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Epidermal langerhans cells rapidly capture and present antigens from C-type lectin-targeting antibodies deposited in the dermis

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Epidermal Langerhans Cells Rapidly Capture and Present Antigens from C-Type Lectin-Targeting Antibodies Deposited in the Dermis

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Antigen-presenting cells can capture antigens that are deposited in the skin, including vaccines given subcutaneously. These include different dendritic cells (DCs) such as epidermal Langerhans cells (LCs), dermal DCs, and dermal langerin[+] DCs. To evaluate access of dermal antigens to skin DCs, we used mAb to two C-type lectin endocytic receptors, DEC-205/CD205 and langerin/CD207. When applied to murine and human skin explant cultures, these mAbs were efficiently taken up by epidermal LCs. In addition, anti-DEC-205 targeted langerin^{+} CD103^{+} and langerin^{−} CD103^{−} mouse dermal DCs. Unexpectedly, intradermal injection of either mAb, but not isotype control, resulted in strong and rapid labeling of LCs in situ, implying that large molecules can diffuse through the basement membrane into the epidermis. Epidermal LCs targeted in vivo by ovalbumin-coupled anti-DEC-205 potently presented antigen to CD4^{+} and CD8^{+} T cells in vitro. However, to our surprise, LCs targeted through langerin were unable to trigger T-cell proliferation. Thus, epidermal LCs have a major function in uptake of lectin-binding antibodies under standard vaccination conditions.

INTRODUCTION

Dendritic cells (DCs) are specialized to take up and present antigens, a feature now being considered in the design of vaccines (Steinman and Banchereau, 2007). Cutaneous DCs, including epidermal Langerhans cells (LCs) and dermal DCs, are ideally positioned to take up skin-administered vaccines, process them, and carry them to the draining lymph nodes, where they stimulate antigen-specific T cells (Romani et al., 2006). The immunogenic potential of LCs in vivo depends on the dose and localization of the antigen (Bennett et al., 2007; Stoitzner et al., 2008; Wang et al., 2008).

C-type lectin receptors facilitate uptake and processing of antigenic proteins, and this ability has been exploited to improve immune responses by targeting antigens to DCs (Tacken et al., 2007). The best-studied example is DEC-205/CD205, which is expressed at highest levels by select subsets of DCs (Henri et al., 2001). When protein antigens are coupled to anti-DEC-205 mAbs and mice are immunized with these conjugates in the presence of DC-activating agents, T-cell-dependent immune responses (Hawiger et al., 2001; Bonifaz et al., 2004; Boscardin et al., 2006) are dramatically enhanced in vivo.

Langerin/CD207 is another C-type lectin, specifically expressed in the skin by epidermal LCs and by a recently described subset of mouse dermal DCs (Valladeau et al., 2000, 2002; Kaplan et al., 2008). Antigen targeting by anti-langerin mAb also results in efficient presentation to CD4^{+} and CD8^{+} T cells in vivo (Idoyaga et al., 2008).

In many of the above-cited studies, immunization with anti-DEC-205 conjugates was performed by subcutaneous injection into the footpad. However, despite extensive research performed with anti-DEC-205 mAbs, it has not been studied whether cutaneous DEC-205^{+} DCs participate in uptake and transport of targeting mAb (Bonifaz et al., 2004; Carter et al., 2006). This question appears important in view of the differential functions that epidermal LCs, dermal DCs, and lymph node-resident DCs seem to have (Allan et al., 2006; Kissenpfennig and Malissen, 2006). Therefore, we examined in more detail in situ uptake and handling of mAbs against C-type lectins by skin DCs, and, surprisingly, observed that intradermal mAbs are captured by epidermal LCs.

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Abbreviations: DC, dendritic cell; LC, Langerhans cell

RESULTS

LCs *in situ* are specifically targeted by mAbs in murine skin explant cultures

Epidermal LCs and dermal langerin$^+$ DCs express DEC-205 and langerin (Kraal *et al.*, 1986; Valladeau *et al.*, 2002; Stoitzner *et al.*, 2003; Kaplan *et al.*, 2008), whereas other dermal DCs lack langerin and display low levels of DEC-205 (Henri *et al.*, 2001). The density of langerin$^+$ cells in the undisturbed dermis was about 5% of LC density in the epidermis ($\sim$50–80 vs $\sim$1000 per mm^2 in BALB/c). Dermal langerin$^+$ cells are emigrating epidermal LCs (Stoitzner *et al.*, 2003) as well as DCs that reside in the dermis (Kaplan *et al.*, 2008).

To determine if these DC subsets would capture anti-receptor antibodies (Abs), we cultured whole-skin explants from BALB/c mice for different time periods in hybridoma supernatant NLDC145 (anti-DEC-205) or control nonreactive IgG2a, LODNP16. Then, epidermal and dermal sheets were prepared. Labeling was evident on epidermal LCs within 30 minutes with NLDC145, was brightest after 2–4 hours, and persisted at least 48 hours (Figure 1a). Conversely, no binding to LCs was evident with LODNP16 (Figure 1b). Similar rapid targeting was observed when anti-langerin Ab L31 was used, and equivalent labeling was seen with whole skin from BALB/c and C57BL/6 mice (Figure 1c).

In keeping with the absence of DEC-205 staining in the dermis (Kraal *et al.*, 1986), dermal cells that had taken up anti-DEC-205 mAb were rarely observed before 18 hours of culture (Figure 1a, bottom; arrowheads). At time points beyond 18 hours, NLDC145 was detected in considerably more dermal cells. Many of them were arranged in typical "cords," which represent dermal lymphatic vessels filled with migratory LCs (Stoitzner *et al.*, 2003) (Figure 1a, bottom; arrowheads).

### Migratory skin DCs transport anti-DEC-205 and anti-langerin mAb after *ex vivo* targeting

To extend our *in situ* observations, we analyzed by FACS migratory cells from whole-skin explants that had been preincubated in targeting mAb for 3 hours, washed extensively, then returned to culture to allow DCs to "crawl out" of the explants. In addition to anti-DEC-205, we used L31, a new mAb to langerin. As opposed to 929F3 mAb, which recognizes an intracellular domain of langerin, L31 binds to the extracellular carbohydrate-recognition domain (Cheong *et al.*, 2007; Idoyaga *et al.*, 2008).

More than 85% of the migratory cells expressed langerin (Figure 2a). NLDC145 was primarily taken up by langerin$^+$ cells, with the exception of a small langerin$^-$ population ($\sim$5% of all migratory cells; Figure 2a, right panel, arrow). As expected, L31 targeting mAb was exclusively detected on langerin$^+$ cells, albeit at lower levels than NLDC145. Of note, the levels of the targeting mAb decreased with time in culture, possibly resulting from a degradation of endocytosed mAb (day 2 vs day 4; data not shown).

To investigate the relative contributions of epidermal LCs and dermal langerin$^+$ DCs, we also studied expression of CD103, an integrin selectively expressed by the latter subset (Kaplan *et al.*, 2008). A small proportion (3.5% $\pm$0.9; $n = 5$) of migratory cells expressed langerin and CD103; these were efficiently targeted by anti-DEC-205 (Figure 2b). Among langerin$^-$ migratory cells, which most likely originate in the dermis, only CD103$^-$ DCs bore the targeting mAb to DEC-205.

Epidermal LCs are specifically targeted *in vivo* by mAb injected intradermally

To examine whether targeting mAb would also reach epidermal LCs in a standard immunization procedure, we
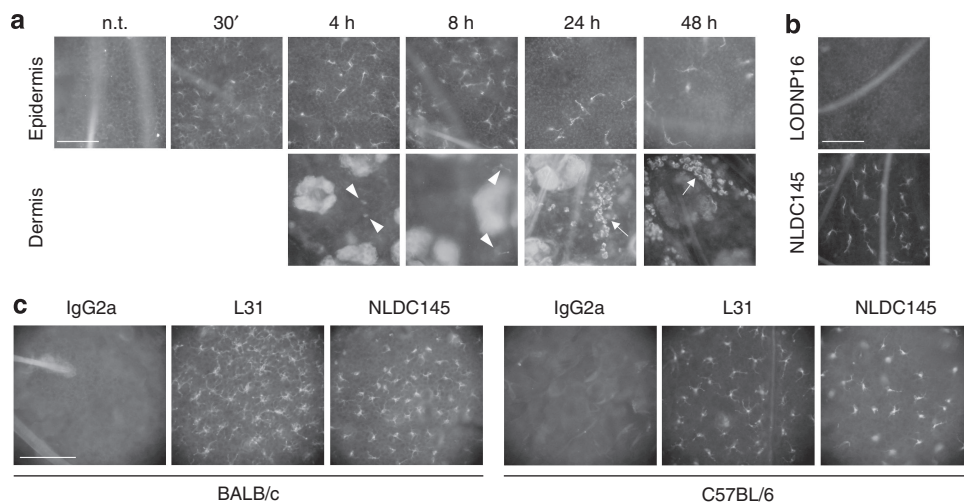
Figure 1. **Langerhans cells *in situ* are specifically targeted by mAb in murine skin explant cultures.** (**a**) Whole-skin explants from BALB/c mice were incubated for 30 minutes to 48 hours in culture medium containing NLDC145 (anti-mouse DEC-205) hybridoma supernatant. Epidermis was then separated from dermis, fixed, and targeting mAb was revealed with anti-rat IgG secondary Ab. Arrowheads indicate dermal cells targeted *in situ*. Arrows point at DC-filled dermal lymphatic vessels. (**b**) Whole-skin explants from BALB/c mice were incubated for 18 hours in culture medium containing NLDC145 or LODNP16 (isotype control) hybridoma supernatants, then epidermis was separated, fixed, and stained with anti-rat IgG Ab. (**c**) Whole-skin explants from BALB/c or C57BL/6 mice were incubated for 4 hours with 5 µg ml^{-1} of purified mAb NLDC145 (anti-mouse DEC-205) or L31 (anti-mouse langerin), then epidermis was separated, fixed, and stained with anti-rat IgG Ab. Bar = 100 µm. Results are representative of three independent experiments.
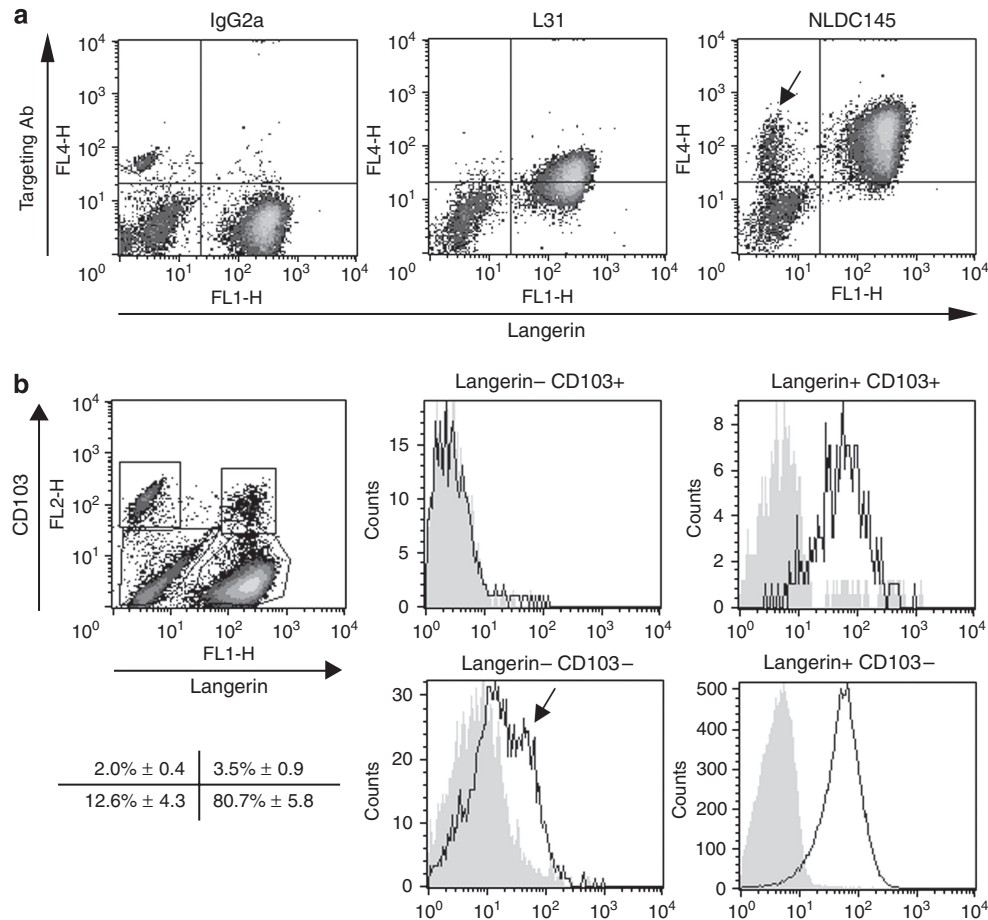
Figure 2. **Langerhans cells and dermal dendritic cells migrating out of murine skin explants transport the targeting mAb.** Whole-skin explants were incubated for 3 hours in medium containing L31 (anti-langerin), NLDC145 (anti-DEC-205), or IgG2a isotype control, washed in PBS and cultured for 3 days. Targeting mAb was detected in permeabilized migratory cells by anti-rat IgG Ab. Cells were counterstained with (**a**) 929F3 mAb to an epitope on the cytosolic domain of langerin or (**b**) 929F3 anti-langerin plus anti-CD103. Migratory cell subsets were gated as depicted in the density plot on the left, and percentages (average ± SEM) of each population are shown in the table below. Histograms display staining intensity of the targeting mAb on the four different subsets. Filled histograms, IgG2a; empty histograms, anti-DEC-205. Results are representative of three independent experiments.

prepared epidermal sheets 18 hours after intradermal injection. As little as 0.2 μg of purified NLDC145 was sufficient to visualize epidermal LCs *in situ* 18 hours after intradermal injection (Figure 3a). For further experiments, we injected 1 μg to obtain a brighter staining. No staining could be observed with IgG2a isotype control (Figure 3b). Interestingly, L31 and NLDC145 were still detectable on LCs for up to 5 days, although labeling was clearly weaker at this late time point (Figure 3b).

Next, we used langerin$^{-/-}$ mice (Kissenpfennig *et al.*, 2005), which have a normal distribution of epidermal major histocompatibility complex class II$^+$ LCs (Figure 3c, top). As anticipated, langerin$^{-/-}$ LCs showed normal binding of intradermally injected anti-DEC-205 mAb, but failed to be targeted by anti-langerin L31 mAb (Figure 3c, bottom).

Finally, whole-skin explants were prepared 4 hours after intradermal injection of targeting mAb, washed in phosphate-buffered saline (PBS), and cultured for 3 days. Langerin$^+$ DCs that had been targeted *in vivo* retained detectable amounts of the L31 and NLDC145 targeting mAb after migrating out of

skin explants (Figure 3d). Targeting intensity was more pronounced for NLDC145, and it declined markedly with time for both targeting mAb (day 2 vs. day 4; data not shown).

Epidermal LCs present DEC-205-targeted antigen after *in vivo* uptake

We next evaluated antigen presentation by epidermal LCs targeted *in vivo*. To achieve this, we used Ab coupled to the model antigen ovalbumin (OVA) and recognizing langerin (L31/OVA), DEC-205 (aDEC/OVA), and an isotype-matched control (Ig/OVA). At 4 hours after intradermal injection of 50 or 500 ng of targeting Ab, we prepared LCs by culturing epidermal sheets for 3 days. Migratory cells consisted largely of LCs (40.6 ± 2.1%; $n = 36$). Other cells in the suspension were T cells and keratinocytes, which are devoid of DEC-205 or langerin (data not shown). Migratory LCs were cultured for 4 days at different ratios with carboxyfluorescein succinimidyl ester (CFSE)-labeled, OVA-specific CD4$^+$ or CD8$^+$ T cells from OT-II or OT-I mice, respectively. Proliferation
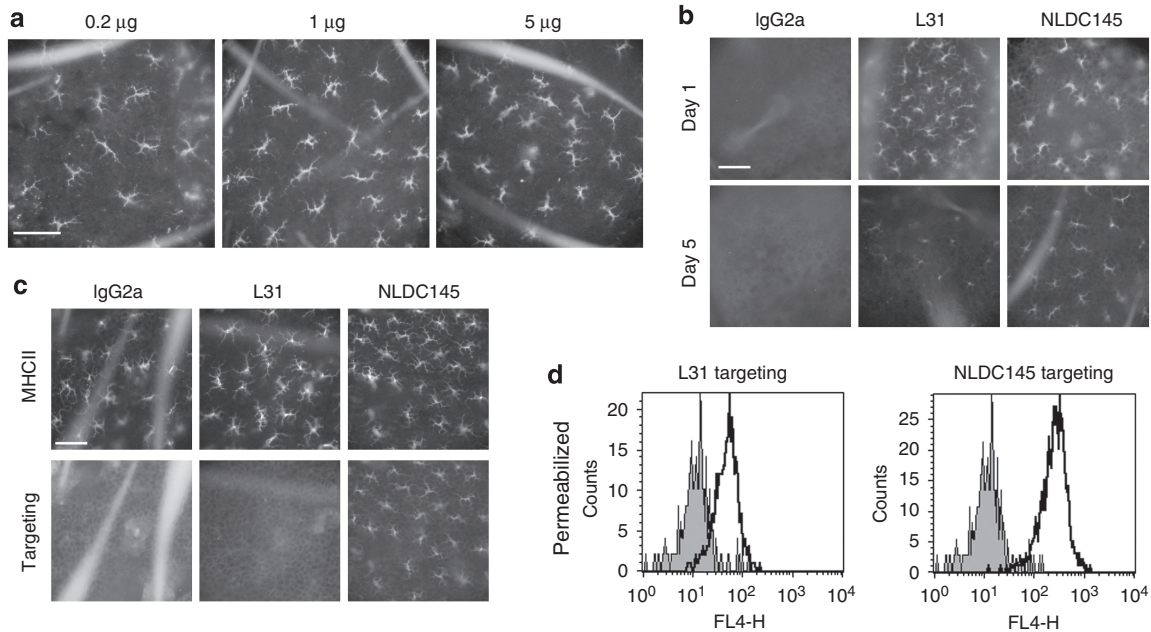
Figure 3. **Langerhans cells are targeted within the epidermis after intradermal injection of anti-lectin Ab *in vivo*.** Different targeting mAbs were injected intradermally into the ear skin of C57BL/6 mice. Epidermal sheets were prepared 18 hours after injection of 0.2, 1, or 5 μg of NLDC145 (**a**) and 24 hours or 5 days after injection of 1 μg IgG2a, L31, or NLDC145 (**b**) The capture of the injected mAb was visualized with anti-rat IgG Ab. (**c**) 18 Hours after injection of 1 μg targeting mAb, major histocompatibility complex class II (upper panel) and targeting mAb (lower panel) were revealed on epidermal sheets from langerin$^{-/-}$ mice. (**d**) At 4 hours after injection of 1 μg targeting mAb into ear skin of BALB/c mice, whole-skin explants were cultured for 3 days. The targeting mAb was detected in permeabilized migratory cells (gated on langerin$^+$ cells; mAb 929F3). Filled histograms, IgG2a; empty histograms, mAb as indicated. Bar = 100 μm. Results are representative of three independent experiments.

was evaluated by the percentage of T cells exhibiting decreased CFSE staining.

Presentation to CD4$^+$ T cells occurred only when aDEC/OVA was the targeting reagent (Figure 4a). With 500 ng of Ab per ear, OVA-specific CD8$^+$ T cells proliferated when cultured with LCs targeted with any OVA-coupled mAb (Figure 4b). As these mAbs are mouse IgG1, this unexpected result likely reflects uptake by Fc-γ receptor I (Romani *et al.*, 1989), which is probably sufficient for antigen presentation given the extremely high sensitivity of CD8$^+$ T cells from OT-I mice (Choi *et al.*, 2009). Still, the use of aDEC/OVA, but not L31/OVA, led to antigen cross-presentation that was significantly stronger than with Ig/OVA control. Moreover, when the injected dose was titrated down to 50 ng per ear, only targeting with aDEC/OVA allowed LCs to present to CD8$^+$ T cells (Figure 4c).

### Human skin DCs bind and transport anti-DEC-205 and anti-langerin mAb
Finally, we extended our observations to human skin DC subsets, which we targeted *in situ* in whole-skin explant cultures. After 4 days, we observed three distinct populations of migratory DCs targeted by anti-human DEC-205 mAb, CD1a$^{neg/low}$, CD1a$^+$, and CD1a^{high} cells (Figure 5, top). Anti-DEC-205 was absent (or very low) on a proportion of the CD1a$^{neg/low}$ cells. Only CD1a^{high} DCs could bind anti-langerin mAb, indicating that they are actually LCs, whereas

CD1a$^+$ langerin$^-$ and CD1a$^-$ langerin$^-$ DCs (Figure 5, bottom) probably originate in the dermis (Ebner *et al.*, 2004).

DISCUSSION
Targeting antigens to DCs with the help of specific mAbs has great potential for the development of effective vaccines (Tacken *et al.*, 2007). We found that anti-langerin and anti-DEC-205 mAbs are captured in a selective manner by mouse and human skin DCs, and that targeted DCs carry mAbs when leaving the skin. Fc receptor-mediated uptake by LCs could be formally excluded, as shown by the lack of binding of nonspecific control mAbs in wild-type mice and of anti-langerin mAbs in langerin$^{-/-}$ mice.

Both anti-langerin and anti-DEC-205 were taken up by epidermal LCs within minutes after intradermal injection. This indicates that even large immunoglobulins (150 kDa) can easily diffuse from the dermis through the basement membrane into the epidermis and gain access to LCs. Of note, the density of LCs in the epidermal sheets remained roughly unchanged, showing that intradermal injection of targeting mAbs in PBS did not result in a degree of inflammation that would have enhanced emigration of LCs. Moreover, targeting mAb persisted on LCs for several days, suggesting that their degradation occurs very slowly after *in vivo* uptake. This remarkable property implies that targeted DCs constitute a pool of antigen-presenting cells that could be exploited throughout an extended period.
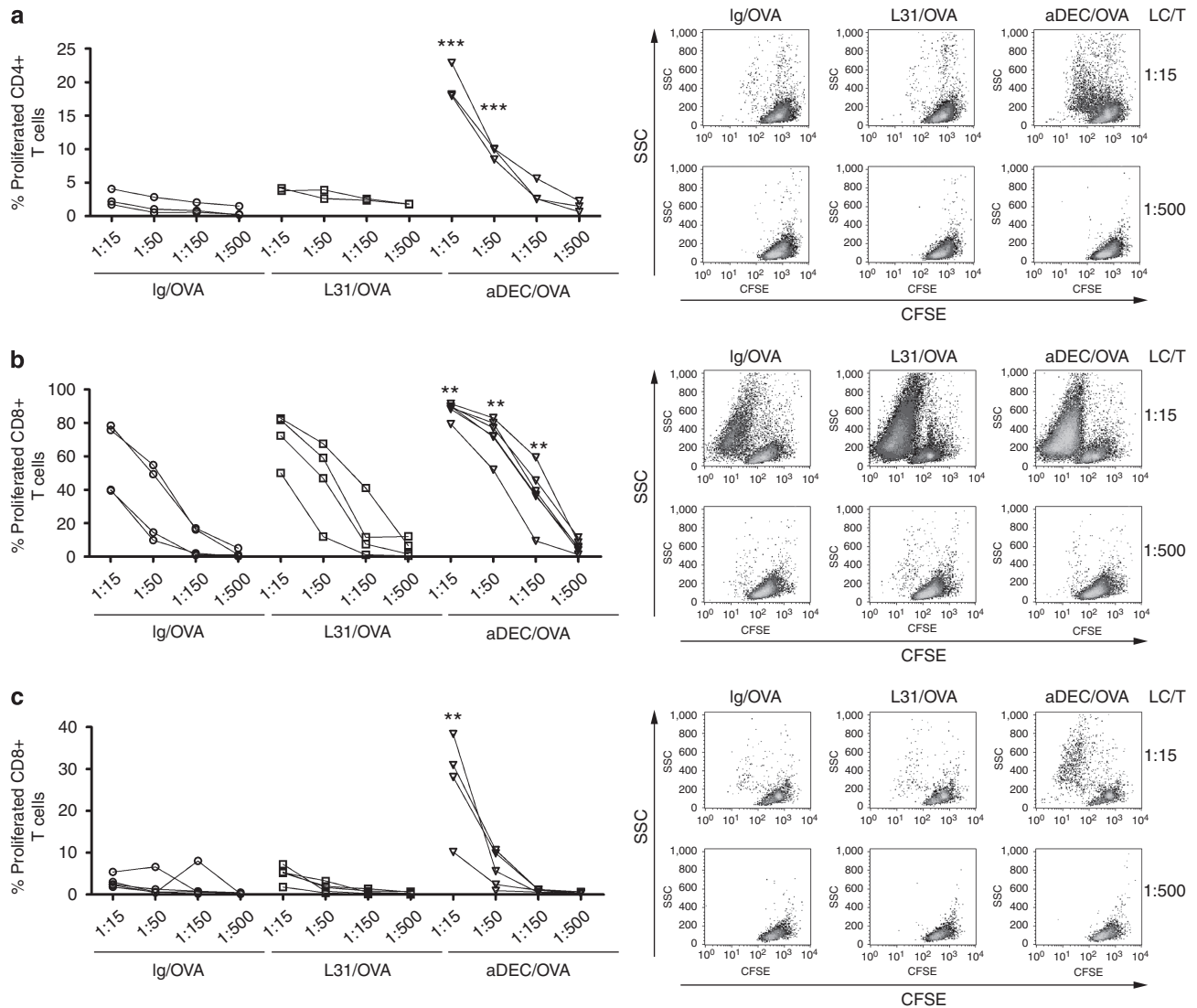
Figure 4. Langerhans cells present DEC-205-targeted antigen after *in vivo* uptake. At 4 hours after intradermal injection of 500 ng (**a**, **b**) or 50 ng (**c**) of OVA-coupled anti-DEC-205, anti-langerin, or isotype control, epidermal sheets were prepared and cultured for 3 days. Migratory epidermal LCs were then cocultured with CFSE-labeled CD4⁺ (**a**) or CD8⁺ (**b**, **c**) T cells purified from OT-II or OT-I mice, respectively. After 4 days, T-cell proliferation was evaluated by dilution of CFSE. Graphs show T-cell proliferation observed at different LC/T ratios; results from three to six independent experiments are shown. Density plots display representative FACS stainings for two different LC/T ratios. Asterisks indicate proliferation rates significantly different (*P*<0.05) from those of cocultures performed at the same T/LC ratio with Ig/OVA-loaded LCs.

In the mouse, the main target of anti-DEC-205 was epidermal LCs. In skin explant cultures, targeting mAb were able to access LsC in the epidermis, whereas relatively few dermal DCs acquired mAbs. However, due to the relatively high background staining of the dermis, we could not exclude the existence of targeted dermal DCs displaying only low levels of NLDC145 *in situ*. In fact, when migratory cells were analyzed by flow cytometry, langerin⁻ CD103⁻ and langerin⁺ CD103⁺ dermal DC subsets had also internalized anti-DEC-205, albeit to a much lower extent in terms of quantity and cell numbers, respectively.

In parallel, human skin explant cultures also revealed efficient labeling of human skin DCs by anti-lectin mAb. Targeting of epidermal LCs was achieved by both anti-langerin and anti-DEC-205 mAb, whereas two distinct DC populations exclusively bound anti-DEC-205. We did not further investigate these two subsets, because human dermal DCs remain less well defined, and their phenotype and distribution differ markedly from what is observed in a murine system (Ebner *et al.*, 2004; Tacken *et al.*, 2007; Zaba *et al.*, 2008). Nevertheless, our findings suffice to indicate that mAb injected into human skin would efficiently target skin DCs, including LCs *in situ*.

Finally, *in vivo* targeted epidermal LCs were able to present the model antigen OVA coupled to anti-DEC-205. As already proposed in the literature, targeting through DEC-205 appeared to preferentially lead to cross-presentation to CD8⁺ T cells, whereas only limited CD4⁺ T-cell proliferation could
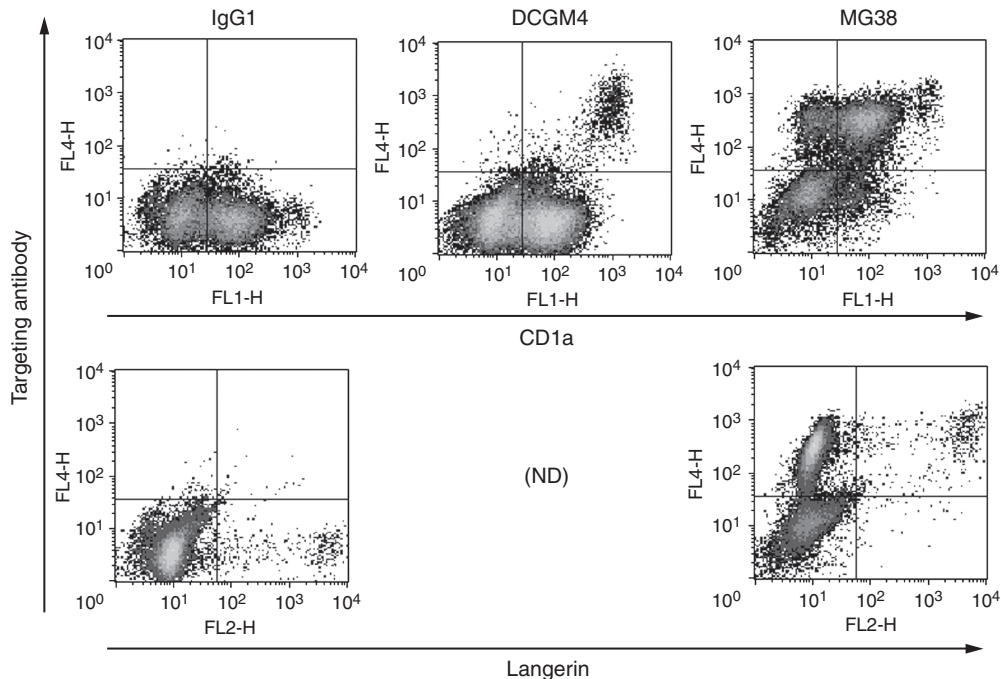
Figure 5. **Human skin dendritic cells bind and transport targeting mAbs.** Human whole-skin explants were incubated for 4 hours in medium containing DCGM4 (anti-langerin), MG38 (anti-DEC-205), or IgG1 isotype control, then cultured for 4 days. Targeting mAbs were detected in permeabilized migratory cells by means of anti-mouse IgG secondary Ab. Cells were counterstained with anti-CD1a or anti-langerin. ND, not determined. Results are representative of two independent experiments.

be induced (Dudziak *et al.*, 2007). Most intriguingly, LCs could not efficiently present to CD4$^+$ or CD8$^+$ T cells *in vitro* when OVA was targeted through langerin, even though L31/OVA injection does induce proliferation of transferred OVA-specific T cells *in vivo* (Idoyaga *et al.*, 2008). Of note, our *in vitro* culture system exclusively yields epidermal LCs. This raises interesting questions about dermal langerin$^+$ DC subsets, where the fate of antigens internalized by langerin may differ as compared with LCs. This may be reflected by the presence or absence of langerin$^+$ Birbeck granules (Douillard *et al.*, 2005) that are a characteristic compartment of epidermal LCs, but were not yet examined in langerin$^+$ dermal DCs.

Maturation probably influences antigen uptake and processing by C-type lectin-targeted DCs, thereby controlling the outcome of T-cell responses. For example, targeting through DEC-205 *in vivo* results in efficient immune responses only when Abs are provided together with adjuvants known to induce DC maturation (Hawiger *et al.*, 2001). However, migratory epidermal LC are already highly mature cells (Stoitzner *et al.*, 2003), making it difficult, if not impossible, to further enhance their activation state. Despite this, we cannot exclude qualitative differences in the maturation that depend on the activation signal. In particular, regarding langerin targeting, tissue damage, which is the likely trigger for LC maturation and migration in our cultures of epidermal sheets, may not be sufficient for presentation of antigens internalized into langerin$^+$ compartments.

We have identified epidermal LCs as potent targets of C-type lectin-binding Abs in human and murine skin. Nevertheless, other DC subsets appear to be involved as well. The complex interactions between different DC populations in the skin, but also in skin-draining lymph nodes, call for detailed *in vivo* investigations. Hence, our future work will focus on resolving these issues, by defining the functional roles of targeted skin DC in cutaneous immune responses *in vivo*.

MATERIALS AND METHODS
Mice
Inbred BALB/c, C57BL/6, OT-I, and OT-II mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and used at 2–10 months of age. Langerin-deficient mice (Kissenpfennig *et al.*, 2005) were kindly supplied by S. Saeland. All experimental protocols were approved by the Austrian Federal Ministry of Science and Research, Department for Genetic Engineering and Animal Experimentation (66.011/16-II/106/2008).

Media and reagents
Complete culture medium was Roswell Park Memorial Institute-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine (Sigma, St Louis, MO), 50 µg ml^{-1} gentamicin (PAA, Linz, Austria), and 50 µM β-mercaptoethanol. Targeting by rat mAb was detected *in situ* by goat anti-rat IgG/FITC (BD-Biosciences, San Diego, CA), or chicken anti-rat IgG/Alexa 594 (Invitrogen, Eugene, OR). For FACS analyses, permeabilization was performed with Cytofix/perm (BD-Biosciences), and targeting

mAb was detected with goat anti-rat IgG/APC in murine samples, or rat anti-mouse IgG/APC in human samples (BD-Biosciences). For analyses of murine cells, we used mAb to major histocompatibility complex class II (anti-I-A/I-E^{diverse}, clone 2G9) (BD-Biosciences), CD103 (clone M290) (BD-Biosciences), and langerin (clone 929F3; Dendritics, Lyon, France), whereas human cells were stained with mAb to CD1a (clone HI149; BD-Biosciences) or langerin (clone DCGM4; Dendritics).

Targeting mAb

L31 mAb recognizing an extracellular portion of murine langerin was used to target langerin as described (Cheong *et al.*, 2007), and NLDC145 mAb (BMA Biomedicals, Augst, Switzerland) to target murine DEC-205 (Kraal *et al.*, 1986). Control mAb was rat IgG2a (R&D Systems, Minneapolis, MN). OVA-coupled anti-langerin (L31/OVA), anti-DEC-205 (aDEC/OVA), or isotype-matched control (Ig/OVA; derived from mouse IgG1) were used in OVA-specific T-cell proliferation assays (Idoyaga *et al.*, 2008). Human skin cells were targeted by anti-human DEC-205 (clone MG38) (Guo *et al.*, 2000) or anti-human langerin (clone DCGM4) (Valladeau *et al.*, 2000). Mouse IgG1 (BD-Biosciences) was used as isotype control.

Determination of the density of dermal langerin$^+$ cells *in situ*

Numbers were determined on dermal sheet specimens from several different C57BL/6 and BALB/c mice, stained by anti-langerin mAb (929F3), and revealed by secondary goat-anti-rat Ig conjugated to Alexa 594. In total, langerin$^+$ cells in 40 randomly selected microscopical fields were counted and areal density determined by means of a calibrated ocular grid.

Mouse skin explant culture and *in vitro* targeting experiments

Briefly, mice were killed, ears were cut off at the base, and ear skin was split into dorsal and ventral halves (Stoitzner *et al.*, 2003). The dorsal halves were cultured in 24-well plates (one per well), for the indicated periods, in complete culture medium containing 10% (v/v) of hybridoma supernatants (NLDC145, or LODNP16/ IgG2a isotype control). Finally, epidermal and dermal sheets were separated with ammonium thiocyanate and fixed in acetone.

For cell migration experiments, ear skin explants from BALB/c mice were exposed to 5 μg ml^{-1} targeting mAb (IgG2a, L31, or NLDC145) for 3 hours at 37 °C, then extensively washed in PBS, to avoid binding of targeting mAbs to migratory cells outside of the skin. Explants were further cultured in complete medium without chemokines. After 2–4 days, emigrant DCs were harvested and investigated by flow cytometry.

Alternatively, 4 hours after *in vivo* targeting (see below), epidermis was separated from dermis with dispase and cultured for 3 days. LCs present in the migratory epidermal cell suspensions were quantified and used in T-cell proliferation assays.

In vivo targeting experiments

Purified rat IgG2a, L31, NLDC145, Ig/OVA, L31/OVA, or aDEC/ OVA (0.05–5 μg) diluted in 25 μl PBS were injected into the ear pinna of anesthetized C57BL/6 or langerin$^{-/-}$ mice.

In vitro T-cell proliferation assays

OVA-specific T cells were obtained by MACS purification (Miltenyi Biotec, Bergisch Gladbach, Germany) of CD4$^+$ and CD8$^+$ T cells from lymphoid organs of OT-II and OT-I mice, respectively. Purified T cells were labeled by CFSE, and seeded in round-bottom microwells (50,000 T cells per well). *In vivo* targeted LCs, obtained from a 3-day culture of epidermis alone (see above), were added at different T/LC ratios. After 4 days of culture, we monitored by FACS the proportion of T cells exhibiting CFSE dilution, which allowed us to evaluate the extent of proliferation. Background proliferation of OT-I and OT-II cells cultured alone was below 0.5% in every experiment (data not shown).

Human skin explant culture and *in vitro* targeting experiments

Healthy human split-thickness skin was obtained from plastic surgery (Ebner *et al.*, 2004), in accordance with the Declaration of Helsinki Principles. Whole-skin explants were floated on culture medium containing 5 μg ml^{-1} targeting mAbs (IgG1, DCGM4, and MG38) for 3 hours at 37 °C. Then, explants were thoroughly washed in PBS and cultured in six-well plates. After 4 days, emigrant DCs were harvested and investigated by FACS.

Statistical analyses

Experiments were performed at least three times with similar results. Error bars are standard error of the mean. *P*-values are from two-tailed Student's *t*-tests.

REFERENCES

Allan RS, Waithman J, Bedoui S *et al.* (2006) Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity* 25:153–62

Bennett CL, Noordegraaf M, Martina CA, Clausen BE (2007) Langerhans cells are required for efficient presentation of topically applied hapten to T cells. *J Immunol* 179:6830–5

Bonifaz LC, Bonnyay DP, Charalambous A *et al.* (2004) *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* 199:815–24

Boscardin SB, Hafalla JCR, Masilamani RF *et al.* (2006) Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. *J Exp Med* 203:599–606

Carter RW, Thompson C, Reid DM, Wong SY, Tough DF (2006) Preferential induction of CD4+ T cell responses through *in vivo* targeting of antigen to dendritic cell-associated C-type lectin-1. *J Immunol* 177:2276–84

Cheong C, Idoyaga J, Do Y *et al.* (2007) Production of monoclonal antibodies that recognize the extracellular domain of mouse Langerin/CD207. *J Immunol Methods* 324:48–62

Choi JH, Do Y, Cheong C *et al.* (2009) Identification of antigen-presenting dendritic cells in mouse aorta and cardiac valves. *J Exp Med* 206:497–505

Douillard P, Stoitzner P, Tripp CH *et al.* (2005) Mouse lymphoid tissue contains distinct subsets of Langerin/CD207+ dendritic cells, only one of which represents epidermal-derived Langerhans cells. *J Invest Dermatol* 125:983–94

Dudziak D, Kamphorst AO, Heidkamp GF *et al.* (2007) Differential antigen processing by dendritic cell subsets *in vivo*. *Science* 315:107–11

Ebner S, Ehammer Z, Holzmann S *et al.* (2004) Expression of C-type lectin receptors by subsets of dendritic cells in human skin. *Int Immunol* 16:877–87

Guo M, Gong SC, Maric S *et al.* (2000) A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. *Human Immunol* 61:729–38

Hawiger D, Inaba K, Dorsett Y *et al.* (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. *J Exp Med* 194:769–79

Henri S, Vremec D, Kamath A *et al.* (2001) The dendritic cell populations of mouse lymph nodes. *J Immunol* 167:741–8

Idoyaga J, Cheong C, Suda K *et al.* (2008) Cutting edge: Langerin/CD207 receptor on dendritic cells mediates efficient antigen presentation on MHC I and II products *in vivo*. *J Immunol* 180:3647–50

Kaplan DH, Kissenpfennig A, Clausen BE (2008) Insights into Langerhans cell function from Langerhans cell ablation models. *Eur J Immunol* 38:2369–76

Kissenpfennig A, Aït-Yahia S, Clair-Moninot V *et al.* (2005) Disruption of the *langerin/CD207* gene abolishes Birbeck granules without a marked loss of Langerhans cell function. *Mol Cell Biol* 25:88–99

Kissenpfennig A, Malissen B (2006) Langerhans cells – revisiting the paradigm using genetically engineered mice. *Trends Immunol* 27:132–9

Kraal G, Breel M, Janse M, Bruin G (1986) Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J Exp Med* 163:981–97

Romani N, Ebner S, Tripp CH, Flacher V, Koch F, Stoitzner P (2006) Epidermal Langerhans cells – changing views on their function *in vivo*. *Immunol Lett* 106:119–25

Romani N, Inaba K, Pure E, Crowley M, Witmer-Pack M, Steinman RM (1989) A small number of anti-CD3 molecules on dendritic cells stimulates DNA synthesis in mouse T lymphocytes. *J Exp Med* 169:1153–68

Steinman RM, Banchereau J (2007) Taking dendritic cells into medicine. *Nature* 449:419–26

Stoitzner P, Green LK, Jung JY *et al.* (2008) Tumor immunotherapy by epicutaneous immunization requires Langerhans cells. *J Immunol* 180:1991–8

Stoitzner P, Holzmann S, McLellan AD *et al.* (2003) Visualization and characterization of migratory Langerhans cells in murine skin and lymph nodes by antibodies against Langerin/CD207. *J Invest Dermatol* 120:266–74

Tacken PJ, de Vries IJ, Torensma R, Figdor CG (2007) Dendritic-cell immunotherapy: from *ex vivo* loading to *in vivo* targeting. *Nat Rev Immunol* 7:790–802

Valladeau J, Clair-Moninot V, Dezutter-Dambuyant C *et al.* (2002) Identification of mouse Langerin/CD207 in Langerhans cells and some dendritic cells of lymphoid tissues. *J Immunol* 168:782–92

Valladeau J, Ravel O, Dezutter-Dambuyant C *et al.* (2000) Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* 12:71–81

Wang L, Bursch LS, Kissenpfennig A, Malissen B, Jameson SC, Hogquist KA (2008) Langerin expressing cells promote skin immune responses under defined conditions. *J Immunol* 180:4722–7

Zaba LC, Krueger JG, Lowes MA (2008) Resident and ''inflammatory'' dendritic cells in human skin. *J Invest Dermatol* 129:302–8