Video Article

Lymphocyte Isolation from Human Skin for Phenotypic Analysis and *Ex Vivo* Cell Culture

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Abstract

Human skin has an important barrier function and contains various immune cells that contribute to tissue homeostasis and protection from pathogens. As the skin is relatively easy to access, it provides an ideal platform to study peripheral immune regulatory mechanisms. Immune resident cells in healthy skin conduct immunosurveillance, but also play an important role in the development of inflammatory skin disorders, such as psoriasis. Despite emerging insights, our understanding of the biology underlying various inflammatory skin diseases is still limited. There is a need for good quality (single) cell populations isolated from biopsied skin samples. So far, isolation procedures have been seriously hampered by a lack of obtaining a sufficient number of viable cells. Isolation and subsequent analysis have also been affected by the loss of immune cell lineage markers, due to the mechanical and chemical stress caused by the current dissociation procedures to obtain single cell suspension. Here, we describe a modified method to isolate T cells from both healthy and involved psoriatic human skin by combining mechanical skin dissociation using an automated tissue dissociator and collagenase treatment. This methodology preserves expression of most immune lineage markers such as CD4, CD8, Foxp3 and CD11c upon the preparation of single cell suspensions. Examples of successful $CD4^+$ T cell isolation and subsequent phenotypic and functional analysis are shown.

Video Link

The video component of this article can be found at https://www.jove.com/video/52564/

Introduction

The skin, as the primary interface between the body and the environment, provides the first line of defence against external physical, chemical and biological insults such as wounding, ultraviolet radiation and micro-organisms. Skin comprises two main compartments, the epidermis and the dermis, and contains a variety of immune cells including Langerhans cells, macrophages, dendritic cells (DCs), and about 20 billion memory T cells, nearly twice the number present in the entire blood volume[1,2]. A growing body of data supports the notion that the skin has essential immunological functions, both during tissue homeostasis and in various pathological conditions. Immune cells resident in normal skin are thought to conduct immunosurveillance[3] and have been shown to play a role in the development of inflammatory disorders such as psoriasis[4]. In psoriatic lesional skin, both $CD4^+$ and $CD8^+$ infiltrated T cells were observed and it was shown that the ratio of the CD4 and CD8 varies depending on the disease status[5]. However, these populations of cells are difficult to study because existing techniques allow the isolation of only few cells.

The currently widely used techniques for T cell isolation from human skin combine mechanical skin dissociation with enzymatic treatment. Human skin biopsies are extensively minced and incubated with enzymes like trypsin, collagenase and/or EDTA[6-8]. Considering that skin is a barrier tissue which is highly resistant to tensile forces and mechanical disaggregation, the established methods of T cell isolation produced very few cells, and even lower numbers of viable cells, which makes *ex vivo* cell culture of these cell populations difficult and challenging.

Here, we report a modified method to isolate lymphocytes from both healthy and involved psoriatic human skin by combining mechanical dissociation of the skin using an automated tissue dissociator instead of the established method of extensively mincing, together with enzymatic digestion using collagenase. Various viable immune cell subsets including DCs and T cells were observed after preparation of a single-cell suspension. Importantly the expression of the surface markers CD3, CD4 and CD8 was well preserved. Cells thus prepared, are ready for use in *ex vivo* cell cultures or flow cytometric analysis. This protocol has been successfully employed for the analysis of single skin biopsies (4 mm) derived from lesional skin of psoriasis patients. Results showed that skin resident patient T cells produced more inflammatory cytokines like IL-17 and IFNγ in comparison to healthy volunteers[9].

Protocol

NOTE: Skin biopsies from healthy individuals were obtained from abdominal skin leftover of individuals undergoing elective plastic surgery after oral or written informed consent for scientific use. The use of human skin was approved and in accordance with the regulations set by the Medical Ethical Committees for human research of the Radboud university medical centre, Nijmegen, the Netherlands and University of Essen, Germany.

1. Preparation of Single Cell Suspensions from Human Skin (Work Sterile in a Flow-cabinet if Subsequent Cell Culture is Required)

1. Prepare cell culture medium: RPMI 1640 + penicillin/streptomycin (final concentrations 100 units/ml and 100 µg/ml, respectively) + pyruvate (0.02 mM) and glutamax (0.02 mM), with no serum added.
2. Prepare complete culture medium: culture medium prepared in step 1.1 + 10% human pooled serum (HPS); store at 4°C. Bring medium to 20 °C ± 2 before using.
3. Obtain the skin biopsy using a 4mm round biopsy punch instrument and keep it in RPMI1640 complete culture medium at 20 °C ± 2 for up to 4 hr or at 4 °C ON. Process the biopsy as soon as possible upon arrival in the laboratory.
   NOTE: Longer storage of skin will influence the cell yield and cell viability.
4. Label a blue-capped dissociation tube and add 5 ml complete culture medium into the labelled tube.
5. Add 2 ml of complete culture medium into each well (in total 3 wells) of a sterile 6-well culture plate. Use sterile tools to place the biopsy into a single well, rinse, move it over to a second well and repeat this step one more time, thus achieving a total of three rinses.
6. Transfer the well rinsed skin biopsy to a sterile Petri dish, add 100 µl of complete culture medium on the top of biopsy, and carefully scrape off the subcutaneous fat tissue using a stainless steel disposable sterile scalpel.
   NOTE: This is a critical step.
7. Cut each skin biopsy into 4 smaller pieces on a sterile Petri dish. Transfer samples (up to four of 4 mm biopsies per tube) to the prepared dissociation tube containing 5 ml of complete culture medium.
8. Tightly close the tubes with the cap, and attach upside down to the sleeve of the automated tissue dissociator. Make sure that all sample material is located in the area of the rotor.
9. Start the dissociation process by running the “program m_spleen _01” (a pre-defined program provided by the instrument’s internal memory or by the accompanied program card) to dissociate the biopsy at the appropriate rotating speed for 56 sec.
10. After processing, detach the dissociation tube from the dissociator and make sure that all the dissociated material is collected at the bottom of the tube.
11. Add 150 µl collagenase I-A (80 mg/ml) into the dissociation tube and incubate the sample in a shaking water bath at 37 °C for 60 min. Add 100 µl of DNase I (5 MU/ml) into thedissociation tube, mix well.
    NOTE: Higher concentration of collagenase or longer incubation time will alter cell viability.
12. Attach the dissociation tube to the sleeve of the automated tissue dissociator and run the “*program m_ spleen _01”* to dissociate the biopsy one more time.
13. Place a 70 µM nylon cell strainer on the top of a 50 ml Falcon tube. Apply dissociated sample materials to this cell strainer to remove cell clumps/tissue debris.
14. Wash cell strainer once with 5 ml of complete culture medium. Centrifuge at 20 °C ± 2, 450 x g for 10 min and aspirate supernatant.
15. Repeat the washing step one more time. Resuspend cell pellets in 300 µl of complete culture medium. Single-cell suspensions are ready for further analysis; continue with protocols for *ex vivo* cell culture or flow cytometry analysis.
16. In case of further intracellular cytokine staining, stimulate cells with PMA (12.5 ng/ml), Ionomycin (500 ng/ml) and Brefeldine A (5 µg/ml) for 4 hr at 37 °C, 5% CO_2 incubator for 4 hr before performing flow cytometry analysis.

2. Polyclonal Activation of Skin-Resident T Cells (*Ex Vivo* Cell Culture)

1. Aliquot 100 µl single-cell suspensions (prepared in step 1.15) into a round-bottom 96-well plate.
   NOTE: For each of 4 mm skin biopsy, the acquired single-cell suspensions can be split into at least two wells of a 96-well plate.
2. Add anti-CD3/anti-CD28 mAb-coated microbeads (25,000 beads per well), recombinant human cytokine rIL-2 (final concentration 25 U/ml) and rIL-1β (final concentration 50 ng/ml).
3. Cover the culture plate with a well-labelled plate lid, incubate in 5% CO_2, 100% humidity, 37 °C incubator. Change medium when the medium colour turns to yellow.
   NOTE: A clear cell colony can be observed from day 8 of cell culture.

3. Flow Cytometry Analysis of Primary/Cultured Skin-Resident T Cells

1. Prepare FACS buffer: PBS + 0.2% BSA.
2. Transfer cells of interest into a v-bottom 96-well plate. Centrifuge at 20 °C ± 2, 450 x g for 2 min, and aspirate supernatant.
3. Resuspend the pellet in 100 µl of PBS. Centrifuge at 20 °C ± 2, 450 x g for 2 min, and aspirate supernatant.
4. Stain cells with 100 µl of prepared eFluorescence780 conjugated fixable viability dye (1:1,000 dilution using PBS) at 4 °C for 30 min. Add 100 µl of FACS buffer, centrifuge at 20 °C ± 2, 450 x g for 2 min, and aspirate supernatant.
5. Select required cell surface marker mAbs, for example: CD45-BV421 (HI30, 1:20), CD3-ECD(UCHT1, 1:50), CD4-PC5.5(1388.2, 1:200), CD8-APC-AlexFluo700(B9.11, 1:400), CD14-FITC(TUK4, 1:50), CD19-APC-AlexFluo750(13-119, 1:50), CD56-PE(MEM-188, 1:50), CD25-PeCy7(BC96, 1:50), CD11c-PeCy7(BU15, 1:10), and CD1c-APC(AD5-8E7, 1:10), prepare the mAbs-mixture using FACS buffer according to each mAb dilution factor tested. Define gate settings using isotype control of antibody together with non-stain sample.
6. Add 25 µl of prepared mAbs-mixture into each well. Incubate 20 min at 20 °C ± 2, protect from light.

7. Add 100 µl of FACS buffer, centrifuge at 20 °C ± 2, 450 x g for 2 min, and aspirate supernatant.
8. For samples that only require cell surface staining, continue with step 3.16.
9. In case of intra-cellular Foxp3 staining:
    1. Prepare fixation and permeabilization buffer by mixing one part of concentrate with 3 parts of diluents.
    2. Prepare 1x permeabilization buffer: 1 part of 10x permeabilization buffer + 9 parts of sterilized H_2O.
10. Resuspend pellets in 100 µl of fixation and permeabilization buffer, mix well, and incubate at 4 °C for 30 min.
11. Add 100 µl permeabilization buffer to each well, centrifuge at 450 x g at RT for 2 min, and aspirate supernatant.
12. Wash cells with permeabilization buffer one more time.
13. Select required intracellular mAbs, for example, Foxp3-eFluo450(PCH101, 1:50), IL-17A-AlexFluo488(eBio64DEC1, 1:50) and IFNγ-PeCy7(4S.B3, 1:400). Prepare the mAbs mixture using permeabilization buffer containing 2% normal rat serum according to each mAb dilution factor tested. Define gate settings using isotype control of antibody together with non-stain sample.
14. Add 20 µl of prepared mAb-mixture into each well. Incubate at 4 °C for 30 min, protect from light.
15. After 30 min incubation, add 100 µl of permeabilization buffer into each well. Centrifuge at 20 °C ± 2, 450 x g for 2 min, and aspirate supernatant.
16. Wash cells with permeabilization buffer one more time. Resuspend pellet in 110 µl of FACS buffer, and transfer cell suspensions into microFACS tube.
    NOTE: Sample is ready for measurement using 10-colour flow cytometry.
17. In case of exact cell number required, add 10 µl of well-mixed uniform suspension of fluorospheres into each sample immediately before performing measurements using flow cytometry.

Representative Results

The protocol presented here will yield between 2,200 ± 615 (mean ± SEM, skin of healthy volunteers) up to 178,000 ± 760 (mean ± SEM, lesional skin of psoriasis patients) viable lymphocytes from human skin when using a single 4 mm skin biopsy.

Different types of $CD45^+$ cells were identified in single-cell suspensions derived from skin of healthy individuals including $CD4^+$ T-cells (~45%), $CD8^+$ T-cells (~30%), and $CD11c^+$ DCs (~5%), whereas few B cells ($CD19^+$), NK cells ($CD56^+CD3^-$), or monocytes/macrophages ($CD14^+$ or $CD1c^+$) were observed (**Figure 1A**). The methodology also allows for the analysis of $CD4^+CD25^+Foxp3^+$ cells in human skin biopsies. By using intra-cellular staining after fixing and permeabilization, it is possible to demonstrate expression of the transcription factor Foxp3 in $CD25^+$ T cells (**Figure 1A**).

Single-cell suspensions derived from human skin biopsies showed a high autofluorescence background in FL1 (FITC), FL2 (PE), FL3 (ECD), FL9 (pacific blue) and FL10 (chrome orange) channels using a cytometer (data not shown). For proper analysis of the lymphocyte population, we therefore recommend the use of an anti-human CD45 mAb conjugated with a Brilliant Violet 421 fluorochrome for gating of the lymphocyte population and exclusion of the autofluorescent cell population (**Figure 1B**). The majority of resident cells in human skin were $CD3^+$ T cells (**Figure 1C**). For cell viability analysis it is recommended to use a fixable viability dye to distinguish viable from dead/dying cells (**Figure 1C**). Typically this protocol results in 65.3% ± 7.7 (Mean ± SD) viable cells. For further analysis of the flow cytometry data, it is advised to gate on the viable cell population, since dead or dying cells lose their cell membrane integrity, and can non-specifically bind conjugated antibody, thereby increasing background staining.

T cells isolated by this protocol are well suited for further functional analysis. By using a single 4 mm biopsy of lesional skin of psoriasis patients, it was shown that the biopsy derived T cells produced IL-17A and IFNγ following 4 hr stimulation with PMA/ionomycin in the presence of Brefeldin A (**Figure 2**).

Freshly prepared single-cell skin suspensions from involved psoriatic skin lesions can be used also for further *ex vivo* cell culture and subsequent functional analysis. Following expansion after polyclonal stimulation with anti-CD3/anti-CD28 mAb-coated microbeads in the presence of the pro-inflammatory cytokines IL-1β or IL-23 for 8 days, the cells can *e.g.* be analysed for their cytokine producing capacity (**Figure 3**). By doing so, a clear difference between the cytokine producing potential of cells derived from skin of healthy and psoriatic individuals was observed. T cells from psoriatic individuals showed a much higher capacity to produce the psoriasis associated cytokines IL17A and IFNγ. This implies that even after *ex vivo* culture a prototypic psoriasis cytokine phenotype is maintained, absent in case of healthy controls.

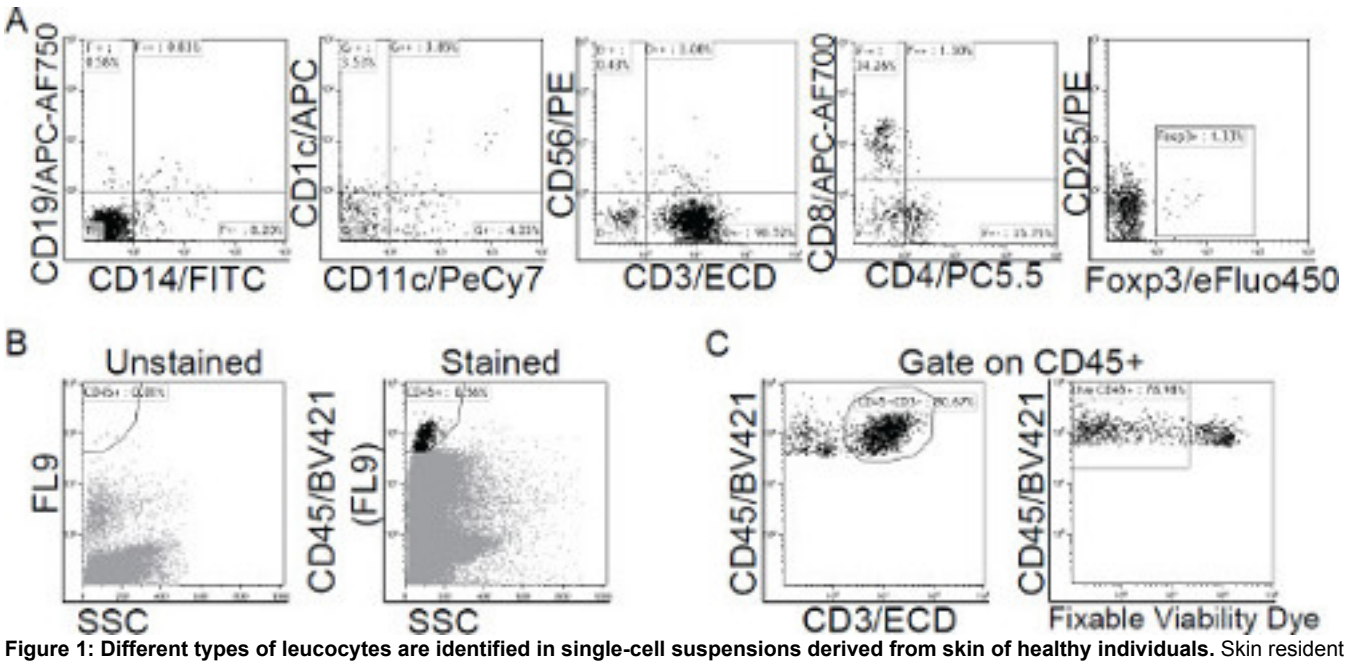

Figure 1: Different types of leucocytes are identified in single-cell suspensions derived from skin of healthy individuals. Skin resident lymphocytes isolated using the protocol described in the text were stained with the fluorochrome-conjugated mAbs of interest plus fixable viability dye and immediately analysed by flow cytometry. (**A**) Flow cytometric detection of the monocytes (CD14), DCs (CD11c), B cells (CD19), NK cells($CD56^+CD3^-$), $CD4^+$, $CD8^+$ and $CD25^+Foxp3^+$ subsets within skin resident lymphocytes. Anti-Human CD45/BV421 mAb distinguishes lymphocytes from autofluorescent cells. The gating strategies used for $CD45^+$ cells is shown in (**B**). (**C**) Percentage of viable $CD45^+CD3^+$ T cells after isolation. Number shows the percentage of positive cells. A representative experiment out of three different experiments/donors is shown. Please click here to view a larger version of this figure.

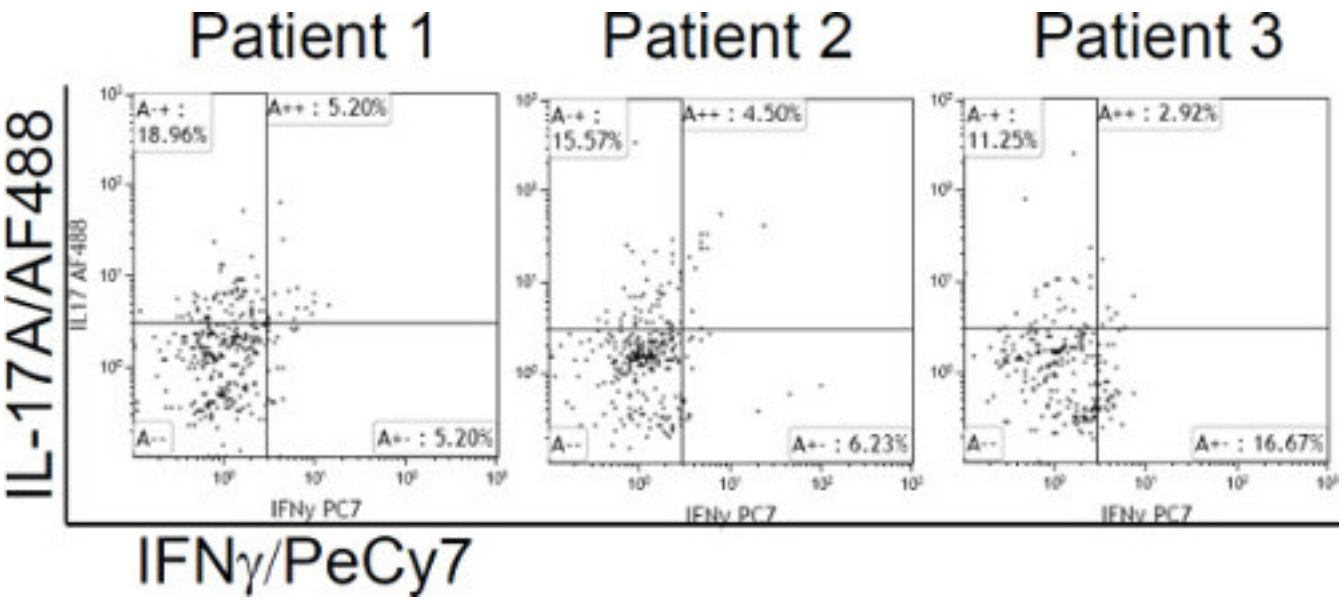

Figure 2: T cells derived from lesional skin of psoriasis patients can produce IL-17A and IFNγ. A single 4 mm skin biopsy was taken from the lesional skin of psoriasis patient upon orally or written informed consent for scientific use. The skin resident T cells were isolated using the protocol described in the text. Production of IL-17A and IFNγ by skin resident T cells. Intracellular accumulation of cytokines in response to 4 hr stimulation with PMA/ionomycin in the presence of Brefeldin A was measured by flow cytometry. Percentage of cytokine-positive cells is shown. Data of three different patients are shown. Please click here to view a larger version of this figure.

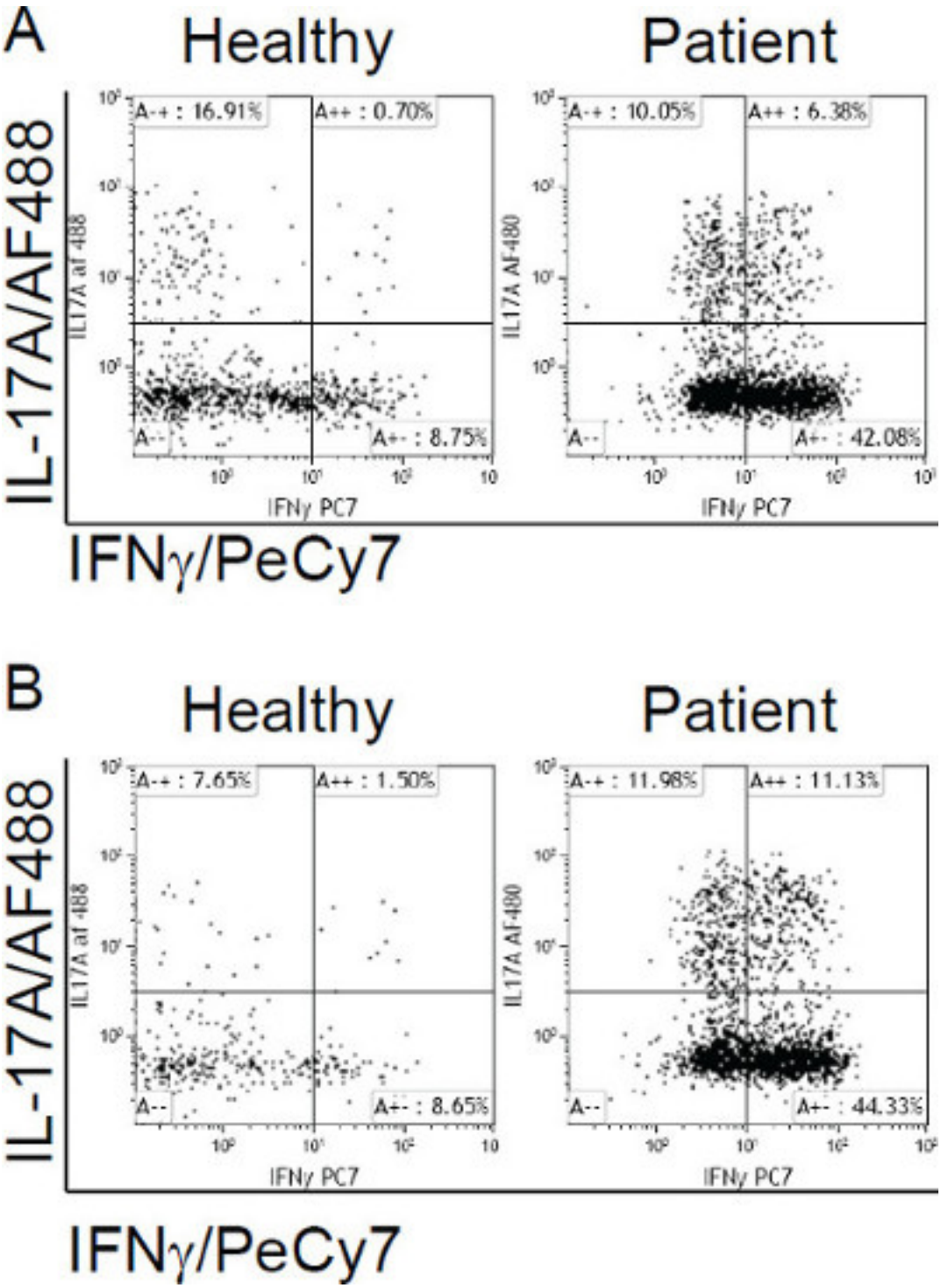

Figure 3: Skin-resident T cells are capable to produce IL-17A and IFNγ following *ex vivo* polyclonal stimulation. Skin resident cells derived from a 4 mm skin biopsy of healthy or psoriasis patient were prepared using the protocol described in the text. Thereafter, cells were stimulated with anti-CD3/anti-CD28 mAb-coated microbeads in the presence of either IL-1β (50 ng/ml, **A**) or IL-23 (50 ng/ml, **B**) for 8 days. Next, the cells were stimulated for 4 hr with PMA/ionomycin in the presence of Brefeldin A and thereafter analyzed for intracellular cytokine production by flow cytometry. A representative example out of three independent experiments/donors is shown. Please click here to view a larger version of this figure.

Discussion

Here, we present a protocol to efficiently isolate skin resident T cells from human skin biopsies. The advantage of this protocol is the isolation of relatively high numbers of viable lymphocytes, and expressing relevant surface markers. The cell subsets identified were: $CD11c^+$ DCs, $CD4^+$

and $CD8^+$ T cells and $Foxp3^+CD25^+$ cells. Importantly, *ex vivo* culture of isolated skin resident T cells was very well feasible and allowed for subsequent functional analysis.

Human skin can be dissociated into single-cell suspensions by combining mechanical dissociation with enzymatic degradation of the extracellular adhesion proteins which function to maintain the structural integrity of tissues. Although dispase based layer separation of the epidermis and dermis is a widely used method to determine cell infiltration in these respective skin layers, we noticed that dispase treatment led to a major reduction of cell surface expression of the CD25 and CD27(not shown). As these markers are important to characterize lymphocyte subsets, we believe that dispase treatment influences subsequent immunophenotyping. It is not likely that the removal of the dermis is responsible for the observed reduction in CD25 or CD27 expressing cells, since in skin of healthy individuals the majority of T cells is localized in the dermis while minute T cell numbers are present in the epidermis[10-12]. The detrimental effect of dispase on the proliferation capacity of isolated keratinocytes has been reported previously[13]. Therefore, prudent use of dispase is warranted if cell function is the aim of the study. In our protocol we have been using Collagenase type I to obtain single cell suspension of skin biopsies. Although Collagenase I has high tryptic activity compared to Collagenase IV and thus is more harsh, we have chosen this particular Collagenase because it has been tested for its suitability for cell culture. The use of a Collagenase IV that is suitable for cell culture might be a future step to further optimize our current protocol.

Due to its nature, the skin is highly resistant to tensile forces and mechanical disaggregation. So far, a comprehensive analysis of immune cells in human skin has been hampered by technical challenges in obtaining enough cell numbers when using relatively harsh digestion protocols. As a consequence, most studies published to date rely on immunohistochemical analyses. The direct isolation of $CD4^+$ T cells from skin biopsies is generally considered cumbersome. An alternative would be the use of skin explant crawl out cultures[10]. However, these take 2-3 weeks of culture, and the skin explant culture medium contains a set selected of cytokines and chemokines, which might lead to preferential crawling out of specific T cell subsets. In addition the culture period might affect the phenotype of the cells. The current protocol does not make use of added cytokines or chemokines, and expression of CD4 (and also other cell markers) is well preserved after the isolation, enabling both phenotypic and functional study of T cell populations directly after isolation.

A commercially available automated tissue dissociator is used for the dissociation of skin fragments before their incubation with collagenase, required for the degradation the extracellular matrix. For the subsequent release of the cells from the extracellular matrix, the automated dissociator is used again. Although extensive manual cutting of skin can yield similar cell numbers (data not shown), the results are less consistent and are more dependent on the performer's experience. Dissociation of skin using the pre-defined procedure offered by the instrument will yield much more reproducible results. Consistent with previous studies showing that skin contains various leukocytes subsets[2,14], $CD11c^+$ DCs, $CD8^+$ and $CD4^+$ T cells, and $Foxp3^+$ cells were observed in single-cell populations prepared by the protocol presented here. Importantly, the protocol yields a relatively high yield of viable lymphocytes. Due to the efficiency of the protocol, it particularly useful when studying patient material, where often only a single 4 mm skin biopsy can be obtained. Using the method, we were able to show that T cells isolated from the lesional skin of psoriasis patients showed a higher capacity to produce IL-17A and IFNγ as compared to healthy skin[9].

Depending on the research question, it may be necessary to further purify certain cell subsets after the preparation of single cell suspensions. In this case, larger pieces of skin can be used to ensure the yield of sufficient cells for subset analyses. When doing so, make sure that the skin should be cut into smaller pieces of around 2 mm x 2 mm before starting the isolation protocol.

In conclusion, the present protocol offers an improved way to isolate skin resident cells, facilitating meaningful phenotypic and functional analysis on a single cell level, thereby improving our insight in skin immune responses.

Critical steps in the current protocol are the proper removal of subcutaneous fat tissue and the cutting of skin into smaller pieces, especially when processing more than one biopsy in a dissociation tube. Fat tissue and/or large tissue pieces will cause the dissociator to work improperly, asking for re-runs. This will seriously harm cell viability. The limitation of the technique is that cell numbers derived from a single 4 mm skin biopsy are not enough for subsequent isolation of subsets of cells.

Disclosures

The authors have nothing to disclose.

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