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⁺ 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 determine 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⁺ T cells, CD8⁺ T cells, γδ 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 reexposition 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, γδ T cells, αβ T cells mainly CD4+ 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).
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 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.


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 (Filcon).

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 cm² 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).

Nicolas Bouladoux et al.

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 μL 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.
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 μL 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).
3. Incubate for 1:45 h at 37 °C in a cell culture incubator (5 % CO₂).

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.
1. Collect the cell suspension from the Medicon using a 20 mL syringe and rinse the Medicon with 8 mL of DNase working solution.

2. Proceed to the filtration in a 15 mL tube using 50 μm syringe filter (Filcon).

3. Centrifuge for 5 min at 450 × g and 4 °C.

4. 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+ Lin−, T cell subsets were further divided according to their expression of γδ and β TCR. DETC correspond to TCRγδ\textsuperscript{high}, whereas dermal γδ cells correspond to TCRγδ\textsuperscript{intermediate}. Conventional CD4+ and CD8+ T cells are found after pre-gating on TCRβ 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 × g.

4. Add Fc block (0.025 μg/mL).
5. Incubate for 10 min on ice.
6. Centrifuge for 5 min at 400 × g.
7. Add 100 μL 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 × 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 μm 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+ cells (see Fig. 3).
4. Gate on DCs and monocyte-related cells as dump channel negative cells (APCCy7-) within CD45+ cells (see Fig. 3a).
5. DC subsets are gated as follows (see Fig. 4 and Tables 1 and 4): within CD45+ Lin- cells, select MHCII+ CD11clow to high. Then plot them using CD24 versus CD11b (see Note 6).

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

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Name</th>
<th>Clone</th>
<th>Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet laser (405 nm)</td>
<td>SytoxBlue</td>
<td>Viability</td>
<td>Lifetechnologies</td>
</tr>
<tr>
<td></td>
<td>BV610</td>
<td>CD11c</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>BV711</td>
<td>CD11b</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Blue laser (488 nm)</td>
<td>FITC</td>
<td>Ly6C</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Green-Yellow laser (561 nm)</td>
<td>Biotin</td>
<td>CD103</td>
<td>Pharmingen</td>
</tr>
<tr>
<td></td>
<td>PECF594</td>
<td>Streptavidin</td>
<td>Pharmingen</td>
</tr>
<tr>
<td></td>
<td>PECy5</td>
<td>CD24</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>PECy5.5</td>
<td>CD45</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>XCR1</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Red laser (633 nm)</td>
<td>Alexa700</td>
<td>MHCII</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>Ly6G</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>CD19</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>NK1.1</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>CD3</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td>APC</td>
<td>CD64</td>
<td>BD</td>
</tr>
</tbody>
</table>

Nicolas Bouladoux et al.
Table 3
mAbs used for FACS analysis of T cell subsets

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Name</th>
<th>Clone</th>
<th>Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet laser</td>
<td>SytoxBlue</td>
<td></td>
<td>Life Technologies</td>
</tr>
<tr>
<td>(405 nm)</td>
<td>BV650</td>
<td></td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>BV421</td>
<td></td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Blue laser</td>
<td>FITC</td>
<td></td>
<td>Pharmingen</td>
</tr>
<tr>
<td>(488 nm)</td>
<td>Ly6C</td>
<td>AL-21</td>
<td>Phamingen</td>
</tr>
<tr>
<td>Green-yellow laser (561 nm)</td>
<td>Biotin</td>
<td>CD103</td>
<td>Phamingen</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td></td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>PECE5.5</td>
<td></td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td></td>
<td>R&amp;D</td>
</tr>
<tr>
<td>Red laser</td>
<td>Alexa700</td>
<td>MHCII</td>
<td>BioLegend</td>
</tr>
<tr>
<td>(633 nm)</td>
<td>APCCy7</td>
<td></td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>Ly6G</td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>CD19</td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>NK1.1</td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>CD3</td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>APCCy7</td>
<td>CD64</td>
<td>BioLegend</td>
</tr>
<tr>
<td></td>
<td>APC</td>
<td></td>
<td>BD</td>
</tr>
</tbody>
</table>

Four distinct populations should be found: (a) the dermal CD24+CD11b− cells which can be further divided using XCR1 and CD103 (see Note 9), (b) The CD24+CD11b+ cells which correspond to the Langerhans cells (LCs), (c) the CD24−CD11b− cells and (D) the CD24−CD11b+ cells which
<table>
<thead>
<tr>
<th></th>
<th>cDCs</th>
<th>Mono</th>
<th>Mo DCs</th>
<th>Macro</th>
<th>T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutro</td>
<td>LCs</td>
<td>CD24+</td>
<td>CD24-</td>
<td>CD11b-</td>
</tr>
<tr>
<td>Naïve</td>
<td>3.6 ± 1</td>
<td>17 ± 6</td>
<td>2 ± 0.03</td>
<td>9 ± 3</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Vehicle</td>
<td>11 ± 5.2</td>
<td>24 ± 3</td>
<td>3 ± 0.3</td>
<td>10 ± 978</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>DNFB</td>
<td>62 ± 3.8</td>
<td>11 ± 3</td>
<td>7 ± 0.3</td>
<td>3 ± 0.6</td>
<td>63 ± 13</td>
</tr>
<tr>
<td>CHS</td>
<td>144 ± 36</td>
<td>19 ± 0.5</td>
<td>2 ± 0.7</td>
<td>12 ± 1.5</td>
<td>21 ± 13</td>
</tr>
</tbody>
</table>
should be further separated using CD64 and Ly6C. The conventional CD24⁻CD11b⁺ dermal DCs correspond to the Ly6C⁺CD64⁻ cells (see Note 10).

6. Monocyte-related cells are gated as follows (see Fig. 5 and Tables 2 and 4): within CD45⁺ Lin⁻ cells, select CD24⁻CD11b⁺ cells. Then plot them using Ly6C versus CD64. Remove the Ly6C⁺CD64⁻ cells, which correspond to the conventional CD11b⁺ dermal DCs. On the remaining cells, plot CD64 versus CCR2 and select CCR2⁺CD64low and CCR2⁻CD64⁺ populations. Within CCR2⁺CD64low cells, by plotting Ly6C versus MHCII, you can distinguish the monocytes (P1, Ly6C⁺MHCII⁻) and monocyte-derived DCs (P2 and P3, Ly6C⁻MHCII⁺ and Ly6C⁺MHCII⁺ respectively), whereas within CCR2⁻CD64⁺ cells, the macrophage populations (P4, Ly6C⁺MHCII⁻ and P5, Ly6C⁺MHCII⁺) can be distinguished.

7. T cells are gated as follows: Remove non T cells using dump channel negative cells (APCCy7⁻) within CD45⁺ cells (see Fig. 3b). γδ and αβ T cells can be separated by plotting TCRβ versus TCRγδ (see Fig. 6 and Tables 3 and 4). TCRγδhi correspond to the dendritic epidermal T cells (DETC), TCRγδintermediate correspond to the dermal γδ T cells and TCRβ⁺ correspond to the conventional αβ T lymphocytes. Conventional αβ T lymphocytes can be further divided into CD4⁺ and CD8⁺ 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⁺ cells, neutrophils can be discriminated as CD11b⁺ Ly6G⁺/Dump⁺ 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 in 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 polymyx grades 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 μl 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 cm² piece of skin, remove the subcutaneous fat using fine forceps and a scalpel. Using a 300 μl 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% CO₂) 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-γ 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 μL 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\(^{-}\)CD64\(^{-}\) 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-MeTaPATH to S.H.

**References**


15. Terhorst D, Chelbi R, Woh C, Malosse C, Tamoutounour S, Jorquera A, Bajenoff M,


Inflammation
Methods and Protocols
Clausen, B.E.; Laman, J.D. (Eds.)
2017, XVI, 464 p. 110 illus., 82 illus. in color., Hardcover
ISBN: 978-1-4939-6784-1
A product of Humana Press