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Chapter 3

Monitoring Skin Dendritic Cells in Steady State and Inflammation by Immunofluorescence Microscopy and Flow Cytometry

Julia L. Ober-Blöbaum, Daniela Ortner, Bernhard Haid, Anna Brand, Christoph Tripp, Björn E. Clausen*, and Patrizia Stoitzner

Abstract

Skin dendritic cells (DC) are strategically positioned at the body's second largest epithelial border to the environment. Hence they are the first antigen presenting cells that encounter invading pathogens and environmental antigens, including contact sensitizers and carcinogens penetrating the skin. Moreover, DC have the unique ability to induce immunity or tolerance and thus take center stage in regulating innate and adaptive immune responses. Skin DC can be divided into several phenotypically and functionally distinct subtypes. The three main subsets are Langerin$^+$ epidermal Langerhans cells (LC) and Langerin$^+$ as well as Langerin^{neg} dermal DC. In the steady state skin DC form a dense network to survey the periphery for pathogens and harmful substances breaching the cutaneous barrier. During inflammation DC become rapidly activated and start their migration to skin-draining lymph nodes where they initiate antigen-specific T cell responses. The homeostasis and mobilization of DC in the skin can be visualized by immunofluorescent staining of epidermal and dermal sheet preparations or skin sections. Here, we describe in detail how inflammation can be induced in the skin with tape stripping or FITC painting and how the skin DC network can be monitored using immunofluorescence microscopy and flow cytometry.

Key words Dendritic cells, Epidermal and dermal sheets, FITC painting, Immunofluorescence microscopy, Langerhans cells, Skin inflammation, Skin cryosections, Tape stripping

1 Introduction

The skin can be divided into an outer epidermis and the underlying dermis that are separated by a basement membrane. Together they form a strong mechanical barrier that protects the host from physical stress and a wide variety of environmental threats, including chemicals and pathogens. In addition, the skin harbors a heterogeneous population of dendritic cells (DC), professional antigen presenting cells that orchestrate the immunological barrier and are central

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* Authors contributed equally.

regulators of innate and adaptive immune responses [1, 2]. In the steady state, DC continuously scrutinize the skin for invading pathogens and along the way sample self- and environmental antigens. Through an unknown mechanism a small fraction of the cells undergoes spontaneous maturation, facilitating their chemokine receptor CCR7-mediated migration to the T cell areas of skin-draining lymph nodes. During migration, the DC upregulate the expression of surface MHC/peptide complexes and cell adhesion molecules enabling efficient recognition and interaction with naïve antigen-specific T cells. Encounter of these *phenotypically mature* DC with T cells recognizing self- or harmless environmental antigens leads to T cell anergy, deletion or induction of regulatory T cells (Treg) (*tolerizing function*) [3, 4]. Pathogen invasion together with proinflammatory signals trigger the *functional maturation* of DC, which now also upregulate expression of costimulatory molecules and, importantly, proinflammatory cytokines. Together, these mediate the activation and proliferation of naïve antigen-specific T cells as well as their polarization towards appropriate T helper (Th) type-1, Th2 or Th17 effector cells (*sensitizing function*) [5–7].

To date, we can distinguish five phenotypically distinct subsets among the $CD11c^+MHC-II^+$ DC that reside in healthy mouse skin using multi-color flow cytometry [8, 9]. The only DC population present in the epidermis are Langerhans cells (LC), which are characterized by the expression of the C-type lectin Langerin (CD207), the β_2-integrin CD11b and the cell adhesion molecule EpCam. In addition to transmigrating LC, the dermis contains a small population of Langerin⁺ DC that can be unambiguously recognized by expression of the chemokine receptor XCR1, lack CD11b and EpCam, and can be further subdivided into a CD103⁺ and negative subset. The by far largest population of DC present in the skin are $Langerin^{neg}CD11b^+$ dermal DC and, finally, the dermis harbors a minor population of $Langerin^{neg}XCR1^{neg}$ DC that are uniquely identified by high expression levels of the chemokine receptor CX_3CR1. During their low-level migration to skin-draining lymph nodes in the steady state, emigrating LC are replaced from a local precursor, while dermal DC are replenished from blood-borne precursors [10, 11]. To compensate for the increased loss of cutaneous DC due to their enhanced mobilization during inflammation, large numbers of monocytes infiltrate the skin where they differentiate into $CD11c^{neg-low}CD11b^+CD64^+$ monocyte-derived DC as well as LC [9, 12]. However, these cells mainly activate skin-resident T cells and disappear after resolution of the inflammation.

In light of their phenotypic diversity, major research efforts have been aimed at dissecting the unique functions of the different skin DC subsets in balancing immunity and tolerance (reviewed in [1]). While there is overwhelming evidence for a functional specialization of the various skin DC populations (*division of labor*) [13], it is becoming increasingly clear that a given DC subset exerts a particular function that may differ depending on the context, i.e., the type of inflammation. For example, the different skin DC populations

exhibit functional redundancy during contact hypersensitivity [14, 15], while LC act as negative regulators of the anti-*Leishmania* immune response [16], are essential to induce antigen-specific Th17 responses after epicutaneous *Candida albicans* infection [17], and mediate cross-tolerance towards epicutaneously applied ovalbumin [18]. On the other hand, Langerin⁺ dermal DC promote *C. albicans*-specific Th1 and efficiently cross-present fungal antigens to activate cytotoxic T cell (CTL) responses [17]; they are also responsible for cross-priming CTL responses following deposition of the model antigen ovalbumin in the skin [18].

In all of these studies, advanced multi-color flow cytometry is essential to distinguish and analyze the small number of cells of individual DC subsets that can be purified from/are present in the skin [8, 9, 11]. However, during their life cycle, the morphology, tissue distribution, mobilization, and migration are important parameters of skin DC function, which can only be visualized by immunofluorescence microscopy. Here we describe in detail how to monitor the skin DC network in the steady state and during inflammation induced by tape stripping or FITC painting.

2   Materials

2.1   Tape Stripping of Murine Ear Skin

1. Sex- and weight-matched mice (*see* **Notes 1** and **2**).
2. Ketamine, xylazine for anesthesia.
3. Syringes.
4. 25–30 gauge needles.
5. 3 M™ Transpore™ Surgical Tape.

2.2   FITC Painting

1. Sex- and weight-matched mice (*see* **Notes 1** and **2**).
2. Ketamine, xylazine for anesthesia.
3. FITC (Fluorescein isothiocyanate isomer I).
4. Dimethyl sulfoxide (DMSO).
5. Dibutyl phthalate (DBP).
6. Acetone.
7. Syringes.
8. 25–30 gauge needles.

2.3   Epidermal and Dermal Sheet Preparation from Murine Ear Skin

2.3.1   Preparation of Epidermal and Dermal Skin Biopsies

1. Sex- and weight-matched mice (*see* **Notes 1** and **2**).
2. Carbon dioxide (CO_2) for euthanizing animals.
3. Scissors.
4. Two surgical splinter forceps.
5. 24-well plates.

6. 0.1 M phosphate buffer (8.9 g $Na_2HPO_4 \times 2\ H_2O$ plus 6.8 g KH_2PO_4 in 500 ml distilled water, pH 6.8).

7. 0.5 M ammonium thiocyanate solution (ATC) (1.9 g ATC in 50 ml 0.1 M phosphate buffer, pH 6.8).

8. Two surgical fine curved forceps.

9. Phosphate buffered saline (PBS).

10. Glass petri dishes.

11. Acetone.

12. PBS supplemented with 1% bovine serum albumin (1% BSA/PBS, dissolve 1 g BSA in 100 ml PBS).

2.3.2 Epidermal Sheet Preparation of Whole Ear on Tape

1. Sex- and weight-matched mice (*see* **Notes 1** and **2**).

2. Carbon Dioxide (CO_2) for euthanizing animals.

3. Scissors.

4. Two surgical fine curved forceps.

5. Depilation cream.

6. 15 ml Falcon tubes.

7. 12-well plates.

8. 20 mM EDTA solution (372 mg in 50 ml phosphate buffer, pH 7.2).

9. Phosphate buffered saline (PBS).

10. 5 cm petri dishes.

11. Tesa tape "crystal clear" (*see* **Note 3**).

12. Optional: Acetone.

13. Glycine solution (3 % BSA/200 mM glycine/PBS, dissolve 3 g BSA and 1.5 g glycine in 100 ml PBS).

14. Optional: 0.1% Sudan black in 70% ethanol.

2.4   Immuno-fluorescence Staining of Murine Ear Skin Sheets

2.4.1 Immuno-fluorescence Staining of Epidermal and Dermal Sheets

1. Freshly prepared or thawed epidermal and dermal sheets.

2. One fine curved forceps.

3. 96-well plate.

4. Primary and secondary antibodies for immunofluorescence staining.

5. 1% BSA/PBS.

6. VECTASHIELD® Antifade Mounting Medium (Vector Laboratories).

7. Glass microscopic slides and coverslips.

2.4.2 Immunofluorescent Staining of Epidermal Sheets on Tape

1. Epidermal sheets on tape, prepared fresh or taken from the fridge.

2. Fine curved forceps (Dumont 7).

3. Phosphate buffered saline (PBS).

4. Glycine solution (3 % BSA/200 mM glycine/PBS, dissolve 3 g BSA and 1.5 g glycine in 100 ml PBS).

5. Fc-block.

6. Saponin solution (10 % w/v saponin in PBS).

7. Parafilm.

8. Humid chamber (an airtight, lightproof plastic box with a wet tissue at the bottom).

9. Blocking solution: 1× PBS containing 3 % FCS and 0.02 % Tween 20.

10. Staining solution: 1× PBS containing 1 % BSA and 0.25 % saponin (saponin is only necessary for intracellular staining, for example, for Langerin).

11. Primary and secondary antibodies for immunofluorescence staining.

12. ProLong® Gold Antifade Mountant with DAPI.

13. Microscope slides, superfrost and coverslips.

2.5   Preparation and Immunofluorescent Staining of Cryosections of Murine Skin

1. Sex- and weight-matched mice (*see* **Notes 1** and **2**).

2. Carbon dioxide (CO_2) for euthanizing animals.

3. Scissors.

4. One splinter forceps.

5. Freezing microtome.

6. Colorless Neg50-Frozen-Section-Medium (Thermo Fischer Scientific).

7. Poly-L-lysine coated slides.

8. Humid chamber.

9. Glass cuvette.

10. Acetone.

11. Hydrophobic slide marker (PAP-pen, Sigma-Aldrich).

12. 1 % BSA/PBS.

13. Primary and secondary antibodies for immunofluorescence staining.

14. VECTASHIELD® Antifade Mounting Medium (Vector Laboratories).

15. Glass microscopic slides and coverslips.

2.6   Preparation of Migratory Skin DC from Lymph Nodes for FACS Analysis

1. Fine curved forceps (Dumont 7).

2. Fine scissors.

3. 1× phosphate buffered saline (PBS).

4. 1× PBS containing 2 mM EDTA.

5. 500 mM EDTA solution.

6. Digestion mix (RPMI 1640, containing 200 U/ml collagenase IV and 0.5 U/ml DNase I).

7. FACS buffer (PBS containing 2 mM EDTA, 3% FCS, and 0.02% thimerosal).

8. 1.5 ml Eppendorf tubes.

9. 50 ml Falcon tubes.

10. 15 ml Falcon tubes.

11. 70 μm cell strainer.

12. Fc-block.

13. Primary antibodies to stain for skin-derived DC subsets in lymph nodes.

3   Methods

3.1   Induction of Skin Inflammation by Tape Stripping

Mice are anesthetized with a mixture of 80–120 mg/kg ketamine and 5–10 mg/kg xylazine in PBS. The dorsal side of the ear is tape stripped by repeated application and removal of 3 M™ Transpore™ Surgical Tape. For each stripping a fresh piece of tape is lightly pressed onto the ear and pulled off [19]. Tape stripping causes
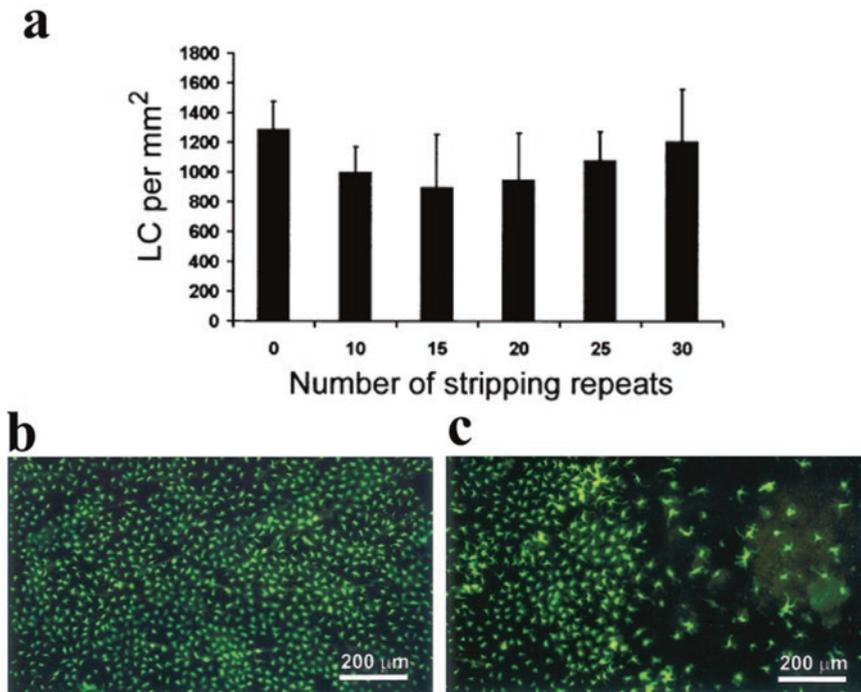

Fig. 1 Tape stripping induces emigration of LC from epidermis. (**a**) Number of stripping repeats affects the network of LC in the epidermis. (**b, c**) Epidermal sheets from steady state skin (**b**) and tape stripped skin (**c**) were stained with antibodies against MHC-class II. Tape stripping induces emigration of LC from epidermis. [Reproduced from Holzmann S, J Invest Dermatol, 2004 with permission from Nature Publishing Group.]

inflammation in the skin and emigration of immune cells like LC and dermal DC (Fig. 1). Experimental parameters e.g., kind of tape and number of stripping repeats, are crucial to the outcome of the procedure. For induction of mild inflammation we recommend 8 strippings, for strong inflammation 12 strippings (*see* **Note 4**).

3.2 Induction of Skin Inflammation by FITC Painting   5 mg FITC are dissolved in 100 ml DMSO. Acetone and DBP are mixed in a 1:1 ratio. The DMSO/FITC is diluted 10-fold in the Acetone/DBP mixture and mixed well. Mice are anesthetized with a mixture of 80–120 mg/kg ketamine and 5–10 mg/kg xylazine in PBS. Both ears (and optional the back) are shaved carefully and painted with 25 µl FITC solution each (optional: paint 100 µl FITC solution on the back, i.e., on the posterior flank, lateral to the spine). After the FITC solution has dried mice are put back into their cages. After 24, 48, 72, and/or 96 h (*see* **Note 5**) mice are sacrificed and the ears are cut off to prepare epidermal sheets as depicted in Fig. 2 (*see* Subheading 3.3 and 3.4), cryosections
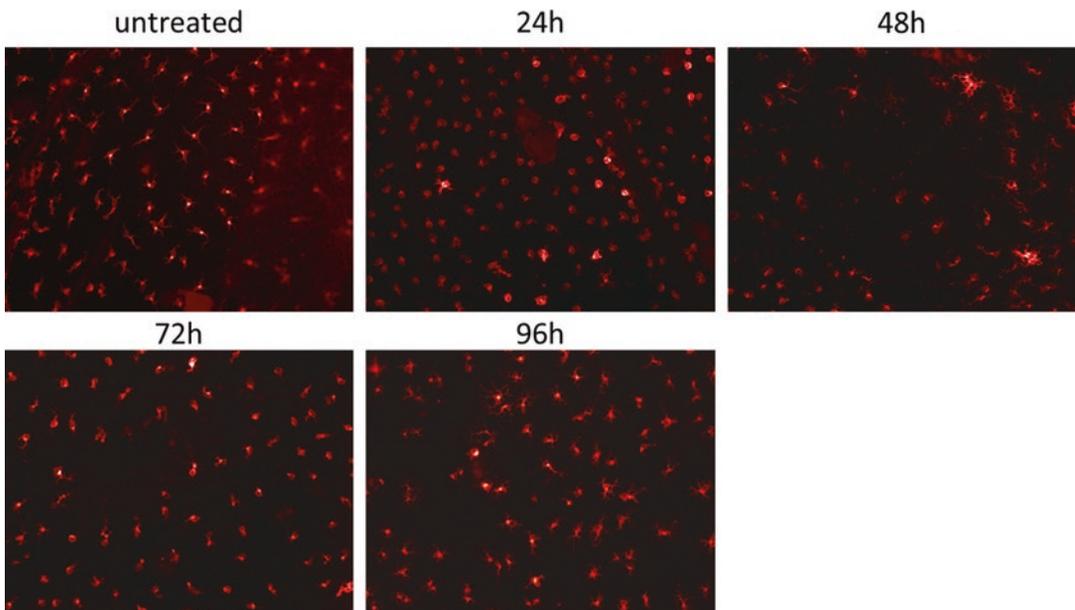

Fig. 2 LC emigration from epidermis after FITC painting. After 24 h LC (stained with MHC-class II in *red*) round up and start to leave the epidermis. 48 h after FITC painting most of the LC have left, while new LC start to repopulate the epidermis. After 72 h new LC have filled in most of the blanks and after 96 h new LC close the gaps by extending their dendrites. The LC network is re-established and the epidermis appears comparable to untreated control samples
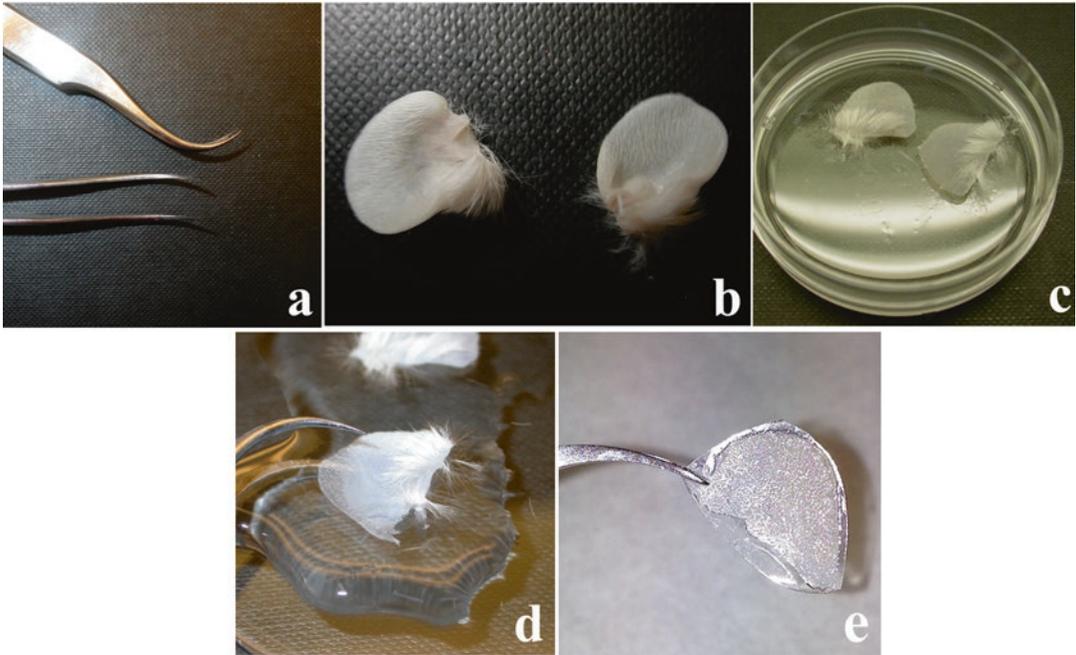
Fig. 3 Epidermal and dermal sheet preparation. Sheets are prepared with thin curved forceps (**a**) from ear skin (**b**). Dorsal ear halves are floated on ATC to split epidermis from dermis (**c**). Epidermis can be peeled off with forceps after incubation with ATC on a drop of PBS (**d**). Alternatively, Tesa tape can be applied to the epidermis before separating from the dermis to obtain whole ear specimens for immune-fluorescence staining (**e**)

(Subheading 3.5) or migratory skin DC from skin-draining lymph nodes for FACS analysis (*see* Subheading 3.6).

3.3 Monitoring of the Epidermal and Dermal DC Network—Epidermal and Dermal Sheet Preparation: Two Alternative Techniques

3.3.1 Preparation of Epidermal and Dermal Sheets

Mice are sacrificed and ears are cut off at their base. The cartilage-containing ventral ear halves can be removed from the dorsal ear halves with two surgical splinter forceps. Then the dorsal skin is floated epidermal side up on 500 µl 0.5 M ammonium thiocyanate solution for 20 min at 37 °C in a 24-well plate (one ear per well) (Fig. 3a–c). This chemically digests the basement membrane and allows the separation of epidermis and dermis [20]. Thereafter the skin is transferred onto a drop of PBS in a petri dish and the epidermis is separated from the dermis using two surgical curved forceps (Fig. 3d, *see* **Note 6**). The epidermal and dermal tissues are fixed in acetone for 20 min at room temperature in glass petri dishes. After fixation tissues are washed in a 24-well plate for 10 min twice in PBS and twice in 1% BSA/PBS to remove the acetone. Epidermal and dermal sheets can be used instantly for immunofluorescence staining or can be frozen in a drop of PBS and wrapped in aluminum foil for storage at −20 °C (*see* **Note 7**).

3.3.2 Epidermal Sheet Preparation of Whole Ear on Tape

Mice are sacrificed and the ears are cut off at their base. Depilation cream is applied onto both sides of the ears and incubated for 3 min at room temperature. Subsequently the ears are washed twice in 10 ml PBS by vortexing vigorously (*see* **Note 8**). Blot the ears on paper to dry and remove any remaining hair. The ears are split into the cartilage-containing ventral side and the dorsal side using two forceps (Fig. 3a–c). Both halves are stretched out on a reversed 5 cm petri dish with the dermal side facing downwards. A small piece (~3–4 cm) of tape is applied onto the epidermal side of each half and rubbed on (*see* **Note 9**). Next, the tape—with the dorsal or ventral half of the ear sticking to it—is carefully taken off the petri dish and is trimmed to leave only a small rim around the ear (Fig. 3e). Now the halves are floated with the dermal sides facing down on 500 µl of 20 mM EDTA solution for 1.5 h at 37 °C in a 12-well plate (one half per well) to digest the basement membrane and to allow the separation of epidermis and dermis. Thereafter, the tape—with the ear halves sticking to it—is transferred with the tape side down into a 5 cm petri dish filled with ~2 ml PBS. The dermis can now be detached from the epidermis by carefully peeling it off. The epidermis remains glued to the tape. For fixation the epidermis is floated—epidermis side down—on 500 µl of either 4 % PFA or ice-cold acetone for 20 min at room temperature (PFA) or −20 °C (acetone) in a 12-well plate (one half per well). After fixation the epidermal sheets are washed twice in PBS. Afterwards 500 µl of Glycine solution is added per well and the sheets are incubated for at least 1 h (and up to 1 week) at 4 °C to reduce potential background fluorescence prior to immunofluorescence staining (*see* **Note 10**).

3.4   Monitoring of the Epidermal and Dermal DC Network— Immunofluorescent Staining of Epidermal Sheets: Two Alternative Techniques

Freshly prepared or thawed epidermal and dermal sheets are cut into smaller pieces of 5 × 5 mm and used for immunofluorescence staining. Stained sheets can be preserved on microscopic slides in VECTASHIELD® Antifade Mounting Medium for long-term storage at 4 °C for up to 1–2 years.

3.4.1 Immunofluorescence Staining of Epidermal and Dermal Sheets

One-Step Staining Procedure/Direct Staining

Sheets can be incubated with one or a mixture of primary antibodies, which are directly conjugated with different fluorescence dyes. Antibody staining solutions are prepared in 1 % BSA/PBS and 100 µl of antibody solution is used per well containing one piece of an epidermal or dermal sheet. The tissue is incubated for 1 h at

37 °C or overnight at 4 °C (*see* **Note 11**). The 96-well plates are covered with a lid or Parafilm during incubation to prevent any fluid evaporation. After incubation with antibody the sheets are washed three times for 10 min in 100 μl 1% BSA/PBS in 96-well plates to remove unbound antibody. The sheets are carefully transferred from well to well with fine curved forceps. After washing steps, the sheets are embedded in VECTASHIELD® Antifade Mounting Medium on glass microscope slides and overlaid with slide coverslips.

Two- and Three-Step Staining Procedure/Indirect Staining

Besides using directly fluorochrome-coupled antibodies one has the option to use unconjugated primary antibodies, which are detected in a two or three step staining procedure. These staining techniques allow amplifying the fluorescent signal in case the detected molecule is only weakly expressed in the tissue. For the two-step staining procedure, sheets are incubated with the primary antibody followed by a fluorochrome-coupled secondary antibody directed against the host species of the primary antibody. For the three step staining a biotinylated secondary antibody against the host species of the primary antibody is used followed by a third amplification step with streptavidin, which binds to biotin, coupled to a fluorochrome (*see* **Note 12**). As described above stainings are performed in 100 μl antibody solution for 1 h at 37 °C. After each staining step the sheets are washed three times for 10 min in 100 μl 1% BSA/PBS in the 96 well plate, followed by embedding in VECTASHIELD® Antifade Mounting Medium on glass microscope slides.

Double Staining

Sometimes it is helpful to counterstain cells to simultaneously visualize two markers on the same cell, e.g., to visualize DC within the pool of CD45+ leukocytes. In order to achieve this, both molecules can be stained concurrently with a mix of two fluorescently conjugated antibodies. When an indirect staining has to be performed, we advise to first stain the molecule that is indirectly labeled. It is important to block open binding sites of the secondary antibody with 10% serum or 100 μg/ml Ig from the host species of the secondary antibody for 15 min at 37 °C (*see* **Note 13**). Then the counterstaining with another antibody can be performed. Species- or isotype-specific secondary antibodies can also be used to avoid cross-reactivity (*see* **Note 12**). An example for a Langerin and MHC-class II double staining is shown in Fig. 4.

3.4.2 Immunofluorescent Staining of Epidermal Sheets on Tape

Epidermal sheets are washed once in PBS and incubated in Glycine solution containing 0.1% saponin and Fc-block for 5 min at room temperature. Meanwhile the antibody mix is prepared in 60 μl staining buffer per sheet (**Note 14**). A layer of Parafilm is placed at the bottom of a saturated humid chamber and a rectangle for each sheet is drawn on the Parafilm (leaving enough space between the rectangles). A drop of 50 μl staining solution is placed into each
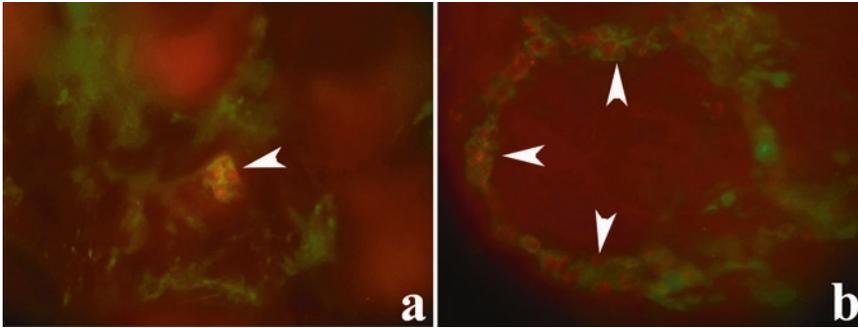
Fig. 4 Dermal sheets with lymphatic vessels containing migratory Langerin+ skin DC. Dermal sheets from steady state skin (**a**) and inflammation induced by tape stripping (**b**) were stained with antibodies against Langerin (*red fluorescence*) and MHC-class II (*green fluorescence*). *Arrowheads* indicate lymphatic vessels filled with migratory skin DC. Double positive cells represent LC and Langerin+ dermal DC, MHC-class II single positive cells represent Langerin[neg] dermal DC and macrophages

rectangle and the sheet is placed carefully on top (tape facing upwards), avoiding air bubbles. The remaining 10 µl staining solution is used to fill up any blank area underneath the sheet if necessary. The epidermal sheets can be incubated for 1 h at room temperature or at 4 °C overnight, depending on the antibodies used (*see* **Notes 10** and **11**). After incubation the sheets are washed three times in PBS and placed on glass slides (tape facing downwards). The sheets are encircled with Pap-Pen and mounted with ProLong® Gold Antifade with DAPI. Slides can be stored like this for up to 2 years at 4 °C.

3.5 Monitoring of Skin DC— Preparation and Staining of Cryosections

Mice are sacrificed and ears are cut off at their base. Tissue is embedded in colorless Neg50-Frozen-Section Medium and 8 µm skin sections are prepared from hardened tissue (−30 °C) in a freezing microtome at −27 °C. Cryosections are placed on poly-L-lysine coated microscopic slides, where they are dried for 1 h at room temperature. Afterwards the microscopic slides with sections can be either stored long-term at −20 °C or used instantly for immunofluorescence staining.

3.5.1 Preparation of Cryosections

3.5.2 Staining of Cryosections

Frozen sections are taken out from the freezer and are placed in a humid chamber for 20 min at room temperature. The sections are fixed with acetone for 10 min at room temperature in glass cuvettes. Afterwards acetone needs to evaporate, so let the sections dry for another 10 min. Before staining draw a circle with a hydrophobic slide marker (Pap Pen) around each tissue sections to prevent spilling of antibody solution. The sections are stained by applying 100 µl of antibody solution onto the tissue, which is then incubated for 30 min at 37 °C or overnight at 4 °C (*see* **Note 11**). Antibody dilutions are prepared in 1% BSA/PBS. As described above for sheet staining, the cryosections can be stained with one or a mixture of primary antibodies in a one, two or three step

staining procedure, depending on the expression levels of the molecule to be detected. Incubations should be performed in a closed humid chamber to avoid liquid evaporation. Following antibody incubation sections are washed three times for 5 min in 1% BSA/PBS to remove unbound antibody. At the end of the staining process sections are embedded in VECTASHIELD® Antifade Mounting Medium on glass microscope slides and covered with slide coverslips. Stained sections can be stored at 4 °C for up to 1–2 years.

3.6 Analysis of Migratory Skin DC from Lymph Nodes by Flow Cytometry

3.6.1 Preparation of a Single Cell Suspension

After FITC painting cervical and, if FITC was also applied onto the back, inguinal lymph nodes are isolated and collected in 1.5 ml Eppendorf tubes. 200 μl of digestion mix are added to each tube and the lymph nodes are cut into very small pieces (clean scissors between samples to avoid cross-contamination). Another 800 μl of digestion mix are added and the samples are incubated for 45 min at 37 °C, shaking at ~1000 rpm. After incubation 20 μl 500 mM EDTA solution per 1 ml digestion mix (final concentration 10 mM) are added and the samples are incubated for additional 5 min at room temperature to separate T cell/ DC clusters. The cell suspension is passed over a 70 μm cell strainer and the strainer is rinsed with 10 ml PBS/ 2 mM EDTA. The cells are centrifuged for 5 min at $400 \times g$ at 4 °C and the supernatant is discarded. The cells are resuspended in an appropriate volume of PBS/2 mM EDTA and counted. The cell concentration is adjusted to 10^6 cells per 50 μl and 10^6 cells (50 μl) are taken per staining.

3.6.2 Flow Cytometric Analysis of Migratory DC in Skin-Draining Lymph Nodes After FITC Painting

10^6 cells are used per staining and are incubated with Fc-block for 10 min at room temperature. For the detection of migratory DC the cells are stained with an antibody cocktail including fixable Life/dead marker in AmCyan (eBioscience), CD103 in PE (clone 2E7), MHC-II in PerCP-Cy5.5 (clone M5/114.15.2), CD11b in PE-Cy7 (clone M1/70), Langerin in Alexa 647 (clone 929 F3.01, Dendritics), CD11c in APC-Cy7 (clone N418), and CD24 in Pacific blue (clone M1769) in FACS buffer. The cells are incubated with the antibody cocktail for 1 h at 4 °C in the dark. Following this incubation, the cells are washed with PBS/2 mM EDTA, resuspended in 100 μl FACS buffer and acquired immediately with a flow cytometer. An example for the FACS analysis of FITC positive migratory cells is provided in Fig. 5 (*see* **Note 15**).

4 Notes

1. Inflammation and emigration of LC from the epidermis can be induced in every inbred mouse strain; nevertheless, the degree of skin inflammation might vary between strains.

2. We recommend not mixing male and female mice in experimental groups. The development of skin inflammation can
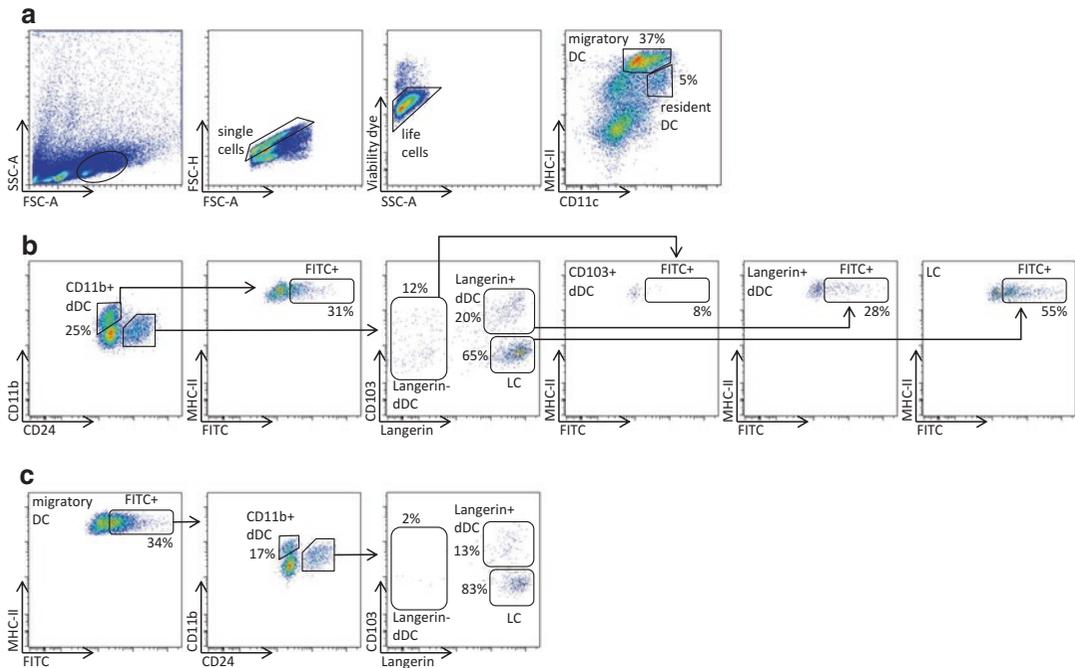
Fig. 5 Flow cytometric analysis of migratory skin DC in skin-draining lymph nodes after FITC painting. (**a**) To identify migratory DC a gate is drawn around mononuclear cells. Single cells are determined with the area and the height of the FSC. Dead cells are excluded by life/dead staining. Migratory DC are defined as MHC-II^{high}CD11c^{int} cells and can be analyzed in two different ways: (**b**) By gating on DC subsets like CD11b$^+$ dermal DC, LC and Langerin$^+$ dermal DC first and then analyzing frequencies of FITC$^+$ cells or (**c**) by gating on FITC$^+$ cells within the migratory DC subset first and then dividing them into the respective skin DC subsets. While CD24 mirrors Langerin expression in the epidermis, this correlation is less pronounced in skin-draining lymph nodes [8]. The indicated percentages represent the frequencies of DC subsets of the respective parental gate

vary between the sexes due to differences in skin thickness and weight.

3. According to our experience Tesa tape crystal clear is the only tape that does not tarnish after contact with acetone or alcohol. Furthermore it does not impair fluorescence microscopy since it stays completely transparent.

4. In early experiments using tape stripping LC were physically extracted with help of the tape [21, 22]. Milder tape stripping induces inflammation followed by emigration of LC from the epidermis [19]. Tape stripping triggers the upregulation of mRNA for pro-inflammatory cytokines like IL-1α, IL-1β, and TNF-α in the skin [23], cytokines that are important for the migration of LC [24].

5. First signs of Langerhans cell emigration can be observed after 24 h, when the cells change their morphology to leave the epidermis (Fig. 2).

6. Epidermis should be easily removable from the dermis in one piece. If this is not the case the digestion might be incomplete,

then incubate the skin for another 5–10 min. Make sure that all epidermal pieces are peeled off from the dermis.

7. Epidermal and dermal sheets can be stored long-term at −20 °C without any loss of quality. Freshly prepared and frozen sheets show comparable staining results.

8. If the ears are still highly inflamed depilation can damage the delicate epidermis, in this case the depilation step should be skipped.

9. In case of strong tissue inflammation the skin will be oozing fluid, which prevents it from sticking to the tape properly. In this case the ears can be pre-fixed in ice-cold acetone for 20 min prior to splitting them into halves to dehydrate the tissue.

10. If high background fluorescence is an issue (e.g., after topical FITC painting), the sheets can be exposed to light for a minimum of 4 h prior to immunofluorescence staining. Alternatively, epidermal sheets can be incubated for 20 min in 0.1 % Sudan black in 70 % ethanol after immunofluorescence staining. This will result in a blackening of the tape, without interfering with the antibody staining.

11. The decision how long primary antibodies are incubated with the sheets depends on the expression levels of the molecules to be detected. We recommend shorter incubation of 1 h at 37 °C for strongly expressed molecules and longer incubation overnight at 4 °C for weakly expressed molecules.

12. Indirect staining in a two or three step procedure can result in unspecific background staining especially in dermal sheets. In order to quench this effect as well as antibody cross-reactivity between species, blocking with normal serum (5 % v/v) from the host species of the secondary antibody can be performed before the staining procedure is started. In addition, secondary antibodies should be carefully titrated.

13. Alternatively, the secondary antibody can be replaced by a F(ab) fragment; a monovalent antibody molecule still binding to its antigen but lacking the Fc portion. Using a F(ab) fragment as secondary antibody can improve the staining results, because (1) it eliminates nonspecific binding between Fc portions of antibodies and Fc receptors on cells and (2) F(ab) fragments do not interfere with specific anti-Fc mediated secondary antibody detection.

14. In case of a weak staining result due to low expression of the target molecule, the signal intensity can be increased by replacing the staining solution with a so-called staining enhancer, such as the Thermo Scientific Pierce Immunostain Enhancer.

15. This basic protocol can be used to generally analyze migratory DC after the induction of skin inflammation, e.g., tape stripping, without the possibility to identify FITC+ recent skin immigrants.

Acknowledgements

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