Experimental concise report

Enhanced isolation of lymphoid cells from human skin

Maryam Salimi¹, Sumithra Subramanian¹, Tharini Selvakumar¹, Xinwen Wang¹², Sophie Zemenides¹, David Johnson³ and Graham Ogg¹*

¹ MRC Human Immunology Unit, NIHR Biomedical Research Centre, Radcliffe Department of Medicine, University of Oxford, UK

² Department of Periodontology and Oral Medicine, School of Stomatology, the Fourth Military Medical University, Xi’an, Shaanxi 710032, People’s Republic of China

³ Department of Plastic and Reconstructive Surgery, John Radcliffe Hospital, Oxford University Hospitals NHS Trust, UK

*correspondence to:

Professor Graham Ogg

graham.ogg@imm.ox.ac.uk

Tel: 0044 1865 222443

Fax: 0044 1865 222502

Key words: Skin, Collagenase, Cell isolation

Manuscript word count: 1000
Abstract

Studying skin immune cells under various pathophysiological conditions is vital for understanding the nature of cutaneous inflammatory responses. Available methods of isolating cells from the skin have relatively low yield or require *in vitro* culture. To increase effective isolation of skin immune cells we used collagenase P treatment. The number of T cells obtained ex vivo using this technique was dramatically greater than conventional methods without the need for long term culture. The phenotype and function of isolated cells were comparable to the cells isolated by EDTA treatment. Collagenase P-based methodology will enhance the ability to investigate lymphoid cell function in healthy and diseased skin.
Introduction

A diverse repertoire of T cells and B cells reside in the skin and indeed it has been estimated that the number of resident T cells in normal skin are almost $2 \times 10^{10}$ which is nearly double the number of T cells in the circulating blood$^{1}$. Established methods using EDTA or collagenase D produce a low yield of cells$^{1, 2}$ and so other approaches have introduced a culture step, for example on the surface of Cellfoam three-dimensional growth matrices for 21 days$^{3}$. Although such culture approaches represent a significant step forward for certain applications, they can introduce potential in vitro changes to the frequency of the isolated cells. Effective isolation of lymphoid cells from the skin is vital for maximising information obtained from skin samples in order to define their role in health and disease. It is clear that T cells play a role in diverse skin surveillance and pathology and understanding their role may contribute to the development of novel therapeutic approaches. Herein we report a new method that gives rise to a far larger number of intact T cells ex vivo from human skin than has previously been possible.

Report

First, T cells were isolated from normal healthy adult skin using EDTA (n=4) and collagenase D digestion (n=4) as described previously$^{2, 4}$ and in the supplementary methods. In addition, we used an alternative approach where skin biopsies were first washed with cold PBS and subcutaneous fat was removed. The biopsies were cut into < 0.5mm pieces, and incubated in RPMI 1640 (Sigma) supplemented with 10% heat inactivated fetal calf serum (FCS) and 2 mM l-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin and 1mg/ml collagenase P (Roche 11213865001) overnight at 37°C. The mixture was then pipetted up and down repeatedly to homogenise the tissue further. To reduce the free DNA fragments,
endonuclease deoxyribonuclease I (DNase I) was added at 200 Kunitz unit/ml (Roche 10104159001) for 15 minutes at room temperature. The tissue was passed through 100μm followed by 70 μm nylon mesh strainers (VWR 734-0004 and 734-0003) and washed with ice-cold 10mM EDTA solution (10x the volume of collagenase solution). After spinning for 20 minutes at 300g at 4°C, the cell pellet was re-suspended in cold RPMI medium containing 10% FCS and passed through a 40μm tissue strainer (VWR 734-0002). Larger samples were further purified using Ficoll density gradient purification.

The cells were then counted using 0.4% trypan blue exclusion. On average, EDTA treatment and collagenase D treatment resulted in the isolation of 2,130±889 and 6,417±927 cells/cm² respectively from the skin biopsies, while the new approach based on collagenase P treatment (n=10) dramatically increased the number of isolated cells to 303,234±68321 cells/cm² (Fig. 1a, Hoechst staining of isolated cells using different protocols is shown on the right).

Depending on naïve T cells first encounter with antigen, activated effector memory cells are imprinted with preferential homing markers. Selective imprinting of T cells is essential for effective recruitment and robust immune responses in tissues and is influenced by the microenvironment and professional antigen presenting cells (APC). To evaluate the phenotype of T cells isolated using collagenase P treatment we compared the expression of skin homing marker cutaneous lymphocyte antigen (CLA) and chemokine receptors CCR4 and CCR10. More than 90% of T cells isolated from the skin expressed CLA and CCR10. CCR4 was expressed on over 70% of T cells. Collagenase P did not alter the expression of homing markers on T cells isolated from the skin (Fig. 1b and 1c).
To ensure that our method did not alter the cell surface expression of CD3, TCR and CD8 markers when compared to EDTA or collagenase D, we investigated expression on the skin resident T cells\(^6\),\(^7\). CD3 and CD8 were expressed at similar proportions in the cells isolated by different methods (Fig. 1d). γδ T cells are another important subset of T cells that are believed to contribute to psoriasis skin inflammation. They are known to be expressed in small numbers in human skin and so we wished to examine whether the new method could detect such a minor population. The percentage of γδ T cells (3.63±0.77% of live cells) detected following collagenase P treatment of healthy skin donors was also similar to earlier reports which used other methods of skin T cell isolation (Fig. 1e)\(^8\),\(^9\).

Further examination of cell surface phenotype showed that most skin resident T cells have a memory phenotype (Fig. 2a). We next compared the ability of cells isolated by collagenase D and collagenase P methods in producing different cytokines after stimulation with PMA/ionomycin for 4 hours by multiplex cytokine array. Cells isolated using collagenase P treatment produced similar or higher amount of cytokines compared to collagenase D treatment (Fig. 2b). Investigation of different sub population of T cells in the skin for their functional integrity after collagenase P digestion showed production of IFNγ, IL-17, IL-22 and IL-13 by activated T cells as determined by ELISA (Fig. 2c). To further demonstrate the efficacy of collagenase P treatment in isolating functionally active T cells, we tested the proliferative capacity of these cells. Greater number of cell divisions was observed in CellTrace Violet-labelled T cells isolated by collagenase P treatment following 5 days of culture with IL-2 (Fig. 2d).

These investigations demonstrate that the cells isolated \textit{ex vivo} from human skin show rapid effector function and that cells producing different cytokines can be identified.
Discussion

Effective isolation of lymphoid cells from human skin *ex vivo* is vital for understanding their role in health and disease. Alternative approaches have been based on cell culture steps, which have been very useful additions to possible methodologies, but require a degree of experience and laboratory infrastructure, and the culture step may introduce potential artefacts. Here we have defined a new method of isolating large number of cells from skin tissue. Using a collagenase P enzymatic treatment step yielded far more cells with intact phenotype and function than conventional collagenase D digestion. Collagenase P is a metalloproteinase which cleaves collagen into smaller peptide fragments. It is secreted by *Clostridium histolyticum*, and has 10 fold higher collagenase activity as well as higher trypic activity. It has been used to isolate pancreatic islet cells, human mesenchymal stem cells (MSC), myocardial fibroblasts. T cells are thought to play a role in the pathogenesis of many common inflammatory skin conditions such as atopic dermatitis, psoriasis and contact dermatitis. Inhibiting T cell function may contribute to the activity of therapeutic intervention. We have developed a novel approach to isolate lymphoid cells from human skin that greatly increases the number of viable cells obtained *ex vivo*. We anticipate that the method will be of value to those studying lymphoid populations of skin resident cells including T cells or innate lymphoid cells in small patient samples.

Learning points

1. Collagenase P treatment isolates a far larger number of T cells *ex vivo* from human skin than has previously been possible.
2. Collagenase P treatment does not modify phenotype and function of isolated T cells from skin biopsies.

3. Collagenase P treatment did not alter the expression of homing markers on T cells isolated from the skin.

4. The percentage of γδ T cells (3.63±0.77% of live cells) detected following collagenase P treatment of healthy skin donors was similar to earlier reports which used other methods of skin T cell isolation.

5. The cells isolated ex vivo from human skin by collagenase P digestion produce cytokines.

6. T cells isolated by collagenase P treatment show high proliferative capacity.

Acknowledgements

We are grateful to the MRC UK, NIHR Biomedical Research Centre Programme, NIHR Clinical Research Network and Barrie Trust for support.
References

1. Clark RA, Chong B, Mirchandani N, et al. The vast majority of CLA+ T cells are resident in normal skin. J Immunol. 2006;176:4431-4439.
2. Campbell JJ, Murphy KE, Kunkel EJ, et al. CCR7 expression and memory T cell diversity in humans. J Immunol. 2001;166:877-884.
3. Clark RA, Chong BF, Mirchandani N, et al. A novel method for the isolation of skin resident T cells from normal and diseased human skin. The Journal of investigative dermatology. 2006;126:1059-1070.
4. Schaefer L, Ebert L, Willimmann K, et al. A skin-selective homing mechanism for human immune surveillance T cells. The Journal of experimental medicine. 2004;199:1265-1275.
5. Ohmori K, Fukui F, Kiso M, et al. Identification of cutaneous lymphocyte-associated antigen as sialyl 6-sulfo Lewis X, a selectin ligand expressed on a subset of skin-homing helper memory T cells. Blood. 2006;107:3197-3204.
6. Mulder MCW, Koenen H, Scheper RJ. Reduced expression of distinct T-cell CD molecules by Collagenase/DNase treatment. Cancer Immunology Immunotherapy. 1994;38:253-258.
7. Abuzakouk M, Feighery C, O'Farrelly C. Collagenase and Dispase enzymes disrupt lymphocyte surface molecules. Journal of immunological methods. 1996;194:211-216.
8. Cai Y, Shen X, Ding C, et al. Pivotal role of dermal IL-17-producing gammagamma T cells in skin inflammation. Immunity. 2011;35:996-1010.
9. Ebert LM, Meuter S, Moser B. Homing and function of human skin gammagamma T cells and NK cells: relevance for tumor surveillance. J Immunol. 2006;176:4331-4336.
10. D. BM, E. VWH. Purification and separation of individual collagenases of Clostridium histolyticum using red dye ligand chromatography. Biochemistry. 1984;23:3077-3085.
11. Qiao AY, Zhang WH, Chen XJ, et al. Isolation and purification of islet cells from adult pigs. Transplant Proc. 2010;42:1830-1834.
12. Xu T, Zhu M, Guo Y, et al. Three-dimensional culture of mouse pancreatic islet on a liver-derived perfusion-decellularized bioscaffold for potential clinical application. J Biomater Appl. 2015.
13. Zhang H, Cai DH, Han JL, et al. [Isolation and purification of human islet cells with semi-automated digestion]. Nan Fang Yi Ke Da Xue Xue Bao. 2007;27:824-826.
14. Ullah M, Hamouda H, Stich S, Sittinger M, Ringe J. A reliable protocol for the isolation of viable, chondrogenically differentiated human mesenchymal stem cells from high-density pellet cultures. Biores Open Access. 2012;1:297-305.
15. Klett CP, Palmer AA, Dirig DM, Gallagher AM, Riosseco-Camacho N, Printz MP. Evidence for differences in cultured left ventricular fibroblast populations isolated from spontaneously hypertensive and Wistar-Kyoto rats. J Hypertens. 1995;13:1421-1431.
16. Salimi M, Barlow JL, Saunders SP, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. The Journal of experimental medicine. 2013;210:2939-2950.
**Figure 1: Collagenase P enzymatic treatment dramatically increases skin lymphoid cell isolation yield with intact expression of CD3 and CD8.**

(a) Skin samples were cut into small pieces and treated with EDTA, collagenase D or collagenase P. Frequencies of isolated cells were measured using 0.4% trypan blue exclusion. EDTA treatment and collagenase D treatment resulted in the isolation of 2,130±889 and 6,417±927 cells/cm² respectively, while collagenase P treatment increased the number of isolated cells to 303,234±68321 cells/cm². Hoechst staining of isolated cells using different protocols is shown on the right. (b) Expression of skin homing markers, CLA, CCR10 and CCR4 were compared on lymphoid cells using the different methods of cell isolation and mean frequencies on CD45⁺ CD3⁺ cells are summarized (c). (d) Live CD45⁺ Cells isolated by EDTA and collagenase P treatment were stained for CD3 and CD8 expression. Similar frequency of CD8⁺ and CD8⁻ cells were observed using the different methods. Frequencies of CD3⁺ γδ T cells isolated by collagenase P treatment were similar to the EDTA method (e). ** P< 0.007

**Figure 2: Skin resident T cells have memory phenotype and are functionally intact.**

(a) Less than 10% of T cells isolated by the new method of collagenase P treatment show naïve phenotype and express CD45RA. (b) Multiplex cytokine analysis of ex vivo PMA/ionomycin activated cells isolated by collagenase P and collagenase D treatments. (c) Expression of IFN-γ, IL-17A, IL-22 and IL-13 by skin resident T cells isolated by collagenase D
as measured by ELISA after stimulation with PMA/ionomycin. (d) Proliferative capacity of CellTrace Violet-labelled T cells isolated by collagenase D and collagenase P treatment following 5 days culture with IL-2.

* P< 0.02 ** P< 0.002 ***<0.0002
Supplementary material and methods

Skin tissue

Normal adult human skin surplus tissue was obtained from surgical procedures according to GCP guidance with ethical approval of the Oxford Research Ethics Committee.

Isolating immune cells using established methods

After removing the subcutaneous fat, skin biopsies were cut into small pieces (0.5x0.5mm) and incubated in 5mM EDTA/HBSS with vigorous shaking at 4°C. After 2 hours the supernatants were spun down and the skin fragments were crushed through a 40μm strainer and pooled to obtain the maximum number of cells. For Collagenase D digestion, skin biopsies were prepared as described above and incubated in RPMI 1640 medium containing 1mg/ml collagenase D on a shaker at 37°C. After 30 minutes the skin fragments were washed with cold 10mM EDTA to stop digestion. The remaining tissue was homogenized through 40μm nylon mesh and spun down.
Flow cytometry studies of isolated skin T cells

Isolated T cells were analysed using anti human CD3 (SK7; BD biosciences), and combination of antibodies specific to skin homing receptors: cutaneous lymphocyte associated antigen CLA (HECA-452 Biolegend), CCR4 (TG6/CCR4 Biolegend), CCR10 (6588 Biolegend). To compare memory and naïve populations, T cells were stained with anti-CD45RA (HI100 BD Biosciences) and anti-CD45RO (UCHL1 BD Biosciences). Monoclonal antibodies against γδ T cell receptor (B1.1) was obtained from eBiosciences. The samples were acquired using FACSDiva or Summit software on LSRFortessa or CyAn flow Cytometer, respectively. FlowJo and Summit software were used for further data analysis.

IFN-γ, IL-22, IL-17 and IL-13 ELISA

IFN-γ, IL-13, IL-22 and IL-17 Enzyme Linked Immunosorbent Assays (ELISA eBiosciences 88-7316-88, 88-7439-86,88-7522 and 88-7176 respectively) were performed according to manufacturers’ instructions in the presence or absence of PMA/ionomycin overnight stimulation. Briefly Coat Corning Costar 9018 (Nunc Maxisorp®) ELISA plates were coated with 100μl/well capture antibody and incubated overnight at 4°C. After 3 washes the plate was blocked for 1 hour with assay buffer and 100μl of supernatant was added to each well and incubated for 2 hours in room temperature. After 5 washes, samples were incubated
with biotinylated detection antibody for 1 hour before Streptavidin-HRP incubation for further 30 minutes. 100μl substrate solution was added to each well for 10 minutes and stopped and read at 450nm wavelength.