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Cutting Edge: CD1a⁺ Antigen-Presenting Cells in Human Dermis Respond Rapidly to CCR7 Ligands

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Recent data from murine models have confirmed that Langerhans cells are not the only population of APCs in the skin involved in initiating immune responses. In healthy human skin, we identify CD1a⁺ dermal APCs located close to the lymphatic vessels in the upper layers of the dermis that are unequivocally distinct from migrating Langerhans cells but exhibit both potent allostimulatory capacity and a chemotactic response to CCR7 ligands. In contrast, CD14⁺ dermal APCs are distributed throughout the dermis and lack a chemotactic response to CCR7 ligands. CD1a⁺ dermal APCs therefore represent an APC population distinct from Langerhans cells that are capable of migrating to lymph nodes and stimulating naive T cells. In humans, CD1a⁺ dermal APCs may fulfill some of the roles previously ascribed to Langerhans cells. The Journal of Immunology, 2006, 176: 5730–5734.

The initiation of immune responses to Ags in the skin has often been attributed to Langerhans cells, although other APCs exist in skin (1, 2). Recent data from murine models have underlined the relative importance of APCs other than Langerhans cells in many immune responses initiated in the skin (3, 4), suggesting that some functions previously attributed to Langerhans cells are conducted by other skin APCs. In particular, Kissenpfennig et al. (4) recently reported that a dermal APC population not only migrated to lymph nodes before Langerhans cells in models of skin inflammation but also homed to areas of the lymph nodes distinct from those colonized by Langerhans cells (4).

Identification of human dermal APCs capable of migrating to lymph nodes to stimulate T cells might improve our ability to efficiently deliver vaccines via human skin and, conversely, to control skin inflammation. Although APCs in human dermis have long been studied, they remain poorly characterized relative to Langerhans cells. In part this is due to the lack of specific phenotypic markers like CD207/Langerin, which adorns Langerhans cells (5). An emerging consensus is that CD1a and CD14 are useful markers for separating functionally distinct populations of human dermal APCs (6–8), but the presence of CD1a on Langerhans cells has meant that the relationship between migrating Langerhans cells and dermal APCs has not always been clear.

Using molecular phenotyping and functional assays, we identify a population of CD1a⁺ dermal APCs unequivocally distinct from migrating Langerhans cells possessing all the properties needed for migration to lymph nodes and stimulation of naive T cells restricted by MHC class I, MHC class II, CD1a, CD1b, and CD1c.

Materials and Methods

Preparation of dermal cell suspensions

Fresh skin samples were obtained from healthy patients undergoing breast resection surgery. Patients gave written informed consent under a protocol approved by the Auckland Ethics Committee and the Clinical Board of the Counties-Manukau District Health Board. Samples were refrigerated and processed no longer than 4 h postsurgery.

Subcutaneous tissue was excised and discarded. Trimmed skin was washed with RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS (RF10). The dermal layer was scored with a scalpel, and the skin was digested with 1 mg/ml collagenase (type I) (Invitrogen Life Technologies) and 1 mg/ml dispase (Invitrogen Life Technologies) in RF10 for 2 h at 37°C. The epidermis was peeled off the dermis. The dermis was incubated at 37°C for a further 16 h in RF10 alone before mechanical disruption by pipetting and filtration through 70-μm cell strainers (BD Biosciences) to obtain single cell suspensions. Single cell preparations were cryopreserved in 50% RPMI 1640, 40% FBS, and 10% DMSO. Cryopreservation and subsequent thawing did not influence cell surface phenotype by flow cytometry when compared with fresh cells (data not shown).

Flow cytometric analysis

Cells suspensions were probed with the following mouse mAbs on ice for 45 min: CD1b (clone 4.A7.6) and CD207 (clone DCGM4) from Beckman Coulter; CD1a (clone HI149), CD1b (clone M-T101), CD1d (clone CD1d42), CD40 (clone 5C3), and HLA-DR (clone L243) from BD Biosciences; CD1c (clone ADS-8E7) from Miltenyi Biotec; CCR7 (clone 150503) from R&D Systems; and CD1a (clone NA1/34-HLK), CD14 (clone UCHM1), CD80 (clone MEM233), and CD86 (clone BU63) from Serotec. Unconjugated primary Abs were tagged using Zenon Alexa 488 (Invitrogen Life Technologies). The nuclear stain 7AAD (BD Biosciences) was included with each stain, and 7-aminoactinomycin D-positive cells were gated out of all analyses to exclude nonviable cells. Stained cells were analyzed using a four-color FACSCalibur flow cytometer (BD Biosciences).

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Enrichment of dermal APC and peripheral blood monocytes

Before CD1a+ APCs enrichment, the CD207+ dermal Langerhans cells were depleted from the dermal single cell suspensions. Dermal Langerhans cells were positively selected using anti-CD207-PE (Serotec), followed by anti-PE-conjugated magnetic beads (Miltenyi Biotec). Thereafter, CD1a+ APCs were positively selected using anti-CD1a-FITC (Serotec), followed by anti-FITC-conjugated magnetic beads (Miltenyi Biotec). CD14+ APCs were positively selected from dermal single cell suspensions using anti-CD14-conjugated magnetic beads (Miltenyi Biotec). Each preparation was passed sequentially through two magnetic columns (Miltenyi Biotec) to improve purity.

Peripheral blood monocytes were positively selected using anti-CD14-conjugated magnetic beads from healthy donor buffy coats.

Allogeneic MLR

Human naive CD4+ T cells were enriched from PBMCs to ≥97% purity using the Naive CD4 T Cell Isolation Kit II (Miltenyi Biotec), followed by depletion of the CD45RO+ cells (Miltenyi Biotec). Naive CD4+ T cells were labeled with 1 μM CFSE (Invitrogen Life Technologies) at 106 cells/ml PBS for 10 min at 37°C. Enriched dermal APCs and monocytes were mixed with 107 naive T cells in RPMI 1640 supplemented with 5% human serum and cultured for 6 days before analysis using flow cytometry.

Three-color immunofluorescence staining

Fresh skin was embedded in TissueTek OCT compound (Sakura Finetek). Sections 5-μm thick were fixed with ice-cold acetone and blocked with serum-free protein block (DakoCytoation). Fixed sections were probed with the following mouseAbs: podoplanin (clone18H5) from Abcam; CD207 (clone DCGM4) from Beckman Coulter; CD1a (clone HI149) from BD Biosciences; CCR7 (clone 159503) from R&D Systems; and CD1a (clone NAI1/34) and CD14 (clone MEM-18) from Serotec. The primary Abs were detected with the corresponding isotype-specific goat anti-mouse (Southern Biotech) or goat anti-FITC (Invitrogen Life Technologies) secondary Abs conjugated to a fluorochrome (Alexa 488, FITC, or tetramethylrhodamine isothiocyanate). When two primary Abs of the same isotype were to be applied to the same section, they were applied sequentially; following application of the first primary Ab and detection of the isotype-specific secondary Ab, the section was blocked with 1% mouse IgG, and the second FITC-conjugated primary Ab was then applied and detected using anti-FITC-Alexa 488.

The slides were mounted using Vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories). Sections were visualized with a Leica DMIRE fluorescent microscope equipped with the following Leica Microsystems epifluorescent filters: UV, 460–490 nm, and 515–660 nm. Images were obtained using a Leica DC500 digital camera and processed using Photoshop (Adobe).

Background staining of keratin in the stratum corneum was observed with some Abs; however, no nonspecific binding occurred within the epidermal or dermal cellular layers.

Chemotaxis assays

Cell migration was assessed using Transwell plates with a 3-μm pore size (Corning). The lower chambers were filled with RPMI 1640 with or without 100 ng/ml test chemokine (CCL19, CCL20, or CCL21) from Peprotech. Dermal single cell preparations in RF10 were applied to the upper chamber. Following an 18-h incubation at 37°C with 5% CO2, the migratory cells in the lower chamber were assessed using other human cells expressing some Abs; however, no nonspecific binding occurred within the epidermal or dermal layers.

Results and Discussion

We developed a rapid enzymatic digestion protocol for the disassociation of human skin into single cell suspensions from the dermis. We confirmed that this protocol preserved expression of all the cell surface markers we studied by using other human cells expressing each marker as positive controls (data not shown).

Flow cytometry was used to distinguish populations of APCs in single cell suspensions from human dermis and to compare these cells with peripheral blood monocytes. As shown in Fig. 1A, HLA-DRlow cells included CD14+CD1a−, CD1a+CD14−, and a minor population of CD14+CD1a+ cells. The CD1a+CD14− cells fell into two groups: CD1abighigh cells that were also CD207−, and CD1a+CD14− cells that were CD207+. Hence, three major populations of MHC class II-high professional APCs were identified in the dermal preparations: 1) CD1abighigh CD207−CD14− dermal Lang-

To confirm that the non-Langerhans dermal APC populations were capable of priming immune responses, they were purified using magnetic beads and compared with monocytes for allostimulatory capacity. Both CD14+ and CD1a+ dermal APCs were capable of stimulating the division of allogeneic naïve CD4+ T lymphocytes whereas monocytes were not, with CD1a+ dermal APCs being more potent (Fig. 1B). This finding was consistent with differences in cell surface phenotypes (Fig. 2, A and B). Both CD14+ and CD1a+ dermal APCs expressed CD40, CD80, and CD86, but the levels of all these markers were higher on CD1a+ than on CD14+ dermal APCs (Fig. 2B). CD1a+ dermal APCs also expressed higher levels of CD1b and CD1c than CD14+ dermal APCs, although HLA-DR levels were similar (Fig. 2A). Hence, CD1a+ dermal APCs were potentially capable of strongly stimulating a far wider range of naïve human T cells than CD14+ dermal APCs, including T cells restricted by all of the group 1 CD1 molecules.
Both CCR7− APCs from the skin into the lymphatic vessels (1, 2, 13–15), and CCR7+ skin. Flow cytometry data show expression by dermal APCs and monocytes of Ag-presenting molecules (4), co-stimulatory molecules and CD40 (8), and the chemokine receptor CCR7 (C). Distinguishing cell surface markers (CD1a and CD14) were included in each stain to identify each individual dermal APC subset. Heat maps indicate the relative density of each molecule on the surface of each cell population. Percentages indicate the proportion of cells expressing CCR7. Data are representative of three independent experiments.

Others have previously noted the presence of CD14+ and CD1a+ APCs in human dermis (6–8), but the relationship between CD1a+ dermal APCs and migrating Langerhans cells has not always been clear in the absence of costaining with CD207. To verify that CD1a+ dermal APCs can be distinguished from Langerhans cells in the dermis in situ, we costained sections of human skin with Abs to CD1a and CD207 (Fig. 3A). All of the CD1a+ cells in the epidermis were CD207+ Langerhans cells, and some Langerhans cells were also detectable in the dermis (Fig. 3A), consistent with their continued surface expression of CD207 while migrating through the dermis and lymphatic vessels (9–11) to the lymph node (12). The majority of CD1a+ dermal cells were, however, CD207−, confirming that CD1a+ dermal APCs are a population distinct from Langerhans cells.

To investigate the likely fate of CD1a+ dermal APCs, we examined their expression of CCR7, their relationship to the lymphatic vessels, and their response to key skin chemokines. There is now broad consensus that CCR7 expression governs the efflux of APCs from the skin into the lymphatic vessels (1, 2, 13–15), and both CCR7+ cells and CCL21, one of its ligands, have previously been demonstrated together in skin lymphatic areas (16). Most CD1a+ dermal APCs expressed surface CCR7 (Figs. 2 C and 3, C–F), whereas only a small percentage of CD14+ dermal APCs expressed surface CCR7 at low levels (Fig. 2C). Immunohistochemistry confirmed that the majority of CD1a+ dermal APCs expressed CCR7 in situ, as did migrating dermal Langerhans cells (Fig. 3, C–F). Some CD14+ dermal APCs also expressed CCR7 in situ (data not shown), although it was not possible to quantitatively evaluate expression levels by immunohistochemistry. The distribution of CD1a+ and CD14+ dermal APCs differed. CD1a+ dermal APCs were only located in the upper dermis (Fig. 3B) and were often associated with podoplanin-positive cells (Fig. 3, G–I), suggesting a proximity to lymphatic vessels. In contrast, CD14+ dermal APCs were distributed throughout the dermis, including the vascular areas of the dermis (Fig. 3B).

Finally, we compared chemotaxis of dermal cells in response to the chemokines CCL19, CCL20, and CCL21. When single cell suspensions from human dermis were placed in a Transwell chamber, only the CCR7 ligands CCL19 and CCL21 increased migration out of the Transwell chamber above background migration (Fig. 4A). When the cells migrating to both CCL19 and CCL21 were examined, they were found to be exclusively CD1a+ dermal APCs; they were CD1a+ but CD14−, and the vast majority were not Langerhans cells because they lacked CD207 (Fig. 4B). To confirm this striking difference in migration, the migratory cells were compared with the starting dermal cell preparation, which

Figure 2. CD1a+ dermal APCs expressed cell surface molecules consistent with more potent T cell stimulatory capacity than CD14+ dermal APCs and peripheral blood monocytes. Flow cytometry data show expression by dermal APCs and monocytes of Ag-presenting molecules (4), co-stimulatory molecules and CD40 (8), and the chemokine receptor CCR7 (C). Distinguishing cell surface markers (CD1a and CD14) were included in each stain to identify each individual dermal APC subset. Heat maps indicate the relative expression levels by immunohistochemistry. The distribution of CD1a+ dermal APCs and migrating Langerhans cells has not always been clear in the absence of costaining with CD207. To verify that CD1a+ dermal APCs can be distinguished from Langerhans cells in the dermis in situ, we costained sections of human skin with Abs to CD1a and CD207 (Fig. 3A). All of the CD1a+ cells in the epidermis were CD207+ Langerhans cells, and some Langerhans cells were also detectable in the dermis (Fig. 3A), consistent with their continued surface expression of CD207 while migrating through the dermis and lymphatic vessels (9–11) to the lymph node (12). The majority of CD1a+ dermal cells were, however, CD207−, confirming that CD1a+ dermal APCs are a population distinct from Langerhans cells.

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Figure 3. CD1a+ dermal APC populations were distributed throughout the upper papilla of the dermis, expressed the lymph node homing receptor CCR7, and were often associated with podoplanin-positive lymphatic endothelium. Three-color fluorescence immunohistochemistry of healthy human skin, with epidermis orientated upwards, was performed. A, CD1a+ cells in the dermis included CD1a+/CD207+ dermal APCs and CD1a+/CD207− dermal Langerhans cells, both of which were located in the papillary area. B, Both CD1a+ and CD14+ dermal APCs were detected in the dermis; C–F, CD1a+CD207− dermal APCs and dermal Langerhans cells expressed CCR7; G–I, CD1a+ dermal populations were often associated with podoplanin-positive lymphatic endothelium. Data are representative of three independent experiments. In A, B and I, blue represents the staining of cell nuclei.
skin processing, and authorities in the field have stated that any manipulation of human skin to derive APCs is likely to induce some maturation (1). However, regardless of this possibility a significant population of CD14<sup>+</sup>CD1a<sup>-</sup> APCs was still present in our single cell suspensions, enabling functional comparisons of CD14<sup>+</sup> and CD1a<sup>-</sup> APCs within the same cell preparations. We conclude that the possibility of some skin processing-induced maturation does not detract from our observation of a striking difference in migratory capacity between CD14<sup>+</sup> and CD1a<sup>-</sup> dermal APCs. Furthermore, our immunohistochemistry data confirm that CD1a<sup>-</sup> dermal APCs express CCR7 in situ and are associated with the lymphatic endothelium, supporting the concept that this population has the capacity to migrate to lymph nodes.

We propose that CD1a<sup>-</sup> dermal APCs represent a skin dendritic cell lineage distinct from Langerhans cells that is capable of carrying Ag to lymph nodes and stimulating a wide range of naive T cells, therefore comprising a second population of lymphoid-homing skin dendritic cells similar to that recently reported in murine models by Kissenpfennig et al. (4). In these models, a dermal APC population distinct from Langerhans cells was not only capable of migrating to lymph nodes and stimulating T cells but also occupied niches within the lymph nodes that were spatially distinct from those colonized by Langerhans cells (4). It will now be intriguing to re-examine coexpression of APC markers in human lymph nodes draining the skin to see whether similar niches are occupied by Langerhans cells and CD1a<sup>-</sup> dermal APCs.

One of the models published by Kissenpfennig et al. also suggested that dermal APCs are more important than Langerhans cells in inducing naive T cell responses to hapten administered via the skin (4). It therefore seems plausible that CD1a<sup>-</sup> dermal APCs and any less mature precursors are likely to be involved in processing vaccines delivered via the skin and may represent an important vaccine target. The pattern recognition receptors expressed by dermal APCs remain poorly defined (2), but presumably their capacity to acquire and process Ags will be of great interest for vaccine design.

The role of CD1a<sup>-</sup> dermal APCs in microbial and inflammatory diseases of the skin also deserves further investigation. In 2000, before mAbs to human CD207 became available, Katou et al. examined human skin flaps that had become severely inflamed by Candida albicans following autotransplantation to the oral cavity (17). In these patients the dermis was heavily infiltrated with cells that were CD1a<sup>+</sup>CD86<sup>+</sup>, presumed at that time to be dermal Langerhans cells (17). Because this molecular phenotype is identical with our observation of CD1a<sup>-</sup> dermal APCs, it will now be important to re-examine such cases to determine whether it is dermal CD1a<sup>-</sup> APCs rather than Langerhans cells that are involved in the dermal response to Candida infection. Similarly, Wollenberg and colleagues have noted that single cell suspensions from shave biopsies of atopic skin contain a large population of CD1a<sub>low</sub>CD1b<sub>low</sub> dendritic cells, lacking Birbeck granules and other phenotypic features of Langerhans cells (18, 19). The relationship between these cells and the CD1a<sup>-</sup> dermal APC counterparts we now report in healthy skin needs clarification, including their anatomical location on full thickness biopsies from atopic skin.

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Disclosures
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