



Research paper

Techniques for time-efficient isolation of human skin dendritic cell subsets and assessment of their antigen uptake capacity

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ABSTRACT

Dendritic cells (DCs) residing in skin are important sentinels for foreign antigens. Methods to facilitate studies of subsets of skin DCs are important to increase the understanding of various pathogens, allergens, topical treatments or vaccine components targeting the skin. In this study, we developed a new DC purification method using a skin graft mesher, clinically used for expansion of skin grafts, to accelerate processing of skin into nets that allowed efficient enzymatic disruption and single cell isolation. The reduction in processing time using the skin graft mesher enabled processing of larger skin samples and also limited the *ex vivo* handling of the specimens which is associated with maturation of DCs. In addition, a skin explant model to functionally monitor early events of antigen uptake by DC subsets *in situ* was developed. DCs isolated from epidermis represented a uniform CD1a⁺ HLA-DR⁺ CD11c⁺ Langerin⁺ DC-SIGN⁺ DC-LAMP^{int} DEC-205^{int} Langerhans cell (LC) population whereas three subtypes of HLA-DR⁺ CD11c⁺ DCs were isolated from dermis based on their varying expression of CD1a. Epidermal LCs showed a significantly higher antigen uptake capacity of fluorescently-labelled ovalbumin (OVA) and dextran as compared to any of the dermal DC (dDC) subsets. In contrast, injection of antigen directly into skin explants followed by *in situ* imaging revealed that the majority of DCs with internalized antigen were localized in the dermis, likely as a consequence of the anatomical site for antigen delivery. These methods offer potency for various applications addressing antigen uptake, microbial DC interactions or other antigenic stimulation targeting the skin and can enhance our knowledge of basic DC biology in human skin.

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1. Introduction

Dendritic cells (DCs) comprise a heterogeneous cell population that plays an essential role in recognition of antigen and induction of primary immune responses (Banchereau and Steinman, 1998; Mellman and Steinman, 2001). DCs in

peripheral tissues sense and capture foreign antigens, which leads to activation and migration to draining lymph nodes where antigen-specific T cell responses are induced. Thus, DCs residing in the skin contribute to the first line of defense against pathogens and are considered immature based on their ability to efficiently take up antigens. Langerhans cells (LCs) were the first subset of DCs to be identified in skin, and are currently the best characterized. LCs are a somewhat homogenous population identified by unique cytosolic structures (i.e. Birbeck granules) and expression of CD1a and the C-type lectin Langerin (Wolff, 1967; Elder et al., 1993; Banchereau and Steinman, 1998; Valladeau et al., 2000; Figdor et al., 2002; Bursch et al., 2007). LCs were long thought to be the

Nonstandard abbreviations: LC, Langerhans cell; dDC, dermal dendritic cell; MDDC, monocyte-derived dendritic cell.

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main antigen-presenting cell population in the skin. However, in recent years several DC subsets in the dermis have been identified, which may have more potent immunostimulatory function, while LCs emerged as possibly also having a tolerogenic and regulatory role (Geissmann et al., 2002; Larregina and Falo, 2005; Berger et al., 2006; Waithman et al., 2007; Fukunaga et al., 2008; Imai et al., 2008; Asahina and Tamaki, 2006; Romani et al., 2006). Epidermal LCs have been described to have a more immature phenotype than dDCs (Santegoets et al., 2008) at steady state, which would facilitate antigen uptake but reduce their antigen-presenting and stimulatory properties. It has also been proposed that LCs are superior inducers of T cell responses, while dDCs are more important for humoral immune responses (Ueno et al., 2007; Klechevsky et al., 2008). In addition, while there is still some ambiguity about the functional specializations of the various DC subsets in skin, the exact phenotypes of dDCs are not yet conclusively defined. Various dermal subsets have been described based on presence or absence of CD1a/c, CD11c, Langerin, DC-LAMP, DC-SIGN and CD14 (Nestle et al., 1993; Ebner et al., 2004; Angel et al., 2007; Zaba et al., 2007; Klechevsky et al., 2008; Ochoa et al., 2008; Figdor et al., 2002; Bursch et al., 2007). Different degrees of maturation and suggested functions are attributed to the subsets based on the expression of the various receptors. Further study of these cells can help us understand how microbes and allergens interact with skin DCs which can generate new treatment strategies targeting the skin, and also be potentially important in defining the mechanisms by which vaccines given through subcutaneous, intradermal or even intramuscular routes are working (Partidos et al., 2004; Rechtsteiner et al., 2005; Tacken et al., 2006; Warger et al., 2007; Stoitzner et al., 2008).

Many of the limitations of performing extensive and comprehensive studies on human skin DCs relate to the lack of experimental tools to either efficiently purify these cells or functionally assess them *in situ*. Many of the existing methods for isolation of skin DCs are labor intensive, which has practical implications on the amount of skin that can be processed, coupled with the fact that prolonged *in vitro* handling of the skin and DCs is associated with increased risk for induction of maturation of these cells. This may potentially alter their functions as compared to when residing in the skin. In this study, we therefore first aimed to refine an isolation protocol for the purification of skin DCs by introducing a skin graft mesher instrument to accelerate the more time-consuming steps in the process. The sorted DC subsets were phenotyped and analyzed for their capacity to take up antigen. Secondly, we developed a skin explant injection model where we could track rapid antigen uptake by these DC subsets *in situ* shortly after antigen administration. We believe that these methods offer advances for various applications addressing targeting of the skin and can contribute to a greater understanding of skin DC biology.

2. Materials and methods

2.1. Isolation of dendritic cells from skin

The Institutional Review Board of Ethics approved this study and informed consent was obtained from all patients. The skin DC isolation is based on our previously described

method with some modifications (Lore et al., 1998; Smed-Sorensen et al., 2008). Healthy skin was obtained from patients with early stage breast cancer who were admitted for mastectomy on the cancer affected breast and simultaneously had reconstructive surgery on the unaffected breast for symmetry (Karolinska University Hospital; Stockholm, Sweden). Discarded skin from the unaffected breast only was used for the experiments. The patients had received no treatment for their cancer prior to surgery, and had an isolated tumour in the cancer affected breast, with no suspected or documented spread of the disease. The skin was collected immediately after surgery and placed in complete media: RPMI 1640 (Sigma-Aldrich, Schellendorf, Germany) supplemented with 2 mM L-glutamine (Sigma), 1% streptomycin and penicillin (Sigma), 10% heat-inactivated fetal calf serum (FCS, Invitrogen, Carlsbad, CA, USA), and 5% Fungizone (Invitrogen). The skin was first cleared of subcutaneous fat and then cut in pieces about 3 × 5 cm in size. The pieces were placed on a skin graft carrier (Zimmer Inc.; Warsaw, Indiana, USA) and run through a skin graft mesher (Zimmer Inc.) that sliced the skin into nets. These nets were incubated in 4U/ml dispase (GIBCO, Grand Island, NY, USA) for 90 min at 37 °C and 5% CO₂. The epidermis could then readily be separated from the dermis using sharp forceps. The epidermis and dermis were washed twice in separate tubes (at 1500 rpm 5 min) in phosphate buffered saline (PBS). Epidermal and dermal sheets were then incubated separately in polystyrene petri dishes in complete media supplemented with 1% Hepes buffer (GIBCO) and 40 ng/ml GM-CSF (Pepro Tech, Rocky Hill, NJ, USA) to maintain a pH-stable physiological medium and to enhance migration, respectively, for 48 h at 37 °C. DCs that migrated into the media were harvested by filtering the media through a 70 µm cell strainer to obtain a single cell suspension. The cells were counted and used for experiments or further enriched by centrifugation using Ficoll-Paque Plus (GE Healthcare Biosciences AB, Uppsala, Sweden) density gradient (Lore et al., 1998). Trypan blue exclusion was used to measure cell viability.

2.2. Generation of monocyte derived dendritic cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation from buffy coats from healthy donor as previously described (Lore et al., 1998; Lore et al., 2001; Smed-Sorensen et al., 2008). Monocytes were isolated by plastic adhesion for 2 h at 37 °C. Adherent cells were washed twice in PBS and subsequently cultured in complete media (as described earlier) supplemented with 100 ng/ml IL-4 (R&D Systems Minneapolis, MN, USA) and 200 ng/ml GM-CSF for six days. Fresh media including cytokines was provided on day 3, and on day 6 >90% of the cells had downregulated CD14, upregulated CD1a and HLA-DR, and exhibited dendritic cell morphology. The contaminating cells consisted of undifferentiated monocytes and CD3⁺ T cells.

2.3. Phenotypic characterization of dendritic cells

Cells were harvested, washed in PBS supplemented with 0.5% FCS and stained for surface or intracellular receptors for 15 min at 4 °C and washed as previously described (Lore et al., 2003). Different combinations of antibodies were used, including DC-SIGN (CD209) PE or DC-SIGN FITC (both clone

120507, R&D Systems), Langerin (CD207) PE (clone DCGM4) and DC-LAMP (CD208) PE (clone 104.G4), (both Immunotech, Marseille, France), CD1a APC (clone HI149), HLA-DR PerCP (clone L243), CD14 FITC (clone M ϕ P9), DEC-205 (CD205) PE (clone MG38), CD11c APC (clone S-HCL-3), CD123 PE (clone 9F5), the maturation markers CD25 APC (clone 2A3), CD83 PE (clone HB15E), CD86 FITC (clone 2331/FUN-1) and lineage CD3 FITC (clone SK7), CD14 FITC (M ϕ P9), CD19 FITC (4G7) and CD56 (NCAM16.2), all from BD Biosciences Pharmingen (San Jose, CA, USA) unless otherwise stated. For intracellular staining of DC-LAMP and DC-SIGN, cells were fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions, prior to staining. The cells were collected on a FACS Calibur flow cytometer (BD Biosciences) and data was analyzed using Flow Jo software (Version 8.5; Treestar Inc., San Carlos, CA, USA).

2.4. Antigens

To evaluate antigen uptake, fluorescently labelled ovalbumin (OVA) (45 kDa, Alexa 488 conjugated, Molecular Probes, Eugene, OR, USA) and dextran (70 kDa, lysine fixable, Oregon Green 488 conjugated, Molecular Probes) were used. The contaminating endotoxin levels were <0.125 EU/ml in the antigen preparations as measured by Limulus Amebocyte Lysate Pyrogen Plus assay (Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA). 10 EU in this assay equals approximately 1 ng.

2.5. Antigen uptake assessment in monocyte derived dendritic cells

The cells were cultured at 1×10^6 cells/ml, with no less than 0.5×10^6 cells per tube in polystyrene round-bottom tubes in complete medium supplemented with IL-4 and GM-CSF. The cells were pulsed with 0.1–1 μ g/ml OVA conjugated with Alexa 488 at 37 °C for up to 48 h. The cells were stained (as described above) for CD1a and maturation markers and analyzed with flow cytometry. Alternatively, cells were transferred to adhesion slides (BioRad Lab, Munich, Germany) as previously described (Lore et al., 1998; Lore et al., 1999; Lore et al., 2001). In short, the cells were allowed to adhere to the slides for 30 min at 37 °C. Excess cells were washed away and cell fixation was performed in 2% formaldehyde (Sigma-Aldrich) for 20 min at RT. The slides were mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) for nuclear staining and analyzed by confocal microscopy (described below) to visualize antigen uptake as evidenced by Alexa 488 fluorescent signals. In indicated experiments, cells were stimulated by 5 μ g/ml of the Toll-like receptor (TLR) 3-ligand Poly I:C (Sigma-Aldrich) or 5 μ g/ml TLR7/8-ligand imidazoquinoline compound, 3 M-012 (Gorden et al., 2005), provided by 3 M Pharmaceuticals, MI, USA for 48 h, or 1 μ g/ml TLR4-ligand LPS (Sigma-Aldrich) or 20 ng/ml TNF- α (R&D Systems) for 24 h, before antigen exposure to induce maturation, indicated by upregulation of CD40, CD80, CD83 and CD86. To assess background binding of the fluorescently labelled antigen, cells were pulsed with antigen at 4 °C in parallel to the uptake assays performed at 37 °C. In addition, the internalization of the OVA uptake was confirmed by examining MDDCs exposed to OVA Alexa 488

and subsequently treated with $1 \times$ trypsin (Sigma-Aldrich) for 10 min at 37 °C to cleave off cell surface protein. To study the pathways of OVA uptake, cells were pre-incubated for 30 min at 37 °C with 5 mg/ml mannan (Sigma-Aldrich) or 3 μ M rottlerin (Sigma-Aldrich) before exposure to 1 μ g/ml OVA Alexa 488 for an additional 10 min incubation at 37 °C. Cells were then washed and either analyzed by flow cytometry, or adhered to slides for confocal microscopy analysis. Untreated cells were also incubated with 1 μ g/ml OVA Alexa 488 at 37 °C for 10, 30 or 60 min, washed and adhered to slides and stained with goat anti-EEA-1 (clone N-19, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or mouse anti-LAMP-1 (clone H5G11, Santa Cruz Biotech. Inc.) as described below in Section 2.8.

2.6. Antigen uptake assessment in skin dendritic cells

OVA Alexa 488, or dextran Oregon green 488, was added to the cells either during migration or after the cells had completed their migration and been harvested. For antigen exposure during migration, OVA or dextran was added directly to the media in which the skin nets were incubated. The migrated cells were harvested after 48 h and analyzed for antigen uptake. Uptake could not be assessed at earlier time points since the migration was not complete until after 48 h. Alternatively, harvested cells (incubated in media without OVA during their migration) were cultured, counted and resuspended at 1×10^6 cells/ml, with no less than 0.5×10^6 cells per tube, in polystyrene round-bottom tubes and pulsed with OVA or dextran. With both exposure procedures, the final concentration of antigen used was 0.1 μ g/ml and the total time of antigen exposure 48 h. Antigen uptake was assessed by flow cytometry.

2.7. Injection of antigen in skin explants

Freshly removed skin samples were cleared of subcutaneous fat and cut manually with a scalpel to pieces of an area about 3×3 cm. The skin was then injected using a BD Plastipak 2 ml syringe and BD Microlance 3 (0.4×19 mm) needle, containing 1 ml of either PBS or 1 μ g/ml of OVA or dextran (conjugated to Alexa 488 and Oregon Green 488, respectively). Each explant was injected several times to make distribution of antigen as uniform as possible. Injections were made as close to the epidermis as possible to minimize leakage of injected antigen from the tissue into the media through the porous deep dermis. The tissue explants were incubated for 48 h in complete media. The migrated cells were harvested and analyzed for antigen uptake by flow cytometry. For *in situ* analysis of antigen uptake in intact tissue, 200–300 μ l of 100 μ g/ml of OVA-Alexa 488 was injected into >1 cm² explants. The pieces were incubated for 90 min at 37 °C. Biopsies of the injection sites were thereafter taken, immediately put in O.C.T. (Sakura Finetek, Torrance, CA, USA) and frozen at -80 °C to be further analyzed for antigen uptake by confocal microscopy.

2.8. *In situ* analysis of antigen uptake in skin explants

Snap frozen skin biopsies taken after injection with antigen were cut to 8 μ m sections with a Micron HM 560

Cryostat (Fisher HealthCare, Houston, TX, USA) and fixed on Superfrost Plus Microscope Slides (Menzel Gmbh, Braunschweig, Germany) in 2% formaldehyde (Sigma-Aldrich), for 15 min at RT and washed thoroughly. Prior to staining, the sections were permeabilized with 0.1% Saponin (Riedel-de Haen, Seelze, Germany) dissolved in 1× Earl's balanced salt solution (EBSS, GIBCO) and supplemented with Hepes buffer (Andersson et al., 1988; Sander et al., 1991; Andersson and Andersson, 1993). Endogenous biotin or biotin-binding proteins were blocked using an Avidin-Biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Primary antibodies, including mouse anti-HLA-DR (clone L243, BD Pharmingen), mouse anti-CD1a (clone NA1/34-HLK, Serotec, Munich, Germany), goat anti-human Langerin (polyclonal, cat no AF2088, R&D Systems), mouse anti-human DC-SIGN (clone 120507, R&D Systems), goat anti-EEA-1 (clone N-19, Santa Cruz Biotechnology Inc.) or mouse anti-LAMP-1 (clone H5G11, Santa Cruz Biotechnology, Inc.) were diluted in EBSS-Saponin with 0.02% sodium azide and applied for incubation overnight. In order to reduce non-specific binding of the secondary biotinylated antibodies, the tissue sections were then blocked for 30 min with 1% rabbit serum (Dako, Glostrup, Denmark). The secondary antibodies (i.e. biotinylated rabbit anti-mouse, rabbit anti-goat or goat anti-mouse (Dako)) were diluted in EBSS-saponin and applied for 30 min. Finally, the sections were incubated with Alexa 488- or Alexa 594-labeled streptavidin (Molecular Probes) diluted in EBSS-saponin for 30 min. All incubations were performed in the dark at RT. After the final incubation, the sections were washed thoroughly with sterile water, dried and mounted using Vectashield mounting medium containing DAPI for nuclear staining. Multiparameter analysis of OVA uptake, cell surface receptor expression and nuclear staining was performed on a laser scanning confocal microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany) using either 20×/0.7 or 63×/1.3 objectives for sequential image capture z-series of each fluorochrome. Separate analyses of the image data were used to verify co-localization with DC cell surface markers and that the OVA Alexa 488 signals were sequestered in an intracellular compartment and not at the cell surface.

2.9. Statistical analyses

Statistical analyses were performed using Student's paired or unpaired *t*-test with Graph Pad Prism software, San Diego, CA and considered significant at * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

3. Results

3.1. Processing using a skin graft mesher facilitates isolation of dendritic cells

Studies on human DCs in the skin are constrained since the existing isolation procedures are often very laborious, limiting the amount of skin that can be processed. Systematic and comprehensive characterization of the DC subsets in the skin requires sufficient cell yields; hence large amounts of skin are needed. One major limitation in most established isolation procedures is the initial step involving processing of intact skin specimens into small pieces, usually strips or slices, which is required to allow for enzymatic disruption of the skin to

enable separation of the epidermis and the dermis and to obtain single cell suspensions. This processing step is usually performed by time-consuming manual slicing of the skin using scalpels and forceps. In this study, we introduced a skin graft mesher which is an instrument used clinically for expansion of skin grafts intended for skin transplantation as treatment of, for example, burn injuries. The skin graft mesher contains built-in blades, which process the skin into a thinly sliced net (Fig. 1A). We found that the processing step using the skin graft mesher was markedly faster, being in the order of minutes, as compared to hours for the standard manual slicing. The mesher method was on average 10 times faster ($n=5$) than the manual method for preparation of small samples (10 g of skin) and this time difference increased even more with larger samples. Due to the faster, and less cumbersome, skin-slicing step using the skin graft mesher, much larger quantities of skin were easily processed. The net structure created by the skin graft mesher also allowed for efficient collagenase treatment of the skin, which preceded the separation of epidermis and dermis. After the collagenase (disperse) treatment the epidermis was easily peeled off the dermis in the meshed skin, often in large intact sheets, using forceps. Thus, repeated peeling of small individual epidermal fragments, as required after manual slicing, was minimized, which helped further accelerating the process. Conclusively, time was saved both in the slicing and in the peeling steps using the skin graft mesher. The reduction in time does not only allow for more skin to be processed, it also reduces the risk of spontaneous DC maturation during the isolation procedure.

The respective epidermal and dermal tissues were then cultured separately to allow migration of DCs into the media. It is well documented that the migration process of DCs from skin starts instantly and spontaneously when the sections are placed in culture (Larsen et al., 1990; Lenz et al., 1993; Ortner et al., 1996; Lore et al., 1998; Kawamura et al., 2000; Sugaya et al., 2004; Flacher et al., 2006; de Witte et al., 2007). We collected significant numbers of migrated DCs after 24 h, however, the highest yield of migrated and viable DCs was harvested after 48 h. Cell counting using a haemocytometer revealed that on average 3.2×10^6 cells/g epidermis were harvested from nets processed by the skin graft mesher while 3.0×10^6 cells/g epidermis of matched donors were harvested from skin processed by manual slicing ($n=5$). In addition, on average 0.7×10^6 cells/g dermis were harvested after skin graft mesher slicing while 0.9×10^6 cells/g donor-matched dermis harvested by manual slicing ($n=4$). These cell numbers were not found to differ statistically. Apart from breast skin samples, we also tested and found the skin graft mesher to be suitable for slicing skin from abdomen and thigh (data not shown).

3.2. Distinct subsets of dendritic cells in epidermis and dermis

The DCs that migrated out of the epidermis and dermis were harvested by filtering the culture supernatants through a cell strainer and collecting the single cells. By flow cytometry, viable cells were gated on by side scatter and forward scatter, and high expression of HLA-DR was then used as the first inclusion criterion to identify DC populations out of the total migrated cells. The proportion of DCs varied between

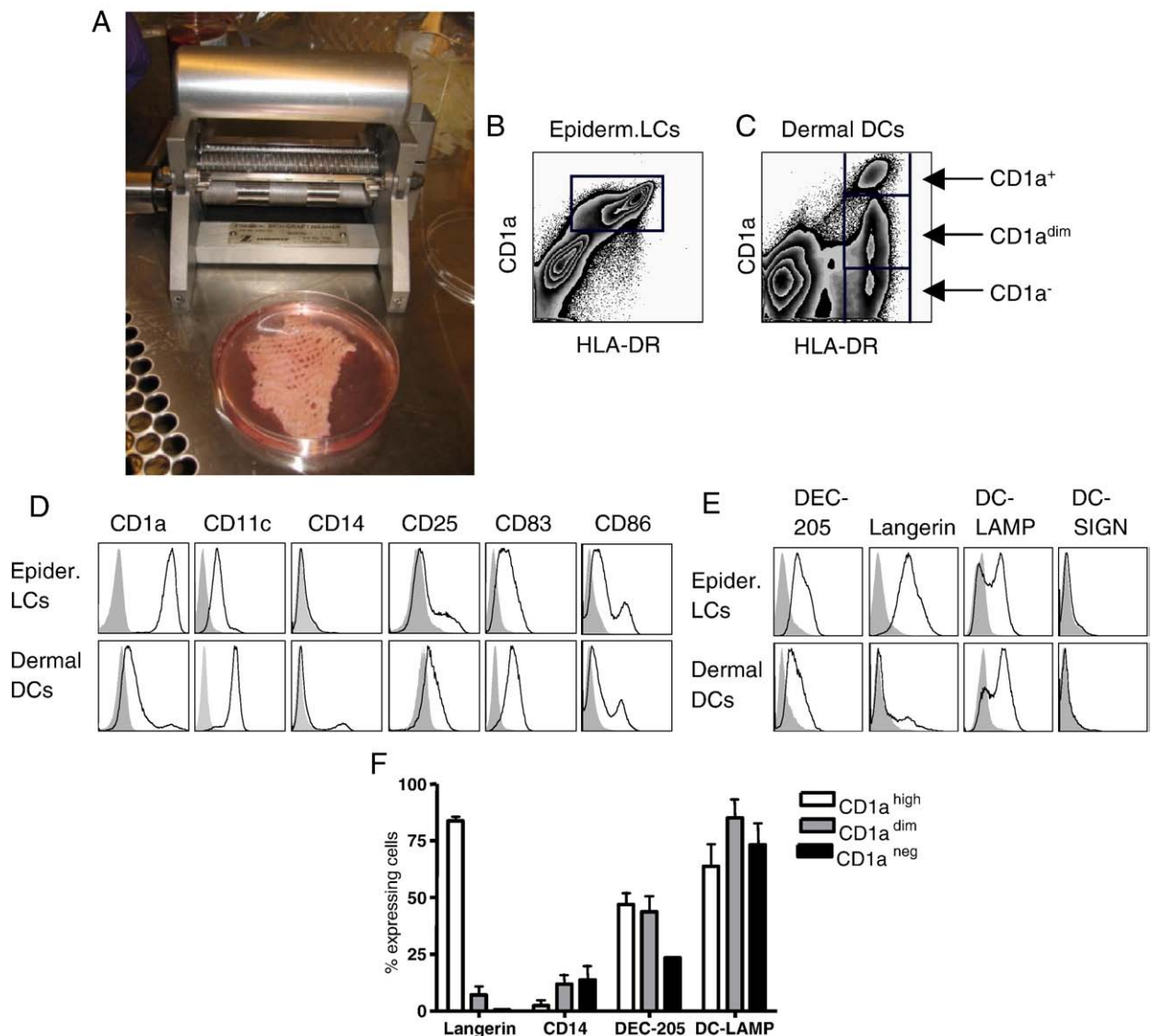


Fig. 1. Phenotype of DC subsets isolated from skin. (A) Skin graft mesher and a piece of processed skin with the characteristic net structure (100 mm × 20 mm polystyrene dish) (B–C) CD1a and HLA-DR expression in cells migrated from epidermis and dermis. (B) Gate includes CD1a⁺ HLA-DR⁺ Langerhans cells migrated from the epidermis. (C) The various gates represent HLA-DR⁺ dDC subsets with different level of CD1a expression. (D) Flow histograms of the phenotypes of harvested DCs from epidermis and dermis, respectively. Solid lines represent indicated marker and grey areas are unstained controls. (E) Flow histograms of C-type lectin expression of the harvested DCs from epidermis and dermis, respectively, after migration. In all figures showing flow cytometric plots, one representative donor of at least three is shown. (F) Expression of Langerin, CD14, DEC-205 and DC-LAMP by the various dDC subsets as divided by their CD1a expression. Graphs show mean values ± SEM of at least three donors.

individuals. The proportion of HLA-DR⁺ DCs from epidermis was $23.2 \pm 2.1\%$ out of all migrated cells collected whereas $9.6 \pm 2.3\%$ of cells harvested from dermis were HLA-DR⁺ DCs. These ratios were also similar between the skin graft mesher and manual slicing procedures. The dominant proportion of contaminating non-DCs consisted of debris and non-viable cells that likely had not actively migrated out from the skin. A minority of the contaminating cells was immune cells such as CD3⁺ T cells, CD19⁺ B cells or CD56⁺ NK cells. Although not confirmed, the remaining contaminating cells were likely to be keratinocytes and fibroblasts as reported earlier (Flacher et al., 2006). The proportion of DCs could be enriched by density gradient centrifugation on Ficoll-Paque Plus leading

to an average of $76.9 \pm 5.5\%$ and $27.8 \pm 7.7\%$ of HLA-DR⁺ DCs from either epidermis or dermis, respectively. The DC viability after the isolation procedure both by manual slicing and skin graft mesher slicing was >90% as determined by trypan blue exclusion.

CD1a and HLA-DR expression was used to define the various DC populations in epidermis and dermis (Fig. 1B–C). The DCs isolated from epidermis consisted of a homogenous population of HLA-DR⁺ CD1a⁺ CD11c⁺ CD123[−] CD14[−] Langerhans cells. Several subtypes of dDCs were isolated which all expressed HLA-DR and CD11c and lacked CD123. We found that the level of CD1a expression could be used to separate distinct dDC populations. A minor but distinct DC

population from dermis expressed high CD1a, while the major populations exhibited either diminished (dim) or no expression of CD1a (Fig. 1C). Most of the emigrated dDCs lacked CD14 with the exception of a minor but distinct population exhibiting CD14 (Fig. 1D). The CD14⁺ population was mainly represented in the CD1a^{dim/-} populations. The harvested DCs both from epidermis and dermis showed evidence of partial maturation as visualized by intermediate but not high expression of CD25, CD83 and CD86, markers associated with DC differentiation (Fig. 1D).

C-type lectin receptor expression was also examined. DEC-205 was expressed on both epidermal Langerhans cells and dermal DCs (Fig. 1E). All the Langerhans cells from epidermis expressed Langerin while there was only a minor subpopulation of Langerin⁺ cells in the dermis, which to a great extent coincided with the CD1a^{high} population (Fig. 1F). DC-LAMP was expressed on a large proportion of the total DCs from epidermis and dermis, respectively. DC-LAMP expression was present in all three CD1a^{high/dim/-} subpopulations from dermis (Fig. 1F). DC-SIGN was not found to be expressed on any of the DCs after migration which has previously been reported (Turville et al., 2002). Skin DCs isolated using the manual slicing or skin mesher techniques presented similar phenotypes (data not shown). Based on these data, we concluded that the DC subsets isolated from skin were of CD11c⁺ myeloid origin and that very few CD123⁺ plasmacytoid DCs resided in healthy skin. There was one uniform LC population in epidermis whereas at least three DC subsets, defined based on their CD1a expression, were present in dermis.

3.3. Analysis of protein antigen uptake in dendritic cells

The relative capacity of antigen uptake by the various DC subsets residing in the skin is poorly understood. To functionally examine the distinct DC subsets from epidermis and dermis, we focused on how efficiently they could internalize antigen. We used ovalbumin fluorescently labeled with Alexa 488 (OVA Alexa 488) in order to track and enumerate the frequencies of DCs that had taken up antigen. We first performed a series of experiments on readily available monocyte-derived DCs (MDDCs) to verify that evidence of OVA⁺ cells found in our assays represented intracellular internalization. MDDCs were exposed to OVA Alexa 488 for various time periods (30 min up to 48 h) using a 100-fold dose range (0.01, 0.1 or 1 µg/ml). In addition, uptake in phenotypically immature MDDCs and mature MDDCs, differentiated with either a TLR7/8-agonist or the TLR3-ligand Poly I:C were compared. The uptake was monitored by flow cytometry. We found that the uptake of OVA by MDDCs was strongly dose-dependent (data not shown) while the OVA exposure time that we investigated had less impact. After 90 min of exposure most of the uptake had occurred and the additional uptake found with longer exposure periods was modest (data not shown). Immature MDDCs had a much more efficient uptake of OVA than mature MDDCs. At a concentration of 0.1 µg/ml the frequencies of OVA⁺ cells were found to be 88.1 ± 3.2% in immature MDDCs versus 14.0 ± 4.3% in MDDCs matured by TLR7/8-ligand stimulation ($p=0.002$), and 15.5 ± 6.3% in cells matured by Poly I:C stimulation ($p=0.006$) ($n=3$; Fig. 2A). In addition to these

maturation stimuli, LPS and TNF- α stimulation also resulted in a significant reduction in OVA uptake capacity (data not shown). To test the specificity of the OVA Alexa 488 signals, the OVA exposure experiments were also performed at 4 °C which is documented to lead to a large reduction of endocytic activity by DCs resulting in inability to engulf antigens (Sallusto et al., 1995; Garrett et al., 2000). MDDCs exposed to OVA for 2 h at 4 °C showed a significant reduction in the number of OVA⁺ cells (Fig. 2B). However, there were still several OVA⁺ cells found after exposure at 4 °C although the intensity of the OVA signal was strongly reduced. Hence, the most dramatic reduction was observed in median fluorescent intensity (MFI) of Alexa 488 of OVA⁺ cells when the exposure was performed at 4 °C as opposed to at 37 °C (Fig. 2C–D). This phenomenon has been described earlier and reflects the fact that binding of OVA to endocytic receptors still occurs at 4 °C but there is less internalization of the antigen in contrast to the efficient and continuous internalization occurring at 37 °C leading to a strong intracellular signal (Sallusto et al., 1995; Garrett et al., 2000; Burgdorf et al., 2006). To ensure that there was minimal cell surface binding of OVA Alexa 488 at later time points when complete OVA internalization supposedly would have occurred, MDDCs exposed to OVA for 24 h were enumerated before and after trypsin treatment which was performed to cleave off cell surface proteins. No decrease in the number of OVA⁺ cells was found after trypsin treatment indicating that the OVA signal was indeed due to internalized OVA and not cell surface-bound OVA (Fig. 2E). Finally, we verified by confocal imaging that the OVA Alexa 488 signals were located intracellularly in the MDDCs (Fig. 2F). The signals were either found in close proximity to the cell nucleus and/or in vesicles in the cytoplasm. Based on these control experiments, we concluded that assessment of OVA uptake using our procedures was validated as a measure of antigen uptake.

3.4. Antigen uptake in skin dendritic cells

Skin DCs were thereafter tested for their capacity to take up OVA. Two different exposure procedures were tested. First, the skin DCs were exposed to OVA during their migration process. To achieve this, OVA was added to the media in which epidermis and dermis, respectively, were incubated during the migration period. The skin DC thereby likely encountered the OVA while still resident in the skin. This was possible since the skin was very thinly sliced and the media-skin interaction surface was substantial. The skin was incubated for 48 h during which time the DCs migrated out into the media to be harvested and analyzed for OVA uptake. Alternatively, DCs were first allowed to migrate from epidermis and dermis, respectively, over 48 h. The cells were then harvested and exposed to OVA, at the same concentration as the cells exposed while still resident in the skin, for 48 h, stained for DC markers and analyzed for uptake. The frequency of OVA⁺ LCs from the epidermis that had internalized OVA during the migration were high (78.2 ± 5.2%) and resembled the high uptake of OVA in immature MDDCs (Fig. 3A). The numbers of OVA⁺ DCs from dermis were significantly lower (30.7 ± 6.1%) than donor-matched epidermal LCs ($p<0.0001$) (Fig. 3A–B). The presence of OVA⁺ cells in the contaminating HLA-DR⁻ non-DC populations was also lower in dermis than in

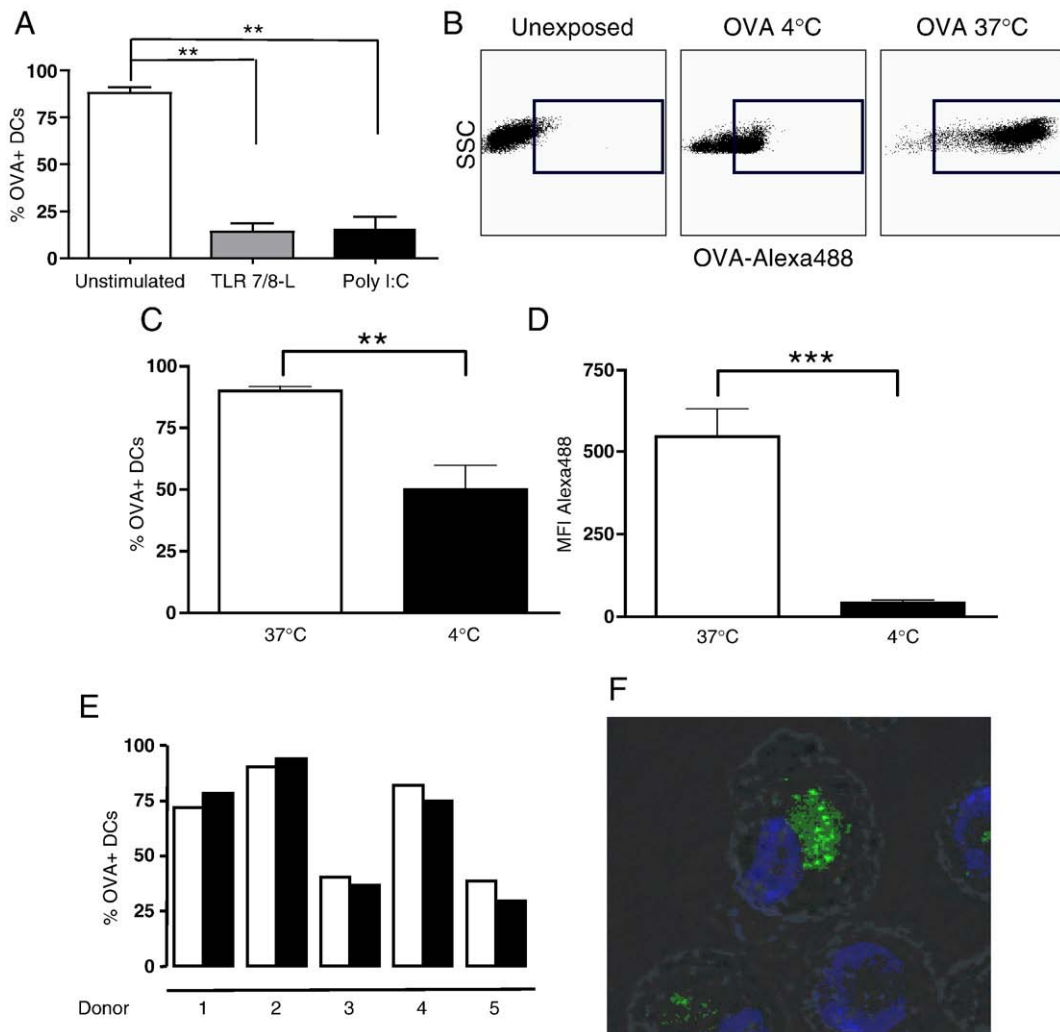


Fig. 2. Antigen binding and internalization by MDDCs. (A) Uptake of OVA-Alexa 488 by immature and mature MDDCs incubated with OVA for 48 h ($n = 3$, matched pairs). (B) Flow plots of OVA binding and/or uptake by MDDCs at 4 °C or 37 °C during a 4 h incubation. (C) Graph shows combined data from separate donors of frequencies of MDDCs binding OVA at 4 °C or 37 °C ($n = 3$, matched pairs). (D) OVA binding presented as MFI of Alexa 488. (E) OVA uptake by MDDCs from five different donors measured in untreated (white bars) or trypsin-treated (black bars) cells after incubation with OVA. (F) Confocal image shows intracellular localization of OVA in MDDCs after 90 min incubation with OVA. OVA (OVA Alexa 488 in green, or bright in black and white version) is seen closely adjacent to the cell nucleus (DAPI, blue). * $p \leq 0.05$, ** $p \leq 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

epidermis, although in both tissues the majority of OVA⁺ cells were indeed DCs (data not shown).

The OVA uptake was much lower both in LCs from epidermis and DCs from dermis when the cells were exposed to OVA after they had been harvested as opposed to exposed during migration ($p < 0.0001$ for LCs and $p \leq 0.029$ for dDCs) (Fig. 3A–B). This likely reflects decreased antigen uptake capacity after maturation associated with incubation and migration. As DCs are likely to be of an immature phenotype when they first encounter the antigen *in vivo*, exposing the cells to OVA during and not after migration is likely more physiologically relevant. We observed a trend, albeit not significant, that OVA exposure led to an increase in DCs migration, as the percentage of DCs out of all migrated cells was higher when the tissue had been exposed to OVA as compared to unexposed ($n = 6$, Fig. 3C). It is possible that any

enhancement in induction of migration caused by OVA exposure was masked by the general and strong migration of DCs that is induced in the skin during culture.

As shown above, using either exposure protocol, LCs from epidermis were more effective at taking up OVA than DCs from dermis. In addition to this, there were clear differences between the different dDC subsets. Surprisingly, the dermal subset that most closely resembled LCs, i.e. the CD1a^{high} Langerin^{high} DCs, consistently showed the lowest antigen uptake (Fig. 3D). The CD1a[−] dDC subset showed an intermediate uptake while CD1a^{dim} were the superior dDC subset for internalizing OVA (Fig. 3D).

In order to expand our analyses on antigen uptake by skin DCs we exposed the cells to an alternative antigen, the polysaccharide dextran, labeled with Oregon Green 488. Like OVA, fluorescently labeled dextrans have been widely used to

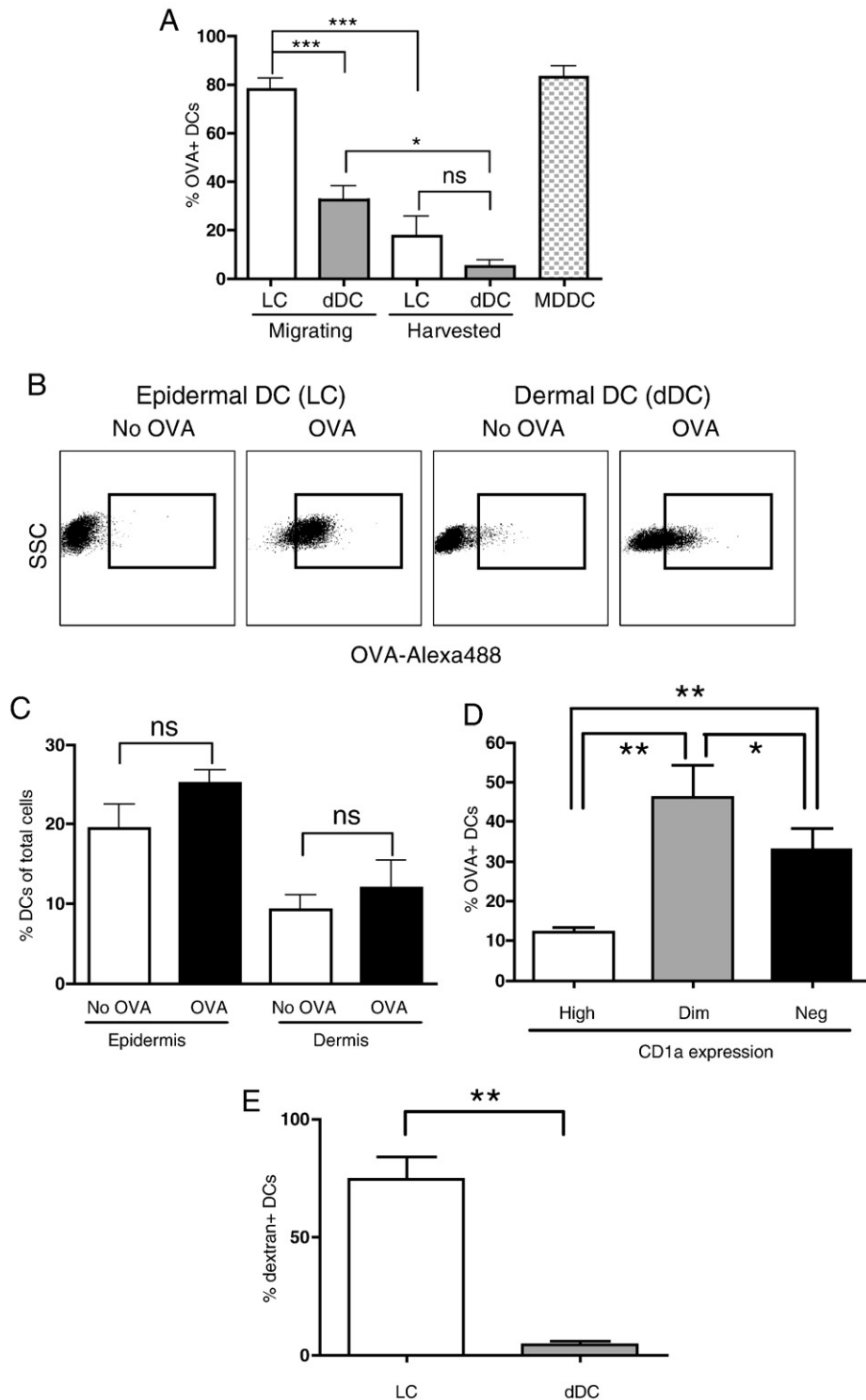


Fig. 3. OVA uptake in DC subsets migrated from meshed skin. Uptake of OVA by skin DCs was monitored by flow cytometry. (A) Epidermal LCs (white bars) and dDCs (grey bars) were exposed to OVA for 48 h either during migration or after harvesting ($n=8$). Immature MDDCs (grey checked bar) were used as positive control ($n=8$). (B) Flow plots of OVA uptake in dermal and epidermal DC populations following exposure during migration. One representative donor of eight is shown. (C) Percentages of DCs out of total emigrated cells from skin in absence (white bars) or presence (black bars) of OVA ($n=6$, unpaired donors). (D) Percentage of OVA⁺ cells in the different dDC subsets divided by level of CD1a expression i.e. CD1a high, dim or neg ($n=4$, matched donors) exposed during migration. (E) Dextran uptake by epidermal LCs (white bar) and dDC (grey bar) exposed to dextran during migration. ($n=4$, matched donors). * $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$.

study the antigen uptake capacity of DCs. Exposure to dextran at the same concentrations as used for OVA (0.1 $\mu\text{g}/\text{ml}$) also resulted in a high uptake by epidermal LCs while DCs from dermis showed a significantly poorer albeit detectable uptake ($p = 0.006$) (Fig. 3E).

3.5. Injection of antigen in skin explants induce antigen uptake by dendritic cells

With the aim of minimizing *in vitro* handling and activation of skin DCs prior to antigen exposure, we developed a skin explant model in which intact skin was exposed to antigen (OVA Alexa 488 or dextran Oregon Green 488) by injection *in situ*. Using a fine needle, multiple and shallow injections were performed directly into the dermis as close as possible to the epidermis of the skin explants (Fig. 4A). As a consequence of the injections, small pouches at the injected

sites were created that were retained for around 4 h (Fig. 4B). Multiple injections per skin explant were performed to distribute the antigen as evenly as possible, and also to expose as many cells as possible to the antigen. With only one single injection the percentage of OVA exposed cells in the explant was very low (data not shown). We administered the antigen as close to the epidermis as possible to minimize leakage through the porous dermis into the media. The injected skin explants were incubated for 48 h, which allowed for migration of cells into the media. High uptake of OVA Alexa 488 was observed in harvested DCs after skin injection (Fig. 4C–D). In total, $67.9 \pm 5.6\%$ of total HLA-DR⁺ DCs showed OVA uptake. Injections with dextran Oregon Green 488 showed lower uptake ($25.8 \pm 6.6\%$) (Fig. 4D).

The absolute number of emigrated cells was much lower in the skin explant model than found with the skin graft mesher isolation protocol described earlier. OVA injection as

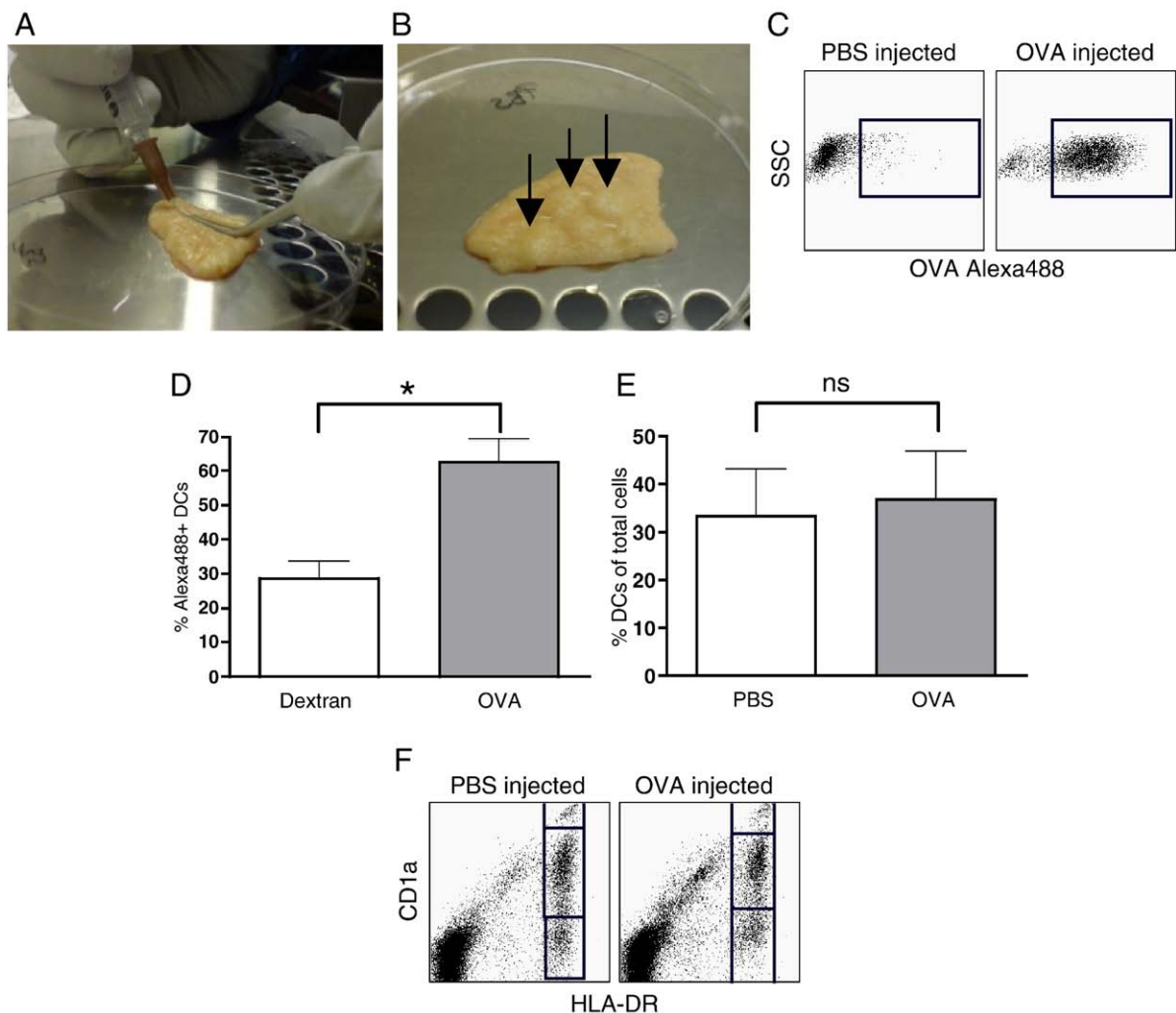


Fig. 4. OVA uptake using a skin explant injection technique. (A) Untreated whole skin explants were injected with PBS or OVA. (B) Pouches in the skin were formed after injection. (C) Emigrated cells were harvested after 48 h and analyzed by flow cytometry. One representative donor of eight is shown. (D) Dextran and OVA uptake in DCs were compared in cells harvested from injected explants ($n = 4$, matched pairs). (E) Percentages of DCs out of total emigrated cells from skin injected with PBS (white bars) or OVA (grey bar) ($n = 6$, unpaired donors). (F) Proportions of the various DC phenotypes as divided by level of CD1a expression were measured after PBS or OVA injection. * $p \leq 0.05$.

opposed to PBS injection did not yield significantly higher proportions of DCs out of all emigrated cells (Fig. 4E). Since the explants consisted of intact skin the harvested populations of emigrated DCs likely contained a mix of DC subsets resident both in dermis and epidermis. Again, we observed that three populations of HLA-DR⁺ DCs including the CD1a^{high}, CD1a^{dim} or CD1a⁻ DCs as described above for dDCs, emigrated from the explants (Fig. 4F). The proportions of these various subsets were rather similar to that of DCs emigrated from dermis in the mesher model suggesting that emigration of epidermal LCs, as compared to that of dermal DC subsets, is inefficient in the injection model. The three dDC populations were harvested at similar proportions regardless of PBS or OVA injection (Fig. 4F). The emigrated DCs from skin explants exposed to PBS alone exhibited a similar mature phenotype as found for the DCs emigrating after processing and collagenase treatment of skin, suggesting that the migration per se is sufficient to cause maturation (data not shown). As observed for dDCs, the lowest and highest proportion of OVA uptake was found in the CD1a⁺ and CD1a^{dim} populations, respectively (data not shown).

3.6. Antigen uptake can be tracked early after injection into skin explants

To examine immediate uptake of antigen by intact skin DCs prior to migration and maturation, we injected skin explants with OVA Alexa 488 or PBS for 90 min and thereafter cut out biopsies which were embedded, cryo-sectioned, stained for DC markers and analyzed for OVA-uptake by confocal microscopy. Epidermal and dermal architectures were still intact 90 min post-injection while some disruption could be observed with longer incubation periods. By *in situ* stainings, we found that bright HLA-DR⁺ cells were most abundant in dermis but also scattered in epidermis (Fig. 5A). Low or undetectable HLA-DR expression on epidermal LCs *in situ* has been described previously (Ebner et al., 2004; Zaba et al., 2007; Klechevsky et al., 2008) and likely reflects the immature state of the cells when residing in peripheral tissue. In contrast to HLA-DR, CD1a expression could readily be used to identify epidermal LCs *in situ*. CD1a⁺ LCs were almost exclusively localized in the epidermis (Fig. 5B). It is possible that the CD1a expressed by the CD1a^{dim} dDC subset found amongst the emigrated cells from dermis, as described above, is below the limit of detection in the *in situ* model and/or that CD1a was first upregulated during migration. While no DC-SIGN expression could be detected on migrated cells by flow cytometry, DC-SIGN expression was clearly detected *in situ* in the dermis (Fig. 5C). Injection of OVA Alexa 488 led to some degree of increased background fluorescence, however, distinct and bright Alexa 488-signal localized to individual cells were easily distinguished from the diffuse background signal. We verified by inspection of the separate sequential confocal image layers in the z-series that the concentrated OVA-signals were sequestered in an intracellular compartment and not at the cell surface. Although most of the diffuse green-fluorescent background was found in the deeper dermis, presumably in proximity to the injection sites, many distinct OVA⁺ cells were seen in the superficial dermis close to the basement membrane (Fig. 5D–H). Also, the disruption of the deeper dermis caused by injection did not allow for

accurate evaluation of the OVA uptake at that site. There are lymphatic capillary plexus (i.e. natural DC migration routes) in both the upper, papillary dermis, and in the deep dermis. After taking up antigen, the DC migrate towards these plexus and it is possible that a portion of the OVA⁺ DCs found in the superficial dermis acquired OVA deeper in the dermis and then relocated in the tissue towards the superficial plexus. Since no or very few OVA⁺ cells were found in the epidermis and the superficial lymphatic plexus would normally be the primary migration route of epidermal DC, migration from deeper dermis could explain the relatively high density of OVA⁺ DC in the upper dermal region. The majority of cells showing OVA uptake expressed HLA-DR and were localized in the dermis (Fig. 5D–E). Several OVA⁺ cells also expressed DC-SIGN (Fig. 5J). Although the vast majority of OVA⁺ cells in the dermis did not express Langerin, some OVA⁺ Langerin⁺ cells were occasionally observed (Fig. 5F, denoted by arrow). The same was true for CD1a⁺ cells, with most abundant CD1a expression in the epidermis, and only few CD1a⁺ OVA⁺ cells observed in the dermis (Fig. 5G–H). Using higher power magnification, a distinct pattern of fluorescent signals in individual cells after internalization of OVA Alexa 488 was observed. The signals were found in distinct vesicles throughout the cytoplasm (Fig. 5I–J).

3.7. OVA is taken up via CLR-mediated endocytosis and by macropinocytosis

To address potential uptake mechanisms of OVA, we performed a series of experiments using mannan to block CLR-mediated endocytosis (Sallusto et al., 1995) and rottlerin to block macropinocytosis (Sarkar et al., 2005) in MDDCs. Both blocking strategies led to a significant reduction of OVA uptake, but mannan was the most potent inhibitor, reducing uptake by $87.2 \pm 4.0\%$ of the control ($n = 4$, $p < 0.0001$) while rottlerin blocked uptake by $55.6 \pm 12.6\%$ ($n = 4$, $p = 0.0037$) (Fig. 6A). Receptor-mediated endocytosis delivers cargo to EEA-1⁺ early endosomes from where it progresses to Lamp-1⁺ lysosomes, whereas cargo internalized via macropinocytosis is delivered to distinct compartments containing Lamp-1 (Sallusto et al., 1995) but not EEA-1. Thus, we stained MDDCs exposed to OVA for 10, 30 or 60 min, for EEA-1 and LAMP-1 and analyzed the cells by confocal microscopy for co-localization with OVA. OVA partially co-localized with EEA-1 after 10 min and increasingly so after 30 min (Fig. 6B–C). OVA also co-localized with Lamp-1 after 60 min but to a lesser extent (Fig. 6D) than with EEA-1. The potent inhibition of OVA uptake by mannan together with this pattern of co-localization with endolysosomal markers confirms that it is taken up via CLR-mediated endocytosis. However, since the co-localization of OVA with EEA-1 was only partial and rottlerin also significantly decreased OVA uptake, we concluded that OVA is also taken up by macropinocytosis in MDDCs, in line with what others have shown (Sallusto et al., 1995; Garrett et al., 2000; Burgdorf et al., 2007).

Blocking studies of OVA uptake in skin DCs were hampered by the requirement of pre-incubation with mannan and rottlerin since there is no way to ensure the inhibitors will efficiently penetrate the skin. However, staining for EEA-1 and LAMP-1 was performed in sectioned skin biopsies taken 90 min after injection of OVA in skin explants. We again

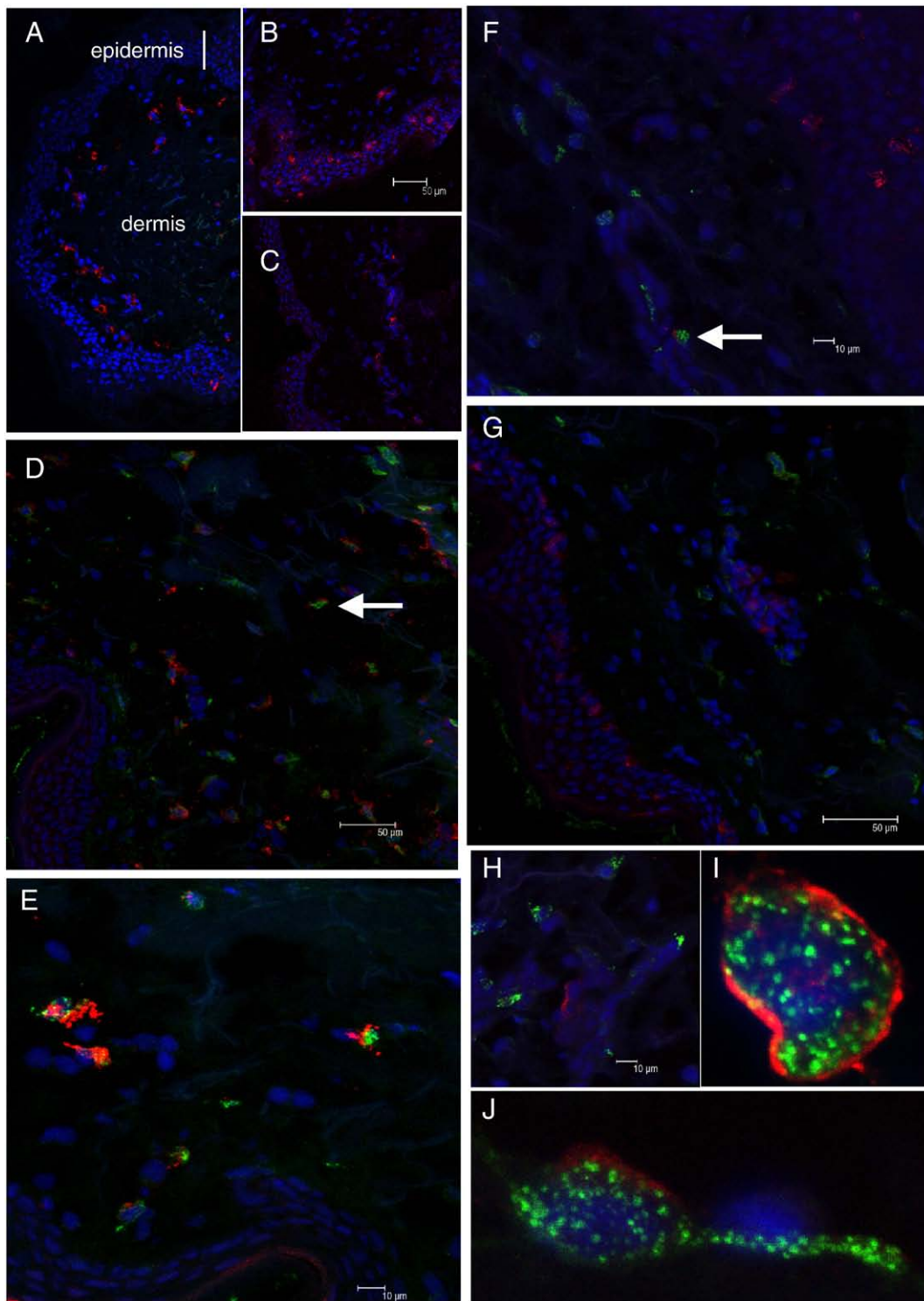


Fig. 5. OVA uptake *in situ* in injected skin explants. Skin explants were injected with OVA or PBS and biopsies collected at 90 min, cryo sectioned, stained and analyzed by confocal microscopy. In all micrographs OVA is visualized in green by Alexa 488 and nuclear staining in blue by DAPI, in addition to the indicated marker in red by Alexa 594. (A) Expression of HLA-DR in un.injected skin showing mainly localization to the dermis and only few cells in epidermis. (B) CD1a expression was mainly localized in the epidermis and (C) DC-SIGN in dermis of un.injected skin. (D and E) After injection, OVA⁺ DCs were almost exclusively found in dermis and frequently co-localized with HLA-DR (arrow). (F) Langerin⁺ epidermal LCs did not show any OVA uptake while rare Langerin⁺ cells in the dermis occasionally showed co-localization with OVA (arrow). (G) CD1a⁺ LCs in epidermis rarely showed OVA uptake (H) CD1a⁺ DCs in the dermis occasionally showed co-localization with OVA (I–J) High power magnification of intracellular localization of OVA in a dermal HLA-DR⁺ (I) and DC-SIGN⁺ (J) DC showing OVA in characteristic vesicular structures.

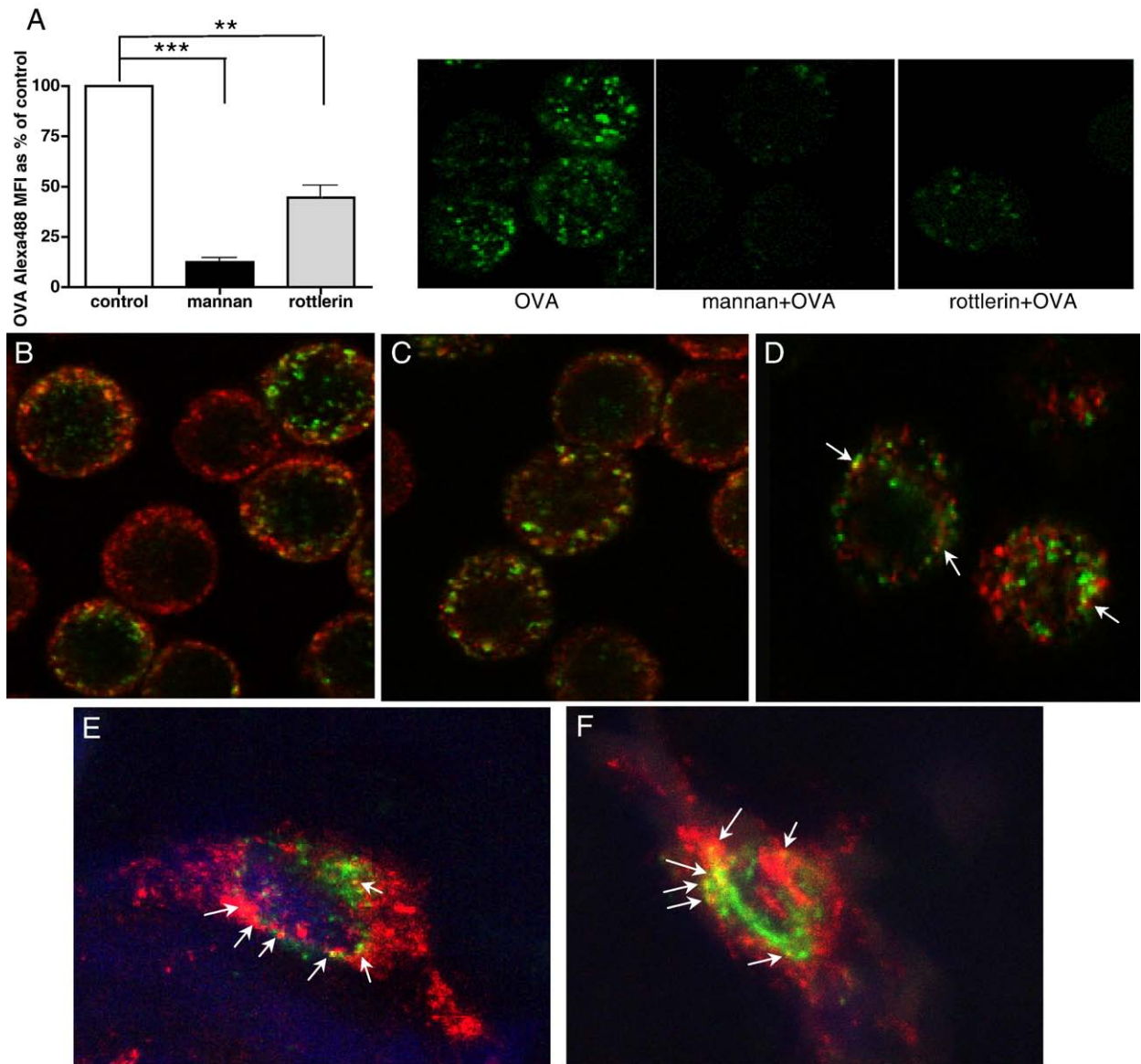


Fig. 6. Mechanisms of OVA uptake. Flow histograms showing uptake of OVA-Alexa488 in MDDCs after 30 min of treatment with CLR inhibitor, mannan or macropinocytosis inhibitor, rottlerin ($n = 4$), with corresponding images taken by confocal microscopy. $**p < 0.01$; $***p < 0.001$. (B and C) OVA partially co-localizes with early endosomes. MDDCs were stained for early endosome marker, EEA-1 (red) for confocal analysis after 10 (B) or 30 (C) min of OVA (green) exposure. Co-localization appears yellow. (D) OVA occasionally co-localizes with LAMP-1. MDDCs were stained for lysosome or mature macropinosome marker, LAMP-1 (red), after 60 min of OVA (green) exposure. Co-localization appears yellow and is indicated by arrows. (E and F) dDC were stained *in situ* with EEA-1 (E, red) or LAMP-1 (F, red) after 90 min exposure to OVA (green). Arrows indicate the partial co-localization between OVA and each marker.

observed co-localization with EEA-1 and Lamp-1 although it was more occasional than in MDDCs (Fig. 6E–F). DCs in the skin would encounter OVA in an asynchronous fashion and the uptake of the antigen may be continuous over the exposure period, thus OVA will naturally accumulate at various stages along both entry pathways, accounting for the incomplete co-localization with either marker at a given time. These data suggest that OVA is taken up by skin DCs via similar mechanisms as MDDCs, however, further studies are needed to more precisely elucidate antigen uptake mechanisms in skin DCs.

4. Discussion

Epidermal and dermal DCs are specialized to take up an array of antigen in the skin, and to control subsequent immune responses. Still, most studies on human DCs are not performed using directly purified primary DCs but instead on DCs derived *in vitro* from precursor cells such as monocytes or CD34⁺ stem cells. The reasons for this include the limitations in efficiency of the purification protocols for primary DC, as well as the challenges in terms of the rarity and vulnerability of these cells. Here, we report that direct isolation of skin DCs

can be accelerated by using a skin graft mesher instrument, which enables fast processing of large skin specimens. The skin graft mesher sliced the skin samples into nets that allow efficient enzymatic disruption of the tissue. The phenotypes and functions exhibited by DCs isolated with this method were indistinguishable from skin DCs isolated with the traditional manual slicing technique.

A homogenous population of Langerhans cells (LCs), with a uniformly high expression of CD1a, Langerin, DEC-205, CD11c, was isolated from the epidermis. Most of the LCs also expressed DC-LAMP, but not CD14 or CD123. Isolated LCs showed a high capacity to take up antigens in our *in vitro* assay, particularly if the antigen was provided early during the migration process from the epidermal sheets. The emigrated DCs showed a mature phenotype characterized by expression of CD25, CD83 and CD86. Presumably due to this mature phenotype, which is associated with reduced antigen uptake, LCs that were exposed to antigen after they had completed their migration showed much lower antigen uptake capacity (Sallusto et al., 1995; Banchereau and Steinman, 1998; Mellman and Steinman, 2001). This phenomenon was clearly evident in our experiments using MDDCs where there was a dramatic decrease in antigen uptake after the cells had been matured by TLR ligand stimulation. We found that the dDCs as a whole had much lower capacity for antigen uptake than LCs. The maturation status of the cells prior to exposure to OVA may correlate with the differences in antigen uptake capacity between epidermal and dermal DCs as well as the differences observed between the various dDC subsets. LCs have been described as more immature cells as compared to dDCs (Angel et al., 2007; Santegoets et al., 2008).

In contrast to epidermal LCs that are relatively well characterized, the phenotypes of the various dDC subsets are not well defined. In our study, we found that the dDCs could clearly, and consistently in all donors, be subdivided into three populations based on their level of CD1a expression. Various levels of CD1a expression in dDCs have been described previously. However, several of the previously reported isolation procedures of DCs from dermis involve exclusion of Langerin or CD14 expressing cells thereby depleting distinct populations. The CD1a^{high} dDCs co-expressed Langerin and thus resembled the phenotype of LCs, which led us to speculate that this dDC subset may represent LCs in transition through the dermis at the time the skin was removed, in line with previously reported hypotheses (Angel et al., 2007; Santegoets et al., 2008). Our finding showing that the CD1a^{high} Langerin⁺ subset isolated from dermis had poor antigen uptake capacity, even if exposed to antigen during migration, supports this theory. This suggests that the cells were of a more mature phenotype already when residing in the tissue. In fact, the low numbers of CD1a^{high} Langerin⁺ dDCs that internalized OVA were very similar to the numbers of OVA⁺ cells found after OVA exposure of emigrated, and thus matured, epidermal LCs. The remaining two dDC subsets (i.e. CD1a^{dim} or CD1a[−]) showed a much higher uptake than the CD1a^{high} Langerin⁺ subset, which indicates that the two former subsets were of a more immature phenotype at the time of exposure to antigen. A high proportion of all dDC subsets when harvested expressed DC-LAMP but no DC-SIGN. It is documented that dDCs can lose DC-SIGN expression during migration (Turville et al., 2002), and that DC-LAMP can

be upregulated on all skin DCs (Santegoets et al., 2008), which is why these particular markers are difficult to use for differentiating between dDC subsets in migrated cells. Some CD14⁺ cells were present in the CD1a^{dim} and CD1a[−] dDC subsets which might represent the DCs which have been described to exhibit superior B cell stimulatory capacity (Klechevsky et al., 2008). All the skin DC subsets found in our study were of a myeloid lineage expressing CD11c and only very few CD123⁺ plasmacytoid DCs were found.

Antigen uptake was also studied after injection into intact skin followed by harvest and analysis of cells that emigrated from the explant. This method offers a means of antigen exposure of cells while still in their tissue environment and may therefore represent a more physiologically relevant model than exposing isolated cells *in vitro*. However, antigen injection into skin explants and subsequent harvesting of emigrated cells does not enable studies of epidermal and dermal DC subsets separately. Lower cell yields were also obtained by the injection model than by the isolation procedure using the skin graft mesher. Since the emigrated DCs from the explants likely consisted of a mix of both epidermal LCs and dDCs, it is not surprising that the numbers of OVA⁺ DCs found were higher than seen in the skin mesher-isolated dDCs, but lower than in the skin-mesher isolated LCs. This may also indicate that the injection technique allows for antigen to be delivered to more phenotypically immature dDCs than the cells processed by the skin graft mesher. In addition to OVA, this model was assessed using dextran-injections. While we could clearly demonstrate dextran-uptake as well, the uptake was not as high as with OVA. It is possible that this discrepancy related to differences in the sizes of the antigens (OVA 45 kDa versus dextran 70 kDa) affecting their diffusion capacities through the skin tissue, a property that is more critical for exposure by injection into intact skin than for exposure of processed skin nets.

The injection technique was successfully used to investigate immediate events of antigen uptake by *in situ* confocal imaging, presumably of DCs that were yet to migrate and were thus immature. Interestingly, even though we found in our *in vitro* assays that epidermal LCs had the highest capacity to engulf antigen, our *in situ* analyses of antigen injection revealed that most of the antigen was detected in dDCs. This disparity may be due to the anatomical distribution of the antigen at injection and thus may mean that most vaccine delivery procedures primarily target dDCs and not epidermal LCs. We occasionally observed a few CD1a⁺ cells in dermis showing OVA uptake, which may represent the migratory LCs described above that had migrated from epidermis when exposed to OVA, but they may also represent dermal resident CD1a⁺ DCs. Although LCs that emigrated from the injected skin explants into the media were collected, they were few in number and it is possible that their migratory ability is reduced in the skin explants hampering their relocation to dermis and ability to capture OVA. It remains to be elucidated if topical application of antigen would instead lead to a predominate uptake by LCs instead of dDCs. To that point, when antigen was provided in the media as opposed to injected into the tissue, poor uptake was observed using *in situ* confocal imaging. Harvested emigrated DCs showed uptake of antigen, but the cells still had a predominantly dermal phenotype (data not shown). Despite possibilities to

study cells in their intact environment using this skin explant model there are still unphysiological constraints. For example, recruitment of new cells not residing in the skin at the time the skin specimen was removed cannot be monitored. Therefore, only a snapshot of events in the skin can be studied which is why focussing on early events after exposure is preferable using this technique.

We chose to perform our analyses using the protein antigen OVA because of its extensive prior documentation. In the MDDC model, we saw a marked reduction in OVA uptake after mannan treatment, but there was also a highly significant reduction after rottlerin treatment, indicating that both CLR-mediated endocytosis and macropinocytosis contribute to the uptake, in line with previous reports (Sallusto et al., 1995; Garrett et al., 2000; Burgdorf et al., 2007; Kato et al., 2000). Our confocal studies of MDDCs confirm this conclusion showing that OVA is delivered to the endolysosomal pathway, and the occasional co-localization seen with Lamp-1 could also be indicative of OVA present in mature macropinosomes. We also observed OVA in vesicular compartments in dDC exposed *in situ* that showed partial co-localization with EEA-1 and LAMP-1. In this respect the uptake of OVA in skin DCs was similar to MDDCs, however more extensive co-localization may be difficult to visualize given the asynchronous nature of exposure to OVA *in situ*. Alternatively, the partial co-localization may reflect a lower dependence on these pathways. While our data indicate that OVA is taken up via CLRs in MDDCs, it should be noted that LCs do not express the same repertoire of CLRs as MDDCs (Turville et al., 2002). Thus given the constraints on blocking studies in skin DCs it is not possible at this point to conclude which, if any, CLRs are mediating OVA uptake or whether macropinocytosis is the dominant uptake mechanism in these cells.

In conclusion, we show that use of the skin graft mesher enables efficient processing of large skin samples, thereby facilitating isolation of skin DCs with less time and effort. The time saved may also facilitate the isolation of more immature skin DCs as extensive *in vitro* handling is associated with differentiation. The antigen uptake capacity of the various skin DC subsets identified provides new clues to the characteristics and functionality of these populations. Furthermore, the skin explant injection model allows for studies of early events of antigen uptake *in situ*. We believe that these methods can contribute to further exploration of the skin DC population.

5. Disclosures

The authors have no financial conflicts of interest.

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