

# Chapter 2

## Hapten-Specific T Cell-Mediated Skin Inflammation: Flow Cytometry Analysis of Mouse Skin Inflammatory Infiltrate

Nicolas Bouladoux, Clotilde Hennequin, Camille Malosse,  
Bernard Malissen, Yasmine Belkaid, and Sandrine Henri

### Abstract

Hapten-specific T cell-mediated skin inflammation also known as contact hypersensitivity (CHS) is characterized by a strong influx of CD8<sup>+</sup> cytotoxic T cells within the skin upon reexposure of sensitized individuals to the same hapten. As many other leukocytes are also recruited during this elicitation phase, we attempted to revisit the skin infiltrate and characterize the inflammatory pattern. Recent improvement in the isolation in conventional as well as inflammatory dendritic cell and macrophage subsets from tissues and in the use of appropriate surface markers unraveling their heterogeneity should allow to determinate their specific functions in the CHS model. Here, we describe procedures to extract those cells from the skin and to analyze them by flow cytometry using a combination of appropriate surface markers allowing further transcriptomic analysis and functional assays.

**Key words** Skin, Contact hypersensitivity (CHS), Dendritic cell subsets, Monocyte-derived cells, Macrophages, Neutrophils, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells,  $\gamma\delta$  T cells, Flow cytometry

---

### 1 Introduction

Hapten-specific T cell-mediated skin inflammation is known under various names: contact hypersensitivity (CHS), allergic contact dermatitis (ACD) or delayed-type hypersensitivity (DTH). This inflammatory skin disease is common in industrialized countries [1] and can be mimicked with animal models by painting a hapten onto the skin allowing the dissection of the pathophysiology of CHS. Using the strong contact sensitizer 2,4-dinitro-1-fluorobenzene (DNFB), it was shown that optimal CHS was a two-step reaction with two temporally and spatially dissociated phases [2, 3]. The first contact with the hapten on the skin is referred to as the sensitization phase also called afferent or induction phase. During this phase, the innate immune system is stimulated leading to the activation of skin dendritic cells (DCs) that migrate to the skin draining lymph nodes and prime naïve T cells

to become skin-tropic, hapten-specific effector T cells. Upon reexposure to the same hapten, those specific T lymphocytes are rapidly activated within the skin, triggering a strong inflammatory process within 24–72 h characterizing the elicitation phase that is also called efferent or challenge phase [4]. At steady-state, the skin contains many cell types of the hematopoietic system including conventional DCs, monocytes, monocyte-derived DCs, macrophages,  $\gamma\delta$  T cells,  $\alpha\beta$  T cells mainly CD4<sup>+</sup> T lymphocytes and very few neutrophils. Conventional DCs were firstly described in lymphoid organs in the early seventies [5], but due to their low numbers and the difficulty to extract them, extensive studies assessing their functions were primarily performed using bone marrow- or monocyte-derived DCs [6–8]. With the use of a few key markers, we could disentangle the complexity of the skin DC network allowing to distinguish phenotypically and functionally distinct subsets [9–11]. Moreover, for many years, there was confusion in the field of DC characterization in tissues as many studies were often mixing conventional tissue DCs derived from DC precursors with monocyte-derived DCs and even tissue macrophages. Such confusion was even worse during inflammatory conditions. Indeed, in models such as CHS, both phases are characterized by a strong influx of neutrophils and monocytes, which will differentiate into inflammatory monocyte-derived DCs, commonly called Tip-DCs for TNF and iNOS producing DCs. We and others contributed to improve cell extraction from mouse skin and unravel the combination of specific surface markers to stain the immune skin infiltrate and distinguish the different conventional DC subsets, the monocytes, monocyte-derived DCs and the macrophages by flow cytometry [12–14]. Upon inflammation, the analysis of the inflammatory response can be completed with the same gating strategy [14, 15]. In the present protocol, we explain how to thoroughly analyze the immune skin infiltrate in a DNFB-induced CHS model. Not only we show how to distinguish the innate cells from the neutrophils to the DC subsets and macrophages but we also show how to follow the adaptive T cell immune response as it ultimately plays a major role within the tissue [16–18].

---

## 2 Materials

### 2.1 Induction of DNFB-Mediated CHS

1. 6–9-week-old, sex- and weight-matched C57BL6 mice.
2. Shaving device and hair depilation cream.
3. Acetone/olive oil vehicle (4:1).
4. 2,4-dinitro-fluorobenzene (DNFB).

## **2.2 Analysis of the Leukocyte Skin Infiltrate by Flow Cytometry**

Keep all the solutions sterile. Do not add sodium azide to the solutions.

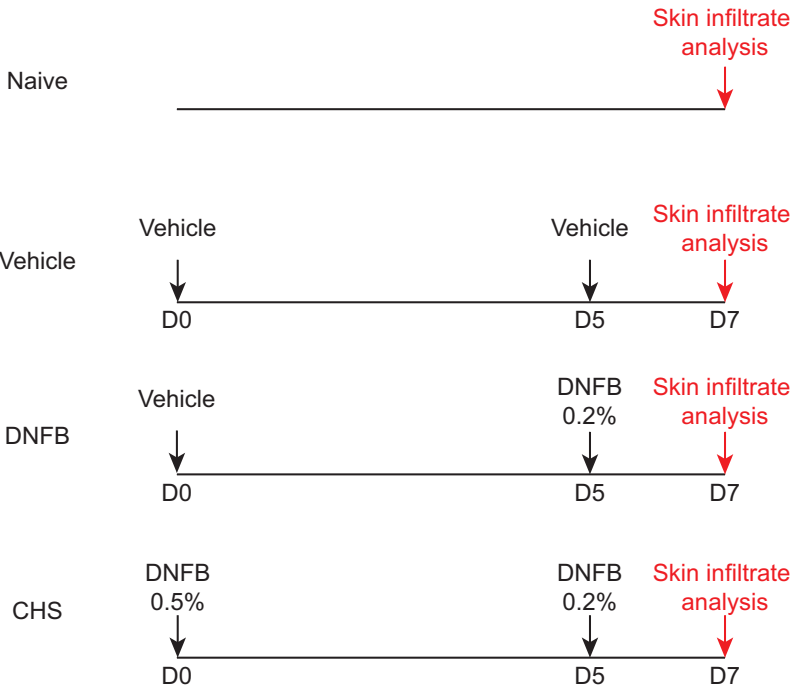
1. Basic medium: To 500 mL of RPMI 1640, add 5 mL of penicillin–streptomycin solution (10,000 I.U., 10,000 µg/mL), 5 mL of 100 mM sodium pyruvate, 5 mL of MEM nonessential amino acids (100×), 5 mL of 200 mM l-glutamine, 500 µL of 55 mM βME, and 10 mL of 1 M HEPES. Store at 4 °C.
2. Complete medium: Basic medium complemented with 10% FBS.
3. FACS Buffer: PBS, 2% FBS, 5 mM EDTA (combine 100 mL of 10× PBS, 10 mL of 0.5 M EDTA (pH8.0), 20 mL of inactivated FBS and complete to 1 L with ultrapure water). Filter through a 0.2 µm filter and store at 4 °C.
4. Sorting FACS Buffer: PBS, 10% FBS, 5 mM EDTA (combine 100 mL of 10× PBS, 10 mL of 0.5 M EDTA (pH8.0), 100 mL of inactivated FBS and complete to 1 L with ultrapure water). Filter through a 0.2 µm filter and store at 4 °C.
5. DNase stock solution (deoxyribonuclease I from bovine pancreas): 10 mg/mL solution in basic medium (resuspend 100 mg of powder in 10 mL of basic medium). Aliquot (1 mL aliquots) and store at –20 °C.
6. DNase working solution: on the day of the experiment, take 500 µL of DNase stock solution at 10 mg/mL and complete to 10 mL with basic medium. Keep the solution on ice.
7. Liberase TL stock solution: 25 mg/mL solution in sterile water (resuspend 5 mg of powder in 200 µL of sterile water). Aliquot (50 µL aliquots) and store at –20 °C.
8. Liberase TL-DNase working solution: on the day of the experiment, combine 100 µL of Liberase TL stock solution at 25 mg/mL and 500 µL of DNase stock solution at 10 mg/mL and complete to 10 mL with basic medium. Keep the solution on ice.
9. Forceps, scissors.
10. Petri dishes.
11. 24-well plates.
12. Automated mechanical disaggregation system (Medimachine).
13. 50 µm sterile disposable chambers (Medicons) to be used on the Medimachine and allowing an efficient cutting of the tissue (microblades).
14. 50 µm sterile syringe filters (Falcon).
15. 20 mL sterile syringes and 19G needles.
16. 10 mL sterile pipettes.
17. 70 µm sterile cell strainers.

- 18. 15 mL sterile polypropylene (PP) tubes.
- 19. 1.5 mL sterile Eppendorf tubes.
- 20. Fc block (clone 24G2).
- 21. RLT Plus Buffer: Lysis buffer for lysing cells prior to RNA isolation.

3 Methods

3.1 Induction of DNFB-Mediated CHS (See Fig. 1)

- 1. Firstly, 2 cm<sup>2</sup> of fur is removed on dorsal skin using the shaving device and the hair depilation cream.
- 2. 24 h later, mice are sensitized by epicutaneous application of 25 µl of 0.5 % DNFB diluted in acetone/ olive oil (4:1), using a 200 µl pipette tip.
- 3. Five days later, mice are challenged on the ear by epicutaneous application of 25 µl of 0.2 % DNFB diluted in acetone/ olive oil (4:1), using a 200 µl pipette tip. This group is called CHS in Figs 1–6 and Table 4 (see Note 1).



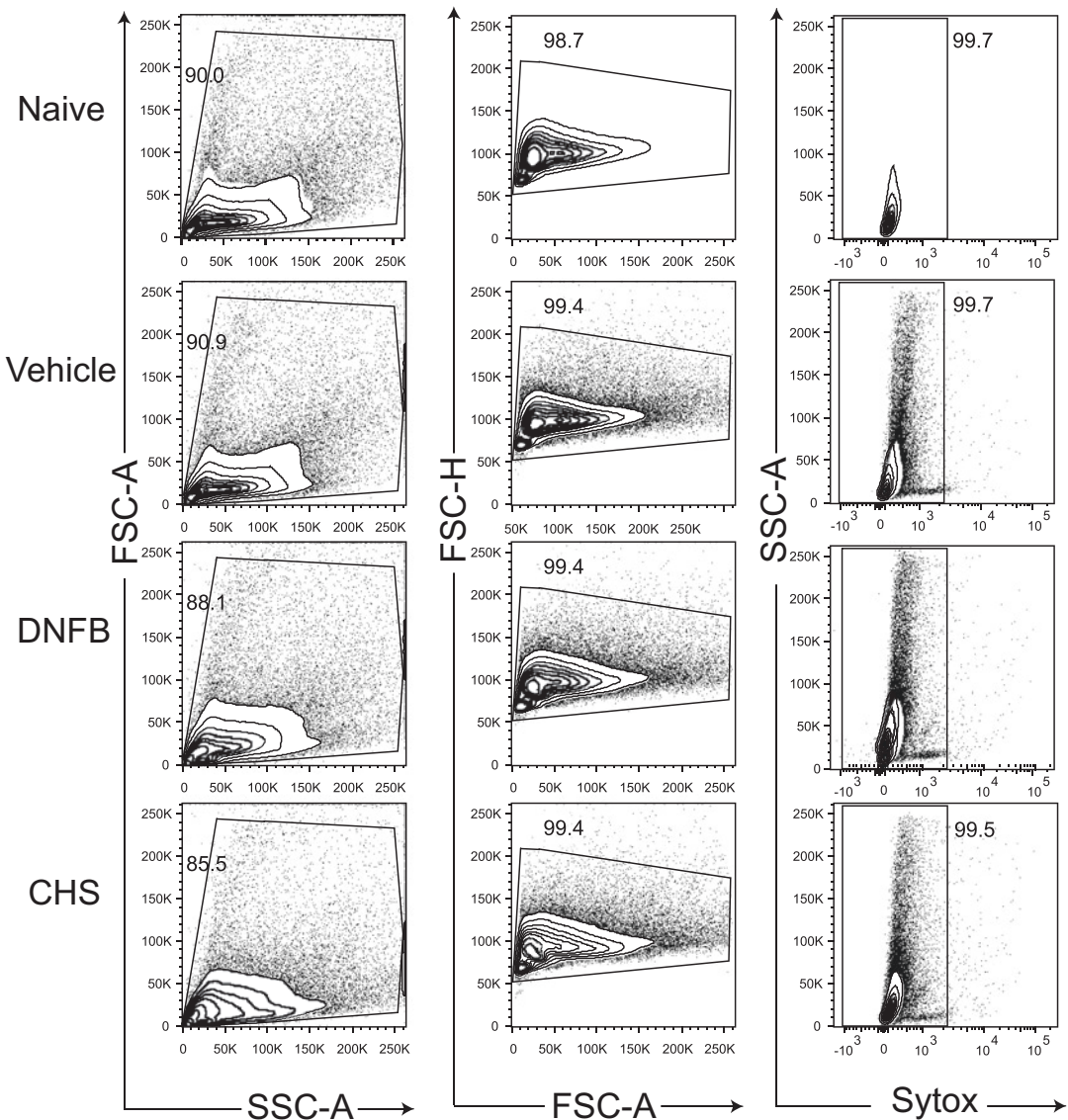
**Fig. 1** CHS induction model CHS was induced as follows: firstly mice were sensitized by topical application of 0.5 % DNFB on the dorsal skin. Five days later, animals were challenged on the ear by topical application of 0.2 % DNFB and the ear skin infiltrate was analyzed 2 days later. Ear skin infiltrate analysis was realized comparing naïve mice (Naïve) to mice that received the vehicle only (Vehicle), to mice that received first the vehicle and then DNFB 0.2 % on the ears (DNFB) and to mice which were sensitized and challenged with DNFB (CHS)

### 3.2 Tissues Collection

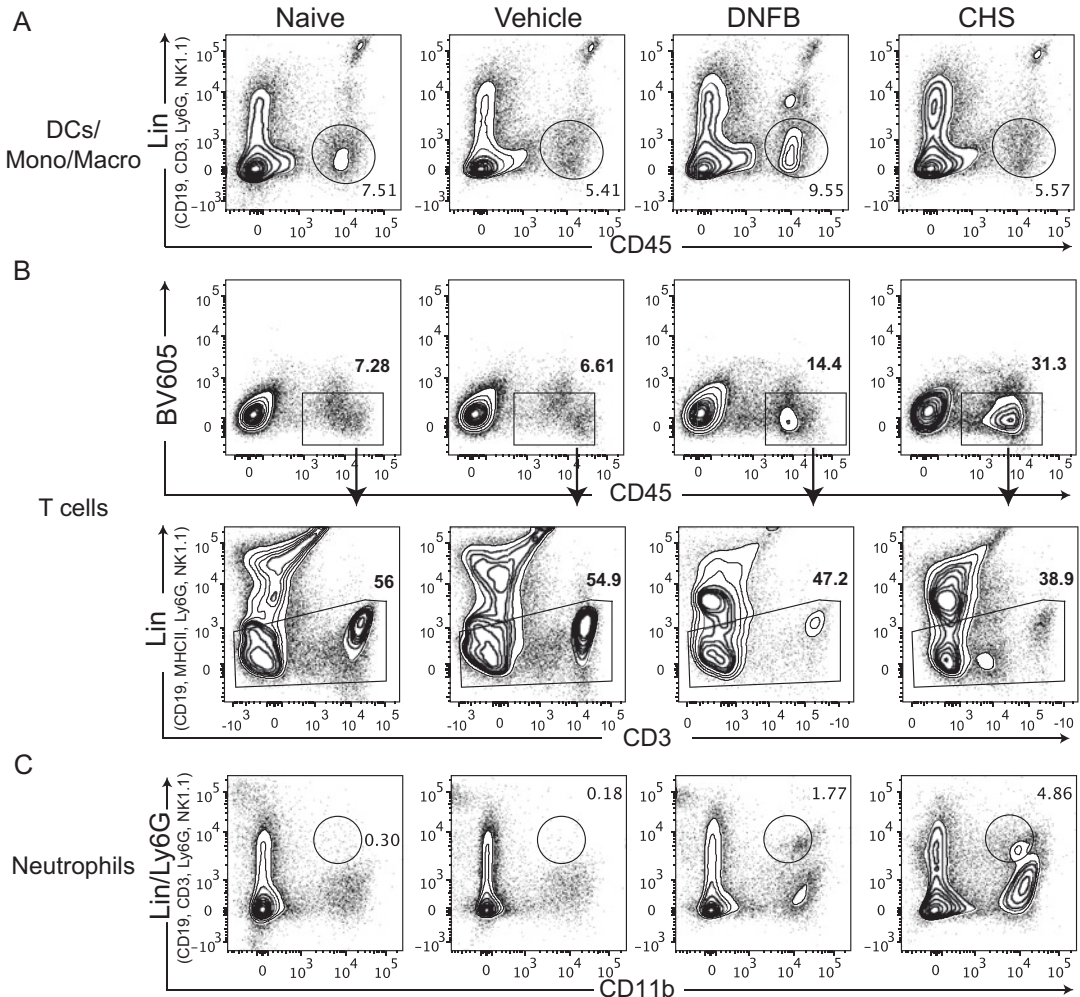
1. Two days after the challenge, sacrifice the mice.
2. Using forceps and small surgery scissors, cut the ears 2 mm from their basis to avoid including hair from the scalp.
3. Add 1 mL of PBS in 24-well plates, place the ears on the PBS and keep on ice.

### 3.3 Enzymatic Treatment

1. In a 24-well plate, distribute 750  $\mu$ L Liberase TL-DNase working solution per well. You will need 2 wells per mouse (if collecting both ears) (*see Note 2*).



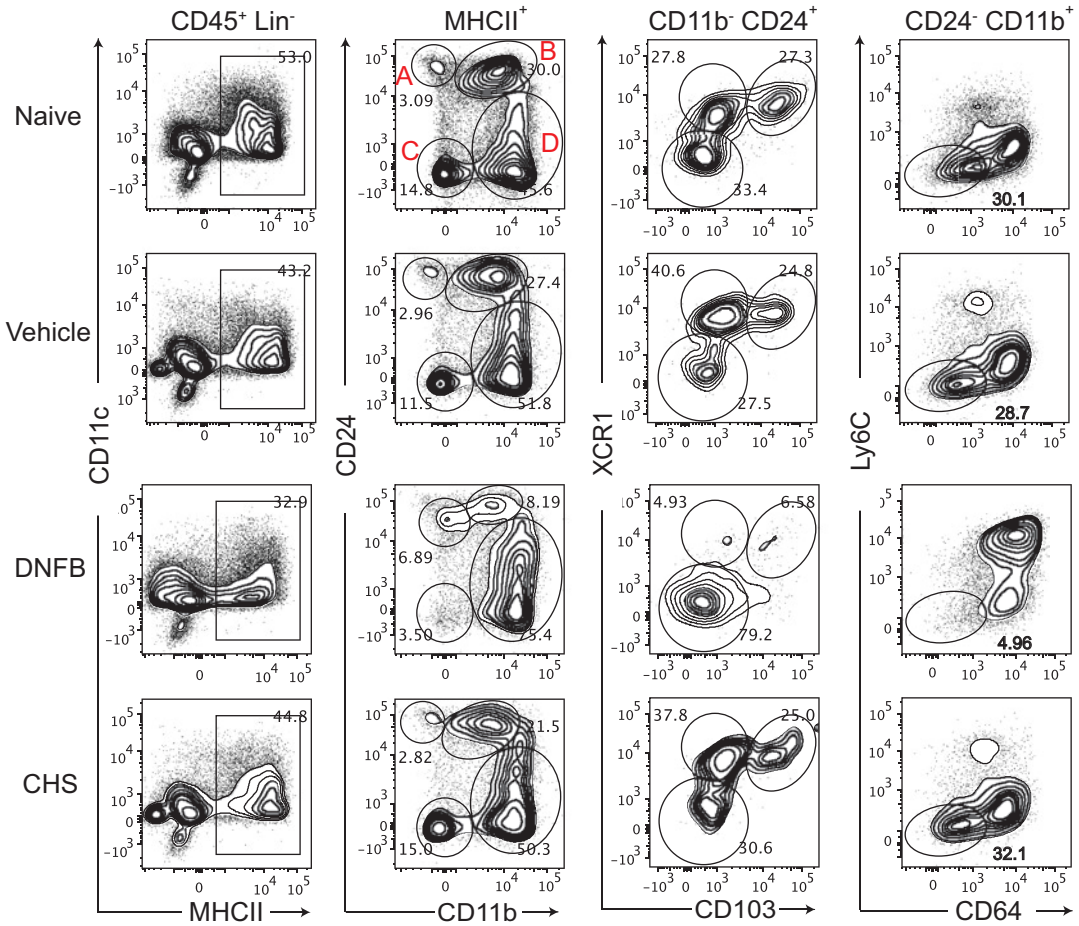
**Fig. 2** Pre-gating strategy of living cells. Cells were prepared from skin and analyzed by flow cytometry. Cells are pre-gated according to their size (FSC-A/SSC-A). Doublets and dead cells are excluded using FSC-H and Sytox blue respectively



**Fig. 3** Pre-gating strategy of hematopoietic cells of myeloid or lymphoid lineage Cells were prepared from skin, run on a flow cytometer, pre-gated on live cells as described in Fig. 1 and further gated to analyze myeloid populations or T lymphocytes or neutrophils. **(a)** Myeloid cells including dendritic cells, monocytes, and macrophages (DCs/Mono/Macro) were selected as hematopoietic cells (CD45<sup>+</sup>) with further exclusion of NK cells, B cells, T cells, and neutrophils (Lin<sup>-</sup>) according to the staining presented in Table 1. **(b)** T lymphocytes were selected as hematopoietic cells (CD45<sup>+</sup>) with further exclusion of NK cells, B cells, myeloid cells and neutrophils (Lin<sup>-</sup>) according to the staining presented in Table 3. **(c)** Neutrophils were selected as CD11b<sup>+</sup> and Ly6G<sup>+</sup>, which is included in the Lin channel according to the staining presented in Table 2

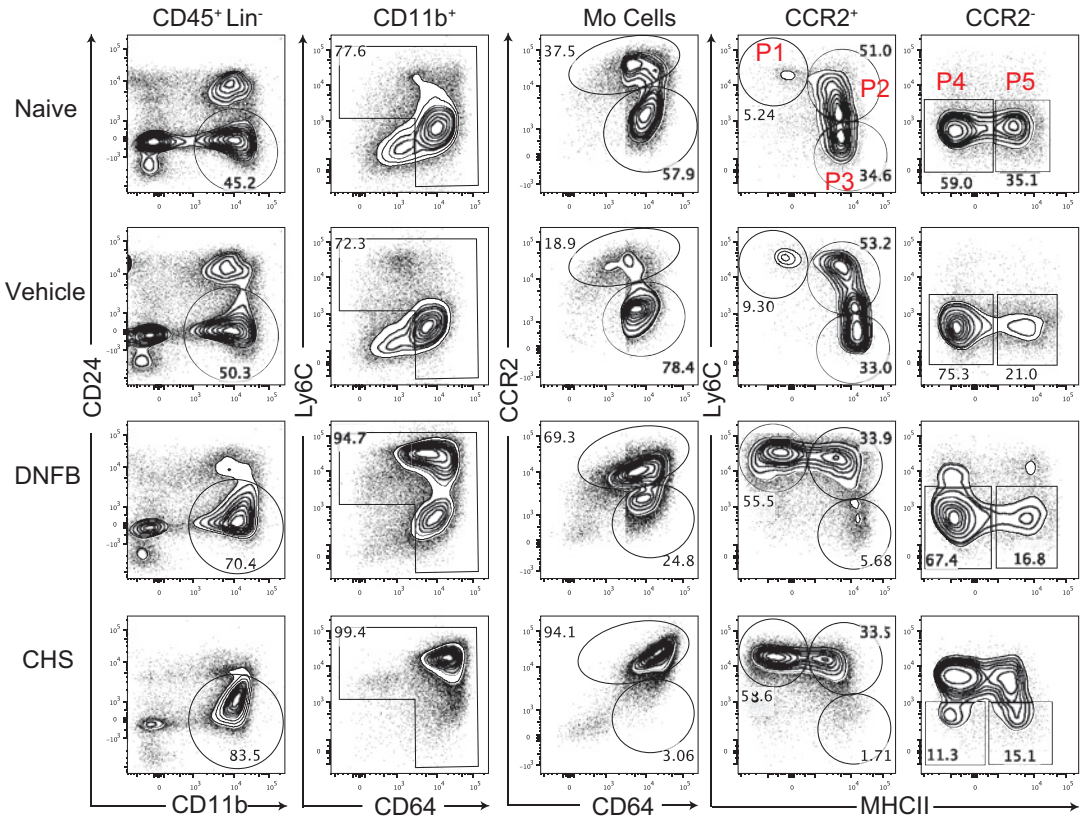
2. With forceps, separate the internal and external faces of the ears (the internal face will come with the cartilage) and lay it down flat on 750  $\mu$ L Liberase TL-DNase working solution. The “outside” (epidermis) of each skin layer should be up, whereas the “inside” (dermis) should be in contact with the solution (*see Note 3*).





**Fig. 4** Conventional DC subset gating strategy Cells were prepared from skin and analyzed by flow cytometry (see Table 1). After pre-gating on CD45<sup>+</sup> Lin<sup>-</sup>, MHCII<sup>+</sup> CD11c<sup>low to +</sup> cells were selected. Conventional DC subsets can then be discriminated using CD24 versus CD11b. CD24<sup>+</sup>CD11b<sup>-</sup>, which correspond to CD24/CD207 dermal DCs can be further divided according to their expression of XCR1 and CD103. CD24<sup>+</sup>CD11b<sup>+</sup> correspond to the Langerhans cells (LCs). The CD24<sup>-</sup>CD11b<sup>-</sup> population is often referred to as DN DCs and finally, within the CD24<sup>-</sup>CD11b<sup>+</sup> population, only the Ly6C<sup>-</sup>CD64<sup>-</sup> cells correspond to the conventional CD11b<sup>+</sup> dermal DC subset

3. Incubate for 1:45 h at 37 °C in a cell culture incubator (5 % CO<sub>2</sub>).
4. To stop the enzymatic treatment, at the end of the incubation, add 750 µL of DNase working solution and 15 µL of 0.5 M EDTA.
5. Using fine forceps, collect the ear halves and place them in the Medicon tissue grinder. Add 1.5 mL of DNase working solution.
6. Place the Medicon in the Medimachine and turn it on for 8 min.



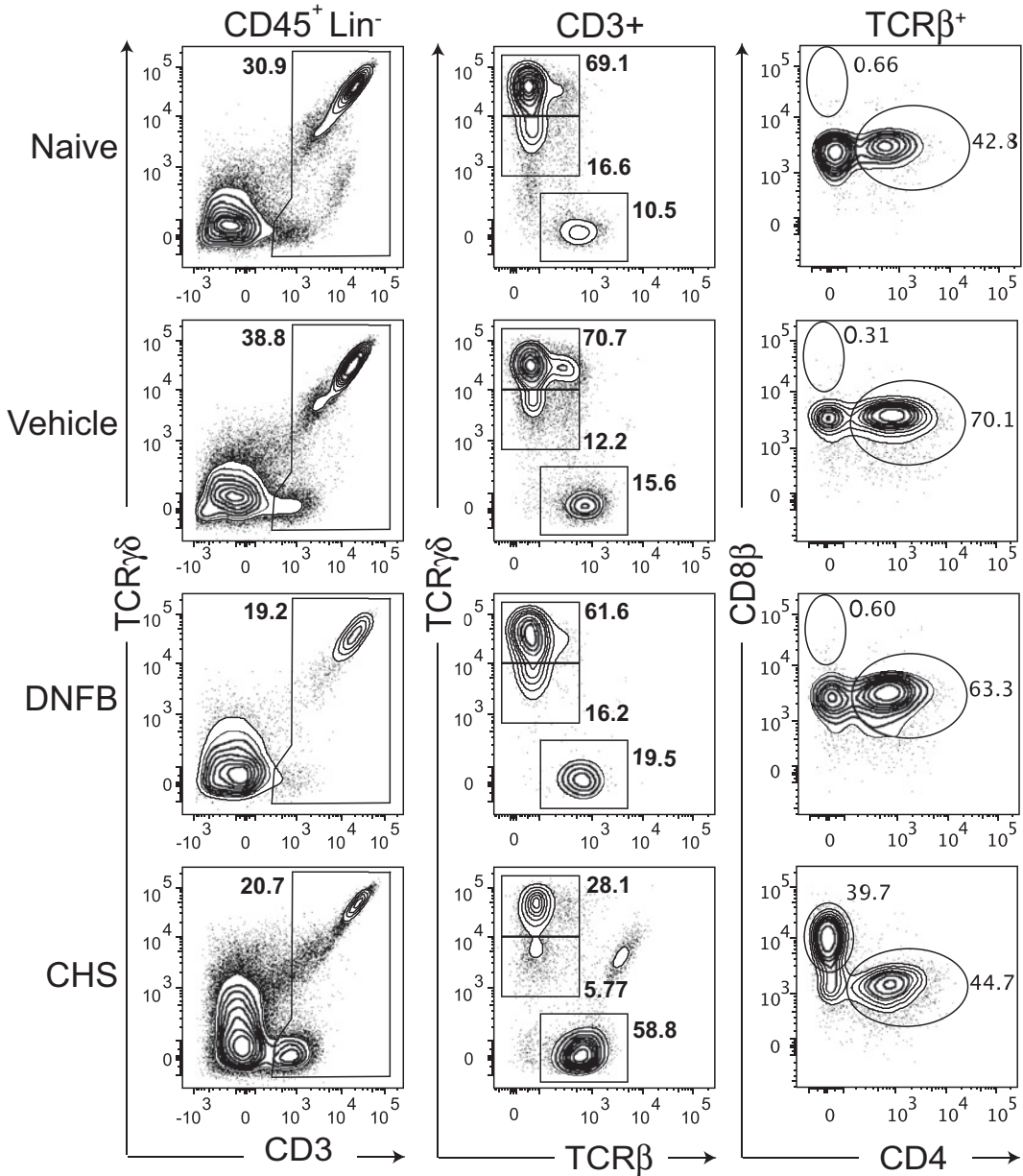
**Fig. 5** Monocyte, monocyte-derived DC, macrophages gating strategy Cells were prepared from skin and analyzed by flow cytometry (see Table 2). After pre-gating on CD45<sup>+</sup> Lin<sup>-</sup>, total CD11b<sup>+</sup> cells were selected. Conventional CD11b<sup>+</sup> DC subset was excluded using Ly6C and CD64. Monocyte-derived cells (Mo-cells) were selected based on their expression of Ly6C and CD64. This population can be further divided into CCR2<sup>+</sup> and CCR2<sup>-</sup>. CCR2<sup>+</sup> cells include dermal monocytes (P1, Ly6C<sup>+</sup>MHCII<sup>-</sup>) and dermal monocyte-derived DCs (P2, Ly6C<sup>+</sup>MHCII<sup>+</sup> and P3, Ly6C<sup>-</sup>MHCII<sup>+</sup>). CCR2<sup>-</sup> cells correspond to the dermal macrophages and can be divided into two populations P4 and P5, which are Ly6C<sup>-</sup>MHCII<sup>-</sup> and Ly6C<sup>-</sup>MHCII<sup>+</sup>, respectively

7. Collect the cell suspension from the Medicon using a 20 mL syringe and rinse the Medicon with 8 mL of DNase working solution.
8. Proceed to the filtration in a 15 mL tube using 50  $\mu$ m syringe filter (Falcon).
9. Centrifuge for 5 min at 450  $\times g$  and 4  $^{\circ}$ C.
10. Resuspend the pellet with 1 mL of FACS Buffer and proceed to counting and staining (see Note 4).

### 3.4 FACS Staining (See Note 5)

1. Prepare the antibody mix as described in Tables 1, 2 and 3 (without the Sytox blue) (see Note 6). The antibodies are diluted in FACS Buffer and require to be titrated before use.





**Fig. 6** T cell gating strategy Cells were prepared from skin and analyzed by flow cytometry (see Table 3). After pre-gating on CD45<sup>+</sup> Lin<sup>-</sup>, T cell subsets were further divided according to their expression of  $\gamma\delta$  and  $\beta$  TCR. DETC correspond to TCR $\gamma\delta^{\text{high}}$ , whereas dermal  $\gamma\delta$  cells correspond to TCR $\gamma\delta^{\text{intermediate}}$ . Conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells are found after pre-gating on TCR $\beta$  expressing cells

2. Cells are distributed in 1.5 mL Eppendorf tubes for staining (with a maximum of 1 million cells per tube).
3. Centrifuge for 5 min at  $400 \times g$ .
4. Add Fc block ( $0.025 \mu\text{g/mL}$ ).

**Table 1**  
**mAbs used for FACS analysis of conventional DC subsets**

	Conjugate	Name	Clone	Purchased from
Violet laser (405 nm)	SytoxBlue	Viability		Lifetechnologies
	BV610	CD11c	N418	Biolegend
	BV711	CD11b	M1/70	Pharmingen
Blue laser (488 nm)	FITC	Ly6C	AL-21	Pharmingen
Green-Yellow laser (561 nm)	Biotin	CD103	M290	Pharmingen
	PECF594	Streptavidin		Pharmingen
	PECy5	CD24	M1/69	Biolegend
	PECy5.5	CD45	30-F11	eBioscience
Red laser (633 nm)	PE	XCR1	ZET	Biolegend
	Alexa700	MHCII	M5/114.15.2	Biolegend
	APCCy7	Ly6G	HK1.4	Biolegend
	APCCy7	CD19	6D5	Biolegend
	APCCy7	NK1.1	PK136	Biolegend
	APCCy7	CD3	145-2C11	Biolegend
	APC	CD64	X54-5/7.1	BD

5. Incubate for 10 min on ice.
6. Centrifuge for 5 min at  $400 \times g$ .
7. Add 100  $\mu$ L of antibody mix.
8. Incubate for 30 min on ice and protected from light.
9. Add 1 mL of FACS buffer to wash the cells.
10. Centrifuge for 5 min at  $400 \times g$ .
11. Resuspend the cell pellet in Sytox blue diluted (1/1000) in FACS buffer.
12. Cells are ready to be run on the FACS or cell sorter (*see* **Notes 7** and **8**). If clumps appear, filter one more time using a 70  $\mu$ m cell strainer.

### 3.5 Gating Strategy for FACS Analysis or Cell Sorting

1. Gate cells according to their size using FSC and SSC, excluding debris (*see* Fig. 2).
2. Remove dead cells by gating on Sytox blue negative cells (*see* Fig. 2).
3. Gate on hematopoietic cells as CD45<sup>+</sup> cells (*see* Fig. 3).
4. Gate on DCs and monocyte-related cells as dump channel negative cells (APCCy7<sup>-</sup>) within CD45<sup>+</sup> cells (*see* Fig. 3a).
5. DC subsets are gated as follows (*see* Fig. 4 and Tables 1 and 4): within CD45<sup>+</sup> Lin<sup>-</sup> cells, select MHCII<sup>+</sup> CD11c<sup>low to high</sup>. Then plot them using CD24 versus CD11b (*see* **Note 6**).

**Table 2**  
**mAbs used for FACS analysis of monocytes, monocytes derived DC, macrophages, and neutrophils**

	Conjugate	Name	Clone	Purchased from
Violet laser (405 nm)	SytoxBlue	Viability		Life Technologies
		CD11c	N418	BioLegend
		CD11b	M1/70	Pharmingen
Blue laser (488 nm)	FITC	Ly6C	AL-21	Pharmingen
Green-yellow laser (561 nm)	Biotin	CD103	M290	Pharmingen
	PECF594	Streptavidin		Pharmingen
	PECy5	CD24	M1/69	BioLegend
	PECy5.5	CD45	30-F11	eBioscience
Red laser (633 nm)	PE	CCR2	475301	R&D
	Alexa700	MHCII	M5/114.15.2	BioLegend
	APCCy7	Ly6G	HK1.4	BioLegend
	APCCy7	CD19	6D5	BioLegend
	APCCy7	NK1.1	PK136	BioLegend
	APCCy7	CD3	145-2C11	BioLegend
	APC	CD64	X54-5/7.1	BD

**Table 3**  
**mAbs used for FACS analysis of T cell subsets**

	Conjugate	Name	Clone	Purchased from
Violet laser (405 nm)	SytoxBlue	Viability		Life Technologies
		CD4	RM4-5	Pharmingen
		TCR $\gamma\delta$	GL3	BioLegend
Blue laser (488 nm)	FITC	TCR $\beta$	H57-597	Pharmingen
Green-yellow laser (561 nm)	PECy5.5	CD45	30-F11	eBioscience
	PE	CD8 $\beta$	H35-17.2	eBioscience
Red laser (633 nm)	APCCy7	MHCII	M5/114.15.2	BioLegend
	APCCy7	Ly6G	HK1.4	BioLegend
	APCCy7	CD19	6D5	BioLegend
	APCCy7	NK1.1	PK136	BioLegend
	Alexa700	CD3 $\epsilon$	500A2	Pharmingen

Four distinct populations should be found: (a) the dermal CD24<sup>+</sup>CD11b<sup>-</sup> cells which can be further divided using XCR1 and CD103 (*see Note 9*), (b) The CD24<sup>+</sup>CD11b<sup>+</sup> cells which correspond to the Langerhans cells (LCs), (c) the CD24<sup>-</sup>CD11b<sup>-</sup> cells and (D) the CD24<sup>-</sup>CD11b<sup>+</sup> cells which

**Table 4**  
**Absolute numbers of cells (10<sup>3</sup>) and standard deviation per mouse (two ears)**

cDCs			Mono			Mo DCs			Macro			T cells		
Neutro	LCs	CD24 <sup>+</sup> CD11b <sup>-</sup>	CD24 <sup>-</sup> CD11b <sup>-</sup>	CD24 <sup>-</sup> CD11b <sup>+</sup>	P1	P2	P3	P4	P5	CD4 <sup>+</sup>	CD8 <sup>+</sup>	$\gamma\delta^{\text{low}}$	DETC	
Naïve	3.6 ± 1	17 ± 6	2 ± 0.03	9 ± 3	16 ± 1	5.2 ± 2.9	6.7 ± 2.5	17 ± 2.3	7 ± 3.3	0.6 ± 0.4	0.007 ± 0.004	1.9 ± 1.6	8.5 ± 5.8	
Vehicle	11 ± 5.2	24 ± 3	3 ± 0.3	10 ± 978	30 ± 3	4.8 ± 0.8	9.5 ± 1.4	49 ± 11	15 ± 2.8	5.2 ± 2.7	0.03 ± 0.0002	5.3 ± 2.9	37 ± 8	
DNFB	62 ± 3.8	11 ± 3	7 ± 0.3	3 ± 0.6	63 ± 13	83 ± 9.6	53 ± 13	32 ± 1.8	8.3 ± 2.1	4.2 ± 2.4	0.2 ± 0.2	4.1 ± 0.7	16 ± 7	
CHS	144 ± 36	19 ± 0.5	2 ± 0.7	12 ± 1.5	21 ± 13	407 ± 31	231 ± 26	4 ± 2.6	3.4 ± 1.1	26 ± 8.6	19 ± 6.8	10 ± 3.1	23 ± 14	

should be further separated using CD64 and Ly6C. The conventional CD24<sup>-</sup>CD11b<sup>+</sup> dermal DCs correspond to the Ly6C<sup>-</sup>CD64<sup>-</sup> cells (*see* **Note 10**).

6. Monocyte-related cells are gated as follows (*see* Fig. 5 and Tables 2 and 4): within CD45<sup>+</sup> Lin<sup>-</sup> cells, select CD24<sup>-</sup>CD11b<sup>+</sup> cells. Then plot them using Ly6C versus CD64. Remove the Ly6C<sup>-</sup>CD64<sup>-</sup> cells, which correspond to the conventional CD11b<sup>+</sup> dermal DCs. On the remaining cells, plot CD64 versus CCR2 and select CCR2<sup>+</sup>CD64<sup>low</sup> and CCR2<sup>-</sup>CD64<sup>+</sup> populations. Within CCR2<sup>+</sup>CD64<sup>low</sup> cells, by plotting Ly6C versus MHCII, you can distinguish the monocytes (P1, Ly6C<sup>hi</sup>MHCII<sup>-</sup>) and monocyte-derived DCs (P2 and P3, Ly6C<sup>+</sup>MHCII<sup>+</sup> and Ly6C<sup>-</sup>MHCII<sup>+</sup> respectively), whereas within CCR2<sup>-</sup>CD64<sup>+</sup> cells, the macrophage populations (P4, Ly6C<sup>low</sup>MHCII<sup>-</sup> and P5, Ly6C<sup>low</sup>MHCII<sup>+</sup>) can be distinguished.
7. T cells are gated as follows: Remove non T cells using dump channel negative cells (APCCy7<sup>-</sup>) within CD45<sup>+</sup> cells (*see* Fig. 3b).  $\gamma\delta$  and  $\alpha\beta$  T cells can be separated by plotting TCR $\beta$  versus TCR $\gamma\delta$  (*see* Fig. 6 and Tables 3 and 4). TCR $\gamma\delta^{\text{hi}}$  correspond to the dendritic epidermal T cells (DETC), TCR $\gamma\delta^{\text{intermediate}}$  correspond to the dermal  $\gamma\delta$  T cells and TCR $\beta^+$  correspond to the conventional  $\alpha\beta$  T lymphocytes. Conventional  $\alpha\beta$  T lymphocytes can be further divided into CD4<sup>+</sup> and CD8<sup>+</sup> T cells (*see* Fig. 6 and Tables 3 and 4).
8. Neutrophils are gated as follows: As Ly6G was included in the dump channel (*see* Table 2), within CD45<sup>+</sup> cells, neutrophils can be discriminated as CD11b<sup>+</sup> Ly6G<sup>+</sup>/Dump<sup>+</sup> cells (*see* Fig. 3c and Tables 2 and 4).

---

## 4 Notes

1. Appropriate controls should be added such as vehicle only for sensitization and elicitation (called Vehicle group on the figures and Table 4), DNFB 0.2% on ear skin with previous sensitization with vehicle on the dorsal skin (called DNFB group on Figs. 1–6 and Table 4) as well as untouched skin (called Naïve in Figs. 1–6 and Table 4).
2. If you wish to separate the dermis from the epidermis prior to the digestion treatment, ears can be treated with dispase II (from *Bacillus polymyxa* grade 2). Briefly, internal and external faces of the ears are separated with forceps and laid on 1 mL of 0.4 mg/mL (or 1000 CU/mL) dispase (diluted in PBS) and incubated 1–2 h at 37 °C or on 1 mL of 0.2 mg/mL dispase and incubated overnight at 4 °C. Take each ear half from the dispase solution, dry it on a paper towel and using forceps pull



off the epidermis from the dermis as well as the cartilage layer from the internal face, before proceeding to the digestion step. To digest further, the epidermis and dermis are laid on 500  $\mu$ L of collagenase D/4 from *Clostridium histolyticum* (5 mg/mL in RPMI with 0.05 % DNase) and incubated 1–2 h at 37 °C. Then tissues are processed using the Medimachine.

3. If your experiments require that you extract cells from flank or back skin of mice, we recommend shaving the skin. Then on a 1 cm<sup>2</sup> piece of skin, remove the subcutaneous fat using fine forceps and a scalpel. Using a 300  $\mu$ L insulin microneedle, inject the skin biopsy five to ten times with Liberase TL-DNase working solution and place it on 1 mL of Liberase TL-DNase working solution in a 24-well plate (dermal side down). Incubate for 2 h at 37 °C in a cell culture incubator (5 % CO<sub>2</sub>) and proceed with the grinding as described for the ears (*see* step 3.3).
4. If the cells need to be restimulated *in vitro* to assess cytokine production, at this step the cell pellet should be resuspended with 1 mL of complete medium (basic medium complemented with 10 % FBS). Cytokine-producing T cell subsets can be easily distinguished using intracellular FACS staining to detect IL-17 and IFN- $\gamma$  cytokines [16, 17].
5. The antibody mix provided in Tables 1, 2 and 3 can be used with the appropriate FACS device with four lasers. In our case, most of the experiments are done using an LSRII for analysis and a FACS-ARIA for sorting.
6. In the past we have used CD207 to discriminate DC subsets but as it requires intracellular staining, we now use CD24 whose expression correlates perfectly with CD207 and whose staining is extracellular. This allows further *ex vivo* functional assays as well as transcriptomic analysis.
7. For *ex vivo* functional assays, cells are sorted using a flow cytometer and collected in 5 mL tubes containing 2 mL of Sorting FACS buffer (10 % FCS EDTA-PBS).
8. For microarray analysis, cells are sorted using a flow cytometer and collected in RNase free Eppendorf tubes containing 90  $\mu$ L of RLT plus buffer for further RNA extraction using a Qiagen microkit plus.
9. XCR1 allows to distinguish cross-presenting DCs in all tissues and lymphoid organs including the skin [19–21]. Until very recently, there was no antibody to track them and instead we were staining them with a dimeric molecule containing XCL1, which is the ligand for XCR1 fused with the fluorescent molecule mCherry (XCL1-vaccibody) which can also be used for very efficient targeting-based vaccine [21, 22].

10. Part of the Ly6C<sup>-</sup>CD64<sup>-</sup> cells may produce aldehyde dehydrogenase (ALDH), which is involved in Treg induction [23, 24].

---

## Acknowledgment

This work is supported by CNRS, INSERM and PIOF-GA-2013-625328-McTaPATH to S.H.

## References

1. Peiser M, Tralau T, Heidler J, Api AM, Arts JH, Basketter DA, English J, Diepgen TL, Fuhlbrigge RC, Gaspari AA et al (2012) Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. *Cell Mol Life Sci* 69(5):763–781
2. Saint-Mezard P, Krasteva M, Chavagnac C, Bosset S, Akiba H, Kehren J, Kanitakis J, Kaiserlian D, Nicolas JF, Berard F (2003) Afferent and efferent phases of allergic contact dermatitis (ACD) can be induced after a single skin contact with haptens: evidence using a mouse model of primary ACD. *J Invest Dermatol* 120(4):641–647
3. Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF (2009) Effector and regulatory mechanisms in allergic contact dermatitis. *Allergy* 64(12):1699–1714
4. Kehren J, Desvignes C, Krasteva M, Ducluzeau MT, Assossou O, Horand F, Hahne M, Kagi D, Kaiserlian D, Nicolas JF (1999) Cytotoxicity is mandatory for CD8(+) T cell-mediated contact hypersensitivity. *J Exp Med* 189(5):779–786
5. Steinman RM, Cohn ZA (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137(5):1142–1162
6. Brasel K, De Smedt T, Smith JL, Maliszewski CR (2000) Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* 96(9):3029–3039
7. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176(6):1693–1702
8. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH (2007) Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* 179(11):7577–7584
9. Henri S, Guillemins M, Poulin LF, Tamoutounour S, Ardouin L, Dalod M, Malissen B (2010) Disentangling the complexity of the skin dendritic cell network. *Immunol Cell Biol* 88(4):366–375
10. Henri S, Poulin LF, Tamoutounour S, Ardouin L, Guillemins M, de Bovis B, Devilard E, Viret C, Azukizawa H, Kissenpfennig A et al (2010) CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med* 207(1):189–206
11. Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N, Tripp CH, Douillard P, Leserman L, Kaiserlian D et al (2005) Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* 22(5):643–654
12. Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, Helft J, Chow A, Elpek KG, Gordonov S et al (2012) Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* 13(11):1118–1128
13. Malissen B, Tamoutounour S, Henri S (2014) The origins and functions of dendritic cells and macrophages in the skin. *Nat Rev Immunol* 14(6):417–428
14. Tamoutounour S, Guillemins M, Montanana Sanchis F, Liu H, Terhorst D, Malosse C, Pollet E, Ardouin L, Luche H, Sanchez C et al (2013) Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* 39(5):925–938
15. Terhorst D, Chelbi R, Wohn C, Malosse C, Tamoutounour S, Jorquera A, Bajenoff M,

- Dalod M, Malissen B, Henri S (2015) Dynamics and transcriptomics of skin dendritic cells and macrophages in an imiquimod-induced, biphasic mouse model of psoriasis. *J Immunol* 195(10):4953–4961
16. Naik S, Bouladoux N, Linehan JL, Han SJ, Harrison OJ, Wilhelm C, Conlan S, Himmelfarb S, Byrd AL, Deming C et al (2015) Commensal-dendritic-cell interaction specifies a unique protective skin immune signature. *Nature* 520(7545):104–108
  17. Naik S, Bouladoux N, Wilhelm C, Molloy MJ, Salcedo R, Kastenmuller W, Deming C, Quinones M, Koo L, Conlan S et al (2012) Compartmentalized control of skin immunity by resident commensals. *Science* 337(6098):1115–1119
  18. Ramirez-Valle F, Gray EE, Cyster JG (2015) Inflammation induces dermal Vgamma4+ gammadeltaT17 memory-like cells that travel to distant skin and accelerate secondary IL-17-driven responses. *Proc Natl Acad Sci U S A* 112(26):8046–8051
  19. Bachem A, Guttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, Salama A, Movassaghi K, Opitz C, Mages HW et al (2010) Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J Exp Med* 207(6):1273–1281
  20. Crozat K, Guiton R, Contreras V, Feuillet V, Dutertre CA, Ventre E, Vu Manh TP, Baranek T, Storset AK, Marvel J et al (2010) The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. *J Exp Med* 207(6):1283–1292
  21. Crozat K, Tamoutounour S, Vu Manh TP, Fossum E, Luche H, Ardouin L, Guillems M, Azukizawa H, Bogen B, Malissen B et al (2011) Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the CD8alpha+ type. *J Immunol* 187(9):4411–4415
  22. Terhorst D, Fossum E, Baranska A, Tamoutounour S, Malosse C, Garbani M, Braun R, Lechat E, Cramer R, Bogen B et al (2015) Laser-assisted intradermal delivery of adjuvant-free vaccines targeting XCR1+ dendritic cells induces potent antitumoral responses. *J Immunol* 194(12):5895–5902
  23. Guillems M, Crozat K, Henri S, Tamoutounour S, Grenot P, Devilard E, de Bovis B, Alexopoulou L, Dalod M, Malissen B (2010) Skin-draining lymph nodes contain dermis-derived CD103(–) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood* 115(10):1958–1968
  24. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204(8):1775–1785

Inflammation

Methods and Protocols

Clausen, B.E.; Laman, J.D. (Eds.)

2017, XVI, 464 p. 110 illus., 32 illus. in color., Hardcover

ISBN: 978-1-4939-6784-1

A product of Humana Press