

Visualizing dendritic cell migration within the skin

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Abstract Dendritic cells (DCs) within the skin are a heterogeneous population of cells, including Langerhans cells of the epidermis and at least three subsets of dermal DCs. Collectively, these DCs play important roles in the initiation of adaptive immune responses following antigen challenge of the skin as well as being mediators of tolerance to self-antigen. A key functional aspect of cutaneous DCs is their migration both within the skin and into lymphatic vessels, resulting in their emigration to draining lymph nodes. Here, we discuss our current understanding of the requirements for successful DC migration in and from the skin, and introduce some of the microscopic techniques developed in our laboratory to facilitate a better understanding of this process. In particular, we detail our current use of multi-photon excitation (MPE) microscopy of murine skin to dissect the migratory behavior of DCs *in vivo*.

Keywords Dendritic cells · Migration · Intravital · Multiphoton · Microscopy

Introduction

The skin is the largest organ in the body, and the home of a vast array of leukocytes that serve as a first line of defense against invading pathogens. Among these leukocytes are dendritic cells (DCs), which continuously migrate from the skin to the draining lymph nodes (LNs) via the lymphatic system, thereby fulfilling their role as sensors for the adaptive immune system (Hemmi et al. 2001; Silberberg-Sinakin et al. 1976). This continuous emigration also enables skin DCs to serve other important functions, such as the maintenance of tolerance (Huang and MacPherson 2001; Waithman et al. 2007). Although lymphatic entry and migration represent a fundamental aspect of DC (and T cell) biology, we have only a limited understanding of the dynamics and molecular mechanisms underlying these processes. While genetic studies have identified a few molecular candidates (and will undoubtedly uncover more) without suitable tools for adequate visualization, we cannot fully appreciate how DCs co-ordinate to achieve what is, by definition, a highly dynamic process. Just as direct visualization significantly facilitated our understanding of leukocyte rolling, adhesion and transmigration through blood vessels (Iparraguirre and Weninger 2003; Springer 1994; von Andrian and Mackay 2000), advances in microscopic techniques promise a far greater appreciation of leukocyte migration into lymphatic vessels. This will be particularly fruitful when utilized in conjunction with some of the newly developed fluorescent markers and transgenic animals. With this comes the hope of greater improvements in a variety of medical applications, particularly DC

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immunotherapy, in which it is well appreciated that DC migration is quite poor (Figdor et al. 2004).

In this review, we discuss our current understanding of the requirements for successful DC migration, particularly from the skin to the draining LN via lymphatic vessels. We also introduce some of the microscopic models and techniques developed in our laboratory to facilitate a better understanding of cutaneous DC behavior and other immune functions within the skin, chiefly the use of multi-photon microscopy.

Dendritic cells in the skin

Broadly speaking, the skin can be divided into two anatomical compartments: The epidermis, a relatively thin layer of cells composed primarily of keratinocytes, and the underlying dermis, which is rich in collagen-producing fibroblasts, and the location of blood and lymphatic vessels. The epidermis and dermis are separated by the basement membrane, a complex mixture of proteins including laminin, type IV collagen and proteoglycans that serve both as an anchoring complex between the two skin compartments, as well as a mechanical barrier against entry into the dermis. DCs can be found in both regions, as Langerhans cells (LCs) in the epidermis and dermal dendritic cells (DDCs) in the dermis.

Langerhans cells, the first type of DCs to be identified and perhaps the best characterized (Jakob et al. 2001; Kissenpfennig et al. 2005; Romani et al. 1989; Ruedl et al. 2001; Stoitzner et al. 2002), are a homogenous population of cells, typically found in tight association with the surrounding keratinocytes. As a result, LCs exhibit a stellate morphology and are largely sessile in the steady-state (Kissenpfennig et al. 2005; Ng et al. 2008a; Nishibu et al. 2006). LCs in mice turn over at a low rate in the steady-state (Kamath et al. 2002; Merad et al. 2002), and these cells are largely radio-resistant (Merad et al. 2002). Furthermore, LC mobilization following inflammatory stimuli is relatively slow, such that LCs reach draining LNs only after 3–4 days (Kissenpfennig et al. 2005; Shklovskaya et al. 2008).

Dermal dendritic cells are a heterogeneous cell population, both in humans (Nestle et al. 1993; Angel et al. 2006; Angel et al. 2007) and mice (Bursch et al. 2007; Ginhoux et al. 2007; Poulin et al. 2007; Shklovskaya et al. 2008). In contrast to LCs, DDCs display an amoeboid morphology (Ng et al. 2008a), more characteristic of a migratory cell (Gunzer et al. 2000). In the mouse, DDCs are replaced every 10–15 days by bone marrow (BM)-derived precursors (Iijima et al. 2007; Kamath et al. 2002; Liu et al. 2007). Furthermore, DDCs mobilize rapidly in response to inflammation, arriving in draining LNs within ~12 h of

stimulation, and peaking at 1–2 days (Kissenpfennig et al. 2005; Shklovskaya et al. 2008). Recently, a number of groups have exploited the radiosensitivity of DDCs to more clearly define this population. These experiments led to the identification of three distinct DDC subsets in mice, one of which expresses langerin/CD207 (Bursch et al. 2007; Ginhoux et al. 2007; Poulin et al. 2007; Shklovskaya et al. 2008). In terms of function, it is thought that both LCs and DDCs are capable of capturing and presenting foreign and self-antigen to naive T cells in vivo (Shklovskaya et al. 2008). However, it is unclear whether antigen presentation to T cells by the different DC subsets leads to distinct functional outcomes during immune responses.

Dendritic cell migration

Dendritic cells, like all leukocytes, use amoeboid cell migration mechanisms to traffic within peripheral and lymphoid tissues (reviewed by Friedl and Weigelin 2008). Amoeboid migration is characterized by the acquisition of cell polarity, which then drives the development of a leading edge followed by the cell body and a posterior tail known as the uropod. Generally, polarization occurs in response to migration-promoting factors such as chemokines, which signal via G protein-coupled receptors (Thelen and Stein 2008).

DC migration has been studied both in three-dimensional (3D) matrices in vitro, as well as a number of organs in vivo (Bouso and Robey 2003; Cavanagh and Weninger 2008; Gunzer et al. 2000; Kissenpfennig et al. 2005; Lammermann et al. 2008; Lindquist et al. 2004; Mempel et al. 2004; Ng et al. 2008a). Depending upon the DC type studied and the specific microenvironment, migratory properties of DCs have been found to differ quite considerably. DC in epithelia, including the epidermis and intestine, are immobile (Chieppa et al. 2006; Kissenpfennig et al. 2005; Nishibu et al. 2006). Similarly, DC in the T cell zones of LNs migrate at low speed (Lindquist et al. 2004). In contrast, we have recently demonstrated that DDCs are constitutively motile, suggesting that they search the dermis for the presence of intruding pathogens (Ng et al. 2008a, see below).

Mechanistically, it was believed that migration of leukocytes, including DCs, relied upon interactions between surface receptors, such as integrins, and their ligands in the extracellular environment. However, a recent study demonstrated that DC locomotion occurred through alignment of the cell body to surrounding cell surfaces and/or extracellular matrix (ECM) proteins independently of integrins (Lammermann et al. 2008). Rather, cell movement is achieved by “squeezing and flowing” of the actin cytoskeleton, a process that appears to utilize weak-to-non-adhesive

interactions and thus propels the DC along the path of least resistance towards the polarizing agent (known as contact guidance, Friedl et al. 1998; Friedl and Weigelin 2008; Lammermann et al. 2008).

Lymphatic vessels

Lymphatic vessels are the structures through which lymph-borne material, including soluble proteins and antigens, migratory DCs and recirculating T cells, travel into LNs. The draining function of the lymphatic system is important for the maintenance of normal tissue homeostasis, as well as in inflammatory situations, when there is increased fluid and cellular efflux (Ryan 1989; Schmid-Schonbein 1990). In the skin (as in most organs), afferent lymphatic vessels begin as a plexus of lymphatic capillaries that drain interstitial fluid. These initial absorbing lymphatic vessels ultimately converge into larger collecting ducts, such that only a few vessels enter and terminate in the subcapsular sinus of the draining LN (reviewed by Randolph et al. 2005).

Traditionally, lymphatic vessels were identified by electron microscopy, using the absence of luminal red blood cells and abluminal pericytes as a means of distinguishing them from blood vessels (Sleeman et al. 2001). The identification of specific lymphatic markers, such as podoplanin (Breiteneder-Geleff et al. 1999; Weninger et al. 1999), prospero-related homeobox 1 (Prox-1) (Wigle and Oliver 1999) and lymphatic endothelium-specific hyaluronic acid receptor 1 (LYVE-1) (Banerji et al. 1999), has significantly improved our understanding of lymphatic vessel architecture and function (Alitalo et al. 2005; Baluk et al. 2007; Randolph et al. 2005).

DC emigration through lymphatic vessels

When considering skin DC mobilization, it is important to separate the requirements for LC emigration from the epidermis from those for DDC emigration. LC emigration to draining LNs involves disassociation from neighboring keratinocytes via the down-regulation of E-cadherin, passage through the underlying basement membrane (Bergstrasser et al. 1980; Larsen et al. 1990), and entry into the dermally located lymphatics (Lukas et al. 1996). In contrast, DDCs are already juxtaposed to dermal lymphatic vessels. Thus, for example, while LC transmigration through the basement membrane is reliant upon $\alpha 6$ integrin binding to laminin (Price et al. 1997), this molecule is unlikely to be required for DDC migration.

Leukocyte entry into lymphatic vessels occurs preferentially at or near the blind-ended tips of the initial lymphatics (Baluk et al. 2007; Randolph et al. 2005). This is an active

and selective process that not only requires the expression of appropriate chemokine receptors, but probably also expression of a number of cell surface and signaling molecules that are only just beginning to be identified.

Chemokine receptors

Dendritic cell migration into lymphatic vessels is dependent upon CCR7. A role for CCR7 in DC migration was first suggested by Lanzavecchia and colleagues following the observation that human monocyte derived-DCs, when stimulated with LPS or TNF α , upregulate this molecule, along with CXCR4 and CCR4 (Sallusto et al. 1998). Following the generation of CCR7-deficient mice, it soon became apparent that CCR7 was essential for DC mobilization to LNs from peripheral tissues (Forster et al. 1999; Ohl et al. 2004). CCR7 recognizes the ligands CCL19 and CCL21, which together coordinate the trafficking of both DCs and T cells to, and within, secondary lymphoid organs under both steady-state and inflammatory conditions (reviewed by Forster et al. 2008). In mice, there exist two copies of CCL21: CCL21-Leu (which contains a leucine residue at position 65) and CCL21-Ser (which contains a serine residue in place of the leucine residue) (Chen et al. 2002). Lymphatic endothelial cells express CCL21-Leu, while CCL21-Ser is expressed by fibroblastic reticular cells within lymphoid organs (including thymus, LNs and spleen) and by high endothelial venules (HEVs) in LNs. CCL19 is primarily expressed by LN fibroblastic reticular cells (Chen et al. 2002; Vassileva et al. 1999; Weninger and von Andrian 2003). In mice that have a naturally occurring deletion of CCL21-Ser and CCL19 (paucity of lymph node T cells, plt/plt), DCs are capable of entering lymphatics in the skin, but accumulate in the superficial cortex of draining LNs (Mori et al. 2001).

It is worth noting that expression of CCR7 alone is not sufficient to ensure responsiveness to CCL19 and CCL21. Rather, CCR7 function is dependent upon intracellular entry of calcium (Ca²⁺), and therefore relies upon additional molecules controlling Ca²⁺ levels. Prostaglandins, particularly PGE₂, have been shown to influence CCR7 responsiveness, since the absence of prostaglandins during anti-CD40-induced activation renders DCs unresponsive to CCR7 ligands (Scandella et al. 2002). PGE₂ appears to exert this effect by increasing intracellular levels of cAMP, thereby increasing intracellular calcium flux (Scandella et al. 2002; Scandella et al. 2004). Similarly, the ADP-ribosyl cyclase CD38 and the Ca²⁺-activated nonselective channel TRPM4 (transient receptor potential melastatin 4) have been shown to promote DC migration through modulation of intracellular Ca²⁺ flux (Barbet et al. 2008; Partida-Sanchez et al. 2004). CCR7 function is also dependent on MRP1 (multidrug resistance-associated protein 1), a

membrane transporter that transports cysteinyl-leukotrienes, which in turn promotes chemotaxis to CCL19 (Robbiani et al. 2000). In addition, while CCR7 appears to be required for DC migration to LNs, additional chemokine receptors may further facilitate this process, particularly in inflammatory conditions. Indeed such a role has been described for CXCR4/CXCL12 (Kabashima et al. 2007).

Integrins

Relatively little is known about the requirement for adhesion molecules in lymphatic entry of DCs. Reduced migration of LCs into draining LNs in ICAM-1-deficient mice suggested a role for integrin binding in the promotion of DC emigration (Xu et al. 2001). It was concluded that the lack of ICAM-1 expression by lymphatic endothelium was responsible for this diminished trafficking, which pointed to a role for DC expression of the α L β 2/ α M β 2 integrins (the counter receptors for ICAM-1) (Xu et al. 2001). In further support of this notion, Johnson et al. (2006) subsequently observed a reduced percentage of fluorescent DCs in draining LNs of oxazolone-treated mice following pre-treatment with anti-ICAM-1. Anti-VCAM-1 was also effective, suggesting an additional role for DC expression of α 4 β 1 integrin (the counter receptor for VCAM-1) in lymphatic entry, at least during inflammation (Johnson et al. 2006). However, a recent report demonstrated that integrin expression by BM-derived DCs was not required for successful migration into the draining LN parenchyma following injection into mouse footpads (Lammermann et al. 2008). Indeed, the authors presented compelling evidence that leukocyte migration within 3D matrices in vitro as well as in the dermis in vivo occurred in the absence of integrin interactions with the extracellular environment, arguing against their role as force transducers (Lammermann et al. 2008). Nevertheless, it is conceivable that other adhesion molecules, such as the hyaluronan receptor CD44, are involved in DC migration within and from the skin (Weiss et al. 1997).

Metalloproteinases

It has been shown that LC emigration from skin explants requires the matrix metalloproteinases MMP-2 and MMP-9 (Ratzinger et al. 2002), which are likely to be involved in promoting migration through the basement membrane by cleavage of ECM proteins, particularly collagen IV (Kobayashi, 1997). Consistent with this role, MMP-9 mediates DC migration through tight junctions in vitro (Ichiyasu et al. 2004). A further role was proposed for MMP-2 and MMP-9 in the migration of both LCs and DDCs within the dermis by “making a path” through the extracellular matrix (Ratzinger et al. 2002). This conclusion is somewhat in disagreement with other reports, which suggest that leukocytes

are capable of migrating through most environments without the need to degrade the surrounding ECM (Wolf et al. 2003).

Other molecules

Recent reports have pointed to additional molecules involved in leukocyte migration through lymphatics. *Jam-A*^{-/-} DCs showed an increase in random motility and in the capacity to transmigrate across lymphatic endothelial cells (Cera et al. 2004), possibly through reduced interactions with α L β 2 integrins (Ostermann et al. 2002). Van et al. (2006) recently reported a requirement for DC expression of CD47 for successful homing, although the mechanism remains unknown. In addition, lymphatic endothelial cell expression of macrophage mannose receptor (Marttila-Ichihara et al. 2008) and CLEVER-1 (Salmi et al. 2004) has been identified to mediate lymphocyte binding, although a role in DC migration has yet to be determined. Sphingosine-1-phosphate is required for T cell entry into lymphatics (Ledgerwood et al. 2008), but its function in DC emigration is yet to be evaluated.

Dendritic Cell Maturation

Another aspect of DC emigration through lymphatic vessels is their maturation state, originally coined to describe the acquisition of antigen presenting capacity by DCs following incubation in culture (Larsen et al. 1990). The term DC maturation has come to encompass a variety of biological processes that are triggered by Toll-like receptor ligands and other microbial products, as well as inflammatory cytokines (reviewed by Trombetta and Mellman 2005). Thus, the hallmarks of maturation are: a reduction in endocytic capacity, upregulation of Major Histocompatibility Complex II (MHC-II) and co-stimulatory molecules, release of immunostimulatory molecules and changes in chemokine receptor expression, including the upregulation of CCR7.

Studies of DC maturation have given rise to two major paradigms. Firstly, it is assumed that all DCs in peripheral tissues, including the skin, are “immature” in the steady-state (Romani et al. 1989), a notion that has been extended to include splenic DCs and their counterparts in LNs (Wilson et al. 2003). By extension, this notion groups LCs and DDCs together functionally, despite clear differences in turnover time (Garg et al. 2003; Kamath et al. 2002), migration kinetics (Kissenpfennig et al. 2005; Shklovskaya et al. 2008) and co-stimulatory molecule expression (Shklovskaya et al. 2008). Secondly, it is thought that “immature” DCs are tolerogenic, while “mature” DCs are immunogenic (Steinman et al. 2003).

With regards to cutaneous DCs, these paradigms present somewhat of a paradox, since migration and maturation were traditionally linked together (de Vries et al. 2003; Weinlich et al. 1998). However, part of the prevailing paradigm of “immature” DCs serving a tolerogenic purpose necessitates that maturation be uncoupled from migration (Banchereau and Steinman 1998; Jiang et al. 2007), so that “immature” DCs in the skin can still reach draining LNs. This raises the question of what drives LC and DDC emigration from the skin during the steady-state. Ample experimental evidence shows that both LCs and DDCs migrate continuously from the skin to the draining LNs under non-inflammatory conditions, as shown by BrdU uptake (Kamath et al. 2002), BM chimeric experiments (Merad et al. 2002), and studies utilizing parabiotic mice (Liu et al. 2007). While the precise mechanisms underlying this phenomenon remain to be determined, a recent study by Mellman and colleagues has described a distinct pathway of DC maturation that can occur in the absence of inflammatory signals (Jiang et al. 2007). Rather, BM-derived DC maturation was induced through the disruption of E-cadherin-mediated contacts (Jiang et al. 2007). It remains to be seen whether such a mechanism plays a significant role in DC migration *in vivo*.

DC vaccination

Based on advances in the generation of DCs for therapeutic purposes, a number of clinical trials over the past decade have employed DC vaccination as a treatment for a variety of cancers, particularly melanoma (Lesterhuis et al. 2008; Ridgway 2003). Typically, this involves the *in vitro* generation of DCs from blood monocytes or CD34⁺ precursor cells using cytokines such as GM-CSF and IL-4, followed by activation using various inflammatory mediators and antigenic loading prior to intradermal (i.d.) or subcutaneous (s.c.) administration. Although a number of variations in DC culture techniques, activation methods and antigen choices have been tested, clinical responses following such “DC vaccination” approaches have been rather disappointing (Lesterhuis et al. 2008).

One explanation for the inefficiency of DC vaccines is that, both in man and mice, less than 5% of inoculated DCs actually reach the draining LN (Adema et al. 2005; de Vries et al. 2003; Eggert et al. 1999). Furthermore, there is an apparent difference in the migratory efficiency of distinct DC subsets. We have found that ~1% of CD8⁻ splenic DCs reach the LN following s.c. injection into the footpad of mice, whereas CD8⁺ DCs do not appear to migrate at all (Smith and Fazekas de St Groth 1999) (Fig. 1a), implying that most of the injected DCs remain within the skin (Fig. 1b).

Central to all DC inoculation protocols is the underlying expectation that the procedure, at least partially, recapitulates the natural process of DC emigration. However, the obvious discrepancy between the excellent migratory ability of DDCs and the exceedingly poor migratory ability of i.d. or s.c. administered DCs (whether they be *in vitro* or *ex vivo* derived) illustrates that we still do not fully understand the requirements for successful homing of these cells to the draining LN. Currently, it is believed that this migratory inefficiency is due to inadequate or poorly timed activation/maturation (Andrews et al. 2008; de Vries et al. 2003; Lesterhuis et al. 2008). However, as discussed above, there are clearly other factors that influence DC emigration (Adema et al. 2005). The failure of CD8⁺ splenic DCs to home to draining LNs, for example, occurs even when these cells are activated with lipopolysaccharide (LPS) (Mempel et al. 2004) and express CCR7 (Fig. 1c). Thus, there is a need for further dissection of the behavior of DCs at the inoculation site, with an emphasis on the roles of some of the aforementioned molecules in mediating their emigration.

Given that endogenous DDCs are in fact constitutively motile cells (Ng et al. 2008a) and can mobilize and emigrate to draining LNs within a few hours of stimulation, it is unlikely that DCs derived from other sources behave in a similar manner when injected into the dermis. Thus, we should focus our efforts on determining the molecular cues directing the locomotion of LCs and DDCs in the skin to decipher the sequence of steps involved in entry into lymphatics. Our data point towards the involvement of chemokine receptors in DDC navigation in the steady-state, as pertussis toxin treatment of mice resulted in a significant decrease in the displacement of these cells (Ng et al. 2008a). Further studies are required to unravel the nature of chemoattractants involved in this process.

LCs and DDCs in the skin *in vivo*: lessons from confocal microscopy

Most studies of the migratory behavior of DDCs have focused on their migration from skin explants *in vitro*. There is very little data on the behavior of DDCs within their natural microenvironment *in vivo*. This is somewhat in contrast to LCs, which have been more extensively studied, due to the relative ease by which they can be isolated and imaged (Jakob et al. 2001; Kissenpfennig et al. 2005; Nishibu et al. 2006; Ruedl et al. 2001; Stoitzner et al. 2002).

One of the problems in studying DDC behavior has been the difficulty in identifying them histologically. Although DCs in the mouse are generally defined by expression of CD11c, in practice the surface expression levels of this marker in the skin are low, which makes identification of

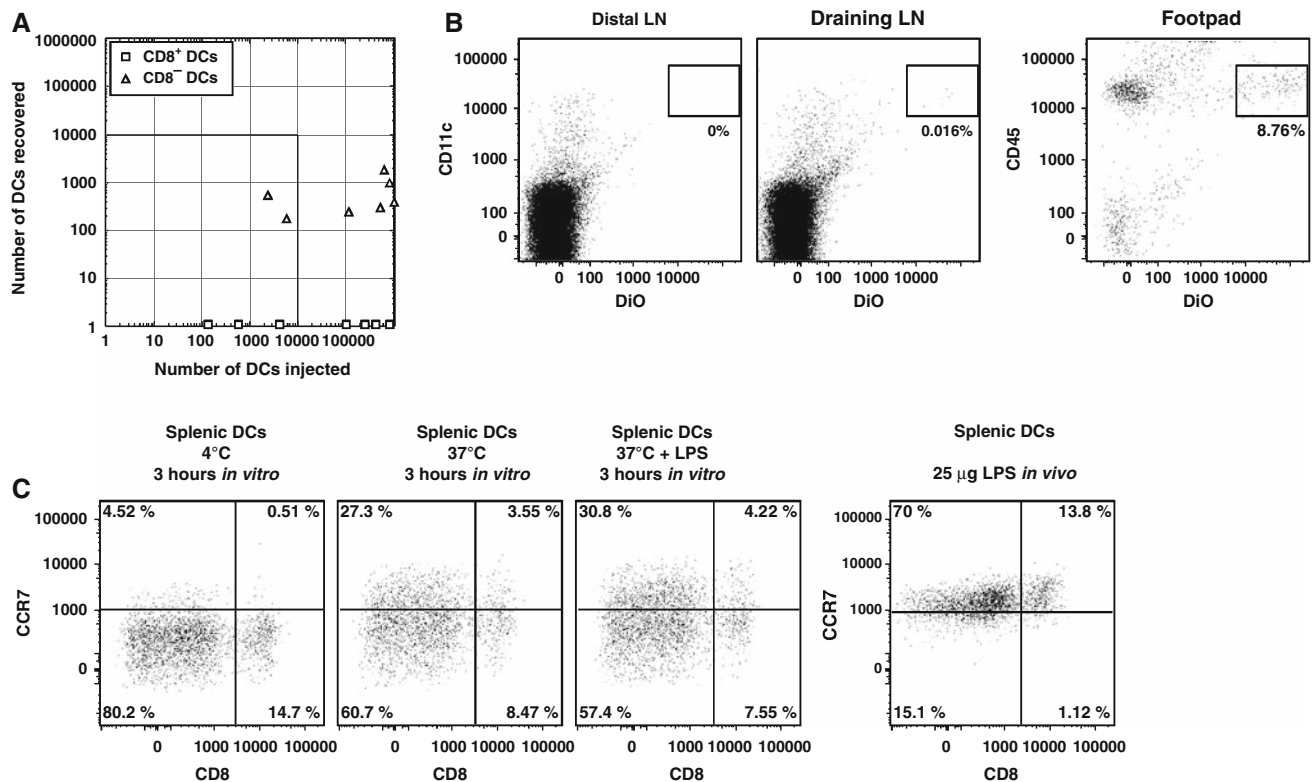


Fig. 1 Poor migratory capacity of s.c. injected splenic DCs. **a** Shown is the relationship between the number of CD8⁺ or CD8⁻ splenic DCs injected s.c. versus the number of DCs recovered in the draining LN 20–24 h later. Squares represent CD8⁺ DCs, and triangles represent CD8⁻ DCs. **b Left panels:** representative flow cytometry dot plots of distal and draining LNs from a mouse that received 2×10^6 DiO-labelled, “mixed” splenic DCs 24 h after s.c. injection into the left hind footpad. **Right panel:** flow cytometry of the footpad of the same mouse: Viable (DAPI⁻) CD45⁺ DiO^{hi} cells—most likely representing the injected DCs—were readily detected, indicating that these DCs had not died or “disappeared” from the site of injection. **c** Analysis of CCR7

expression by splenic DCs in response to LPS *in vitro* and *in vivo*. **Left panels:** freshly isolated splenocytes were incubated for 3 h at 4 or 37°C, in the presence or absence of LPS (10 ng/ml) before staining and examination by flow cytometry. Both CD8⁻ and CD8⁺ subsets of splenic DCs (gated on DAPI exclusion, forward scatter, CD11c expression, B220 exclusion and differential CD8 expression) expressed minimal levels of CCR7 when maintained at 4°C, but were capable of upregulating CCR7 when incubated at 37°C. **Right panel:** mice were injected i.v. with 25 µg of LPS and their spleens were harvested 4 h later for analysis by flow cytometry. Both CD8⁻ and CD8⁺ DCs upregulated CCR7 in response to LPS

CD11c⁺ cells problematic. In contrast to CD11c, DDC expression of MHC-II is much higher and more readily identifiable by sectional and whole-mount immunostaining. However, MHC-II is also expressed by macrophages, a more abundant cell population in the skin (Dupasquier et al. 2004). Thus, distinguishing non-migratory macrophages from DDCs becomes a tedious task histologically, often requiring the use of multiple markers (Dupasquier et al. 2004). Furthermore, identification of DDCs by flow cytometry is equally troublesome, since it is difficult to release these cells into solution without the use of enzymes such as dispase or trypsin, which frequently cleave CD11c.

A notable advance in the field has been the generation of transgenic (Tg) mice in which enhanced yellow fluorescent protein (EYFP) expression is driven by a CD11c promoter (hereafter referred to as CD11c-EYFP Tg mice) (Lindquist et al. 2004). DCs in these mice exhibit high fluorescence, and are readily identifiable by both fluorescence microscopy and flow cytometry. These mice have not only greatly

improved our understanding of the behavior of endogenous DCs in the LN (Lindquist et al. 2004; Shakhari et al. 2005), but also their roles in various models of infection (Aoshi et al. 2008; Hapfelmeier et al. 2008; Veres et al. 2007). They also enabled the visualization of intestinal DCs (Flores-Langarica et al. 2005) and the identification of a previously uncharacterized population of DCs in the brain (Bullock et al. 2008). In the skin of these mice, both LCs and DDCs express the transgene (although expression is generally higher in DDCs compared to LCs), and can therefore be identified with minimal sample preparation (Ng et al. 2008).

Confocal microscopy of whole mount preparations of ears from CD11c-EYFP Tg mice has enabled us to characterize the distribution of DCs within the skin. LCs within the epidermis are distributed relatively evenly, at a concentration typically counted as 1,000–1,200 LCs/mm² (Ratzinger et al. 2002). In contrast, DDCs assume a non-random distribution within the dermis, where they are found as

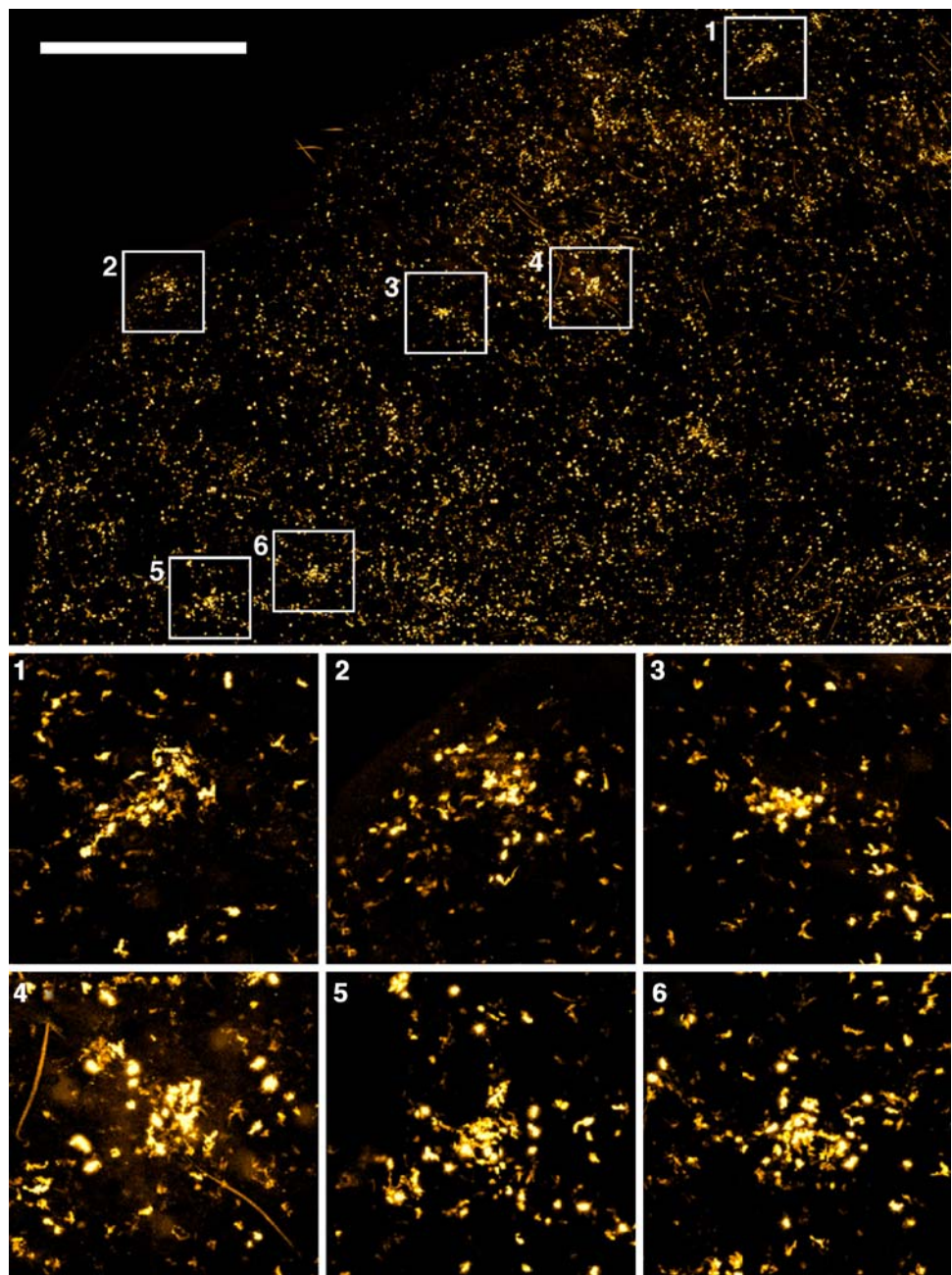
single cells or within clusters of 20–40 cells (Fig. 2). As a result, the relative density of DDCs varies considerably, depending on the area examined, from 100 DDCs/mm² in some of the sparser regions, to 2,500 DDCs/mm² within clusters. This makes estimation of overall DDC density difficult, although it seems clear that there are at least twice as many LCs as DDCs/mm² of skin.

The origin and function of the DDC clusters is currently under investigation, although they do not appear to be associated with lymphatic vessels (Fig. 3a). We draw this conclusion because, although the lymphatic vessels within the skin are usually within 150 μ m of each other, and are thus

never far from any anatomical structure, these clusters do not appear to aggregate around the blind-ended tips that serve as the preferential site of leukocyte entry (Fig. 3b). It is possible that they represent sites of preferred entry and/or proliferation of DC precursors, but this remains to be determined. It is also possible that some of these cells are not DCs, but rather DC-precursors, since it has been shown that pre-DCs in the spleen express CD11c prior to differentiation into bona fide MHC-II⁺ DCs (Diao et al. 2006; Naik et al. 2006).

These results show that DCs are a prominent population of leukocytes in the skin. It is noteworthy that while DDCs

Fig. 2 Confocal imaging of DDCs within the skin of a CD11c-EYFP Tg mouse. Mouse ears were mechanically separated into dorsal and ventral halves, and the epidermis was enzymatically removed using dispase. The entire dermal tissue was then optically sectioned (from the epidermal side) and a maximum-intensity, extended focus image generated. EYFP⁺ DDCs were scattered throughout the dermis as single cells or within tight clusters. Boxes 1–6 DDC clusters. Scale bar 1 mm



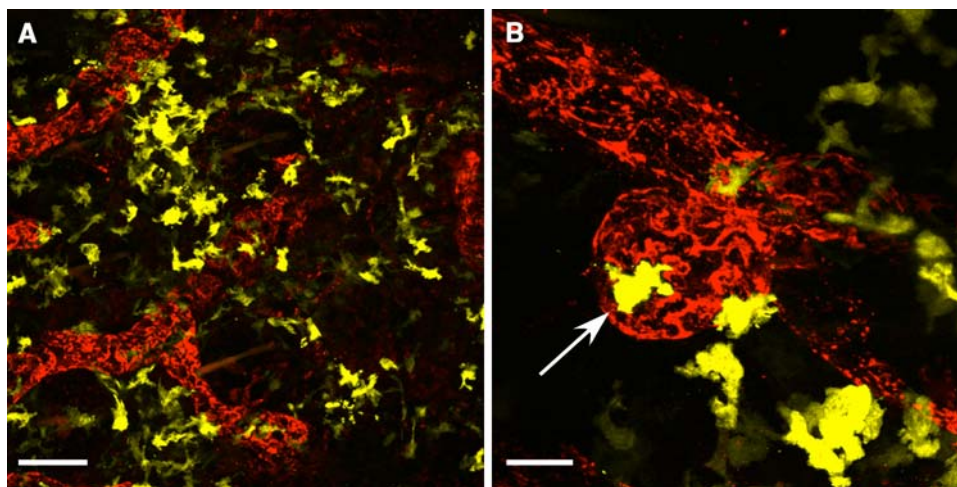


Fig. 3 Confocal imaging of lymphatic vessels within the skin of a CD11c-EYFP Tg mouse. Mouse ears were mechanically separated into dorsal and ventral halves, and the cartilage-free half was incubated overnight in rat anti-LYVE-1 followed by washing and a further 1 h incubation in anti-rat-Alexa594. Tissue was imaged from the dorsal

side. **a** EYFP⁺ DDC clusters (yellow) did not appear to be associated with lymphatic vessels (red). Scale bar 80 μ m. **b** Few DDCs were observed within the lymphatics, although they were occasionally found associated with the lymphatic endothelial cells at the blind-ended regions of the initial lymphatics (arrow). Scale bar 24 μ m

are half as frequent (or less) as LCs, they are three times as abundant within the skin-draining LNs (Shklovskaya et al. 2008). Thus, the degree of cellular flux through the dermis in the steady-state greatly exceeds that of the epidermis. This is consistent with studies of DC lifespan, which report a complete replacement of DDCs by BM-derived precursors within 2 weeks (Iijima et al. 2007; Kamath et al. 2002; Liu et al. 2007), while only 20–60% of LCs turn over in this same period (Henri et al. 2001).

Technical difficulties associated with conventional microscopy techniques

Despite the relative ease of DDC identification in CD11c-EYFP Tg mice, there are still a number of components of the skin that make imaging these cells difficult by conventional fluorescence or confocal microscopy. The most obvious hurdle is the depth. Unlike LCs, that sit prominently in the epidermal layer, DDCs lie further below the surface of the skin (from 20 to 200 μ m). And since the skin is an optically dense tissue, the reduced penetrance of laser light in the visible range due to absorption/scattering hinders the clear identification of these cells. Although it is still possible to image some way into the dermis using confocal microscopy, this requires a high laser power, which increases the probability of tissue damage. Furthermore, the image quality rapidly diminishes at greater penetration depths (Fig. 4).

Secondly, while the most efficient immunostaining is achieved on frozen sections, this provides only a 2D view, and makes it difficult to place the cells into anatomical context. Whole mount staining provides a significant advancement over this technique, since it enables much better

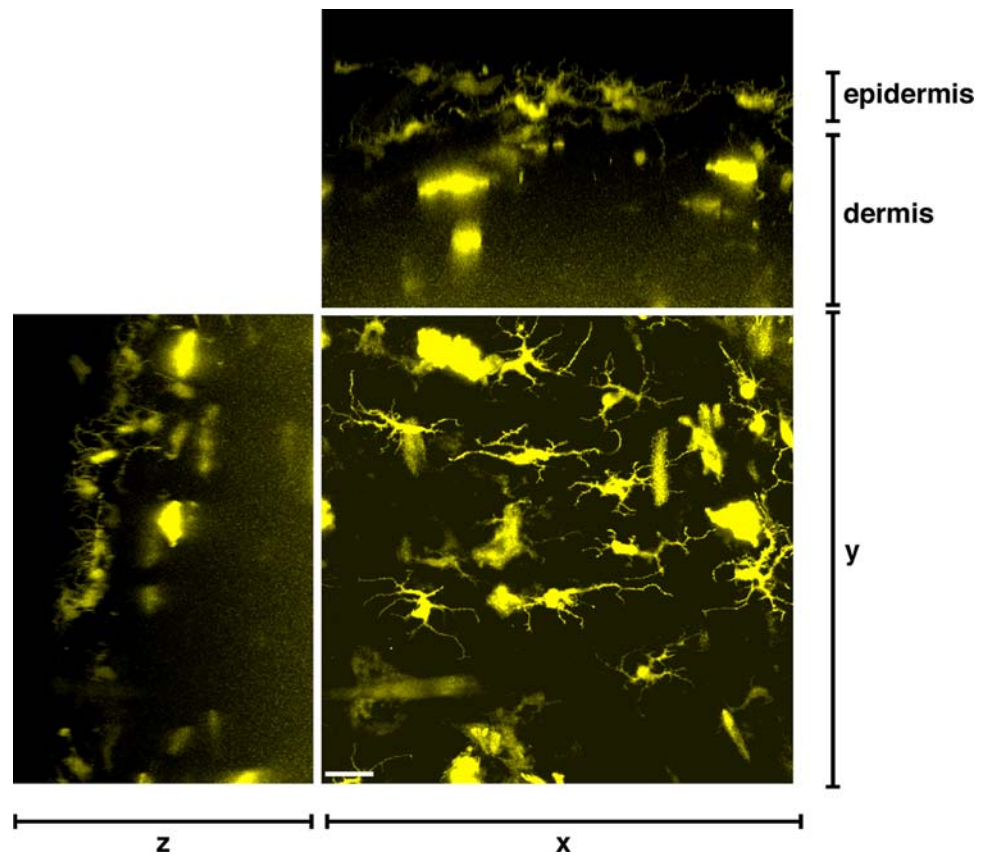
orientation of skin cells within their 3D environment. However, whole mount immunostaining is not without its limitations. For example, the epidermis is relatively impervious to antibody, thereby restricting antibody penetration. Similarly, other components underlying the dermis (cartilage, muscle, fat) can also restrict antibody penetration. One solution to these problems is to enzymatically remove the epidermis prior to staining and/or imaging (see Fig. 2). However, as noted previously, this may result in the loss of certain cell surface antigens, such as CD11c. Furthermore, one runs the risk of disrupting the microarchitecture of the remaining tissue, introducing staining artifacts related to higher “non-specific” binding of antibody as well as increased autofluorescence.

Ultimately, despite the impressive resolution provided by confocal microscopy, in this setting it is unsuitable for intravital imaging of DDCs. This is of considerable importance when examining DDC migration both within the skin and through lymphatics, since not only is DC migration a dynamic process, but the draining function of the lymphatic vessels is reliant upon intact circulation.

Multi-photon excitation microscopy

The development of multi-photon excitation (MPE) microscopy has provided unprecedented insight in many research fields, including immunology, by enabling dynamic visualization of cells within their natural microenvironment deep underneath the surface of organs. In the past 5 years, a plethora of reports from various groups have described the cellular dynamics of immune cells during primary and effector phases of immune responses (e.g. Bousso

Fig. 4 Confocal imaging of intact ear of a CD11c-EYFP Tg mouse. An intact mouse ear was imaged from the epidermal side. LCs within the epidermis displayed their characteristic stellate shape, while the DDCs exhibited a more amoeboid morphology. EYFP⁺ DDCs could be observed as far as 100 μ m below the epidermis, although the resolution at this depth was greatly reduced. Scale bar 25 μ m



and Robey 2003; Lindquist et al. 2004; Mempel et al. 2004; Mrass et al. 2006; Ng et al. 2008b). Furthermore, several recent reports of direct visualization of host cell-pathogen interactions add to these findings (Aoshi et al. 2008; Chtanova et al. 2008; Ng et al. 2008a; Peters et al. 2008), and illustrate the potential of MPE microscopy in unraveling new (and old) questions in biomedical research. Despite these advances, relatively little is known about the cellular and interactive behavior of immune cells within the peripheral, non-lymphoid organs such as the skin, although this will undoubtedly change over the next few years.

For MPE, a femtosecond-pulsed, near-infrared laser beam is generated by a tunable laser [usually Titanium (Ti)-Sapphire, tuning range from approximately 700 to 1,020 nm] and utilized to excite fluorophores. The Ti:Sapphire laser can also be coupled with a synchronously pumped optical parametric oscillator (OPO), which can be tuned to even longer wavelengths (approximately 1,080–1,500 nm) for excitation of red and far-red fluorophores (Niesner et al. 2008). MPE microscopy features the following advantages over conventional microscopy: (1) the (near) infrared beam penetrates tissue better than visible wavelengths used in conventional microscopy, thereby enabling optical sectioning of living tissues up to several hundreds of microns deep; (2) phototoxicity and photobleaching are reduced, permitting longer-term imaging of

living tissues; (3) MPE enables the simultaneous excitation and detection of several fluorophores with a single wavelength due to the broad MPE spectra; (4) emission signals from the sample can be detected in both forward (transmitted) and backward (reflected) directions; and (5) components of the ECM, i.e. α -helical proteins (e.g. collagen and elastin), can be visualized without the need for fluorescent labeling due to second and third harmonic generation (SHG and THG) signals (Friedl et al. 2007).

A MPE model for intravital imaging of mouse ear skin

In the past, the penetration limits of conventional microscopy have confined real-time imaging of immune responses in the skin to the study of leukocyte interactions with microvessels (Auffray et al. 2007; Weninger et al. 2000), and the behavior of cells within the epidermis (Kissenpfennig et al. 2005; Nishibu et al. 2006). We have recently established a model that permits intravital imaging of the skin over relatively long periods of time (>4 h) by MPE microscopy (Ng et al. 2008a). We have selected the dorsal surface of the mouse ear as the imaging site, due to its accessibility and the ease of avoiding respiratory movements. Using this approach, we have been able to visualize the migratory behavior of immune cells at the single cell

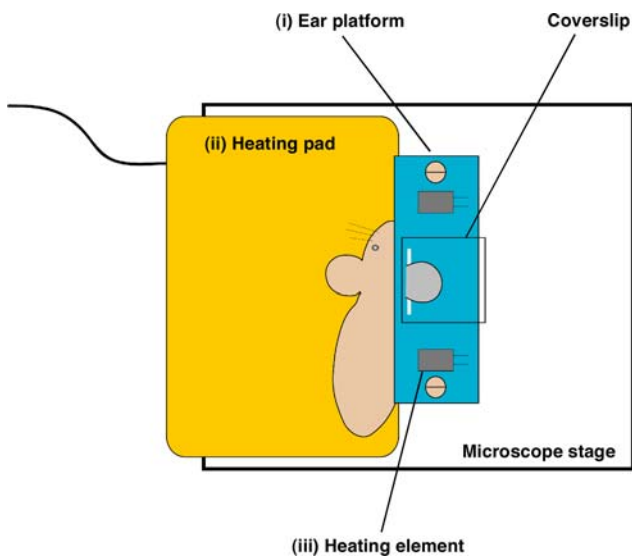
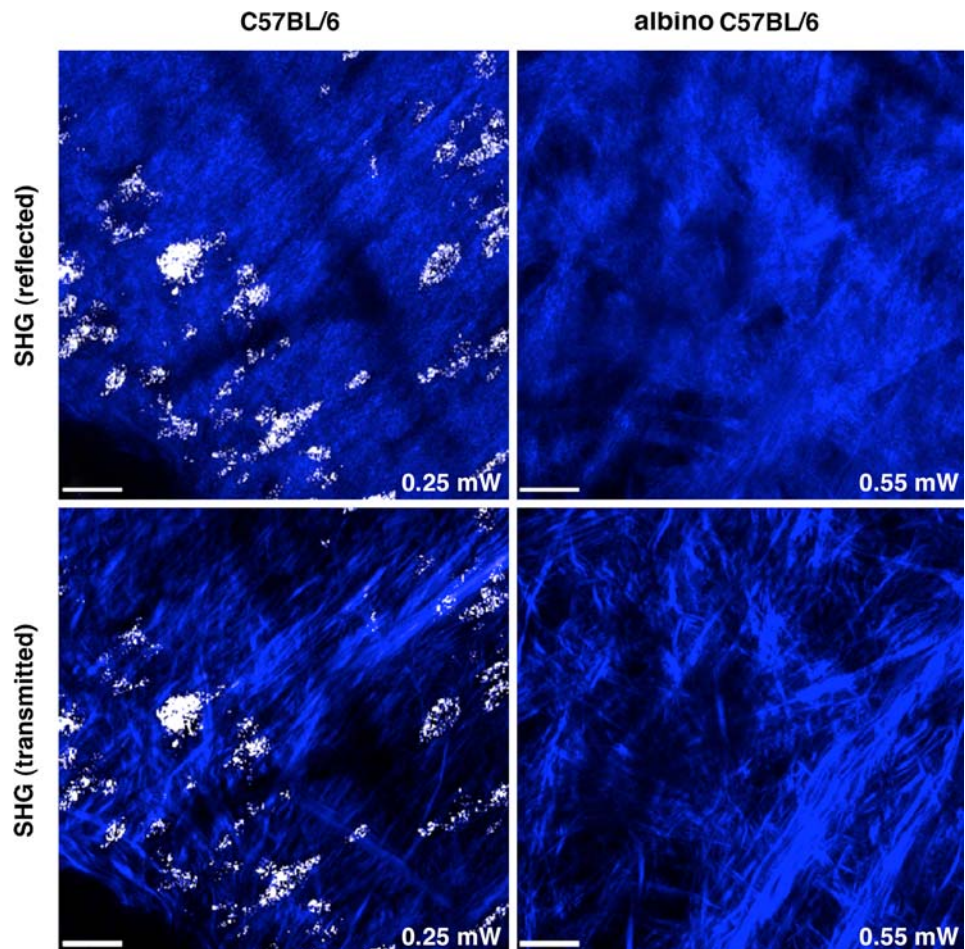


Fig. 5 Schematic representation of a microscope stage for intravital MPE imaging of mouse ear skin. This stage consists of: *i* a metal platform that can be fitted onto the microscope; *ii* a heating pad for maintaining body temperature of the test animal at 37°C; and *iii* heating elements that maintain the platform temperature at 36°C

Fig. 6 “Speckling” in the skin of melanin-producing mice during multiphoton microscopy. SHG signal (*blue*) in the ear of a wild-type C57BL/6 mouse and an albino mouse carrying a mutation in tyrosinase (C57BL/6-C^{2J}) following MPE excitation at 840 nm. Laser power is indicated in each figure. At high laser power, high intensity signals are detected in all channels (*white*) in wild type, but not mutant mice (even when laser power was doubled). Photomultiplier tubes captured SHG signals either in the forward or reflected direction. Note that the resolution of individual ECM fibers is superior in the forward direction. Scale bars 20 μm



level, as well as their interactive behavior with their micro-environment (e.g. ECM).

In the following section, we provide details on the experimental setup for intravital imaging of mouse ear skin and describe features that may be generalized to visualizing other cell types/structures in the skin. In addition, we discuss some of the limitations of applying MPE for the dynamic study of skin cells.

Animal preparation

After hair removal, the anaesthetized mouse is placed onto a custom-built microscope stage (Fig. 5), the ear is immersed in PBS/glycerin (70:30 vol:vol) and covered with a coverslip. During these procedures, extreme care is taken to avoid mechanical trauma that may result in the obstruction of blood flow or inflammation. We have found that even short periods of hypoxia as a result of an interrupted blood flow have significant impact on the migration of cells in the dermis, which is consistent with previous reports showing migratory arrest of naïve T cells in LN after the death of an experimental animal (Mempel et al. 2004).

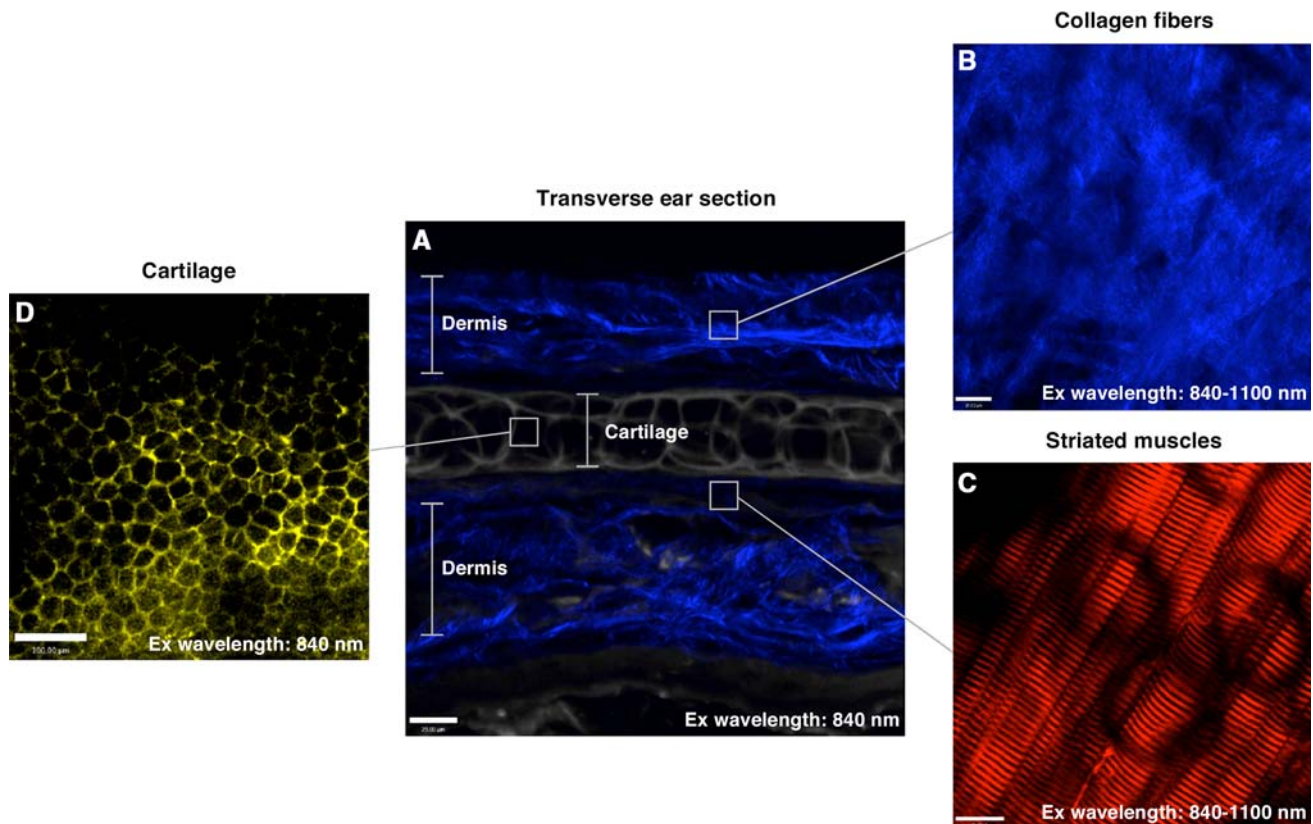


Fig. 7 Multi-photon excitation for the identification of anatomical structures within the skin. **a** Two colour representation of collagen fibers (SHG) and cartilage (autofluorescence) in a transverse frozen section of mouse ear. *Scale bar* 25 μm . **b–d** Extended focus view of images from MPE imaging of intact ear in vivo. **b** Dermal collagen

fibres (SHG). *Scale bar* 15 μm . **c** Striated muscles (SHG). *Scale bar* 20 μm . **d** Cartilage (autofluorescence). *Scale bar* 100 μm . Excitation wavelengths used to detect each individual structure is indicated within the figure

Since interstitial leukocyte migration within tissue is dependent on temperature (Miller et al. 2002), the temperature of the ear platform is regulated independently and maintained at 36°C, while the body temperature is kept at 37°C through a heating pad underneath the mouse. Body temperature is monitored through a rectal probe.

Challenges associated with MPE imaging of mouse skin

A clear advantage of the ear skin model is accessibility and the avoidance of potentially artifact-ridden surgical procedures. However, even a relatively simple model is not without pitfalls that require special considerations.

Autofluorescence from hairs

Optimal imaging conditions are only achieved after hair removal, given that hair is highly autofluorescent and can obscure effective visualization of the cells/structures of interest within the skin. Hair is removed using commercially available depilation creams (e.g. Nair™ Church & Dwight). We have found no difference in the migration of DDCs within ears with or without hair removal, indicating

that, when appropriately applied, this procedure does not induce significant disturbance of the microenvironment (Ng et al. 2008a).

Pigmentation of mouse ear skin

A commonly observed phenomenon in the ear skin of pigmented mice, such as C57BL/6, is the occurrence of high-intensity signals (“speckles”) from dermal cells that appear in all collection channels and can reduce image quality (Fig. 6). These speckles appear to arise from pigmented cells, such as melanophages, since they are not present in albino mice, including BALB/c and C57BL/6-C^{2J} (albino C57BL/6, Townsend et al. 1981), even at high laser power (Fig. 6). Therefore, imaging in C57BL/6 mice must be performed at lower laser power, which concomitantly results in reduced tissue penetration and decreased resolution in deeper regions of the skin.

Intracutaneous injection

Undoubtedly, a strength of dynamic intravital imaging is in studying the consequence of applying reagents such as

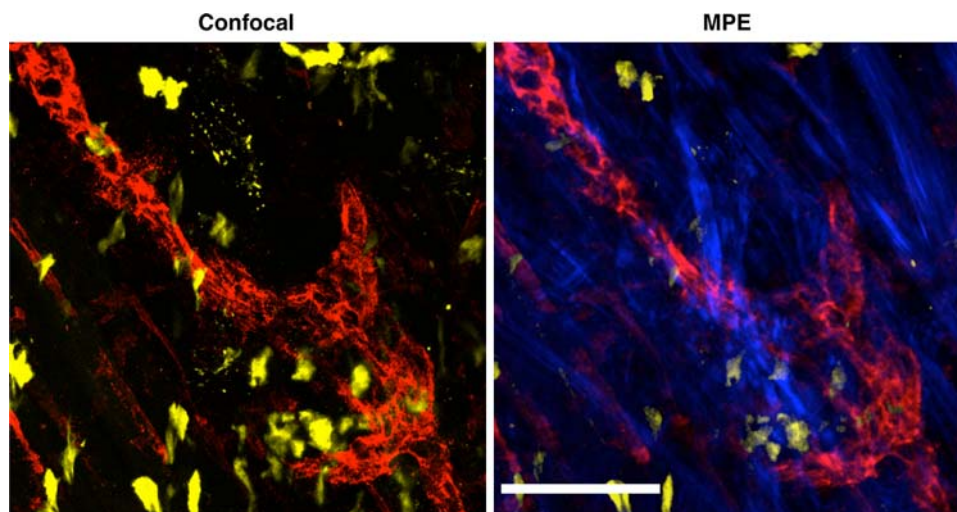


Fig. 8 Comparison of confocal and MPE imaging in a tissue explant of CD11c-EYFP Tg mice. Mouse ears were mechanically separated in dorsal and ventral halves, and the cartilage-free half was incubated overnight in rat anti-LYVE-1 followed by washing and a further 1 h incubation in anti-rat Alexa594. *Left*: confocal imaging of EYFP⁺ DDCs (yellow) in association with LYVE-1⁺ lymphatic vessels (red). EYFP was excited using the 514 nm line of a multi-line Argon Laser. Alexa594 was excited using a 561 nm diode laser. *Right*: MPE imaging

of the same region. In addition to the confocal image, MPE differs for the visualization of ECM fibers (blue). Simultaneous excitation of EYFP and Alexa594 was achieved using a Ti-Sapphire laser tuned to 890 nm, coupled with a synchronously pumped OPO (tuned to 1120 nm). This ‘proof-of-principle’ experiment indicates that the OPO can be utilized to simultaneously image GFP⁺ or YFP⁺ cells with other cells/structures labeled with red and far-red fluorophores, such as mCherry or mKate

inflammatory mediators or pathogens on the cells of interest. However, the direct deposition of any substance into the skin carries the risk that the mechanical trauma resulting from injection itself induces artifacts. Indeed, we have found that larger injection volumes (particularly >5 μ l) cause a disruption in the local microenvironment of the ear skin. This can lead to migratory arrest of DDCs even when physiologic saline solution is injected into the skin. Consequently, it is critical to use small injection volumes, i.e. 1–2 μ l, administered by means of a Hamilton syringe with a 33G needle.

Orientation within tissues

Since intravital imaging of intact tissues is performed in a 3D space, and often only a specific subset of cells is fluorescently tagged, it is important to identify tissue “landmarks” that facilitate orientation. This can, for example, be achieved by the intravenous injection of fluorescent dyes, such as labeled dextran or Qdots. In addition, using the right filter sets, it is possible to visualize sources of non-linear signals by virtue of SHG and THG (Friedl et al. 2007). In the ear, connective tissue fibers, striated muscle and cartilage can all be imaged by this means (Fig. 7). Thus, because the epidermis is collagen-free, while the dermis is densely composed of collagen, elastic and reticular fibers, we are able to distinguish epidermal and dermal compartments simply based on SHG signals. It is noteworthy that SHG signals generated from collagen fibers have a distinct

scattering pattern, dependant upon their orientation. It has been shown that vertically orientated collagen fibers scatter mostly in forward direction, while horizontally orientated collagen fibers scatter bi-directionally (Zipfel et al. 2003). As shown in Fig. 6, this results in distinct reflected and transmitted SHG signals in the mouse ear.

Choice of fluorescent probes

Maximizing the potential for multi-parameter analysis in vivo requires us to utilize the entire gamut of available fluorescent proteins. In this regard, the OPO provides a valuable extra dimension to MPE microscopy. Since the laser wavelengths extend further into the infrared spectrum, the OPO promises to not only enable greater penetration of samples but also visualization of red and far-red fluorochromes. Thus, the Ti-Sapphire:OPO configuration can be used to excite lower wavelength targets and higher wavelength targets simultaneously. We have confirmed this capability using EYFP plus Alexa594 (Fig. 8) or mCherry (not shown), demonstrating the exciting possibility that the OPO can be used for simultaneous excitation of a large panel of fluorescent probes.

DDCs are constitutively migratory

Using our MPE ear skin model in CD11c-EYFP Tg mice, we have recently found that DDCs are, in contrast to immobile

LCs, constitutively migratory (Ng et al. 2008a). These cells appear to migrate at a velocity of approximately 3 $\mu\text{m}/\text{min}$ in the absence of inflammation. However, after exposure to an inflammatory signal such as LPS, or the presence of the parasite *L. major*, DDCs cease to migrate for several hours, which may facilitate the uptake of intruding microbes (Ng et al. 2008a). These results point to a fundamental difference in the biology of interstitial DCs as compared to their epithelial counterparts. Future studies will focus on the dynamics and mechanisms of DC entry into the lymphatic system, and on visualizing antigen transport from the skin to draining LNs.

Conclusion

Over the past few years, our knowledge of DC populations has dramatically increased. Nevertheless, we still have an incomplete understanding of the real-time behavior of LCs and DDCs during inflammatory and infectious diseases, as well as in skin neoplasms. In addition, we have only limited information as to the mechanisms regulating the exit of DCs from the skin. MPE microscopy is a promising new technology for unraveling many of these unanswered questions. Although there are a number of technical difficulties associated with skin imaging, they are not insurmountable. Provided the appropriate precautions are taken, meaningful, functionally relevant data can be obtained. Thus, we anticipate that the model described above will reveal further insights into not only DC biology, but also a range of immunological processes within the skin.

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