blood were prepared as described in Sections 2.2.2. For staining of cell surface antigen, fluorochrome-labelled antibodies were incubated with the cells at 4°C for 20 mins, washed and resuspended in PBS 2% for analyses. For intracellular staining for Ki-67, it was done according to manufacturer's instructions (eBioscience). Briefly, after staining procedure for cell surface antigens, cells were fixed, permeabilized before staining for Ki-67 at 4°C for 20 mins after which the cells were washed and resuspended in PBS 2% for analyses. For detection of intracellular cytokines, cells from Sections 2.2.8 were first stained for cell surface antigen CD3, CD4 and CD8, then fixed and permeabilized before staining with anti-IFN-γ and IL-10 antibodies diluted in 0.5% saponin. Stained cells were subsequently washed and resuspended in PBS 2% for analyses.
To stain for influenza-specific CD8 T cells, PE-labeled H-2Db MHC class I Dextramer™ of Influenza A Nucleoprotein epitope ASNENMETM (NP
366-374) was used. Briefly, single cell suspensions from BALs, lung, MLN and blood from Sections 2.2.2 were stained for fixable dead cell stain Live/Dead Fixable® Violet Dead Cell stain or Zombie Aqua™ Fixable Viability Dye (procedure done according to manufacturer's instruction), after which the cells were stained for H-2Kb MHC Dextramer™ NP
366-374 (procedure done according to manufacturer's instruction) followed by staining for other cell surface antigens. Stained cells were subsequently washed with PBS 2% and resuspended in fixation buffer for analyses. The MHC class I Dextramer™ comprises a dextran polymer backbone harboring optimized number of MHC and fluorochrome molecules. It carries more MHC and fluorochrome molecules than conventional MHC multimers hence increasing their avidity for the specific T cells, enhancing signal intensity and improving signal-to-noise ratio.

For detection of cell death and apoptosis, single cell suspensions from lung from Sections 2.2.2 were first stained for Annexin V followed by staining for H-2Kb MHC Dextramer™ NP
366-374 (both procedures done according to manufacturers' instructions) followed by staining for other cell surface antigens. Stained cells were subsequently washed with PBS 2% and resuspended in PBS 2% containing PI for analyses. Annexin V is specific for phosphatidylserine (PS) which normally present in the intracellular leaflet of plasma membrane. During early apoptosis, PS translocates to the external leaflet and this exposure allows binding of annexin V. Early apoptotic cells exclude PI, while late stage apoptotic cells (or dead cells) and necrotic cells permit entrance of PI for DNA staining.

Samples were acquired on a cytometer (BD FACSCalibur, BD LSRII or BD LSRFortessa). Leukocytes were gated based on forward- and side-scatter properties (FSC, SSC) and live cells were gated based on exclusion of cells staining positive for PI, Live/Dead Fixable® Violet Dead Cell stain or Zombie Aqua™ Fixable Viability Dye.
2.2.5 Serum passive immunization

Clec9a-DTR, Clec4a4-DTR and wild type mice were infected with 8 PFU influenza virus PR8. After 10 days of infection, retro-orbital bleeding was conducted on all the infected mice. Collected blood was centrifuged at 11,000 g for 10 mins (4ºC). After centrifugation, upper layer serum solution was transferred to clean tube. One dose of infected mice sera (50 µl each) were transferred i.v to naive mice one day prior to intranasal challenge with 32 PFU or 64 PFU influenza virus PR8.

2.2.6 Cytokines Enzyme Linked Immunosorbent Assay (ELISA)

BALs were collected from mice after day 6 and 10 P.I. BALs were collected by flushing lung airways and alveolar space 3 times with 0.5 ml PBS each after which they were centrifuged at 11,000 g for 10 mins (4ºC). Supernatants were transferred into clean tube for storage (-20ºC). ELISA for cytokines IFN-γ, IL-6, TNF-α and IL12-p40 were conducted.

One day prior conducting the assay, 100 µl of capture antibody (diluted in 1X PBS) was added into each well of a 96-well Nunc Maxisorp plate. After overnight coating in 4ºC, the plate was washed 3 times with wash buffer and added with 100 µl assay buffer per well for 1 hour at room temperature. This step reduces the non-specific binding and improves signal-to-noise ratio. Assay buffer was decant, washed 4 times with washing buffer and added with 100 µl of BAL samples into each well. After 2 hours incubation at room temperature, the plate was washed 4 times, and incubated with 100 µl per well of detection antibody for 1 hour at room temperature. Subsequently, the plate was washed again for 4 times and incubated with 100 µl per well of Avidin-HRP for 30 mins before another 5 times washing and incubation with 100 µl per well of TMB substrate. Optical Density (OD) at 370 nm was measured and recorded every 5 mins for a period of 30 mins.
2.2.7 DT-mediated ablation
For DT-induced ablation of targeted DC subpopulations, Clec9a-DTR and Clec4a4-DTR mice were injected i.p with 20ng/body weight of DT (in PBS supplemented with 1% mouse serum) for 2 consecutive days. For DC ablation profilings, mice were sacrificed 24 hours after the second dose of DT. For DC turnover experiment, mice were sacrificed 1, 3 or 5 days after the second dose of DT. For influenza virus infection experiments, DT injection was routinely given after the first 2 doses DT in which Clec9a-DTR mice were given DT once in every 3 days whereas Clec4a4-DTR mice were given DT once in every 2 days until the end of experiments. For homosubtypic and heterosubtypic infection experiments, DT injection was maintained in Clec9a-DTR and Clec4a4-DTR mice for 2 weeks after the primary infection.

2.2.8 Preparation of bone marrow-derived dendritic cells (BMDCs)
Bone marrow cells were isolated by flushing femurs and tibias with PBS 2%. Isolated cells were centrifuged and then resuspended in RBC lysis buffer for 10 mins at room temperature. Cells were centrifuged again, resuspended in IMDM 2% supplemented with 10ng/ml GM-CSF at density 1 x 10^6 cells/ml before plating on 10 cm petri dish. Cells were incubated at 37°C with 10% CO2. After 6-7 days, a mixture of cells were generated in the culture with a majority being DCs. To obtain for BMDCs, cells were collected, stained and sorted for CD11c+B220-CD11b+ cells.

2.2.9 Preparation of cells for intracellular cytokine staining
*T cells restimulation with bone marrow-derived dendritic cells (BMDCs)*
GM-CSF-derived BMDCs from Sections 2.2.7 were incubated with influenza virus PR8 virus at approximately 2 PFU per cell for 5-6 hours. Influenza infected BMDCs were then collected, enumerated and mixed with total lung single cell suspensions collated at day 10 P.I at a 1:3 ratio in the presence of
Monensin (10 µg/ml) for 5-6 hours after which the cells were collected and resuspended in PBS 2%.

*T cell restimulation with PMA/Iono or SIINFEKL peptide*

Single cell suspensions from lung collected 10 days P.I were incubated with or without PMA/Iono (PMA 10 ng/ml, Ionomycin 1 µg/ml) for 6 hours with the addition of Brefeldin A (10 µg/ml) in the last 3 hours after which the cells were collected and resuspended in PBS 2%. Single cell suspensions from MLN collected 6 days P.I (harvested from OTI-PR8 infected mice) were incubated with or without PMA/Iono or SIINFEKL (10 µM) peptide for 6 hours with the addition of Brefeldin A (10 µg/ml) in the last 3 hours after which the cells were collected and resuspended in PBS 2%.

Monensin and Brefeldin A are proteins transport inhibitors. Hence they prevent cytokine secretion, facilitate accumulation of cytokines in the cells and increase the signal detection intensity.

**2.3 Statistical Analysis**

Statistical significance was tested by Student's t-test with Graphpad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Survival curves were analyzed by the Mantel-Cox long-rank test. Statistical significance was accepted if p value < 0.05.
Chapter 3: Results I

Characterization of Clec9a-DTR and Clec4a4-DTR transgenic mice

In this section, we illustrate a number of distinct DC subpopulations in the lung and mLN compartments and subsequently show the DC ablation profile in Clec9a-DTR and Clec4a4-DTR mice, the two mouse models which we used for the entire study. Lung and their draining MLN were the focus of our study considering the highly localized nature of influenza infection in the lung and the initiation of anti-viral T cell response in the draining MLN.

3.1 Gating strategies for the analysis of 2 distinct DC populations in the lung and 4 distinct DC populations in the mLN.

In the steady state, there are 2 major DC subpopulations in the lung CD103+ cDCs and CD11b+ cDCs. Recent study reported that lung CD11b+ cDC subset are not entirely DCs, instead the CD11b+ cDC subset contains a genuine DC population and non-DC population [173]. Consistent with this report, we illustrated in the following that the genuine DC population in CD11b+ cDC subset can be distinguished from the non-DC population by expression of surface antigen CD24. We referred to this genuine DC subset as CD24+CD11b+ cDCs. The contaminating non-DC population in CD11b+ cDC subset which do not express CD24, has been suggested as macrophages [173]. Our focus was DC subpopulations in the lung, therefore in this experiment we demonstrated how CD103+ cDCs and CD24+CD11b+ cDCs can be identified.
Figure 3.1 Gating strategy for lung migratory CD103+ DC and CD24+CD11b+ DC subsets in lung

(A) Flow cytometry of lung cell suspensions. MHCII+CD11c+ identifies all lung DCs. MHCII+CD11c+B220+ identifies pDCs while MHCII+CD11c+B220- identifies cDCs. In MHCII+CD11c+B220- gating, 2 major lung DC subpopulations can be identified by mutually exclusive expression of CD103 and CD11b. CD103+ cDC subset express high level of CD24 and low level of CX3CR1(G1). CD11b+ cDC subset can be subcategorized into CD24+CX3CR1^{hi} genuine DCs (G2) and CD24-CX3CR1^{hi} non-DC (G3).

Multiple surface antigen markers are required to specifically identify the lung DC populations in the flow cytometry (Table 3.1). CD103+ cDCs can be identified as MHCII+CD11c+B220-CD103+CD24+CX3CR1^{lo} whereas CD24+CD11b+ cDCs can be identified as MHCII+CD11c+B220-CD11b+CD24+CX3CR1^{hi} (Table 3.1). MHCII+ and CD11c+ identify all lung
DCs. In this gating, lung DCs can be categorized into B220+ DC subpopulation which identifies pDCs and B220- DC subpopulation which identifies cDCs. In the MHCII+CD11c+B220- gating, lung cDCs can be further categorized into CD103+ cDCs and CD11b+ cDCs subpopulations (Figure 3.1A) [13]. Consistent with the recent report, we show here that CD11b+ cDC subset can be subcategorized into 2 subpopulations based on CD24 expression, the genuine DCs which expressed CD24 (CD24+CD11b+ cDCs) and the non-DCs which did not express CD24 (CD24-CD11b+ Mφ) (Figure 3.1A). In addition, expression of surface marker CX3CR1 is a convenient marker to identify CD24+CD11b+ cDCs owing to the distinctly high expression of CX3CR1 on this subpopulation [2]. The authenticity of this subset was confirmed in CX3CR1-eGFP mice in which high CX3CR1 expression was found on CD24+CD11b+ cDCs (Figure 3.1A). Figure 3.1A shows the dot plot representations of these various cell subpopulations in the lung.

In the uninfected mice, there are 4 major DC subpopulations in the MLN. Two of these DC subpopulations in the MLN are DCs that constantly migrate from the lung collectively known as lung migratory cDCs. These migrant lung-derived DCs are made up of CD103+ cDCs and CD24+11b+ cDCs, the DC populations that we just discussed above (Figure 3.1B). It is very important to note that only the genuine DCs, CD24+CD11b+ cDCs migrate from the lung to the MLN whereby the non-DCs CD24-CD11b+ Mφ are non-migratory and reside in the lung. The remaining two DC subpopulations in the MLN are mLN-resident cDCs, CD8+ cDCs and CD8- cDCs (Figure 3.1B). Similarly, multiple surface antigen markers are required to specifically identify these DC populations in the MLN by flow cytometry (Table 3.1). In this experiment, we illustrated how these 4 distinct DC subpopulations in the MLN can be identified.
Figure 3.1 Gating strategy for lung migratory CD103+ DCs, CD24+CD11b+ DCs and mLN-resident CD8+ DCs, CD11b+ DCs in mLN.

(B) Flow cytometry of mLN cell suspensions. MHC\textsuperscript{hi}CD11c\textsuperscript{int} identifies lung migratory cDCs where 2 DC populations can be identified based on mutually exclusive expression of CD103 and CD11b. CD103+ cDCs express high level of CD24 and low level of CX3CR1 (G4). Unlike in the lung, CD11b+ subset in the MLN is almost entirely DCs with dominant presence of CD24+CX3CR1\textsuperscript{hi} (G5) whereas very few numbers of CD24-CX3CR1\textsuperscript{hi} (G6). MHC\textsuperscript{int}CD11c\textsuperscript{hi} identifies mLN-resident cDCs where 2 DC populations can be identified based on mutually exclusive expression of CD8 (G7) and CD11b (G8). Data representative of 3 experiments.

In the MLN, MHC\textsuperscript{II} and CD11c expressions were used to distinguish between lung migratory cDCs (or lung-derived cDCs) and mLN-resident cDCs in which lung migratory cDCs are MHC\textsuperscript{II}CD11c\textsuperscript{int} whereas mLN-resident cDCs are MHC\textsuperscript{II}CD11c\textsuperscript{hi} (Figure 3.1B) [13]. In MHC\textsuperscript{II}CD11c\textsuperscript{int} gating, lung migratory cDCs can be subcategorized into CD103+ cDCs and CD11b+ cDCs (Figure 3.1B). Consistent to what has been reported; we have shown here that almost all the lung migratory CD11b+ cDCs in the MLN are genuine DCs,
CD24+CD11b+ cDCs, whereas very few non-DCs, CD24-CD11b+ Mφ can be spotted in MLN (Figure 3.1B). In MHCII^{int}CD11c^{hi} gate, mLN-resident cDCs can be subcategorized into CD8+ cDCs and CD8- cDCs (Figure 3.1B). Table 3.1 summarizes the combination of surface antigens that were used to specifically identify these various distinct DC subpopulations in the lung and mLN compartment.

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<td>MHCII^{lo}CD11c^{+}CD8^{-}/CD11b^{+}</td>
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hi = high, + = positive, int = intermediate, − = negative, lo = low

Table 3.1: Summary of fluorescent antibody combinations used for FACS analysis of DC subsets in the lung and MLN

3.2 In lung, DT efficiently ablates lung migratory CD103+ DCs and CD24+CD11b+ DCs in the Clec9a-DTR and Clec4a4-DTR mice respectively.

Clec9a molecule is abundantly and specifically expressed on LT CD8+ cDCs and NLT CD103+ cDCs. In our Clec9a-DTR mice, whereby DTR expression is driven by clec9a gene promoter, CD103+ cDCs in the lung compartment should be susceptible to DT-mediated cytotoxicity. Clec4a4 expression can be found on LT CD8- cDCs, but its expression on NLT DC population is uncertain therefore it is unknown which NLT DC subpopulation will be ablated in DT administered Clec4a4-DTR mice. Hence, in this experiment we assessed the ablation efficiency as well as the specificity of DT-mediated cell ablation that takes place in our Clec9a-DTR and Clec4a4-DTR mice. To achieve this, Clec9a-DTR and Clec4a4-DTR mice were injected i.p with 20ng/gbw DT for 2 consecutive days (Figure 3.2A). 24 hours after the second
In lung, DT efficiently ablates lung migratory CD103+ and CD24+CD11b+ DCs in Clec9a-DTR and Clec4a4-DTR mice respectively. (A) DT injection scheme. DT injection via i.p for 2 consecutive days and harvest 24 hours after 2nd DT injection. (B) Representative dot plots showing the profile of lung migratory cDCs ablation. (C) Absolute number of CD103+ cDCs and CD11b+ cDCs in the lung of Wild Type, DT-treated Clec9a-DTR and DT-treated Clec4a4-DTR (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, * p < 0.05 *** p < 0.001.

DT injection, lung were harvested, processed and ablation efficiency was analyzed by flow cytometry. Figure 3.2B indicates that CD103+ cDCs were efficiently ablated in Clec9a-DTR mice whereas there was only partial ablation of CD11b+ cDCs in Clec4a4-DTR mice. Total cell numbers were enumerated and our data show that the ablation efficiency is >95% in Clec9a-DTR mice and 50% in Clec4a4-DTR mice (Figure 3.2C). In previous section, we illustrated that lung-derived CD11b+ cDC subset comprises 2 subpopulations, CD24+CD11b+ cDCs and CD24-CD11b+ Mφ (Figure 3.1A). Hence, in the next experiment, we sought to investigate which subpopulation was ablated in the Clec4a4-DTR mice. Our data clearly show that only CD24+CD11b+ cDCs were ablated in Clec4a4-DTR mice whereas CD24-CD11b+ Mφ population
was unaffected (Figure 3.2D). In terms of absolute number, the ablation efficiency of CD24+CD11b+ cDCs in Clec4a4-DTR mice was >95% (Figure 3.2E). Together, our data have shown that >95% ablation efficiency can be achieved for CD103+ cDCs in our DT treated Clec9a-DTR mice. Similarly, >95% of CD24+CD11b+ cDCs were ablated in our DT treated Clec4a4-DTR mice (Figure 3.2C, E left panel). More importantly, the non-DCs (CD24-CD11b+ Mφ) population in the CD11b+ subset was spared from DT-mediated cytotoxicity indicating the selectivity and specificity of our targeted ablation towards DC populations (Figure 3.2E right panel).

Figure 3.2 In lung, DT efficiently ablates lung migratory CD103+ and CD24+CD11b+ DCs in Clec9a-DTR and Clec4a4-DTR mice respectively.

(D) Representative dot plots showing CD24+CD11b+ cDCs ablation.

(E) Absolute number of CD24+ CD11b+ cDCs and CD24- CD11b+ cDCs in the lung of Wild Type, DT-treated Clec9a-DTR and DT-treated Clec4a4-DTR (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, *** p < 0.001. Data representative of 3 experiments.
3.3 In mLN, DT efficiently ablates lung migratory CD103+ DCs and mLN-resident CD8+ DCs in Clec9a-DTR mouse while lung migratory CD24+CD11b+ DCs and mLN-resident CD11b+ DCs (33D1+) are both ablated in Clec4a4-DTR mouse.

Figure 3.3 In mLN, DT efficiently ablates lung migratory CD103+ DCs and mLN-resident CD8+ DCs in Clec9a-DTR mouse while lung migratory CD24+CD11b+ DCs and mLN-resident CD11b+ DCs (33D1+) are both ablated in Clec4a4-DTR mouse.
(A) Representative dot plots showing the profile of lung migratory cDCs ablation in the mLN. (B) Absolute number of CD103+ cDCs and CD11b+ cDCs in the MLN of Wild Type, DT-treated Clec9a-DTR and DT-treated Clec4a4-DTR (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, ** p < 0.01 *** p < 0.001. (C) Representative dot plots showing CD8+ cDCs (G5) and CD8- cDCs (G6) ablation. (D) Absolute number of CD8+ cDCs and CD8- cDCs in the MLN of Wild Type, DT-treated Clec9a-DTR and DT-treated Clec4a4-DTR. (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, ** p < 0.01 *** p < 0.001. Data representative of 3 experiments.

To assess DC ablation efficiency in the MLN, MLN were harvested from DT treated Clec9a-DTR, Clec4a4-DTR and wild type mice, processed and analyzed by flow cytometry. Our data show that lung migratory CD103+ cDCs and CD11b+ cDCs were efficiently ablated in Clec9a-DTR and Clec4a4-DTR mice respectively (Figure 3.3A). Based on the absolute cell numbers, the ablation efficiencies were >95% and >70% in the MLN of DT treated Clec9a-DTR and Clec4a4-DTR mice respectively (Figure 3.3B). As we have demonstrated in Figure 3.1B, CD11b+ cDCs in the MLN are made up of entirely CD24+CD11b+ cDCs, we concluded that the CD24+CD11b+ cDCs were efficiently ablated in the MLN of DT treated Clec4a4-DTR mice (Figure 3.3B). Next, we assessed the ablation efficiency of mLN-resident CD8+ cDCs and CD8- cDCs in the MLN (Figure 3.3C). The ablation efficiency of CD8+ cDCs was >90% in Clec9a-DTR mice whereas the ablation of CD8- cDCs was approximately 50% in Clec4a4-DTR mice (Figure 3.3D).
In mLN, DT efficiently ablates lung migratory CD103+ DCs and mLN-resident CD8+ DCs in Clec9a-DTR mouse while lung migratory CD24+CD11b+ DCs and mLN-resident CD11b+ DCs (33D1+) are both ablated in Clec4a4-DTR mouse.

(E) **Upper panel.** Representative dot plots showing CD8- cDCs (G1) consists of CD8-33D1+ (G2) and CD8-33D1- (G3) populations. **Lower panel** Representative dot plots showing CD8-33D1+ cDCs in WT mice and the ablation of corresponding DC population in DT-treated Clec9a-DTR and DT-treated Clec4a4-DTR mice. Absolute number of CD8-33D1+ cDCs in Wild Type, DT-treated Clec9a-DTR and DT-treated Clec4a4-DTR. (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, ** p < 0.01 *** p < 0.001. Data representative of 3 experiments.

It is well-documented that CD8- cDCs comprise 2 subpopulations based on 33D1 expression [2, 172]. 33D1 is a monoclonal antibody that recognizes clec4a4 [2, 172]. Consistent with these reports, we identified 2 populations, CD8- 33D1+ and CD8- 33D1- subsets (Figure 3.3E upper panel). Figure 3.3E lower panel clearly shows that only CD8- 33D1+ cDCs, the clec4a4-expressing subset, were efficiently ablated in Clec4a4-DTR mice. Collectively,
these experiments show to us that the ablation of targeted DC populations in the MLN was highly efficient.

### 3.4 Non-targeted cells are not affected by DT

![Figure 3.4 Non-targeted cells are not affected by DT](image)

**Figure 3.4 Non-targeted cells are not affected by DT**

(A) Representative dot plots showing the profile of lung interstitial macrophages (F4/80+CD11b\(^{int}\)), Neutrophils (CD11b+Ly6G\(^+\)), Monocytes (CD11b+Ly6C\(^hi\)), NK cells (CD49b+CD3\(-\)), CD4 T cells (CD3+CD4\(+\)), CD8 T cells (CD3+CD8\(+\)), B cells (B220\(+\)) and pDCs (CD11c\(^{int}\)SiglecH\(+\))

Previously our lab observed depletion of non-targeted macrophage population (F4/80+CD11b\(^{int}\)) in the spleen and gut. To confirm the specificity of both DTR transgenic strains, we analyzed the profile of various immune cells including macrophages, neutrophils, monocytes, T and B lymphocytes and pDCs upon DT treatment (Figure 3.4A). Increase in the number of neutrophils (neutrophilia)
and monocytes (monocytosis) have been observed in other DTR- transgenic mice upon DT administration [174, 175]. It is therefore important to assess various immune cell subsets in our Clec9a-DTR and Clec4a4-DTR mice after

(B) Figure 3.4 Non-targeted cells are not affected by DT
Absolute number of lung interstitial macrophages (F4/80+CD11b\textsuperscript{int}), Neutrophils (CD11b+Ly6G+), Monocytes (CD11b+Ly6C\textsuperscript{hi}), NK cells (CD49b+CD3-), CD4 T cells (CD3+CD4+), CD8 T cells (CD3+CD8+), B cells (B220+) and pDCs (CD11c\textsuperscript{int}SiglecH+). (C) Representative dot plots showing alveolar macrophages (SiglecF+CD11b\textsuperscript{int}) profile in the BAL. Absolute number of alveolar macrophages (SiglecF+CD11b\textsuperscript{int})(n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, * p < 0.05. Data representative of 2 experiments.
DT administration to see if any non-targeted population was ablated or if there is any neutrophilia/monocytosis or any other alterations that could potentially affect our study in influenza infection. Similar to what have been observed in the spleen and gut, but to a lesser extent there was slight depletion of macrophages (interstitial) (F4/80+CD11b\(^{\text{int}}\)) in the lung of Clec4a4-DTR mice (Figure 3.4A, B). In addition, there was hardly significant neutrophilia and monocytosis in both Clec9a-DTR and Clec4a4-DTR mice (Figure 3.4A, B). Furthermore, we also observed 30% pDCs reduction in Clec9a-DTR mice. It is known that pDCs express low level of clec9a hence the reason these cells are susceptible to DT cytotoxicity (Figure 3.4A, B) [176]. Other immune cells including T and B lymphocytes, NK cell numbers were unaffected in the DT treated Clec9a-DTR and Clec4a4-DTR mice (Figure 3.4A, B).

Next, we proceeded to monitor macrophages in the alveolar space (alveolar macrophages) of our Clec9a-DTR and Clec4a4-DTR upon DT administration (Figure 3.4C). It is well-documented that absence of alveolar and interstitial macrophages has been shown to cause more severe influenza disease [60]. To isolate alveolar macrophages, we performed bronchoalveolar lavage (BAL) which is a technique to collect cell populations from the conducting airways and alveolar space. The cell population isolated, we hereafter referred to as BAL, contained predominantly alveolar macrophages during the uninfected steady state. We compared the alveolar macrophages among Clec9a-DTR, Clec4a4-DTR and untreated control mice 24 hours after the second dose of DT and observed that alveolar macrophages in Clec4a4-DTR were perhaps slightly decreased than that of Clec9a-DTR and untreated control mice (Figure 3.4C).

Collectively, this experiment shows to us that slight reduction in alveolar and interstitial macrophages in Clec4a4-DTR mice, weak neutrophilia in both Clec9a-DTR and Clec4a4-DTR mice, and partial pDCs ablation in Clec9a-DTR mice should be carefully considered when interpreting results obtained from influenza infected Clec9a-DTR and Clec4a4-DTR mice.
3.5 In lung, CD103+ cDCs’ turnover is substantially slower than CD24+CD11b+ cDCs’.

Figure 3.5 Turnover of CD103+ cDCs and CD24+CD11b+ cDCs
(A) DT injection scheme. Lung are harvested at 3 different time points.
(B) Representative dot plots showing Upper panel CD103+ cDCs and lower panel CD24+CD11b+ cDCs. (C) Absolute number of CD103+ cDCs. (D) Absolute number of CD24+CD11b+ cDCs. (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, *** p < 0.001. Data representative of 2 experiments.
CD8+ cDCs are known to have a turnover of 3-4 days in the spleen [177]. Determining the turnover of the DCs in the lung would inform us the frequency of DT administration required during the influenza infection in order to ensure steady depletion throughout the course of infection. To assess CD103+ cDCs and CD24+CD11b+ cDCs turnover in Clec9a-DTR and Clec4a4-DTR mice respectively, we administered DT for 2 consecutive days and harvested the lung for flow cytometry analyses at 1, 3 and 5 days after 2nd DT injection (Figure 3.5A). CD103+ cDCs exhibited a slow recovery in which there was no robust recovery even after 5 days (Figure 3.5B, C). On the other hand, the turnover of CD24+CD11b+ cDCs was more rapid whereby 50% of the ablated DCs were restored after 5 days (Figure 3.5B, D).
3.6 Discussion

DCs are professional APCs that are endowed with exquisite antigen presentation capacity to activate naïve T cells. This remarkable feature has been well-established for conventional cDCs but less so for pDCs [178]. The introduction of CD11c-DTR transgenic mouse which depletes all cDCs has enabled investigators to define in vivo role of DCs for the first time whether these cells are indeed critical to the initiation of immune response [179]. Numerous studies using CD11c-DTR mice demonstrated that cDCs-deficiency can significantly compromise CD4 and CTL responses [163, 180]. Increased mortality, uncontrolled virus replication, defective accumulation of CTLs in the lung were observed in influenza infected CD11c-DTR mice which is a clear indication that cDCs are critically required for orchestrating influenza immunity. To initiate anti-viral adaptive immunity, cDCs in the lung acquire viral antigen and rapidly move to draining MLN to activate naïve influenza specific T cells for expansion and differentiation which subsequently move to infected lung to clear the virus.

There is a great need to assess the contribution of distinct lung DC subpopulations in the generation of influenza immunity, a requirement which CD11c-DTR mice cannot fulfil. Functional study of CD103+ cDCs, one major DC subpopulation in the lung, has been greatly benefited from the availability of CD103+ cDCs-deficient mouse models. Batf3 -/- and Langerin-DTR mice enable specific depletion of CD8+ cDCs in the lymphoid organs and CD103+ cDCs in the non-lymphoid organs. Based on west nile virus infection model, Hildner et al noted defective CTL responses in the spleen of batf3 -/- mice [159]. GeurtsvanKessel et al and Helft et al both groups demonstrated reduction of CTL responses in influenza infected Batf3 -/- and Langerin-DTR mice [70, 181]. Langerin-DTR mouse model was simultaneously introduced by Bennett et al and Kissenpfennig et al in 2005. Our Clec9a-DTR mice represents another mouse model to investigate in vivo role of CD103+ cDCs because we have demonstrated that our DT treated clec9a-DTR mice
displayed specific and efficient ablation of CD103+ cDCs in the lung (Figure 3.2A, B) [182, 183].

CD11b+ cDCs, another major DC subpopulation in the lung were not as extensively studied compared to CD103+ cDCs. All the functional roles assigned to CD11b+ cDCs were based on the observations from in vitro studies due to lack of mouse model to specifically deplete this population in vivo. One major concern is regarding the accuracy of these in vitro studies on CD11b+ cDCs because it has been strongly suggested for very long time that this subset contains bona fide DC and other non-DC population [173]. As such, outcomes of these in vitro studies were likely to be influenced by the presence of contaminating non-DC cells. Only very recently, Schlitzer et al illustrated how bona fide cDCs could be identified in the CD11b+ cDC subset, that is to use CD24 surface marker to accurately distinguish CD24+CD11b+ cDCs from the CD24-CD11b+ non-DC population [173]. In this same report, they described a novel mouse model that was deficient in transcription factor IRF4 [173]. IRF4-/- mice displayed constitutive loss of CD24+CD11b+ cDCs in the lung and CD103+CD11b+ in the gut lamina propria [173]. IRF4-/- mice could potentially be a useful tool to investigate the in vivo role of CD24+CD11b+ cDCs in influenza immunity. However, Schlitzer et al noted that during the steady state condition, there was reduced proportion of Th17 response and corresponding increase in Th1 cells in IRF4-/- mice [173]. Hence, this poses a concern over the use of IRF4-/- mice in influenza virus infection model considering the fact that Th1 and Th17 responses greatly influence the disease progression [39, 184, 185].

In our study, we show that the bona fide CD24+CD11b+ cDCs in the lung were specifically and efficiently depleted upon DT administration and more importantly CD24-CD11b+ non-DC population was unaffected (Figure 3.2D, E). Therefore, our Clec4a4-DTR mice offer a unique opportunity to investigate the in vivo role of CD24+CD11b+ cDCs without the concern regarding the contribution from CD24-CD11b+ non-DC population that could potentially lead to inaccurate conclusion. Using both Clec9a-DTR and Clec4a4-DTR mice in our influenza virus infection setting, for the first time in vivo roles of CD103+
cDCs and CD24+CD11b+ cDCs can be compared side by side in particular their impact to the generation of influenza immunity. To our knowledge, Clec4a4-DTR is the first mouse model that depletes CD24+ CD11b+ cDCs and hence a useful tool to dissect the in vivo role of this subset that has never been explored.

The multiple DC subpopulations in the lung coupled with the fact that these various DC subsets express distinct endocytic receptors, cell surface molecules and populate different anatomical location within the lung indicates the functional specification of each of these subpopulations in the lung [186]. In the influenza infection setting, it is important to understand that lung migratory cDCs are the most efficient APCs that prime naïve CD8 T cell activation when they arrive in mLN. Some studies however suggest that mLN-resident CD8+ cDCs participated in the activation of naïve CD8 T cell after influenza infection [79, 80, 187]. Given the current mouse model (Langerin-DTR and our Clec9a-DTR) that deplete both lung migratory CD103+ cDCs and mLN-resident CD8+ cDCs, the relative contribution of these DCs in CD8 T cells cross-priming could not be assessed. Based on ex vivo assay that compared purified CD103+ cDCs and CD8+ cDCs from the MLN of influenza infected mice in CD8 T cells proliferation study, CD103+ cDCs appear to be much more efficient. CD8+ cDCs do not exist in the lung; therefore how and where these CD8+ cDCs in the MLN acquire virus antigen from is still unknown. The contribution of mLN-resident CD8+ cDCs aside, in this study we focused on assessing the relative contribution of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs to influenza-specific CD8 T cells generation. This is an outstanding question which needs to be addressed given the lack of consensus on the relative importance of these two subsets in the initiation of indispensable CTL responses against influenza infection.

In the CD11c-DTR mice generated by Jung et al, repetitive DT injection caused death within a week [163]. This observation raised concern in our infection setting in which the assessment of primary influenza infection usually goes up to 2 weeks. Our lab has addressed this issue with our DTR transgenic mice by monitoring for 4 weeks after DT administration and DT did not cause
lethality in our DTR mice. One important issue which we should take note for our Clec4a4-DTR mice in the influenza infection setting is that the slight yet statistically significant reduction in alveolar/interstitial macrophages in this mouse model upon DT administration (Figure 3.4C). Since we have previously shown that complete loss of these macrophage populations (95-100% ablation efficiency achieved in our CD169-DTR mice) resulted in more rapid morbidity and increased mortality [60]. In view of the our DC turnover experiment, we noted the faster kinetics of turnover in CD24+CD11b+ cDCs compared to CD103+ cDCs in the lung, an indication that more frequent DT administration is required for Clec4a4-DTR mice(Figure 3.5).
Chapter 4: Results II

Impact of myeloid DCs to influenza virus immunity

In our study, we used mouse adapted influenza A virus strain H1N1 (PR8) to induce influenza infection to our Clec9a-DTR, Clec4a4-DTR and wild type control mice.

DT was injected via i.p for 2 consecutive days to the DTR mice prior to the intranasal delivery of PR8. DT injection was given once in 3-4 days in Clec9a-DTR mice while Clec4a4 mice were given once in 2-3 days throughout the infection (Figure 4.1A). This DT injection scheme was applied to all the experiments except otherwise stated.

4.1 Influenza infection induces massive recruitment of inflammatory innate cells to the lung

Immediately after intranasal delivery of influenza virus, various myeloid immune cells could be seen rapidly attracted to the infected lung [45, 140, 188]. In this experiment, we examined whether the ablation of targeted DC populations in our DTR transgenic mice would influence the recruitment of these early infiltrates to the infected lung. To do this, we first compared the infiltration of these cells into the lung among Clec9a-DTR, Clec4a4-DTR and wild type mice after the infection. On day 3 of infection, BALs and lung were collected, processed and analyzed by flow cytometry.

We used CD45 surface marker to track all immune cells because of its exclusive expression on all hematopoietic cells and we found no difference in the numbers of total CD45+ cells. Expectedly, influenza infection induced massive recruitment of inflammatory infiltrates to the lung including neutrophils, monocytes and natural killer cells (Figure 4.1B, C). One exception being interstitial macrophages (F4/80+ CD11bint), in which their numbers decreased after the infection (Figure 4.1C). One study suggests that interstitial
Influenza infection induces massive recruitment of inflammatory innate cells (neutrophils, monocytes and NK cells). Ablations of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs are maintained during the course of infection. (A) DT injection scheme, lung harvested on day 3 of infection. (B) Absolute number of lung CD45+ cells. (C) Absolute number of lung interstitial macrophages, F4/80+CD11b\textsuperscript{int} (Interstitial M\textsubscript{φ}), neutrophils (CD11b+Ly6G+), monocytes (CD11b+Ly6C\textsuperscript{hi}), natural killer cells (CD49b+CD3-, NK cells). Influenza PR8 infection dose, 16 pfu. (n = 4 per group). Absolute numbers are expressed as mean ± SD. Student t test, * p < 0.05 ** p < 0.01. Data representative of 2 experiments.

macrophages change their usual surface marker expression after infection as such they seem to disappear from the lung as the disease progresses (Figure 4.1C) [189]. Infected clec4a4-DTR mice displayed somewhat less interstitial macrophages compared to the infected Clec9a-DTR and wild type mice (Figure 4.1C). In addition, both infected Clec9a-DTR and Clec4a4-DTR mice exhibited slightly increased neutrophilia. However, there was no difference in the numbers of monocytes and natural killer cells among the infected DTR and wild type mice (Figure 4.1C).

To prove the efficiency of the DT-mediated targeted cell ablation over the infection period, next we monitored the number of CD103+ cDCs and CD11b+
cDCs in the lung over the course of 10 days after infection (Figure 4.1D). First, we found no difference in total CD45+ cells in all the infected mice over this period and more importantly ablation of CD103+ cDCs in Clec9a-DTR mice was maintained throughout the infection (Figure 4.1E, F). In wild type and Clec4a4-DTR mice, there was gradual decrease in the numbers of CD103+ cDCs after infection which was expected because it is known that this DC population migrates away from the lung to the MLN after the infection (Figure 4.1F) [13, 70]. On the other hand, there was no reduction in CD11b+ cDCs in the Clec4a4-DTR mice after infection (Figure 4.1F). Figure 4.1F shows that there was massive increase in CD11b+ cDCs after influenza infection. This increment was due to massive recruitment of infiltrating monocytes (also known as inflammatory monocytes-derived dendritic cells, iDCs) to the influenza infected lung (Figure 4.1G, left panel G1 gating). To exclude these contaminating monocytes during infection, we gated on population that did not express Ly6C (Figure 4.1G, left panel G2 gating) and demonstrated that on day 6 of infection, CD24+CD11b+ cDCs were selectively ablated in the Clec4a4-DTR mice (Figure 4.1G, right panel). This result showed that during influenza infection, CD103+ cDCs and CD24+CD11b+ cDCs ablation was maintained in both Clec9a-DTR and Clec4a4-DTR mice.

Similar massive recruitment of cells was observed in the alveolar space (Figure 4.1H). Number of alveolar macrophages decreased gradually as the disease progressed, similar to what we observed for lung interstitial macrophages, however no difference was observed between the groups (Figure 4.1I).
Figure 4.1 Influenza infection induces massive recruitment of inflammatory innate cells (neutrophils, monocytes and NK cells). Ablations of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs are maintained during the course of infection.

(D) DT injection scheme, lung harvested on day 3, 6 and 10 of infection.
(E) Absolute number of lung CD45+ cells. (F) Absolute number of lung migratory CD103+ cDCs and CD11b+ cDCs. CD103+ cDCs (MHCII+CD11c+B220-CD103+), CD11b+ cDCs (MHCII+CD11c+B220-CD11b+).

Influenza PR8 infection dose, 16 pfu. (n = 4 per group) Absolute numbers are expressed as mean ± SD. Student t test, ** p < 0.01. Data representative of 2 experiments.
Influenza infection induces massive recruitment of inflammatory innate cells (neutrophils, monocytes and NK cells). Ablations of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs are maintained during the course of infection.

(G) Left panel: Representative dot plots showing the profile of infiltrating monocyte-derived dendritic cells, iDCs (G1 gating) and lung migratory CD11b+ cDCs (G2 gating). Note that during uninfected state, there is very little iDCs (G1) reason why it does not greatly affects our staining strategy for CD11b+ cDCs (MHCII+CD11c+B220-) even though we do not include staining for Ly6C. After infection (at day 6 P.I), there is massive increase in iDCs (G1), and therefore it appears that there is massive increase in CD11b+ cDCs if we do not gate on Ly6C-non expressing population (G2). Representative dot plots showing the profile of lung migratory CD24+CD11b+ cDCs (MHCII+CD11c+B220-Ly6C-CD24+CX3CR1hi). CX3CR1 expression is useful in differentiating iDCs and CD24+CD11b+ cDCs in which the former is CX3CR1int whereas the latter is CX3CR1hi, as discussed in Sections 3.1. CX3CR1int population is not present after G2 gating indicating we gate out contaminating iDCs in our analysis of CD24+CD11b+ cDCs. Right panel: Absolute numbers of CD24+CD11b+ cDCs and CD24+CD11b+ Mφ. Influenza PR8 infection dose, 16 pfu. (n = 4 per group) Absolute numbers are expressed as mean ± SD. Student t test, ** p < 0.01. Data representative of 2 experiments.
Figure 4.1 Influenza infection induces massive recruitment of inflammatory innate cells (neutrophils, monocytes and NK cells). Ablations of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs are maintained during the course of infection.

(H) Left panel Absolute number of CD45+ cells in the BAL. Right panel Absolute number of alveolar macrophages in the BAL, SiglecF+CD11b^{int} (Alveolar Mφ).

Influenza PR8 infection dose, 16 pfu. (n = 4 per group) Absolute numbers are expressed as mean ± SD. Student t test, **p < 0.01. Data representative of 2 experiments.
4.2 Ablations of lung migratory cDCs increase susceptibility of the DTR transgenic mice to influenza infection.

We were naturally interested to know whether loss of either CD103+ cDCs or CD24+CD11b+ cDCs would increase the susceptibility to mice to influenza A virus PR8 infection. The extent of susceptibility, in our context, is defined based on the weight loss of the infected mice. We subjected the mice to 3 different doses of PR8, 8 pfu, 16 pfu and 32 pfu and monitored their weight loss for 2 weeks (Figure 4.2A). The mice infected with the lowest dose of 8 pfu displayed similar weight loss over the course of 2 weeks and survived the infection (Figure 4.2B, C). Figure 4.2B shows that the mice began to lose weight at 3-4 days P.I and their weight loss reached maximum at 8-9 days P.I after which they began to recover and complete recovery could be seen 2 weeks after the infection. If mice were infected with a higher dose of 16 pfu, initial weight loss until day 8-9 P.I among the DTR and wild type mice was similar (Figure 4.2D). After this point, wild type control mice slowly gained weight and completely recovered in a few days time (Figure 4.2D). In contrast, after 8-9 days P.I Clec9a-DTR and Clec4a4-DTR mice did not regain weight and could not recover from the infection (Figure 4.2D). While wild type control mice survived at this dosage, all Clec9a-DTR and Clec4a4-DTR mice succumbed to the infection (Figure 4.2E). At highest dose of 32 pfu used, all mice including wild type control mice died showing rapid weight loss and succumbing to infection within 1 week (Figure 4.2F, G). These observations demonstrated that CD103+ cDCs and CD24+CD11b+ cDCs can be protective against primary influenza A virus PR8 infection. Importantly, we observed that Clec4a4-DTR mice succumbed to infection more rapidly than Clec9a-DTR mice (Figure 4.2D, E).
Figure 4.2 Ablations of lung migratory cDCs increase susceptibility of the DTR transgenic mice to influenza infection

(A) DT injection scheme. (B, D, F) Weight loss monitoring for mice infected with 8 pfu, 16 pfu and 32 pfu PR8 respectively. (C, E, G) Survival curve of mice infected with 8 pfu, 16 pfu and 32 pfu PR8 respectively. Mantel-Cox log-rank test (survival curve), *p <0.05, ***p <0.001. Student t test (weight), *p <0.05, **p <0.01. In B and F, There is no statistical significant difference in the weight loss between WT and Clec9a-DTR or Clec4a4-DTR mice. In D, * (blue), statistical analysis between WT and Clec9a-DTR mice, * (red), statistical analysis between WT and Clec4a4-DTR mice. (n = 4-5 per group). Data representative of 3 experiments.
Next, we sought to investigate whether DC ablation in Clec9a-DTR and Clec4a4-DTR mice that was induced only after the infection started would similarly increase the susceptibility of these mice to the infection (Figure 4.2H). In one of the groups, DC ablation in Clec9a-DTR and Clec4a4-DTR mice was induced later at day 3 of infection and the ablation was maintained day 3 P.I onwards (Figure 4.2H, Group 1). In another group, as per what we did previously, DC ablation was induced before the infection started and the ablation was maintained throughout the infection (Figure 4.2H, Group 2). Regardless of DC ablation which was induced prior to the infection or induced on day 3 after the infection, all Clec9a-DTR and Clec4a4-DTR mice succumbed to PR8 16 pfu infection dose (Figure 4.2D-G). This result indicates to us that the presence of lung migratory cDCs for the first 3 days was not sufficient to induce protection against primary influenza PR8 infection.
Figure 4.2 Ablations of lung migratory cDCs increase susceptibility of the DTR transgenic mice to influenza infection

(H) DT injection scheme. Group 1 mice DT injection starts on day 3 after infection (Day+3), Group 2 mice DT injection starts 2 days before infection (Day-2). 

(I, J) Weight loss monitoring and survival curve of Clec9a-DTR (Group 1), Clec9a-DTR (Group 2) and wild type mice infected with 16pfu PR8.

(K, L) Weight loss monitoring and survival curve of Clec4a4-DTR (Group 1), Clec4a4-DTR (Group 2) and wild type mice infected with 16pfu PR8.

Mantel-Cox log-rank test (survival curve). *p < 0.05, **p<0.01. Student t test (weight). **p <0.01, ***p <0.001. In I, * and * statistical analyses between WT and Clec9a-DTR mice (group 1, orange) or Clec9a-DTR mice (group 2, blue). In K, * and * statistical analyses between WT and Clec4a4-DTR mice (group 1, green) or Clec4a4-DTR mice (group 2, red).

Data representative of 3 experiments. (n = 4-5 per group).
4.3 Loss of lung migratory cDCs reduces total and influenza virus-specific CD8 T cell population in the lung.

Lung migratory cDCs are required for the initiation and expansion of CD8 T cell response during influenza virus infection [186]. Using batf3 −/− and Langerin-DTR mice, recent studies indicate that loss of lung CD103+ cDCs resulted in greatly reduced number of influenza-specific CD8 T cells [190]. Due to lack of specific cell ablation mouse model, in vivo contribution of lung CD24+CD11b+ cDCs to the generation of influenza-specific T cell response has never been studied. In the following series of experiments, we assessed how the loss of either CD103+ cDCs or CD24+CD11b+ cDCs would impact the CTL response in the lung, virus antigen cross-presentation in the MLN, development of influenza-specific memory CD8 T cells, and the proliferative and functional abilities of effector CD8 T cells.

DT was administered for 2 consecutive days prior to infection and followed by DT administration once in every 3-4 days for Clec9a-DTR and once in every 2-3 days for Clec4a4-DTR. This DT injection scheme was applied to all subsequent experiments unless otherwise stated.

4.3.1 Loss of lung migratory cDCs reduces total CD8 T cell population.

To assess the contribution of lung migratory cDCs to the generation of CD8 T cell response, we compared the absolute number of CD8 T cells in the lung, MLN and BAL over the course of infection among wild type, Clec9a-DTR and Clec4a4-DTR mice.

In the wild type mice, significant expansion of CD8 T cells was observed in the lung and BALs on day 10 of infection (Figure 4.3.1A, E) [191]. Different kinetics of CD8 T cell expansion was observed in the MLN whereby the expansion became distinct on day 3 of infection (Figure 4.3.1C). These observations were in line with previous reports demonstrating that the
expansion of CD8 T cells in the MLN preceded those in the lung and airways [90, 93]. During uninfected condition, CD8 T cells usually are not present in the BAL (refers to CD8 T cells in the alveolar space or airway) in which alveolar macrophages constitute 90-95% of the BAL immune cell population [18]. As our data indicate, influenza infection triggered dynamic change in BAL cell populations in which total number and frequencies of CD8 T cells rose by 4-5 folds after 10 days (Figure 4.3.1E, F). Similar dynamic change was observed in the lung in which total numbers and frequencies of lung CD8 T cells increased by 8-12 folds over the course of 10 days (Figure 4.3.1A, B). However, the number and proportion of CD8 T cells in the MLN were unperturbed by the ongoing infection (Figure 4.3.1C, D).

In the influenza infected Clec9a-DTR and Clec4a4-DTR mice, there was significantly reduced number of CD8 T cell population in the lung and BALs (Figure 4.3.1A, B, E, F). Comparatively, there was only a slight reduction in CD8 T cell numbers in the MLN (Figure 4.3.1C, D). These observations indicate that CD103+ cDCs and CD24+CD11b+ cDCs dictate optimal accumulation of CD8 T cell populations in the influenza virus infected sites, the lung parenchyma and airways.
Figure 4.3.1 Loss of lung migratory cDCs reduces total CD8 T cell population.

(A, C, E) Absolute number of CD8 T cells in the lung, MLN and BALs respectively after influenza PR8 infection. (B, D, F) Frequency of CD8 T cells in the lung, MLN and BALs respectively after influenza PR8 infection.

Influenza PR8 infection dose, 16 pfu. (n = 4-5 per group except uninfected) Absolute numbers and frequencies are expressed as mean ± SD. Student t test, * p < 0.05, ** p < 0.01. Data representative of 3 experiments.
4.3.2 Loss of lung migratory cDCs reduces influenza-specific CD8 T cell population.

A very important feature of influenza-specific CD8 T cell response is that there are only a few virus peptide-derived epitopes (approximately 6) recognized by CD8 T cells [192]. The CD8 T cells specific for these epitopes are collectively known as influenza-specific CD8 T cells. NP\textsubscript{366-374} (8 amino acids short-peptide epitope derived from influenza virus NP protein) is the most immunodominant epitope among all the identified virus epitopes [193]. NP\textsubscript{366-374}-specific CD8 T cells alone accounts for approximately 20% and 40% of total influenza-specific CD8 T cells in the lung and MLN respectively during the peak CD8 T cell response (~ day 10 P.I) [192]. Lack of influenza-specific CD8 T cells is correlated with aggravated disease severity [181, 194]. Influenza-specific CD8 T cells are functionally important effector cells that combat the infection for clearance. In this experiment, we assessed the relative contribution of the lung migratory cDCs to the accumulation of NP\textsubscript{366-374}-specific CD8 T cells in the lung, MLN and BALs after influenza virus infection.

4.3.2 Loss of lung migratory cDCs reduces influenza-specific CD8 T cell population.

(A) Representative dot plots showing the gating strategy from the total lung cells to tetramer+ CD8 T cells (NP\textsubscript{366-374}-specific CD8 T cells). LD, Fixable Live/dead cell dye.

HLA/peptide tetramers are commonly used to detect antigen-specific T cells. These tetramers detect antigen specific T cells by binding to specific T cell receptors (TCRs). Tetramers can be generated to detect either antigen
specific CD4 or antigen specific CD8 T cells. In this experiment, we used tetramer agent called H-2Db Influenza NP Tetramer-ASNENMETM. This tetramer agent detects NP\textsubscript{366-374}-specific CD8 T cells. ASNENMETM refers to the virus peptide sequence in NP\textsubscript{366-374} epitope. CD8 T cells which are stained positive by this tetramer refers to the CD8 T cells harboring TCR that are capable of recognizing viral peptide-ASNENMETM displayed on MHC class I H-2Db by the influenza-infected cells (Figure 4.3.2A).

Maximum number of influenza-specific CD8 T cells can be seen accumulated in the lung on day 9-11 of infection [195]. We observed that, in our study, the NP\textsubscript{366-374}-specific CD8 T cells in the lung were detectable only starting from day 6 P.I, however at this period their numbers remained low (Figure 4.3.2D). Their numbers increased exponentially and tremendously for the next 4 days thereby reaching a significantly expanded population at day 10 P.I after which the attrition began in a gradual fashion (Figure 4.3.2B, D). Substantial amount of NP\textsubscript{366-374}-specific CD8 T cells can still be found in the lung on day 15 of infection (Figure 4.3.2D). Similar profile of NP\textsubscript{366-374}-specific CD8 T cells in the MLN was observed after infection though the expansion from day 6 to day 10 was less drastic (Figure 4.3.2C, E).
4.3.2 Loss of lung migratory cDCs reduces influenza-specific CD8 T cell population.

(B, C) Representative dot plots showing tetramer staining of CD8 T cells in the lung and MLN respectively. (D, E) Absolute number of NP366-374-specific CD8 T cells in the lung and MLN respectively. (F) Representative dot plots showing tetramer staining of CD8 T cells in the BALs. (G) Absolute number of NP366-374-specific CD8 T cells in the BALs.

Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Absolute numbers are expressed as mean ± SD. Student t test, *p < 0.05, **p < 0.01, ***p < 0.001. Data representative of 3 experiments.
In infected Clec9a-DTR and Clec4a4-DTR mice, there was clearly reduced number of NP\textsubscript{366-374}-specific CD8 T cells in the lung on day 10 and 15 of infection (Figure 4.3.2B, D). Lowest number of NP\textsubscript{366-374}-specific CD8 T cells was reported in the Clec9a-DTR mice (Figure 4.3.2D). We have also examined the recruitment of these effector T cells in the BALs and similarly there was much reduced number of NP\textsubscript{366-374}-specific CD8 T cells in the alveolar space and airways (Figure 4.3.2F, G). Collectively these results indicate to us that lung migratory CD103+ cDCs and CD24+CD11b+ cDCs are required to achieve optimal accumulation of influenza-specific CD8 T cells in the lung in which CD103+ cDCs deficiency had the greatest impact.

In MLN, we were surprised to observe similar number and frequency of NP\textsubscript{366-374}-specific CD8 T cells in all influenza infected mice regardless of the presence or absence of lung migratory cDCs (Figure 4.3.2C, E). The number of influenza-specific CD8 T cells in MLN is an important parameter to determine the extent of DC–T cells cross-presentation. Lack of antigen cross-presentation by lung migratory cDCs would result in reduced number of influenza-specific CD8 T cells. This observation suggests to us that cross-priming of naïve CD8 T cells in the MLN are not dependent on CD103+ cDCs and CD24+CD11b+ cDCs during influenza virus infection. However, we were aware of the discrepancy between our study and other numerous ex vivo and in vitro studies that strongly propose the importance of lung migratory cDCs in cross-presenting virus antigen to naïve CD8 T cells in the MLN [70, 80, 194]. Therefore, in the next experiment, we set out to address this discrepancy in particular whether lung migratory cDCs participated in naïve influenza-specific CD8 T cells cross-presentation.
4.4 Ablation of lung migratory CD103+ cDCs, but not CD24+CD11b+ cDCs, impairs antigen presentation in the mLN.

In this experiment, we sought to compare the cross-presentation events in the Clec9a-DTR, Clec4a4-DTR and wild type mice during influenza virus infection. To do this, we quantified the frequencies and absolute numbers of CD8 T cells which were undergoing division as a measurement of cross-presentation activities in the MLN. Lower frequency of dividing cells means lower counts of cross-presentation activities. We used anti-Ki67 fluorescence antibody to identify the cells which were undergoing proliferation. Ki-67 is a nuclear protein that regulates cell division in which it is expressed abundantly during all active phases of cell division and absent in quiescent cells. Therefore, CD8 T cells which are stained positive by anti-Ki67 refer to actively dividing CD8 T cells (Figure 4.4A).

In influenza infected wild type mice, 20% of the total CD8 T cells were positive for Ki-67 at day 6 P.I indicating one fifth of mLN CD8 T cells were actively dividing (Figure 4.4B). Influenza virus infection rapidly enhances the migration of lung migratory cDCs from lung to mLN in the early phase (day 1-6 P.I) of infection [196]. This massive influx leads to huge number of lung migratory cDCs in the MLN ready to cross-present virus antigen to influenza-specific naïve CD8 T cells. Recognition of cognate virus antigen presented by the APCs lead to activation, proliferation and differentiation of influenza-specific naïve CD8 T cells in the MLN [90]. Using BrdU incorporation approach, Flynn et al noted higher frequency of proliferating cells in the early phase (day 0-8 P.I) of infection. As the disease progressed, the frequency of BrdU-labelled CD8 T cells dropped steeply [197]. In our study, frequency of dividing CD8 T cells on day 6 of infection was the highest compared to the frequencies observed at day 10 and day 15 P.I (Figure 4.4B). In fact, the frequencies at day 10 and day 15 P.I were approximately the same level as that observed in uninfected mice (Figure 4.4B).
Figure 4.4 Ablation of lung migratory CD103+ cDCs impairs antigen presentation in the mLN.

Analysis of the Ki-67 expression on total CD8 T cell population in the mLN. (A) Representative dot plots showing Ki-67 staining of CD8 T cells in the mLN. (B) Frequency of Ki-67+ (proliferating) CD8 T cells in the mLN. (C) Absolute number of Ki-67+ (proliferating) and Ki-67- (non-proliferating) CD8 T cells in the MLN. (D) Absolute number of Ki-67+ (proliferating) CD8 T cells only in the MLN.

Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Absolute numbers and frequencies are expressed as mean ± SD. Student t test, *p < 0.05, **p < 0.01, ***p < 0.001. Data representative of 2 experiments.
In Clec9a-DTR mice, 8-10% of CD8 T cells were undergoing active division on day 6 of infection which was half of the frequencies observed in Clec4a4-DTR and wild type mice (Figure 4.4B). Similar trend was observed in terms of the absolute count of total non-dividing and dividing CD8 T cells in the MLN after infection (Figure 4.4C, D). This result strongly suggests that CD103+ cDCs, but not CD24+CD11b+ cDCs, cross-present virus antigen to naïve CD8 T cells in MLN (Figure 4.4B-D).

The conclusion that CD24+CD11b+ cDCs do not mediate significant cross-presentation in the MLN is consistent with our previous result that loss of this DC subpopulation did not affect the number of NP<sub>366-374</sub>-specific CD8 T cells in the MLN (Figure 4.3.2C, E). Observation from the previous result also suggests that CD103+ cDCs are not effective cross-presenting DCs considering the fact that loss of this subpopulation did not affect the number of NP<sub>366-374</sub>-specific CD8 T cells (Figure 4.3.2C, E). But, based on the reduced frequency of proliferating CD8 T cells in the MLN of Clec9a-DTR mice, the conclusion is clear that CD103+ cDCs are dominant APCs that cross-present virus antigen to CD8 T cells in the MLN (Figure 4.4B-D).

In view of the demonstration from Ray et al that circulating influenza-specific CD8 T cells required the presence of VLA-1 to migrate to the infected lung tissue, it is likely that influenza-specific CD8 T cells require additional signal or perhaps VLA-1 to migrate from the MLN into the blood circulation [198]. If this is true, lack of such signal would trap the influenza-specific CD8 T cells in the MLN. Hence similar scenario applies; it is possible that the NP<sub>366-374</sub>-specific CD8 T cells in the Clec9a-DTR mice are lacking this signal to move from the MLN to the circulation. Our argument is that, though loss of CD103+ cDCs led to lack of cross presentation and fewer number of NP<sub>366-374</sub>-specific CD8 T cells in the MLN, these influenza-specific CD8 T cells were unable to migrate out of MLN and as such gradually accumulated in the MLN. Consequently, in the MLN, total number of NP<sub>366-374</sub>-specific CD8 T cells in Clec9a-DTR mice built up to the level comparable to wild type mice (Figure 4.3.2C, E).
NP\textsubscript{366-374}-specific CD8 T cells move from MLN to the blood before they get disseminated to infected lung or other lymphoid/non-lymphoid organs. If there is defective migration of Clec9a-DTR mice’ NP\textsubscript{366-374}-specific CD8 T cells from MLN to blood, then the frequency of NP\textsubscript{366-374}-specific CD8 T cells in the blood would be lower than that of the wild type. We proceeded to quantify the NP\textsubscript{366-374}-specific CD8 T cells in the blood with an aim to demonstrate whether NP\textsubscript{366-374}-specific CD8 T cells in the Clec9a-DTR mice were indeed ‘trapped’ in MLN.
4.5 Ablation of lung migratory CD103+ cDCs, but not CD24+CD11b+ cDCs, leads to inefficient NP_{366-374}-specific CD8 T cell migration from the mLN.

On day 10 of infection, we isolated the blood from the infected Clec9a-DTR, Clec4a4-DTR and wild type mice and stained the PBMCs for flow cytometry analyses (Figure 4.5A). Our result shows that there was much reduced frequency of NP_{366-374}-specific CD8 T cells in the blood of Clec9a-DTR mice (1.5-2%) compared to the wild type mice (5-6%) indicating that there was indeed mobilization defect of the influenza-specific CD8 T cells generated in the absence of CD103+ cDCs (Figure 4.5B). On the other hand, loss of CD24+CD11b+ cDCs did not compromise the egress of NP_{366-374}-specific CD8 T from the MLN (Figure 4.5B).

Figure 4.5 Ablation of lung migratory CD103+ cDCs, but not CD24+CD11b+ cDCs, leads to inefficient NP_{366-374}-specific CD8 T cell migration from the mLN.
(A) Representative dot plots showing NP_{366-374}-specific CD8 T cells in the blood. (B) Frequency of NP_{366-374}-specific CD8 T cells in the blood. Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Frequencies are expressed as mean ± SD. Student t test, *p < 0.05. Data representative of 3 experiments.
4.6 Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced viability of NP\textsubscript{366-374}-specific CD8 T cells in the lung.

The size of influenza-specific CD8 T cell population in the infected lung is critically dependent on the delivery of survival signals to these effector T cells [199]. IL-2 and IL-15 cytokines, co-stimulatory receptor (CD70, GITR and 4-1BB) mediated signals have recently been demonstrated to promote the survival of influenza-specific CD8 T cells in the lung [200-203]. In this experiment, we were interested to know whether the reduced size of NP\textsubscript{366-374}-specific CD8 T cell population in Clec9a-DTR and Clec4a4-DTR mice is due to survival defect in these cells. To achieve this, we assessed the number of apoptotic cells in the infected Clec9a-DTR, Clec4a4-DTR and wild type mice at day 10 P.I. We included Annexin V fluorescence antibody and PI dye in the tetramer staining for NP\textsubscript{366-374}-specific CD8 T cells. Cells which are stained positive for Annexin V are apoptotic cells while cells which are stained positive for PI are dead cells.

Our results indicate that 45-50%, 40-45%, 18-20% of NP\textsubscript{366-374}-specific CD8 T cells in Clec9a-DTR, Clec4a4-DTR and wild type mice respectively were apoptotic (Figure 4.6B, D). The frequencies of apoptotic NP\textsubscript{366-374}-specific CD8 T cells (Annexin V+) in the Clec9a-DTR and Clec4a4-DTR mice were double that of the wild type mice indicating that viability of influenza-specific CD8 T cells contributed to the diminished size of NP\textsubscript{366-374}-specific CD8 T cell population in Clec9a-DTR and Clec4a4-DTR mice. In the total CD8 T cells, there was slightly higher frequency of apoptotic cells in Clec4a4-DTR compared to the wild type (Figure 4.6A, D). In the CD8- population, we did not observe any viability difference across all groups demonstrating that absence of lung migratory cDCs specifically affected the survival of CD8 T cells (Figure 4.6C, D). Collectively, our results show that the CD103+ cDCs and CD24+CD11b+ cDCs are important to promote the survival of NP\textsubscript{366-374}-specific CD8 T cells in the lung.
Figure 4.6 Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced viability of NP<sub>366-374</sub>-specific CD8 T cells in the lung.

(A-C) Representative dot plots showing apoptotic (Annexin V+) and dead (PI+) cells gated on total CD8 T cells, NP<sub>366-374</sub> specific CD8 T cells and CD8- cells respectively. (D) Frequency of apoptotic (Annexin V+) cells in the lung.

Analysis on lung cells day 10 P.I. Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Frequencies are expressed as mean ± SD. Student t test, *p < 0.05, **p < 0.01. Data representative of 2 experiments.
4.7 Reduced frequency of proliferating $\text{NP}_{366-374}$-specific CD8 T cells in Clec9a-DTR and Clec4a4-DTR mice during CD8 T cells contraction phase.

Most of the influenza-specific CD8 T cells which are recruited from the MLN to the lung continue to divide after arriving in the lung [93]. As much as 70% of influenza-specific CD8 T cells undergo active division in the lung on day 4 of infection [93]. This lung-resident secondary proliferation (or local proliferation) phase contributes significantly to the magnitude of the influenza-specific CD8 T cell response. It is still unknown which cell types or what factors promote this continued proliferation. However, it has been suggested that lung DCs are involved [93]. Though McGill et al showed that lung DCs played a direct role in promoting influenza-specific CD8 T cell survival in the lung via IL-15 trans-presentation; the same mechanism did not influence the proliferative capacity of these effector T cells [201]. An interesting study by Brincks et al recently demonstrated the involvement of TRAIL in regulating the proliferative capacity of influenza-specific CD8 T cells. In this study, they reported that influenza-specific CD8 T cells in Trail-/- mice displayed tremendous increase in proliferative potential [110].

Contraction of the influenza-specific CD8 T cells typically occurs immediately after virus clearance which is between day 9-11 of infection [204]. In our study, frequencies of dividing $\text{NP}_{366-374}$-specific CD8 T cells reached the maximum (80%) at day 10 P.I in both MLN and lung and decreased thereafter (Figure 4.7A-C). The frequencies of dividing $\text{NP}_{366-374}$-specific CD8 T cells in the lung of Clec9a-DTR (20-25%) and Clec4a4-DTR (20-25%) mice plummeted at day 15 P.I while a substantial amount (60%) of $\text{NP}_{366-374}$-specific CD8 T cells in the wild type mice were still undergoing robust proliferation (Figure 4.7B, C). However, in the MLN, there was no difference in terms of the frequency of dividing $\text{NP}_{366-374}$-specific CD8 T cells (Figure 4.7A, D). Together, this set of data indicate that in the absence of either lung migratory DC subpopulation, some unknown factors contributed to the temporal defect in the proliferation capacity of pulmonary $\text{NP}_{366-374}$-specific CD8 T cells.
Figure 4.7 Reduced frequency of proliferating NP$_{366-374}$-specific CD8 T cells in Clec9a-DTR and Clec4a4-DTR mice during CD8 T cells contraction phase.

(A, B) Representative dot plots showing Ki-67+ NP$_{366-374}$ specific CD8 T cells in MLN and lung respectively (Gated on NP$_{366-374}$-specific CD8 T cells).

(C) Frequencies of Ki-67+ NP$_{366-374}$-specific CD8 T cells in MLN and lung. Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Frequencies are expressed as mean ± SD. Student t test, **p < 0.01. Data representative of 2 experiments.
4.8 Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced number of IFN-γ-producing and IL-10-producing cells.

Using influenza-specific TCR transgenic mice, Wiley et al noted that IFN-γ production by effector CD8 T cells was required for the early migration of effector CD8 T cells to the lung and to prevent excessive inflammatory response in the influenza infected lung [100]. However, as most studies have shown IFN-γ is ineffectual against influenza virus and it does not impact significantly on the virus clearance [99, 101, 205-207]. Effector CD8 T cells are the major producers of IL-10 in the influenza infected lung at the peak anti-viral T cell response [104]. Presence of IL-10 is required to prevent lethal lung injury and to alleviate inflammation caused by influenza infection [104]. In this experiment, we assessed whether lack of CD103+ cDCs or CD24+CD11b+ cDCs would affect the generation of IFN-γ- and IL-10-secreting effector CD8 T cells in the influenza infected lung.

On day 10 of infection, we collected, isolated and processed the lung tissues to single cell suspensions. Subsequently, lung cells were re-stimulated with influenza-infected bone marrow derived DCs (BMDCs) for the induction of IL-10 production. For the induction of IFN-γ secretion, T cell stimulant PMA/Ionomycin was added to lung cell culture. After 6 hours in culture, we did extracellular staining for surface markers CD8 and CD3 followed by intracellular staining for cytokines IL-10 or IFN-γ (Figure 4.8A, C). The frequencies of IL-10-secreting CD8 T cells in Clec9a-DTR, Clec4a4-DTR and wild type mice at day 10 P.I were 2-3%, 4-5% and 10% respectively indicating that the ablation of CD103+ cDCs had the most impact on the number of IL-10-secreting CD8 T cells in the infected lung (Figure 4.8B). We also observed lower frequency of IFN-γ-secreting CD8 T cells in Clec9a-DTR (20-25%) and Clec4a4-DTR (20-25%) mice compared to the wild type (40-45%) mice (Figure 4.8D). Apart from the huge contribution from effector CD8 T cells to IL-10 production, Sun et al also noted that effector CD4 T cells were accounted for a substantial production of IL-10 [104]. Consistent with this report, we observed 12-15% of IL-10-secreting CD4 T cells in the wild type mice. Again, loss of
Figure 4.8 Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced number of IL-10-producing cells in the lung. Ablation of lung migratory CD103+ cDCs leads to reduced number of IFN-γ producing cells in the mLN. (A, C) Representative dot plots showing profile of IL-10+ CD8 T cells and IFN-γ+ CD8 T cells respectively. (B) Frequency of IL-10+ CD4 and IL-10+ CD8 T cells after 6 hours incubation with influenza infected BMDC and final 4 hours of Monensin. (D) Frequency of IFN-γ+ CD8 T cells after 6 hours incubation with PMA/ionomycin and final 3 hours of Brefeldin A. Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Frequencies are expressed as mean ± SD. Student t test, **p < 0.01, ***p < 0.001. Data representative of 3 experiments.
CD103+ cDCs or CD24+CD11b+ cDCs had a negative impact on the number of IL-10-secreting CD4 T cells (Figure 4.8A, B). Collectively these results showed that the ablation of either CD103+ cDCs or CD24+CD11b+ cDCs significantly abrogated the number of IFN-γ- and IL-10-secreting cells in the infected lung. Reduction in IFN-γ and more importantly IL-10 production may incapacitate the hosts to suppress the inflammation caused by influenza infection [208].

Next, we investigated the functional ability of CD8 T cells in the MLN after influenza infection. At day 6 P.I., we harvested MLN from the infected Clec9a-DTR, Clec4a4-DTR and wild type mice, processed, and cultured the cells in the presence of either PMA/ionomycin or OVA257-264-SIINFEKL peptide. In this experiment, we infected the mice with recombinant PR8 virus, OTI-PR8. OTI-PR8 carries chicken OVA257-264-SIINFEKL peptide in the virus NA segment. Following OT1-PR8 infection, the infected mice generate OVA257-264-specific CD8 T cells among other influenza-specific CD8 T cells [209]. The presence of OVA257-264-specific CD8 T cells allows us to assess the activation of CD8 T cells in antigen-specific manner. On the other hand, PMA/ionomycin activates T cells in TCR-independent manner that is PMA/ionomycin-mediated T cells activation is unspecific. After 6 hours in the presence of either PMA/ionomycin or OVA257-264-SIINFEKL peptide, we did extracellular staining for surface markers CD8 and CD3 followed by intracellular staining for cytokine IFN-γ and compared the frequencies of IFN-γ-secreting CD8 T cells in the MLN of OTI-PR8 infected Clec9a-DTR, Clec4a4-DTR and wild type mice (Figure 4.8E). In both PMA/ionomycin and OVA257-264-SIINFEKL peptide stimulated cultures, the frequencies of IFN-γ-secreting CD8 T cells in Clec9a-DTR mice were significantly lower than those in Clec4a4-DTR and wild type mice hence this observation strongly suggests that CD103+ cDCs are required for the induction of antigen-specific CD8 T cell response in the MLN (Figure 4.8E-G). Together this ex vivo re-stimulation experiment outcome lends further support to our previous conclusion that CD103+ cDCs, but not CD24+CD11b+ cDCs, are important cross-presenting APCs that cross-present antigen to and induce the activation of antigen-specific CD8 T cell response in the MLN.
Figure 4.8 Loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs results in reduced number of IL-10-producing cells in the lung. Ablation of lung migratory CD103+ cDCs leads to reduced number of IFN-γ producing cells in the mLN.

Mice are infected with recombinant OTI-PR8 virus, MLN are collected on day 6 of infection and single cell suspensions are prepared. Isolated mLN cells are stimulated with either PMA/ionomycin or SIINFEKL peptide. (E) Representative dot plots showing profile of IFN-γ+ CD8 T cells in the presence of PMA/ionomycin stimulation. (F) Representative dot plots showing profile of IFN-γ+ CD8 T cells in the presence of OVA257-264-SIINFEKL peptide stimulation. (G) Frequency of IFN-γ+ CD8 T cells after 6 hours incubation with PMA/ionomycin or OVA257-264-SIINFEKL peptide and final 3 hours of Brefeldin A.

Influenza OTI-PR8 infection dose 50 pfu. (n = 4 per group except uninfected). Frequencies are expressed as mean ± SD. Student t test, *p < 0.05. Data representative of 3 experiments.
4.9 CD8 T cells surface marker expression profiles

4.9.1 NP\textsubscript{366-374}-specific CD8 T cells in Clec9a-DTR mice display T\textsubscript{CM}-like property.

CD8 T\textsubscript{CM} (T central-memory) population has been defined as memory cells that express both CCR7 and CD62L. Memory CD8 T cell population is critical for the memory protection against secondary influenza virus challenge due to their lower threshold of activation and rapid upregulation of effector functions. Memory CD8 T cell population is not homogenous; it can be classified into several categories, including CD8 T\textsubscript{CM} and T\textsubscript{EM} populations (T effector-memory). In the systemic infection model e.g. LCMV infection, it has been demonstrated that CD8 T\textsubscript{CM} population mediates effective memory protection. For peripheral organs as the main infection sites, e.g. influenza infection, CD8 T\textsubscript{EM} population has been suggested as the major memory effector cells that contribute to the memory protection against secondary influenza infection. In influenza infection model, the developmental fate of memory CD8 T cells can be determined by a number of factors, one of them being the density of antigen-bearing lung migratory cDCs in the MLN. This fate decision determines the eventual composition of the memory CD8 T cell population as either dominated by CD8 T\textsubscript{CM} or CD8 T\textsubscript{EM} population [210, 211]. Low number of antigen-bearing cDCs in MLN tends to give rise to CD8 T\textsubscript{CM}-like cells. Our previous result on the frequency of proliferating CD8 T cells in the mLN strongly suggests that there was lower number of influenza virus antigen-bearing cDCs in Clec9a-DTR mice compared to Clec4a4-DTR and wild type mice. We were therefore interested to know whether CD8 T cells differentiated in Clec9a-DTR mice displayed a T\textsubscript{CM} phenotype.

We included CD62L, CCR7 and IL-7R (CD127) fluorescence antibodies to our tetramer staining for cells isolated from blood 5 weeks after the infection. Though we found no difference in the frequency of NP\textsubscript{366-374}-specific CD8 T cells in the blood across the group, we observed that more influenza-specific CD8 T cells in Clec9a-DTR mice were CD62L\textsuperscript{hi} and these cells expressed higher level of CCR7 than those from Clec4a4-DTR and wild type mice (Figure 4.9.1A-C). In contrast, CD127 expression on the blood NP\textsubscript{366-374}-specific CD8
T cells was comparable in all the mice examined (Figure 4.9.1D). Our observation on CD62L and CCR7 expression profiles suggests that the memory CD8 T cells development in the Clec9a-DTR mice were T_{CM}-like.

**Figure 4.9.1 NP\textsubscript{366-374}–specific CD8 T cells in Clec9a-DTR mice display T_{CM}-like property.**

(A) Frequency of NP\textsubscript{366-374} specific CD8 T cells. (B) Frequency of CD62L+ NP\textsubscript{366-374} specific CD8 T cells. (C) Expression of CCR7 on NP\textsubscript{366-374} specific CD8 T cells. (D) Expression of IL-7R (CD127) on NP\textsubscript{366-374} specific CD8 T cells.

Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). MFI and frequencies are expressed as mean ± SD. Student t test, **p < 0.01. Analyses on blood PBMCs on day 35 of infection. MFI, Mean Fluorescence Intensity (Median). Data representative of 2 experiments.
4.9.2 NP\textsubscript{366-374}-specific CD8 T cells in Clec4a4-DTR mice lack KLRG1 expression.

Joshi et al have recently proposed the idea of early delineation of memory precursors using systemic virus LCMV infection model [212]. As early as 1 week after the infection, LCMV-specific CD8 T effector cell population can be divided to at least 2 subsets, the short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). According to Joshi et al, SLECs and MPECs are effector CD8 T cells that possess different potential in becoming long-lived memory CD8 T cells. SLECs, defined as KLRG1+ and IL-7R-, have limited potential to become memory CD8 T cells. Comparatively, MPECs, defined as KLRG1- and IL-7R+, have greater potential to become long-lived memory CD8 T cells [212]. We were interested to know whether the ablation of lung migratory DCs would influence the expression of KLRG1 and CD127 on the anti-viral CD8 T cells in which the expression profiles would inform us on the role CD103+ cDCs and CD24+CD11b+ cDCs in memory CD8 T cells formation.

To this end, we collected lung on day 10 of infection and stained for NP\textsubscript{366-374}-tetramers, CD8, KLRG1 and CD127 expression. It is noteworthy to point out that the effector CD8 T cells in influenza infection model show a different expression profile compared to the LCMV infection model in which there is distinct expression of KLRG1 but no marked CD127 expression at day 10 P.I [213]. Consistent with this report, the NP\textsubscript{366-374}-specific CD8 T cells in lung and MLN at day 10 P.I did not stain positive for CD127 but we did observe two distinct populations resolved by KLRG1 expression (Figure 4.9.2A, B). In Clec4a4-DTR mice, there was only 4-8% KLRG1\textsuperscript{hi} influenza-specific CD8 T cells compared to 20-30% in Clec9a-DTR and wild type mice. This result shows that there was a selective loss of KLRG1\textsuperscript{hi} influenza-specific CD8 T cells in Clec4a4-DTR mice (Figure 4.9.2A, B).
Figure 4.9.2 NP\textsubscript{366-374}-specific CD8 T cells in Clec4a4-DTR mice lack KLRG1 expression.

(A) Frequency of KLRG1+IL-7R (neg) lung cells gated on either total CD8 T cells or NP\textsubscript{366-374} specific CD8 T cells. (B) Frequency of KLRG1+IL-7R (neg) mLN cells gated on either total CD8 T cells or NP\textsubscript{366-374} specific CD8 T cells.

Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Frequencies are expressed as mean ± SD. Student t test, *p < 0.05, ***p < 0.001. Analyses on day 10 of infection. Data representative of 2 experiments.
4.9.3 Significantly higher frequency of CD62L+ CD8 T cell population in Clec9a-DTR while significantly lower frequencies of CD69+ CD8 T cell populations are observed in Clec9a-DTR and Clec4a4-DTR mice.

CD44, CD69 and CD62L are commonly used surface markers to identify activated CD8 T cells. CD44 is an adhesion receptor required for lymphocyte extravasation from the blood into inflammatory site [214]. During influenza virus infection, CD44 is highly expressed on activated T cells and its expression is sustained into memory T cells therefore CD44 is commonly known as effector/memory T cell marker [191, 215-217]. CD62L is important for lymphocyte entry into lymph nodes, acting as a ligand for MAdCAM-1 that is abundantly expressed on endothelium lining the high endothelial venules in the lymph node [218, 219]. Activated CD8 T cells express high level of CD44 and low level of CD62L. TCR stimulation induces CD69 expression [220, 221]. Upregulated CD69 expression on T cells has been commonly used as an early activation marker or an indication of antigen encounter [222].
Figure 4.9.3 Significantly higher frequency of CD62L+ CD8 T cell population in Clec9a-DTR while significantly lower frequencies of CD69+ CD8 T cell populations are observed in Clec9a-DTR and Clec4a4-DTR mice.

(A) Frequency of CD44hi CD8 T cells. (B) Frequency of CD62L+ CD8 T cells. (C) Frequency of CD69+ CD8 T cells. Influenza PR8 infection dose 16 pfu. (n = 4 per group except uninfected). Frequencies are expressed as mean ± SD. Student t test, *p < 0.05 **p < 0.01. Analyses on lung cells on day 6 and 10 of infection. Data representative of 2 experiments.

In this experiment, we examined and compared the activation marker expression on the pulmonary effector CD8 T cells during peak anti-viral T cell response (day 10 P.I). Figure 4.9.3A shows that the frequencies of CD44+ CD8 T cells in the lung were comparable across the group. Compared to the wild type, Clec9a-DTR mice exhibited lower frequency of CD69+ CD8 T cells and high frequency of CD62L+ CD8 T cells whereas Clec4a4-DTR mice displayed lower frequency of CD69+ CD8 T cells (Figure 4.9.3B, C). These results show that certain activation marker expression was differentially affected in the absence of these lung-derived DCs. CD62L is an important LNs homing ligand, higher frequency of CD62L+ CD8 T cells in Clec9a-DTR mice suggests that the ablation of CD103+ cDCs increases the propensity of CD8 T cells to recirculate through LNs.
4.11 Higher levels of pro-inflammatory cytokines in Clec9a-DTR and Clec4a4-DTR mice.

Elevated level of pro-inflammatory cytokines is a hallmark of human influenza disease [43]. Clinical studies have shown that human H1N1 pandemic virus and highly pathogenic avian H5N1 induce dysregulated and exaggerated production of pro-inflammatory cytokines in which the level of pro-inflammatory cytokines correlate with increased disease severity. We were interested to know whether the enhanced susceptibility of our DTR transgenic mice was associated with increased levels of pro-inflammatory cytokines. To this end, we monitored the levels of IL-6 and TNF-α on day 6 and day 10 P.I and noted that on day 6 P.I, all the infected mice displayed similar level of IL-6 and TNF-α (Figure 4.10A, B). As the disease progressed, levels of IL-6 and TNF-α in the wild type mice were reduced but the levels of these two pro-inflammatory cytokines remained high in Clec9a-DTR and Clec4a4-DTR mice (Figure 4.10A, B). This result indicates that there was sustained level of pro-inflammatory cytokines IL-6 and TNF-α in both Clec9a-DTR and Clec4a4-DTR mice during primary influenza infection.
In the in vitro culture, both CD103+ cDCs and CD11b+ cDCs produce IL-12 when they are infected in vitro with influenza virus. These in vitro studies showed that CD103+ cDCs are the major producers of IL-12 while CD11b+ cDCs secrete significantly lower amount of IL-12 [223, 224]. In our study, Clec9a-DTR mice displayed a drastically low level of IL-12 whereas level of IL-12 in Clec4a4-DTR mice was comparable to the wild type mice after influenza infection hence confirming that CD103+ cDCs are the major producers of IL-12 (Figure 4.10C, D). CD8 T cells and NK cells are major producers of IFN-γ during influenza infection (Figure 4.10C, D). Absence of either subset can lead to severe reduction in IFN-γ level [225]. In this experiment we show that IFN-γ level was reduced in both infected Clec9a-DTR and Clec4a4-DTR mice, an
indication that this reduction is either caused by decreased CD8 T cell or NK cell populations or their capacity to produce IFN-γ was compromised (Figure 4.10C, D).
4.12 Lung migratory cDCs are dispensable for the generation of homosubtypic immunity.

Although all mice survived the infection with mild PR8 infection dose of 8 pfu, we examined whether these recovered Clec9a-DTR, Clec4a4-DTR and wild type mice would be protected from re-infection with the same virus, but at significantly higher dose (Figure 4.11A).

In this experiment, we infected the mice with 8 pfu PR8 infection dose to ensure all mice survived the primary infection (Figure 4.11A). 4 weeks after the primary infection, all mice were challenged with lethal dose of PR8 during the secondary infection (Figure 4.11A). We attempted a number of infection doses for secondary infection, ranging from 10 to 30,000 folds of the primary infection dose. All the primed-mice survived the high dose secondary challenge (Figure 4.11B). Importantly, these mice did not lose weight like they did during the primary infection. There was initial 5% drop in weight for the first 3 days followed by rapid weight gain thereafter (Figure 4.11B). In parallel, the virus and infection dose used for secondary infection were tested on naïve non-immuned mice. All the naïve mice succumbed to infection within 1 week thus showing that the primed-mice which survived the secondary infection were not due to non-viable/weakened virus (Data not shown). The absence of substantial weight loss in primed-mice during secondary infection is likely because the anti-HA neutralizing antibodies which were generated during and after primary infection, bound to the influenza virus particles and prevented most of them from giving rise to productive infection in the lung. As such, the productive infection events were limited and therefore no distinct weight loss.

One important thing to take note is the DT injection scheme whereby DT injection was maintained for the first 2 weeks after which there was no DT administration (Figure 4.11A). Therefore, throughout the course of primary infection which usually lasted for 2 weeks, DC ablation was maintained in the infected Clec9a-DTR and Clec4a4-DTR mice. By the end of 4th week in which they was no DT administration for 2 weeks, the DC populations were fully recovered in the primed-Clec9a-DTR and Clec4a4-DTR mice. In other words,
Lung migratory cDCs are dispensable for the generation of homosubtypic immunity.

(A) Experiment design for homosubtypic challenge. 1° challenge (primary challenge) 8pfu PR8. 2° challenge (secondary challenge) 80,000 pfu PR8.

(B) Weight loss during primary and secondary homosubtypic infection (n = 4 per group). Student t test, *p<0.05. There is no statistical significant difference in the weight loss between WT and Clec9a-DTR mice as well as between WT and Clec4a4-DTR mice throughout the course of primary and secondary infection. Data representative of 2 experiments.

Figure 4.11

lung migratory DCs were present in the primed-wild type as well as in the primed-Clec9a-DTR and Clec4a4-DTR mice when all the primed-mice were challenged with secondary infection. In this experiment setting, we ensured that the presence or absence of lung migratory cDCs was not contributing to the outcome of secondary infection.
This result suggests to us that lung migratory cDCs are dispensable for the generation of homosubtypic immunity. Homosubtypic immunity, the protection against re-infection with the same virus, is mediated by anti-HA neutralizing antibodies. GCs and long-lived B cells which produce neutralizing antibodies, require activated CD4 T cells provision of CD40 signals [185, 226, 227]. Hence, there is potential risk of compromising influenza-specific neutralizing antibodies in Clec9a-DTR and Clec4a4-DTR mice whereby activation of CD4 T helper cells requires the presentation of cognate antigen by lung migratory DCs. Using CD11c-DTR mice, it has been previously demonstrated that lack of lung DCs down-regulated the level of influenza-specific antibodies in the lung (local humoral immunity) after influenza infection [228]. However, level of anti-HA neutralizing antibodies in the serum was comparable between influenza infected CD11-DTR and wild type mice [181].

Because primed-Clec9a-DTR and Clec4a4-DTR mice survived homosubtypic challenge as readily as primed-wild type mice, this observation suggests to us that loss of lung migratory cDCs did not compromise the neutralizing antibodies-mediated homosubtypic protection. To assess the robustness of influenza-specific neutralizing antibodies between Clec9a-DTR, Clec4a4-DTR and wild type mice, we collected serum from uninfected naïve mice and PR8 (8 pfu) infected wild type, Clec9a-DTR and Clec4a4-DTR at day 10 P.I followed by i.p transfer of these serum to naïve mice (Figure 4.11C). One day after serum transfer, recipient mice were infected with 15 pfu or 80 pfu of PR8 (Figure 4.11C). Recipient mice, which were transferred with serum isolated from either infected wild type, Clec9a-DTR or Clec4a4-DTR mice, all showed improved protection against intermediate (15 pfu) and high (80 pfu) infection dose in which there was no distinct weight loss difference among these recipients mice (Figure 4.11D). Comparatively, serum from naïve mice conferred much weaker protection in which the recipient mice lost more weight and recovery was delayed when infected with 15 pfu whereas high dose PR8 of 80 pfu killed all these mice (Figure 4.11D). Mice without any prior serum transfer lost the most weight and took longest period to recover when infected with intermediate dose (15 pfu) whereas these mice rapidly succumbed to high dose (80 pfu) infection (Figure 4.11D).
Collectively, these results suggest to us that CD103+ cDCs and CD24+CD11b+ cDCs are dispensable in homosubtypic immunity formation.

Figure 4.11Lung migratory cDCs are dispensable for the generation of homosubtypic immunity.

(C) Experiment design for serum transfer from PR8 8pfu infected wild type, Clec9a-DTR and Clec4a4-DTR mice to naïve mice one day prior to PR8 15 pfu or 80pfu infection. (D) Weight loss of PR8 15 pfu or 80 pfu infected mice. (n = 4 per group). Student t test, *p < 0.05. * refers to statistical analyses between mice receiving serum from WT, Clec9a-DTR or Clec4a4-DTR mice and mice that did not receive any serum. Data representative of 2 experiments.
4.13 Lung migratory cDCs regulate heterosubtypic immunity.

Unlike homosubtypic immunity, heterosubtypic immunity is mediated mainly by cellular T cell responses [229, 230]. Lung migratory cDCs are important in the generation of cross-protective CD8 T cell memory. Co-stimulatory signal delivered by lung DCs are pivotal programming signal for memory CD8 T cells. It has been shown that lack of either OX40 or 4-1BB signaling led to defective CD8 T cell memory formation and these memory cells displayed severely compromised proliferative potential [200, 231]. Study from Kim et al suggests that distinct lung DC subpopulations regulated different influenza-specific memory CD8 T cell lineage development in which these distinct memory CD8 T cells differed in their protection capacity against secondary infection [190]. Considering the crucial role of lung DCs in memory CD8 T cell generation, it is likely that loss of lung DCs would have considerable impact on the heterosubtypic immunity.

The DT injection scheme was identical to the schedule used for homosubtypic challenge study so as to ensure that lung migratory cDCs were present in x-31-primed Clec9a-DTR and Clec4a4-DTR mice during secondary infection (Figure 4.12A). As such this experiment setting made sure the presence or absence of lung migratory cDCs was not contributing to the outcome of secondary infection.

In this experiment, we subjected the mice to X-31 (H3N2) virus during primary infection followed by PR8 (H1N1) virus in secondary challenge. X-31 and PR8 are serotypically different influenza A virus strains whereby X-31 and PR8 virus are coated with HA3 and HA1 proteins respectively. In terms of humoral immunity, anti-HA3 specific antibodies generated during the X-31-induced primary infection cannot recognize HA1-typed PR8 and thus HA3-specific neutralizing antibodies cannot prevent PR8 virus from infecting X-31-primed hosts. However, X-31 and PR8 share identical internal proteins in which CD8 T cell immunodominant epitopes are known to derive from virus internal proteins. In other words, X-31-specific CD8 T cells generated in the primary
Figure 4.12 Lung migratory cDCs regulate heterosubtypic immunity.

(A) Experiment design for heterosubtypic challenge. 1° challenge (primary challenge) 5 pfu x-31. 2° challenge (secondary challenge) 1,500 pfu PR8. (B) Weight loss during primary and secondary heterosubtypic infection. (n = 4 per group). Student t test, *p < 0.05, **p < 0.01. * (red) refers to statistical analysis between WT and Clec4a4-DTR mice. * (blue) refers to statistical analysis between WT and Clec9a-DTR mice. Data representative of 2 experiments.

Infection are able to detect the presence of PR8-infected cells, eliminate these infected cells and prevent virus spreading and propagation. As such, in this experiment setting, the protective immunity against secondary infection is derived from influenza-specific CD8 T cell memory, with no contribution from HA-specific neutralizing antibodies.
We infected the mice with low dose X-31 (5 pfu) during primary infection to ensure all mice survived (Figure 4.12B). 4 weeks after the primary infection, X-31-primed mice were challenged with PR8 virus. After secondary infection with PR8, we observed rapid weight loss in all X-31-primed mice (Figure 4.12B). X-31-primed wild type and Clec4a4-DTR mice suffered weight loss until 3-4 days P.I after which they recovered and Clec4a4-DTR mice displayed a more protracted recovery than the wild type (Figure 4.12B). In contrast, X-31-primed Clec9a-DTR mice were quickly overcome by secondary infection and died within 1 week (Figure 4.12B). This observation suggests to us that both CD103+ cDCs and CD24+CD11b+ cDCs influence the formation heterosubtypic immunity and loss of CD103+ cDCs led to total loss of this cross-protection.
4.13 Discussion
Accumulation of influenza-specific CD8 T cells in the lung after influenza virus infection dictates the resolution of the infection. To achieve optimal CTL response in the lung, lung DCs are indispensable. It has long been fully appreciated that indispensability of lung DCs lies in their capacity to initiate expansion of naive influenza specific CD8 T cells in the MLN after influenza infection. However, in contention is the relative contribution of distinct lung DC subpopulations to this CTL induction after influenza virus infection. Using our Clec9a-DTR and Clec4a4-DTR mice, we sought to address the relative contribution of two major lung DC subpopulations CD103+ cDCs and CD24+CD11b+ cDCs in this aspect. Recent advances in this field revealed that the optimal accumulation of anti-viral CD8 T cells in the lung is also dependent on the ability of influenza-specific CD8 T cells to migrate and localize to the lung as well as additional signals in the lung that guide further expansion and promote survival of these effector CD8 T cells. Investigations from several reports provided some indications of lung DCs involvement in these aspects suggesting that lung DCs, besides indispensable for the induction of anti-viral CD8 T cells response in the MLN, are also involved in the maintenance of these effector CD8 T cells in the lung. In this report, we investigated the potential involvement of CD103+ cDCs and CD24+CD11b+ cDCs in these areas.

Dysregulated formation of CTL response in the lung is associated with increased disease severity and delayed virus clearance. Using Clec9a-DTR and Clec4a4-DTR transgenic mice infected with mouse-adapted Influenza A virus H1N1/PR8, we demonstrated that loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs led to significantly smaller number of influenza-specific CD8 T cells in the lung that correlated with the lack of protection against primary influenza infection (Figure 4.3.2B, D and Figure 4.2D, E). Our data indicate that CD103+ cDCs deficiency led to reduction of cross-presentation in the MLN, inefficient mobilization of influenza-specific CD8 T cells away from the MLN and lower viability of these anti-viral effector CD8 T cells in the lung (Figure 4.4B, Figure 4.5 and Figure 4.6D). Together these
multiple defects exert enormous negative impact on the accumulation of influenza-specific CD8 T cells in the lung of infected Clec9a-DTR mice. In parallel, we have shown that CD24+CD11b+ cDCs deficiency resulted in decreased survival of influenza specific CD8 T cells in the lung but in the absence of this DC subpopulation, both cross-presentation and mobilization of anti-viral effector CD8 T cells remained unaffected (Figure 4.6D, Figure 4.4B and Figure 4.5). Hence, viability defect represents chief contributing factor to the sup-optimal accumulation of pulmonary influenza-specific CD8 T cells in the infected Clec4a4-DTR mice. Another paramount factor that determines overall size of anti-viral CD8 T cell population in the lung is the proliferation of the influenza specific CD8 T cells in situ. In this regard, we have shown that loss of either CD103+ cDCs or CD24+CD11b+ cDCs during the peak T cell response (day 10 P.I) did not undermine the proliferation capacity of the pulmonary influenza specific CD8 T cells (Figure 4.7). However, we noted that there was substantial reduction in the proportion of proliferating influenza specific CD8 T cells in both DTR transgenic mice late into the infection (day 15 P.I) (Figure 4.7).

Influenza A virus infection can result in the generation of cross-protective immunity against subsequent exposure to influenza A virus of distinct serotype, this immunity is known as heterosubtypic immunity. This cross-reactive immunity is conferred predominantly by the memory CD8 T cells. Our results demonstrated that both lung migratory CD103+ cDCs and CD24+CD11b+ cDCs are critically required for the generation of effective heterosubtypic immunity suggesting that for the elicitation of cross-protective CD8 T cell responses, lung migratory cDCs are indispensable (Figure 4.12).

Cytokine secretion by effector CD8 T cells is important in influenza immunity and influenza-induced lung pathology. IL-10 is a crucial immunosuppressive cytokine that improves the lung pathology by restricting excessive inflammation during influenza infection. Our investigation revealed that loss of either lung migratory CD103+ cDCs or CD24+CD11b+ cDCs resulted in significantly reduced number of IL-10 and IFN-γ secreting effector CD8 T cells in the infected lung (Figure 4.8). Concurrently, we observed that the levels of
proinflammatory cytokines (TNF-α and IL-6) were significantly higher in influenza infected Clec9a-DTR and Clec4a4-DTR mice compared to the wild type mice (Figure 4.10) indicating higher level of inflammation in the DTR transgenic mice. We reasoned that one of the contributing factors to this enhanced level of inflammation in the infected lung might be due to impaired production of anti-inflammatory IL-10 in our lung migratory cDC-depleted mice.

On ablation of lung migratory cDCs increases susceptibility of our DTR transgenic mice to influenza infection and massive reduction of influenza-specific CD8 T cell population in the lung. The importance of DCs in the generation of influenza-specific CD8 T cell responses was first demonstrated in studies using CD11c-DTR transgenic mice in which these studies observed diminished size of influenza-specific CD8 T cells in DC-deficient CD11c-DTR compared to DC-sufficient wild type mice [181]. A defined lung DC subpopulation, CD103+ cDCs, was subsequently demonstrated to be strictly required to achieve high number of influenza-specific CD8 T cells in the influenza infected lung [80, 181, 190, 194]. However, it has not been explored in vivo whether the accumulation of these effector CD8 T cells in the influenza infected lung is CD24+CD11b+ cDCs dependent.

Using our Clec9a-DTR and Clec4a4-DTR mice infected with influenza virus PR8, we confirmed the role of CD103+ cDCs and demonstrated for the first time that the presence of CD24+CD11b+ cDCs is required in vivo to achieve optimal number of influenza-specific CD8 T cells in the infected lung (Figure 4.3.2B, D). In most circumstances, the size of influenza-specific CD8 T cell population in the lung positively correlates with the protection against influenza induced infection [40]. By transferring in vitro generated virus antigen primed-effector CD8 T cells, Cerwenka et al showed that the increased presence of these transferred effector CD8 T cells afforded the recipient mice with enhanced protection against lethal influenza infection [232]. However, when the influenza infected mice displayed suboptimal number of influenza-specific CD8 T cells in the lung, Kandasamy et al noted that these mice displayed increased disease severity and susceptibility [194]. Using H3N2 influenza A
virus strain, X-31, GeurtsvanKessel et al showed that more severe weight loss was observed in CD11c-DTR mice than in wild type mice after infection [181]. In this study, they further showed that the loss of CD103+ cDCs were responsible for the increased sensitivity to influenza infection because X-31 infected CD103+ cDC deficient-Langerin-DTR mice similarly exhibited more severe weight loss [181]. Consistent with these reports, we show that our Clec9a-DTR mice which specifically lacked CD103+ cDCs were more susceptible to the infection (Figure 4.2). Our investigations on Clec4a4-DTR mice show that loss of CD24+CD11b+ cDCs also negatively impact the protection against primary influenza infection. In fact, Clec4a4-DTR mice were more sensitive than Clec9a-DTR mice to influenza infection (Figure 4.2).

**On the lung migratory CD103+ cDCs as predominant APCs that prime naïve CD8 T cells in the mLN.**

The mLN is the main site where lung migratory cDCs induce influenza-specific CD8 T cell response during influenza virus infection. There are 2 possible scenarios as to how lung DCs acquire virus antigens in the infected lung, through direct infection by the virus or through acquisition of exogenous antigens by phagocytosis of virus infected cells. By isolating lung CD103+ cDCs and CD11b+ cDCs from the MLN on day 2 of infection and injecting these cells to virus-permissive embryonated chicken eggs, Helft et al showed that these lung migratory cDCs were not productively infected [70]. Moreover, they also showed that these lung DCs did not express virus HA proteins on the cell surface in which HA expression signifies productive virus infection. In addition, microscopic images illustrated that virus proteins were kept in the phagocytic compartment hence collectively these observations indicated that lung DCs acquire virus antigen through phagocytosis of virus infected cells and not through direct virus infection. In another study, Moltedo et al established that lung CD103+ cDCs, CD11b+ cDCs to a lesser extent, in the MLN were productively infected with influenza virus [80]. This deviation from the report by Helft et al was probably due to different timing of analyses in which the analyses from Moltedo et al investigation were based on the cells isolated on day 3 and 4 of infection [80]. Together these two studies suggest that CD103+ cDCs were not infected in the first two days of infection but were
productively infected as the disease progressed whereas CD11b+ cDCs showed few signs of being infected over the course of infection.

Because CD11b+ cDCs are not productively infected and the fact that virus proteins reside in phagocytic compartment, this lung DC subpopulation appears to engage predominantly cross-presentation pathway to prime naïve CD8 T cells in the MLN [234]. In order to engage cross-presentation pathway, it requires the APCs to be adept in phagocytosis. However, unlike CD103+ cDCs, CD11b+ cDCs express very little CD36 and DNGR-1 which are two essential phagocytic receptors that facilitate uptake of dying cells suggesting that CD11b+ cDCs are not adept in phagocytosis of virus infected cells. In addition, it has been shown that those phagocytosed virus antigens were not well-preserved in the phagocytic compartments of CD11b+ cDCs whereby a well-preserved virus antigen supports efficient cross-presentation to naïve CD8 T cells. Therefore, though lung CD11b+ cDCs predominantly engage cross-presentation pathway to prime naïve CD8 T cells, the efficiency is likely to be low.

On the other hand, CD103+ cDCs appear to engage both direct MHC-I antigen presentation and cross-presentation pathway to induce naïve CD8 T cells activation in the MLN based on the susceptibility of this lung DC subpopulation to infection as well as the demonstration that these DCs phagocytose virus infected cells [234]. Regardless of how these 2 distinct lung DC subpopulations present virus antigens to naïve influenza-specific CD8 T cells in the MLN, outcomes of the in vitro experiments from these two reports suggest that CD103+ cDCs were more efficient than CD11b+ cDCs in the induction of anti-viral CD8 T cell response. One interesting point from Molteado et al study was the claim that the readiness of virus to replicate in lung DC population correlates with the virus antigen presentation capacity [80]. Specifically they showed that by abrogating IFN-α/β receptor (IFNAR) signaling and hence rendering lung CD11b+ cDCs permissive to virus infection/replication, the capacity to induce CD8 T cell activation in the MLN become as potent as that of lung CD103+ cDCs [80].
Using Langerin-DTR and batf3 -/- mice, several groups have demonstrated that CD103+ cDCs are the major APCs that cross-prime influenza-specific naïve CD8 T cells to expand and differentiate in the MLN [14, 79, 80, 181, 190, 194]. By transferring CFSE-labeled TCR transgenic CD8 T cells to the mice followed by influenza PR8 infection, Kandasamy et al demonstrated that the donor cells proliferation was less robust in the Langerin-DTR mice compared to the donor cells in wild type [194]. In another study, Moltedo et al demonstrated that CD103+ cDCs isolated from the MLN of influenza infected wild type mice were capable of cross-priming and initiating naïve CD8 T cells proliferation in culture [80].

The in vivo contribution of CD24+CD11b+ cDCs to CD8 T cell cross-presentation in the MLN after influenza infection is unknown. However, several studies have attempted to study the role of this subset in cross-presentation using ex vivo CD8 T cell proliferation assay. Kim et al reported that CD11b+ cDCs isolated from the MLN of influenza infected mice on day 4 P.I were capable of inducing naïve TCR transgenic CD8 T cells to undergo extensive proliferation in culture [14]. Using co-culture of purified DCs and NP366-374-specific CD8 T cells, Ballesteros-Tato et al provided evidence that CD103+ cDCs and CD11b+ cDCs isolated from the MLN of infected mice on day 3, 5 and 7 P.I had comparable ability to expand NP366-374-specific CD8 T cells in the culture [13]. Investigations by Moltedo et al have arrived at different conclusion, their study showed that CD11b+ cDCs isolated from MLN of mice on day 4 of infection were inefficient in cross-priming naïve influenza-specific TCR transgenic CD8 T cells expansion [80]. Moltedo et al is not the only group that argued against the importance of CD11b+ cDCs in cross-presentation, observations from a number of investigators supported similar claim [70, 81]. Therefore, attempts from in vitro studies did not reach a consensus regarding the role of CD11b+ cDCs in cross-presentation. Using our Clec9a-DTR and Clec4a4-DTR mice, we did head-to-head comparison between the 2 DC subpopulations CD103+ cDCs and CD24+CD11b+ cDCs in particular to address their in vivo contribution to cross-presentation in the MLN after influenza infection (Figure 4.4). Despite the claim from several groups that CD11b+ cDCs mediate efficient influenza virus antigen cross-presentation, our
results revealed that lack of CD24+CD11b+ cDCs did not significantly influence the expansion of influenza-specific CD8 T cells in the MLN (Figure 4.4B). Based on our results, we propose that CD24+CD11b+ cDCs in the mLN do not play a significant role in the priming of naïve CD8 T cells. However, it is possible that CD24+CD11b+ cDCs function mainly to support the expansion of primed/differentiated CD8 T cells. It has been demonstrated that CD27-expressing primed/differentiated CD8 T cells in mLN require CD27 ligand, CD70, for optimal proliferation and survival. CD24+CD11b+ cDCs, which migrate to the mLN, express CD70 during influenza infection. Hence the presence of CD70-expressing CD24+CD11b+ cDCs seems to suggest its role in promoting expansion of primed/differentiated CD8 T cells rather than priming the naïve CD8 T cells. At the same time, CD103+ cDCs as the dominant cross-presenting APCs in the MLN after influenza infection were further confirmed by our study (Figure 4.4B).

**On loss of lung migratory CD103+ cDCs that led to inefficient Influenza-specific CD8 T cell migration from the mLN.**

Influenza-specific CD8 T cells generated in the mLN are required to move into the circulation and exit to the infected lung. Either defective mobilization from the mLN into the blood circulation or the subsequent dysregulated migration to the infected lung could result in suboptimal number of influenza-specific CD8 T cells in the lung. Ray et al demonstrated that deficiency in VLA-1 led to severe loss of influenza-specific CD8 T cells in the lung in which they subsequently showed that VLA-1 is an important lung homing receptor [198]. Another molecule, CXCR3, has also been shown to be required for CD8 T cell homing to the influenza infected lung. [235] VLA-1 and CXCR3 are the prominent lung tissue homing molecules but their role in mediating migration of CD8 T cells from mLN into the blood circulation have not been explored. Richards et al identified that CD62L expression regulated trafficking of influenza-specific CD8 T cells away from the mLN in which they showed that transgenic expression of CD62L (protease resistant form) on the CD8 T cells resulted in the accumulation of CD8 T cells in the mLN [236]. In other words, the constitutive expression of CD62L is able to ‘trap’ CD8 T cells in the mLN as such these T lymphocytes are unable to mobilize from the mLN into the blood circulation. In
view of these studies, perhaps the mobilization defect of the CD8 T cells differentiated in Clec9a-DTR mice is not due to lack of required signal to migrate away from the mLN, but rather is due to the excessive expression of signal that is instructing these effector CD8 T cells to remain in the mLN (Figure 4.5 and Figure 4.3.2C, E).

On the requirement of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs for the optimal survival of influenza-specific CD8 T cells in the lung.

Our results demonstrated that influenza-specific CD8 T cells in the lung of Clec9a-DTR and Clec4a4-DTR mice were less viable compared to those in the wild type mice (Figure 4.6B, D). Reduced viability of influenza-specific CD8 T cells is likely due to deprivation of pro-survival signal. Recently it has been demonstrated that maintenance of influenza-specific CD8 T cell population in the lung requires pro-survival signal [93]. The pro-survival signal is delivered by lung DCs without which a sizable number of influenza-specific CD8 T cells would undergo apoptosis [237]. McGill et al showed that trans-presentation of cytokine IL-15 by lung DCs promoted the survival of influenza-specific CD8 T cells in the lung [201]. In support of this observation from McGill et al, Yadava et al reported that TSLP, which is an upstream cytokine that upregulates the expression of IL-15, is pivotal to maintain the survival of influenza-specific CD8 T cells in the influenza infected lung [238]. Observations from these two studies suggest that pDCs, iDCs and CD11b+ cDCs in the lung are involved in IL-15 trans-presentation. Involvement of CD103+ cDCs and CD24+CD11b+ cDCs as IL-15 trans-presenter has not been reported. Since these lung-derived DCs are ablated in our mouse models and yet they are potential IL-15 trans-presenters, therefore it is possible that the influenza-specific CD8 T cells are deprived of IL-15-mediated survival signal in our Clec9a-DTR and Clec4a4-DTR mice. This could be one of the reasons why the influenza-specific CD8 T cells were less viable in our Clec9a-DTR and Clec4a4-DTR mice (Figure 4.6A-D).
On the reduced proliferative potential of influenza-specific CD8 T cells during CD8 T cell contraction phase in Clec9a-DTR and Clec4a4-DTR mice.

Cross-presentation by lung migratory cDCs in the mLN leads to proliferation of rare influenza-specific naïve CD8 T cells that ultimately generate massive number of Influenza-specific differentiated CD8 T cells in the mLN. Influenza-specific CD8 T cells that arrive in the lung from mLN continue to proliferate. Using BrdU (a label that tags proliferating cells) incorporation, McGill et al showed that 70% of the influenza-specific CD8 T cells in the lung at day 4 P.I were undergoing division [93]. In another study, Flynn et al identified 80% of NP\(_{366-374}\)-specific CD8 T cells were labelled by BrdU in the lung on day 8 of influenza infection [197]. Consistent with these reports, we have shown high frequency of dividing (Ki-67+) NP\(_{366-374}\)-specific CD8 T cells in the lung after influenza infection (Figure 4.7C). McGill et al showed that by using FTY720, a drug agonistic for S1P receptor, to inhibit recruitment of new influenza-specific CD8 T cells from the mLN to lung, the role of CD8 T cell proliferation within the lung independent of new cell recruitment can be assessed. They reported that even if new recruitment was blocked at day 6 P.I onwards, the CD8 T cell proliferation within the lung was sufficient to sustain high numbers of T cells found in the infected lung at the later time points, indicating that the lung-resident CD8 T cell division contributed significantly to the overall magnitude of effector CD8 T cells responses in the lung [93]. In our study, we noted that loss of either CD103+ cDCs or CD24+CD11b+ cDCs did not affect the frequency of dividing NP\(_{366-374}\)-specific CD8 T cells on day 6 and 10 P.I in the lung, an indication that the overall reduced number of NP\(_{366-374}\)-specific CD8 T cells in the Clec9a-DTR and Clec4a4-DTR mice was not due to reduced capacity to proliferate in situ (Figure 4.7C).

Specifically in the lung, our results indicate a notable reduction in the frequency of dividing influenza-specific CD8 T cells in Clec9a-DTR and Clec4a4-DTR mice during the contraction phase (day 15 P.I) but not during the earlier stage of infection (day 6 and day 10 P.I) (Figure 4.7C). Such defect that occurred late into the infection suggests that the contributing factors may lie between days 10-15 of infection. In our lung cytokine ELISA data, we noted that on day 10 of infection, there were significantly higher levels of
proinflammatory cytokines in Clec9a-DTR and Clec4a4-DTR mice (Figure 4.10A, B). This observation implies that the influenza-specific CD8 T cells in wild type, Clec9a-DTR and Clec4a4-DTR mice were exposed to different cytokine milieu in the later stage of infection. The importance of cytokines that regulates the expansion/proliferation of CD8 T cells has been established by in vitro and in vivo studies in several virus infection models [239, 240]. LCMV, vaccinia virus and versicular stomatitis virus infection models have singled out the pivotal role of IFN-α- and IL-12-mediated signals without which the CD8 T cells failed to undergo active proliferation. However, the role of cytokine that regulates CD8 T cells proliferation in influenza infected mice has not been fully investigated. Using mice immunized with peptide-coated mature DC, Miller et al showed that IL-12 and IFN-α signals are required to prolong division of activated CD8 T cells via maintaining a high affinity IL-2 signaling [241]. Perhaps the lack of these signals may account for the reduced capacity of influenza-specific CD8 T cells to proliferate in our Clec9a-DTR and Clec4a4-DTR mice observed on day 15 of infection (Figure 4.10A, B). However, it is unlikely that the defect to proliferate is intrinsic given the observation that this dysregulated capacity to proliferate was not found throughout the infection period. Considering the importance of local proliferation within the lung that contribute significantly to the overall magnitude of influenza-specific CD8 T cell responses in the lung, further study is warranted to investigate the role of cytokine in the regulation of lung-resident CD8 T cell proliferation during influenza-induced infection.

On the preponderance of CD8 T<sub>CM</sub> development in Clec9a-DTR transgenic mouse strain.

Following the eradication of influenza virus from the lung, majority of the influenza-specific CD8 T cells die, leaving behind a heterogeneous pool of memory cells [242]. Based on the effector function, proliferative capacity, migration pattern and localization, memory CD8 T cell population can be broadly classified into two groups, systemic memory T cell (circulating) and tissue-resident memory T cell (non-circulating) populations [242]. Systemic memory T cell population can be further classified into two subgroups, CD8 T<sub>CM</sub>, which expresses CCR7 and CD62L and CD8 T<sub>EM</sub>, which do not express
these molecules. CD8 T<sub>CM</sub> recirculates among blood, spleen and LNs whereas CD8 T<sub>EM</sub> recirculates among blood, spleen and non-lymphoid tissue (or peripheral tissues e.g. lung) [242]. In contrast, tissue-resident memory CD8 T cell (CD8 T<sub>RM</sub>) population do not recirculate but rather reside in the peripheral tissues after infection is cleared [242].

The efficacy of memory CD8 T cell-mediated protection against secondary influenza infection is directly linked to the number of influenza-specific memory CD8 T cells in the lung before the secondary challenge [243]. More importantly, it has been proposed that CD8 T<sub>CM</sub>, T<sub>EM</sub> and T<sub>RM</sub> confer varying degree of protections against heterosubtypic influenza challenge [242, 244, 245]. In influenza immunity, the evidence is emerging that CD8 T<sub>EM</sub> and T<sub>RM</sub> are accountable for the significant part of memory protection whereas CD8 T<sub>CM</sub> confer limited protection against heterologous influenza challenge [246, 247]. In general, effective heterosubtypic immunity lasts approximately 6 months, coinciding to the period when the presence of CD8 T<sub>EM</sub> and T<sub>RM</sub> in the lung become negligible [84, 243, 246, 248, 249]. The arguments that CD8 T<sub>CM</sub> is insignificant are based on the fact that reactivation of this subset takes place in the mLN and it takes at least 7 days before the CD8 T<sub>CM</sub>-derived secondary influenza-specific CD8 T cells to arrive in the lung after secondary infection [250]. The delayed presence of CD8 T<sub>CM</sub> in the influenza infected lung is thought to require CD8 T<sub>RM</sub> and T<sub>EM</sub> for the early containment [250-252]. In addition, due to the expression of CD62L and CCR7, CD8 T<sub>CM</sub> preferentially re-circulates through spleen, blood and LNs in which this migration pattern limits its presence in the lung [88]. In our study, we have shown that the circulating pool of influenza-specific memory CD8 T cells in the Clec9a-DTR mice displayed T<sub>CM</sub> phenotype (Figure 4.12). This revelation does not bode well for Clec9a-DTR mice in which CD8 T<sub>CM</sub> preponderance may partially explain the compromised efficacy of heterosubtypic immunity in this transgenic mouse strain (Figure 4.12).

Density of DCs, levels of antigen-bearing DC during cross-presentation and T cell precursor frequency have been demonstrated to influence memory CD8 T lineage commitment, that is, commitment of effector CD8 T cells into either
TEM or TCM is influenced by the level of antigen stimulation and clonal competition [210, 211, 253]. By adoptively transferring additional peptide-loaded DCs, injecting virus peptide or Flt3L treatment to increase DC density in order to achieve enhanced antigen stimulation in mLN during influenza virus infection, Shen et al and Marzo et al showed that the resultant memory CD8 T cell generated were preferentially TEM, indicating that excess of antigen signals in mLN inhibited the development of TCM. Conversely, a reduced level of antigen stimulation in the mLN of influenza infected CD11c-DTR mice skewed the development to TCM phenotype [210, 211, 253]. Consistent with these reports, we have shown that in Clec9a-DTR mice in which there was reduced level of virus antigen stimulation in the mLN after influenza virus PR8 infection, the commitment to CD8 TCM lineage was enhanced (Figure 4.4 and Figure 4.9.1). On the other hand, Clec4a4-DTR and wild type mice displayed relatively more intense cross-presentation indicating a higher level of virus-antigen stimulation and therefore influenza-specific CD8 T cells in Clec4a4-DTR and wild type mice were relatively more resistant to the development of TCM-like phenotype (Figure 4.4 and Figure 4.9.1). To demonstrate that antigen stimulation strength in mLN is indeed a decisive factor, more investigations will be required in particular to assess whether increasing DC density in the mLN (hence increasing the level of antigen stimulation) by adoptive transferring BMDC or Flt3L treatment will inhibit influenza-specific CD8 T cells in Clec9a-DTR mice from developing TCM-like property.

**On the selective loss of KLRG1+ influenza-specific CD8 T cell population in Clec4a4-DTR transgenic mouse strain.**

Effector CD8 T cell population in the primary response during virus infection are heterogeneous. There are 2 major subsets distinguished by KLRG1 and IL-7R (CD127) expression. KLRG1hiCD127lo cells represent short-lived effector cells (SLECs) and KLRG1loCD127hi cells represent memory precursor effector cells (MPECs) [254]. SLECs and MPECs identification is important as investigators demonstrated that these cells differ in their potential to develop into long-lived memory cells [255]. This classification approach is based on systemic virus LCMV infection model. SLECs and MPECs can be distinctly found in the blood of mice infected with systemic virus LCMV as early as 8
Using influenza virus infection model, Fang et al demonstrated that the effector CD8 T cells on day 10 P.I were KLRG1$^{hi}$CD127$^{lo}$ (SLECs) and KLRG1$^{lo}$CD127$^{lo}$ (Double Negative-DN effector CD8 T cells) but there was no KLRG1$^{low}$CD127$^{hi}$ (MPECs) cells, indicating a different expression profile compared to those cells in LCMV-infected mice [213]. Consistent with what Fang et al have shown, NP$^{366-374}$-specific CD8 T cells in our influenza infected mice were stained KLRG1$^{hi}$CD127$^{lo}$ (SLECs) and KLRG1$^{lo}$CD127$^{lo}$ (DN) referred to as KLRG1$^{hi}$ and KLRG1$^{lo}$ cells respectively (Figure 4.9.2). And there were no KLRG1$^{low}$CD127$^{hi}$ (MPECs) cells at this time point (day 10 P.I) that we investigated (Figure 4.9.2). We noted that there was very little or no KLRG1$^{hi}$ cells in Clec4a4-DTR mice compared to Clec9a-DTR and wild type mice (Figure 4.9.2A, B). Previous studies on LCMV infected mice demonstrated that KLRG1$^{hi}$ cells gave rise to the majority of T_{EM} population. As discussed above, T_{EM} play a more crucial role than T_{CM} is conferring cross-protection against heterosubtypic infection. A lack of KLRG1$^{hi}$ cells in Clec4a4-DTR mice would mean a lack of important source for T_{EM} and possibly compromising heterosubtypic immunity in Clec4a4-DTR mice (Figure 4.9.2A, B). Importantly, both LCMV and influenza infection model noted that some KLRG1$^{lo}$ cells regained KLRG1 expression over time and subsequently developed into memory cells exhibiting phenotypic markers characteristics of T_{EM}. These studies therefore suggest that KLRG1$^{lo}$ cells are capable of contributing to a minority of T_{EM} pool. However, whether this KLRG1$^{lo}$-derived T_{EM} are able to compensate for the loss of KLRG1$^{hi}$-derived T_{EM} require further investigation. More importantly, it is necessary to assess the significance of KLRG1$^{lo}$ and KLRG1$^{hi}$ –influenza specific CD8T cells in the heterosubtypic influenza immunity. Though we do not know what contribute to the selective loss of KLRG1$^{hi}$ cells in Clec4a4-DTR mice, the fact that memory potential is imprinted on effector CD8 T cells during the early primary response especially during CD8 T cell cross-presentation suggests that DCs may play a significant part [210].
On the absence of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs that resulted in significant reduction of IL-10-producing T cell population.

IL-10 is an immune-regulatory cytokine that inhibits inflammatory response [208, 256]. Sun et al showed that in the influenza infected lung, both effector CD4 T,1 cells and effector CD8 T cells are the major producers of IL-10 [104]. When IL-10R signaling was abrogated, there was an increased and accelerated mortality as well as over-production of proinflammatory cytokines illustrating that IL-10 is crucial in controlling the inflammation caused by influenza infection [104]. In our Clec9a-DTR and Clec4a4-DTR mice, there was significantly lower frequency of IL-10-secreting effector CD8 T cells in the infected lung. This impaired source of IL-10 might have led to the eventual enhanced levels of proinflammatory cytokines in Clec9a-DTR and Clec4a4-DTR mice (Figure 4.8A, B and Figure 4.10A, B). Considering the importance of IL-10 in the amelioration of influence-induced lung pathology, there is a need to investigate the reason behind this impairment. It has been reported that CD4 T cells-derived IL-2 and inflammatory infiltrates (macrophages or neutrophils)-derived IL-27 synergistically induce IL-10 production of effector CD8 T cells [257]. In view of this demonstration, it will be of great interest to investigate of possible involvement of IL-2 and IL-27.

On the dispensability of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs in the elicitation of homosubtypic immunity.

Vaccine or infection (primary) induced neutralizing antibodies protect the host from re-infection (secondary infection) with the identical virus strain [258]. Homosubtypic immunity is mediated by antibody response (primarily anti-HA neutralizing antibodies) in which passive transfer of these HA-specific antibodies prior to infection has been shown to confer full protection against serotypically identical virus strain [259, 260]. In our study, we show that loss of either CD103+ cDCs or CD24+CD11b+ cDCs did not affect homosubtypic immunity (Figure 4.11B). We further demonstrated that serum from infected Clec9a-DTR, Clec4a4-DTR and wild type mice conferred equivalent protection in naive mice against influenza infection induced by identical virus (Figure 4.11D). Collectively, these observations suggest to us that lung migratory cDCs are not important for the elicitation of homosubtypic immunity. However,
there is a need to quantify the level of HA-specific neutralizing antibodies between influenza infected wild type, Clec9a-DTR and Clec4a4-DTR mice by using a more sensitive approach called influenza hemagglutination inhibition (HI) assay. Basically virus bind to red blood cells and lead to formation of lattice, a process called hemagglutination. Presence of neutralizing antibodies will bind the virus and prevent hemagglutination. High abundance of neutralizing antibodies will efficiently inhibit hemagglutination. This assay will better inform us the level of neutralizing antibodies in the serum.

**On the requirement of lung migratory CD103+ cDCs and CD24+CD11b+ cDCs for the generation of effective cross-protection (heterosubtypic immunity).**

To our knowledge, the in vivo role of lung migratory cDCs in influenza immunity cross-protection (heterosubtypic immunity) has not been assessed. Heterosubtypic immunity is predominantly dependent on memory CD8 T cell response. Virus HA protein is highly prone to mutation, as such HA-specific antibodies are potent in conferring homosubtypic protection but generally do not cross-react with serotypically distinct influenza virus strains. Influenza-specific CD8 T cells target virus proteins which are conserved and thus capable of cross-reacting with various influenza virus subtypes.

In our study, we show that lack of CD103+ cDCs, but not CD24+CD11b+ cDCs led to complete loss of heterosubtypic immunity (Figure 4.12). This is unexpected considering that Clec4a4-DTR mice in fact displayed higher sensitivity than Clec9a-DTR mice to primary influenza infection. There is good evidence from animal models and humans that indicate the involvement of cross-reactive CD8 T cells in heterosubtypic immunity [261-263]. Christensen et al reported that when mice were challenged with highly lethal H7N7 influenza A virus, memory CD8 T cells which were established by previous encounter with H1N1 PR8 provided substantial protection [261]. Hillaire et al demonstrated that virus-specific memory CD8 T cells which were isolated from mice previously infected with seasonal H3N2 virus afforded cross-protection against H1N1 virus [264]. Given the observation that there was severe lack of cross-protection in our Clec9a-DTR mice, it is likely that the ablation of CD103+ cDCs had considerable impact on influenza-specific memory CD8 T cell pool (Figure 4.12).
Chapter 5: General Conclusion and Implication

In this study, we investigated the relative in vivo contribution of lung distinct DC subpopulations to the generation of influenza immunity through the use of our Clec9a-DTR and Clec4a4-DTR mice. We have shown for the first time that lung-derived CD24+CD11b+ cDCs do not contribute to the induction of influenza-specific CD8 T cell response in the mLN indicating that the presence of this subset in the mLN does not impact the initiation and expansion of mLN-residing naive influenza-specific CD8 T cells. In stark contrast, the number of influenza-specific CD8 T cells in the lung is CD24+CD11b+ cDC-dependent in which this DC subset is required to maintain the survival of anti-viral effector CD8 T cells. Together our results strongly propose that the presence of this lung-derived DC subset in the lung, rather than in the mLN, impact the overall size of influenza specific CD8 T cells in the lung. On the other hand, we have also confirmed the importance of lung CD103+ cDCs in the induction of influenza-specific CD8 T cell response in the mLN. The mechanism behind the regulation of influenza-specific CD8 T cells in the lung by lung-derived CD103+ cDCs is unclear. We have shown that lack of CD103+ cDCs reduced the egress of influenza-specific CD8 T cells from the mLN and CD103 DC deficiency led to enhanced survival defect of these effector CD8 T cells in the lung. Therefore in the absence of CD103+ cDCs, the overall accumulation of influenza-specific CD8 T cells in the lung is affected by compromised cross-presentation in the mLN, defective mobilization from the mLN, and enhanced survival defect in the lung indicating that the presence of this subset in the mLN as well as in the lung impact the accumulation of pulmonary anti-viral effector CD8 T cells.

Influenza-specific CD8 T cells are central to the design of flu vaccine that aims to induce cross-reactive immunity against influenza virus of distinct serotype. In order to exploit the DC potential for the induction of these cross-protective CD8 T cells, a thorough understanding of various distinct lung DC subpopulations is critically required. It is widely believed that each distinct DC
subset is ascribed with non-redundant property that controls different type of immune response. Hence it is important to activate the appropriate DC subset that will give rise to the intended responses. In the context of influenza infection, lung DC which excels at cross-presenting and inducing CTL response should be an ideal candidate. Lung migratory CD103+ cDCs, as demonstrated by our results and other studies, are the dominant lung DC subset that present virus antigen for the induction and expansion of influenza-specific CD8 T cells during the influenza infection. Henceforth, by targeting virus antigen to lung migratory CD103+ cDC population, a robust CD8 T cell response can be efficiently induced in the host. Antigen targeting to specific DC population has been demonstrated by Bonifaz et al whereby in this experiment they conjugated antigen of interest to anti-DEC205 antibody [269]. DEC205 is an endocytic receptor expressed rather uniquely by the specific DC subset they intended to target. Using this strategy, they were able to direct the antigen to the intended DC subset for activation and subsequent induction of robust T cell response for the increased protection against vaccinia virus infection.

There is another important consideration when it comes to T-cell based flu vaccine design. On top of how competent lung DCs are in terms of priming and expanding influenza-specific CD8 T cells in the mLN, the second wave proliferation of these effector CD8 T cells in the lung are equally crucial for their accumulation to high number in situ. We have shown that lung DCs are important regulators of this second wave effector T cell proliferation in the lung. In the absence of CD103+ cDCs, the frequency of proliferating effector CD8 T cells was significantly lower on day 15 P.I a period when the virus-specific CD8 T cells undergo rapid contraction. This data suggests that CD103+ cDCs are required to sustain the proliferative potential of these highly protective influenza-specific CD8 T cells. The exact mechanism how DCs enable proliferating CD8 T cells to persist is not clear at this stage. But more importantly, our results suggest that in order to promote robust influenza-specific CD8 T cell response, not only antigen presenting capacity but the ability to regulate proliferation potential of the effector T cells should also be considered in the design of effective T-cell based flu vaccine. If we are able to
tap into the T cell-proliferation-modulating property of CD103+ cDCs, it will likely benefit the design of future vaccine that aims to maximize the anti-influenza CD8 T cell response. Also, we were brought to attention of the selective lost of KLRG1+ CD8 T cells in Clec4a4-DTR mice. Though the exact functional significance of KLRG1+ CD8 T cells is unclear, our study has highlighted the potential of distinct DCs to differentially regulate CD8 T cell property and the possibility to increase the presence of KLRG1+ CD8 T cells by specifically targeting vaccine antigen to CD24+CD11b+ cDCs. So far, our study has facilitated and expanded the understanding the lung DC biology in the mouse and more importantly human DCs equivalents have recently been identified [270]. It has been shown that human CD141hi DCs and CD1c+ DCs are functional homologs of mouse CD103+ cDCs and CD24+CD11b+cDCs respectively an indication that mouse functional DC biology can be translated to the human setting.

A very interesting study recently showed that human CD1c+ DCs, isolated from humanized mouse model intranasally vaccinated with attenuated influenza virus, uniquely induced the expression of CD103 on naive and memory CD8 T cells in ex vivo culture assay [271]. CD103+ memory CD8 T cells are CD8 T_{RM}, is a very important memory CD8 T cell subset that contributes significantly to heterosubtypic immunity owing to the fact that CD103 expression retains memory CD8 T cells in the lung tissue and hence boost the presence of influenza-specific memory CD8 T cells in the lung. This report concluded that CD1c+ and CD141+ DCs generate CD8 T cells with different properties, a conclusion which our result resonates with in regard to our result suggesting the unique ability of CD24+ CD11b+ cDCs in driving KLRG1+ expression on CD8 T cells. Unfortunately, in vivo significance of human CD1c+ DCs in this aspect cannot be pursued. In light of these findings, our Clec4a4-DTR mice may potentially be a useful model to study the in vivo contribution of CD24+ CD11b+ cDCs to the generation of memory CD8 T_{RM} that may ultimately provide answer to the mechanisms behind the elicitation of long term heterosubtypic influenza immunity. One major concern regarding the effectiveness of targeting vaccine antigen to CD24+CD11b+ cDCs is that this population does not mediate significant cross-presentation in vivo, a pre-
requisite to generate effective CD8 T cell response. To conclude, we have shown that Clec9a-DTR and Clec4a4-DTR mouse models are useful tools to study the in vivo functions of two major lung DC subpopulations CD103 cDCs and CD24+CD11b+ cDCs respectively. Using experimental mouse-adapted influenza virus strain H1N1/PR8, our study has since expanded the understanding of the biology of these two distinct DC populations. This knowledge will benefit the development of more efficient T cell based flu vaccine.
References


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APPENDIX

Red blood cells (RBC) lysis buffer
0.89% ammonium chloride
Distilled H₂O

IMDM 2%
Iscove’s Modified Dulbecco’s Medium (IMDM)
2% Fetal Calf serum (FCS)

PBS 2%
1X PBS
2% FCS

Cell fixation buffer
1X PBS
1% paraformaldehyde
pH adjusted to 7.4

Permeabilization Buffer
PBS 2%
0.5% saponin

Diphtheria Toxin (DT)
1X PBS
1% mouse serum
2 ng/µl DT

3% Sodium Citrate Solution
70 µl of 20% Sodium Citrate solution (Distilled H₂O)
500 µl 1X PBS

70% Percoll solution
10X PBS, 35 ml
Percoll, 315 ml
IMDM, 150 ml

40% Percoll solution
10X PBS, 20 ml
Percoll, 180 ml
IMDM, 300 ml

ELISA wash buffer
1X PBS
0.05% Tween-20

ELISA assay buffer
1X PBS
1% Bovine Serum Albumin (BSA)
**Supplementary Figure 1**

Supplementary Figure 1: Targeting construct for the generation of Clec4a4-DTR knock-in mouse.
Author’s Publication

Christina Purnama, See Liang Ng, Piotr Tetlak, Yolanda Aphrilia Setiagani, Matheswaran Kandasamy, Sivasankar Baalasubramanian, Klaus Karjalainen and Christiane Ruedl. Transient ablation of alveolar macrophages leads to massive pathology of influenza infection without affecting cellular adaptive immunity. Eur J Immunol, 2014. (Co-author paper)
**Posters**

3rd Advanced Singaporean Immunology PhD student Retreat, Singapore
August 2012.
Poster presentation: “Inflammation Reporter Mouse”
See Liang, Ng., Karjalainen, K.

5th International Singapore Symposium of Immunology, Singapore,
June 2013.
Poster presentation: “Functional Roles of Pulmonary CD103+ and CD11b+
Dendritic cells in Influenza A virus infection”
See Liang, Ng., Ruedl, C., Karjalainen, K.

FEBS 17th International Summer School on Immunology. Rabac, Croatia.
September 2013
Poster presentation: “Functional Roles of Pulmonary CD103+ and CD11b+
Dendritic cells in Influenza A virus infection”
See Liang, Ng., Ruedl, C., Karjalainen, K.