The Role of Dendritic Cell Subsets in Cross-presentation and Stimulation of Homing Marker Expression

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The Role of Dendritic Cell Subsets in Cross-presentation and Stimulation of Homing Marker Expression

A DISSERTATION PRESENTED
BY
Suzanne Josette Taghap Nizza
TO
The Division of Medical Sciences
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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Cell and Developmental Biology

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The Role of Dendritic Cell Subsets in Cross-presentation and Stimulation of Homing Marker Expression

Abstract

Topical antigen (Ag) application mimics natural Ag exposure across the skin. Soluble Ag introduced through this route requires cross-presentation by dendritic cells (DCs) to generate CD8 T cell responses, including skin-homing T cells. DCs process Ag for display to other immune cells, and stimulate T cells to release cytokines or directly lyse infected cells. Some T cells are further stimulated to express homing markers allowing them to enter non-lymphoid tissue such as the skin or the gut.

In the last decade, DCs have been divided into subsets based on differential surface marker expression. The differences in development and function of these DC subsets are an area of active investigation. Some subsets may specialize in cross-priming T cells or imprinting skin homing, for example. Determining how different DC subsets interact with T cells would inform vaccine design and potentially lead to the development of DC-targeted vaccines.

The studies described in this dissertation were undertaken to determine the contribution of various skin-derived DC subsets to cross-priming and skin-selective imprinting. We developed a co-culture system where DCs loaded in vivo with soluble Ag from skin could be isolated and used to stimulate T cell proliferation and homing marker
expression in vitro. Using this co-culture system, we found that CD11b+ MHCIIhi migratory DCs are responsible for the cross-presentation of soluble OVA protein Ag applied to skin. Both Langerin+ and Langerin- CD11b+ migratory DCs cross-present Ag, but only the Langerin+ subset can imprint the skin-selective homing marker E-selectin ligand on proliferating CD8 T cells. From these data, we conclude that the ability of skin-derived DCs to stimulate proliferation varies greatly and DCs that can stimulate proliferation do not necessarily imprint homing markers. Our findings significantly advance our understanding of the cell population responsible for producing skin-homing T cells and provide a possible target for vaccines targeting skin-applied Ag or skin-specific immune responses. We also identified a previously unknown role for CCR4 in DC subset development, opening the door for further research into the role of chemokine receptors in DC development and subsequent T cell stimulation capacity.
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This dissertation is dedicated to my mother Tessie,

my father Joe, and my husband Peter
LIST OF ABBREVIATIONS

ADH  alcohol dehydrogenases
Ag   antigen
APC  antigen-presenting cell
ATL  adult T cell leukemia
BATF3 basic leucine zipper transcriptional factor ATF-like 3
BM-DC bone marrow-derived dendritic cell
cDC conventional dendritic cell
CFSE carboxyfluorescein succinimidyl ester
CT   cholera toxin
CTCL cutaneous T-cell lymphoma
CTL  cytotoxic T lymphocyte
DC   dendritic cell
DMSO dimethyl sulfoxide
DT   diphtheria toxin
E-lig E-selectin ligand
EAE  experimental autoimmune encephalitis
ER   endoplasmic reticulum
Flt3 fms-related tyrosine kinase 3
Flt3L Flt3 ligand
FucT-VII Fucosyltransferase-VII
GALT gut-associated lymphoid tissue
GM-CSF granulocyte macrophage colony-stimulating factor
HSV-1 herpes simplex virus 1
HTLV-1 human T cell leukemia virus type 1
i.c. intracutaneous
IEL intra-epithelial lymphocyte
i.l. intra-lymphatic
i.p. intraperitoneal
i.v. intravenous
L-CTCL leukemic cutaneous T-cell lymphoma
Lang Langerin
LC   Langerhans cell
LCMV lymphocytic choriomeningitis virus
LN   lymph node
LP   lamina propria
LPS  lipopolysaccharide
MDC  human macrophage-derived chemokines
MHC I major histocompatibility complex I
MHC II major histocompatibility complex II
MLN  mesenteric lymph nodes
OVA chicken ovalbumin
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<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
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<td>PLN</td>
<td>peripheral lymph nodes</td>
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<td>PP</td>
<td>Peyer’s patches</td>
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<td>PRR</td>
<td>pattern recognition receptor</td>
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<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
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<td>RA</td>
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<td>retinal dehydrogenase</td>
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<td>RAR</td>
<td>retinoic acid receptor</td>
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<td>RSV</td>
<td>respiratory syncytial virus</td>
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<td>RXR</td>
<td>retinoid X receptor</td>
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<tr>
<td>s.c.</td>
<td>sub-cutaneous</td>
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<td>sdLN</td>
<td>skin-draining LN</td>
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<td>SI-LP</td>
<td>small intestine lamina propria</td>
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<td>SIEC</td>
<td>small intestinal epithelial cells</td>
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<td>TAP</td>
<td>transporter associated with antigen processing</td>
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<td>TARC</td>
<td>thymus and activation regulated chemokine</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>WT</td>
<td>wildtype</td>
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CHAPTER ONE

Introduction

Suzanne Nizza

Attributions: Previous data (Figure 1.3) generated by N. Tubo. This chapter was written by S. Nizza with critical input from J. Campbell.
Dendritic cells (DCs) are the primary antigen (Ag)-presenting cells responsible for initiating T cell responses. After encountering, processing, and displaying Ag, DCs can stimulate T cells and B cells to trigger the immune response. T cells can then release cytokines that will further activate other immune cells or directly lyse infected cells in order to fight off an infection. Understanding how different DCs stimulate T cell responses could inform the design of vaccines to target specific DCs. We hypothesized that certain DC subsets could cross-present Ag, while others could imprint homing markers onto CD8 T cells, allowing T cells to move into the skin. In this first chapter, I introduce the two major cell types involved in our experiments: dendritic cells and T cells.

Dendritic Cells

Dendritic cells are responsible for antigen presentation

In the immune system, DCs comprise a population of Ag-presenting cells that initiate T cell responses. Immature DCs can take in Ag through macropinocytosis, receptor-mediated endocytosis, or phagocytosis of larger particles, cell fragments, viruses, bacteria, and intracellular parasites (Albert et al., 1998; Engering et al., 1997; Inaba et al., 1993; Jiang et al., 1995; Reis e Sousa et al., 1993). Upon taking up Ag, the DC matures from an Ag-capturing cell to an Ag-presenting cell (APC). Mature APCs express CCR7, a chemokine receptor involved in directing APCs to the draining lymph node to stimulate T cells. DCs process acquired Ag into peptides for display on major histocompatibility complexes I or II (MHC I or MHC II), as reviewed in (Trombetta and Mellman, 2005). Maturing DCs also express high levels of MHC II on their surface due
to inflammatory stimuli (Cella et al., 1997; Inaba et al., 2000; Pierre et al., 1997). DCs can process and display endogenous Ag (self Ag and intracellular pathogens) as well as exogenous Ag (Ag acquired externally) (Banchereau et al., 2000). DCs were first recognized for presenting exogenous Ag to CD4 T cells via MHC II, and endogenous Ag to CD8 T cells via MHC I (Banchereau et al., 2000; Burgdorf and Kurts, 2008) – so-called “classical presentation.” MHC I presentation of endogenous Ag is useful for the display of intracellular peptides after viral infection. MHC II presentation of exogenous Ag is useful for the activation of helper CD4 T cells.

**Dendritic cells cross-present exogenous antigen to CD8 T cells**

Tumor cells and viruses or intracellular pathogens that do not infect DCs do not generate endogenous peptides within DCs. Therefore, those Ags cannot be classically presented on MHC I to CD8 T cells. Cross-presentation, a more recently recognized DC function, is the process of presentation of *exogenous* Ag to CD8 T cells via MHC I (Kurts et al., 2010). This allows for viral Ag to be presented on MHC I without first infecting DCs, and for the presentation of exogenous Ag to CD8 T cells.

The exact intracellular processes within DCs that drive cross-presentation are not well understood. Ag moves from phagosomes into the cytosol, where it is degraded by proteasomes (Pfeifer et al., 1993). In the most well-studied pathway, Ag peptides are transported by TAP (transporter associated with antigen processing) from the cytosol into the endoplasmic reticulum (ER). Alternatively, early phagosomes containing TAP contain machinery for processing Ag for cross-presentation (Ackerman et al., 2003) and
process Ag on their own, or fuse with the ER (Guermonprez et al., 2003). Ag peptides are then bound to MHC I and then displayed on the cell surface (Burgdorf et al., 2007; Houde et al., 2003). Another, less well understood, mechanism is TAP-independent and involves the generation of peptides within endocytic compartments, so transportation to the cytosol is unnecessary (Pfeifer et al., 1993; Song and Harding, 1996; Wick and Pfeifer, 1996). Cathepsin S plays a key role in this pathway (Shen et al., 2004).

A significant component of the work presented in this dissertation focuses on the cross-presentation capability of different DC subsets. Cross-presentation is a key function of DCs, but exploring cross-presentation requires one to be sure that no classical presentation is occurring. This requires careful choice of Ag and Ag-application method.

Additional dendritic cell signaling beyond antigen receptors

A DC encountering Ag is alone not enough to trigger a further immune response. DCs need additional signals to up-regulate co-stimulatory molecules and enhance their response to Ag. Most commonly, these signals come from the activation of Toll-like receptors (TLRs) as reviewed in (Kawai and Akira, 2007), though other adjuvants can also trigger a response. TLRs are type I integral membrane glycoproteins that are part of the group of pattern-recognition receptors (PRRs) that recognize molecules from pathogens (Kumar et al., 2009). TLRs are highly expressed by DCs and can trigger downstream signaling cascades resulting in chemokine production and/or immune cell activation when bound to their agonists.
Here, we used the bacterial cell wall component lipopolysaccharide (LPS) and the bacterial protein cholera toxin (CT) as adjuvants. LPS is detected by TLR4 in association with MD-2 (Shimazu et al., 1999). CT is a toxin secreted by *Vibrio cholerae* that is frequently used as an adjuvant. CT can induce functional DC migration, as DCs matured with CT express functional CCR7 (Gagliardi et al., 2000). CT is thought to activate DCs through the GM1 ganglioside receptor (Kawamura et al., 2003). CT has been seen to enhance epicutaneous immune responses to co-administered Ag (Kahlon et al., 2003).

In some cases, additional signals to the DC from CD4 T cells are required for DC cross-priming. In this situation, the CD4 T cell signals through CD40 ligand, “licensing” the DC and enabling it to further activate CD8 T cells, also known as cytotoxic T lymphocytes (CTLs). Some examples of DC licensing can be seen in CTL priming in response to herpes simplex virus 1 (HSV-1) (Smith et al., 2004) and CTL-mediated tumor immunity (van Mierlo et al., 2004).

**Development of dendritic cells**

DCs in the spleen develop from precursors that circulate in the blood and develop into immature CD8α⁻ and CD8α⁺ DC subsets after entering the spleen (Liu et al., 2007; Shortman and Naik, 2007). DCs found in the lymph nodes (LN) are thought to travel there through the afferent lymph from non-lymphoid tissues. Some DCs and DC precursors are also thought to reach LN across the high endothelial venules (Randolph et al., 2005). Epidermal DCs are also known as Langerhans cells (LCs). The LC
population is maintained by local precursor cells in the steady state (Merad et al., 2002). During severe skin inflammation or other major LC depletion, LC can be repopulated by circulating precursors in the blood, namely Gr-1^{hi} monocytes (Ginhoux et al., 2006).

**Dendritic cell subsets**

The population of DCs within a tissue can be divided into multiple subsets, based on their surface marker expression and their positioning within tissues. There are two large categories of DC in mouse lymphoid organs – conventional DCs (cDC) and plasmacytoid DC (pDCs). pDC are mainly known for their unique ability to secrete type I interferons, and are B220^{+} and CD11c^{int}. pDC are considered poor presenters of exogenous Ag, but can present endogenous Ag (Villadangos and Young, 2008). cDC are the main studied DC population and can be divided into migratory DC and resident DC (Villadangos and Schnorrer, 2007). Resident DCs are MHC II^{int}CD11c^{hi} and can be divided into three subsets: CD4^{+}CD8^{-}, CD4^{+}CD8^{+}, and CD4^{+}CD8^{-} (double-negative) DCs (Henri et al., 2001; Vremec et al., 2000). The CD4^{+} and the double-negative resident DCs are also CD11b^{+}. Migratory DC develop in peripheral tissues, such as the skin and the gut, and migrate constitutively into lymph nodes. Migratory DCs include various subsets that can be divided largely on the basis of Langerin, CD11b, and CD103 expression (Figure 1.1).

Below, I detail some of the many different DC subsets identified with a variety of surface markers. The most studied non-lymphoid tissues are the skin, gut, and more
Figure 1.1: There are a number of DC subsets that can be differentiated on the basis of surface markers. Adapted from Heath, W.R. and Carbone, F. R. (2009) Nature Immunology, 10(12), 1237-1244.

Nature Immunology, 10(12), 1237-1244.
recently, the lung. Some of the subsets found in different tissues share common surface markers, but are not necessarily of the same type of DC. The work presented herein mainly focuses on the DC subsets found in the skin and skin-draining LNs.

**Dendritic cell subsets in the skin**

Most DCs within murine skin-draining lymph nodes (sdLN) are either resident or migratory DCs. LN-resident DCs are MHC II^{int}CD11c^{hi}, and can be divided into CD8α^{+}CD11b^{-} and CD8α^{-}CD11b^{+} subsets (López-Bravo and Ardavín, 2008; Shortman *et al.*, 2002). Migratory DCs transport Ag from the skin to the sdLN via afferent lymphatics (Allan *et al.*, 2006; Lee *et al.*, 2009). Migratory DC are MHC II^{hi}CD11c^{int} and require functional CCR7 expression for migration to sdLN. This population can be further divided into CD11b^{-}CD103^{+} and CD11b^{+}CD103^{-} subsets. Langerin (CD207) is expressed by essentially all DC originating from the epidermis (i.e., Langerhans cells) and by some of the migratory DC originating from dermis (Bursch *et al.*, 2007; Ginhoux *et al.*, 2007; Poulin *et al.*, 2007). Epidermal-derived Langerhans cells (Langerin^{+}CD11b^{+}CD103^{-}) and dermal-derived Langerin^{+}CD11b^{-}CD103^{+} cells can both be found within the sdLN (del Rio *et al.*, 2010). Langerin^{+} dermal DCs were identified and differentiated from LCs in late 2007 (Bursch *et al.*, 2007; Poulin *et al.*, 2007).

DCs traveling from the skin take varying amounts of time to reach the draining LN. LC arrive late in the LN, taking up to four days post-immunization to reach their peak presence in the LN, while Langerin^{-} dermal DCs take two days post-immunization to reach their peak in the LN (Kamath *et al.*, 2002; Kissenpfennig *et al.*, 2005). Langerin-
EGFP mice express EGFP under the control of the Langerin promoter, allowing detection of Langerin\(^+\) DCs \textit{in vivo} and \textit{in vitro} (Kissenpfennig \textit{et al.}, 2005).

Kissenpfennig \textit{et al.} found that Lang\(^+\) and Lang\(^-\) DCs localize in different areas of the draining LN. Lang\(^+\) DCs (assumed to be LC-derived) were found in the inner paracortex of the T cell zones and excluded from B cell zones, while dermal DCs were in the outer paracortex, closer to the B cell follicles (Kissenpfennig \textit{et al.}, 2005).

\textbf{Dendritic cell subsets in the gut}

DCs in the intestinal tract are found in the lamina propria (LP) and lymphoid organs in the intestine (Peyer's patches (PP) and mesenteric lymph nodes (MLN)). These lymphoid organs contain varying DC subpopulations. The LP contains CD103\(^-\)CD11b\(^+\) DCs located near the gut epithelium (Niess \textit{et al.}, 2005; Rescigno \textit{et al.}, 2001), and CD103\(^+\)CD11b\(^-\) and CD103\(^+\)CD11b\(^+\) DCs deeper in the LP (Bogunovic \textit{et al.}, 2009; Denning \textit{et al.}, 2011; Jaensson \textit{et al.}, 2008; Schulz \textit{et al.}, 2009; Varol \textit{et al.}, 2009). CD11b\(^+\) DCs in the LP can be further subdivided based on their level of CX\(_3\)CR1 expression (Schulz \textit{et al.}, 2009; Varol \textit{et al.}, 2009). The MLN and PP contain resident CD11b\(^+\)CD8\(\alpha\)\(^-\), CD11b\(^-\)CD8\(\alpha\)\(^+\), and CD11b\(^-\)CD8\(\alpha\)\(^-\) DCs, along with migratory CD103\(^+\) DCs (Annacker \textit{et al.}, 2005; Iwasaki and Kelsall, 2001; Jaensson \textit{et al.}, 2008).

\textbf{Dendritic cell subsets in the lung}

In the lung and the respiratory tract, CD11c\(^+\)MHCII\(^+\) DCs can be divided into CD103\(^+\)CD11b\(^-\) and CD103\(^+\)CD11b\(^hi\) populations. The CD103\(^+\) DCs localize to the lung
mucosa and pulmonary vessels (Sung et al., 2006), while the CD11b^{hi} DCs localize to the airway submucosa and the lung parenchyma (Kim and Braciale, 2009; von Garnier et al., 2005). After intra-tracheal transfer of in vitro-derived DCs, Ag presentation and T cell responses can be observed in the draining mediastinal LN (Lambrecht et al., 2000). Both the CD11b^{hi} DCs and the CD103^{+} DCs can migrate and transport Ag from the airway mucosa to the mediastinal LNs (also called the pulmonary or thoracic LNs) (Vermaelen et al., 2001). Some of the CD103^{+} CD11b^{-} DCs in the lung express Langerin (Sung et al., 2006), and may be part of the same subset as the CD103^{+} Lang^{+} DCs seen in the dermis of the skin (Bursch et al., 2007). The migration of CD103^{+} DC from the lung to the bronchial LN is CCR7-dependent (Hintzen et al., 2006). Migratory CD103^{+} lung DCs can transfer Ag to LN-resident CD8{\alpha}^{+} DCs in mediastinal LNs (Belz et al., 2007; Belz et al., 2004).

**Dendritic cell subsets and cross-presentation**

It is currently thought that only certain DCs (those expressing CD24, CD8{\alpha}, or CD103) are able to cross-present Ag under non-inflammatory conditions (Bedouli et al., 2009; del Rio et al., 2007; den Haan et al., 2000; Pooley et al., 2001). Under inflammatory conditions, other DC subsets may be able to cross-present (Ballesteros-Tato et al., 2010; Chung et al., 2005; Den Haan and Bevan, 2002; Iyoda et al., 2002; Mcdonnell et al., 2010; Schulz and Reis E Sousa, 2002). The work I present in this dissertation demonstrates the varying cross-presentation capacity of DC subsets from sdLN under inflammatory conditions.
There are various hypotheses as to why the capability for cross-presentation may be restricted among DC subsets (Villadangos and Schnorrer, 2007). Specific Ag-processing machinery may be necessary for cross-presentation and only produced in certain DC subsets (Dudziak et al., 2007). Work with splenic DCs showed that all subsets were capable of taking up Ag, but only the CD8⁺ DC cross-presented efficiently (Pooley et al., 2001; Schnorrer et al., 2006). Cross-presentation may also be restricted to Ags taken up by specific endocytosis receptors, such as mannose receptors, Fc receptors, and some C-type lectin receptors (Burgdorf et al., 2007; Burgdorf and Kurts, 2008; Burgdorf et al., 2006; Caminschi et al., 2008; Dudziak et al., 2007; Lin et al., 2008; Weck et al., 2008), thus restricting cross-presentation to DC subsets expressing those receptors. However, recent work in humans implies that a wide variety of DC subsets may be capable of cross-presentation (Segura et al., 2013), with differences in actual cross-presentation activity influenced on another level (such as access to Ag, or localization of the DC within the tissue). Supporting this idea, Lee et al. found that HSV-1 was primarily cross-presented by different DCs after different infection methods. HSV-1 was cross-presented by resident DCs after epicutaneous infection, but by migratory DCs after vaginal infection (Lee et al., 2009).

It should be noted that some studies using viral Ag to study cross-presentation have used viruses that may directly infect DC at some level (de Graaff et al., 2005; Goldwich et al., 2011; Sevilla et al., 2000). Viral Ag displayed by a DC after infection of that DC would be classically presented, not cross-presented. As a result, some of the
following findings may be at least partially attributable to the classical presentation of endogenous Ag by MHC I after DC infection, not cross-presentation.

**Resident dendritic cells associated with cross-presentation**

Resident CD8α⁺ DC were found to be the sole cross-presenting DCs after intravenous (i.v.) infection with *L. monocytogenes*, i.v. or intraperitoneal (i.p.) infection with lymphocytic choriomeningitis virus (LCMV) (Belz *et al.*, 2005), or cutaneous infection with HSV (Allan *et al.*, 2006; Allan *et al.*, 2003; Lee *et al.*, 2009). In some cases, Ag was carried by migratory DCs to the lymph node, where it was transferred to CD8α⁺ DCs (Lee *et al.*, 2009). CD8α⁺ DCs are hypothesized to possess specialized intracellular machinery for processing and presenting exogenous Ag on MHC I (Dudziak *et al.*, 2007; Schnorrer *et al.*, 2006). CD8α⁺ DCs were one of a few cross-presenting DC subsets in pulmonary or cutaneous flu infections (Belz *et al.*, 2004; GeurtsvanKessel *et al.*, 2008). CD8α⁻ DCs, on the other hand, have been found to classically present Ag to CD4 T cells under certain conditions (Iezzi *et al.*, 2006; Mount *et al.*, 2008).

**Migratory dendritic cells associated with cross-presentation**

Many migratory DCs found in non-lymphoid tissue are able to ferry Ag to the draining LN after infection or Ag application. The particular migratory DC subset largely thought to be responsible for cross-presentation in the draining LN is the CD103⁺ migratory DC subset. The migratory DCs most studied are those in the skin, gut, and more recently, lung.
In the skin, CD103⁺ dermal DCs have been seen to present Ag to both CD8 and CD4 T cells (Mount et al., 2008). After cutaneous lentiviral infection, skin-derived DCs were responsible for CD8 T cell priming (He et al., 2006). Langerin⁺ CD103⁺ dermal DCs have been seen to cross-present keratinocyte-derived Ag (Henri et al., 2010) and to present HSV-1 Ag to CD8 T cells after secondary viral skin infection (Bedoui et al., 2009). Langerin⁺ CD103⁺ dermal DCs harvested from the sdLN of K5-mOVA mice were also able to present skin-derived self-antigen to Ag-specific CD8 cells and trigger proliferation (Bedoui et al., 2009). The transcription factor BATF3 (basic leucine zipper transcriptional factor ATF-like 3) is expressed by CD8α⁺ DCs and CD103⁺ DCs, and is thought to be necessary for development of those DC subsets and subsequent cross-presentation. Hildner et al. found that Batf3⁻/⁻ mice lacked CD8α⁺ DCs in the spleen, LN, and thymus, while CD8α⁻ DCs were normally present (Hildner et al., 2008). Batf3⁻/⁻ mice also had a reduced number of CD103⁺ CD11b⁻/⁻ CD8α⁻ dermal DCs in skin-draining LN (Hildner et al., 2008).

In the gut, CD11b⁺ DCs in the MLN have been seen to promote cross-tolerance to dietary proteins (Chung et al., 2005). CD103⁺ and CD103⁻ DCs in MLN are both able to prime CD8 T cells, but differ in their ability to stimulate homing molecules on CD8 T cells, as will be discussed later in this chapter (Johansson-Lindbom et al., 2005).

In the lung, the CD103⁺ migratory DCs can cross-present Ag (Belz et al., 2004; GeurtsvanKessel et al., 2008), and could cross-present OVA injected intranasally, while CD11b⁺ DCs were focused on classical MHC II presentation (del Rio et al., 2007). After influenza infection, both CD103⁺ and CD11b⁺ DCs from the mediastinal LN could
stimulate Ag-specific CD4 T cell proliferation, but the CD103+ DC were more efficient at activating naïve Ag-specific CD8 T cells (Kim and Braciale, 2009). After influenza virus infection, CD103+ DCs induced virus-specific CD8 T cells and were protected from influenza virus infection (Helft et al., 2012). CD103+ DCs, but not CD11bhi DCs, are responsible for apoptotic cell clearance and cross-presentation of apoptotic cell-associated Ag in the lung (Desch et al., 2011).

Some work has shown that lung CD11b+ DCs play a role in cross-presenting certain viral Ag. After respiratory syncytial virus (RSV) infection, both CD11bhi and CD103+ DCs migrate to the lung-draining mediastinal LN, and both are able to trigger IFNγ production by CD8 T cells in mediastinal LN (Lukens et al., 2009). After pulmonary influenza infection, both CD11bhi and CD103+ DCs migrate to the mediastinal LN (Ballesteros-Tato et al., 2010). In this case, CD11bhi DCs accumulate more than CD103+ DCs in the mediastinal LN, and cross-prime CD8 T cells with exogenous Ag (Ballesteros-Tato et al., 2010). However, another group showed that, despite the influx of CD11bhi DCs into the mediastinal LN, CD103+ DCs were responsible for inducing influenza virus-specific CD8 T cells (Helft et al., 2012).

Langerhans cells (LC) and cross-presentation

The ability of LC to cross-present Ag has been controversial. Bursch et al. showed that CD8 T cell responses to epidermal self-Ag did not require LCs (Bursch et al., 2009). Igyártó et al. used a Candida albicans skin infection model to demonstrate that LCs were important for Th17 generation, but not CD8 proliferation (Igyártó et al.,
2011). This study used huLang-DTR mice, which express DTR exclusively on LCs (Bobr et al., 2010), allowing the specific elimination of LC in response to DT without affecting Lang+ dermal DCs. CD8 T cells were able to proliferate in DT-treated huLangerin-DTR mice just as well as in WT mice, so LCs were considered dispensable for the CD8 T cell response (Igyártó et al., 2011). Other work with LC ablation models has produced mixed results on the role of LCs in contact hypersensitivity, with some groups finding a positive role for LCs, some groups finding a negative role, and some finding a redundant role. This work has been reviewed in (Kaplan, 2010; Kaplan et al., 2008). The finding that LCs do not directly present HSV Ag to CD8 T cells, but transfer Ag to CD8+ DCs that subsequently prime CD8 T cells, supported the notion that LCs were not directly involved in cross-presentation (Allan et al., 2006).

However, other studies suggested that Langerhans cells may in fact have the ability to cross-present Ag. For example, Stoitzner et al. pulsed skin explants with ovalbumin protein ex vivo and allowed LCs to migrate out of the skin, finding these LCs capable of cross-priming OT-I cells (Stoitzner et al., 2006). LCs were able to take up HPV-like particles (HPV-VLP) and prime CD8 T cells (Yan et al., 2004), and in vitro-generated LC were also able to cross-prime CD8 T cells (Cao et al., 2007). These studies involve loading LCs with Ag ex vivo, a non-physiological method, but are still useful for demonstrating that LCs do have the capacity to cross-prime CD8 T cells. In Chapter 3, I demonstrate the ability of Langerhans cells to cross-present Ag using my co-culture system.
**T cells and homing**

T cells proliferate in response to activation signals from Ag-loaded DCs. There are two types of T cells: CD4 T cells and CD8 T cells. CD4 T cells recognize peptides bound to MHC class II, and CD8 T cells recognize peptides bound to MHC class I. CD4 T cells are commonly thought of as “helper” T cells, instructing other immune cells to act to fight infections. Through direct stimulation and the release of cytokines and chemokines, they stimulate B cells to make antibodies, recruit basophils and neutrophils to infection sites, and stimulate macrophage activity.

Our work mainly focuses on CD8 T cells, or CTLs. CTLs destroy cells that have become infected with viruses or have become cancerous. CD8 T cells are activated by APCs displaying their cognate Ag on MHC I. Proinflammatory cytokines, such as IL-12, further lead to the maturation and differentiation of a naïve CD8 T cell into an effector CTL (Curtsinger et al., 2003). After identifying a target cell, CD8 T cells release perforin and granzymes to trigger apoptosis.

For study of T cell proliferation and activity, mice containing T cells with a transgenic TCR have been produced. These T cells respond to a particular peptide of a particular Ag. Use of these T cells allows the production of a strong immune response by applying Ag to mice that have been injected with T cells with the corresponding TCR. Chicken ovalbumin protein (OVA) is a commonly used experimental Ag. CD8 T cells from OT-I mice express a transgenic TCR specific for the H2-K\(^b\)-restricted peptide OVA\(_{257-264}\) (Hogquist et al., 1994). CD4 T cells from OT-II mice express a transgenic TCR specific for the IAb-restricted peptide OVA\(_{323-339}\) (Barnden et al., 1998). DO11.10
mice have a MHC II-restricted TCR specific for the peptide OVA\textsubscript{323-339} on the H\textsuperscript{2d} background (Murphy \textit{et al.}, 1990; Robertson \textit{et al.}, 2000). K5-mOVA mice are sometimes used to study responses to self-Ag. These mice express a membrane-associated form of OVA, under the keratin 5 promoter (Azukizawa \textit{et al.}, 2003). Our work in the lab has focused on OT-I and OT-II T cells. For studies using viral Ag, the commonly used P14 mice have a MHC I-restricted TCR specific for the P14 peptide from LCMV on the H-2D\textsuperscript{b} background (Pircher \textit{et al.}, 1989).

\textbf{T cells can express surface markers allowing them to enter non-lymphoid tissues}

Naïve T cells can migrate into secondary lymphoid organs, but do not express the surface molecules allowing them to enter non-lymphoid tissues until they encounter Ag (Butcher \textit{et al.}, 1999; von Andrian and Mackay, 2000). There are many surface molecules associated with T cell rolling, tethering, and extravasation out of the bloodstream. Some of these surface molecules are tissue-specific. Some Ag-experienced CD4 and CD8 T cells express these tissue-specific molecules, allowing them to accumulate in certain peripheral tissues such as the skin, gut, or lung (Figure 1.2). The conditions controlling the expression of these homing molecules are the main point of study of our lab. We hypothesized that only certain DC subsets would be able to trigger the expression of surface markers allowing T cell entry into the skin.

There are multiple steps involved in the movement of T cells from blood into tissues. First, adhesion receptors on leukocytes, namely L-selectin (CD62L), P-selectin...
Figure 1.2: Different surface molecules are associated with homing to the skin and to the gut. Adapted from Agace, W.W. (2006). *Nat Rev Immunol*, 6(9), 682-692.

Various surface molecules are commonly associated with skin- or gut-homing, due to their roles in the entry of naïve T cells into extralymphoid tissues.
(CD62P), and E-selectin (CD62E), bind to oligosaccharide ligands on the blood vessel wall (Kansas, 1996). This binding is not intended to permanently stop the T cell’s movement; the bonds formed are not strong enough to fully resist the shear stresses of flowing blood. The T cell begins to roll along the blood vessel wall, as old bonds break and new bonds form in the direction of blood flow. This rolling slows the T cell down, allowing additional adhesion molecules to form bonds. These secondary adhesion molecules are integrins, specifically LFA-1, α4β1 (VLA-4), and α4β7 (Alon et al., 1995; Berlin et al., 1995; Lawrence and Springer, 1991). Integrins, unlike selectins, must first be activated before forming bonds. Chemokines released from the endothelial cells of the blood vessel activate integrins on the rolling T cell (Campbell et al., 1998; Cyster, 1999). The bonds formed by the activated integrins are much stronger than those formed by the selectins, bringing the T cell to a firm stop. Other chemokines released in the tissues form gradients which direct the T cells after extravasation (Kim and Broxmeyer, 1999).

T cell expression of E- and P-selectin ligands is associated with migration into skin. E-selectin ligands (E-lig) are important for the interaction of skin-selective T cells with the endothelium of cutaneous vasculature and the subsequent entry of these cells into skin from the blood (Butcher et al., 1999). CD4 and CD8 T cells lacking E- and P-selectin ligands are unable to migrate into the skin (Erdmann et al., 2002; Tietz et al., 1998). CCR4 was found to be expressed by skin-homing memory T cells in the blood that also expressed cutaneous lymphocyte antigen (CLA) (Campbell et al., 1999). CLA is related to an E-lig (Fuhlbrigge et al., 1997). CCR4 is also thought to be associated
with skin homing. CCL17, a ligand of CCR4, is expressed on skin venules, and this expression is increased during inflammation (Campbell et al., 1999). CCR4 is necessary for Ag-driven cutaneous accumulation of CD4 T cells (Campbell et al., 2007), and is also expressed on skin-homing CD8 T cells (Dudda et al., 2004). In chapter 4, I demonstrate a previously unknown role for CCR4 in DC subset composition.

CCR10 is also thought to be associated with skin homing, as CCR10 is expressed by some CLA-expressing memory CD4 and CD8 T cells. CCR10’s ligand CCL27 is found in the skin (Hudak et al., 2002). CCR6 is also thought to be a skin-homing T cell molecule, as it is expressed by some E-lig⁺ memory T cells (Homey et al., 2000), and by many skin-resident CD4 T cells in humans (Clark et al., 2006). However, recent work has shown that deficiency of CCR6 or CCR10 has no effect on accumulation of memory CD4 T cells in skin, suggesting that CCR6 and CCR10 are unrelated to skin-specific trafficking (Tubo et al., 2011). Many human skin-resident T cells also express CCR8 (Clark et al., 2006; McCully et al., 2012), though the specific significance of CCR8 in skin homing is unknown.

Expression of integrin α4β7 and CCR9 are associated with T cell migration to the gut. α4β7 on T cells binds with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed by blood vessels in the intestinal epithelium (Butcher et al., 1999; Campbell and Butcher, 2002; Kantele et al., 1999; Rott et al., 1996). Lymphocytes in the small intestine and lamina propria migrate towards CCL25 (TECK, thymus-expressed chemokine). This migration is mediated by lymphocyte-expressed CCR9 (Kunkel et al., 2000; Svensson et al., 2002).
Models of T cell tissue homing

Initially, it was not known how T cells were stimulated to express homing markers appropriate for traveling to the site of Ag presence. Some suggested that the initial expression of homing surface markers was stochastic (Davenport et al., 2000). A wide variety of T cells expressing different homing markers would be produced, and selection would favor the further proliferation of T cells with appropriate homing markers. T cells that entered areas with little to no Ag would not be stimulated and so die off.

Currently accepted is an instructional model of lymphocyte homing, where factors in the environment (other immune cells, tissue cells, etc.) directly instruct the T cell what homing markers to express. This model is supported by work done in the last several years showing that dendritic cells (and possibly other tissue cells) are able to stimulate T cell expression of homing markers in vitro (described in the following pages).

Dendritic cells and T cell tissue homing

DCs are thought to be the main Ag-presenting cells capable of activating developmental programs within primed naïve T cells that cause proliferating T cells to express homing molecules – a process known as imprinting (Butcher et al., 1999; Campbell and Butcher, 2000). This imprinting directs T cells to preferentially enter the target tissues where Ag was initially taken up by DCs (Butcher et al., 1999; Campbell and Butcher, 2000; Dudda et al., 2004; Mora, 2005; Mora et al., 2003). T cells in peripheral blood use E-lig (e.g., CLA in humans) to enter skin, and integrin α4β7 to
enter intestinal tissues (Butcher et al., 1999). Early work demonstrated that peptide-pulsed DCs from sdLN or Peyer’s patches could stimulate CD8 T cells to express E-lig or α4β7, respectively (Mora, 2005).

A variety of methods have been used to study the influence of DCs on tissue homing, to generate/isolate DCs and to load them with Ag. Below, I categorize them based on the type of DC used: DCs generated in vitro from bone marrow precursors (BM-DCs); DCs isolated from either spleen or LNs and used as a complete population; and DCs isolated from lymphoid tissue but divided into DC subsets.

Studies of gut-homing imprinting using varying DC types

I. DCs generated from bone marrow precursors

Bioluminescence imaging in mice has shown that i.v. injected BM-DCs accumulate in the spleen, PLN, and tracheal LN, while i.p. injected BM-DCs accumulate in peritoneal LN (especially pancreatic LN), and in omentum-associated lymphoid tissue (Creusot et al., 2009). After injection of peptide-pulsed BM-DCs i.c., i.v., and i.p., adoptively transferred P14 CD8 T cells upregulate E-lig and α4β7 depending on the mode of injection: E-lig is expressed only after i.c. injection, and α4β7 only after i.p. injection. Intravenous injection did not generate either homing molecule (Dudda et al., 2004). In a similar experiment, OVA-peptide pulsed BM-DCs were injected i.p., i.v., or subcutaneously (s.c.) after adoptive transfer of OT-I T cells. CD8 T cells in the MLN expressed α4β7 after i.p. injection. A substantial number also expressed P-lig, a possible skin-homing marker. Intravenous injection also led to α4β7 expression in the
MLN, but at lower levels (Ferguson and Engelhard, 2010). *In vitro* work with BM-DCs is inconsistent. While peptide-pulsed BM-DC were able to stimulate α4β7 expression on P14 T cells *in vitro* (Dudda *et al.*, 2004), Ag-loaded BM-DCs used by Hammerschmidt *et al.* were unable to induce any homing marker expression *in vitro*. However, Ag-loaded BM-DCs injected directly into the afferent lymphatics of the mesenteric LN were able to stimulate α4β7 and CCR9 expression on adoptively transferred OVA-specific DO11.10 (Hammerschmidt *et al.*, 2008).

**II. Bulk/unsorted lymph node dendritic cells**

OVA peptide-loaded splenic DCs were able to induce CCR9 and α4β7 expression on T cells after intralymphatic (i.l.) injection (Hammerschmidt *et al.*, 2008). After expansion by s.c. injection of Flt3L, OVA peptide-pulsed DCs from the lamina propria were also able to stimulate CCR9 and α4β7 expression by CD8 T cells (Johansson-Lindbom *et al.*, 2005).

DCs harvested from gut-draining LN were also successfully used to stimulate gut homing marker expression on T cells. MLN DC in the presence of anti-CD3 Ab stimulated more CD8 T cell expression of α4β7 and CCR9 than PLN DC (Stagg *et al.*, 2002). DCs isolated from MLN, when loaded *in vitro* with OVA peptide, stimulate CD8 T cells and expression of CCR9 and α4β7 (Dudda *et al.*, 2005; Dudda *et al.*, 2004; Johansson-Lindbom *et al.*, 2003). After transferring OT-I cells in recipient mice, i.p. immunization with OVA protein produced T cells in MLN that expressed CCR9 and α4β7 (Johansson-Lindbom *et al.*, 2003). Peptide-pulsed DCs from Peyer’s patches (PP)
can also stimulate naïve CD8 T cell proliferation and expression of α4β7 and CCR9 (Dudda et al., 2005; Dudda et al., 2004; Mora, 2005; Mora et al., 2003).

III. Dendritic cell subsets

In the gut, CD103+ DCs seem to be responsible for gut homing marker imprinting. Peptide-pulsed CD103+ small intestine lamina propria (SI-LP) DCs were able to stimulate both α4β7 and CCR9 expression on Ag-specific CD8 T cells (Jaensson et al., 2008). After oral OVA immunization, sorted CD103+ MLN DCs, but not CD103− MLN DCs, were able to stimulate OT-I and OT-II T cell proliferation (Jaensson et al., 2008). After i.p. OVA immunization, CD103+ DCs stimulated significantly higher CCR9 and high α4β7 expression compared to CD103− DCs (Jaensson et al., 2008).

Using Ag-pulsed DCs in vitro, however, has produced conflicting results. All subsets of PP DCs were able to induce α4β7 expression but not CCR9 expression after being pulsed with peptide (Jaensson et al., 2008; Mora, 2005). In vitro, Ag-pulsed CD103+ or CD103− MLN DCs were both able to stimulate CD8 T cell proliferation and α4β7 expression, but only CD103+ DCs were able to stimulate CCR9 expression (Jaensson et al., 2008; Johansson-Lindbom et al., 2005). Annacker et al. found that CD103+ MLN DCs were better than CD103− DCs at stimulating CCR9 expression when cultured with OT-II CD4 T cells and OVA peptide (Annacker et al., 2005).

Vitamin A and imprinting of gut-homing
After identification of α4β7 and CCR9 as the key surface molecules for gut-homing, the next major discovery in the gut-homing field was that the vitamin A (retinol) metabolite, retinoic acid (RA), stimulated α4β7 and CCR9 expression on T cells (Iwata et al., 2004). Vitamin A deficiency leads to fewer α4β7+ T cells in lymphoid organs (Iwata et al., 2004).

RA is formed through the intracellular metabolism of retinol into retinal, then into RA (Duester, 2000). This pathway is catalyzed by certain alcohol dehydrogenases (ADH) and retinal dehydrogenases (RALDH) (Duester, 2000). Iwata et al. found that DCs from GALT (gut-associated lymphoid tissue) converted retinol into retinoic acid, leading to the production of gut-homing T cells (Iwata et al., 2004). ADH5 was expressed by DCs from all the secondary lymphoid organs examined. PP DC also expressed ADH1 and ADH4. PP DC expressed RALDH1, and MLN DC expressed RALDH2, while both weakly expressed RALDH3 (Iwata et al., 2004).

Iwata et al. found that adding 10nM all-trans-RA (the major physiologic RA) to stimulated naïve CD4 T cells enhanced α4β7 expression and suppressed E-lig expression on the CD4 T cells (Iwata et al., 2004). They also found RA to enhance α4β7 expression and suppress E-lig expression on activated CD8 T cells. All-trans-RA also induced CCR9 mRNA expression and suppressed CCR4 mRNA expression. RA-treated CD4 T cells could home efficiently to MLN and PP, but not to PLN (Iwata et al., 2004). Overall, Iwata et al. concluded that RA is able to imprint CD4 T cells with gut-homing markers. RA has since been shown to stimulate α4β7 and CCR9 expression on B cells as well (Mora et al., 2006).
Studies of skin-homing imprinting using varying DC types

I. DCs generated from bone marrow precursors

Peptide-pulsed BM-DC stimulated proliferation of P14 T cells in vitro, but were unable to stimulate E-lig expression (Dudda et al., 2004). As previously mentioned, peptide-pulsed BM-DCs injected intracutaneously stimulate E-lig expression on adoptively transferred P14 CD8 T cells, while BM-DCs injected i.v. or i.p. do not generate E-lig-expressing T cells (Dudda et al., 2004). After s.c. injection of OVA-peptide pulsed BM-DCs, adoptively transferred OT-I T cells in axillary and brachial sdLN expressed E-lig (Ferguson and Engelhard, 2010).

II. Bulk/unsorted lymph node dendritic cells

Bulk PLN DCs have been found to stimulate T cell proliferation and skin-homing marker expression. Peptide-pulsed Langerhans cells and PLN DC were able to stimulate E-lig expression on P14 T cells in vitro; MLN DC were able to do so as well, but at much lower levels. All three DC types were able to stimulate P14 T cell proliferation (Dudda et al., 2004). Naïve CD8 T cells from P14 transgenic mice were co-cultured with Ag-pulsed DCs from PLN, and subsequently expressed higher levels of E- and P-selectin ligands, and higher levels of CCR4 mRNA (Mora, 2005). After using Flt3L to expand DCs, peptide-pulsed LC and PLN DC also stimulated significant E-lig expression on CD8 P14 T cells (Dudda et al., 2005).
III. Dendritic cell subsets

Little work has been performed using sorted DCs from PLN to stimulate T cell E-lig or P-lig expression. DC from PLN were divided into CD8αCD11bhi, CD8αhiCD11blo, and CD8αlo/CD11blo subsets, then pulsed with peptide Ag and co-cultured with naïve CD8 T cells. All PLN DC subsets were able to stimulate E-lig and P-lig expression on CD8 T cells (Mora, 2005). However, this work used DCs pulsed ex vivo with peptide Ag. We hypothesized that after in vivo administration of Ag, only certain DC subsets would be responsible for stimulating E-lig expression on CD8 T cells. In Chapter 3, I identify a specific sdLN DC subset responsible for imprinting E-lig expression on CD8 T cells after an epicutaneous immunization.

Vitamin D and imprinting of skin-homing

Parallel to the role of vitamin A in generating gut-homing T cells, Sigmundsdottir et al. found that vitamin D3 may play a role in generating CCR10+ T cells that migrate towards CCL27 in the skin (Sigmundsdottir et al., 2007). In vitro, T cells activated with anti-CD3 and anti-CD28 in the presence of the active form of vitamin D3 (1,25(OH)2D3) expressed CCR10 (Sigmundsdottir et al., 2007). Vitamin D3 is generated in the skin in response to UVB radiation, converted to 25(OH)D3 in the liver by the enzyme CYP27A1, then 1,25(OH)2D3 in the kidney by the enzyme CYP27B. Sigmundsdottir et al. found that DCs exiting from skin expressed CYP27A1 and could convert vitamin D3 to 1,25(OH)2D3 in culture. Subsequently, DCs cultured with T cells in the presence of vitamin D3 or 25(OH)D3 generated CCR10+ T cells (Sigmundsdottir et al., 2007).
However, the significance of CCR10 in skin-homing is still questionable, as CCR10 deficiency has no effect on accumulation of memory CD4 T cells in skin (Tubo et al., 2011). In addition, the active form of vitamin D3 inhibits CLA expression, which is associated with T cell homing to skin (Yamanaka et al., 2008).

**Skin-homing – the default condition?**

Interestingly, E-lig expression on T cells and subsequent homing to skin is thought by some to be a default condition that occurs in the absence of gut signaling (Mora, 2008). Retinoic acid suppresses the expression of the skin-homing marker CCR4 on T cells after activation (Iwata et al., 2004). Mouse CD8 T cells stimulated by fixed Ag-pulsed PP DCs (which are no longer producing retinoic acid) do not express the gut-homing markers CCR9 and α4β7, but instead express the skin-homing markers E-lig and P-lig (Mora, 2005). These data were interpreted as implying that skin-homing marker expression was the result of a pathway that was actively suppressed by gut signals (namely retinoic acid). In the absence of gut signals, the default skin-homing markers would be displayed.

**Does the ability to imprint skin-homing markers vary among DC subsets?**

Work done in (Mora, 2005) seemed to imply that all DC subsets from skin-draining LNs were able to stimulate E-lig expression on CD8 T cells. However, we hypothesized that DC subsets have differing capabilities for stimulating proliferation and homing marker expression. Previous work done by Noah Tubo, a former graduate
student in our laboratory, led us to suspect that there may be differences in the ability of DCs to stimulate proliferation and trigger E-lig expression on T cells. In these experiments, Dr. Tubo applied increasing amounts of OVA Ag to recipient mice ears after OT-II T cell transfer. He found that increasing OVA application led to increased OT-II proliferation (Figure 1.3). However, a concurrent increase in E-lig expression was not observed; the number of OT-II T cells expressing E-lig reached a maximum (Figure 1.3). We hypothesize that this could be occurring because certain DC subsets are only able to stimulate proliferation, while other subsets could both stimulate proliferation and imprint E-lig expression. In this work, we develop a co-culture system that we use to test the abilities of different DC subsets to stimulate E-ligand expression.

Co-culture systems and the importance of proper Ag application

There are at least three ways in which DCs can obtain cutaneous Ag from skin in vivo, for presentation in the draining LN (Figure 1.4). Skin-resident migratory DCs can obtain Ag in the skin and transport it to the sdLN (Shklovskaya et al., 2008). LN-resident DCs in the sdLN could also obtain soluble Ag directly from afferent lymph (Anderson and Shaw, 2005). Migratory DCs are able to transfer Ag to LN-resident DC (Allan et al., 2006). Similar Ag-acquisition mechanisms can be applied to acquiring oral or intestinal Ag.

Given this variety of methods in which Ag can be acquired by DCs for presentation to T cells, it can be understood how avoiding physiological Ag presentation by applying Ag to DCs after their isolation from the LN, and/or using peptide Ag instead
of protein Ag could obscure the true role of different DC subsets in presenting or cross-presenting Ag. It is thought that only certain DC subsets can cross-present because only they contain the necessary Ag-cross-presentation machinery. However, applying peptide Ag allows the DC to skip the process of breaking down protein Ag, and the peptide Ag can be immediately displayed on surface MHC, making any special machinery irrelevant. In addition, DCs are known to localize to different areas of the LN (Kissenpfennig et al., 2005), which can affect their access to different Ag. Applying Ag to DCs after isolating them from the LN ignores differences in Ag access and gives all DCs an artificially equal exposure to Ag.

Clearly, a physiological method of applying Ag is key for accurately determining the role of DCs in cross-presenting Ag and in stimulating homing marker expression on T cells. This method should be robust enough to accommodate a wide repertoire of Ag, as well as a variety of different adjuvants. In Chapter 2, I describe the development of such a co-culture system. This co-culture system in then used to test our hypothesis of varying DC subset capacity to stimulate T cell proliferation and homing marker expression.
Figure 1.3: Production of E-lig\textsuperscript{hi} OT-II cells is “saturated.” Mice were immunized on the ears with varying doses of OVA protein Ag, causing the proliferation of transferred OT-II cells in the CLN. As the protein dose increased, the number of OT-II cells in the CLN increased. The number of Elig\textsuperscript{hi} skin-homing OT-II T cells, however, did not continuously increase, but seemed to reach a maximum. This implies that there is a “limiting factor” controlling the number of E-lig\textsuperscript{hi} T cells that can be produced. Figure from N. Tubo.
Figure 1.4: Ag can reach the LN in a number of ways. Adapted from Segura, E. and Villadangos, J.A. (2009). *Curr Op in Immunol*, 21(1), 105-110. Ag can be picked up by migratory DCs in peripheral tissues and brought to draining LN. In the LN, migratory DC can directly present Ag to T cells. They can also transfer Ag to LN-resident DCs, which then present Ag to T cells. Additionally, Ag can travel in the lymph to the LN directly (not shown), and be picked up by DCs in the LN (either LN-resident or migratory DCs).
Summary

In this project, our first goal was to design a co-culture system for the application on the skin of soluble protein Ag. Next, we sought to use this system to explore the abilities of different DC subsets to stimulate CD8 T cell proliferation and homing marker expression.

Chapter 2 below details the design and testing of this co-culture system. Chapter 3 details the use of this co-culture system in determining the activity of DC subsets in stimulating CD8 T cell proliferation, and identifies a particular DC subset that is responsible for triggering E-lig expression proliferating T cells. This work identifies the CD11b+ MHCIIhi DC subset as being responsible for cross-presentation of soluble OVA protein Ag applied to the skin, stimulating CD8 T cell proliferation. In addition, the Lang+ CD11b+ MHCIIhi DC subset is primarily responsible for imprinting E-lig expression on the proliferating CD8 T cells. This data furthers the investigation into the cells responsible for producing skin-homing T cells, and provides a possible target for vaccines targeting skin-applied Ag or skin-specific immune response. Chapter 4 investigates the role of CCR4 on DCs in stimulating T cell proliferation and in DC development. We found a previously unknown role for CCR4 in DC subset development, opening the door for further research in the role of chemokine receptors in DC development and subsequent T cell stimulation capacity. In chapter 5, we discuss the implications of this work and further research possibilities. The work presented in this dissertation sheds light on the differing functions of various DC subsets and illuminates the role of CCR4 in regulating DC subset composition.
References


CHAPTER TWO

Co-culture system design, development, and optimization

Suzanne Nizza

Attributions: J. Campbell assisted in conceptualizing experiments. All experiments were performed by S. Nizza. This chapter was written by S. Nizza with critical input from J. Campbell.
Summary

Studying the interaction of dendritic cells (DCs) and T cells can be difficult in the whole mouse, as there are structures and other cells that may obscure the DC-T cell interaction. Applying antigen (Ag) to the whole animal can also be difficult, as Ag-loaded DCs must be isolated in sufficient numbers for co-culture while avoiding the use of artificial expansion means. However, examining the DC-T cell interaction in vitro leads to a new set of challenges. Applying Ag to isolated DCs ignores the mechanisms that deliver Ag to particular areas of the LN and to certain DCs, resulting in DC activity that is not relevant to that which occurs in vivo.

We developed a novel co-culture system that would allow us to administer Ag in vivo, then isolate Ag-charged DCs to stimulate T cell proliferation in vitro. This co-culture system could then be used to determine the importance of different DCs and DC subsets in stimulating T cell proliferation and homing marker expression under multiple immunization conditions, as discussed in Chapter 3. In this current chapter, I detail the steps taken to design and test this co-culture system.

Introduction

DCs are key antigen-presenting cells (APCs) known for their ability to process and present Ag to both CD8 and CD4 T cells. There are two categories of Ag presentation: cross and classical. Cross-presentation is the presentation of exogenous Ag on MHC I. Classical presentation is the presentation of exogenous Ag on MHC II or the presentation of endogenous Ag on MHC I. Cross-presentation is a unique ability of
DCs that is not shared by other APCs (Heath and Carbone, 2009). The processes involved in cross-presenting Ag are still under study (Burgdorf et al., 2007). The ability of DCs to cross-present Ag is thought to vary among DC subsets, possibly requiring specialized molecular machinery (Dudziak et al., 2007).

Experimental Ag application

There are at least three ways in which DCs can obtain cutaneous Ag from skin in vivo for presentation in the skin-draining lymph nodes (sdLN): migratory DCs obtain Ag from the skin and transport it to the sdLN, LN-resident DCs obtain Ag in the sdLN from afferent lymph, or migratory DCs obtain Ag and transfer it to LN-resident DC (Figure 1.4). Culturing DCs and T cells together in vitro is a relatively straightforward method to test the ability of those DCs to stimulate T cell proliferation and homing marker expression. However, there are a number of possible issues in co-culture design that can affect results. In some cases, peptide Ag is loaded onto DCs for presentation to T cells (Dudda et al., 2005; Dudda et al., 2004). Using peptide Ag avoids the key DC functions of breaking down and processing whole protein Ag. As one possible reason for the limitation of DC cross-presentation capability is the need for DCs to contain specific Ag-processing machinery (Dudziak et al., 2007), using peptide and skipping protein processing may obscure any differences in DC capacity to cross-present Ag.

Other groups have described loading protein Ag onto DCs ex vivo for presentation to T cells (den Haan et al., 2000; Pooley et al., 2001). This is an improvement over the use of peptide Ag, as DCs must process protein Ag before
displaying it. However, applying Ag to DCs \textit{ex vivo} ignores differences in DC localization or Ag access \textit{in vivo}. Different skin-derived DC subsets localize to different areas of the LN (Kissenpfennig \textit{et al.}, 2005). DC subsets in the gut also localize to different areas in the LN and harvest Ag from different locations (Jaensson \textit{et al.}, 2008; Niess \textit{et al.}, 2005; Varol \textit{et al.}, 2009). Applying Ag to DCs after isolation from LN delivers Ag to all DC subsets, regardless of where those subsets normally acquire Ag \textit{in vivo} after immunization through a barrier tissue. As a result, the most representative way to deliver Ag to DCs for subsequent \textit{in vitro} T cell stimulation is through the normal barrier tissue, so that the Ag is applied as physiologically as possible. Varying types of Ag and immunization methods have been utilized to investigate the identity of the cross-presenting DC subset (Allan \textit{et al.}, 2006; Allan \textit{et al.}, 2003; Belz \textit{et al.}, 2005; Belz \textit{et al.}, 2004; GeurtsvanKessel \textit{et al.}, 2008).

More recent work has examined DC cross-presentation using physiological methods of applying Ag. Even with physiological Ag application, there are still possible confounding issues. Some have used viral Ag to stimulate T cell proliferation (Bedoui \textit{et al.}, 2009). While cross-presentation is important for presenting Ag from viruses that do not infect DCs, the viruses used in these co-cultures may infect DCs (Goldwich \textit{et al.}, 2011). Under these circumstances, experiments would be testing classical presentation by infected DCs, not true cross-presentation. Our co-culture system uses soluble OVA protein Ag, which does not have the same risk of direct presentation as viruses do, ensuring cross-presentation.
Homing marker expression

Homing marker expression is also affected by the method of Ag application. Homing markers on T cells allow them to enter non-lymphoid tissues to fight local Ag. E-lig and CCR4 are associated with homing to the skin (Campbell et al., 1999; Campbell et al., 2007; Erdmann et al., 2002; Tietz et al., 1998). CCR6, CCR8, and CCR10 are also thought to be possibly associated with skin homing (Homey et al., 2000). However, work on these CCRs is inconclusive; recent work has shown that CCR6 and CCR10 may not play a role in skin-homing (Tubo et al., 2011). α4β7 and CCR9 are associated with gut homing (Campbell and Butcher, 2002; Svensson et al., 2002), and vitamin A is a key intermediate in the gut-homing pathway (Iwata et al., 2004). Co-cultures with Ag added in various ways have been used to stimulate homing marker expression on T cells in co-culture (as described in the introduction). However, the method of Ag application may play a role in homing marker expression, as local inflammation is necessary for observing homing marker expression in vivo (Tubo, 2011). Methods of Ag-loading DCs that do not involve local inflammation – e.g., the loading of Ag on DCs ex vivo - may not necessarily lead to homing marker expression resembling that which would occur in vivo. Some experiments have used Ag applied to the whole animal with local inflammation to examine gut-homing marker expression (Jaensson et al., 2008; Johansson-Lindbom et al., 2003), but not for studying skin-homing marker expression.

Notably, when examining homing marker expression in vivo, there is specificity in the homing response: gut immunizations stimulate production of gut-homing T cells, and skin immunizations stimulate production of skin-homing T cells (Butcher et al., 1999).
This helps prevent misdirected immune responses, which can cause damage to other regions of the body. However, pulsing DCs post-isolation with Ag, then culturing them with T cells, does not result in the same specificity. Expression of both skin- and gut-homing markers can be observed when using Ag-pulsed DCs isolated from draining LNs (Mora, 2005); these results do not replicate what is observed in vivo.

In this chapter, I describe our development of a reliable co-culture system that uses physiologically relevant skin application of soluble protein Ag application to trigger in vitro T cell proliferation and homing marker expression. This co-culture system can be used to test our hypothesis that only certain DC subsets are able to trigger homing-marker expression on T cells, as described in Chapter 3. We also present data showing that DCs harvested from skin-draining LN differ in their stimulation of OT-I and OT-II cell proliferation. Lastly, we demonstrated that our co-culture system does not produce non-specific homing marker expression, and will thus be quite useful in examining the true contribution of different DC subsets to homing marker expression on T cells.

Materials and Methods

Mice

C57Bl/6 CD45.2 mice were purchased from Charles River Labs (Wilmington, MA). CD45.1 OT-I (Hogquist et al., 1994) and CD45.1 OT-II (Barnden et al., 1998) mice were from our colony, the founders obtained from Jackson Labs. CD8 T cells from OT-I mice express a transgenic TCR specific for the H2-Kb-restricted peptide OVA257-264. CD4 T
cells from OT-II mice express a transgenic TCR specific for the IAb-restricted peptide OVA_{323-339}.

**Topical Skin Immunization**

Topical immunization of ear skin was performed as described in (Baekkevold, 2005; Campbell *et al.*, 2007). The stratum corneum on each side of each ear was gently stripped with ten applications of adhesive tape (Scotch matte finish magic tape, 3M), without breaking the skin or causing bleeding. 25μl of acetone was spread over each ear to remove any cutaneous lipids that would repel Ag in aqueous solution. After the acetone evaporated, 25μl of an aqueous mixture containing 1mg/ml cholera toxin (CT) adjuvant (List Biological Labs, Campbell, CA) was applied to each ear and uniformly spread with a small paintbrush. Control mice received only the CT adjuvant, while experimental mice also received 50μl of an aqueous mixture containing 100mg/ml ovalbumin protein (OVA) Ag (Sigma-Aldrich product #A5503) on each ear.

**Oral Immunization**

Oral immunization was performed by gavage. Control mice received 10μg of CT in 500μl of distilled water. Experimental mice received 10μg of CT and 25mg of OVA protein in 500μl of distilled water.

**Dendritic Cell Isolation**
After immunization, skin-draining lymph nodes (sdLN) were harvested, disrupted between frosted microscope slides, and filtered through 80μm mesh. Any solid stroma remaining on the mesh was then incubated for 30 min at 37°C with 1mg/ml collagenase (Sigma) in RPMI media. The digested product was again filtered through 80μm mesh and added to the rest of the sdLN prep prior to washing and counting.

**Dendritic Cell Sorting**

DC were enriched from the sdLN prep by exclusion of T and B cells. The sdLN cell suspension was incubated with anti-B220 (clone RA3.3A1/6.1, ATCC hybridoma supernatant) and mThy-1.2 Ab (BioXCell, West Lebanon, NH) antibodies. After spinning and washing, the cell suspension was incubated with mouse anti-rat Igκ microbeads (Miltenyi Biotec, Auburn, CA) and then negatively depleted on an AutoMACS Separator.

**T Cell Preparation**

Spleens were harvested from CD45.1 OT-I mice and dissociated into a single cell suspension. Red blood cells were lysed, and remaining cells were washed and loaded with CFSE. 50mM CFSE in DMSO was diluted 1:10 in PBS with 0.5% bovine serum albumin and 0.05% sodium azide. Diluted CFSE was added to cells to a final 10uM concentration. Cells were then incubated at 37°C for 10 minutes, followed by quenching with cold RPMI media, 5 minutes on ice, and then a wash. CD8⁺ T cell selection was performed using the “CD8⁺ T cell isolation kit II, mouse” (Miltenyi).
Co-culture

After sorting, isolated populations were resuspended in 5ml RPMI with 10% fetal bovine serum. A sample was taken and stained to identify DC or CD8 T cells by FACS analysis. A known number of 5μm beads was added to the sample for accurate counting. DC and T cells were plated for co-culture in 96-well round-bottom plates. A 1:1 DC:T cell ratio was found to provide the most reproducible T cell proliferation and was maintained for all co-cultures. This high ratio was likely required due to the relative rarity of DC carrying in-vivo-acquired Ag. Co-cultures were incubated for six days, after which cells were stained for FACS analysis.

Flow Cytometry

Directly conjugated mAbs were purchased from eBioscience (La Jolla, CA) or BD Pharmingen (San Jose, CA). Flow cytometry was performed on a BD FACS Canto (Becton Dickinson) and analyzed by FlowJo software version 8.8.6 (Treestar, Inc., Stanford, CA).

Statistics

All statistics were performed using one-tailed Mann-Whitney U-tests using Prism software version 5.0a (GraphPad, Inc., La Jolla, CA).
Results

Development of co-culture system

Our goal was to develop a co-culture system that would allow us to apply Ag in vivo, then isolate Ag-loaded DCs for T cell stimulation ex vivo. In doing so, we would preserve physiological Ag acquisition and Ag processing, while still being able to examine the cross-presentation capability of DCs and or individual DC subsets. The sole source of Ag in these co-cultures would be OVA protein applied topically to the ear skin of the DC donor mice.

The initial plan was to apply OVA protein to mouse ears along with CT as an adjuvant, harvest the sdLN a few days later, then isolate DCs and co-culture them with OVA-specific T cells (Figure 2.1). Preserving physiological Ag application in this manner would give us a more accurate picture of which DCs are relevant for cross-presentation. To design a reliable co-culture system, we first needed to develop an optimal DC isolation process and determine how many days prior to DC harvest mice should be immunized on their ear skin.

Methodology for isolating dendritic cells

We chose a negative selection method for DC isolation to avoid any DC activation that could occur by using CD11c beads for positive selection. We used B220 and Thy1.2 Abs, followed by incubation with Ab-specific beads, to remove the B and T
Figure 2.1: Brief outline of co-culture system. After topically immunizing mice on the ears, DCs are sorted from skin-draining LNs. T cells are sorted from OT-I mouse spleens. DCs are cultured with OT-I T cells. After a period of incubation, cells are harvested and stained for FACS analysis.
cells from the DC prep – the largest groups of non-DC cells. In addition, prior to Ab treatment, we tested the use of collagenase to break down the LN stroma after disrupting the LN with microscope slides. We did not want to lose any DCs that were trapped in the stroma, as that would alter the DC subset composition of our LN prep in future experiments. We found that collagenase use caused the release of additional DCs (likely migratory DCs that were trapped in the LN stroma) (Figure 2.2). We therefore included a collagenase treatment step in all subsequent co-cultures.

An additional refinement was calculating the exact number of cells of interest (DCs or T cells) before resuspension in culture. DCs would not always comprise the same percentage of cells in the LN prep due to variations in mouse age and size. To allow the comparison of results from multiple experiments, we wanted to ensure maintenance of the 1:1 DC:T cell ratio. We chose a 1:1 DC:T cell ratio as we were unable to stimulate robust replicable T cell proliferation with fewer DCs per T cell. This is likely because in our DC prep, a small number of DCs is actually presenting Ag after the in vivo immunization. We added a known number of 5μm beads to a 1% sample of the DC or T cell preparations. These beads are easily distinguishable from cells on a FACS plot (Figure 2.3). Staining and analyzing this sample with flow cytometry allowed us to calculate exactly how many cells of interest were in the original preparation, and then plate the DCs and T cells in co-culture at the desired 1:1 ratio. Briefly, the number of cells of interest (DCs or T cells) is divided by the number of beads of interest in the sample population. The result is multiplied by the number of beads added and the
dilution factor (in this case, 100). This calculation gives the total number of DCs or T cells in the original preparation.

**Chosen Immunization Technique**

For this co-culture system, we used a cutaneous immunization technique that was previously described in Kahlon *et al.* 2003. This technique involves tape-stripping of the skin, removing the stratum corneum and also inducing local DC maturation (Seo *et al.*, 2000). Use of cholera toxin as adjuvant has been seen to enhance epicutaneous immune responses (Glenn *et al.*, 1998). Kahlon demonstrated that immunization with OVA peptide alone led to minimal proliferation of OVA-specific CD8 T cells in the draining LN (Kahlon *et al.*, 2003). In addition, Kahlon *et al.* were unable to generate a CD8 T cell response to epicutaneously-applied OVA peptide using CT alone without tape-stripping, and using tape-stripping alone without CT (Kahlon *et al.*, 2003). As a result, we included both tape-stripping and the use of CT in our immunization process.

In order to preserve the DC processes of breaking down whole protein Ag, we immunized our mice with whole ovalbumin protein instead of OVA peptide. As the ability of DCs to process and present or cross-present protein Ag to T cells may vary among DC subsets, we wanted to ensure that DCs in our co-culture system would be required to break down whole protein Ag before displaying peptides on surface MHC. As a result, our co-culture system would be useful for both examining whole DC populations from LN, and also for examining individual DC subsets.
Figure 2.2: Digesting stroma with collagenase. Comparison of DC yield with and without stromal digestion. There is a population of MHCII$^{\text{int}}$ CD11c$^{\text{hi}}$ DCs present after stroma digestion (circled), likely released from stroma by the collagenase. All further co-cultures in this dissertation use collagenase digestion, in order to isolate the most complete DC population possible. FACS plots were gated on CD45.1$^+$ B220$^-$ cells. 29000 cells depicted.
Figure 2.3: Microbeads are easily distinguishable on FACS plots. Plot of CLN cell suspension with circled microbead population. Microbeads can be differentiated from cells based on forward vs side scatter, as they are approximately 1 log higher in side scatter. 200000 events shown.
Choosing the day of immunization

DCs travel post-immunization from the skin to the LN at different rates. Dermal DCs take at least two days to travel to the LN, while Langerhans cells (LCs) take at least four days (Kissenpfennig et al., 2005). To determine the optimal day for DC harvest post-immunization, we tested DCs harvested 1d, 2d, and 4d after immunization. Waiting four days would ensure that LCs from the skin had sufficient time to reach the LN (Kissenpfennig et al., 2005). Any differences in T cell proliferation stimulated by DCs harvested on different days could also hint at the DC subsets involved.

Interestingly, CD4 and CD8 T cells proliferated differently when stimulated with DCs harvested on varying days. We found that DCs harvested on day 4 after immunization stimulated the most OT-I T cell proliferation. We confirmed this by testing DCs harvested 6d after immunization; these 6d DCs stimulated less proliferation than those harvested on day 4 (Figure 2.4). This was not the case for OT-II cells, however, as DCs harvested on day 2 after immunization stimulated the most CD4 T cell proliferation (Figure 2.5). We also tested this co-culture system with DCs harvested after oral OVA immunization. Peak OT-I T cell proliferation was stimulated by MLN DCs harvested one day post-immunization (Figure 2.6).

Specificity of homing marker expression

We found that our co-culture system offered increased specificity when examining homing marker expression (Figure 2.7). Other work using Ag-pulsed DCs to stimulate T cells found some gut-homing marker expression triggered by PLN DCs and
Figure 2.4: DCs harvested from sdLN four days after topical immunization stimulate the most OT-I T cell proliferation. DCs were harvested from sdLN one, two, four, and six days after topical ear skin immunization with CT and OVA protein. After sorting, DCs were co-cultured with CFSE-labeled naïve OT-I cells for six days. Cells were then stained and analyzed with FACS. CD45.1+ CD3+ CD8+ cells were gated for assessment of CFSE loss and proliferation. The number of proliferated T cells per 1000 input T cells is shown. Black bars: Immunization with CT only. White bars: Immunization with CT+OVA protein. sdLN from 3-4 mice per condition were pooled before DC isolation. Results are from two (1d, 6d) or three (2d, 4d) independent experiments. One-tailed Mann-Whitney $p$ values shown. * $p < .05$; *** $p < .0001$; n.s. = not significant.
Figure 2.5: DCs harvested from sdLN two days after topical immunization stimulate the most OT-II T cell proliferation. DCs were harvested from sdLN one, two, and four days after topical ear skin immunization with CT and OVA protein. After sorting, DCs were co-cultured with CFSE-labeled naïve OT-II cells for six days. Cells were then stained and analyzed with FACS. CD45.1^+ CD3^+ CD4^+ cells were gated for assessment of CFSE loss and proliferation. The percentage of total OT-II cells that have proliferated is shown. Black bars: Immunization with CT only. White bars: Immunization with CT+OVA protein. sdLN from 3-4 mice per condition were pooled before DC isolation. Results are from six independent experiments. One-tailed Mann-Whitney p values shown. * p < .05; *** p < .0001; n.s. = not significant.
Figure 2.6: DCs harvested from mesenteric LN one day after immunization by oral gavage stimulate the most OT-I T cell proliferation. DCs were harvested from MLN one, two, and four days after oral gavage immunization with CT and OVA protein. After sorting, DCs were co-cultured with CFSE-labeled naïve OT-I cells for six days. Cells were then stained and analyzed with FACS. CD45.1+ CD3+ CD8+ cells were gated for assessment of CFSE loss and proliferation. The percentage of total OT-I cells that have proliferated is shown. Black bars: Immunization with CT only. White bars: Immunization with CT+OVA protein. MLN from 3-4 mice per condition were pooled before DC isolation. Results are from two independent experiments. One-tailed Mann-Whitney p values shown. * p < .05.
Figure 2.7: Our co-culture system demonstrates specificity in homing marker expression. When OVA is added after DC isolation, proliferating T cells stimulated by MLN DCs express E-lig, which is not seen when mice are immunized with OVA orally before DC isolation. DCs were harvested from MLN one day after oral gavage immunization with CT and OVA protein and co-cultured with CFSE-labeled naïve OT-I cells for six days. Some wells had OVA protein added to culture media at the beginning of the six-day incubation. FACS plots are gated on CD45.1⁺ CD3⁺ CD8⁺ cells. 50000 events are shown.
some skin-homing marker expression triggered by MLN DCs (Dudda et al., 2004; Mora, 2005). However, this non-specific homing marker expression is not seen \textit{in vivo}. We wanted our co-culture system to reflect \textit{in vivo} homing marker expression.

Using this co-culture system with \textit{in vivo}-applied Ag resulted in homing marker expression that closely replicated what we see \textit{in vivo} – namely, no non-specific E-lig expression when using MLN DCs harvested after oral immunization (Figure 2.7). When we added Ag \textit{ex vivo} directly to the culture wells, we did observe significant non-specific E-lig expression. This demonstrated the utility of our co-culture system for examining the homing marker imprinting capability of different skin-resident DC subsets.

We ultimately focused this project on using DC after skin immunization to stimulate OT-I T cells, as these conditions resulted in the most consistent T cell proliferation. For all subsequent experiments, we used DCs harvested four days after immunization to stimulate T cells.

\textbf{Summary of co-culture system}

We developed a method for studying the DC/T cell interactions that lead to cross-priming of naïve CD8 T cells via the topical introduction of soluble Ag. Specifically, we created a co-culture system that allows for \textit{in vivo} Ag application, preserving the intricate cellular interplay involved, while still allowing DC isolation for \textit{in vitro} T cell stimulation and subsequent proliferation and homing marker expression. Our final co-culture protocol is as follows (Figure 2.8): mice are topically immunized with CT and whole OVA protein on the ear skin, after tape stripping and acetone wash, as previously
**Figure 2.8: Completed co-culture system details.** For stimulation of OT-I T cells, mice are topically immunized on the ears with CT and OVA protein. Four days later, DCs are enriched from sdLN preparations through negative sorting of B and T cells. Splenocytes from OT-I transgenic mice are loaded with CFSE and CD8+ T cells are sorted. DCs and T cells in a 1:1 ratio are co-cultured in round-bottom wells for six days. Cells are then harvested and stained for FACS analysis.
described (Baekkevold, 2005; Campbell et al., 2007; Kahlon et al., 2003; Tubo et al., 2011). After immunization, “Ag-charged” DCs are isolated from the skin-draining cervical LNs (sdLN). LNs are first digested with collagenase, then B- and T-cell depleted. At the same time, naïve CD8 T cells are purified from the spleens of OT-I mice, using an AutoMACS sorting kit. The DC-enriched sdLN cell populations and OT-I T cells are co-cultured ex vivo at a 1:1 DC/ T cell ratio. Using this system, DCs obtain Ag only from in vivo immunization, preserving key aspects of DC biology and Ag transport.

Discussion

Here, we describe the development of a novel co-culture system that robustly stimulates T cell proliferation in vitro after immunization and Ag-loading onto DCs in vivo. After design and optimization of the system, we used these co-cultures to stimulate proliferation of CD4 and CD8 T cells after skin immunization and CD8 T cells after gut immunization. We tested DCs harvested various days post-immunization. We found that DCs harvested on the fourth day after skin immunization stimulated the most CD8 T cell proliferation, while DCs harvested on the second day after skin immunization stimulated the most CD4 T cell proliferation. Additionally, DCs harvested on the first day after gut immunization stimulated the most CD8 T cell proliferation, compared to day 4 for post-skin immunization.

One possible explanation for the difference in peak proliferation between CD4 and CD8 T cells lies in the time needed for different DC subsets to travel to the LN. LN-resident DCs may be able to access soluble Ag that travels through the lymph to the LN.
within one day after immunization. Dermal DCs take at least two days to reach the LN, while epidermal Langerhans cells take as many as four days to reach the LN (Kissenpfennig et al., 2005). It may be the case that the DC subset that reaches the LN on day 2 (likely the dermal DCs) primarily stimulates CD4 T cells, while the DC subset that reaches the LN on day 4 (likely the Langerhans cells) primarily stimulates CD8 T cells. We further investigate this question with regards to CD8 T cells in Chapter 3.

We also observed a difference in proliferation timecourse for OT-I T cells after gut or skin immunization. This is most likely due to differences in the kinetics of Ag access and Ag movement to the LN in the skin and gut. OVA protein Ag applied orally may be able to quickly reach the MLN through the lymph – thus, DCs harvested as soon as one day after immunization are already loaded with Ag and can stimulate strong T cell proliferation. If most of the Ag reaches the LN within the first 24 hours, DCs harvested on following days are loaded with less Ag and so stimulate less T cell proliferation. Further developing this system for stimulating OT-II T cells after gut immunization would serve as an interesting comparator to OT-I T cells. We ultimately chose to focus on using our co-culture system after skin immunization to stimulate OT-I cells, as that data was consistent and robust.

Implications of immunization technique

We used a topical immunization technique that had been previously described in Kahlon et al. 2003, and used in Baekkevold 2005 and Campbell et al. 2007. We have
been using that technique frequently in the lab for the last several years, as it elicits a strong response after T cell transfer.

This method of disrupting skin through tape-stripping does lead to IL-1α release by keratinocytes (Nishijima et al., 1997) and maturation of local DCs (Seo et al., 2000). It is possible that this technique may shift results to responses promoted by IL-1α and local DCs in the skin. This demonstrates that the method of Ag exposure plays a role in determining the scale of the immune response and the specific cell subsets involved. This is especially relevant in vaccine design, where different methods of Ag application – intradermal vs subcutaneous vs epidermal – may lead to different immune responses. Use of the developed co-culture method for studying the role of dendritic cell subsets in eliciting CD8 T cell responses would be most relevant for epidermally applied vaccines, possibly in patch form.

An immunization technique that replicates the manner of Ag application would be vital to further elucidate the dendritic cell subsets involved in other methods of vaccine administration (e.g., intradermal or subcutaneous). Ag access and cytokine release by skin cells likely differs under those conditions. Mimicking the effects on the cytokine milieu in vitro that are caused by a particular Ag application method would provide a representative illustration of what is occurring in vivo.

In addition, different immunization methods may lead to Ag presentation by different DC subsets. This has been observed in the case of HSV-1, which is cross-presented by LN-resident DCs after epicutaenous infection, but by migratory DCs from the skin after vaginal infection (Lee et al., 2009). It is possible that intradermal or
subcutaneous immunizations would lead to a different timecourse of CD8 proliferation. Before this co-culture system is used for another immunization method, it should therefore be refined and tested to find the best conditions for that method.

Physiological Ag presentation is necessary for accurate homing marker expression in co-culture

Co-cultures involving non-physiologically applied Ag may lead to results inconsistent with those from co-cultures in which Ag was applied to DCs in vivo. For example, studies of gut homing markers using BM-DCs or Ag-pulsed DCs have led to conflicting results. Ag-loaded BM-DCs were unable to induce homing marker expression in vitro, but were able to stimulate α4β7 and CCR9 expression on adoptively transferred OVA-specific DO11.10 when injected directly into the afferent lymphatics of mesenteric LN (Hammerschmidt et al., 2008). After oral OVA immunization, sorted CD103+ MLN DCs were unable to stimulate CD8 T cell proliferation, but Ag-pulsed CD103+ MLN DCs were able to stimulate CD8 T cell proliferation in vitro (Johansson-Lindbom et al., 2005). Our co-culture system avoids the issues associated with inaccurate Ag application as it uses physiological Ag application.

Specificity in homing marker expression

Our co-culture system offers improved specificity in homing marker expression. Previous work suggested that all DC subsets were capable of stimulating α4β7 and E-lig expression (Dudda et al., 2004; Mora, 2005). However, these experiments were
performed with non-physiological Ag application (pulsing DCs with Ag) and there was no local inflammation in the area of DC harvest. Both factors could influence homing marker expression, generating results not representative of what occurs in vivo. Applying Ag after DC isolation ignores how DCs encounter Ag in vivo, as not all DCs may have access to Ag in vivo. In addition, the act of applying Ag through the skin or gut could stimulate release of certain factors from the epithelial cells or draining LN that also influence the expression (or non-expression) of homing markers by T cells, and/or the ability of DCs to imprint those markers.

It may instead be the case that DC subsets have differing capabilities for stimulating proliferation and homing marker expression, as we have hypothesized in this work based on previous data generated in our lab. Here, we have presented a novel coculture system that we will use to answer this question in Chapter 3. This system preserves in vivo Ag acquisition by DCs, and this should result in a more accurate picture of the responsibilities of different DC subsets in vivo.

In summary, we set out to more clearly define the in vivo DC subsets that actually cross-present cutaneous soluble Ag and/or imprint naïve CD8 T cells with skin-selective homing profiles. To do so, we designed a murine in vivo co-culture system in which cutaneous DCs acquired Ag within the skin under inflammatory conditions. We then isolated these Ag-charged DC from the sdLN of skin-immunized mice and used these cells to prime Ag-specific naïve CD8 or CD4 T cells ex vivo. We were also able to isolate DC after oral immunization and use them to prime CD8 T cells.
In the next chapter, we utilize our new co-culture system to examine the capacity of DC subsets to stimulate T cell proliferation. Our system allows us to preserve physiological Ag application, which will be important for examining DC subsets as different DC subsets can be found in different areas of non-lymphoid tissue. After traveling to the LN, these varying DC subsets can localize to different areas of the LN (Kissenpfennig et al., 2005). This approach will allow us to test the contribution of various DC subsets to the cross-priming process by direct sorting of DC subsets or by immunizing mouse strains that lack a given DC subtype.
References


CHAPTER THREE

DC subsets have differing capabilities to stimulate
proliferation and homing marker expression

Suzanne Nizza

Attributions: J. Campbell assisted in conceptualizing experiments. All experiments were performed by S. Nizza. Staining assistance for some co-cultures was provided by S. King. This chapter was written by S. Nizza with critical input from J. Campbell.
Summary

Only in the last 15 years has the field begun to differentiate dendritic cells (DCs) into subsets based on surface marker expression. DCs of different subtypes have been found to have varying functionality in vivo. Clearly understanding the roles of DC subsets, however, can be difficult, as can be preserving their in vivo roles when examining them in vitro. Many studies have sorted DCs and applied antigen (Ag) in vitro to co-cultures with T cells, which do not necessarily reflect the true access of DCs to in vivo Ag, and therefore their true functions may not be observed. We hypothesized that skin-derived DC subsets differed in their ability to stimulate skin-homing markers such as E-lig. In this chapter, I present evidence that CD11b+ migratory DCs are the DCs most capable at cross-presenting skin-applied soluble protein Ag and that the Lang+ CD11b+ migratory DC subset is responsible for imprinting E-ligand (E-lig) expression on T cells. We utilized the co-culture system described in Chapter 2, which has demonstrated robustness in stimulating CD8 T cell proliferation with DCs harvested from skin-draining lymph nodes (sdLN) post-immunization, to use DC subsets to stimulate T cells. Our co-culture system preserves differential Ag loading among DC subsets and gives a truer representation of which DCs are responsible for stimulating proliferation or homing marker expression in vivo, compared to loading Ag on all DCs after isolation from LN. Using this method, we were able to identify particular DC subsets that were responsible for stimulating T cell proliferation and E-lig expression after skin immunization with OVA protein.
Introduction

Dendritic cell subsets

Dendritic cells can be divided into multiple subsets, based on surface marker expression and tissue localization. DCs can be divided into conventional DCs (cDC) and plasmacytoid DC (pDCs). pDC are poor presenters of exogenous Ag (Villadangos and Young, 2008). cDC can be divided into migratory DC and resident DC (Villadangos and Schnorrer, 2007). In the skin-draining LN, resident DCs are MHC II^{int}CD11c^{hi} and can be divided into CD8α^+ CD11b^- and CD8α^- CD11b^+ subsets (López-Bravo and Aravín, 2008; Shortman et al., 2002). Migratory DC are MHC II^{hi} CD11c^{int} and can transport Ag from the skin to the sdLN (Allan et al., 2006; Lee et al., 2009). This population is divided into CD11b^-CD103^+ and CD11b^+ CD103^- subsets. Langerin^+ and Langerin^- DCs can be found within each of these subsets. Langerhans cells are found in the epidermis and are Lang^+ CD11b^- CD103^-; dermal-derived Lang^+ DCs are CD11b^- CD103^+ (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007).

Cross-presentation by DC subsets

The most well-studied types of Ag presentation by DCs are the presentation of endogenous Ag on MHC I and exogenous Ag on MHC II. However, tumor cells and viruses or intracellular pathogens that do not infect DCs do not generate endogenous peptides and need an alternate pathway in order to be displayed on MHC I. Cross-presentation allows the presentation of exogenous Ag on MHC I (Kurts et al., 2010) and the production of a CD8 T cell response to those viruses and intracellular pathogens. It
is thought that only DCs expressing certain surface markers are able to cross-present Ag (Heath and Carbone, 2009). Among the resident DC subsets, CD8α+ are considered the key cross-presenting subset (Allan et al., 2006; Allan et al., 2003; Belz et al., 2005; Lee et al., 2009). In the skin, CD103+ dermal DCs have been observed to cross-present Ag to CD8 T cells (Mount et al., 2008).

DCs may require additional signals from other DC subsets or from CD4 helper T cells before being able to stimulate proliferation of CD8 T cells. This concept is known as “DC licensing” (Kurts et al., 2010) (Figure 3.1). Some examples of DC licensing are CD8 T cell priming in response to HSV-1 (Smith et al., 2004) and CTL-mediated tumor immunity (van Mierlo et al., 2004).

Tissue homing by T cells

When stimulated by APCs, T cells can proliferate and may also express surface molecules that allow them to enter different body tissues. Expression of E-lig and CCR4 by T cells is associated with homing to the skin (Campbell et al., 1999; Erdmann et al., 2002). Expression of α4β7 and CCR9 is associated with migration to the gut (Butcher et al., 1999). Ag-pulsed DCs from MLN or Peyer’s patches (PP) can stimulate α4β7 and CCR9 expression on T cells in culture (Johansson-Lindbom 2003, (Dudda et al., 2005; Dudda et al., 2004; Johansson-Lindbom et al., 2003; Mora, 2005). Similarly, Ag-pulsed PLN DCs or LCs are able to trigger E-lig expression and higher levels of CCR4 mRNA in T cells in culture (Dudda et al., 2005; Dudda et al., 2004; Mora, 2005).
Figure 3.1: DC licensing – DCs may require additional signals from other DCs or CD4 helper T cells before being able to cross-prime CD8 T cells. Adapted from Kurts, C. et al. (2010). Nat Rev Immunol, 10(6), 403-414.
The capacity of DC subsets to stimulate gut homing marker expression has been characterized in much greater detail than for skin homing marker expression. In the gut, peptide Ag-pulsed CD103+ DCs from the small intestine lamina propria stimulate both α4β7 and CCR9 expression on CD8 T cells (Jaensson et al., 2008). All subsets of PP DCs are able to induce α4β7 expression but not CCR9 expression (Jaensson et al., 2008; Mora, 2005). After oral or intraperitoneal OVA immunization, CD103+ DCs were able to stimulate significantly higher CCR9 and α4β7 expression than CD103− DCs (Jaensson et al., 2008). In vitro, only peptide-pulsed CD103+ MLN DCs were able to stimulate CCR9 expression, but not CD103− DCs (Jaensson et al., 2008; Johansson-Lindbom et al., 2005). For skin DCs, the sole work published in this area found that all peptide Ag-pulsed PLN DC subsets were able to stimulate E-lig and P-lig expression on CD8 T cells (Mora, 2005).

Most other studies have ignored homing marker expression or physiological application of Ag in co-culture design and data acquisition. Using peptide Ag ignores DC Ag processing capabilities, while pulsing with protein Ag ex vivo ignores the steps involved in Ag transport to the LN. Loading Ag onto DCs in vivo preserves physiological Ag access and may give a more accurate demonstration of the role of different DC subsets. Our immunization method uses CT to trigger local inflammation, which is necessary for homing marker expression in vivo (Tubo, 2011) and preserves Ag application through the barrier of the skin.

Much work on cross-presentation has used viral Ag (Lee et al., 2009; Lukens et al., 2009). Some viruses may infect DCs, resulting in classical presentation, not cross-
presentation (de Graaff et al., 2005; Goldwich et al., 2011). Our use of soluble OVA protein Ag should eliminate the possibility of accidental, unintended classical presentation. Another common method when studying DC subsets is to use Flt3L or Flt3L-expressing cells to expand the number of DCs. As the frequency of DCs is quite low (~1% on average in LNs), increasing DC number is desirable and enables the use of fewer experimental animals. However, this method does not enlarge all DC subsets equally (Masten et al., 2004; Waskow et al., 2008), possibly hiding the function of smaller DC subsets behind larger, more expanded subsets. Our co-culture system does not use Flt3L, making our model more physiologically relevant.

We first hypothesized that only certain DC subsets would be able to stimulate both T cell proliferation and skin-homing marker expression. The experiments described in this chapter seek to test this hypothesis in the context of epicutaneous soluble protein Ag application. The co-culture system developed in Chapter 2 enabled us to obtain a more accurate picture of the physiological roles of different DC subsets, especially with regards to E-lig expression. Here, we used our co-culture system to examine the DC subsets found in the sdLN after ear skin immunization with OVA protein. We identified the CD11b+ migratory DC subset as responsible for stimulating T cell proliferation and the Lang+ CD11b+ migratory DC subset as responsible for imprinting E-lig expression.

**Materials and Methods**

*Mice*
C57Bl/6 CD45.2 mice were purchased from Charles River Labs (Wilmington, MA). Lang-DTREGFP (Lang-DTR) and Lang-EGFP mice were a generous gift from Bernard Malissen, Centre d’immunologie de Marseille Luminy (Kissenpfennig et al., 2005). CCR7⁻/⁻ (Förster et al., 1999) and CD45.1 OT-I (Hogquist et al., 1994) mice were from our colony, the founders obtained from Jackson Labs. (TCRα⁻/⁻ OT-I T cells were used for some experiments, but no differences in proliferation were seen with respect to TCRα⁺ OT-I T cells). CD8 T cells from OT-I mice express a transgenic TCR specific for the H2-Kb-restricted peptide OVA257-264.

**Topical Skin Immunization**

Topical immunization of ear skin was performed as described in (Baekkevold, 2005; Campbell et al., 2007). The stratum corneum on each side of each ear was gently stripped with ten applications of adhesive tape (Scotch matte finish magic tape, 3M), without breaking the skin or causing bleeding. 25µl of acetone was spread over each ear to remove any cutaneous lipids. After the acetone evaporated, 25µl of an aqueous mixture containing 1mg/ml cholera toxin (CT) adjuvant (List Biological Labs, Campbell, CA) was applied to each ear and uniformly spread with a small paintbrush. Control mice received only the CT adjuvant, while experimental mice also received 50µl of an aqueous mixture containing 100mg/ml ovalbumin protein (OVA) Ag (Sigma-Aldrich product #A5503) on each ear.

**Treatment of Lang-DTR mice with Diphtheria Toxin**
For some co-cultures (as indicated), WT or Lang-DTR mice were treated with diphtheria toxin (DT) (List Biological Labs, Campbell, CA). Mice were injected intraperitoneally with 1μg DT in 100μl PBS. Mice were treated one day preceding immunization and one day after immunization.

**Dendritic Cell Isolation**

After immunization, skin-draining lymph nodes were harvested, disrupted between frosted microscope slides, and filtered through 80μm mesh. Any solid stroma remaining on the mesh was then incubated for 30 min at 37C with 1mg/ml collagenase (Sigma) in RPMI media. The digested product was again filtered through 80μm mesh and added to the rest of the sdLN prep prior to washing and counting.

**Dendritic Cell Sorting**

DC were enriched from the sdLN prep by exclusion of T and B cells. The sdLN cell suspension was incubated with anti-B220 (clone RA3.3A1/6.1, ATCC hybridoma supernatant) and mThy-1.2 Ab (BioXCell, West Lebanon, NH) antibodies. After spinning and washing, the cell suspension was incubated with mouse anti-rat Igκ microbeads (Miltenyi Biotec, Auburn, CA) and then negatively depleted on an AutoMACS Separator. CD8α+ DC selection was done using the CD8+ dendritic cell isolation kit, mouse (Miltenyi). Isolation of individual DC subsets was performed on a BD FACSARIA (Becton Dickinson, San Jose, CA).
**T Cell Preparation**

Spleens were harvested from CD45.1 OT-I (or OT-I TCRα−/−) mice and dissociated into a single cell suspension. Red blood cells were lysed, and remaining cells were washed and loaded with CFSE. 50mM CFSE in DMSO was diluted 1:10 in PBS with 0.5% bovine serum albumin and 0.05% sodium azide. Diluted CFSE was added to cells to a final 10uM concentration. Cells were then incubated at 37°C for 10 minutes, followed by quenching with cold RPMI media, 5 minutes on ice, and then a wash. CD8+ T cell selection was performed using “CD8+ T cell isolation kit II, mouse” (Miltenyi).

**Co-culture**

After sorting, isolated populations were resuspended in 5ml RPMI with 10% fetal bovine serum. A sample was taken and stained to identify DC or CD8 T cells by FACS analysis. A known number of 5μm beads was added to the sample for accurate counting. DC and T cells were plated for co-culture in 96-well round-bottom plates. A 1:1 DC:T cell ratio was found to provide the most reproducible T cell proliferation and was maintained for all co-cultures. This high ratio was likely required due to the relative rarity of DC carrying *in-vivo*-acquired Ag. Co-cultures were incubated for six days, after which cells were stained for FACS analysis.

**In vivo Transfers**

CD45.1 OT-I spleen and peripheral LN were harvested and single cell suspensions prepared. Red blood cells were lysed and remaining cells were washed and loaded with
CFSE as described above. After counting, approximately $1.5 \times 10^7$ T cells were retro-orbitally injected into anesthetized recipient mice. WT and Lang-DTR mice were used as recipients. The next day, recipient mice were immunized on ear skin and LNs were harvested and analyzed for T cell proliferation five days later. DT-treated mice were injected with DT one day before and one day after T cell transfer. **Timeline:** day -2, first DT treatment; day -1, OT-I cells transferred IV to recipients; day 0, ear skin immunized and second DT treatment given; day 5, skin-draining LN harvested.

*Flow Cytometry*

Directly conjugated mAbs were purchased from eBioscience (La Jolla, CA) or BD Pharmingen (San Jose, CA). Flow cytometry was performed on a BD FACS Canto (Becton Dickinson) and analyzed by FlowJo software version 8.8.6 (Treestar, Inc., Stanford, CA).

*Statistics*

All statistics were performed using one-tailed Mann-Whitney *U*-tests using Prism software version 5.0a (GraphPad, Inc., La Jolla, CA).

*Results*

**Migratory DCs are necessary for CD8 T cell proliferation**

We used the co-culture system described in Chapter 2 to examine the abilities of different DC subsets to cross-present Ag and imprint homing. Before sorting individual
DC subsets, we first used other methods available to us to narrow down the subsets of interest. Briefly, we used Langerin-DTR-EGFP mice and CCR7−/− mice to gauge the necessity of Langerin+ DCs and LN-resident DCs, respectively. A CD8α+ DC magnetic bead sorting kit was used to isolate CD8α+ DCs.

To determine whether Langerin (Lang)-expressing DCs are necessary players in the cross-presentation process, we examined T cell proliferation stimulated by Langerin+ DC-depleted DC populations. We did so using Langerin-DTR-EGFP (Lang-DTR) mice (a generous gift from Bernard Malissen, Marseille-Luminy), a knock-in strain that loses its Lang+ DC populations after treatment with diphtheria toxin (DT) (Poulin et al., 2007). DT is not otherwise toxic to mice. Wild-type (WT) or Lang-DTR mice were treated with DT in parallel and then immunized on ear skin as described above. When compared side-by-side, we found that DC populations depleted of Lang+ DC were only about half as efficient as DC from WT mice at stimulating OT-I T cell proliferation (Figure 3.2). All co-cultures were set up with a 1:1 DC:T cell ratio; the expected isolation of fewer total DCs from the Lang+ DC-depleted mice did not affect how many DCs were plated per culture well. Interestingly, the WT and Lang+ DC-depleted populations were equally capable of cross-presenting exogenous Ag added directly to co-culture wells, demonstrating that the purified DCs remained alive and functional and that any defect in T cell stimulation observed in Lang+ DC-depleted populations was restricted only to Ag acquired in vivo (Figure 3.2).

Next, we examined the CD8α+ subset of the “LN-resident” DC population, which is considered to be specialized for cross-presentation (Dudziak et al., 2007; Kurts et al.,
A small sample of DCs was negatively depleted of B and T cells (as in Chapter 2) and considered “unsorted” for comparison to the CD8α+ sorted DCs. We used a magnetic bead sorting kit to positively select CD8α+ DCs. The kit involves a two-step process: depletion of B and T cells followed by positive selection with CD8α microbeads. We found that CD8α+ DCs alone were unable to stimulate CD8 T cell proliferation, while unsorted DCs did stimulate normal T cell proliferation (Figure 3.3). Sorted CD8α+ DCs were able to cross-present OVA protein added to the culture wells (Figure 3.3), demonstrating their functionality and viability post-sort.

Lastly, we examined the total LN-resident DC population, as CD8α+ DCs may require assistance from other LN-resident DCs to be able to cross-present. We used DC from CCR7−/− mice, as the sdLN of these mice do not contain migratory DCs. CCR7 is required for DCs to migrate from the skin to the draining LN via afferent lymphatics (Förster et al., 1999). We immunized WT and CCR7−/− mice, harvested the sdLN, and set up co-cultures as previously described. We found that DCs from CCR7−/− mice were unable to cross-prime OT-I cells (Figure 3.4). These DC were still able to cross-present OVA protein added directly to co-culture wells (Figure 3.4).

As the DC from CCR7−/− mice were unable to stimulate T cell proliferation, this suggests that the presence of other LN-resident DC subsets was not sufficient for CD8α+ DC to cross-present in vivo-acquired Ag. It has been suggested that a migratory DC subset might work together with CD8α+ DC to achieve cross-presentation via DC licensing (Kurts et al., 2010). Before testing migratory DC subsets together with CD8α+
Figure 3.2: Langerin\(^+\) DC depletion results in decreased OT-I proliferation. WT and Lang-DTR mice were topically immunized with CT+OVA protein or CT alone. Mice were injected with diphtheria toxin (DT) five and three days prior to cell isolation. On day 4 post-immunization, sdLN cells were isolated, enriched for DCs, and co-cultured with CFSE-labeled OT-I T cells. sdLN from 4-5 mice per condition were pooled before DC isolation. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1\(^+\)CD3\(^+\)CD8\(^+\) cells. The number of proliferated (CFSE\(^\text{lo}\)) T cells per 1000 input T cells is depicted. Adj = adjuvant. Tx = treatment. N = 4 experiments. **Left:** The only Ag present in co-culture wells was that carried by DC from immunized mice. **Right:** Exogenous OVA protein was added to wells to confirm DC viability and functionality. One-tailed Mann-Whitney \(p\) values shown. *** \(p < .0001\); n.s. = not significant.
**Figure 3.3:** CD8α+ DCs are unable to independently stimulate OT-I proliferation.

CD8α+ DCs were isolated from immunized WT mice using an AutoMACS kit. Unsorted DCs were treated as previously described. DCs were co-cultured with CFSE-labeled OT-I T cells. sdLN from 8-10 mice were pooled before CD8α+ DC isolation. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1+ CD3+ CD8+ cells. The number of proliferated (CFSElo) T cells per 1000 input T cells is depicted. N = 3 experiments. **Left:** The only Ag present in co-culture wells was that carried by DC from immunized mice. **Right:** Exogenous OVA protein was added to wells to confirm DC viability and functionality. One-tailed Mann-Whitney *p* values shown.

* *p* < .01; *** *p* < .0001; n.s. = not significant.
**Figure 3.4: DCs from CCR7<sup>−/−</sup> mice are unable to stimulate OT-I proliferation.**

WT and CCR7<sup>−/−</sup> mice were topically immunized with CT+OVA protein or CT alone. On day 4 post-immunization, sdLN cells were isolated, enriched for DCs, and co-cultured with CFSE-labeled OT-I T cells. sdLN from 4-5 mice per condition were pooled before DC isolation. T cell proliferation was analyzed on day 6 of *ex vivo* culture. Flow cytometry plots were gated on CD45.1<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> cells. The number of proliferated (CFSE<sup>lo</sup>) T cells per 1000 input T cells is depicted. N = 3 experiments. **Left:** The only Ag present in co-culture wells was that carried by DC from immunized mice. **Right:** Exogenous OVA protein was added to wells to confirm DC viability and functionality. One-tailed Mann-Whitney *p* values shown. *** *p* < .0001; n.s. = not significant.
DCs, we first decided to test the cross-priming capability of migratory DCs independently.

**Migratory DCs have the ability to cross-prime naïve CD8 T cells with *in vivo*-acquired Ag**

In the absence of genetic techniques to isolate migratory DCs for co-culture, we turned to fluorescence-activated cell sorting to purify DC subsets from immunized mice. We sorted DCs from sdLN into MHC II$^{\text{hi}}$CD11c$^{\text{int}}$ migratory DC and MHC II$^{\text{int}}$CD11c$^{\text{hi}}$ LN-resident DC sub-populations. Migratory DCs were then sorted into CD11b$^+$CD103$^-$ and CD11b$^-$CD103$^+$ subsets (Figure 3.5). LN-resident DCs were isolated for comparison with migratory DCs, as well as to confirm our data from CCR7$^{-/-}$ mice showing that LN-resident DCs were unable to stimulate T cell proliferation.

Surprisingly, we found that the CD11b$^+$CD103$^-$ migratory DCs were the only subset to induce appreciable T cell proliferation in co-culture. The CD11b$^-$CD103$^+$ migratory DCs and the LN-resident CD11c$^{\text{hi}}$ DCs stimulated negligible amounts of T cell proliferation (Figure 3.6). Again, all DC populations were demonstrated to be alive and functional post-sort, as they were able to cross-present exogenous OVA Ag added directly to co-culture wells (Figure 3.6).

It should be noted that the observed cross-presentation capability of CD11b$^+$ DCs, could be particular to our selected adjuvant (CT), selected day of immunization (four days prior to LN harvest), or selected length of co-culture (six days). We addressed those issues with the following set of experiments.
Figure 3.5: Migratory DCs can be sorted based on CD11b and CD103 expression.

To isolate DC subsets for co-culture experiments, sdLN were harvested and prepared as previously described (including B and T cell depletion). Remaining cells were stained with CD11c, MHCII, CD11b, and CD103, and sorted on a BD FACSARIA. CD11c$^{hi}$ MHCII$^{int}$ (“LN-resident”) dendritic cells were sorted as one population. CD11c$^{int}$ MHCII$^{hi}$ migratory DCs were sorted into CD11b$^+$ and CD103$^+$ sub-populations.
Figure 3.6: CD11b$^+$ migratory DCs are responsible for stimulating OT-I proliferation in co-culture. Sorted DCs were isolated from immunized WT mice as described in Figure 3.5 and co-cultured with CFSE-labeled OT-I T cells. Mice were immunized four days before lymph node harvest with CT and OVA protein. sdLN from 8-10 mice were pooled before DC sorting. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1$^+$ CD3$^+$ CD8$^+$ cells. The number of proliferated (CFSE$^{lo}$) T cells per 1000 input T cells is depicted. N = 3 experiments.

**Left:** The only Ag present in co-culture wells was that carried by DC from immunized mice. **Right:** Exogenous OVA protein was added to wells to confirm DC viability and functionality. One-tailed Mann-Whitney p values shown. **p < .001; ***p < .0001; n.s. = not significant.
To determine whether CD11b+ DC cross-presentation was specific to the cholera toxin used as our adjuvant, we tested an alternative adjuvant, lipopolysaccharide (LPS). LPS is detected by TLR4 (Shimazu et al., 1999), while CT is thought to activate DCs through the GM1 ganglioside receptor (Kawamura et al., 2003). We found that the CD11b+CD103- migratory DCs remained the sole cross-presenting population when using LPS as the immunization adjuvant (Figure 3.7). Again, all DC populations were to be alive and functional post-sort, capable of cross-presenting exogenous OVA Ag added to co-culture wells (Figure 3.7).

It was also necessary to confirm that CD11b+ DC cross-presentation capability was not an artifact of the selected DC harvest day. For all previous co-cultures, DCs were harvested from sdLN four days after immunization, as that resulted in the most CD8 T cell proliferation (Figure 2.4). When we harvested DCs from sdLN two days post-immunization instead of four days, CD11b+ migratory DCs were still the only DC subset to induce significant T cell proliferation (Figure 3.8). All DCs were alive and capable of cross-presenting exogenous OVA Ag post-sort (Figure 3.8).

In addition, the observed CD11b+ DC cross-presentation capability may have been an artifact of co-culture length, as all previous co-cultures were incubated for six days. Other DC subsets may have been cross-presenting at earlier timepoints in culture. To test this, we incubated some co-cultures for three days instead of six days. We still found that CD11b+ migratory DCs were the only DC subset to induce significant T cell proliferation (Figure 3.9). Again, all DCs were alive and capable of cross-presenting exogenous OVA Ag post-sort (Figure 3.9).
Figure 3.7: CD11b⁺ migratory DCs are still responsible for stimulating OT-I proliferation when LPS is used as the adjuvant. Sorted DCs were isolated from immunized WT mice as described in Figure 3.5 and co-cultured with CFSE-labeled OT-I T cells. Mice were immunized four days before lymph node harvest with LPS and OVA protein. sdLN from 8-10 mice were pooled before DC sorting. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1⁺ CD3⁺ CD8⁺ cells. The number of proliferated (CFSE⁻) T cells per 1000 input T cells is depicted. N = 3 experiments. Left: The only Ag present in co-culture wells was that carried by DC from immunized mice. Right: Exogenous OVA protein was added to wells to confirm DC viability and functionality. One-tailed Mann-Whitney p values shown. * p < .05; ** p < .001; *** p < .0001.
Figure 3.8: CD11b⁺ migratory DCs are still responsible for stimulating OT-I proliferation when sdLN are harvested two days post-immunization. Sorted DCs were isolated from immunized WT mice as described in Figure 3.5 and co-cultured with CFSE-labeled OT-I T cells. Mice were immunized two days before lymph node harvest with CT and OVA protein. sdLN from 10 mice were pooled before DC sorting. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1⁺ CD3⁺ CD8⁺ cells. The number of proliferated (CFSE(lo)) T cells per 1000 input T cells is depicted. N = 3 experiments. **Left:** The only Ag present in co-culture wells was that carried by DC from immunized mice. **Right:** Exogenous OVA protein was added to wells to confirm DC viability and functionality. One-tailed Mann-Whitney $p$ values shown.

* $p < .05$; ** $p < .001$; *** $p < .0001$; n.s. = not significant.
Figure 3.9: CD11b\textsuperscript{+} migratory DCs are still responsible for stimulating OT-I proliferation when co-cultures are incubated for three days. Sorted DCs were isolated from immunized WT mice as described in Figure 3.5 and co-cultured with CFSE-labeled OT-I T cells. Mice were immunized four days before lymph node harvest with CT and OVA protein. sdLN from 10 mice were pooled before DC sorting. T cell proliferation was analyzed on day 3 of ex vivo culture. Flow cytometry plots were gated on CD\textsuperscript{45.1}\textsuperscript{+} CD\textsuperscript{3}\textsuperscript{+} CD\textsuperscript{8}\textsuperscript{+} cells. The number of proliferated (CFSE\textsuperscript{lo}) T cells per 1000 input T cells is depicted. \textbf{Left}: The only Ag present in co-culture wells was that carried by DC from immunized mice. N = 3 experiments. \textbf{Right}: Exogenous OVA protein was added to wells to confirm DC viability and functionality. N = 1 experiment. One-tailed Mann-Whitney \( p \) values shown. * \( p < .01; ** \( p < .001; *** \( p < .0001; \) n.s. = not significant.
The CD11b⁺ CD103⁻ migratory DC population can be further subdivided on the basis of Langerin expression. We used Langerin-EGFP mice for our DC source, allowing us to sort EGFP⁺ and EGFP⁻ populations (Kissenpfennig et al., 2005) (Figure 3.10). Langerin-EGFP mice express GFP under the control of the Langerin promoter. After sorting, both Lang⁺ and Lang⁻ CD11b⁺ migratory DCs were able to stimulate significant T cell proliferation, though the Lang⁺ DCs stimulated more proliferation than the Lang⁻ DCs (Figure 3.11). All DC subsets were able to cross-present exogenous OVA Ag after sorting (Figure 3.11).

Lang⁺ CD11b⁺ migratory DCs imprint E-lig expression

Previous experiments had demonstrated that unsorted DCs from the sdLN of skin-immunized mice were able to induce E-lig expression on proliferating CD8 T cells in culture. To confirm whether our sorted CD11b⁺ migratory DCs were capable of imprinting skin homing, we stained our co-cultures for E-lig expression. We found that sorted CD11b⁺ migratory DCs were able to stimulate E-lig expression on CD8 T cells just as well as unsorted DCs (Figure 3.12). We decided to compare the E-lig imprinting capability of Lang⁺ and Lang⁻ CD11b⁺ migratory DCs. Interestingly, while both subsets could cross-prime CD8 T cells (Figure 3.11), the ability to induce E-lig expression resided almost exclusively within the Lang⁺ population (Figure 3.13).

The observed E-lig⁺ proliferating T cells after stimulation with sdLN DCs contrast with previous work that suggested that CD8 T cells activated by sdLN DCs express E-lig
Figure 3.10: CD11b+ migratory DCs can be further sorted based on Langerin expression. sdLN were harvested and prepared as previously described (including B and T cell depletion). Remaining cells were stained with CD11c, MHCIi, CD11b, and CD103, and sorted on a BD FACSARIA as in Figure 3.5. CD11b+ migratory DCs were further divided based on Langerin-EGFP expression.
Figure 3.11: Both Langerin+ and Langerin− CD11b+ migratory DCs are able to stimulate OT-I proliferation. DCs were sorted from WT sdLN as described in Figure 3.9 and co-cultured with CFSE-labeled OT-I T cells. CD11b+ migratory DCs were subdivided into Lang+ and Lang− subsets. Mice were immunized four days before lymph node harvest with CT and OVA protein. sdLN from 9-10 mice were pooled before DC sorting. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1+CD3+CD8+ cells. The number of proliferated (CFSElo) T cells per 1000 input T cells is depicted. N = 4 experiments. **Left:** The only Ag present in co-culture wells was that carried by DC from immunized mice. **Right:** Exogenous OVA protein was added to wells to confirm DC viability and functionality. One-tailed Mann-Whitney p values shown. * p < .05; ** p < .001; *** p < .0001; n.s. = not significant.
Figure 3.12: E-ligand expression on OT-I cells is stimulated by CD11b+ DCs. **Top:** Representative example of E-lig expression on CD8 T cells after co-culture with unsorted DCs or sorted CD11b+ MHCII^hi^ migratory DCs. Control was stained in the presence of EDTA (which disrupts Ca^{++}-dependent E-selectin binding) to determine non-specific binding of the E-selectin-Ig chimera. Flow cytometry plots are gated on CFSE-low CD45.1^+^ CD3^+^ CD8^+^ T cells. Vertical axis: side scatter. Plots representative of 3 different experiments of 5-8 wells each (depending on DC yield). 3433 cells shown per panel. **Bottom:** E-lig expression depicted as the percentage of proliferated OT-I T cells that are E-lig^{hi}. sdLN from 10 immunized mice were pooled before DC sorting. N = 3 experiments. One-tailed Mann-Whitney p values shown. n.s. = not significant.
Figure 3.13: Langerin+, but not Langerin−, CD11b+ MHCIIhi migratory DCs are able to stimulate E-ligand expression on OT-I cells. **Top:** Representative example of E-lig expression on CD8 T cells after co-culture with Lang+ or Lang− CD11b+ MHCIIhi DCs. Control was stained in the presence of EDTA (which disrupts Ca++-dependent E-selectin binding) to determine non-specific binding of the E-selectin-Ig chimera. Flow cytometry plots are gated on CFSE-low CD8+ T cells. Vertical axis: side scatter. Plots are representative of samples from 4 different experiments of 3-8 wells each (depending on DC yield). 1623 cells shown per panel. **Bottom:** E-lig expression depicted as the percentage of proliferated OT-I T cells that are E-lighi. sdLN from 9-10 immunized mice were pooled before DC sorting. N = 4 experiments. One-tailed Mann-Whitney p values shown. * p < .01; *** p < .0001.
“by default” unless they receive gut-tropism signals, possibly from retinoic acid (Iwata et al., 2004; Mora, 2005). Other groups have suggested that imprinting homing markers on T cells is independent of activation site or tissue of origin (Masopust et al., 2004). The variation in E-lig imprinting that we see after co-culture with CD11b+ DC subsets strongly indicates that the induction of E-lig expression (i.e. skin-selective imprinting) is an instructive process that requires signals from Lang+ DCs that are independent of signals that induce proliferation. The Lang+ CD11b+ migratory DCs in the sdLN are likely Langerhans cells that have migrated from the epidermis, while the Lang- CD11b+ migratory DCs are dermal DCs (del Rio et al., 2010).

Our co-culture assays suggest that CD11b+ Lang+ DCs are the key subset that stimulates E-lig expression by naïve CD8 T cells after topical immunization. This result implies that a mouse lacking Lang+ DC would have an impaired ability to produce E-lig+ T cells after topical immunization. To test this hypothesis, naïve OT-I cells were transferred into mice bearing normal numbers of Lang+ DC and DT-treated Lang-DTR mice. We found that OT-I proliferation and E-lig expression in the sdLN were reduced significantly when Lang+ DC were depleted (Figure 3.14).

Discussion

Using the co-culture system developed in Chapter 2, we examined the abilities of different sets of DCs to stimulate CD8 T cell proliferation and imprint E-lig expression. First, we used several alternative methods to determine the DC subsets on which to focus. Langerin-DTR mice were used to determine that Lang+ DC played a role in
Figure 3.14: Proliferation and E-ligand expression are reduced in vivo after depletion of Langerin⁺ DCs. **Left:** The percentage of OT-I T cells harvested from the sdLN that are proliferated (CFSElo) is shown. Langerin⁺ DC-depletion has reduced T cell proliferation by ~40%. **Right:** The percentage of proliferated T cells that are Elighi is shown. Langerin⁺ DC-depletion has reduced E-lig expression by ~50%. OT-I splenocytes were loaded with CFSE and retro-orbitally injected into WT and Lang-DTR recipient mice. The next day, recipient mice were immunized on ear skin. LNs were harvested and analyzed for T cell proliferation five days later. DT-treated mice were injected with DT one day before and one day after T cell transfer. **Timeline:** day -2, first DTX treatment; day -1, OT-I cells transferred IV to recipients; day 0, ear skin immunized and second DTX treatment given; day 5, skin-draining LN harvested. Adj = adjuvant. Tx = treatment. N = 4 experiments. One-tailed Mann-Whitney p values shown. * p < .05; ** p < .001.
stimulating CD8 T cell proliferation in our co-culture system, as DC preparations depleted of Lang⁺ DCs stimulated decreased proliferation. As CD8α⁺ DCs are often cited as the archetypal cross-presenting DC due to special Ag-processing machinery (Dudziak et al., 2007), we used an autoMACs sorting kit to isolate these DCs for testing in our co-culture system. We found that CD8α⁺ DCs by themselves were unable to stimulate T cell proliferation. Additionally, CCR7⁻/⁻ mice allowed us to examine the ability of LN-resident DCs alone to stimulate CD8 T cell proliferation. LN-resident DCs by themselves did not stimulate proliferation, implying that migratory DCs played a major role in cross-priming CD8 T cells. Taken together, this data led us to focus on the role of migratory DCs in stimulating CD8 T cell proliferation.

Sorting the MHC II⁺CD11c⁺ migratory DCs into CD11b⁺ and CD103⁺ subsets allowed us to identify the CD11b⁺ DCs as the only DC subset that stimulated CD8 T cell proliferation. The cross-presentation ability of the CD11b⁺ DC subset was shown to be independent of adjuvant used, day of DC harvest, and length of co-culture. The CD11b⁺ DCs were further divided based on Langerin expression. While both Langerin⁺ CD11b⁺ and Langerin⁻ CD11b⁺ subsets could stimulate T cell proliferation (with the Lang⁺ subset stimulating more proliferation), the two subsets significantly differed in their ability to imprint E-lig expression on proliferating T cells. Lang⁺ CD11b⁺ migratory DCs stimulated approximately 6x as much E-lig expression as Lang⁻ CD11b⁺ migratory DCs, suggesting that the Lang⁺ CD11b⁺ migratory DCs are largely responsible for the production of skin-homing CD8 T cells after soluble Ag application. This data supports our original hypothesis that DC subsets differed in their ability to stimulate T cell proliferation and
expression of homing markers. We have identified a DC subset able to stimulate both T cell proliferation and E-lig expression, and a DC subset that is only able to stimulate T cells proliferation, but not E-lig expression.

The next step in this work would be to test the functionality of these proliferating CD8 T cells – whether they were tolerogenic or immunogenic. One way would be to test the cytokine production of the CD8 T cells after DC stimulation. Alternatively, testing the cytolytic activity of the CD8 T cells would be helpful to understand whether they are functional after our *ex vivo* stimulation, or merely proliferating without functionality.

**Significance of CD11b⁺ DCs in cross-presentation**

Our results indicate that CD11b⁺ CD103⁻ migratory DCs are the relevant subset for cross-presentation in the context of soluble protein Ag applied epicutaneously to skin. All other DC subsets – including the CD8α⁺ LN-resident DCs and the CD103⁺ migratory DCs – were incapable of cross-priming CD8 T cells. This DC population has an immunophenotype consistent with that of Langerhans cells (*i.e.*, CD11b⁺ CD103⁻ DC within the MHC II⁺ CD11c⁺ migratory DC population). These results are supported by the data presented in Chapter 2, wherein CD8 T cells were stimulated best by DCs harvested from sdLN on day four after immunization. As Langerhans cells take four days to reach the draining LN (Kissenpfennig *et al.*, 2005), these cells may be responsible for the bulk of CD8 T cell proliferation in this co-culture system. This possibility is especially intriguing given previous work demonstrating that all PLN DCs were able to imprint E-lig expression (Mora, 2005).
The observed importance of CD11b+ migratory DCs could be model-dependent, specific to our epidermal immunization method utilizing tape-stripping. These DCs could be the relevant cross-presenting DCs in our co-culture system due to their Ag access through the epidermis. However, our findings are still generally relevant, as Ag access is a key consideration when designing vaccines. The planned method of vaccine administration must be tested to determine which DC subsets are encountering the vaccine, in order to understand what DC subsets to target, or what adjuvants to use to activate those DCs. As mentioned in Chapter 2, use of this co-culture system with other immunization techniques could help us understand possible differences in DC cross-presentation function \textit{in vivo}. Avoiding tape-stripping of the skin and using a subcutaneous or intradermal injection of Ag may lead to the maturation of and/or Ag access by alternate DC subsets. It may be the case that, after an i.d. Ag injection, DC in the dermis are the predominant cross-presenting DC, and the epidermal Langerhans cells play a smaller role.

Interestingly, the present study demonstrates that each of the DC subsets we isolated from sdLN is capable of cross-presentation when pulsed with OVA protein antigen \textit{ex vivo} (Figure 3.11, right panel). Thus, it may be the case that cross-presentation capability \textit{in vivo} is not restricted to only certain DC subsets based on inherent differences in their function, as has been previously thought. Cross-presentation by DCs may instead be restricted based on type of Ag encountered or access to Ag. Here, it may be the case that LN-resident DCs have decreased access to epidermal Ag, leading to their inability to stimulate CD8 T cell proliferation (Figures 3.3
and 3.4). Additionally, certain DC subsets may only cross-present certain types of Ag, Ag delivered through a specific method, or Ag delivered with a particular adjuvant. Therefore, when testing the cross-presentation abilities of different DC subsets ex vivo it is crucial to deliver Ag as similarly to the in vivo conditions as possible, and to test a range of Ag and adjuvant before declaring all or only a few DC subsets the “cross-presenting DC.”

Our results may not be extendable to all Ag and adjuvant combinations; testing of other combinations would be vital for understanding how Ag and adjuvant selection affect DC cross-presentation capability. In addition, repetition of these experiments with OT-II cells would indicate whether LCs were only important for CD8 T cell stimulation under our immunization conditions, or whether other DC subsets would play a role in stimulating CD4 T cell proliferation under these conditions.

In addition, testing alternative immunization methods (such as subcutaneous or intradermal injection) would indicate whether details inherent to our tape-stripping immunization method are skewing our results towards certain DC subsets. As tape-stripping causes the release of certain cytokines from skin cells such as keratinocytes, the observed function of the CD11b+ migratory DCs in this co-culture system may be dependent on those cytokines. It may be that these cytokines lead to either specific activation of the CD11b+ migratory DCs, or the inhibition of other DC subsets present. Using other immunization methods that do not involve tape-stripping would result in a different cytokine milieu in the skin, and possibly the activation of different DC subsets. This information would help us understand why the CD11b+ migratory DCs have such a
key role in our co-culture system, and possibly identify alternative methods of activating specific DC subsets.

**Restriction of DC ability to imprint E-lig expression**

Previous work indicated that all sdLN DC subsets are capable of stimulating expression of the skin homing marker E-lig (Mora, 2005). In contrast, we showed that only Lang+ subset of CD11b+ migratory DCs is responsible for imprinting T cells with skin homing ability, in the form of E-lig expression (Figure 3.13). This suggests that not all DC subsets are capable of imprinting skin homing, that this ability may be restricted to only certain DCs.

Our results also indicate that expressing skin-homing markers is not necessarily a default outcome in the absence of gut-homing imprinting signals. It had been previously hypothesized that, without suppressing signals from retinoic acid, activated T cells would express E-lig (Mora, 2005; Mora, 2008). Additionally, proliferating T cells activated by PLN DCs would be predicted to express E-lig (Dudda et al., 2004). Essentially, the conditions for skin-homing according to this hypothesis are as follows: 1) absence of gut homing signals, and 2) stimulation with DCs from sdLN after skin immunization. However, in our experiments, we observed T cells proliferating after stimulation with DCs from sdLN without expressing E-lig. If skin-homing were in fact a default outcome, we would expect to find skin-homing markers expressed by all proliferating T cells, whether stimulated by Lang+ or Lang- CD11b+ migratory DCs. Instead, E-lig expression was not observed on T cells stimulated by Lang- CD11b+
migratory DCs. These results demonstrate that a particular DC subset, the Lang⁺ CD11b⁺ migratory DCs, actively stimulates E-lig imprinting.

Our data implies that homing marker expression is an instructive process, relying on signals from DCs (and possibly other cells as well) to determine which homing markers, if any, to express. It may be the case that cells in the epidermis or dermis, such as keratinocytes, release factors that cause DCs in those areas to be able to imprint skin-homing on T cells. Some work has demonstrated that lymph node stroma and the non-lymphoid tissue microenvironment are a key source of factors that stimulate the expression of homing markers (Edele et al., 2008; Hammerschmidt et al., 2008). One way to narrow down where signals are being released is to use DCs migrated from skin ex vivo to stimulate T cell proliferation. Those DCs have not traveled to the LN, and have only been exposed to local skin signals. If those DCs are able to imprint skin-homing markers on T cells, then local epidermal or dermal factors may be capable to giving DCs the ability to imprint T cells, in addition to lymph node stromal cells.

In the experiments presented here, DCs were able to provide imprinting signals independently of any simultaneous exposure to afferent lymph or LN stroma cells, although those factors may have influenced the imprinting capabilities of the DCs themselves in vivo prior to isolation from the LN and ex vivo culture. Future work could explore the capacity of CD11b⁺ migratory DCs and other DC subsets to imprint E-lig expression in the context of LN stromal cells or other factors, to determine whether co-culture with other factors can change the E-lig imprinting capability of the Lang⁺ CD11b⁺ migratory DCs or enable the Lang⁻ CD11b⁺ migratory DCs to imprint E-lig.
Future directions

Further investigation into the differences between Lang⁺ and Lang⁻ CD11b⁺ migratory DCs would provide insight into what makes one subset capable of stimulating E-lig expression, but not the other. Identification of the difference in E-lig imprinting capability between the Lang⁺ and Lang⁻ subsets opens new avenues for examining the cause of increased fucosyltransferase-VII (FucT-VII) mRNA production in T cells as well as E-lig expression on T cells. FucT-VII is an enzyme required for E-selectin ligand synthesis (Malý et al., 1996), generating binding sites for E-selectin on the P-selectin glycoprotein ligand-1 (PSGL-1) backbone. This generation is regulated by IL-12, TGFβ-1, and IL-4, among other factors (Lim et al., 1999; Wagers and Kansas, 2000; Wagers et al., 1998). Interestingly, retinoic acid suppresses FucT-VII expression (Iwata et al., 2004). Vitamin D3 has also been seen to suppress CLA expression on T cells (Yamanaka et al., 2008). CD8 T cells activated with Ag-pulsed PLN DCs were found to express higher levels of FucT-VII mRNA than those activated with Ag-pulsed PP DCs (Mora, 2005).

One first step toward understanding what controls E-lig expression on T cells would be to examine the levels of FucT-VII mRNA in CD8 T cells after culture with Lang⁺ or Lang⁻ CD11b⁺ DCs. If FucT-VII mRNA levels are similar between CD8 T cells cultured with either Lang⁺ or Lang⁻ CD11b⁺ migratory DCs, the DCs must be affecting E-lig expression pathways in the T cell downstream of FucT-VII mRNA production. However, if Lang⁺ DCs trigger high levels of FucT-VII mRNA in proliferating T cells, then
we could investigate known factors affecting FucT-VII mRNA production, and compare production of those factors by Lang⁺ and Lang⁻ CD11b⁺ migratory DCs. Examining differences in gene expression, surface marker expression, and secreted factor production between these two subsets could identify candidates involved in imprinting E-lig on T cells.

Another question to consider is where these findings fit into the context of previous work on DCs and cross-presentation. Previous work may need to be reexamined to determine whether cross-presentation is being observed, or whether it is instead direct presentation by a small population of infected DCs. In addition, different DC subsets may be responsible for cross-presenting different classes of Ag under varying inflammatory conditions. If so, exploring how environmental conditions affect the DC’s ability to cross-present would be especially important for designing DC vaccines. Targeting a vaccine to one specific DC subset would be useful only if that DC subset is responsible for cross-presentation under the conditions of vaccine application.
References


CHAPTER FOUR

CCR4 influences DC subset composition

Suzanne Nizza

Attributions: J. Campbell assisted in conceptualizing experiments. Co-culture experiments and in vivo transfers were performed by S. Nizza. Transwell experiments to test DC migration were performed by J. Campbell. ELISA experiments to measure factor secretion were performed by S. Nizza. Staining assistance for the DC subset work was provided by S. King. This chapter was written by S. Nizza with critical input from J. Campbell.
Summary

CCR4 is commonly associated with T cell homing to the skin and is thus often studied in the context of T cells. However, recent studies have brought attention to the function of CCR4 with regards to dendritic cells. CCR4 was found to regulate GM-CSF and IL-23 production in dendritic cells (DCs), leading to delayed and decreased experimental autoimmune encephalitis (EAE) incidence in CCR4\(^{-/-}\) mice (Poppensieker et al., 2012). Here, we compared DCs and T cells from CCR4\(^{-/-}\) mice to those of wild-type (WT) mice in our co-culture system. While we saw no difference in proliferation between CCR4\(^{-/-}\) T cells and WT T cells, we did find an unexpected decrease in the T cell proliferation stimulated by CCR4\(^{-/-}\) DCs compared to that of WT DCs. We then investigated the mechanism underlying this reduction in T cell proliferation. After examining several possibilities, we found a difference in DC subset distribution between CCR4\(^{-/-}\) and WT mice, with CCR4\(^{-/-}\) mice containing fewer CD11b\(^{+}\) migratory DCs – the DC subset relevant for cross-presentation in our system. This new finding indicates that CCR4 modulates the composition of DC subsets, adding to our understanding of the processes involved in the development of varied DC subsets.

Introduction

CCR4 is expressed on a variety of T cells

CCR4 is a G-protein coupled receptor for the chemokines CCL2, CCL4, CCL5, CCL17, and CCL22. CCL17 and CCL22 are also known as thymus and activation regulated chemokine (TARC) and human macrophage-derived chemokine (MDC),
respectively. CCR4 was first identified as a receptor for CCL17 (Imai et al., 1999). It was subsequently shown to be predominantly present on Th2, not Th1 cells (Sallusto et al., 1998a; Sallusto et al., 1998b). CCR3 and CCR8 expression were also associated with Th2 cells (Kim and Broxmeyer, 1999; Zlotnik and Yoshie, 2000). More recent work has demonstrated that CCR4 is not Th2-restricted (Freeman et al., 2006). IL-4 enhanced CCR4+ T cell generation, while IL-12 suppressed the level of CCR4 in responding T cells (Kim et al., 2003). Surface expression of CCR4 is dependent on the IL-4/STAT6 pathway, and functionality of CCR4 is thought to occur late in the Th2 differentiation process (Morimoto, 2005). CCR4 also plays a role earlier in T cell development as a factor in T cell localization in the thymus (Campbell et al., 2003). More recently, regulatory T cells have also been observed to express CCR4 (Sather et al., 2007). Human Th17 cells co-express CCR6 and CCR4 (Acosta-Rodriguez et al., 2007).

**CCR4 mediates T cell attraction to antigen-presenting cells**

CCR4 is known to regulate T cell attraction to antigen-presenting cells (APCs). Specifically, CCR4 on T cells may play a role in bringing T cells into the vicinity of APCs (Kim et al., 2003; Wu et al., 2001). The CCR4 ligands CCL17 and CCL22 were found to recruit CCR4-expressing Th2 cells towards APCs (Imai et al., 1999). CCL17 is produced by monocytes treated with GM-CSF or IL-3 (Imai et al., 1999) and by BM-DCs, especially after stimulation with LPS (Lieberam and Förster, 1999). Langerhans cells migrating from skin upregulated CCL22 and Ag-specific T cells acquired CCL22 responsiveness *in vivo* after subcutaneous injection of antigen (Ag) (Tang, 1999).
CCL22 produced by cervical LN DCs contributes to the retention in the LN of CCR4-expressing iTreg cells (Vitali et al., 2012). Splenic CD8α+ DCs licensed by NK T cells are able to produce CCL17 that attracts naïve CCR4-expressing CTLs (Semmling et al., 2010). Expression of CCR4 by effector T cells enhances their migration to Hodgkin lymphoma cells producing CCL17 and CCL22 (Di Stasi et al., 2009). Interestingly, CCR4 may be involved in regulating the movement of other cell types as well. Activated B cells produce chemokines that may attract CCR4-expressing T cells (Schaniel et al., 1998).

CCR4 and skin homing

CCR4 and T cell skin homing have been a major area of investigation in our laboratory. A potential role for CCR4 in skin homing was first hinted at when the Yoshie group found that CCR4 is expressed on one-fifth of adult peripheral blood effector/memory CD4 T cells (Imai et al., 1999). Our lab showed that CCR4 was important in the regulation of movement to skin vasculature, but not gut vasculature (Campbell et al., 1999), and thus held significance in directing skin-homing (reviewed in Campbell and Butcher, 2000). CCR4 also plays a role in the migration of Th2 effector T cells (von Andrian and Mackay, 2000) and is necessary for Ag-driven cutaneous accumulation of CD4 T cells (Campbell et al., 2007). The ligands for CCR4 and CCR10 can be found in cutaneous sites (Hudak et al., 2002), suggesting that one or both of these receptors is involved in skin-homing. E-lig+ T cells (known to be efficient at homing to the skin) migrate towards CCL17 and CCL27, further supporting the idea that CCR4 and CCR10
are involved in T cell homing to skin (Reiss et al., 2001). Recent work reinforces the importance of CCR4 in homing to skin. Specifically, we showed that CCR4 deficiency reduces the accumulation of memory CD4 T cells in the skin, while CCR10 deficiency has no effect (Tubo et al., 2011).

CCR4 was found to be involved in the migration of memory T cells to skin (von Andrian and Mackay, 2000), and CCR4 deficiency reduces the accumulation of memory CD4 T cells in skin by approximately 20-fold (Tubo et al., 2011). This is supported by some interesting work using competitive bone marrow chimeras. Baekkevold et al. reconstituted RAG-1−/− hosts with CCR4+/+ and CCR4−/− mixed bone marrow. Both types of bone marrow were able to develop into B cells, naïve CD8 and CD4 T cells, IFNγ+ Th1 cells, and IL-4+ Th2 cells (Baekkevold, 2005). However, cutaneous memory E-lig+ CD4 T cells were more than four times as likely to be CCR4+/+ than CCR4−/− (Baekkevold, 2005).

In the experiments described in this chapter, we used CD8 T cells in our co-culture experiments based on their robust proliferation in our co-culture system. Importantly, CCR4 is expressed on skin-homing CD8 T cells, in addition to CD4 T cells (Dudda et al., 2004). CD8 T cells expressed significantly higher levels of CCR4 mRNA after four days of culture with Ag-pulsed peripheral LN DCs, compared to culture with Ag-pulsed Peyer’s patch DCs (Mora, 2005). Retinoic acid, known to play a key role in the expression of gut-homing markers, suppresses CCR4 expression on CD8 T cells, directing them to home to gut instead of to skin (Iwata et al., 2004). These findings suggest that CCR4 may serve an important role in CD8 T cell skin homing.
CCR4 and dendritic cells

The role of CCR4 on DCs has not been studied as extensively as the role of CCR4 on T cells. This is likely due to a lack of adequate murine antibody (Ab) for detecting CCR4 surface expression. While the absence of CCR4 had no effect on cutaneous DC migration in an atopic dermatitis model (Stutte et al., 2010), CCR4 on DCs may have a role in influencing effector cell action. Freeman et al. analyzed CCR4’s role in models of Th1 and Th2 cell-mediated pulmonary granulomas, intravenously challenging primed mice with either mycobacterial purified protein derivative or schistosomal egg Ag-coated beads (Freeman et al., 2006). CCR4+/− mice received CD4 T cells from sensitized CCR4+/+ donor mice and were then challenged. They found that CCR4−/− mice did not support a secondary response, but instead responded similarly to naïve mice (Freeman et al., 2006). This implies a role for CCR4+ cells in the recipient mice. They also found that CCR4−/− DCs triggered impaired IFNγ production in wild-type CD4 T cells (Freeman et al., 2006).

More recently, it was observed that CCR4-expressing DCs play a role in experimental autoimmune encephalitis (EAE). EAE is a Th1/Th17-mediated autoimmune disease of the CNS that is used as a mouse model of human multiple sclerosis (MS). EAE had a delayed and decreased disease incidence in CCR4−/− mice, as a result of diminished CNS infiltrate possibly caused by reduced macrophage function (Forde et al., 2011). Later work found that CCR4 regulates GM-CSF and IL-23 production in DCs (Poppensieker et al., 2012). CCR4−/− DCs produced less IL-23 and
GM-CSF in the CNS, and were less efficient in Th17 maintenance (Poppensieker et al., 2012). These data establish that CCR4 promotes Th17 T cell maintenance via induction of IL-23 and GM-CSF production in DCs.

Our work on CCR4 was initiated after we used CCR4−/− mice as dendritic cell donors while developing our co-culture system. We were surprised to find that CCR4−/− DCs stimulated less CD8 T cell proliferation in our co-culture system than did wild-type DCs after skin immunization. In this chapter, I present evidence that CCR4 deficiency on DCs leads to a smaller, less functional CD11b+ migratory DC subset, establishing a crucial role for CCR4 in regulating DC subset differentiation and function.

Materials and Methods

Mice

C57Bl/6 CD45.2 mice were purchased from Charles River Labs (Wilmington, MA). CCR4−/− (Chvatchko et al., 2000) and CD45.1 OT-I (Hogquist et al., 1994) mice were from our colony, the founders obtained from Jackson Labs. (TCRα−/− OT-I T cells were used for some experiments, but no differences in proliferation were seen with respect to TCRα+ OT-I T cells). CD8 T cells from OT-I mice express a transgenic TCR specific for the H2-Kb-restricted peptide OVA257-264.

Topical Skin Immunization

Topical immunization of ear skin was performed as described in (Baekkevold, 2005; Campbell et al., 2007). The stratum corneum on each side of each ear was gently
stripped with ten applications of adhesive tape (Scotch matte finish magic tape, 3M), without breaking the skin or causing bleeding. 25μl of acetone was spread over each ear to remove any cutaneous lipids that would repel Ag in aqueous solution. After the acetone evaporated, 25μl of an aqueous mixture containing 1mg/ml cholera toxin (CT) adjuvant (List Biological Labs, Campbell, CA) was applied to each ear and uniformly spread with a small paintbrush. Control mice received only the CT adjuvant, while experimental mice also received 50μl of an aqueous mixture containing 100mg/ml ovalbumin protein (OVA) Ag (Sigma-Aldrich product #A5503) on each ear.

*Dendritic Cell Isolation*

After immunization, skin-draining lymph nodes were harvested, disrupted between frosted microscope slides, and filtered through 80μm mesh. Any solid stroma remaining on the mesh was then incubated for 30 min at 37C with 1mg/ml collagenase (Sigma) in RPMI media. The digested product was again filtered through 80μm mesh and added to the rest of the sdLN prep prior to washing and counting.

*Dendritic Cell Sorting*

DCs were enriched from the sdLN prep by exclusion of T and B cells. The sdLN cell suspension was incubated with anti-B220 (clone RA3.3A1/6.1, ATCC hybridoma supernatant) and mThy-1.2 Ab (BioXCell, West Lebanon, NH) antibodies. After spinning and washing, the cell suspension was incubated with mouse anti-rat Igκ microbeads (Miltenyi Biotec, Auburn, CA) and then negatively depleted on an AutoMACS Separator.
**T Cell Preparation**

Spleens were harvested from CD45.1 OT-I (or OT-I TCRα⁻⁻) mice and dissociated into a single cell suspension. Red blood cells were lysed, and remaining cells were washed and loaded with CFSE. 50mM CFSE in DMSO was diluted 1:10 in PBS with 0.5% bovine serum albumin and 0.05% sodium azide. Diluted CFSE was added to cells to a final 10uM concentration. Cells were then incubated at 37°C for 10 minutes, followed by quenching with cold RPMI media, 5 minutes on ice, and then a wash. CD8⁺ T cell selection was performed using “CD8⁺ T cell isolation kit II, mouse” (Miltenyi).

**Co-culture**

After sorting, isolated populations were resuspended in 5ml RPMI with 10% fetal bovine serum. A sample was taken and stained to identify DC or CD8 T cells by FACS analysis. A known number of 5μm beads was added to the sample for accurate counting. DC and T cells were plated for co-culture in 96-well round-bottom plates. A 1:1 DC:T cell ratio was found to provide the most reproducible T cell proliferation and was maintained for all co-cultures. This high ratio was likely required due to the relative rarity of DC carrying *in-vivo*-acquired Ag. Co-cultures were incubated for six days, after which cells were stained for FACS analysis.

**In vivo Transfers**
CD45.1 OT-I spleen and peripheral LN were harvested and single cell suspensions prepared. Red blood cells were lysed and remaining cells were washed and loaded with CFSE as described above. After counting, approximately $1.5 \times 10^7$ T cells were retro-orbitally injected into anesthetized recipient mice. WT and CCR4$^{-/-}$ mice were used as recipients. The next day, recipient mice were immunized on ear skin (as described above). Three days later, lymph nodes were harvested and analyzed for T cell proliferation.

**Flow Cytometry**

Directly conjugated mAbs were purchased from eBioscience (La Jolla, CA) or BD Pharmingen (San Jose, CA). Flow cytometry was performed on a BD FACS Canto (Becton Dickinson) and analyzed by FlowJo software version 8.8.6 (Treestar, Inc., Stanford, CA).

**Statistics**

All statistics were performed using one-tailed Mann-Whitney *U*-tests using Prism software version 5.0a (GraphPad, Inc., La Jolla, CA).

**Results**

CCR4 deficient DCs stimulate less T cell proliferation in our co-culture system

We first aimed to determine the effect of CCR4 deficiency on T cells or DCs with regards to T cell proliferation using the our co-culture system (described in Chapter 2).
Our co-culture system has the advantage of isolating the DC-T cell interaction, allowing us to examine the importance of CCR4 on just those two cell types, independent of the structure of the whole LN. We first compared the proliferation of CCR4−/− T cells and wild-type (WT) T cells when stimulated by Ag-loaded WT DCs. We found no significant differences in the proliferation and E-lig expression of CCR4−/− T cells compared to WT T cells (Figure 4.1). This result was surprising, given historical data demonstrating a role for CCR4 in helping T cells interact with DCs (Semmling et al., 2010).

We also tested the ability of WT versus CCR4−/− DCs to stimulate WT T cells. Surprisingly, we found that the CCR4−/− DCs stimulated less T cell proliferation relative to WT DCs (Figure 4.2). We confirmed these co-culture results by using CD11c beads to isolate DCs for co-culture. In previous co-culture experiments, we had avoided positive DC selection, as we did not want to artificially activate or stimulate the DCs by using beads that would bind directly to DCs. In this case, however, we wanted to ensure that the observed proliferation decrease was not merely a side effect of a non-DC CCR4−/− cell that was included in the DC preparation. Using CD11c beads, we confirmed that CCR4−/− DCs continued to stimulate less T cell proliferation than WT DCs when positively selected (Figure 4.3).

We also confirmed this decrease in T cell proliferation in vivo using adoptive transfer experiments. CFSE-labeled OT-I T cells were injected into WT and CCR4−/− mice. Recipient mice were immunized the following day with CT and OVA protein on the ears. After three days, the skin-draining LN were harvested and stained for FACS...
Figure 4.1: WT and CCR4$^{-/-}$ T cells proliferate similarly when co-cultured with WT DCs. WT mice were topically immunized with CT+OVA protein. sdLN DC were harvested on day 4 post-immunization and co-cultured with CFSE-labeled OT-I or CCR4$^{-/-}$ OT-I T cells. sdLN from 8-10 WT mice were pooled before DC isolation. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1$^+$ CD3$^+$ CD8$^+$ cells. The number of proliferated (CFSE$^{lo}$) T cells per 1000 input T cells is depicted. Adj = adjuvant. N = 3 experiments. One-tailed Mann-Whitney $p$ values shown. n.s. = not significant.
**Figure 4.2: CCR4<sup>−/−</sup> DCs stimulate less OT-I proliferation than WT DCs.** Ag-loaded CCR4<sup>−/−</sup> DCs stimulated ~60% less T cell proliferation than Ag-loaded WT DCs. WT and CCR4<sup>−/−</sup> mice were topically immunized with CT+OVA protein or CT alone. On day 4 post-immunization, sdLN cells were isolated, enriched for DCs, and co-cultured with CFSE-labeled OT-I T cells. sdLN from 4-5 mice per condition were pooled before DC isolation. T cell proliferation was analyzed on day 6 of *ex vivo* culture. Flow cytometry plots were gated on CD45.1<sup>+</sup> CD3<sup>+</sup> CD8<sup>+</sup> cells. The number of proliferated (CFSE<sup>lo</sup>) T cells per 1000 input T cells is depicted. Adj = adjuvant. N = 5 experiments. One-tailed Mann-Whitney *p* values shown. *** *p* < .0001.
Figure 4.3: CCR4−/− DCs isolated by positive selection stimulate less OT-I proliferation than similarly-isolated WT DCs. Ag-loaded CCR4−/− DCs stimulated ~68% less T cell proliferation than Ag-loaded WT DCs. WT and CCR4−/− mice were topically immunized with CT+OVA protein or CT alone. On day 4 post-immunization, CD11c+ DCs were isolated from sdLN preps using an AutoMACS kit. DCs were then cultured with CFSE-labeled OT-I T cells. sdLN from 4-5 mice per condition were pooled before DC isolation. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1+ CD3+ CD8+ cells. The number of proliferated (CFSElo) T cells per 1000 input T cells is depicted. Adj = adjuvant. N = 3 experiments. One-tailed Mann-Whitney p values shown. ** p < .001; *** p < .0001.
analysis. We found that CCR4−/− mice had fewer proliferated CD8 T cells in their sdLN than WT mice three days after immunization (Figure 4.4).

These results were intriguing, as we had no reason to expect CCR4−/− DCs to be less capable of stimulating T cell proliferation than WT DCs. To investigate the cause of this reduction in T cell proliferation, we first assessed whether CCR4−/− DCs were competent to process whole protein Ag. We found that CCR4−/− DCs cultured with exogenous OVA protein are able to take up, process, and display Ag and stimulate T cell proliferation (Figure 4.5). Next, we determined that CCR4 was not required for DC migration to skin-draining LNs. Previous work had briefly shown that CCR4−/− had no effect on DC migration (Stutte et al., 2010). We confirmed those findings using a transwell system, in which DCs placed in the top section of the transwell were allowed to migrate towards chemokines in the bottom section. We observed no DC migration towards the CCR4 ligand CCL22, while DCs migrated as expected towards CCR7 ligands (Figure 4.6).

Inconclusive effects of CCR4 deficiency on DC factor secretion

We then investigated whether CCR4−/− DCs had altered cytokine secretion. Some work has shown that CCR4−/− DCs are impaired in the production of certain chemokines and in their ability to stimulate T cell chemokine production. CCR4−/− DCs were less efficient in GM-CSF and IL-23 production, and Th17 maintenance (Poppensieker et al., 2012). CCR4−/− DCs also trigger impaired IFNγ production in wildtype CD4 T cells (Freeman et al., 2006).
Figure 4.4: OT-I proliferation is reduced *in vivo* in CCR4<sup>−/−</sup> mice. The percentage of OT-I T cells harvested from the sdLN that are proliferated (CFSE<sup>lo</sup>) is shown.

Transferred OT-I T cell proliferation in CCR4<sup>−/−</sup> mice is ~20% less than in WT mice. OT-I splenocytes were loaded with CFSE and retro-orbitally injected into WT and CCR4<sup>−/−</sup> recipient mice. The next day, recipient mice were immunized on ear skin with CT and OVA protein. sdLN<sub>s</sub> were harvested and analyzed for T cell proliferation three days later.

Adj = adjuvant. N = 4 experiments. One-tailed Mann-Whitney *p* values shown. ** *p* < .001; *** *p* < .0001.
Figure 4.5: CCR4<sup>−/−</sup> DCs are competent to take up, process, and display whole OVA protein Ag. Both WT and CCR4<sup>−/−</sup> DCs were similarly able to display whole OVA protein added to the co-culture media. WT and CCR4<sup>−/−</sup> mice were topically immunized with CT+OVA protein or CT alone. On day 4 post-immunization, sdLN cells were isolated, enriched for DCs, and co-cultured with CFSE-labeled OT-I T cells. Exogenous OVA protein was added to co-culture wells. sdLN from 4-5 mice per condition were pooled before DC isolation. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1<sup>+</sup> CD3<sup>+</sup> CD8<sup>+</sup> cells. The number of proliferated (CFSE<sup>lo</sup>) T cells per 1000 input T cells is depicted. Adj = adjuvant. N = 5 experiments. One-tailed Mann-Whitney p values shown. n.s. = not significant.
Figure 4.6: DC expression of CCR4 is not required for DC migration. CCR4−/− DCs did not migrate towards the CCR4 ligand CCL22, but did migrate as expected towards the CCR7 ligand CCL21. CCR4−/− DCs were placed into the top part of a transwell chamber. Media containing either CCL22 (also known as MDC) or CCL21 (also known as SLC) were placed in the bottom part of the chamber. After incubation, the cells in the bottom part of the chamber were stained and FACS analyzed. Migrated cells as a percentage of the input population was calculated. N = 2 independent experiments.
We hypothesized that a decrease in GM-CSF production by CCR4<sup>-/-</sup> DCs from sdLN was also responsible for the observed decrease in T cell proliferation. To test this hypothesis, we first attempted to measure the GM-CSF produced by stimulated BM-DCs, as previously described (Poppensieker et al., 2012). Unfortunately, we were unable to generate detectable levels of GM-CSF, as measured with ELISA, when stimulating with LPS. We then stimulated isolated sdLN DCs, but were again unable to generate measurable GM-CSF production in this case as well. We did observe a difference in GM-CSF levels in co-culture media harvested from wells of T cells and either WT or CCR4<sup>-/-</sup> DCs (Figure 4.7), but higher GM-CSF production in the WT wells may be due to T cell production of GM-CSF by the larger number of T cells in the WT DC wells, rather than differences in the DCs.

**CCR4 deficiency decreases the size of the CD11b<sup>+</sup> migratory DC subset**

Inspired by the DC subset work detailed in Chapter 3, we then turned to analyzing the DC subset makeup of CCR4<sup>-/-</sup> mice. We hypothesized that differences in DC composition in CCR4<sup>-/-</sup> mice could be responsible for the altered T cell proliferation stimulated by CCR4<sup>-/-</sup> DCs. Surprisingly, we found that the DC subset composition in CCR4<sup>-/-</sup> mice was measurably different than that of WT mice. After skin immunization, a significantly smaller percentage of CD11c<sup>int</sup> MHC II<sup>hi</sup> migratory DCs was present in the CCR4<sup>-/-</sup> sdLN than in WT sdLN. Consequently, there was a smaller percentage of CD11b<sup>+</sup> MHC II<sup>hi</sup> migratory DCs in the CCR4<sup>-/-</sup> sdLN compared to WT sdLN (Figure 4.8). This significant difference in CD11b<sup>+</sup> MHC II<sup>hi</sup> migratory DC subset size was also
Figure 4.7: Media taken from CCR4⁻/⁻ DC co-cultures contains less GM-CSF than media from WT co-cultures. After DC-T cell coculture as described in Figure 4.5, co-culture plates were spun down and media was harvested before T cell staining. An ELISA was used to determine the concentration of GM-CSF in the co-culture media. The results of two independent co-cultures are shown.
Figure 4.8: The size of the CD11b+ migratory DC subset is reduced in both unimmunized and immunized CCR4−/− mice. sdLN from WT and CCR4−/− mice were harvested and collagenase-treated. Cell suspensions were then stained for DC subsets and FACS analyzed. The size of the migratory subsets as compared to the whole DC population with and without skin immunization was calculated. The size of the CD11b+ migratory DC subset is about 16% smaller in CCR4−/− unimmunized mice than their WT counterparts. The size of the CD11b+ migratory DC subset is about 40% smaller in CCR4−/− immunized mice than their WT counterparts.
Figure 4.8 (Continued): The size of the CD11b+ migratory DC subset is reduced in both unimmunized and immunized CCR4−/− mice.
present in unimmunized mice (Figure 4.8). In light of the results from Chapter 3 demonstrating that CD11b+ migratory DCs are responsible for cross-priming T cells in this co-culture system (Figure 3.6), we confirmed that CD11b+ migratory DCs from CCR4−/− mice were also responsible for cross-priming T cells (Figure 4.9).

Interestingly, when we compared the T cell proliferation stimulated by CD11b+ DCs from WT and CCR4−/− mice, we saw that CD11b+ DCs were not only present in smaller numbers in CCR4−/− mice, but less able to stimulate T cell proliferation than CD11b+ DCs from WT mice (compare CD11b+ DC bars from Figures 3.6 and 4.9).

Please note that in these co-cultures, the DC:T cell ratio is kept constant in the WT and CCR4−/− co-cultures, even though there are fewer CD11b+ migratory DCs overall harvested from the CCR4−/− mice. CD11b+ migratory DCs from CCR4−/− mice stimulate less T cell proliferation than an equivalent number of CD11b+ migratory DCs from WT mice.

Taken together, our data shows that CCR4−/− DCs are deficient in activating CD8 T cells both because of a decrease in number of CD11b+ migratory DCs present, and a reduced ability of those key DCs to stimulate T cell proliferation. As other DCs from the CCR4−/− mouse do not stimulate T cell proliferation in our co-culture system, the overall ability of DCs from CCR4−/− mice to stimulate CD8 T cell proliferation is reduced.
Figure 4.9: CD11b⁺ migratory DCs from CCR4⁻/⁻ mice are responsible for stimulating OT-I proliferation in co-culture. Sorted DCs were isolated from immunized CCR4⁻/⁻ mice as described in Figure 3.5 and co-cultured with CFSE-labeled OT-I T cells. Mice were immunized four days before lymph node harvest with CT and OVA protein. sdLN from 8-10 mice were pooled before DC sorting. T cell proliferation was analyzed on day 6 of ex vivo culture. Flow cytometry plots were gated on CD45.1⁺ CD3⁺ CD8⁺ cells. The number of proliferated (CFSElo) T cells per 1000 input T cells is depicted. N = 6 experiments. One-tailed Mann-Whitney p values shown. * p < .05; *** p < .0001; n.s. = not significant.
Discussion

In this chapter, we demonstrated that CCR4−/− DCs activate less T cell proliferation than WT DCs, owing to altered DC subset composition. Specifically, we observed a decrease in size of the CD11b+ migratory DC subset. These CD11b+ migratory DCs were also less able to stimulate T cell proliferation than their WT counterparts.

Altered DC subsets

We examined DC subset distribution in CCR4−/− mice. The CD11b+ migratory DC subset was significantly smaller in CCR4−/− mice than in WT mice, both with and without skin immunization (Figure 4.8). Since the CD11b+ DCs are the DCs responsible for stimulating CD8 T cell proliferation, having fewer of these DCs present would lead to the decreased T cell proliferation that we saw in our in vitro and in vivo experiments.

First, we should confirm our findings by examining the DC populations in the skin of CCR4−/− and WT mice. It may be the case that the CD11b+ migratory DCs are less able to migrate from the skin, and are therefore less represented in the skin-draining LN. This is not likely, as we found that CCR4 was not necessary for DC migration (Figure 4.6), but there may be other effects of CCR4 deficiency that indirectly reduce DC migration from skin, such as decreased CCR7 production.

Further examination of the DCs in CCR4−/− mice could indicate other alterations in DC composition. In chapter 3, we showed that WT Lang+ CD11b+ migratory DCs stimulated greater T cell proliferation than their Lang− counterparts (Figure 3.11).
smaller Lang⁺ CD11b⁺ subset would contribute to the decreased T cell stimulation capability of CD11b⁺ DCs from CCR4⁻/⁻ mice. In addition, CCR4⁻/⁻ CD11b⁺ DCs were less able to stimulate T cell proliferation than their WT counterparts. Thus, we have demonstrated that CCR4⁻/⁻ DCs are deficient in activating T cells for two reasons: (1) a decrease in the relevant DC subset, and (2) a decreased ability of that DC subset to stimulate T cell proliferation.

Only recently have studies focused on the possible functions of CCR4 on dendritic cells, mostly with regards to cytokine secretion. The data we presented in this chapter is intriguing because it points to a previously unknown role for CCR4 in directing DC development. Other chemokine receptors are also known to influence developmental processes. For example, CX₃CR1 promotes the differentiation of myeloid precursors into DCs, monocytes, and macrophages (Łyszkiewicz et al., 2011). It may be the case that CCR4 plays a similar role in the development of CD11b⁺ migratory DCs from DC precursors. Lang⁺ CD11b⁺ migratory DCs have the phenotype of Langerhans cells; reduction in the size of this subset could indicate that CCR4 plays a specific role in LC development. Determining whether the Lang⁺ CD11b⁺, the Lang⁻ CD11b⁺, or both CD11b⁺ DC subsets is altered in size could indicate which stage of DC development is altered in CCR4⁻/⁻ mice.

It remains to be determined whether the observed reduction of the CD11b⁺ DC population in CCR4 deficient mice is cell autonomous or non-cell autonomous – i.e. whether this is caused by CCR4 expressed by DC precursors or an effect of CCR4 expressed by other cell types in the surrounding environment. To examine this question,
we will generate bone marrow chimeras, injecting either CCR4+/+ or CCR4−/− bone marrow into lethally irradiated recipient mice. If we observe that CCR4+/+ bone marrow is able to generate a normal complement of CD11b+ DCs in CCR4−/− recipients, then DC precursor-expressed CCR4 must cell autonomously induce DC differentiation. However, if CCR4−/− bone marrow can generate a normal complement of CD11b+ DCs in CCR4+/+ recipients, then we would conclude that CCR4 expressed by another cell population non-cell autonomously drives DC development.

**CCR4 in T cell attraction to DCs**

CCR4 is thought to play an *in vivo* role in aiding T cell movement and binding to DCs (Imai *et al.*, 1999; Kim *et al.*, 2003; Semmling *et al.*, 2010; Vitali *et al.*, 2012). Our *in vitro* co-culture system does not require CCR4 to bring T cells and DCs together for T cell proliferation, as we saw no difference in proliferation between WT and CCR4−/− T cells when stimulated with WT DCs loaded with Ag *in vivo* (Figure 4.1). This may be because our *in vitro* co-culture does not fully recapitulate the complex architecture of the LN. DCs are also present in higher number relative to T cells in the co-culture, allowing interactions between DCs and T cells to occur more frequently. However, this does not affect the validity of our finding that CCR4−/− DCs stimulate less T cell proliferation than WT DCs, as they are each present in the same DC:T cell ratio in their co-culture wells.

**Future analysis of factor secretion**
We have preliminary data showing a reduction in GM-CSF content in media from CCR4\(^{-/-}\) DC co-culture wells compared to WT controls (Figure 4.7). Future work will further examine altered factor secretion by CCR4\(^{-/-}\) DCs, as it is a possible mechanism for the observed altered DC development, or for the observed reduced T cell proliferation.

Cytokines released by proto-DCs or surrounding cells may play a role in DC development. There are several cytokines that are known to play a role in DC development and maturation. After TGF-\(\beta1\)\(^{-/-}\) mice were found to have no epidermal Langerhans cells (Borkowski \textit{et al.}, 1996), it was confirmed that TGF-\(\beta1\) promotes the development of DCs from CD34\(^{+}\) progenitors (Caux \textit{et al.}, 1999; Strobl \textit{et al.}, 1996) and the development of Langerhans-type DCs \textit{in vitro} (Strobl \textit{et al.}, 1997). IL-4 stimulates the development of DCs from CD34\(^{+}\) progenitors (Romani \textit{et al.}, 1994; Sallusto and Lanzavecchia, 1994), specifically non-LC DCs (Caux \textit{et al.}, 1999). IL-10 blocks LC maturation (Ozawa \textit{et al.}, 1996) and general human DC maturation (De Smedt \textit{et al.}, 1997; Morel \textit{et al.}, 1997; Steinbrink \textit{et al.}, 1997). GM-CSF plays a key role in DC development (Kingston \textit{et al.}, 2009; van de Laar \textit{et al.}, 2012; Xu \textit{et al.}, 2007; Zhan \textit{et al.}, 2012). Alterations in the production of any of these cytokines could affect DC development into different subsets. A cytokine/chemokine array would enable us to define potential factors for further study.

Factors whose release from other cells is stimulated by DCs could also be produced aberrantly in CCR4\(^{-/-}\) mice. CCR4\(^{-/-}\) DCs trigger impaired IFN\(\gamma\) production in WT CD4 T cells (Freeman \textit{et al.}, 2006). We are interested in determining whether this...
extends to CD8 T cells – whether IFNγ production, or production of any other cytokine, by CD8 T cells is affected by CCR4−/− DC stimulation. Cytokines released by T cells can stimulate additional T cell proliferation, so changes in CD8 T cell cytokine production could be a secondary effect of CCR4−/− DCs further contributing to decreased T cell proliferation.
References


CHAPTER FIVE

Summary and Discussion

Suzanne Nizza

Attributions: This chapter was written by S. Nizza with critical input from J. Campbell.
Dendritic cells (DCs) respond to and activate immune responses to a wide variety of antigen (Ag). The capacity of DCs to stimulate CD8 T cells make them a key player in responding to soluble Ag, tumor cells, and viruses that do not infect Ag-presenting cells (APCs). The continued discovery and evaluation of new DC subsets and new markers to identify them indicate that skin immunity is quite complex. Different types of DCs from different areas in the body may each respond differently to various Ag. Currently, it is thought that CD8α+ DCs are the lymph node-resident DCs that cross-present and CD103+ DCs are the migratory DCs that cross-present. These findings, however, have been reached using a number of different antigens and antigen-application methods, which at times make it difficult to conclusively state that cross-presentation, not classical presentation, is truly taking place.

Here, we set out to determine which DC subsets are important for activating CD8 T cells using our co-culture system. We hypothesized that DC subsets able to stimulate T cell proliferation would differ in their ability to stimulate homing marker expression on proliferating T cells. To answer this question, we developed a novel co-culture system enabling in vivo Ag uptake by, digestion by, and loading on skin DCs, thus preserving the physiological methods by which DCs – in lymphoid or non-lymphoid tissue – encounter soluble Ag. This co-culture system allowed us to eliminate the issues that arise when non-protein Ag is delivered directly to isolated DCs. In this dissertation, I presented evidence that the CD11b+ migratory DCs in skin-draining lymph nodes (sdLN) are the critical DC subset for triggering CD8 T cell proliferation, and that the Lang+ subset of these DCs is responsible for the majority of E-lig imprinting onto proliferating
CD8 T cells. In addition, I showed that CCR4 deficiency decreases the size and impairs the function of the CD11b+ migratory DC subset in the sdLN, leading to decreased T cell proliferation and indicating a role of CCR4 in the proper development of CD11b+ migratory DCs.

**CD8α and CD103 – the only cross-presenting DCs?**

CD8α+ DCs were first shown to be superior at cross-presentation than CD8α- DCs in the early 2000s (den Haan et al., 2000; Pooley et al., 2001). However, this work was performed under steady-state conditions with no local inflammation, which greatly differ from the inflamed conditions of infection in which DC-T cell interactions are most relevant. CD8α+ DCs were later shown to play a role in viral immunity, specifically in response to herpes simplex virus type 1, influenza virus, vaccinia virus, and lymphocytic choriomeningitis virus (LCMV) (Belz et al., 2005; Belz et al., 2004a). It has been proposed that CD8α+ DCs are particularly specialized to present exogenous Ag on MHC I (Dudziak et al., 2007). Many studies have demonstrated the ability of CD8α+ DCs to cross-present in the lung (Belz et al., 2004b; GeurtsvanKessel et al., 2008) and in the skin (Allan et al., 2006; Allan et al., 2003; Lee et al., 2009).

More recent studies indicated that CD103+ DCs are also capable of cross-presentation. After intranasal (i.n.) infection with influenza virus expressing the OVA peptide recognized by OT-I T cells, Lang+ CD103+ CD11b- intraepithelial DCs in the lung presented influenza virus to CD8 T cells (GeurtsvanKessel et al., 2008). After influenza infection, CD103+ DCs from mediastinal LN were more efficient at activating naïve CD8
T cells (Kim and Braciale, 2009). In the skin, Langerin+ CD103+ dermal DCs cross-present keratinocyte-derived Ag (Henri et al., 2010), to present HSV-1 Ag to CD8 T cells after secondary viral skin infection (Bedoui et al., 2009), and to present skin-derived self Ag to OT-I (Bedoui et al., 2009). CD103+ Lang+ dermal DCs were found to promote CD8 T cell responses after plasmid DNA immunization or gene gun vaccination (Elnekave et al., 2010; Stoecklinger et al., 2011). On the other hand, Langerhans cells have been shown to have no role in CD8 T cell priming after cutaneous HSV infection; this work used a flank abrasion method of HSV inoculation (Allan et al., 2003). This body of work has led researchers in the field to consider CD8α+ and CD103+ DCs the archetypical cross-presenting DCs.

**Necessity for a physiological co-culture system**

However, the relevance of this previous work to cross-presentation by DC subsets is dependent on whether these viruses directly infect DC. It is assumed that dead cells post-viral infection provide a source of exogenous viral Ags for DCs to cross-present. However, if viruses infect DCs, DCs would be directly presenting endogenous Ag, not cross-presenting exogenous Ag. As a result, it can be difficult to determine whether cross-presentation is truly taking place when using viral Ag. A recent paper found that HSV-1 can replicate within mature DCs (Goldwich et al., 2011), and persistent LCMV strains have also been seen to infect DCs (Sevilla et al., 2000), so there is the possibility that some earlier findings have derived from direct presentation, instead of cross-presentation as was thought. This demonstrates the need for truly
exogenous Ag to be used in determining the importance of DC subsets in cross-presentation. An exogenous soluble protein does not have the same risk of being directly presented, thus avoiding those issues inherent with viral antigens.

We designed a novel co-culture system that eliminates the confounding factors present in previous work. Our co-culture system allowed us to preserve physiological Ag application, DC composition, and in vivo Ag access and acquisition by DCs, while still allowing us to evaluate the capabilities of individual DC subsets. We used this co-culture system along with sorted DCs to determine which DC subsets were capable of stimulating T cell proliferation. We wanted to have a definitive example of cross-presentation, so we used a soluble protein Ag instead of a virus with the potential to infect DCs.

In our experiments, we administered whole protein Ag in vivo, not post DC-isolation, to keep the DC’s encounter with and processing of Ag as physiological as possible. Other studies have provided exogenous Ag (in protein or peptide form) to DCs post-isolation from lymph nodes or other tissue. This method hides any effects from differing DC Ag access or Ag transportation methods, and does not replicate in vivo physiological Ag access. In addition, pulsing with Ag allows all DC subsets to have access to Ag, which may allow the DC subset that is actually relevant for cross-presentation in vivo to be out-competed for Ag by another DC subset that is normally unable to access Ag. Our co-culture system preserves the Ag access and digestion of in vivo DCs, giving us confidence that the DC subsets observed to be relevant in our co-culture do have relevance in vivo as well.
We also avoided the use of Flt3L or any other DC growth factors in our co-culture system. Flt3L can be used to expand the overall DC population allowing the use of fewer mice per experiment. However, the use of Flt3L to expand the DC population does not account for the fact that not all DC subsets are equally affected by Flt3L. For example, Langerhans cells (LCs) are unaffected in mice that lack Flt3L or the receptor Flt3, while other DCs are affected (Ginhoux et al., 2009). As a result, using Flt3L could alter the DC subset makeup within a LN, making it difficult to notice the activity of smaller DC subsets. In addition, DCs that normally do not encounter a certain Ag in their area of the lymph may, when their population is expanded, leave their usual niche and access Ag located in other areas. Our co-culture system enables physiological Ag processing by an unaltered DC cohort, so that the experimental conditions more closely emulate in vivo conditions.

**New importance of CD11b⁺ migratory DCs in CD8 T cell stimulation**

Using our co-culture system, we identified CD11b⁺ migratory DCs from the skin as being responsible for cross-presenting soluble protein Ag to CD8 T cells (Figure 3.6). All other DCs – LN-resident DCs and CD103⁺ migratory DCs – were unable to stimulate CD8 T cell presentation. This demonstrates the importance of the CD11b⁺ migratory DC subset and the irrelevance of CD103⁺ DCs, for cross-presenting epidermally-applied protein Ag. This role was still held by the CD11b⁺ MHC II⁺ migratory DCs when DCs were harvested from the sdLN on a different day (Figure 3.8) and when immunization was stimulated with a different adjuvant (Figure 3.7). These findings are intriguing, as
CD11b+ DCs have only been previously associated with triggering T cell tolerance or stimulating CD4 T cells, not CD8 T cell activation. CD11b+ DCs in mesenteric LN promote cross-tolerance to dietary proteins (Chung et al., 2005). CD11b+ DCs in the lung and mediastinal LN take part in classical MHC II presentation to CD4 T cells (del Rio et al., 2007; Kim and Braciale, 2009). No CD11b+ stimulation of CD8 T cells in the skin or skin-draining LN had been shown before this present work.

Some groups had reported that lung CD11b+ migratory DCs were able to provide viral Ag to resident CD8α+ DCs (Ballesteros-Tato et al., 2010; Lukens et al., 2009). However, RSV, used by Lukens et al. (2009), has been seen to infect monocyte-derived dendritic cells (de Graaff et al., 2005); this group may have been observing classical presentation rather than cross-presentation. In addition, despite the large influx of CD11b+ DCs into the mediastinal LN shown in (Ballesteros-Tato et al., 2010), it was later shown that CD103+ DCs, not CD11b+ DCs, were responsible for inducing influenza virus-specific CD8 T cells (Helft et al., 2012). Helft et al. also showed that GFP expressed by a recombinant influenza virus was almost undetectable in the CD11b+ DCs entering the mediastinal LN, further supporting the idea that CD11b+ were not responsible for cross-priming CD8 T cells (Helft et al., 2012).

We are not asserting that CD11b+ DCs are the only cross-presenting DC subset. For example, it is known that DCs from Batf3−/− mice are unable to cross present West Nile virus, due to their lack of CD8α+ DCs (Hildner et al., 2008). Under those conditions, CD8α+ DCs are the relevant cross-presenting DC. Our data suggests that CD11b+ DCs are the relevant cross-presenting DCs in the case of soluble protein Ag delivered
through the skin, a situation in which CD8α⁺ DCs and CD103⁺ DCs are irrelevant. It is quite possible that other DC subsets are primarily responsible for cross-presentation under the conditions of alternate adjuvants, antigens, or immunization methods. It is therefore inaccurate to call any subset the sole cross-presenting DC subset; rather, a new combination of adjuvant and Ag must be tested before declaring which DC subset to focus on. This is especially important in the case of vaccine design. Instead of choosing a DC subset to target with a vaccine based on existing research indicating that particular DC is a cross-presenting DC, one must first test the vaccine administration method and adjuvant being used, as that particular combination of factors may instead target a different DC subset.

We also question the idea that certain DC subsets are uniquely able to stimulate T cell proliferation due to specialized machinery. Indeed, we found that all DC subsets were able to stimulate CD8 T cell proliferation when exogenous OVA protein Ag was added to co-culture wells (Figure 3.6, right). Instead, differences in observed cross-presentation capability may instead stem from Ag type used or Ag access by different DC subsets, for example. Our work shows that CD11b⁺ migratory DCs are the relevant subset for cross-priming CD8 T cells in the case of OVA protein Ag topically applied to the skin. This may be the result of easier access to epidermal Ag, or preferential activation by the cytokines released in our immunization process (i.e., tape-stripping). Other methods of applying Ag (subcutaneous, intradermal, scarification) or other types of Ag may lead to presentation by alternate DC subsets.
Langerhans cells and cross-presentation

We demonstrated that LCs (Lang+ CD11b+ migratory DCs) cross-present soluble OVA Ag in our co-culture system (Figure 3.11). Whether LCs have the ability to cross-present Ag has been an open question. Studies using various LC ablation models have produced mixed results regarding the role of LCs in contact hypersensitivity and general CD8 T cell responses to Ag (Bursch et al., 2009; Igyártó et al., 2011; Kaplan et al., 2008). Investigating the importance of DC subsets using depletion models may be informative, but cannot provide a complete answer because other DC subsets may take over Ag cross-presentation in the absence of competing LCs. It is possible that a certain DC subset does not typically access or cross-present Ag under normal conditions, but has the opportunity to do so when a competing DC subset (the LCs) is removed. Depletion models can be useful, however, for narrowing down DC subsets for further investigation, as we have done in the present work. In addition, it is possible that LCs directly present some types of Ag (e.g. soluble protein Ag) and transfer other types of Ag to LN-resident DCs for presentation (e.g. HSV Ag).

The question of Langerhans cell cross-presentation is quite interesting, as LCs are commonly considered to be tolerogenic, not immunogenic. The first evidence for a tolerogenic function for LCs was based on the finding that LCs internalize self-Ag during the steady state without causing autoimmune responses (Morelli et al., 2001). Another line of thinking points to the late arrival of LCs in sdLN (on day four after skin immunization) and their relatively inefficient induction of the primary costimulator molecules CD80 and CD86 (Shklovskaya et al., 2008). Our data demonstrates a strong
role for LCs (Lang+ CD11b+ migratory DCs) in cross-presentation and imprinting of homing markers (Figures 3.11 and 3.13), which argues against the idea that LCs are solely tolerogenic. It is possible, however, that the tolerogenicity of LCs depends on their maturation state (Lutz et al., 2010) as immature LCs can migrate to draining LN (Geissmann et al., 2002). It should also be noted that early studies did not consistently distinguish LCs from Langerin+ dermal DCs, Langerin+ dermal DCs being a relatively recently discovered DC subset (Ginhoux et al., 2007). Langerin+ dermal DCs are known to be capable of cross-presenting self Ag (Bedoui et al., 2009). Dermal Langerin+ DCs may in fact be responsible for some tolerogenic activities currently ascribed to LCs, as reviewed in (Lutz et al., 2010; Mutyambizi et al., 2009). LC may transfer Ag to Langerin+ dermal DCs, which then trigger tolerance to that Ag (Allan et al., 2003). It is possible that LCs are tolerogenic in the case of certain adjuvant and Ag combinations. However, we have found conditions in which LCs actively stimulate T cell proliferation and imprint E-lig homing – the conditions of our immunization protocol, involving tape-stripping and cholera toxin as adjuvant. Therefore, we assert that LCs are not solely tolerogenic, but may be tolerogenic under certain conditions and immunogenic under others.

**Langerin+ CD11b+ DCs are responsible for imprinting skin homing**

DCs are capable of imprinting homing marker expression on naïve T cells, allowing T cells to enter certain tissues. Early work demonstrated that peptide-pulsed DC from skin-draining LN stimulated E-lig expression on CD8 T cells, and peptide-pulsed DC isolated from Peyer’s patches stimulated α4β7 expression on CD8 T cells.
(Mora, 2005). This group also sorted DCs by CD11c, B220, CD8α, and CD11b expression and examined their ability to trigger homing marker expression on co-cultured CD8 T cells. They found all peptide-pulsed DC subsets to be capable of stimulating E-lig expression, with CD11b<sup>hi</sup> DCs stimulating the lowest percentage of T cells (Mora, 2005). However, using peptide Ag<sup>in vitro</sup> to charge DCs ignores how Ag is physiologically encountered<sup>in vivo</sup> and the Ag-processing abilities of DCs.

Our co-culture system preserves<sup>in vivo</sup> Ag administration while still allowing us to isolate different sets of migratory DCs. As a result, we were able to use this co-culture system to test our hypothesis that different DC subsets would have varying abilities to trigger skin-homing markers on proliferating T cells. We did demonstrate distinct differences in the abilities of DC subsets to stimulate E-lig expression. While the CD11b<sup>+</sup> migratory DC subset as a whole was already found to be capable of stimulating CD8 T cell proliferation, we found that, of the CD11b<sup>+</sup> migratory DCs, the Lang<sup>+</sup> DC subset was significantly better than the Lang<sup>-</sup> DC subset at triggering E-lig expression on CD8 T cells (Figure 3.13). This contrasts with (Mora<sup>et al.</sup> 2005), which stated that all sdLN DC subsets stimulated E-lig expression. Our work shows that not all sdLN DC subsets have the ability to imprint E-lig expression, and that the ability to stimulate T cell proliferation is separate from the ability to imprint skin homing.

**Do T cells express E-lig by default?**

What triggers E-lig expression on T cells is a complex question. Previous work has suggested that CD8 T cells activated by DC express E-lig “by default” unless they
receive gut-tropism signals (possibly from retinoic acid) (Iwata et al., 2004; Mora, 2005). Some have said that T cells may be biased toward expressing skin-homing markers when activated in the absence of gut-tropism signals such as RA (Mora, 2004). Peptide-pulsed PLN DCs can stimulate E-lig expression on Ag-specific CD8 T cells, and stimulate FucT-VII mRNA expression in T cells as well (while PP DCs are unable to) (Mora, 2005). Mora et al. also found that PLN DCs continued to stimulate E-lig expression, even after fixation, implying that E-lig can be imprinted on T cells without the DC being metabolically active (Mora, 2005). Interestingly, fixed PP-DCs were also able to stimulate E-lig expression on T cells, with or without the presence of Ag-pulsed PLN DCs. Non-fixed PP DCs, whether peptide-pulsed or not, were able to reduce the ability of peptide-pulsed PLN DCs to imprint E-lig (Mora, 2005). These data were interpreted to mean that PP DCs suppress E-lig expression, and that in the absence of other signals, E-lig expression occurs.

While it is possible that PP DCs are releasing factors that suppress E-lig expression on T cells, this does not necessarily mean that in the absence of those factors, E-lig expression on T cells would take place. It may merely be that the default for PLN DCs is imprinting E-lig, and PP DCs interfere with that imprinting, or that the signaling from PP DCs imprinting gut-homing markers is stronger than the signals imprinting skin-homing markers, so in the case where both are present, gut homing “wins.” This does not mean that a lack of gut signals necessarily leads to expression of skin homing markers.
Additionally, it is unclear whether the observed E-lig expression in Mora et al. (2005) is physiological and directly relatable to *in vivo* processes. As the Ag in the above experiments is peptide and pulsed with already-isolated DCs, these DCs may be stimulating the T cells much stronger than they would be under normal immunization conditions *in vivo*. It may be the case that high levels of stimulation can trigger alternate pathways or mis-expression of surface markers that would not be seen under physiological conditions. In addition, as DCs may localize to different areas of the lymph node, it may be the case that different dendritic cells in different areas have different access to antigen. Administering exogenous Ag to dendritic cells ignores the differences in DC access to Ag, and could obscure which DC subset is relevant.

In Chapter 3, we presented findings demonstrating that DC subsets have varying capabilities to stimulate E-lig expression. We identified a DC subset – the Lang\(^{\dagger}\) CD11b\(^{+}\) migratory DCs – that stimulates T cell proliferation, yet does not imprint E-lig on T cells, even in the absence of RA (Figure 3.13). In contrast, Mora *et al.* (2005) found that all PLN DC subsets were able to stimulate E-lig expression, with the CD11b\(^{hi}\) DCs stimulating the least. Our results suggest a more complex model, wherein imprinting capability varies among DC subsets. The variation in E-lig imprinting that we see after co-culture with CD11b\(^{+}\) migratory DCs indicates that E-lig expression does not occur by default. It further suggests that not all DCs are equally capable of stimulating E-lig expression. The induction of E-lig expression (*i.e.* skin-selective imprinting) seems to be an instructive process that requires signals independent of those that induce
proliferation. These signals can come from skin-derived DCs, and may also be released by cells in sdLN or local cells in the skin.

Our findings prompt a more detailed examination of the function of this subset under other Ag application conditions. Further study of DC subsets in skin-draining LNs and their abilities to stimulate T cell proliferation and E-lig expression will help us determine ways to control wanted and unwanted skin immune responses. Use of our co-culture system with OT-II T cells would further add our knowledge of the factors that control E-lig expression. We already suspect that there are different DC subsets responsible for stimulating OT-I and OT-II proliferation, based on the proliferation stimulated by DCs harvested at different timepoints (Figures 2.4 and 2.5). It would be interesting to confirm that on the DC subset level, and then to determine whether a different DC subset is responsible for E-lig imprinting of OT-II cells.

**Other factors that may affect E-lig imprinting**

Besides DCs, other cell types and factors may be involved in determining T cell homing marker expression. Some groups have suggested that imprinting homing markers on T cells is independent of activation site or tissue of origin. Masopust *et al.* (2004) found that CD8 T cells responding to a local infection were able to migrate widely within the body, not just to the site of infection. Oral infection with rotavirus (which preferentially infects intestinal epithelial cells (Arias *et al.*, 2002)) resulted in activated cells specific for viral peptides present in the LP and intra-epithelial lymphocyte (IEL) compartments, in addition to the spleen, lung, and liver (Masopust *et al.*, 2004).
Infection with sendai virus infection, which preferentially infects lung cells, resulted in Ag-specific CD8 T cells being found in the spleen, blood, intestinal mucosa, and liver, in addition to the lung (Masopust et al., 2004). However, this may be the result of Ag presentation outside of the initial infection site (even with efforts made to ensure local immunization).

The local microenvironment may also play a role in dictating homing marker expression. Culturing Ag-pulsed BM-DCs with CD8 P14 T cells leads to E-lig expression on T cells co-cultured with dermal fibroblasts, or CCR9 and α4β7 expression on T cells co-cultured with small intestinal epithelial cells (SIEC) (Edele et al., 2008). Soluble factors from SIEC seemed to be responsible, as culturing activated P14 T cells with conditioned media from SEIC cultures also resulted in α4β7 and CCR9 expression (Edele et al., 2008). On the other hand, Edele et al. found that dermal fibroblasts needed direct contact to trigger E-lig expression on T cells (Edele et al., 2008). It may be the case that epithelial cells release factors that license DCs in those areas to imprint the appropriate homing markers on T cells. This would be logical – cells in non-lymphoid tissues would stimulate DCs to be able to direct an immune response back to those non-lymphoid areas. Investigating whether DCs harvested directly from skin or from gut epithelium are able to imprint T cells without traveling to the draining LN should shed light on this question.

Other work has shown a role for lymph node stroma in dictating T cell homing marker expression. Ag-loaded BM-DCs injected into the skin (s.c. or i.c.) are able to trigger E-lig expression in the PLN (Dudda et al., 2004; Ferguson and Engelhard, 2010).
E-lig expression is observed in the PLN after i.c. injection of peptide-pulsed PLN DC or MLN DC and high α4β7 expression was restricted to the MLN after i.p. injection of either peptide-pulsed PLN DC or MLN DC (Dudda et al., 2005). In these experiments, it seems that the DC environment (within gut or skin) in which T cell activation is taking place is the determinant of what homing markers are imprinted, instead of the origin of the injected DC. Hammerschmidt et al. (2008) took this idea one step further, transplanting PLN fragments into MLN areas in mice, which allowed them to connect to gut-draining afferent and LN efferent lymphatics (Hammerschmidt et al., 2008). CFSE-labeled OT-I and OT-II T cells were transferred into these mice, and OVA immunization was administered orally. Interestingly, while T cells in transplanted PLN did proliferate, they did not upregulate α4β7 and CCR9, unlike T cells in transplanted MLN or endogenous MLN (Hammerschmidt et al., 2008). These data further support the idea that DC instruction to T cells can be “overridden” by local factors released from the microenvironment. Additionally, DCs may be influenced by local lymph node factors, affecting the type of homing they are able to imprint on T cells.

Our co-culture system could be adapted to examine the relations between non-DCs and DCs in imprinting homing markers. It is possible that Lang+ CD11b+ migratory DCs receive signals from dermal fibroblasts as they pass through the dermis that enable them to imprint E-lig on T cells. Alternatively, Lang+ CD11b+ migratory DCs could receive signals from stromal cells in their area of localization within the LN. One way to examine these possibilities would be to harvest skin and allow migratory DCs located in the skin to migrate out of the skin. After isolating the Lang+ CD11b+ DCs and applying
Ag, either in a skin immunization before harvest, or pulsed with the DCs after isolation, DCs would be co-cultured with T cells and E-lig expression would be determined. If Lang$^+$ CD11b$^+$ DCs isolated from skin could not imprint E-lig on T cells, this would support the necessity of external factors from skin or LN stroma. We could also modify the co-culture system to include stroma cells from sdLN and determine whether the presence of these cells leads to E-lig expression by T cells in co-cultures lacking Lang$^+$ CD11b$^+$ DCs.

**CCR4 regulates the size of the CD11b$^+$ migratory DC subset**

In addition to our work studying the functions of DC subsets, we utilized our co-culture system to identify a novel role for CCR4 in DC subset composition. We identified CCR4 as a chemokine receptor of interest after we observed how DCs from CCR4$^{-/-}$ mice stimulated less T cell proliferation than DCs from WT mice in our co-culture system (Figure 4.2); the altered DC subset composition of CCR4$^{-/-}$ mice might have otherwise gone unnoticed. We found that the size of the CD11b$^+$ migratory DC subset was decreased in CCR4$^{-/-}$ mice (Figure 4.8). Understanding what role CCR4 plays in DC subset composition will add to our understanding of DC development.

Detailed examination of factor secretion by CCR4$^{-/-}$ DCs as well as T cell factor secretion stimulated by CCR4$^{-/-}$ DCs is an area of future study. A cytokine panel could identify multiple chemokines of interest, which could then be further examined by testing their effects on T cell proliferation when added to co-culture. Observing decreased production of cytokines which promote T cell proliferation would indicate that our
findings stem from more than just altered DC development. CCR4<sup>−/−</sup> DCs are already known to have difficulty stimulating IFN<sub>γ</sub> production in WT CD4 T cells (Freeman <i>et al.</i>, 2006).

In addition, these factors could play a role in DC development, leading to the altered DC subset composition we observe in CCR4<sup>−/−</sup> mice. CX<sub>3</sub>CR1 has been found to promote the development of myeloid precursors into DCs, monocytes, and macrophages in the steady-state (Łyszkiewicz <i>et al.</i>, 2011). TGF-β1 and IL-4 have been shown to promote DC development and maturation (Caux <i>et al.</i>, 1999; Romani <i>et al.</i>, 1994; Sallusto and Lanzavecchia, 1994; Strobl <i>et al.</i>, 1997; Strobl <i>et al.</i>, 1996). Flt3 and Flt3-ligand (Flt3L) are key players in the differentiation of DCs (Ginhoux <i>et al.</i>, 2009; Onai <i>et al.</i>, 2007; Waskow <i>et al.</i>, 2008), though LCs are not affected by the loss of Flt3L or Flt3 (Ginhoux <i>et al.</i>, 2009). CCR4 deficiency may alter production of these factors or others by either DCs or cells in the lymphoid organs where these DCs develop.

**Concluding remarks**

This project was first conceived when our lab observed that increased protein application during immunization led to increased proliferation, but not increased E-lig expression on T cells. Our data shows that sdLN DC subsets do have differing capabilities to stimulate E-lig expression - that proliferation does not necessarily lead to E-lig expression on T cells, and that E-lig expression is not a default condition for T cells. Overall, the question of what regulates E-lig expression on T cells may be more complex than a single DC subset or stromal cell type. How the different factors – DCs,
stromal cells, soluble factors, tissue microenvironment interact in vivo, and which are
dominant, is an area for further study.

We have only begun to explore the different DC subsets and their varying functions. It seems as though new surface markers and new methods of segmenting DCs are discovered regularly. Understanding these subsets and their development, localization, and function in the organism is vital for complete understanding of the immune response. Studying the primary function of different DCs subsets will help us to identify mechanisms of controlling and limiting the function of these DCs. This knowledge can be applied to the development of vaccines intended to target specific DC subsets. Especially important is the development of homing imprinted T cells, to facilitate the immune response at the desired site. Identifying the DC subsets most able to imprint T cells for homing to certain areas of the body would be key for proper targeting of vaccines to those areas. The co-culture system described in this work, along with those developed and used by others, will aid in further characterizing the roles of these different DC subsets.
References


