Increasing mechanical stimulus induces migration of Langerhans cells, and impairs the immune response to intracutaneously delivered antigen

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Short title: Pressure induces Langerhans cell migration

Abbreviations used: LC=Langerhans cells

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Abstract

Skin is subjected regularly to mechanical stimulus. Surprisingly, when studying the use of microneedle arrays to introduce antigen into skin, we observed that a mechanical stimulus to skin achieved by application of the arrays or a flat metal plate of up to 2.5 MPa stress induced by the patch application process resulted in temporary depletion of up to 80% of Langerhans cells from 1 to 10 days after patch application, with the degree of depletion related to the applied stress, while whereas no depletion was seen in the interspersed dendritic epidermal T cell population. Further, a significantly impaired immune response to intracutaneous antigen administration was observed in skin exposed to recently subjected to low level mechanical stimulus. This observation has may have implications for selection of sites of skin immunisation and for immunogenicity of infections at skin sites routinely subjected to mechanical stimuli.

Key Words Immune function; stress; Langerhans Cells; skin; Pressure; mechanical stimulus
Background

Intracutaneous routes of immunisation have been of increasing interest, as there are high densities of antigen presenting cells including Langerhans cells (LC) within the epidermis and dendritic cells (DC) in the dermis (1). We wished specifically to determine the behaviour of LC following presentation of antigen administered on arrays of microneedles applied to the skin (2)—that penetrate only into the epidermis (3).

Experimental Design

A microneedle array, or a flat plate of corresponding size, was applied to skin under controlled pressure. Microneedle patch arrays, measuring 5mm x 5mm in total size, with 3364 projections each 40 μm in length tapering down to tips <500 nm in diameter—were synthesised from silicon using a process of deep reactive ion etching (4). Fluorescein Isothiocyanate (FITC) was coated on microneedle patches using a nitrogen-jet drying coating method (4). Briefly, 7 μl of coating solution, containing FITC in acetone, was applied to each patch. A nitrogen gas jet (6-8 m/s) was used to localize the FITC on the projections.

Epidermal sheets prepared as described from skin (5) were fixed in acetone for 15 minutes, held in 5% bovine serum albumin (Sigma) for 1 hour, and then exposed to fluorochrome conjugated monoclonal antibodies specific for murine MHC II (16-5321, BD Biosciences), CD207 (ab49730, Abcam) or vγ3-TCR (12-5711, BD Biosciences) for 1 hour at 37°C.

Groups of mice (n=4 unless otherwise stated) were immunised with hen egg ovalbumin (OVA) (Sigma) and Quil A (Superfos Biosector, Vedbaek, Denmark). Intramuscular immunisation of mice was performed using 60µg OVA and 6µg Quil A—intramuscularly or intracutaneously in ear skin. Immunogens used for intracutaneous immunisation were FITC—(Sigma)—(20mg/ml— in acetone: dibutylphthalate (1:1)) or with OVAovalbumin (Sigma)—(1.2 mg/ml) in 0.02 M phosphate buffered 0.15 M NaCl pH 7.4 (PBS) with Quil-A (Superfos Biosector, Vedbaek, Denmark), 0.12mg/ml. Intracutaneous immunisation was undertaken with 4 microneedle arrays applied through immunogen solution applied to the skin and held in place for 20 sec with 750g static weight. The microneedle projections penetrate about 9μm into skin (3), less than the 13-18μm thickness of the murine epidermis. Antibody to OVA was measured by ELISA as previously described (6).

Results
After application of microneedle patches to mouse ear, ear thickness increased from 199 ± 9 µm (mean ± SEM) to 265 ± 29 µm at 24 hours, and to 265 ± 66 µm at 48 hours (Supp Fig 1 a-d), and marker dye, FITC, was distributed in a pattern corresponding to the microneedle grid (Supp Fig 1e). Langerin + MHC Class II + cells of dendritic morphology decreased in number over time after patch application, whereas there was no significant change for another epidermal resident bone marrow derived cell, the dendritic epidermal T cell (Fig 1a) (Supp Fig 1f,g). However, morphology of the dendritic epidermal T cells was altered following patch application as described in response to skin injury (7).

To test whether the observed reduction in Langerhans cell numbers was could be induced by the simple application of mechanical pressure, we exposed skin to differing quasi-static loads, applied either with a flat plate, or with a patch bearing microneedle projections. For a given applied load (Fig 1 b), depletion of LC was less following application of a flat plate than following application of a similar sized microneedle patch. However, when we calculated the stresses experienced by the LC under different loading conditions using a validated finite element model (Supp Methods, Supp Fig 2), a linear relationship was observed between decline in LC population and calculated stress, until LC depletion became maximal at about 80% with an applied stress of 2 MPa.

To examine whether pressure induced loss of APC from skin prior application of the microneedle patch might impact on the impair the immune response to a subsequent microneedle patch immunisation at the site, immune response generated following intracutaneous introduction of antigen, previously untreated mice and mice treated with an uncoated microneedle patch with 500 g of static load 3 days previously were immunised with ovalbumin by applying a microneedle patch to ear skin through a solution of ovalbumin (OVA) and Quil-A adjuvant. For the mice previously treated with a microneedle patch, the immunisation was undertaken at the site of previous patch application. After immunisation of previously untreated animals, a significant antibody response to OVA was induced (Fig 2) patch application, and there was a significant increase in the cellularity of the draining lymph node, presumably in response to adjuvant or to endotoxin in the OVA preparation, as the cellularity increased only with antigen bearing patches (Supp Fig 3a). An increased number of MHCII+ CD11c+ Langerin CD207+ cells (Supp Fig 3b) was also observed in the node, confirming that at least some LC migrate to the draining lymph node in response to patch application. stress.

A significant humoral response to OVA was generated (Fig 2). When the antibody response following antigen-ovalbumin administration through previously untreated skin was compared with the response where skin had been pre-treated with 500 g of static load using a patch 3 days
previously, which resulted in partial depletion of LC, the antibody response induced to OVA was markedly reduced (Fig 2).
Conclusions

Microneedle insertion provides a mechanical stimulus to the skin (3), and LC migrate away from the skin following some forms of physical stimulus (8-10). In this study we have identified that increasing pressure from a flat plate or mechanical stimulus from a microneedle patch promotes increased migration of LC away from the epidermis, and that application of a microneedle patch three days before immunisation is also associated with loss of immune response to subsequent local introduction of antigen by microneedle patch at the same site. Migration of mature LC occurs largely in response to direct signalling from proinflammatory cytokines, particularly TNFα and IL-1β (11;12), and can be inhibited indirectly by cytokines constitutively produced in skin including IL-10 (13) and IL-4 (14). Infection is a major driver of LC migration (15), although physical damage from heat (16) and ultraviolet and ionising radiation (17;18) will also promote migration, as will appropriate chemokine stimuli on which migration is dependent (19) or exposure to toll-like receptor agonists (20;21). While pressure might, as for other stimuli (22), encourage LC migration through induction of proinflammatory cytokines, absence of an evident inflammatory infiltrate in the dermis in response to pressure (Supp Fig 1 a-c) argues against this mechanism, and greater pressure for much longer than was used in this study is required to induce the relevant cytokines (23). Skin baroreceptors stimulate autonomic activity, and LC migration is promoted by sympathetic activation (24), a potential alternate explanation for the observed LC migration.

LC have long been implicated in hapten induced contact sensitivity reactions. However, their relevance to induction of immune responses has recently been questioned (25) as a consequence of apparently normal cellular immune responses to antigen presented in skin in animals depleted of LC. Our data are consistent with the hypothesis that the observed pressure or mechanical stimulus induced migration of LC contributes to the observed impairment of antibody response, though alteration in antigen presentation capacity of other APC populations in the skin in response to pressure or injury (26) cannot be excluded. Whatever the mechanism, pressure induced impairment of immune responses to local antigen may contribute to invasive infection in skin subjected to pressure stress, and hence enhance the risk of pressure ulceration. Further, skin sites subjected to routine pressure might best be avoided for intracutaneous immunisation.

Conflict of interest: The authors state no conflict of interest

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Figure Legends

Figure 1

Microneedle patch application is associated with loss of LC from skin.

a: Epidermal sheets from mouse ear were stained for MHC Class II positive cells (LC) and for dendritic epidermal T cells (DETC) at various times after patch application.

b: LC in epidermal sheets, identified as MHC Class II + cells, were quantitated 48 hours after application of different static loads as shown, using a microneedle patch or of a flat plate of the same area without needles.

Each dot represents mean cell count per unit area ± SEM for one animal. Statistical comparisons by Student’s t test are with pre-treatment results. * p<0.05, **p<0.01, ***p<0.001. Differences not marked were non-significant.

Figure 2

Immune response induced by intracutaneous administration of ovalbumin is reduced by prior exposure of skin to pressuremicroneedle patch application.

Mice were immunised intracutaneously by pressing a microneedle patch through a solution of ovalbumin and Quil-A. Immune response was measured as serum antibody reactive with ovalbumin at 1/100 dilution as measured by ELISA O.D. 14 days after immunisation. The immunisation site was either untreated prior to immunisation (Patch), or pre-treated with 500 g static load using an uncoated patch 3 days prior to immunisation (Patch pre). Data are also provided for intramuscularly immunised (IM) and unimmunised (Control) animals. Comparison of Patch and pre-patch results by Student’s t test **p<0.01.


**Supplementary Figures**

Supplementary Figure 1

Microneedle patch application to ear skin induces local swelling and MHC-II expression on LC. Sections of ear were examined histologically at (b) 2 days and (c) 4 days after patch application, and compared to untreated ear skin (a) (H&E staining). d: Ear thickness was measured by spring loaded callipers at various time points after patch application. Statistical comparisons with Student’s t test: * p<0.05, **p<0.01, ***p<0.001. e: Distribution of fluorescent dye (green) amongst MHC-II staining cells (red) across an mouse ear epidermal sheet 16 hours after application of a patch coated with FITC (indirect immunofluorescence). Epidermal sheets from control untreated (f) and patch treated (g) mouse ear 48 hours after patch application were stained for LC (MHC II - red) and for dendritic epidermal T cells (TCR-Vy3 : green). Results representative of 3 mice per group.

Supplementary Figure 2

Langerhans cell loss from ear skin is sensitive to pressure or mechanical stimulus applied to skin. Correlation of Langerhans cell number per unit area with computed stress induced in epidermal skin by a microneedle patch (E-G) or by a flat surface (A-D), with the indicated static load. Commercial finite element analysis software (ABAQUS 6.8, DS Simulia) (27) was used. The needle array and flat surface were modelled as mixed tri and quad elements; the ear model consisted of quad-elements. Smallest element sizes were used at projection penetration regions and flat surface corners. The mouse ear was modelled as a linearly elastic and viscoelastic deformable solid made of seven layers: three layers (stratum corneum, viable epidermis and dermis) on each side of the cartilage. We assigned thicknesses of 5, 10, 55 and 30 µm to the stratum corneum, viable epidermis, dermis and cartilage layers based on measurement. Young’s moduli were 90 MPa (28), 2.9 MPa (29), 60 MPa (30), 5.11 MPa (31) respectively for stratum corneum, viable epidermis, dermis and cartilage. We considered the skin as nearly incompressible; hence the Poisson’s ratio used was 0.45 (32). The stress in skin is composed of an elastic and viscoelastic component. In ABAQUS we defined the viscoelastic stress relaxation function from experimental data on human skin using a second order Prony series with g1 = 0.148, g2 = 0.252, τ1 = 2.123 and τ2 = 9.371 (33). A pure master-slave predictor/corrector (kinematic contact) algorithm was used to manage the contact between the skin and the interacting object: nodes of the master surface can penetrate into the slave surface, but not vice versa. A classical Coulomb model with constant coefficient simulated the friction effect. A static uniform load was applied on the top edge of the patch on a flat surface.
This model was validated by evaluation of stress on the surface of freshly excised mouse ear measured using a piezoelectric transducer, in response to impact of a flat surface on the ear with velocities of 0.592 or 0.644 m/s, as used for applying patches. Measured stress was calculated by dividing averaged measured force by impact area, and compared with the weighted average computed stress across the entire contact region. The observed errors between measured and calculated stress were between 2% and 13%.

Supplementary Figure 3

Lymph node cellularity increases after delivery of antigen by microneedle patch.

a: Total cell numbers isolated from draining auricular lymph nodes 2, 3 and 4 days after application of a microneedle patch with (patch + OVA) or without (patch) ovalbumin antigen, compared with untreated mice (control).

b: CD207+ cells as a percentage of total lymph node antigen presenting cells (CD11c+) 24 to 72 hours after patch application. Statistical comparisons with Student's t test: * p<0.05, **p<0.01, ***p<0.001.

Cells prepared from lymph nodes as previously described (34) were stained with CD11c, MHCII, CD45.2 and 7AAD (BD Biosciences). Intracellular staining for CD207 (Langerin) (Abcam) used a Cytofix/Cytoperm kit (BD Biosciences). Results were analysed using a BD Biosciences FACSCalibur or FACSCanto flow cytometer and CellQuest (BD Biosciences) software.