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Dermal Dendritic Cells, and Not Langerhans Cells, Play an Essential Role in Inducing an Immune Response

Atsushi Fukunaga, Noor M. Khaskhely, Coimbatore S. Sreevidya, Scott N. Byrne, and Stephen E. Ullrich

Langerhans cells (LCs) serve as epidermal sentinels of the adaptive immune system. Conventional wisdom suggests that LCs encounter Ag in the skin and then migrate to the draining lymph nodes, where the Ag is presented to T cells, thus initiating an immune response. Platelet-activating factor (PAF) is a phospholipid mediator with potent biological effects. During inflammation, PAF mediates recruitment of leukocytes to inflammatory sites. We herein tested a hypothesis that PAF induces LC migration. Applying 2,4-dinitro-1-fluorobenzene (DNFB) to wild-type mice activated LC migration. In contrast, applying DNFB to PAF receptor-deficient mice or mice injected with PAF receptor antagonists failed to induce LC migration. Moreover, after FITC application the appearance of hapten-laden LCs (FITC⁺, CD11c⁺, Langerin⁺) in the lymph nodes of PAF receptor-deficient mice was significantly depressed compared with that found in wild-type mice. LC chimerism indicates that the PAF receptor on keratinocytes but not LCs is responsible for LC migration. Contrary to the diminution of LC migration in PAF receptor-deficient mice, we did not observe any difference in the migration of hapten-laden dermal dendritic cells (FITC⁺, CD11c⁺) into the lymph nodes of PAF receptor-deficient mice. Additionally, the contact hypersensitivity response generated in wild-type or PAF receptor-deficient mice was identical. Finally, dermal dendritic cells, but not LCs isolated from the draining lymph nodes after hapten application, activated T cell proliferation. These findings suggest that LC migration may not be responsible for the generation of contact hypersensitivity and that dermal dendritic cells may play a more important role. The Journal of Immunology, 2008, 180: 3057–3064.

Dendritic cells (DCs) are professional APCs that play a crucial role in activating adaptive immune responses. Langerhans cells (LCs) are a subset of immature DCs that reside in the epidermis. DCs are distinguished from other DCs by the presence of cytoplasmic organelles, known as Birbeck granules (1). LCs are characterized by expression of the type II transmembrane Ca²⁺-dependent lectin, langerin/CD207 (2). Because of their specialized location, LCs are thought to constitute the first immunologic barrier to pathogens. LC migration is triggered via a variety of stimuli, including hapten application, TNF-α, and UV irradiation (3–6). LCs have traditionally been thought to play a role in the induction of contact hypersensitivity (CHS). However, three different groups reported three distinct results after applying hapten to mice genetically engineered to be deficient in LCs: a diminished reaction (7), an enhanced reaction (8), and an unchanged response (9). Moreover, recent findings suggest that LCs are not required for the induction of humoral and cell-mediated immunity following gene gun immunization (10). Therefore, it remains controversial whether LCs are dispensable for the induction of skin immunity.

Dermal DCs (dDCs) were identified more than 120 years after LCs were discovered (11). There is no exclusive marker for dDCs comparable to langerin/CD207, making dDCs difficult to track in vivo, and they have often been overlooked in studies of skin immunity. Recently, Kissesphenfennig et al. demonstrated that CD11c⁺ CD8α⁺ CD205⁺ epidermal growth factor-negative, hapten-positive DCS migrating to draining lymph nodes of mice are dDCs, and their data suggested that dDCs are responsible for inducing CHS (9).

Platelet-activating factor (PAF) is a phospholipid mediator with potent biological effects (12). PAF binds to a specific receptor (PAFR), which is found on a wide variety of cells, including platelets, monocytes, mast cells, polymorphonuclear lymphocytes, DCs, and keratinocytes (13). PAF is produced by physical stress, including UV light and trauma (14). PAF is involved in UVB, psoralen plus UVA light, and jet fuel-induced immune suppression (13, 15, 16). PAF also plays a role in regulating the immune response to microbial pathogens (14). PAF binding results in the production of numerous cytokines (13, 17, 18). During inflammation, PAF mediates recruitment of leukocytes to inflammatory sites (12, 19), but its role in LC or dDC migration is unclear.

Based primarily on the role of PAF in UV and psoralen plus UVA light-induced immune suppression, we hypothesized that PAF receptor binding induces LC migration. We used PAFR-deficient mice and...
PAFR antagonists to test the role of PAF in LC migration. We found that PAF is involved in LC migration from the skin to the draining lymph nodes, but it does not appear to play a role in dDC migration. Regardless, we did not observe any difference in the CHS response generated in wild-type (WT) mice vs PAFR-deficient mice, suggesting that the dDCs and not LCs play an essential role in the induction of CHS.

Materials and Methods

**Mice**

PAFR-deficient mice, backcrossed onto a C57BL/6 background, originally described by Ishii and colleagues (20), were provided to us by Dr. Jeffrey B. Travers (Indiana University School of Medicine, Indianapolis, IN), with Prof. T. Shimizu’s permission (20). C57BL/6 mice that express the CD45.2 allele were purchased from the U.S. National Cancer Institute. OT-II mice (Vv2 C57BL/6J.Tg(CD4-DsRedII)1) were provided by Dr. Y.-J. Liu (University of Texas M.D. Anderson Cancer Center). C57BL/6 mice that express the CD45.1 allele (C57BL/6-Ly5.1.Peb3b) were purchased from The Jackson Laboratory. Within each experiment, all mice were age and sex matched. All procedures were reviewed and approved by the University of Texas M.D. Anderson Cancer Center Animal Care and Use Committee.

**Abs and reagents**

Abs (FITC, PE, PerCP-Cy5.5, and/or allophycocyanin) specific for CD45.1, CD45.2, CD8α, CD11c, PE-conjugated mouse anti-rat IgG2a, corresponding isotype controls, and secondary reagents (FITC-conjugated streptavidin, PerCP-conjugated streptavidin, and allophycocyanin-conjugated streptavidin) were purchased from BD Biosciences. Abs (biotin) specific for anti-mouse MHC class II I-A/I-E and Langerin (CD207; clone 205C1) was purchased from AbCys (assay ID: Mm00443258_m1) expression was quantified using TaqMan gene expression assays (Applied Biosystems). Murine TNF-α (assay ID: Hs00174134_m1) was purchased from Qiagen. cDNA was reverse-transcribed from total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Murine TNF-α (assay ID: Mm00443258_m1) expression was quantified using TaqMan gene expression assays (Applied Biosystems) in an ABI PRISM 7000 sequence detection system (Applied Biosystems). GAPDH gene expression was measured using the TaqMan rodent GAPDH reagents (Applied Biosystems). Real-time amplifications were analyzed using SDS 7000 v1.1 software (Applied Biosystems). Threshold cycle (Ct) values for TNF-α were normalized to GAPDH using the following equation: 2^[(ΔCtGAPDH − ΔCtTNF-α)] / 10,000, where GAPDH is the Ct of the GAPDH control, TNF-α is the Ct of the TNF-α, and 10,000 is an arbitrary factor to bring all values above 1.

**CHS response**

WT and PAFR-deficient mice were immunized by applying 50 μl of 0.5% DNFB solution solubilized in acetone/olive oil (4:1, v/v) on their shaved abdomens. Six days later, 10 μl of 0.25% DNFB solution was applied to both ear surfaces. On day 7, the mice were sedated, and the thickness of each ear was measured with an engineer’s micrometer (Mitutoyo). The data are expressed as the mean change in ear swelling (left ear + right ear/2) for each animal (n = 5).

**Preparation and analysis of LC chimeric mice**

LC chimeric mice were generated as described previously (23). Briefly, 7- to 8-wk-old recipient mice (CD45.2^-/- PAFR-deficient or CD45.1^-/- control) were lethally irradiated with two doses of 500 rads each 3 days before irradiation, and injected i.v. with bone marrow (BM) cells (5 x 10^7) obtained from CD45.1^-/- or CD45.2^-/- donors. Seven weeks after BM transplantation, >90% of blood cells were of donor origin. The dorsal hair of the chimeric mice was removed with electric clippers, and then mice were exposed to 10 kJ/m^2 (CD45.1^-/- mice) or 20 kJ/m^2 (CD45.2^-/- PAFR-deficient mice) of UVB (290 – 320 nm, FS-40 sunlamps) radiation as measured with an IL-1700 research radiometer (International Light). The presence of donor- and host-derived LCs in the epidermis was evaluated by flow cytometric analysis of epidermal cell suspensions and immunofluorescence analysis of epidermal sheets 9 wk after UVB irradiation (24). Murine dorsal skins were incubated for 16 h at 4°C in 0.3% trypsin HBSS (Life Technologies), and epidermal sheets were separated from the dermis. The epidermal sheets were incubated with 0.3% trypsin/DNase 1 HBSS (Sigma-Alrich) for 20 min at 37°C, shaken vigorously, filtered through a 70-μm filter, and then resuspended into 5% FCS/PBS for flow cytometry.

**T cell proliferation assay**

OXA (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one) was dissolved in acetone/dibutylphthalate (1:1). Two days after applying 400 μl of 2% OXA, the draining lymph nodes were removed, single-cell suspensions were prepared, and the CD11c^-/- CD8α^-/- Langerin^-/^- (skin-derived LCs) and CD11c^-/- CD8α^-/- Langerin^-/- (skin-derived dDCs) cells were selected by sorting. CD4^+ T cells were isolated from OT-II mice by negative selection with magnetic beads (Miltenyi Biotec) as described previously (25). To monitor cell proliferation, CD4^+ T cells were incubated with CFSE at final concentration of 5 μM in 1% FCS/PBS for 10 min at 37°C. CFSE-labeled T cells (1 x 10^6), LCs, and OVA peptide 323–339 (10 μg/ml) were incubated in 96-well plates in complete RPMI 1640. Proliferation in wells with plate-bound anti-mouse CD3 Abs (10 μg/ml; BD Biosciences) and soluble anti-mouse CD28 Abs (5 μg/ml; BD Biosciences) served as the positive control. After 4 days, the cells were stained with allophycocyanin-conjugated anti-mouse CD4 (eBioscience), and CFSE content was analyzed by flow cytometry. For intracellular staining, a BD Cytofix/Cytoperm (BD Biosciences) kit was used according to the manufacturer’s recommendations. Permeabilized fixed cells were incubated with permeabilized with anti-Langerin at 4°C overnight, washed with BD Perm/Wash, and incubated with PE-conjugated mouse anti-rat IgG2a at 4°C for 40 min. After washing, flow cytometric analysis was performed (FACSCalibur, BD Biosciences).

**Real-time quantitative RT-PCR**

Murine keratinocytes (PAM 212) were plated and treated with 5 μM of c-PAF. Total RNA was extracted from the PAM 212 cells using RNeasy Mini Kit (Quiagen). cDNA was reverse-transcribed from total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Murine TNF-α (assay ID: Mm00443258_m1) expression was quantified using TaqMan gene expression assays (Applied Biosystems) in an ABI PRISM 7000 sequence detection system (Applied Biosystems). GAPDH gene expression was measured using the TaqMan rodent GAPDH reagents (Applied Biosystems). Real-time amplifications were analyzed using SDS 7000 v1.1 software (Applied Biosystems). Threshold cycle (Ct) values for TNF-α were normalized to GAPDH using the following equation: 1.8(GAPDH − TNF-α) x 10,000, where GAPDH is the Ct of the GAPDH control, TNF-α is the Ct of the TNF-α, and 10,000 is an arbitrary factor to bring all values above 1.
Statistical analysis

Statistical differences between the positive control and the experimental groups were determined by Student’s two-tailed t-test. Probabilities < 0.05 were considered to be significant.

Results

PAF is involved in LC migration from the epidermis

We first tested the hypothesis that PAF plays a role in LC migration. Applying hapten to WT mice had two effects. First, fewer LCs were found in the epidermal sheets. Second, there was a noticeable increase in the size of the remaining LCs (Fig. 1A–C). In contrast, applying DNFB to PAFR-deficient mice failed to induce the migration of LCs (Fig. 1A–C). Next, mice were injected with PCA4248, CV3988, two PAF receptor antagonists, and then treated with hapten. LC migration was measured 24 h later. As expected, applying DNFB induced LC migration (Fig. 1, D and E).

**FIGURE 1.** PAF is involved in LC migration from the epidermis. A, DNFB was applied to the ears of WT mice and PAFR-deficient mice. Epidermal sheets were collected 1 day after DNFB application and stained with Langerin. B, After staining, the number of LCs found in the epidermis was determined by counting. *Statistically significant difference (p < 0.001; n = 5) from no treatment group of WT mice. C, Epidermal sheets were collected 1 day after DNFB application and stained with I-A. *Statistically significant difference (p < 0.001; n = 5) from no treatment group of WT mice. D, The PAFR antagonists PCA4248 and CV3988 (500 nmol per mouse) or respective vehicle control was injected i.p. immediately before DNFB application. Epidermal sheets were collected 1 day after DNFB application and stained with Langerin. E, After staining, the number of LCs found in the epidermis was determined by counting. *Statistically significant difference (p < 0.001; n = 5) from no treatment group. The pinnae of the ear was injected with 150 pmol of c-PAF or respective vehicle control. Epidermal sheets were collected 1 day after injection and stained with Langerin. *Statistically significant difference (p < 0.001; n = 5) from vehicle group. A representative experiment is shown; each experiment was performed at least twice.

**FIGURE 2.** PAF is involved in LC migration from the skin to the draining lymph nodes. A, FITC was applied onto the backs of WT mice. Two days after FITC application, draining lymph nodes were collected and stained with CD11c and Langerin. We gated on the FITC+CD11c+ population and counted the Langerin+ and CD8α+ cells. In the Langerin panel, the blue line indicates Langerin staining, and the red line indicates the isotype control. In the CD8α panel, the red line indicates CD8α expression in whole cells DLN (positive control), the blue line indicates CD8α expression in the FITC+CD11c+ population, and the green line indicates the isotype control. B, The numbers of FITC+CD11c+Langerin+ cells (LCs) and FITC+CD11c−Langerin− cells (dDCs) found in draining lymph nodes were counted at various times after FITC application. C, Cell numbers of the lymph nodes were increased after hapten application. D, FITC was applied onto the backs of WT mice and PAFR-deficient mice. Two days later, the numbers of FITC+CD11c+Langerin+ cells (LCs) in the draining lymph nodes were counted. *Statistically significant difference (p < 0.05; n = 5) from WT mice. E, FITC was applied onto the backs of WT mice and PAFR-deficient mice. Two days later, the inguinal lymph nodes were collected and stained with Langerin (red). In PAFR-deficient mice, fewer FITC+Langerin+ cells (yellow; LCs) were found compared with those in WT mice. Bar = 200 μm. A representative experiment is shown; each experiment was performed at least twice.
the ear of CD45.2. Langerin

and anti-Langerin. Flow cytometric analysis indicated that two

HAotype, matching that expected for skin-derived DCs (9, 21) (Fig.

CD45.1

9). The second subset of FITC

rightmost panel

A

in LC migration. Two groups of chimeric mice were prepared: group 1

(Fig. 2A, rightmost panel). The second subset of FITC CD11c+ cells

were Langerin+ (Fig. 2A), matching that expected for migrating

hapt-en laden DCs (9, 21) (Fig.

CD45.1 mice BM/PAFR−/− UVB 10 kJ/m2

CD45.1 mice BM/PAFR−/− UVB 10 kJ/m2

PAFR on keratinocytes but not LCs plays an important role in LC migration. Two groups of chimeric mice were prepared: group 1

FITC

CD45.1 mice BM/PAFR−/− UVB 10 kJ/m2

CD45.1 mice BM/PAFR−/− UVB 10 kJ/m2

PAFR−/− mice; keratinocytes are

PAFR−/−). A, Representative experiment showing the expression of CD45.1 and CD45.2 on gated I-A+CD11c+ LCs isolated from CD45.1 recipients reconstituted with PAFR−/− cells. B, Epidermal sheets obtained from CD45.1 mice, reconstituted with BM from PAFR−/− mice and then irradiated with 10 kJ/m2 of UVB. Epidermal sheets were stained with CD45.1 or CD45.2 and Langerin. C, Representative experiment showing the expression of CD45.1 and CD45.2 on gated I-A+CD11c+ LCs isolated from PAFR−/− mice reconstituted with CD45.1 BM. D, Epidermal sheets obtained from PAFR−/− mice reconstituted with BM from CD45.1 mice and then irradiated with 20 kJ/m2 of UVB. E, DNFB was applied onto the ear of CD45.1− control mice and CD45.1− mice reconstituted with PAFR+ BM (group 1). One day after application, epidermal sheets were stained with Langerin. The cell density of the no treatment group was adjusted to 100%. *, Statistically significant difference (p < 0.001; n = 5) from no treatment group. F, DNFB was applied onto the ear of CD45.2 PAFR−/− mice and CD45.2 PAFR−/− mice reconstituted with BM from CD45.1 mice (group 2). One day later, epidermal sheets were stained with Langerin (n = 5). A representative experiment is shown; each experiment was performed at least twice.

FIGURE 3. PAFR on keratinocytes but not on LCs plays an important role in LC migration. Two groups of chimeric mice were prepared: group 1 (PAFR−/− CD45.2 BM cells into CD45.1 recipients; LCs are PAFR−/−); group 2 (CD45.1 bone marrow into CD45.2 PAFR−/− mice; keratinocytes are PAFR−/−). A, Representative experiment showing the expression of CD45.1 and CD45.2 on gated I-A+CD11c+ LCs isolated from CD45.1 recipients reconstituted with PAFR−/− cells. B, Epidermal sheets obtained from CD45.1 mice, reconstituted with BM from PAFR−/− mice and then irradiated with 10 kJ/m2 of UVB. Epidermal sheets were stained with CD45.1 or CD45.2 and Langerin. C, Representative experiment showing the expression of CD45.1 and CD45.2 on gated I-A+CD11c+ LCs isolated from PAFR−/− mice reconstituted with CD45.1 BM. D, Epidermal sheets obtained from PAFR−/− mice reconstituted with BM from CD45.1 mice and then irradiated with 20 kJ/m2 of UVB. E, DNFB was applied onto the ear of CD45.1− control mice and CD45.1− mice reconstituted with PAFR+ BM (group 1). One day after application, epidermal sheets were stained with Langerin. The cell density of the no treatment group was adjusted to 100%. *, Statistically significant difference (p < 0.001; n = 5) from no treatment group. F, DNFB was applied onto the ear of CD45.2 PAFR−/− mice and CD45.2 PAFR−/− mice reconstituted with BM from CD45.1 mice (group 2). One day later, epidermal sheets were stained with Langerin (n = 5). A representative experiment is shown; each experiment was performed at least twice.

In contrast, applying DNFB immediately after injecting PCA4248

or CV3988 failed to induce LC migration (Fig. 1, D and E). To further test the role of PAF in LC migration, c-PAF, a metabolic stable analog of PAF, was injected into the skin. Injecting 150 pmol of c-PAF induced LC migration (Fig. 1F). These results indicate that PAF signaling activates LC migration.

PAF is involved in LC migration from the skin to the draining lymph nodes

The previous findings indicate that PAF is involved in inducing LCs to leave the skin. To determine whether PAF is responsible for inducing LCs to migrate to the lymph nodes, the following was done. WT mice were painted with FITC. One to 5 days later, the draining lymph node was removed, a single-cell suspension was prepared, and the cells were stained with anti-CD11c, anti-CD8α, and anti-Langerin. Flow cytometric analysis indicated that two subsets of CD11c+ FITC+ cells were found in the draining lymph nodes (Fig. 2A). The main subset was Langerin+, matching the phenotype expected for migrating hapt-en laden DCs (9). More than 90% of the FITC+ CD11c+ cells exhibited a CD8αlow phenotype, matching that expected for skin-derived DCs (9, 21) (Fig. 2A, rightmost panel). The second subset of FITC CD11c+ cells were Langerin− (Fig. 2A), matching that expected for migrating hapt-en laden LCs (9). We observed a time-dependent increase in DC migration and cell number in the lymph node, which peaked 2–3 days after FITC application and then gradually decreased (Fig. 2, B and C). PAFR-deficient mice then were painted with FITC to test a role for PAF in LC migration. The appearance of FITC+ CD11c+ Langerin+ cells in draining lymph nodes of PAFR-deficient mice was depressed compared with that found in WT mice (Fig. 2, D and E). These data indicate that PAF is involved in LC migration from the skin to draining lymph nodes.

PAF on keratinocytes but not LCs plays an important role in LC migration

LC migration is regulated by keratinocyte-derived cytokines, such as TNF-α or IL-1β produced by LCs (26). PAF binds to a specific receptor found on a wide variety of cells, including DCs and keratinocytes (13, 27). To determine whether PAF binding to its receptor on keratinocytes drives LC migration, we made LC chimeric mice. The first group (PAFR−/− CD45.2 BM cells into CD45.1 recipients) is characterized by PAFR+ keratinocytes and PAFR− LCs. The second group (CD45.1 BM cells into CD45.2 PAFR−/− mice) is characterized by PAFR− keratinocytes and PAFR+ LCs. We observed that high-dose UV irradiation (10–20 kJ/m2) of both groups depleted the host LCs and resulted in LC chimeric mice. We found that Langerin+ LCs in mice exposed to 10 kJ/m2 of UV express CD45.1 (host phenotype) 9 wk after UV irradiation (Fig. 3, A and B). Similarly, when PAFR−/− BM cells (CD45.2) were used to reconstitute X-irradiated CD45.1 mice, and these mice were subsequently exposed to 10 kJ/m2 of UVB 9 wk post-UV irradiation, all the LCs in the skin were of donor origin.
the ears of WT or PAFR

Injecting TNF-

These results indicate that PAFR on keratinocytes, but not on LCs,

A

B

C

FIGURE 4. TNF-α activates LC migration in PAFR−/− mice. A, PAM 212 cells were treated with 1 μM of c-PAF, and TNF-α expression was determined by real-time PCR. The levels of TNF-α mRNA were normalized to GAPDH expression. *, Statistically significant difference (p < 0.02; n = 4) from nontreated (0 h) group. B, c-PAF (150 pmol) was injected into the ears of WT or PAFR−/− mice. Epidermal sheets were collected 1 day after injection and stained with Langerin. *, Statistically significant difference (p < 0.02; n = 5) from vehicle treated WT mice. C, Recombinant TNF-α (50 ng) was injected into the ears of WT or PAFR−/− mice. Epidermal sheets were collected 1 h after injection and stained with Langerin. +, Statistically significant difference (p < 0.001; n = 5) from vehicle-treated mice. A representative experiment is shown; each experiment was performed at least twice.

(CD45.2) (Fig. 3, A and B). We also could produce LC chimera in PAFR-deficient recipient mice; however, higher doses of UVB radiation were required (Fig. 3, C and D). We then applied DNFB to the ears of the LC chimeric mice to activate LC migration. There was no significant difference in LC migration between CD45.1 control mice and group 1 mice (PAFR−/− CD45.2 bone marrow into CD45.1 recipients) (Fig. 3E). However, LC migration was totally abrogated when group 2 mice (CD45.1 bone marrow cells into CD45.2 PAFR−/− mice) were treated with DNFB (Fig. 3F). These results indicate that PAFR on keratinocytes, but not on LCs, plays an important role in LC migration after hapten application.

Injecting TNF-α overcomes the defect in LC migration in PAFR−/− mice

Cumberbatch et al. demonstrated that injecting TNF-α into the dermis, or TNF-α produced by keratinocytes, induces LC migration (26, 28). Because others have noted that treating PAM-activated human epidermal cells with the c-PAF resulted in increased TNF-α mRNA and protein secretion (18), we sought to determine whether treating PAM 212 cells induces TNF-α expression. Similar to human epidermal cells, c-PAF treatment increased expression of TNF-α RNA in PAM 212 cells in a time-dependent manner (Fig. 4A). Whereas s.c. injection of c-PAF induced significant LC migration from the epidermis in WT mice, it did not induce LC migration in PAFR-deficient mice (Fig. 4B). In contrast, injecting recombinant TNF-α induced equivalent LC migration from the epidermis of WT mice and PAFR-deficient mice (Fig. 4C). These data indicate that PAF may be activating LC migration via a TNF-α dependent mechanism, and they suggest that PAF is upstream of TNF-α.

CHS and dDC migration in WT and PAFR-deficient mice

Although traditional evidence suggests that LCs play a critical role in induction of CHS (29), more recent studies have led to questions regarding the role of LCs in CHS (30). Based on the data presented above, we tested the CHS response in PAFR-deficient mice. We did not observe any difference in the magnitude of the CHS response generated in WT or PAFR-deficient mice (Fig. 5A). These data indicate that PAF signaling is not essential for inducing CHS.

Failure of LCs to migrate from the skin of PAFR-deficient mice, coupled with the observation that CHS was equal in these mice, suggests that LCs are not the relevant APC for CHS. We therefore decided to focus on the role of dDCs in this reaction. First we measured dDC migration after hapten application. In contrast to LC migration, the migration of dDCs (FITC+/CD11c+ Langerin+) into the draining lymph nodes of PAFR-deficient mice was equivalent to that found in WT mice (Fig. 5B). Additionally, the increase in lymph node cell numbers found after dermal hapten application of PAFR-deficient mice was equivalent to that found in WT mice (Fig. 5C). These results indicate that PAF does not appear to play a role in dDC migration.

Lymph node dDCs but not LCs have an essential role in CD4+ T cell proliferation

Our data imply that dDCs and not LCs play an essential role in the generation of CHS in vivo. To directly test the ability of each cell
type to present Ag, we performed the following experiment. An Ab specific for the extracellular domain of Langerin (clone 205C1) was used to sort LCs from the draining lymph nodes of hapten-sensitized mice. We observed that both LCs (CD11c+CD8αlow/Langerin−) and dDCs (CD11c+CD8αlow−/Langerin+) were located in the draining lymph nodes 2 days after hapten application (Fig. 6A). Moreover, we noted the dDCs outnumbered the LCs by a ratio of 10:1 (Fig. 6A). This physiological ratio of LCs to dDCs was used in a functional assay. CFSE-labeled OT-II CD4+ T cells and either LCs or dDCs were cocultured with OVA peptide 323–339 for 6 days (Fig. 6B). As a positive control, some T cells were stimulated with anti-CD3 + anti-CD28, which induced significant T cell proliferation. Using physiological conditions, migrating dDCs induced a stronger proliferative response than did migrating LCs (Fig. 6B, right two panels). Even when the number of dDCs was reduced to one-half that seen in physiological conditions, they induced stronger T cell proliferation than did LCs (Fig. 6C). We also asked whether the LCs could suppress the stimulation of T cells by dDCs in a mixing experiment. Coculture of dDCs and LCs at physiological concentration did not affect T cell proliferation, as the response of this group was similar to the response found when an equal number of dDCs was used (Fig. 6C). To confirm these observations, we repeated these experiments using the classical technique of [3H]thymidine incorporation to measure T cell proliferation. Note that when nonphysiological concentrations of dDCs and LCs were used, and the cells were added to the wells in equal numbers, dDCs presented Ag whereas the by LCs did not (Fig. 6D). These data indicate that dDCs but not LCs activate T cell proliferation.

**Discussion**

Conventional wisdom suggests that upon encountering Ag in the skin, epidermal LCs carry that Ag to the draining lymph nodes, where they initiate an immune reaction. Generally, the signal that induces LCs to migrate from the skin to the lymph node is physiological stress. Because stress induces keratinocytes to release PAF (31), a lipid mediator of inflammation that plays a role in leukocyte migration (32), we wanted to determine whether PAF contributes to LC migration in vivo. We observed the following. First, we found that in PAF-deficient mice, hapten-induced LC migration was significantly impaired. Second, we observed that hapten-induced LC migration from the epidermis was blocked by pretreatment with selective PAFR antagonists. Third, s.c. local injection of c-PAF, a structural analog of PAF, induced LC migration from the epidermis. Finally, studies with chimeric mice indicated that PAF receptor on the keratinocytes, and not the LC, is more important for the induction of migration. These results suggest that PAF is involved in hapten-induced LC migration. In contrast, we noted no difference in the magnitude of the CHS reaction generated in PAFR-deficient and WT mice. This last finding suggested that LCs are not involved in initiating an immune reaction. Using sorted LCs and dDCs from the lymph nodes of hapten-sensitized mice, we observed that only the dDCs, and not the LCs, could present Ag to CD4+ T cells.

CHS is a classic delayed-type hypersensitivity reaction to topically applied hapten. Conventional wisdom concerning LC function would predict that the defect in LC migration we noted in PAFR-deficient mice would result in a diminished CHS response (7, 33). We observed, however, no difference in the magnitude of the CHS response generated in WT or PAFR-deficient mice. This observation is consistent with data reported by Kissenpfennig et al. that the absence of LCs at the time of sensitization does not affect CHS in genetically engineered LC knockout mice (9). Also, our data are consistent with the conclusion arrived at by others (7–9) that the dDCs play a critical role in initiating CHS and migrate to draining lymph nodes in greater numbers and more quickly in response to hapten application when compared with LCs. Our findings extend these observations in two important ways. First, we
used FACS to isolate LCs and dDCs from the lymph nodes of hapten-sensitized mice and asked directly whether they could present Ag to T cells. As shown in Fig. 6, only the dDCs and not the LC could present Ag. Second, we used unmanipulated WT mice. Although gene knockout mice provide the most rigorous way to measure the function of any particular gene, it must be kept in mind, as pointed out by Olson and colleagues nearly 10 years ago, that “such manipulations (necessary to create a knockout) may disrupt the expression of other genes located near the intended target and therefore confound the interpretation of phenotypes” (34). This may contribute to the fact that mice genetically engineered to be LC deficient show diminished (7), enhanced (8), or unchanged (9) CHS reactions. Our findings confirm that the dDC and not the LC is the primary Ag-presenting cell in CHS. Finally, Kaplan et al. proposed that LCs served to regulate the CHS response (8). In our co-mixing experiment, we could not find any evidence that LCs regulate the function of dDCs. However, we may not have used the proper conditions to activate immune modulatory LCs. For example, one well-known way to activate LCs to become immune regulatory is through the use of UV radiation (35). In this study we simply used UV as a tool to remove the LCs from the skin and then allow reconstitution of the skin 9 wk later by donor-derived LCs. We did not ask whether LCs found in the lymph nodes of UV-irradiated mice 2–3 days after UV induce tolerance. Studies are currently in progress, using sorted lymph node LCs, to address this question.

Our findings also illustrate the importance of PAFR on keratinocytes in LC migration. The literature indicates that both keratinocytes and dDCs express functional PAFR (27). Although our initial findings (Fig. 1) indicated the importance of PAFR signaling in LC migration, we did not know whether PAFR on either LCs or keratinocytes (or both) was more important. To test this, we produced LC chimeric mice whose keratinocytes were PAFR−/− or whose LCs were PAFR+/+. In these mice, we observed that PAFR on keratinocytes, but not LCs, plays an important role in hapten-induced LC migration. We assume that cytokines from keratinocytes affect LC migration. This is supported by the fact that PAF is a known regulator of transcription, and it has been shown to up-regulate the secretion of a variety of cytokines (32), including TNF-α (Fig. 4). These results imply that PAF produced by keratinocytes after hapten application binds to keratinocytes in an autocrine fashion, and activates them to produce TNF-α, which then induces LC migration.

In summary, our results reveal that PAF is involved in LC migration. However because PAFR−/− mice generate a CHS reaction different from controls, our findings support the hypothesis that LCs are not involved in activating CHS. By demonstrating that dDCs, but not LCs, sorted from the lymph nodes of hapten-sensitized mice can present Ag to T cells, we confirm the critical, but not well-appreciated, role for these cells in skin immunity. These findings suggest that further studies into the role of dDCs in other cutaneous immune diseases such as atopic dermatitis, psoriasis, and UV-induced carcinogenesis (35–37) have merit.

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Disclosures
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References


