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Langerhans cells and dermal dendritic cells capture protein antigens in the skin: Possible targets for vaccination through the skin

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Abstract

Dendritic cells capture and process antigen and present it to T lymphocytes in the lymphoid organs. Dendritic cells of the skin, including epidermal Langerhans cells, langerin⁺ and langerin^{negative} dermal dendritic cells are ideally positioned to take up pathogens that enter the body through the skin or vaccines that are administered into (intradermal) or onto (epicutaneous) the skin. The antigen uptake properties of skin dendritic cells have not thoroughly been studied yet. We therefore investigated the uptake of the fluorochrome-conjugated model antigen ovalbumin (OVA) by skin dendritic cells in the mouse. OVA was readily taken up by immature Langerhans cells both *in situ* and in cell suspensions. When offered to Langerhans cells *in situ* either by “bathing” skin explants in OVA-containing culture medium or by intradermal injection they retained the captured OVA for at least 2–3 days when migrating into the culture medium and, importantly, into the draining lymph nodes. Also langerin⁺ and – to a larger extent – langerin^{negative} skin dendritic cells took up and transported OVA to the lymph nodes. Interestingly, mature Langerhans cells were still capable of ingesting substantial amounts of OVA, indicating that predominantly receptor-mediated endocytosis is operative in these cells. Unlike macropinocytosis, this pathway of endocytosis is not shut down upon dendritic cell maturation. These observations indicate that in intradermal vaccination schemes, Langerhans cells from the epidermis are prominently involved. They were recently shown to possess the capacity to induce functional cytotoxic T lymphocytes. Furthermore, the potential to markedly enhance antigen uptake and processing by targeting antigen to c-type lectin receptors on Langerhans cells was also recently demonstrated. Our data provide a rationale and an incentive to explore in more detail antigen targeting to Langerhans cells with the aim of harnessing it for immunotherapy.

Keywords

Dendritic cells; Langerhans cells; Antigen

Introduction

The mammalian immune system displays two distinct key features, which are important to meet challenges by potentially dangerous exogenous microbes. First, it has the ability to

rapidly recognize pathogens and second, it is capable to react with an immune response, tailored against the specific pathogens by means of the adaptive immune system. Although the effector cells, like T and B lymphocytes, respond with high specificity to the pathogens, they alone are not capable of inducing and regulating the response. The decision, whether and how the immune system reacts is largely depending on a distinct cell type called dendritic cell. Dendritic cells are specific antigen-presenting cells with the unique ability to induce a primary immune response in the host. To fulfill this task dendritic cells are capable of capturing, transferring and presenting exogenously derived antigens to the effector cells of the adaptive immune system (Steinman, 2007, 2008). Dendritic cells possess at least three different mechanisms by which antigen uptake can occur (Trombetta and Mellman, 2005): One mechanism called macropinocytosis, implicates the formation of endocytotic vesicles based on cytoskeleton rearrangement and fusion of distinct areas of the cell surface. However, in context with antigen uptake, it is still not clear whether skin dendritic cells really make use of this mechanism, which is rather unspecific. The fact, that there is hardly any fluid phase to be taken up in the intracellular space within the epidermis also argues against this form of antigen uptake. The other two mechanisms can be described as “phagocytosis” and “receptor-mediated endocytosis”. Both depend on distinct cell surface receptors and thus are antigen-specific. Upon maturation of dendritic cells, macropinocytosis and phagocytosis are largely down-regulated (Sallusto et al., 1995), whereas “receptor-mediated endocytosis” is just partially down-regulated. The mechanisms involved in these changes are unclear.

The purpose of our study was to investigate the uptake of protein antigen by skin dendritic cells, especially by epidermis-derived Langerhans cells. Ovalbumin (OVA), the main protein in chicken egg white, is a widely used model antigen for the investigation of many immunological questions. Nevertheless, protein uptake into Langerhans cells has not been studied in much detail (Reis e Sousa et al., 1993). Yet, this is an important question since in most immunization protocols antigens are applied into (or sometimes onto) the skin, where they likely encounter Langerhans cells (Stoitzner et al., 2008; Nicolas and Guy, 2008; Flacher et al., 2009, 2010). Hence, we performed experiments using OVA conjugated with two different variants of fluorescent Alexa fluor tracer molecules (Alexa Fluor 488 and Alexa fluor 647) to visualize the incorporation of this protein antigen.

Experimental procedures

Mice

Mice of inbred strain BALB/c were purchased from Charles River Laboratories (Sulzfeld, Germany) and were used at 2–8 months of age. Furthermore transgenic *langerinEGFP* mice expressing a fusion protein of langerin/CD207 and enhanced green fluorescence protein (EGFP) (Kissenpfennig et al., 2005) were used for some experimental approaches at 2–8 month of age. All experimental protocols were approved by the Austrian Animal Ethics Committee and performed according to the institutional guidelines.

Antibodies and antigens

For flow cytometry the following antibodies were used: MHC class II-APC (clone M5/114.15.2), CD11c-PE-Cy5 (clone N418), CD103-PE (clone M290), CD40-PE (clone 3/23), CD86-PE (clone GL-1), CD8 α -PE (clone Ly-2). For immunofluorescence we used antibodies against langerin/CD207 (clone 929F.3, hybridoma supernatant, kindly provided by Dr. Sem Sealand, Lyon, France) and MHC II (clone B21.2, hybridoma supernatant, kindly provided by Dr. Ralph M. Steinman, Rockefeller University, NY, USA), LAMP2/CD107b (hybridoma supernatant, R.M. Steinman), CD86 (clone GL1), CD40 (clone 3/23) (all from BD Biosciences, San Diego, USA). For secondary antibody anti-rat Alexa fluor

488 and anti-rat Alexa fluor 594 (Invitrogen/Molecular Probes, Eugene, Oregon, USA) were used. As a model antigen we used OVA conjugated with either Alexa fluor 488 or Alexa fluor 647 (Invitrogen). Application and defined concentrations of the antigens were dependent on the respective experimental protocols and are described below.

Skin explant culture

The culture medium was RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FCS), 50 μ M gentamycin (all from PAA, Linz, Austria), 2 mM L-glutamin (Invitrogen-Gibco, Paisley, Scotland) and 50 μ M 2-beta-mercaptoethanol (Sigma, St. Louis, MO). Mice were killed by CO₂ inhalation and ears were cut off at the base. Ear skin was mechanically split into dorsal and ventral halves with two forceps and dorsal (i.e. cartilage free) whole skin explants consisting of epidermis and dermis were cultured in 24-well plates (one ear per well) for 24 to 96 h at 37 °C (Stoitzner et al., 2010a). Skin explant cultures were set up to expose skin dendritic cells to the fluorescent OVA conjugate *in situ*. The whole skin explants were placed with the epidermal side upwards onto culture medium containing a defined concentration of the OVA protein as indicated in the figure legends. Incubation time with the antigen was 3 h for all experiments at 37 °C.

Preparation of epidermal cell suspension

Pieces of mouse ear skin were incubated on 0.8% trypsin (Merck, Darmstadt, Germany) for 25–45 min. Epidermis was peeled off and incubated for 30 min at 37 °C in a shaking water bath. Resulting epidermal cell suspensions contained 1–3% LC. For *in vitro* treatment of Langerhans cells with the fluorescent OVA protein, the cells were resuspended in fresh culture medium containing defined concentrations of the antigen and incubated at 37 °C agitating in a water bath. The antigen concentration for the *in vitro* approach was 160 μ g/ml. The incubation time was 3 h at 37 °C.

Cytospin preparation

The number of viable cells of a particular cell suspension was determined by adding Trypan-Blue to the cell suspension and counting the cells with a Hemocytometer (@Bright-Line). Subsequently, the cells were resuspended in an appropriate volume of PBS to obtain series of dilutions from 3×10^5 to 6×10^5 cells/ml. 200 μ l of the cell suspension were used for the preparation of Cytospin slides. For immunofluorescence staining the cells were fixed in acetone (Merck, Darmstadt, Germany) for 5 min at room temperature. Antibody incubations were performed for 30 min for each antibody at 37 °C.

Preparation of lymph node cell suspension

48 h after intradermal injection of 100 μ g of OVA–Alexa fluor 647, auricular skin-draining lymph nodes were removed and transferred into Hank's balanced salt solution, supplemented with 2% FCS, and mechanically disrupted with forceps. Lymph nodes were further digested with 0.5 mg/ml of collagenase P (Roche, Indianapolis, USA) and 120 μ g/ml of DNase I (Boehringer-Mannheim, Mannheim, Germany) for 25 min at 37 °C. Digestion was stopped by adding EDTA to a final concentration of 10 mM (AccuGene, Inc. Rockland, ME, USA). Single cell suspensions were obtained by pressing the digested tissues through a 70 μ m cell strainer (@BD Falcon).

Epidermal “sheet” preparation

Epidermis was detached from dermis by incubation in ammoniumthiocyanate (Merck, Darmstadt, Germany). Dorsal ear skin explants were placed with the epidermis facing upwards onto 0.5 M ammoniumthiocyanate and incubated for 15–20 min at 37 °C. Afterwards the skin was transferred onto PBS (PAA, Linz, Austria) supplemented with 1%

bovine serum albumin (Biomex, Mannheim, Germany) and the epidermis was separated from the dermis with two forceps. For immunofluorescence staining the tissue was fixed in acetone (Merck) for 15 min at room temperature.

Flow cytometry

Before staining, cells were preincubated at 4 °C for 10 min with anti-CD32 (clone 2.4G2, hybridoma supernatant) antibody to block unspecific binding to Fc receptors. Four-color multiparameter FACS analysis was performed by using a FACS Calibur (BD Biosciences, San Jose, CA, USA). FlowJo software (Tree Star, Inc., Oregon, USA) was used for data analysis.

Results

Freshly isolated Langerhans cells take up OVA antigen *in vitro*

Since it was not clear whether the incorporation of the fluorescent model antigen OVA–Alexa fluor 488/647 into Langerhans cells could be visualized *in situ* by immunofluorescence imaging, as well as by the flow cytometry, we chose a “straight-forward” approach and exposed isolated Langerhans cells to the OVA antigen *in vitro*. Langerhans cells, obtained by enzymatic digestion of epidermal skin, were resuspended in culture medium and were pulsed with OVA–Alexa fluor 488 at a final concentration of 160 µg/ml. The cells were incubated for 3 h at 37 °C. In order to visualize the uptake of the antigen by microscopy, epidermal cells were stained on cytocentrifuge smears with an antibody against langerin/CD207. As shown in Fig. 1A, fluorescently labeled OVA antigen was taken up into langerin⁺ cells in the epidermal cell suspensions. Interestingly, some of the OVA and langerin co-localized in the same compartments as judged by conventional fluorescence microscopy (Fig. 1A).

To quantify the uptake of OVA antigen by Langerhans cells, the mixed population of epidermal cells was analysed by flow cytometry. This revealed that MHC class II-positive Langerhans cells showed an increase in fluorescence after incubation with OVA–Alexa fluor 488 indicating that antigen had been incorporated (Fig. 1B). Although this experimental approach does not directly correspond to the physiological situation in which Langerhans cells encounter foreign antigen, our data clearly show that Langerhans cells are capable of incorporation of OVA antigen and that this process can be visualized by fluorescence microscopy and flow cytometry analysis.

In vitro matured Langerhans cells can still incorporate OVA-antigen

Although it is known that dendritic cells downregulate phagocytosis and macropinocytosis upon maturation (Sallusto et al., 1995; Trombetta and Mellman, 2005), we were interested whether Langerhans cells are still able to take up OVA–Alexa fluor 488 after maturation *in situ*. In order to address this issue, we set up whole skin explant cultures for 3 days to allow skin dendritic cells (i.e. Langerhans cells and dermal dendritic cells) to emigrate into the culture medium, thereby undergoing maturation. At day 3 of skin explant culture we harvested the emigrated dendritic cells and resuspended them in fresh culture medium. These dendritic cells were exposed for 3 h to 160 µg/ml of fluorescent OVA at 37 °C. After removing excess antigen by thoroughly washing the cells, Langerhans cells were identified by langerin/CD207 on cytospin preparations (Fig. 2A). We know from earlier experiments that most of the emigrated skin dendritic cells represent langerin⁺ Langerhans cells and only few langerin⁺CD103⁺ dermal dendritic cells and langerin[−] dermal dendritic cells can be found (Stoitzner et al., 2010a). Microscopy analysis showed that almost all langerin⁺ cells took up detectable amounts of OVA–Alexa fluor 488. The localization of the antigen was perinuclear in distinct areas containing a high number of small vesicles (Fig. 2A). Certain

areas showed co-localization with lysosomal vesicles as shown with a counterstaining against the LAMP II antigen. This unique staining pattern could be found in almost every langerin⁺ dendritic cell (Fig. 2A). To confirm the maturation state we additionally stained for the maturation markers CD40 and CD86. The majority of langerin⁺ cells expressed CD86 as well as CD40 indicating that they were mature. Besides the majority of langerin⁺ dendritic cells, there were also few langerin⁻ dendritic cells, which took up the antigen. Due to the fact, that whole skin was used for the experiments, these cells were considered to be dermal langerin⁻ dendritic cells. For the purpose of confirming the qualitative data, the same experiment was carried out and analysed with flow cytometry. For these experiments we made use of the langerinEGFP transgenic mice that allow omission of permeabilization of the cells. This would be necessary for intracellular langerin staining, but would lead to a potential loss of fluorescent OVA antigen. To allow the simultaneous detection of langerin-EGFP and OVA, we used the OVA–Alexa fluor 647 conjugate for these experiments. Again, we could observe a clear increase in fluorescence intensity of OVA–Alexa fluor 647 in mature Langerhans cells. The expression of CD40, as observed by immunofluorescence analysis, could be confirmed by flow cytometry. CD40 expression was significantly increased compared to freshly isolated Langerhans cells supporting the fact that the cells are mature (data not shown). Thus, mature Langerhans cells are still able to take up the antigen *in vitro*. The different staining patterns of the antigen between immature and mature Langerhans cells could be the result of different uptake pathways used by the cells at different stages of maturation.

Immature Langerhans cells incorporate OVA-antigen *in situ*

Our *in vitro* data clearly showed, that by using the OVA–Alexa conjugates, we were able to study antigen uptake by Langerhans cells in a qualitative as well as in a quantitative way. Thus, we were interested, whether it is possible to analyse the uptake of the antigen *in situ* by setting up an experimental approach, which resembles the physiological situation more closely. In order to accomplish this, we used whole skin, which was transferred with the epidermal side facing upwards on culture medium, containing 250 µg/ml of OVA–Alexa fluor 488. The tissue was incubated for 3 h at 37 °C. The majority of Langerhans cells took up OVA–Alexa fluor 488 as shown by immunofluorescence analysis of epidermal “sheets” (Fig. 3A). We repeated the experiments using the above described flow cytometry approach to receive quantitative results about the efficiency of antigen uptake *in situ*. Due to the more sensitive method we could use a lower concentration of 160 µg/ml of OVA–Alexa fluor 488. After 3 h of skin explant incubation on medium containing fluorescently labeled OVA, epidermal cell suspensions were prepared. Analysis showed an efficient uptake of OVA–Alexa fluor 488 by Langerhans cells as identified by the expression of MHC class II *in situ* (Fig. 3B). Additionally, we analysed the expression of the maturation marker CD40 by MHC class II⁺ Langerhans cells. As shown in Fig. 3B, the mean fluorescence intensity (MFI) of CD40 is almost similar to the isotype control indicating that, as expected, the Langerhans cells can be considered immature at the time point of antigen uptake.

Mature Langerhans cells take up OVA-antigen *in situ*

Corresponding to the analysis of antigen uptake by mature Langerhans cells *in vitro*, we were interested to investigate whether Langerhans cells, which had undergone maturation *in situ* would still be able to ingest antigen. Therefore, whole skin explants were cultured in medium for 48 h to induce maturation of Langerhans cells. Subsequently, the skin tissue was transferred onto fresh medium containing 250 µg/ml of OVA–Alexa fluor 488 for 3 h at 37 °C. Immunofluorescence analysis of epidermal “sheets” showed a reduced, partially discontinuous network of Langerhans cells, which is likely the result of cell emigration starting within two days of culture (data not shown). Furthermore, the remaining Langerhans cells showed a typical mature phenotype indicated by an increased cell size with enlarged

dendrites. Despite the reduced number of Langerhans cells, we were still able to find cells, which took up substantial amounts of OVA–Alexa fluor 488 (Fig. 4A). The qualitative data could be further confirmed by flow cytometry analysis of epidermal cell suspensions. The experimental setup was similar to the microscopy analysis, except that the skin explant culture period was shortened from two days to one day, in order to avoid the massive emigration of maturing Langerhans cells. Skin explants were incubated with an antigen concentration of 160 µg/ml for 3 h before preparation of Langerhans cells from epidermis. As expected, we could observe antigen uptake by MHC class II⁺ Langerhans cells after treatment with OVA–Alexa fluor 488, as indicated by an increased MFI in the antigen detecting channel. Additionally, we could confirm the advanced maturation status of Langerhans cells based on the increased expression of CD86 and CD40 (Fig. 4B).

OVA antigen is detectable in all migratory skin dendritic cell subsets

Next we wished to investigate whether the antigen, which was ingested by immature Langerhans cells *in situ*, is still detectable in migratory Langerhans cells that had emigrated from the skin. To fulfill this task, whole skin explants were exposed to a defined antigen concentration (160 µg/ml) for 3 h at 37 °C. Afterwards, excess antigen was removed by careful rinsing and the tissue was further cultured for 72 h in medium to obtain migratory skin dendritic cells. At the end of culture, cells were harvested and used for cytospin preparations. Langerhans cells were visualized by antibodies directed against CD207/ Langerin. We detected antigen inside the cells, but the staining pattern of the antigen seemed to be different compared to the previously analysed immature Langerhans cells. The antigen appeared to be more concentrated in a few areas of the cytoplasm of the cells, represented by a lower number of big dots (Fig. 5A). To quantify the fluorescence intensity of the antigen inside the migratory Langerhans cells, the experimental setup was repeated and the cells were analysed by flow cytometry. To this end, we applied the same OVA concentration of 160 µg/ml and incubated the tissue for 3 h at 37 °C. As shown in Fig. 5B, the fluorescence intensity of the antigen in migratory cells obtained from antigen-treated tissue is higher than the fluorescence of the untreated cells, meaning that after 3 days antigen is still detectable in the cells. Nevertheless, comparing the average fluorescence of the migratory cells (Fig. 5B) with the one of the freshly isolated Langerhans cells pulsed with antigen *in vitro* (Fig. 1B) demonstrated a decreased amount of antigen in the Langerhans cells. This fits to the microscopical observation that there was less antigen detectable in migratory Langerhans cells compared to immature Langerhans cells in suspension. When we further analysed the skin dendritic cell subsets, we observed that all three subsets, i.e., Langerin⁺ CD103[−] Langerhans cells, Langerin⁺ CD103⁺ dermal dendritic cells and Langerin[−] dermal dendritic cells incorporated/transported similar amounts of fluorescently labeled antigen. All skin dendritic cells were mature as judged by the high CD40 expression (Fig. 5B).

Langerhans cells, but also dermal dendritic cells, take up OVA antigen in situ and transport it to the skin draining lymph node

Since Langerhans cells were efficient in antigen incorporation and we could still detect antigen in emigrated skin dendritic cells, we next focused on the clinically most relevant application, which is intradermal injection. For this purpose, we injected 100 µg of OVA–Alexa fluor 647 in 25 µl of PBS into the ear skin of langerinEGFP transgenic mice. No dendritic cell maturation stimulus was added to the injection. On day 2 post-injection we digested the draining auricular lymph nodes of the mice and analysed the cells by the flow cytometry. As a control, we injected the same volume of PBS into the ear skin of langerinEGFP mice. The discrimination of skin-derived dendritic cells from lymph node resident dendritic cells was based on the expression of high levels of CD40 on skin dendritic cells. Both subpopulations i.e., CD40⁺ langerin⁺ as well as CD40⁺ langerin[−] skin dendritic cells were able to transport the antigen to the skin draining lymph nodes. However, part of

the langerin⁺ dermal dendritic cells seemed to take up more antigen as reflected by the second peak in the FACS analysis (Fig. 6A). For a more detailed discrimination of the skin-derived langerin⁺ dendritic cell subsets we split the cell populations based on their expression of langerin and CD103. Again, there was no significant difference in case of antigen delivery between the langerin⁺ CD103⁺ epidermal Langerhans cells and langerin⁺, CD103⁺ dermal dendritic cells (Fig. 6B). Both subsets were able to transport the antigen to the lymph node. Interestingly, blood derived langerin⁺ dendritic cells also showed an increase in fluorescence based on the OVA uptake (Fig. 6B). This can be explained by passive diffusion of the antigen from the skin to the skin draining lymph nodes.

Discussion

It was the purpose of this work to visualize and quantify the uptake of protein antigen by skin dendritic cells, especially epidermal Langerhans cells. To this end we used OVA coupled to a fluorescent tracer (Alexa fluor 488/647). Using different experimental approaches, we were capable of analyzing the uptake of our model antigen by skin dendritic cells at various stages during maturation as well as in the context of intradermal immunization. All together we found that Langerhans cells are efficient in incorporating soluble protein antigen at an immature stage. Moreover, mature Langerhans cells can still take up antigen in relevant amounts indicating that migrating Langerhans cells do not completely lose their ability to incorporate antigen on their way to the lymph nodes.

In an immature stage dendritic cells are prone to take up antigen via several different mechanisms, such as (macro)pinocytosis, phagocytosis and endocytosis (Trombetta and Mellman, 2005). In order to confirm this finding for Langerhans cells, we first investigated freshly isolated immature Langerhans cells. We observed that immature Langerhans cells take up copious amounts of ovalbumin, when exposed to the antigen *in vitro*. Although this does not immediately correspond to a physiological *in vivo* situation, this observation indicates that the endocytosis mechanism as described for dendritic cells from other sources operates well in immature Langerhans cells independent of the physiological environment. Studies performed in a physiologically more relevant situation, in which Langerhans cells were exposed to the antigen *in situ*, showed that they take up the antigen at least as well as compared to the *in vitro* approach. One could assume that floating whole skin tissue with the epidermal side facing upwards on antigen-containing medium would somehow reduce the accessibility of Langerhans cells to the antigen that has to diffuse all the way through the dermis to reach the epidermis. This was not the case, however, since similar amounts of antigen in Langerhans cells that had been exposed to antigen *in vitro* (i.e., in suspension) or *in situ* (i.e., within the intact epidermis) were detected by flow cytometry.

It has been reported that OVA protein is taken up through receptor-mediated endocytosis either via the mannose receptor in CD8⁺ dendritic cells or the scavenger receptor in macrophages (Burgdorf et al., 2007). Although it has been shown that receptor-mediated endocytosis is partially down-regulated upon maturation of dendritic cells (Sallusto et al., 1995), we observed uptake of ovalbumin by mature Langerhans cells exposed to the antigen *in vitro*. Furthermore, like in the *in vitro* approach we could show that pre-matured Langerhans cells are still able to take up antigen, when pulsed *in situ*. Therefore, we conclude that Langerhans cells, independent of their maturation stage, are able to ingest soluble protein antigen *in vitro* and *in situ*. Similar findings were reported by Ruedl et al. showing that migratory skin dendritic cells isolated from the lymph nodes were still well able to incorporate and present soluble ovalbumin to CD4⁺ T cells. In contrast, skin dendritic cells matured *in vitro* by addition of the cytokines GM-CSF and TNF- α or by the toll-like receptor ligands LPS or CpG were no longer capable of taking up and presenting soluble antigen to T cells (Ruedl et al., 2001). This indicates that skin dendritic cells

matured *in situ* during migration out of skin or during *in vitro* culture without defined maturation stimuli like TNF- α or TLR ligands are not comparable in their function to dendritic cells that have been exposed to experimentally added maturation stimuli. In accordance with these results, Langerhans cells and dermal DC derived from skin explants were able to present *in vitro* administered free ovalbumin antigen and ovalbumin delivered in liposomes to Fc-receptors (Henri et al., 2007). This suggests that receptor-mediated endocytosis is not switched off in migratory skin dendritic cells although they start to mature. Underlining this observation, Platt et al. (2010) recently reported that mature dendritic cells can still incorporate antigen via receptor-mediated endocytosis whereas constitutive macropinocytosis and phagocytosis are down-regulated. The chemokine receptor CCR7 has been described to be important for receptor-mediated endocytosis. After ligation of CCR7, enhanced incorporation of mannosylated-bovine serum albumin by mature dendritic cells but not immature dendritic cells was observed (Kikuchi et al., 2005).

Since the mannose receptor (CD206) has been implicated in the uptake of soluble ovalbumin antigen (Burgdorf et al., 2006), we investigated its expression on Langerhans cells in murine skin. Immature Langerhans cells *in situ* in steady-state epidermis lack expression of mannose receptor. Upon inflammation some mannose receptor-positive cells appeared in the epidermis, however, these cells did not express langerin indicating that they might be the murine counterpart to the inflammatory dendritic epidermal cells (“IDECs”) described for inflamed human skin (Alban Millonig, unpublished observations). This is in line with observations in human skin that showed expression of mannose receptor in inflammatory dendritic epidermal cells but not in Langerhans cells (Wollenberg et al., 2002). In murine skin, mannose receptor expression was demonstrated in MHC-class II⁺ cells, most probably macrophages in the dermis, which appeared to acquire expression of CD11c, CD11b, DEC-205, MHC-class II and co-stimulatory molecules upon migration to skin-draining lymph nodes (McKenzie et al., 2007). In human skin, mannose receptor expression was observed on macrophages and dermal dendritic cells (Zaba et al., 2007). In another study, mannose receptor was found expressed on dermal macrophages in murine skin besides other macrophage markers (Dupasquier et al., 2006).

Another receptor that is discussed in context of ovalbumin uptake is the scavenger receptor/CD163 on macrophages (Burgdorf et al., 2007). However, so far nothing is known about the expression of this receptor on murine Langerhans cells. Macrophages present in the human dermis also express the scavenger receptor CD163, however, dermal dendritic cells were negative for this marker, and also Langerhans cells appeared negative (Zaba et al., 2007). The appearance of fluorescently labeled ovalbumin in late endosomes, as indicated by co-localization with LAMP-1 suggests that scavenger receptor-mediated endocytosis and pinocytosis are involved in ovalbumin uptake into Langerhans cells. A correlation between pinocytosis/scavenger receptor-mediated endocytosis and localization in late endosomes has previously been shown for bone marrow-derived dendritic cells (Burgdorf et al., 2007).

We were further interested whether we could still detect our model antigen in skin dendritic cells which were allowed to migrate out of cultured whole skin explants incubated with antigen for a few hours before onset of culture. Indeed, we were able to detect the antigen in migratory skin dendritic cells indicating that they can retain it for prolonged periods. This held true for all subsets that can be defined by langerin and CD103 expression (Stoitzner et al., 2010a; Romani et al., 2010a). All three subsets seemed to incorporate ovalbumin to a similar level. This finding was important with regard to our subsequent experiments, which studied the clinically most relevant approach, namely intradermal injection of OVA–Alexa into the ear skin of recipient mice. Transport of antigen to the draining lymph nodes by the different skin dendritic cell subsets was assessed. Using the MFI of the antigen to measure the amount of ovalbumin incorporated, we noted that langerin⁺ dermal dendritic cells

incorporated more ovalbumin than langerin⁺ dendritic cells. This is probably due to the fact that the antigen is mainly deposited in the dermis by the intradermal injection and thus, more easily accessible for dermal dendritic cells than epidermal Langerhans cells. However, the CD103⁺ langerin⁺ dermal dendritic cells also incorporated similar amounts of antigen than Langerhans cells. An alternative explanation would be that the mannose receptor (CD206), which has been described on human langerin⁺ dermal dendritic cells, enables dermal dendritic cells to acquire ovalbumin more efficiently by receptor-mediated endocytosis (Turville et al., 2002). Regarding the Langerhans cells, there are two possible ways how these cells gain access to the intradermally deposited antigen. Either, epidermal Langerhans cells take up the antigen while passing through the dermis on their way to the skin draining lymph node or the antigen is able to diffuse right into the epidermis. The second possibility is quite likely since we could show that after bathing of skin explants in medium containing the protein, antigen can be detected in Langerhans cells in the epidermis. Moreover, intradermally injected mAbs (as an antigen surrogate) bind to Langerhans cells in the overlying epidermis as was shown recently by our group (Flacher et al., 2010). When we further subdivided the langerin⁺ dendritic cells in the skin-draining lymph nodes into Langerhans cells, dermal langerin⁺ and CD8⁺ langerin⁺ dendritic cells, all subsets demonstrated similar antigen incorporation after intradermal immunization. It has been shown that CD8⁺ dendritic cells express the mannose receptor and are able to incorporate ovalbumin protein (Burgdorf et al., 2007). After intradermal injection of ovalbumin antigen passive diffusion of the antigen to the skin draining lymph node, rather than active antigen transfer between subsets of dendritic cells is the likely way how langerin⁺CD8⁺ lymph node resident dendritic cells access antigen.

Another issue, which should be considered in this case, is the fact that in order to avoid loss of fluorescence of our reporter molecule we could not wait longer than two days after intradermal injection until analyzing the skin draining lymph node. It is known that dermal dendritic cells migrate faster than Langerhans cells to the skin-draining lymph node after FITC/TRITC painting (Kissenpfennig et al., 2005; P.Stoitzner, unpublished observations). Thus, to clarify the antigen transport by Langerhans cells, this should be tested with a more stable tracer molecule and by analyzing the lymph node three or four days post injection. The proportion of antigen-carrying Langerhans cells within the lymph nodes might possibly increase.

In summary, we observed that Langerhans cells are efficient in the incorporation of soluble ovalbumin antigen, most probably via various mechanisms, such as receptor-mediated endocytosis, which is maintained during *in situ* maturation upon migration from skin, and pinocytosis which is down-regulated during maturation. The continued uptake of antigen during migration to the skin-draining lymph nodes ensures that Langerhans cells have ample time to acquire enough antigen for presentation to T cells in lymphatic tissue. The uptake of antigen and its processing and presentation by Langerhans cells can be harnessed for immunotherapeutic purposes (Ueno et al., 2010; Stoitzner et al., 2010b). In this regard it is important to remember, that uptake of, for instance a vaccine by Langerhans cells can be made more than 100-times more efficient if that antigen is coupled to antibodies that direct it to antigen uptake receptors like DEC-205/CD205 on Langerhans cells (Flacher et al., 2010). Such “antigen targeting” would also lead to greatly enhanced immune responses which are desirable in tumor immunotherapy (Romani et al., 2010b).

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Abbreviations

OVA	ovalbumin
APC	allophycocyanin
PE	phycoerythrin
MFI	mean fluorescence intensity

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Fig. 1.

In vitro uptake of OVA antigen by freshly isolated Langerhans cells. (A) Microscopy analysis of cytospin preparations: Freshly isolated Langerhans cells from BALB/c mice were incubated with 160 $\mu\text{g}/\text{ml}$ of OVA–Alexa fluor 488 for 3 h at 37 °C. Langerhans cells stained for langerin/CD207 (*red fluorescence*), incorporated OVA–Alexa fluor 488 (*green fluorescence*). (B) Flow cytometry analysis of antigen uptake: Freshly isolated Langerhans cells from BALB/c mice were treated with 160 $\mu\text{g}/\text{ml}$ of OVA–Alexa 488 (*bottom row*) or with PBS (*upper row*) for 3 h at 37 °C. Langerhans cells were identified as MHC class II⁺ cells. Antigen incorporation was visualized by increased MFI in the antigen detecting channel FL1 (histogram: black: OVA–Alexa fluor 488, dotted: PBS control). One representative out of 3 experiments is shown; 3–5 mice were pooled in each experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

In vitro uptake of OVA antigen by mature Langerhans cells. (A) Microscopy analysis of cytopsin preparations: Dendritic cells emigrated from whole skin explants of BALB/c mice were incubated with 160 $\mu\text{g}/\text{ml}$ of OVA–Alexa 488 for 3 h at 37 °C. Langerin⁺ skin dendritic cells, mostly representing Langerhans cells stained for langerin/CD207 (*red fluorescence*, upper row), incorporated OVA–Alexa fluor 488 (*green fluorescence*). Additional staining for lysosomal vesicles with LAMP II /CD107b (*red fluorescence*, second row) is shown. Maturation status of langerin/CD207⁺ cells was validated based on staining for maturation markers CD86 and CD40 (*red fluorescence*, third and fourth row). (B) Flow cytometry analysis of antigen uptake: Dendritic cells emigrated from whole skin explants from langerinEGFP mice were incubated with 160 $\mu\text{g}/\text{ml}$ of OVA–Alexa 647 for 3 h at 37 °C. Langerin⁺ skin dendritic cells were identified as EGFP⁺ cells. Antigen incorporation was visualized by increased MFI in the antigen detecting channel FL4 (histogram: black: OVA–Alexa fluor 647, dotted: PBS control). One representative out of 3 experiments is shown; 10–15 mice were pooled in each experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

In situ uptake of OVA antigen by immature Langerhans cells. (A) Microscopy analysis of sheet preparations: Whole skin explants from BALB/c mice were treated with 250 $\mu\text{g/ml}$ of OVA–Alexa 488 for 3 h at 37 °C and epidermal sheets prepared. Langerhans cells stained for langerin/CD207 (*red fluorescence*), incorporated OVA–Alexa fluor 488 (*green fluorescence*) *in situ*. One representative out of 4 experiments is shown. (B) Flow cytometry analysis of antigen uptake: Whole skin explants from BALB/c mice were treated with 160 $\mu\text{g/ml}$ of OVA–Alexa 488 for 3 h at 37 °C and Langerhans cells were isolated from epidermis. Langerhans cells were identified as MHC class II⁺ cells. Antigen incorporation was visualized by increased MFI in the antigen detecting channel FL1 (histogram: black: OVA–Alexa fluor 488, dotted: PBS control). Maturation status of Langerhans cells was analysed by expression of CD40 (histogram: black: CD40; grey: isotype control). One representative out of 3 experiments is shown; 3–5 mice were pooled in each experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

In situ uptake of OVA antigen by mature Langerhans cells. (A) Microscopy analysis of sheet preparations: Whole skin explants from BALB/c mice were cultured for 2 days, incubated with 250 $\mu\text{g/ml}$ of OVA–Alexa 488 for 3 h at 37 °C followed by epidermal sheet preparation. Langerhans cells, stained for langerin/CD207 (*red fluorescence*), incorporated OVA–Alexa fluor 488 (*green fluorescence*). One representative out of two experiments is shown. (B) Flow cytometry analysis of antigen uptake: Whole skin explants from BALB/c mice were cultured for 1 day, incubated with 160 $\mu\text{g/ml}$ of OVA–Alexa 488 for 3 h at 37 °C followed by Langerhans cells isolation. Langerhans cells were identified as MHC class II⁺ cells. Antigen incorporation was visualized by increased MFI in the antigen detecting channel FL1 (histogram: black: OVA–Alexa fluor 488, dotted: PBS control). Maturation status of MHC class II⁺ Langerhans cells) was analysed by expression of CD86 and CD40 (histogram: black: CD86/CD40; grey: isotype control). One representative out of two experiments is shown; 3–5 mice were pooled in each experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

In situ uptake of OVA antigen by different skin dendritic cell subsets. (A) Microscopy analysis of cytopsin preparations: Whole skin explants from BALB/c mice were treated with 160 $\mu\text{g/ml}$ of OVA–Alexa 488 for 3 h at 37 °C and after extensive washing skin explants were further cultured on fresh medium for 3 days. Most of the emigrated skin dendritic cells stained for langerin/CD207 (*red fluorescence*) and had incorporated OVA–Alexa fluor 488 (*green fluorescence*). (B) Flow cytometry analysis of antigen uptake: Whole skin explants from langerinEGFP mice were treated with 160 $\mu\text{g/ml}$ of OVA–Alexa 488 for 3 h at 37 °C and after extensive washing skin explants were further cultured on fresh medium for 3 days. Emigrated dendritic cells were identified by expression of CD11c. Skin dendritic cell subsets were dissected based on the additional expression of langerin/CD207 and CD103. Antigen incorporation was visualized by increased MFI in the antigen detecting channel FL4 (histogram: black: OVA–Alexa fluor 647 dotted: PBS control). Maturation status of all CD11c⁺ cells was validated by expression of CD40 (histogram: black: CD40; grey: Isotype control). One representative out of 3 experiments is shown; 10–15 mice were pooled in each experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

In situ uptake and transport of OVA antigen by skin dendritic cells after intradermal injection. 100 μ g of OVA–Alexa fluor 647 was injected intradermally into the ear skin of langerinEGFP mice. Auricular lymph nodes were harvested 48 h post-injection. (A) Analysis of migratory CD11c⁺ CD40⁺ dendritic cells obtained from skin draining lymph node two days post injection. The CD40⁺ population was further split into langerin/CD207⁺ and langerin/CD207⁻ subpopulation. Antigen incorporation was visualized by increased MFI in the antigen detecting channel FL4 (histogram: black: OVA–Alexa fluor 647, dotted: PBS control). (B) Analysis of langerin/CD207⁺ subpopulations obtained from skin draining lymph node two days post-injection. CD11c⁺ dendritic cells were dissected based on the expression of langerin/CD207, CD103 as well as CD8. Antigen incorporation was visualized by increased MFI in the antigen detecting channel FL4 (histogram: black: OVA–Alexa fluor 647, dotted: PBS control). One representative out of 3 experiments is shown; 3 mice were pooled in each experiment.