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Topical steroid therapy induces pro-tolerogenic changes in Langerhans cells in human skin

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Summary

We have investigated the efficacy of conditioning skin Langerhans cells (LCs) with agents to promote tolerance and reduce inflammation, with the goal of improving the outcomes of antigen-specific immunotherapy. Topical treatments were assessed *ex vivo*, using excised human breast skin maintained in organ bath cultures, and *in vivo* in healthy volunteers by analysing skin biopsies and epidermal blister roof samples. Following topical treatment with a corticosteroid, tumour necrosis factor-α levels were reduced in skin biopsy studies and blister fluid samples. Blister fluid concentrations of monocyte chemoattractant protein-1, macrophage inflammatory proteins -1α and 1β and interferon-γ inducible protein-10 were also reduced, while preserving levels of interleukin-1α (IL-1α), IL-6, IL-8 and IL-10. Steroid pre-treatment of the skin reduced the ability of LCs to induce proliferation, while supernatants showed an increase in the IL-10/interferon-γ ratio. Phenotypic changes following topical steroid treatment were also observed, including reduced expression of CD83 and CD86 in blister-derived LCs, but preservation of the tolerogenic signalling molecules immunoglobulin-like transcript 3 and programmed death-1. Reduced expression of HLA-DR, CD80 and CD86 were also apparent in LCs derived from excised human skin. Topical therapy with a vitamin D analogue (calcipotriol) and steroid, calcipotriol alone or vitamin A elicited no significant changes in the parameters studied. These experiments suggest that pre-conditioning the skin with topical corticosteroid can modulate LCs by blunting their pro-inflammatory signals and potentially enhancing tolerance. We suggest that such modulation before antigen-specific immunotherapy might provide an inexpensive and safe adjunct to current approaches to treat autoimmune diseases.

Keywords: human dendritic cells; skin; tolerance; topical steroid.

Introduction

The skin has been used for over two centuries as a route of antigen administration to induce immune responses.[1] More recently, the skin has been recognized as a route to down-regulate unwanted immune responses including allergy and autoimmunity.[2,3] The attraction of the dermal route is that it is easy and convenient to access and the skin is rich in dendritic cells (DCs), particularly in the upper layers.[4] Although some success has been achieved with desensitization in allergy using the intradermal[5] or subcutaneous[6] routes of antigen administration, the ability of tolerance protocols to down-regulate autoimmune responses has been modest in the published literature.[7–12]

Tolerogenic DCs undoubtedly exist in the skin,[13–17] presumably with a role in promoting self-tolerance, as originally proposed by Steinman and Nussenzweig.[18] Several subsets of DCs are capable of uptake and presentation of self-antigen.[19] Recently a specific tolerogenic $CD14^+$ $CD141^+$ DC subset has been identified in

Abbreviations: CD, cluster of differentiation; cDNA, complementary deoxyribonucleic acid; DC, dendritic cell; IFN-γ, interferon-γ; IL, interleukin; ILT3, immunoglobulin-like transcript 3; LC, Langerhans cell; MECLR, mixed epidermal cell lymphocyte reaction; PBMCs, peripheral blood mononuclear cells; PD-1, programmed death-1; TNF-α, tumour necrosis factor-α

humans.[20] The role of epidermal Langerhans cells (LCs) in this process has been controversial, with some confusion arising from the existence of dermal langerin-positive DCs; however, the evidence from murine studies supports their tolerogenic potential, at least in the uninflamed state.[21–23] Current evidence suggests that, in the absence of pro-inflammatory signals, resting antigen presenting cells in many sites, including the skin, present self-antigen in a pro-tolerogenic manner and indeed, this may be part of the general system of maintenance of peripheral tolerance to self.[24] It therefore seems likely that the limited tolerance induction resulting from skin antigen administration to date is not due to a lack of uptake by DCs in the skin, but is a result of the failure to selectively target those with the most tolerogenic potential, a pro-inflammatory effect of the antigen administration process on immature DCs or dissemination of the antigen to distant sites (lymph nodes, spleen, bone marrow) where tolerance is not sufficiently promoted.

To overcome these limitations, and develop a method of skin administration that can reliably induce antigen-specific tolerance, we reasoned that conditioning of the LCs with agents to promote tolerance and reduce inflammation might improve outcomes. The accessibility of the skin permits conditioning with topical agents, so reducing the effects of systemic exposure. The optimal induction of tolerance might then be expected if successful pre-conditioning was combined with subsequent antigen administration into the superficial layers of the skin. The outermost skin layer, the epidermis, is the most accessible for conditioning treatment, is richest in DCs[25] and is likely to facilitate localized conditioning of local DCs without excessive dosing of the systemic immune system. Furthermore, by using a topical treatment to condition all DCs in the local area, we may circumvent the need to subsequently target the 'most tolerogenic' DCs with an antigen immunotherapy.

Local conditioning of DCs for tolerance is made more feasible in skin rather than other sites (e.g. gut, nasal epithelium), as not only can the site of conditioning treatment and antigen administration be accurately targeted, but the skin can be sampled to monitor the effects of treatment. Here we report the examination of a range of potential conditioning therapies in human skin, as well as more extensive studies of the most promising of these (topical corticosteroid), using a combination of *ex vivo* and *in vivo* approaches.

Materials and methods

Subjects and human samples

The studies were conducted in accordance with the International Conference on Harmonization/World Health Organization Good Clinical Practice standards. All subjects provided written informed consent before enrolment in the study, according to the Declaration of Helsinki (revision 2013).

Skin biopsies for the in vivo assessment of the topical immunomodulatory treatment. This study, approved by the Bath Research Ethics Committee, UK, recruited healthy volunteers aged 18–50 years with no history of skin conditions and no current or recent use of topical or systemic corticosteroids, vitamin D preparations or any other potentially immunomodulatory therapy. One group of untreated subjects had two 4-mm skin punch biopsies removed from the medial aspect of one arm (Kai Medical, Seki, Japan). A second group of subjects received two 50-μl intradermal injections of 0·9% saline, and a skin punch biopsy was taken from the treatment site 6 or 9 hr later. A third group applied Dovobet® ointment [calcipotriol + betamethasone, 0·005% weight/weight (w/w) + 0·05% w/w; Leo Pharma Inc., Ballerup, Denmark], every 12 hr for 4 days, to the medial aspect of their arm before receiving two 50-μl intradermal injections of 0·9% saline. Each treatment site was then punch biopsied 6 hr later. Skin biopsy samples were immediately snap frozen in liquid nitrogen and stored for future use at −80°.

Skin organ bath culture for the ex vivo assessment of the topical steroid treatment. Skin samples were obtained from female patients aged 19–82 years, following mastectomy or breast reduction. Skin without obvious pathological findings that was surplus to diagnostic histopathology requirements was excised and transported to the laboratory on ice in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK). The study received full ethical approval from South East Wales Research Ethics Committee, UK.

Subcutaneous adipose tissue was removed by blunt dissection to yield full-thickness skin, which was topically treated with DiproSone® cream (0·005% w/w betamethasone, as dipropionate; MerckSharp&Dohme Ltd., Hoddeson, UK) or PBS (Invitrogen). Without removal of the steroid or PBS, the skin was cut into smaller circular samples (diameter of 0·8 cm) and cultured at the air–liquid interface on Transwell® (Lifesciences, Tewksbury, MA) inserts in 24-well plates containing Dulbecco's modified Eagle's medium supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen) at 37° and 5% volume/volume (v/v) CO_2/95% (v/v) air. After a 24-hr incubation the skin was removed from culture and immersed in enzyme solution containing Dispase II (Roche, Burgess Hill, UK), collagenase I and DNase I (Invitrogen) for 1 hr at 37°. Epidermal sheets were mechanically separated from the dermis and processed to obtain an epidermal cell suspension.

Blister formation for the in vivo assessment of the topical steroid treatment. This study, approved by the Bath Research Ethics Committee, UK (Part 1), and South West 3 Research Ethics Committee, UK (Part 2) involved

healthy volunteers aged 18–45 years with no history of skin conditions and no current or recent use of topical or systemic steroid, vitamin D preparation or any other potentially immunomodulatory therapy.

Subjects in the Part 1 study group applied topical treatment, 12 hr for 4 days, followed by intradermal injection of 50 μl of 0·9% saline to the medial aspect of the arm. Blister formation was performed 6–18 hr after injection. Five sub-groups were created, depending on the topical treatment that was applied before injection: (I) DiproSone® cream (0·05% w/w betamethasone as dipropionate, MerckSharp & Dohme Ltd.); (II) Dovobet® ointment (calcipotriol + betamethasone dipropionate, 0·005% w/w + 0·05% w/w, Leo Pharma Inc.), (III) Dovonex® ointment (calcipotriol 0·005% w/w, Leo Pharma Inc.), (IV) Retin-A® gel (tretinoin 0·01% w/w, Janssen-Cilag Ltd., High Wycombe, UK) and (V) Control group – saline injection only, before the blister formation, without a previous topical treatment.

Participants in Part 2 of the study applied DiproSone® cream, every 12 hr for 4 days, to the medial aspect of one arm (with the other arm left untreated) before receiving two 50 μl intradermal injections of 0·9% saline, one in the pre-treated arm and one in the untreated arm. Blister formation was performed 6–18 hr after injections, in both arms.

Skin suction blisters were performed by gradually applying negative pressure (up to 50 kPa) from a suction pump machine VP25 (Eschmann, Lancing, UK) through a suction blister cup with a 15-mm hole in the base (UHBristol NHS Foundation Trust Medical Engineering Department, Bristol, UK) for 2–6 hr until a unilocular blister had formed within the cup. The blister fluid was aspirated after blister formation and stored at −20° for cytokine measurement at a later date. The blister roof was removed using a surgical blade (Swann Morton Ltd., Sheffield, UK) and used to prepare an epidermal cell suspension the same day (see below).

Preparation of epidermal cell suspension from skin organ bath culture or blister roofs. Epidermal sheets obtained from the skin organ bath culture or blister roof were transferred into 0·25% w/v trypsin solution (Sigma-Aldrich, Poole, UK) in PBS for 30 min at 37°. After the incubation, epidermal cells were re-suspended in 0·025% (w/v) DNase I in 10% (v/v) fetal calf serum (PAA, Pasching, Austria) in PBS for 1 hr. Epidermal cell suspensions were filtered through a 70-μm cell strainer and subsequently stained for flow cytometry analysis or used in the mixed epidermal cell lymphocyte reaction (MECLR).

Peripheral blood samples. Blood samples were collected from healthy volunteers aged 18–55 years, after full ethical approval by Research Ethics Committee for Wales, Bath Research Ethics Committee and South West 3 Research Ethics Committee, UK.

Polymerase chain reaction (PCR)

Ribonucleic acid (RNA) was extracted from frozen skin biopsy samples using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's Spin Protocol for Animal and Plant Tissue. Depending on the quantity of RNA obtained from the skin biopsy sample, cDNA synthesis was performed using either the SuperScript™ First-Strand Synthesis System for real-time PCR (Invitrogen) or the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen), again according to the manufacturer's protocols. Quantitative PCR was performed using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen) and TaqMan® Gene Expression Assays Hs00174128_m1, for tumour necrosis factor-α (TNF-α), and Hs00174086_m1, for interleukin-10 (IL-10) (Applied Biosystems/Life Technologies, Paisley, UK) in a 7500 Realtime PCR Systems machine (Applied Biosystems/Life Technologies). Reactions contained 12·5 μl of the SuperMix-UDG solution, 0·05 μl of ROX reference dye and 1·25 μl of primer probe (the assay gene) and were made up to a total volume of 25 μl with the cDNA sample and diethylpyrocarbonate-treated water. Samples were analysed in triplicate for each assay gene and for the endogenous control gene, non-primer limited and 6-carboxyfluorescein-labelled glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems/Life Technologies). Experiments were conducted using the standard curve method.

Mixed epidermal cell lymphocyte reaction and mixed lymphocyte reaction

Peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Paque Plus (1·077 ± 0·001 g/ml, +20°; GE Healthcare Biosciences, Uppsala, Sweden) gradient by centrifugation from the peripheral blood of healthy donors.

Epidermal cells (50 000 live cells/well, containing DCs) or irradiated PBMCs (1·2 Rad/s; 200 000 live cells/well), were incubated with allogeneic PBMCs (200 000 live cells/well) in 200 μl/well of 10% (v/v) human AB serum (PAA, Pasching, Austria) in RPMI-1640 (Gibco, Paisley, UK) supplemented with 0·5 mM of L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0·25 μg/ml of amphotericin B (Gibco) in a 96-well plate, at 37° in 5% CO_2/95% (v/v) air. After 96 hr of culture, 100 μl of supernatant was collected and stored at −20° for cytokine detection at a later date. [^{3}H]Thymidine (1 μCi; Perkin-Elmer, Cambridge, UK) was added to each well and the incorporation of radioactivity was measured after 16 hr using a MicroBeta2 plate scintillation counter (Perkin-Elmer).

Co-culture of epidermal cells with allogeneic donor PBMCs is referred to as MECLR and the co-culture of

irradiated PBMCs with allogeneic donor PBMCs is referred to as mixed lymphocyte reaction.

Cytokine measurements

Cytokines, interferon-γ (IFN-γ) and IL-10, in the supernatants of the MECLR driven by DCs originating from *ex vivo* skin cultures, were measured by ELISA (eBiosciences, Hatfield, UK).

Cytokines from both blister fluid samples and from the supernatants from MECLR driven by DCs originated from blister roofs, were measured by using a human Milliplex cytokine 96-well plate assay MPXHCYTO-60K-26 (Merck-Millipore, Billerica, MA), analysed using Luminex 100TM Luminex Corp, Austin, MI).

Flow cytometry analysis

The following monoclonal antibodies were used for the analysis of DCs: eFluor450-anti-human-CD1a (clone HI149), FITC-anti-human-CD40 (clone 5C3), phycoerythrin-anti-human-CD83 (clone HB15e), eFluor710-anti-human-CD83 (clone HB15e), phycoerythrin-anti-human-immunoglobulin-like transcript 3 (ILT3; clone ZM4.1), PECy7-anti-human-CD11c (clone 3.9) all from eBiosciences; biotin-anti-human-CD80 (clone L307.4, BD Pharmingen, Oxford, UK) with streptavidin peridinin chlorophyll protein-Cy5.5 (eBiosciences) or anti-biotin Pacific Orange (Invitrogen), allopycocyanin-anti-human-CD86 (clone 2231 FUN-1), peridinin chlorophyll protein-Cy5.5-anti-human-HLA-DR (clone G46-6), both from BD Pharmingen, Oxford, UK; Alexa Fluor® 488 anti-human programmed death-1 (PD-1; clone EH12.2H7) from Biolegend, London, UK and near IR fixable Live/Dead stain (Invitrogen, Paisley, UK). After gating on live and single cells, LCs were defined as $CD1a^+$ $HLA\text{-}DR^+$ cells.[21]

The cells were analysed on a FACS Canto II flow cytometer (BD Biosciences) and analysed with FlowJo software version 8.8.6 (Leland, Stanford, CA).

Confocal imaging

Participants applied DiproSone® cream, twice daily for 4 days, to the medial aspect of one arm with the other arm left untreated. Epidermal sheets originated from blister roofs were mounted on silane prep slides (Sigma-Aldrich), fixed with 2% paraformaldehyde (Sigma-Aldrich) for 15 min, permeabilized with 0·1% Triton X-100 (Sigma-Aldrich) and stained with a one-step monoclonal anti-human, mouse Langerin-Alexa Fluor® 488 (Dendritics, Lyon, France), after blocking with human Fc receptor binding inhibitor (eBiosciences). Cover glass was adhered over the stained blister sheets with Prolong Gold (Invitrogen, Paisley, UK), and the slides were placed horizontal in the slides box and left to adhere in the fridge (2–8°) overnight.

Confocal Images were acquired using a Radiance 2000-MP CLSM mounted on a Nikon TE 300 inverted microscope (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Dual channel images were collected (1024 × 1024 pixels) with the ×10 CFI Plan Fluor; N.A. 0·3. Alexa488 was detected using 488 nm excitation/500 nm LP-HQ 530 nm SP emission and including a transmission image, which enabled evaluation of tissue integrity and presentation (for counting, pixel calibration was set at 1·04 μm). Next analysis was conducted using the Metamorph Offline software (version 7.6.00) (MDS Analytical Technology, Sunnyvale, CA) to detect and count 'Langerhan' objects. The approach was based on a threshold detection of a cell object and an object was designated a Langerhan object if it was > 3 pixels to ensure that objects counted were cell bodies only within a random square that had a minimum of 400 pixels and a maximum of 800 pixels on centroid (x, y). This detection approach was applied to all epidermal sheets studied.

The numbers of Langerin-stained DCs were then calculated in 160 000-pixel squares from the four sides of each sample and the average number of LCs from the four sides of the sample was compared between control and matching steroid-treated skin specimens. The numbers were calculated in 1 mm^2 by using a conversion factor from pixels to 1 mm^2.

Statistics

The Wilcoxon Signed Rank test and the Mann–Whitney U-test were performed to test differences between paired and unpaired measurements, respectively. One sample t-test was used to test the significance of the percentage of the change in relation to the baseline value. For comparison of three or more samples one-way analysis of variance was used. Analysis was performed using Prism GraphPad 4.0a (GraphPad Software, La Jolla, CA, USA) for Macintosh. P-values of $< 0{\cdot}05$ were considered significant. Results were expressed as mean ± standard deviation (SD).

Results

Intradermal injection of 50 μl of isotonic saline in healthy volunteers was found to induce local pro-inflammatory cytokine release, as demonstrated by an increase in TNF-α mRNA (Fig. 1a). This effect was abrogated by pre-conditioning of the skin with a topical corticosteroid (betamethasone dipropionate) and a vitamin D analogue (calcipotriol), which led to significant reduction of the TNF-α expression ($P = 0{\cdot}04$, $n = 8$, Fig. 1b). Interleukin-10 mRNA was detected in three out of eight individuals both before and after 4 hr of topical steroid and vitamin

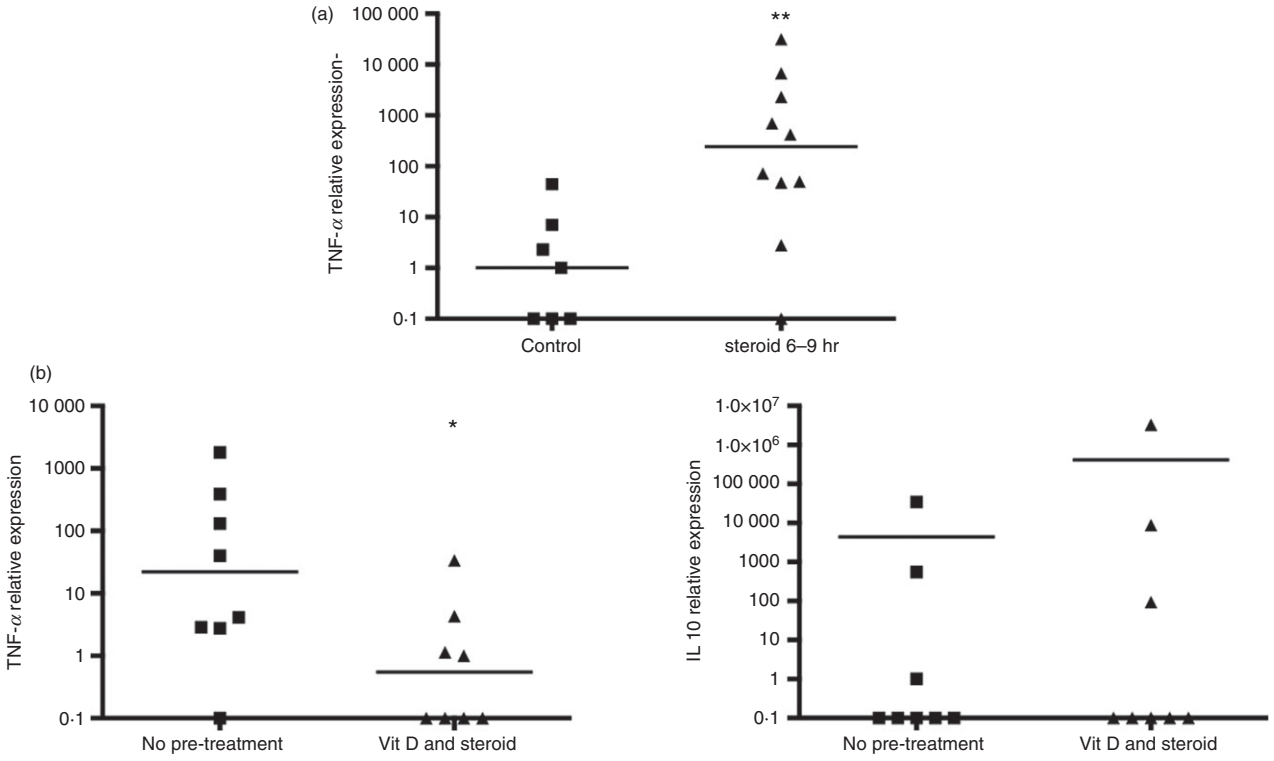

Figure 1. Cytokine mRNA levels in skin biopsies. (a) Tumour necrosis factor-α (TNF-α) expression by RT-PCR in skin biopsy samples taken from untreated subjects and from subjects 6–9 hr following intradermal injection of 0·05 ml 0·9% saline (n = 7 and 10, respectively; P = 0·005). (b) TNF-α and interleukin-10 (IL-10) expression by RT-PCR in skin biopsy samples taken from subjects 6 hr following intradermal injection of 0·05 ml 0·9% saline without (n = 8) and with 4 days of 12 hourly topical vitamin D/steroid (calcipotriol + betamethasone, 0·005% w/w + 0·05% w/w) pre-treatment (n = 8); P = 0·04 and non-significant for TNF-α and IL-10, respectively. Expression was shown as relative to the expression of the endogenous control gene *GAPDH* for glyceraldehyde-3-phosphate dehydrogenase. Each point represents one subject. $^*P < 0·05$, $^{**}P < 0·01$.

D therapy, suggesting that IL-10 production was unaffected by topical treatments (Fig. 1b).

To determine if the cytokine changes in whole skin following steroid therapy reflected changes in LC function, a human skin organ culture system was used. Excised breast skin from mastectomy procedures was treated with or without topical steroids and was subsequently cultured overnight before enzyme digestion, separation of the epidermal layer and preparation of an epidermal cell suspension. As expected, the epidermal cell suspensions contained a high number of dead cells (differentiated keratinocytes), but a $CD1a^+$ population was consistently identified among the live cell population (see Supplementary material, Fig. S1a). $CD1a^+$ $HLA\text{-}DR^+$ and $CD1a^+$ $Langerin^+$ DCs represented 1–2% of the live epidermal cells (see Supplementary material, Fig. S1b) with similar percentages in both untreated and corticosteroid-treated samples (see Supplementary material, Fig. S1c). The epidermal cell population showed significant reduction in HLA-DR, CD80 and CD86, with a trend towards reduction in CD83 levels and a trend towards an increase in PD-1, following topical steroid therapy. This is consistent with a more immature or tolerogenic phenotype (Fig. 2a). Consistent with this, there was a reduction in the ability of the epidermal cell suspensions to stimulate an MECLR (Fig. 2b). Supernatants from these cultures demonstrated an increase in the IL-10/IFN-γ ratio (Fig. 2c), additionally consistent with a more protolerogenic state in the LCs.

The skin organ culture methodology permits the effects of topically applied corticosteroid therapy to be studied in human skin, but may not be wholly reflective of the effects when applied *in vivo*. We therefore developed a modification of the induced skin blister technique, described by Reed *et al.*,[26] in which topical corticosteroid therapy was applied to arm skin *in vivo*, followed by suction blister formation and an aspiration of blister fluid to sample interstitial cytokine and chemokine levels.[27] Epidermal cells from excised blister roofs were also used to study LC function. Fifteen individuals were studied, including seven in whom blisters were induced in both arms with one arm steroid pre-treated and another (control arm) left untreated. Blister cytokine levels in individuals in which paired samples from two arms were studied (n = 7), showed differences in blister fluid levels of macrophage inflammatory protein-1α (P = 0·03) and trends (P = 0·05) towards differences in macrophage-in-

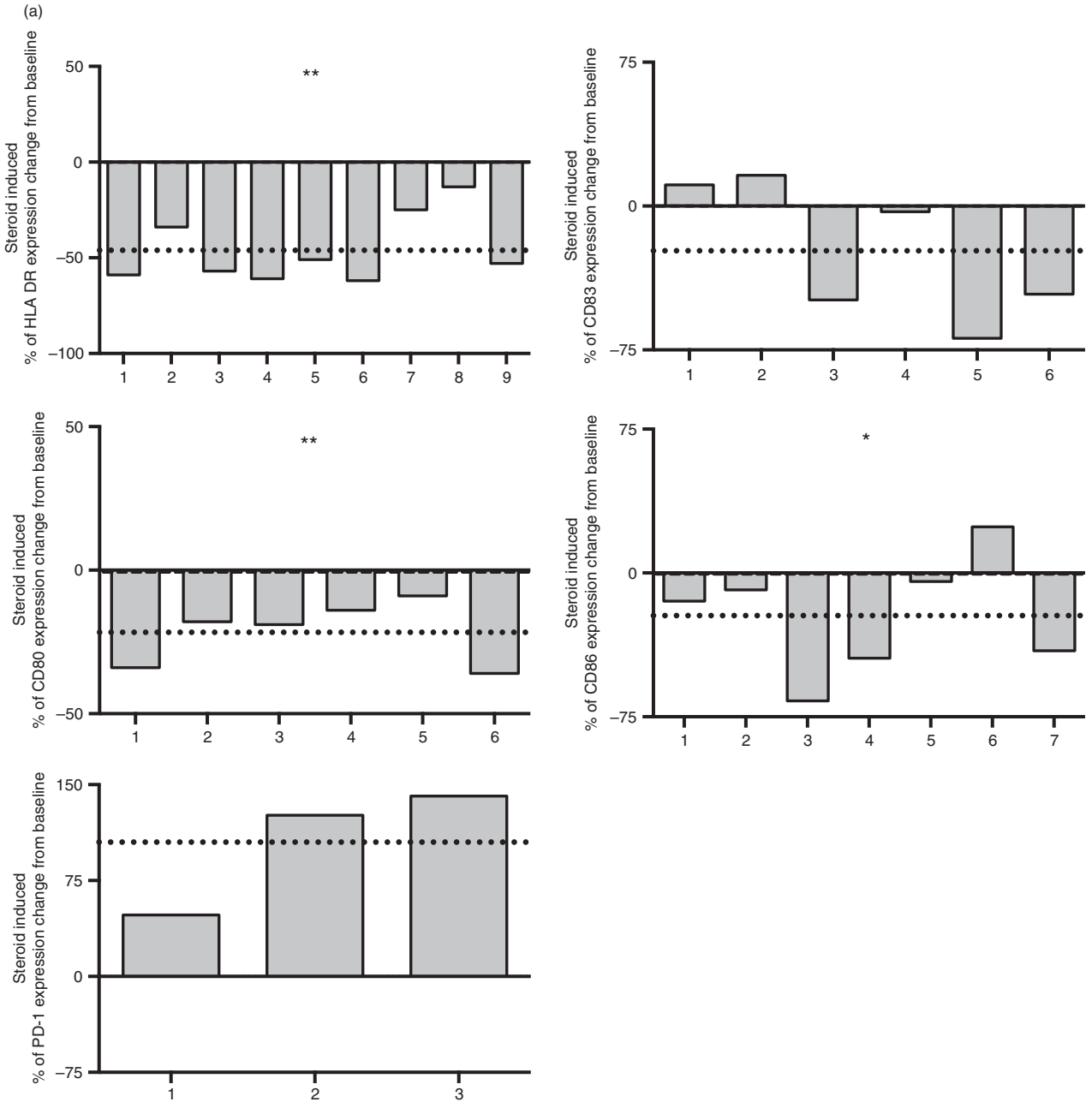

Figure 2. Phenotype and functional capacity of Langerhans cells (LCs) with and without *ex vivo* exposure to betamethasone dipropionate cream (0·05% w/w) in the human skin organ culture. Comparisons were made to untreated skin from the same skin donor (baseline). (a) Percentage of change in mean fluorescence intensity (MFI) measured by flow cytometry, of the HLA-DR ($n = 9$, $P = 0{\cdot}002$), CD80 ($n = 6$, $P = 0{\cdot}016$), CD86 ($n = 6$, $P = 0{\cdot}05$), CD83 ($n = 6$, ns) and programmed death 1 (PD-1); $n = 3$, ns) is shown in the bar charts, each representing the skin sample from the individual donor (dotted line across histograms marks the mean). Representative examples of flow cytometry histograms are shown below (black line – untreated, grey line – steroid-treated, solid grey – unstained control). (b) Proliferation in the mixed epidermal cell lymphocyte reaction (MECLR), expressed as proliferation index, i.e. ratio of the MECLR/peripheral blood mononuclear cell (PBMC) [^{3}H]thymidine uptake ($n = 9$, $P = 0{\cdot}03$). Baseline responder PBMC proliferation was 459 ± 463 cpm. (c) Cytokine profile in the MECLR, expressed as the ratio of interleukin-10 (IL-10) to interferon-γ (IFN-γ) measured in the supernatants of MECLRs ($n = 9$, $P = 0{\cdot}02$). Mean absolute values: IFN-γ untreated 1928 ± 2656 pg/ml, steroid-treated 1224 ± 1804 pg/ml, IL-10 untreated 803 ± 958 pg/ml, steroid-treated 507 ± 444 pg/ml. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ns, not statistically significant.

flammatory protein-1β and TNF-α (data not shown). These differences were not apparent with the unpaired samples alone. The samples were pooled for greater power ($n = 15$). The reduction in TNF-α levels in the skin biopsy studies (Fig. 1) was replicated in the blister fluid following corticosteroid treatment for 4 days

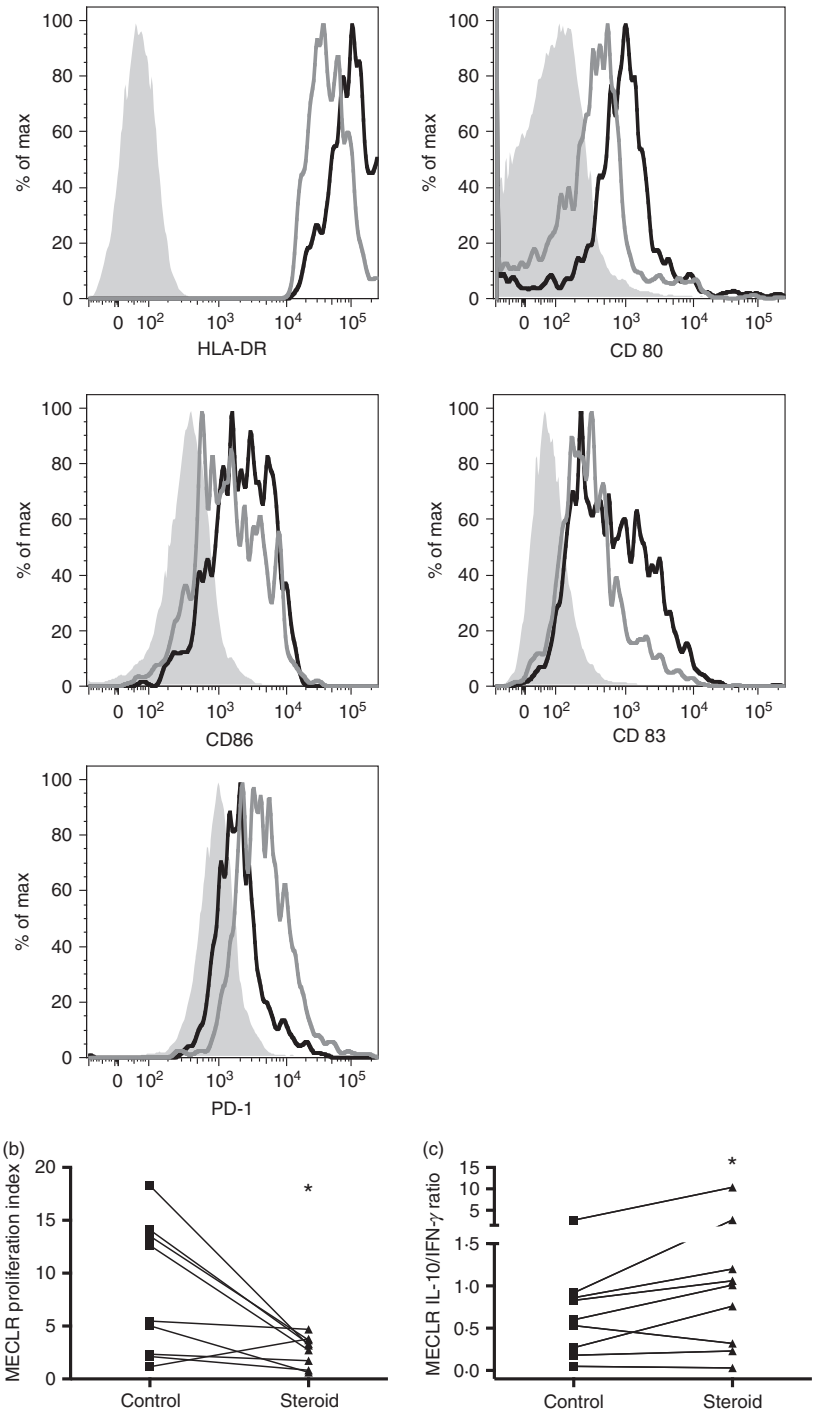

Figure 2. Continued

(Fig. 3a). Topical corticosteroid also significantly reduced blister interstitial fluid levels of the chemokines monocyte chemoattractant protein-1, macrophage inflammatory proteins 1α and 1β and IFN-γ-inducible protein 10 (Fig. 3a), while preserving levels of IL-1α, IL-6, IL-8, IL-10 and granulocyte colony-stimulating factor (Fig. 3b). Epidermal cells isolated from blister roofs were up to 10-fold more potent at stimulating an MECLR compared with the skin-organ-bath-derived cells, presumably due to the reduced exposure to *in vitro* culture. As few as 500 epidermal cells (comprising around 10 DCs) were consistently able to stimulate an MECLR (Fig. 4a). Consistent with skin organ cultures, corticosteroid pre-treatment of the skin reduced the ability to induce an MECLR. This effect was apparent in all volunteers, including those in whom blisters were obtained from both arms, so provid-

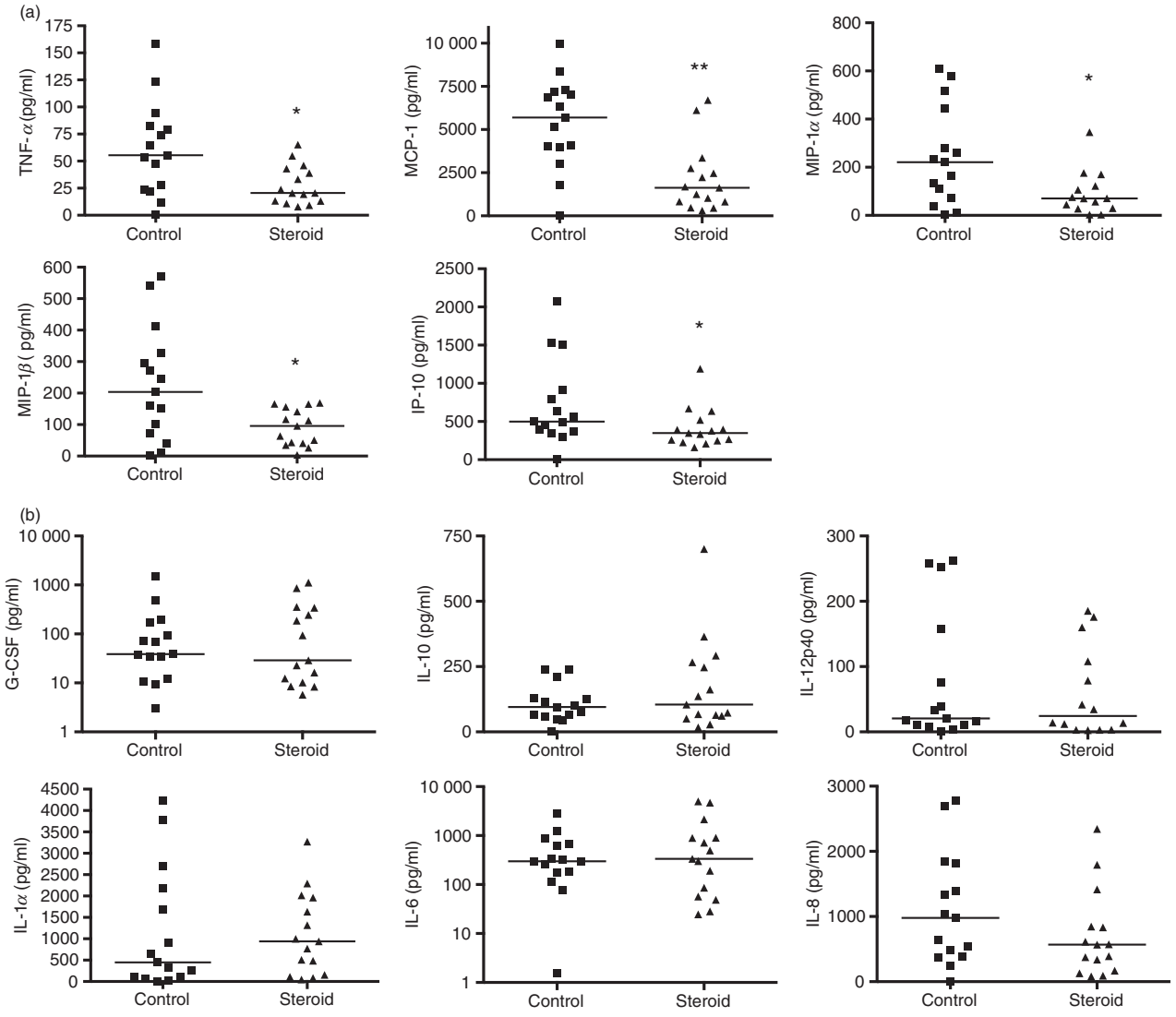

Figure 3. Cytokine profile in blister fluid with and without previous *in vivo* exposure to betamethasone dipropionate cream (0·05% w/w). (a) Statistically significant changes in the level of tumour necrosis factor-α (TNF-α) ($P = 0{\cdot}01$), monocyte chemoattractant protein-1 (MCP-1) ($P = 0{\cdot}001$), macrophage inflammatory proteins MIP-1α ($P = 0{\cdot}03$), MIP-1β ($P = 0{\cdot}04$) and interferon-γ inducible protein 10 (IP-10) ($P = 0{\cdot}04$) were observed after topical steroid treatment for 4 days. Analysis was performed using a human Milliplex cytokine 96-well plate assay. (b) Four days of topical steroid treatment did not cause significant changes in the levels of granulocyte colony-stimulating factor (G-CSF), interleukin-10 (IL-10), IL-12p40, IL-1α, IL-6 and IL-8. $n = 15$. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

ing an internal control (Fig. 4b) and confirming the consistency of the effect. It is unlikely that this was simply a result of the depletion of LCs following steroid therapy as the overall numbers of epidermal cells obtained were similar (see Supplementary material, Fig. S2), as previously observed in the human skin organ culture model (see Supplementary material, Fig. S1). In addition, confocal microscopy did not show any difference in LC density (Fig. 4c) Phenotypic changes in blister-derived LCs following topical treatment with corticosteroid included reduced expression of CD83 and CD86 in $CD1a^{+}$ cells, with preservation of the tolerogenic signalling molecule ILT3 (Fig. 5). In contrast to the excised human skin culture model, no difference in HLA-DR or CD80 was detected in the human skin blister system, which may be related to the different steroid treatment regimens in these two experimental systems (4 days versus 1 day).

To determine whether other topical agents could have a similar effect on LC phenotype and function, the blister cell experiments were repeated using a vitamin D analogue (calcipotriol), vitamin D analogue plus steroid, and vitamin A (tretinoic acid), which did not result in any significant changes in the parameters studied (see Supplementary material, Fig. S3).

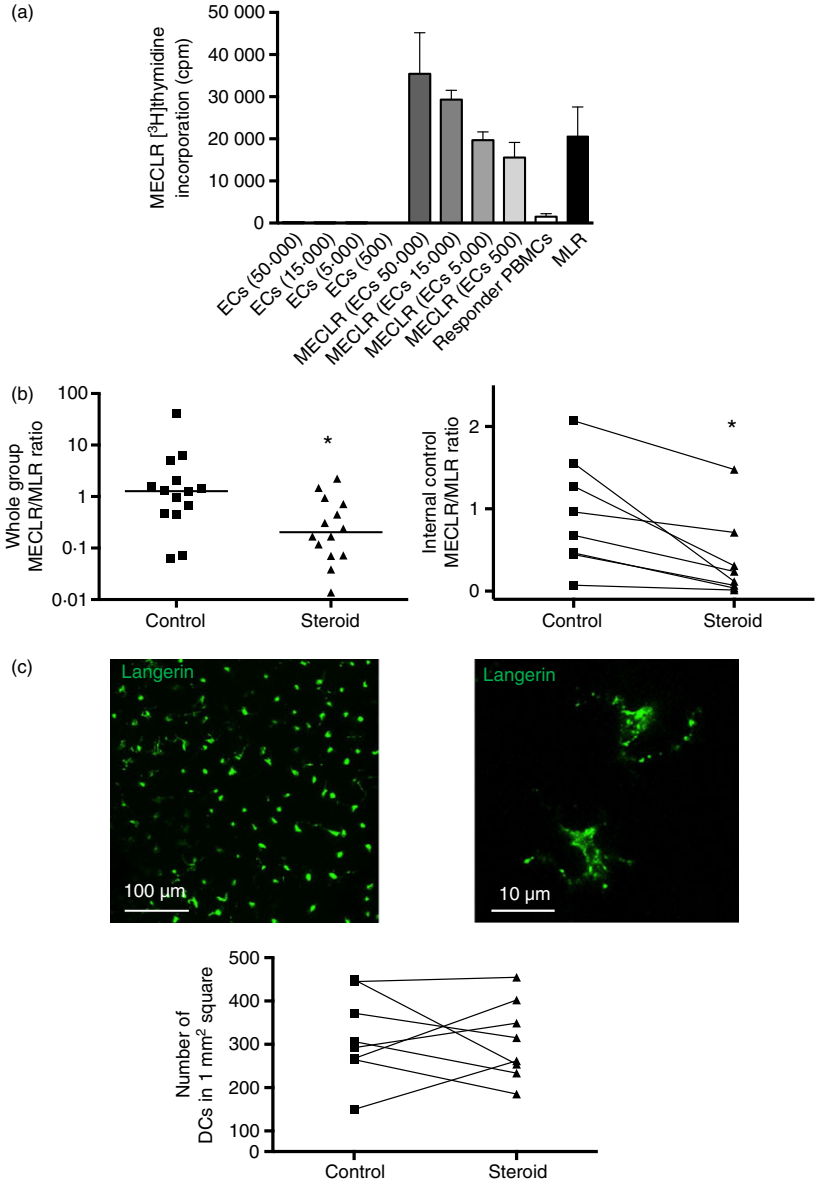

Figure 4. Functional capacity of Langerhans cells (LCs) with and without previous *in vivo* exposure to betamethasone dipropionate cream (0·05% w/w). (a) Proliferation in mixed epidermal cell lymphocyte reaction (MECLR) stimulated by a variable number of untreated epidermal cells (ECs), compared with the proliferation in mixed lymphocyte reaction (MLR) from the same donor–responder pair. Data from previous experiments (not shown) indicated a low proliferation rate of epidermal cells measured by ^{3}[H]thymidine uptake (below 100 cpm). (b) Proliferation in the MECLR expressed as ratio of the MECLR/MLR from the same donor–responder pair; whole group ($n = 14$, $P = 0{\cdot}016$), group with the internal control ($n = 7$, $P = 0{\cdot}008$). Baseline responder peripheral blood mononuclear cell proliferation was 403 ± 267 cpm in the group with the internal control and 495 ± 386 cpm in the whole group. Number of ECs per well was 15 000. (c) Analysis of the LCs in the blister roof by confocal microscopy: langerin-stained epidermal dendritic cells (DCs) (green spots) are shown in the two upper figures; lower figure: exposure to betamethasone dipropionate cream (0·05% w/w) did not change the mean number of langerin-stained DCs in the blister roof ($n = 8$, ns). The number of langerin-stained DCs was calculated in 1 mm^2. The mean number of LCs was compared between control and steroid-treated skin specimens from the same donor. Lines in the graph are connecting the data from the same individual (untreated and steroid treated). *$P < 0{\cdot}05$.

Discussion

This study has shown that injection of an inert liquid (isotonic saline) in volumes as small as 50 µl into human skin induces increased expression of TNF-α with the potential for pro-inflammatory effects, which may antagonize the beneficial effects the intradermally delivered immunomodulating agent administered to achieve tolerance. This change was negated by conditioning the skin with topically applied corticosteroids, which have been shown to be as effective as steroid plus vitamin D analogue. In addition, topical corticosteroids induced multiple changes that might be expected to promote tolerance including a reduction in HLA-DR, CD83 and CD86 expression in LCs, and reduced proliferation and IFN-γ production in T cells activated by these LCs. Interleukin-10 production and the expression of the tolerogenic co-stimulatory molecule ILT3 were not affected by steroid therapy, whereas there was a trend towards increase in PD-1. Additional changes in the skin environment were observed, including reduction in chemokine expression including monocyte chemoattractant protein-1, macrophage inflammatory proteins and IL-10. No significant effect on any of these parameters was seen with topical vitamin D analogue (calcipotriol) or vitamin A alone (isotretinoin).

Strengths of the current study include the use of *in vivo* studies and live human skin-derived cells, concordance of data from different techniques (skin biopsies, skin organ bath cultures, blister fluid studies) and the use of control cells from the same subject in later experiments. A potential weakness is the fact that due to

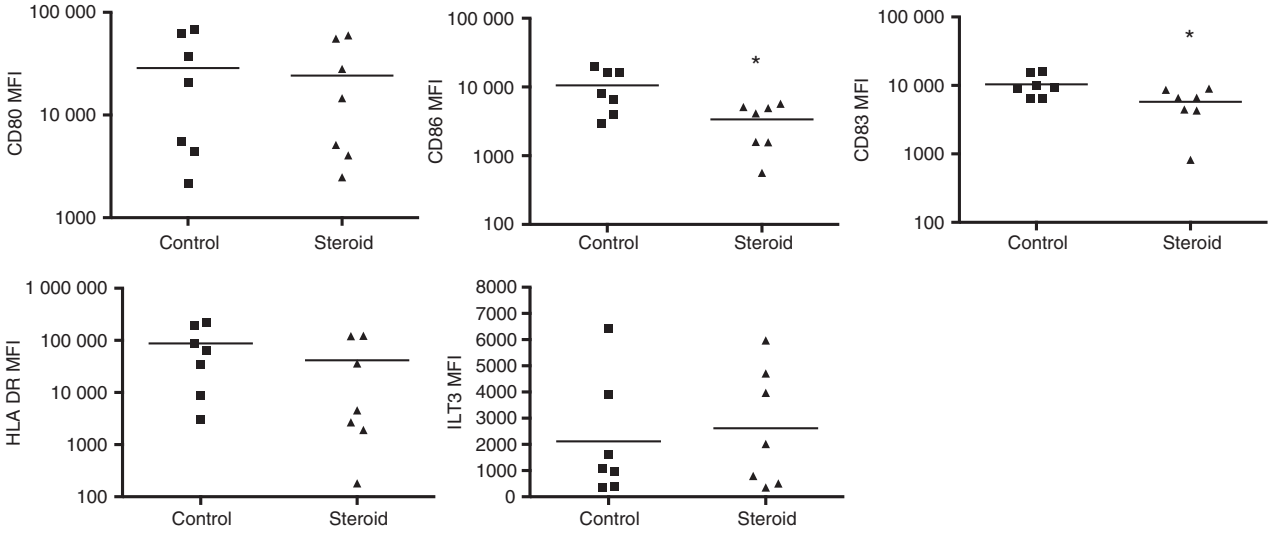

Figure 5. Phenotype of blister-roof-derived Langerhans cells (LCs) with and without *ex vivo* exposure to betamethasone dipropionate cream (0·05% w/w). The effect of topical corticosteroid was assessed by the measurement of the expression of surface markers, illustrated by mean fluorescence intensity (MFI) measure by flow cytometry, of CD80 ($n = 7$, ns), CD86 ($n = 7$, $P = 0{\cdot}04$), CD83 ($n = 7$, $P = 0{\cdot}04$), HLA-DR ($n = 7$, ns) and immunoglobulin-like transcript 3 (ILT-3) ($n = 7$, ns). *$P < 0{\cdot}05$; ns, not statistically significant.

limited cell numbers, LCs were not purified, so other cell types such as keratinocytes may also have contributed to the functional changes. In addition, although many potentially relevant changes following steroid therapy were defined, the key mechanism of action resulting in a reduction in induced T-cell proliferation and IFN-γ production was not identified. Topical steroid therapy has previously been shown to reduce T-cell proliferation induced by skin cells extracted from treated skin, but the phenotype of the cells and the regulatory potential was not examined.[28] The phenotype of the steroid-treated LCs observed in the current study – low CD80, CD83, CD86 HLA-DR with preserved ILT3 and trend towards increase in PD-1, is similar to that reported by others to be associated with tolerogenic DCs.[29–31]

Our method of LC enumeration in epidermal sheets employed an automated pixel recognition technique (see Materials and methods) designed to objectively compare numbers of LC between samples. Only cells with more than a minimum pixel density were counted and as a result the absolute number of LCs per mm^2 was in some cases lower than previously reported in studies of epidermal sheets.[27,32] However, comparison between samples enumerated consistently by this approach did not reveal any significant change in LC number following *in vivo* steroid pre-treatment, and this was consistent with the findings from our *ex vivo* treatment studies enumerated by flow cytometry. Note that previous studies suggest that saline injection does not affect LC number in epidermal sheets derived from suction blisters, but the suction blister method itself results in approximately 30% fewer LCs than in the epidermal sheets derived from punch biopsies.[32]

We were surprised that neither topical treatment with vitamin D nor vitamin A appeared to promote a regulatory phenotype in LCs. Vitamin D has been reported to induce regulatory potential when used to treat psoriatic skin,[33] when used to locally target dermal DCs in skin explants[34] and in combination with steroid to treat mature monocyte-derived DCs.[29] DCs used in the skin blister model are mainly of epidermal origin and therefore likely to exhibit an immature phenotype.[21–23] This and the fact that they are derived from non-inflamed skin may be an explanation for the lack of vitamin D effect. It remains possible that these agents could be of benefit, if used in an alternative protocol.

It is open to speculation what is the right exposure of the LCs to corticosteroid to induce tolerance. Future experiments with different potency of topical corticosteroids and titration of the optimal dose and the duration of the treatment, together with tolerance assays to define the mechanism of the reduction of T-cell proliferation and IFN-γ production, are necessary to answer this question. Although murine models can be used to address some of these questions and explore *in vivo* effects on tolerance induction[35–37] murine epidermis is 1/10th of the thickness of human skin, increasing the likelihood of systemic absorption of steroid after topical therapy and making comparisons with human skin using this approach difficult. We have therefore instituted a clinical trial to compare tolerance induction after intradermal autoantigen administration with and without topical steroid pre-conditioning in humans.

To our knowledge, this is the first study exploring the effect of topical steroid on the functioning of LCs, through three different, but concordant, translational and

clinical models. Taken together, our experiments, suggest that topical pre-conditioning of the skin with steroid for 1–4 days modulates LCs by blunting their pro-inflammatory signals and potentially enhancing tolerance. Although these findings require confirmation in clinical trials, we suggest that steroid pre-treatment before the injection of antigens for antigen-specific immunotherapy might provide an inexpensive, readily available and safe adjunct to current approaches to treat autoimmune disease.

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Author contributions

MAA, SLT and DT designed, performed experiments and analysed data. SJH contributed to the data analysis and interpretation. SC, JCB, FSW and CMD oversaw the research. CMD and DT conceptualized and wrote the manuscript.

Disclosures

The authors do not have any conflict of interest to report with regards to this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Viability of Langerhans cells (LCs) in the *ex vivo* human skin organ culture.

Figure S2. Viability of Langerhans cells (LCs) in the blister roof.

Figure S3. Effect of other topical agents (calcipotriol 0·005% w/w, calcipotriol + betamethasone 0·005% w/w + 0·05% w/w and tretinoin 0·01% w/w) on blister fluid and phenotype and function of blister-roof-derived Langerhans cells (LCs).

