The Journal of Immunology

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J Immunol 2007; 179:4535-4541; ;
doi: 10.4049/jimmunol.179.7.4535
http://www.jimmunol.org/content/179/7/4535

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Skin-Derived Dendritic Cells Can Mediate Deletional Tolerance of Class I-Restricted Self-Reactive T Cells[1]

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Skin-draining lymph nodes contain a number of dendritic cell (DC) subsets of different origins. Some of these are migratory, such as the skin-derived epidermal Langerhans cells and a separate dermal DC subset, whereas others are lymphoid resident in nature, such as the CD8$^+$ DCs found throughout the lymphoid tissues. In this study, we examine the DC subset presentation of skin-derived self-Ag by migratory and lymphoid-resident DCs, both in the steady state and under conditions of local skin infection. We show that presentation of self-Ag is confined to skin-derived migrating DCs in both settings. Steady state presentation resulted in deletional T cell tolerance despite these DCs expressing a relatively mature phenotype as measured by traditional markers such as the level of MHC class II and CD86 expression. Thus, self-Ag can be carried to the draining lymph nodes by skin-derived DCs and there presented by these same cells for tolerization of the circulating T cell pool. ***The Journal of Immunology,*** **2007, 179: 4535–4541.**

There are two broad dendritic cell (DC)[3] populations within the skin: Langerhans cells found in the skin epidermis and a separate pool of DCs in the underlying dermis (1–5). Langerhans cells and dermal DCs are migratory subsets that are mobilized by infection, injury, or inflammation of the skin and, as such, they have long been considered key mediators of T cell immunity during skin infection. Originally, skin immunity was thought to result from the coordinated maturation of these cells to an immunogenic form, combined with the presentation of skin-acquired Ag. However, it now emerges that the situation is far more complicated than described in this paradigm, with presentation extending beyond the migratory DC population (6–10). Although certain pathogens mediate T cell activation via skin emigrants (8, 10), immunity to other types of skin infection appears driven by purely lymphoid-resident DCs, namely the CD8$^+$ DC subset. For example, infection with cytopathic viruses like herpes simplex and vaccinia viruses result in class I-restricted presentation that is almost exclusively found within this nonmigratory population. Moreover, recent data suggests that skin DCs can drive tolerogenic or regulatory type responses (11, 12). This latter observation is particularly intriguing because examination of skin-draining lymph nodes (LNs) shows the migratory DCs to be those that are most mature, as measured by phenotypic markers such as the costimulatory molecules CD80 and CD86 (2, 13). Indeed, Mayerova et al. (14) argued that presentation of skin-expressed self-peptide by migratory DCs induced a predominantly autoimmune CD8$^+$ T cell response as a direct consequence of the maturational state of the migratory population, which they assume arises as a consequence of continuous environmental challenge. Given these conflicting notions about the function of skin-derived DCs, we have re-examined the issue of DC subset presentation of skin-derived self-Ag. Our results show that, unlike what is seen in skin infection with cytopathic virus, presentation of self-Ag occurs by the mature migratory DCs of both dermal and epidermal origin and this presentation results in long-term deletional tolerance.

Materials and Methods

Mice

C57BL/6 (B6), B6.SJL-Ptprc^{a}Pep3^b/BoyJ (B6.Ly5.1), B6.C-H-2^{bm-1} (bm1) (15), RIP.OVA^{high} (16), K5m.OVA (17), K5.mOVA × bm1, K5.mOVA × B6.Ly5.1, OT-I × B6.Ly5.1, and gBT-I × B6.Ly5.1 mice were bred and maintained at the Department of Microbiology and Immunology, University of Melbourne or at the Walter and Eliza Hall Institute of Medical Research.

Preparation and adoptive transfer of CFSE-labeled OT-I or gBT-I T cells

OT-I or gBT-I T cells were prepared by generating a single-cell suspension of LNs (axillary, brachial, inguinal, cervical, and mesenteric) and/or spleen cells from either OT-I or gBT-I transgenic mice. In some experiments, OT-I or gBT-I T cells were purified. Single-cell suspensions were incubated for 30 min with predetermined optimal concentrations of the following purified mAbs: anti-Mac-1α (M1/70), anti-F4/80 (F4/80), anti-erythrocyte (TER-119), anti-GR-1 (RB68C5), anti-I-A/I-E (M5114), and anti-CD4 (GK1.5). The Ab-coated cells were then removed by incubation with sheep anti-rat IgG-coupled magnetic beads (Dynabeads: Dynal Biotech) or goat anti-rat IgG-coupled magnetic beads (Qiagen). Purity was determined by propidium iodide (PI) exclusion of cells stained with anti-CD8 and Vα2.

For CFSE labeling, OT-I or gBT-I T cells were resuspended in PBS containing 0.1% BSA (Sigma-Aldrich) and labeled with 2.5 μM CFSE (Molecular Probes) for 10 min at 37°C. Cells were then washed twice in HEPES MEM. A total of 10^6 CFSE-labeled OT-I T cells (Ly5.1$^+$, CD8$^+$,

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Received for publication February 9, 2007. Accepted for publication July 17, 2007.

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[1] This work was supported by grants from the Australian National Heath and Medical Research Council, The Wellcome Trust, and the Howard Hughes Medical Institute.

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[3] Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; PI, propidium iodide.

and Vα2$^+$) were adoptively transferred by i.v. injection into recipient mice. After 42 h, pooled brachial and inguinal LNs from individual mice were analyzed by flow cytometry for proliferation of Ly5.1$^+$CD8$^+$CFSE$^+$PI$^-$ cells. For in vivo experiments aimed at analyzing proliferation, unenriched CFSE-labeled OT-I T cells from the LNs were used. For experiments involving in vivo deletion and naive CD8$^+$ T cell stimulation by DCs in vitro, purified OT-I or gBT-I T cells were used.

Viral infections

K5.mOVA mice, 6–12 wk old, were inoculated with 1×10^6 PFUs of HSV (KOS strain of HSV-1) on both sides via the flank scarification model as previously described (18). K5.mOVA or C57BL/6 mice, 6–12 wk old, were inoculated with 500 PFU of a recombinant influenza virus carrying the MHC class I-restricted OVA injected s.c. into each hind footpad.

Flow cytometric analysis of OT-I T cell expansion

K5.mOVA or C57BL/6 mice that received 1×10^5 OT-I $\times$ B6.Ly5.1 LN cells 4 wk earlier were inoculated with a recombinant influenza virus carrying the MHC class I-restricted OVA via foothock infection. Seven days after infection, mice were sacrificed and their spleens were removed. Single-cell suspensions were stained with anti-Ly5.1-FITC (A20), anti-Vα2-PE (B20.1), and anti-CD8α-allophycocyanin (53-6.7). Stained cell solutions were analyzed by flow cytometry with PI used to exclude dead cells.

DCs isolation from LNs

The s.c. draining LNs (brachial, inguinal, and cervical) were removed from mice. The LN were cut into small fragments and digested for 20 min at room temperature in a collagenase/DNase solution (1 mg/ml collagenase type II; Worthington Biochemicals) and 0.1% grade II bovine pancreatic DNase I (Boehringer Mannheim) in mouse tonicity RPMI 1640 containing 10% FCS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete RPMI medium). To disrupt T cell-DC complexes, 0.1 M EDTA was added to the suspension and digested for a further 5 min at room temperature. All subsequent procedures were in balanced salt solution/EDTA/5% FCS. DCs were purified from the single-cell suspensions as described previously (7). In brief, the single-cell suspensions were incubated for 30 min with predetermined optimal concentrations of the following purified mAbs: anti-CD3ε (KT3), anti-Thy-1 (T24/31.7), anti-CD19 (ID3), and anti-erythrocyte (TER-119). The Ab-coated cells were then removed by incubation with sheep anti-rat IgG-coupled magnetic beads (Dynabeads; Dynal Biotech). For purification of LN DCs into their different phenotypic populations, DCs were labeled with anti-CD11c-FITC (N418), anti-CD205-PE (NLDC-145), and anti-CD8α-allophycocyanin (53-6.7; BD Pharmingen) specific mAbs and sorted on a MoFlo flow cytometer (Cytomation).

DCs that expressed langerin were identified by incubating cells for 30 min in anti-CD16/32 (2.4G2) supernatant before staining with anti-mouse CD207 (langerin, 205C1; AbCys). Ab-bound cells were detected using anti-mouse IgM^a-PE (AF6-78; BD Pharmingen). PI was used to exclude dead cells from the sorts.

Coculture of DC subpopulations with CFSE-labeled OT-I or gBT-I T cells

Two-fold serial dilutions starting at 2.5×10^4 of each DC population were cocultured in vitro with 5×10^4 CFSE-labeled OT-I or gBT-I T cells in 200 μl of complete RPMI medium in V-bottom tissue-culture plates (Costar). Proliferation was measured as a loss of CFSE staining of CD8$^+$Ly5.1$^+$PI$^-$ cells as determined by flow cytometric analysis (FACSCalibur; BD Biosciences) after 60 h of culture.

Analysis of T cell deletion

A total of 5×10^6 OT-I T cells (Ly5.1$^+$, CD8$^+$, Vα2$^+$) were adoptively transferred into recipient mice and, after 6 wk, the mice were analyzed. Cells were pooled from the axillary, brachial, inguinal, cervical, and mesenteric LNs and spleen of each recipient mouse. Cells were stained with anti-Ly5.1-FITC (A20) and anti-CD8α-allophycocyanin (53-6.7), together with either anti-Vα2-PE (B20.1) or H-2K^b-OVA$_{257–264}$-PE tetramer. Live lymphocytes were determined by forward and side scatter profiles together with PI exclusion. Analysis was performed on a FACSCalibur flow cytometer. A total of 2×10^5 live cells were collected for analysis. The number of OT-I cells per animal was enumerated using a known number of small nonfluorescent Sphero beads (BD Biosciences) added to a known volume of the cell sample. Therefore cells per milliliter = number cells collected $\times$ (number beads in sample/number beads collected)/sample volume (19).

Generation of bone marrow chimeras

Chimeric mice were generated by irradiation of recipient mice with two doses of 550 cGy, 3 h apart, and reconstituted with 5×10^6 T cell-depleted donor bone marrow cells. Donor bone marrow cells were depleted of T cells by labeling cell suspensions with the following mAbs: anti-CD4 (RL172), anti-CD8 (3.168), and anti-Thy-1 (J1J). The Ab-coated cells were removed by incubation with rabbit complement for 30 min at 37°C. The following day, residual radio-resistant T cells were depleted with 100 μl of T24 (anti-Thy-1) ascites i.p. The mice were allowed to reconstitute for 8–10 wk before use.

DCs isolation from skin biopsies

Dermal DCs and/or epidermal DCs were isolated from either whole skin or epidermal sheets, respectively, by culture in the presence of 6Ckine (R&D Systems) as described previously (7). Mice were clipped along the flank and depilated with Veet (Reckitt Benckiser). Full-thickness skin was harvested from the flank region. The s.c. tissue was removed using a scalpel blade and the skin was cut into small pieces. The skin was floated (dermal side down) on 1 ml of complete RPMI medium containing 0.1 μg of recombinant mouse 6Ckine to promote DC migration. After 24 h incubation at 37°C, the emigrant cells were collected and placed at 4°C. The skin was transferred into fresh medium containing 6Ckine and incubated for a further 24 h at 37°C. Cells that migrated into the culture medium over the first and second incubations were pooled before staining for FACS analysis.

For DCs derived from the epidermal sheet crawlouts, the skin pieces were floated dermal side down in complete RPMI medium containing 2.5 mg/ml dispase II (Roche) for 90 min at 37°C. Epidermal sheets were peeled from the dermis and treated the same as whole skin crawlouts. The phenotype of DCs isolated from skin was analyzed by staining isolated cells with various combinations of the following Abs: anti-CD45.2-FITC (104; BD Pharmingen), anti-CD205-PE (NLDC-45) or anti-I-A^b-PE (AF6-120.1; BD Pharmingen), and anti-CD11c-biotin (HL3; BD Pharmingen) or anti-H-2K^b-biotin (5F1), followed by an allophycocyanin-streptavidin (BD Pharmingen). Dead cells were excluded with PI. Analysis was performed on a FACSCalibur flow cytometer.

Direct isolation of epidermal DCs from skin biopsies

For direct isolation of epidermal DCs, skin was excised as described above. The small skin pieces were floated dermal side down in complete RPMI medium containing 2.5 mg/ml dispase II for 90 min at 37°C. The epidermal sheets were peeled from the dermis and homogenized using a scalpel blade. Samples were then digested in 3 ml of collagenase (3 mg/ml collagenase type III; Worthington Biochemicals) in mouse tonicity RPMI 1640 containing 2% FCS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) per mouse for 30 min at 37°C. Samples were preincubated with anti-CD16/32 (2.4G2 supernatant) to block nonspecific Ab binding. All subsequent procedures were performed in balanced salt solution/EDTA/5% FCS. Samples were stained with anti-CD11c-biotin (HL3; BD Pharmingen) and anti-langerin (205C1; AbCys) followed by allophycocyanin-conjugated streptavidin (BD Pharmingen) and anti-mouse IgM^a-PE (AF6-78; BD Pharmingen). Other mAbs used were anti-I-A^b-FITC (AF6-120.1; BD Pharmingen), anti-CD86-FITC (GL1), and anti-CD40-FITC (FGK45.5). PI was included to exclude dead cells. Analysis was performed on a FACSCalibur flow cytometer.

Results

Dermal DCs and Langerhans cells present skin-derived self-Ag

Azukizawa et al. (17) generated a transgenic mouse expressing a membrane-bound form of OVA under the control of the keratin K5 promoter. In this mouse, Ag expression was confined to selected tissues including the skin epidermis and this resulted in preferential proliferation of CFSE-labeled OVA-specific OT-I T cells in skin draining LNs (Fig. 1*A*). We wanted to examine the DC subset presentation of Ag in these LNs. Given that transgene expression is confined to epidermis of the skin, we first assessed whether DCs originating from this location, the Langerhans cells, presented class I-restricted OVA peptide. To this end, CD11c$^+$ cells from skin-draining LNs were separated on the basis of expression of a Langerhans cell-specific marker, CD207 (langerin (20)) and CD8 (Fig. 1*B*). The latter was included because CD8$^+$ DCs can present

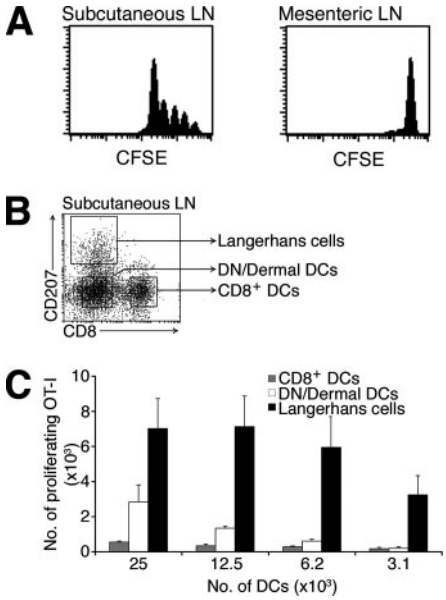

FIGURE 1. OT-I T cell proliferation is confined to the s.c. LN in K5.mOVA mice and Langerhans cells present skin-derived self-Ag. *A*, A total of 10^6 CFSE-labeled OT-I × Ly5.1 cells were adoptively transferred into K5mOVA recipients. After 42 h, the skin-draining inguinal and brachial LNs as well as the non-skin-draining mesenteric LNs were analyzed by flow cytometry for proliferating $Ly5.1^+CD8^+CFSE^+PI^-$ cells. *B*, DCs were isolated from the s.c. LNs of K5mOVA mice. $CD11c^+$ DCs were flow cytometrically sorted into $CD8^-CD207^{high}$ Langerhans cells, $CD8^-CD207^-$ double-negative (DN)/dermal DCs and $CD8^+CD207^-$ DCs. *C*, Two-fold dilutions of purified DC subsets were cocultured with 5×10^4 CFSE-labeled OT-I T cells in vitro at 37°C. After 60 h of culture, $CD8^+$ OT-I T cells were analyzed by flow cytometry for proliferation. Data are pooled from three independent experiments showing the mean ± SEM. DCs isolated from nontransgenic C57BL/6 (B6) mice did not induce proliferation of $CD8^+$ OT-I T cells (data not shown).

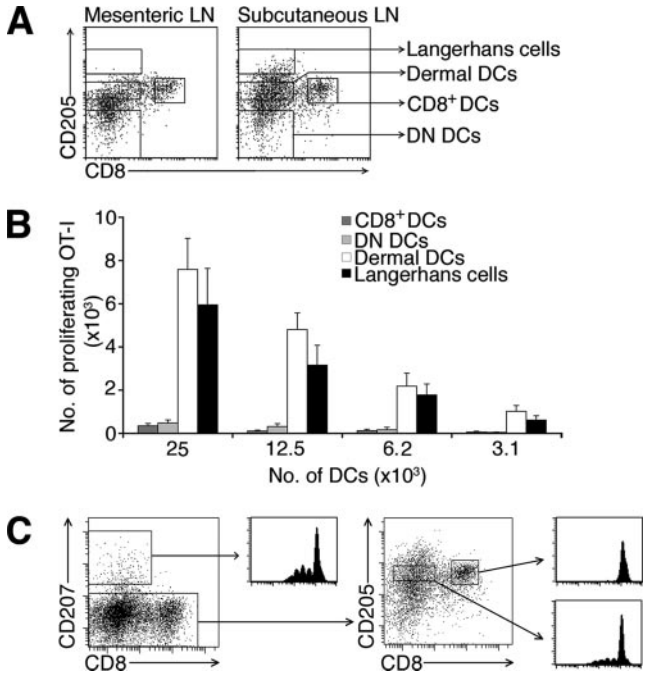

FIGURE 2. Skin-derived self-Ag is presented by DCs of skin origin. *A*, DCs were isolated from the s.c. or mesenteric LNs of K5mOVA mice. $CD11c^+$ DCs were flow cytometrically sorted into $CD8^-CD205^{high}$ Langerhans cells, $CD8^-CD205^{int}$ dermal DCs, $CD8^-CD205^-$ double-negative DCs, and $CD8^+CD205^{int}$ DCs. *B*, Two-fold dilutions of purified DC subsets were cocultured with 5×10^4 CFSE-labeled OT-I T cells in vitro at 37°C. After 60 h of culture, $CD8^+$ OT-I T cells were analyzed by flow cytometry for proliferation. Data are pooled from four independent experiments showing the mean ± SEM. *C*, $CD11c^+$ DCs were flow cytometrically sorted into $CD8^-CD207^+$ Langerhans cells and $CD207^-$ DCs were further segregated into $CD8^-CD205^{int}$ dermal DCs and $CD8^+CD205^{int}$ DCs. Two-fold serial dilutions of purified DC subsets (6.25×10^4 DC shown) were cocultured with 5×10^4 CFSE-labeled OT-I T cells in vitro at 37°C. After 60 h of culture, $CD8^+$ OT-I T cells were analyzed by flow cytometry for proliferation.

Ag after cutaneous viral infection (6, 7, 9). The purified DC populations were used to stimulate CFSE-labeled OVA-specific T cells from the OT-I transgenic mouse (21) in an in vitro proliferation assay. Fig. 1*C* shows that while purified Langerhans cell progeny can effectively present the class I-restricted Ag, there is little activity within the $CD8^+$ DC subset. Interestingly, some presentation is also seen in the $CD207^-CD8^-$ population, which consists of a number of distinct DC subsets including the dermal and myeloid, nonmigratory resident DCs.

We thought that the presenting population within the $CD207^-CD8^-$ subset seen in Fig. 1 would predominantly consist of dermal DCs. To show this, the $CD11c^+$ DCs were separated on the basis of CD205 (DEC205) and CD8 expression. In this situation, the different skin-derived CD8-negative DCs can be differentiated on the basis of CD205 expression levels (2, 3). Langerhans cell progeny express the highest levels of this marker, clearly seen when comparing the profile of DCs originating in the skin (which contain the $CD205^{high}$ Langerhans cells) with those from non-skin-draining LNs such as the mesenteric LNs (where Langerhans cells are absent) (Fig. 2*A*). In contrast, interstitial DCs, such as the dermal DCs, give rise to a population of $CD205^{int}$ cells found in skin-draining and non-skin-draining LNs. Fig. 2*B* shows that the $CD8^+$ DCs presented little class I-restricted Ag, consistent with the results from Fig. 1. In contrast, dermal DCs and Langerhans cells drove good OT-I T cell proliferation. To further exclude that the dermal DC presentation in the preceding assays was not in part due to contaminating Langerhans cells, we showed that the dermal DCs purified as $CD207^-CD205^{int}CD8^-$ cells could drive OT-I proliferation (Fig. 2*C*). Thus, both skin-derived DC populations, the dermal DCs and Langerhans cells, appeared to carry OVA from the site of its expression for presentation in a class I-restricted manner within the skin-draining LNs.

Radio-resistant skin DCs can drive T cell proliferation in transgenic mice with skin-specific Ag expression

Langerhans cell precursors are known to be radiation resistant (7, 22), although at the start of this study it had been unclear to what extent the dermal DCs are replaced in these bone marrow chimeras. We resolved this by comparing migrating DCs in explant cultures of epidermal sheets (which should contain exclusively Langerhans cells) and whole skin (which also have the dermal-derived DC component) from bone marrow chimeric mice differing in expression of CD45 allotypes (Fig. 3). Again, Langerhans cells can be distinguished from dermal DCs by their higher expression of CD205 (2, 3). Fig. 3 shows that the $CD205^{high}$ Langerhans cells migrating from the epidermal sheets were almost exclusively of host (CD45.2) origin (Fig. 3*A*), confirming their resistance to irradiation. In contrast, a large proportion of whole skin migrants were of donor (CD45.1) origin (Fig. 3*B*). The $CD205^{int}$ dermal DCs in these emigrating populations had undergone ~90% replacement, while most of the $CD205^{high}$ cells were of host origin reflecting their Langerhans cell classification. These are slightly lower levels of dermal DC replacement than recently reported by

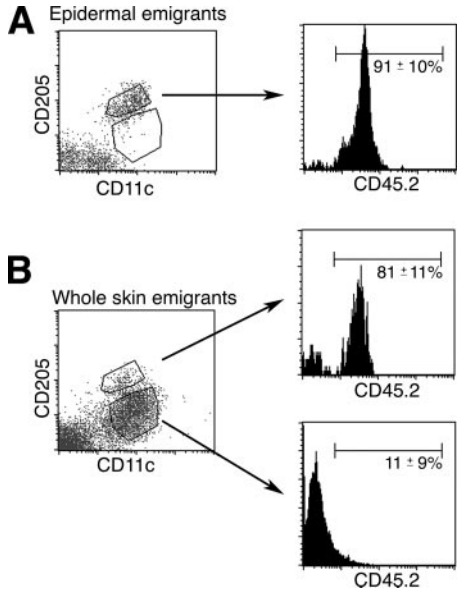

FIGURE 3. Discrimination between Langerhans cells and dermal DCs using bone marrow-irradiation chimeras. *A*, Epidermal sheets predominantly containing Langerhans cells or whole skin containing Langerhans cells and dermal DCs (*B*) isolated from B6.Ly5.1 (CD45.1)→B6 (CD45.2) bone marrow chimeric mice were cultured for 48 h in complete medium containing 6Ckine. Cells migrating into the medium were collected in separate 24 h pools and combined for staining and analysis. Cells were stained for their expression of CD11c, CD205, and CD45.2 and the percent ± SD of Langerhans cells ($CD11c^+CD205^{high}$) or dermal DCs ($CD11c^+CD205^{int.}$) that expressed CD45.2 was determined.

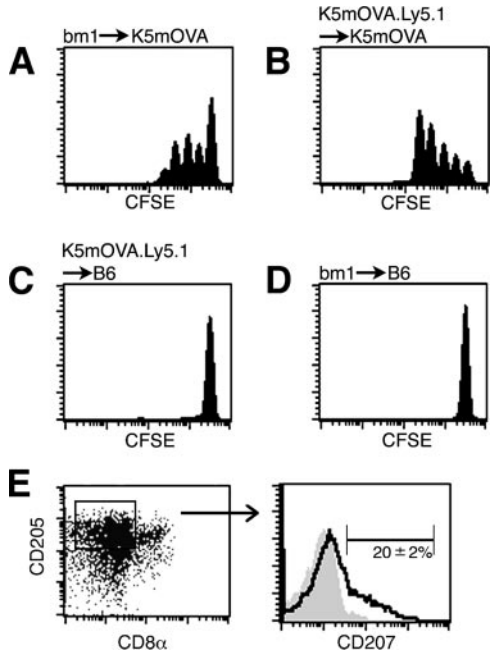

FIGURE 4. Skin-derived self-Ag is presented by radiation-sensitive and -resistant APCs. A total of 10^6 CFSE-labeled OT-I T cells were adoptively transferred into K5mOVA mice grafted with either bm1 (*A*) or K5mOVA.Ly5.1 (*B*) bone marrow and C57BL/6 (B6) mice grafted with K5mOVA.Ly5.1 (*C*) or bm1 (*D*) bone marrow. After 42 h, the pooled inguinal and brachial LNs were analyzed by flow cytometry for proliferating $CD8^+CFSE^+$ cells. *E*, Flow cytometric analysis of the percent of $CD207^+$ Langerhans cells that make up the pool of $CD205^+CD8^-CD11c^+$ skin-derived DCs in the cutaneous LNs of naive C57BL/6 (B6) mice.

Bogunovic et al. (23), who found around 25% of dermal DCs survive irradiation. All other non-skin-derived DCs in the draining LNs are of host origin (data not shown). Thus, irradiation chimeras contain a population of largely donor-derived skin and non-skin origin DCs compared with host-derived Langerhans cells.

DC contribution to in vivo T cell stimulation can be assessed within the context of the OVA Ag system by taking advantage of bone marrow chimeras using donor cells from the bm1 mouse, which carries a class I molecule incapable of presenting the determinant recognized by the OT-I T cells (24). Given that Fig. 2 shows presentation is by dermal and epidermal derived DCs and that a considerable proportion of skin DCs survive irradiation (Fig. 3), one would predict that class I-restricted presentation would persist in bm1→K5mOVA chimeric animals. Put simply, these chimeras have most of their Langerhans cells and even a small proportion of dermal DCs still capable of presenting the skin-acquired Ag. However, these particular chimeras were originally described as lacking in vivo stimulatory capacity (17). It should be noted that the original data showed some stimulatory capacity remained in these animals and it was not appreciated that this residual activity could result from the surviving radio-resistant Langerhans cells. The level of residual presentation is shown here in Fig. 4. The bm1→K5mOVA animals are seen to support much weaker proliferation of the transferred CFSE-labeled OT-I cells (Fig. 4*A*) than the K5mOVA→K5mOVA chimeric animals (Fig. 4*B*). Although stimulation of transferred OT-I cells is reduced in the bm1→K5mOVA chimeras, it is clearly stronger than seen in negative controls such as the non-Ag-expressing K5mOVA→B6 or bm1→B6 animals (Fig. 4, *C* and *D*). In retrospect, attenuated but not absent stimulation of OT-I T cells is to be expected because the K5mOVA→K5mOVA animals have both dermal DCs and Langerhans cells capable of presenting the K^b-restricted OVA peptide, whereas presentation is predominantly confined to the Langerhans cell subset in bm1→K5mOVA chimeras. Overall, $CD207^+$ Langerhans cells make up <20% of all skin-derived DCs in the LNs (where the latter is measured as the $CD205^+CD8^-CD11c^+$ population) (Fig. 4*E*), explaining the weaker stimulation seen in these latter chimeric animals where Langerhans cells and relatively few radio-resistant dermal DCs are the only cells capable of Ag presentation. Finally, robust stimulation of OT-I T cells in K5mOVA. Langerin-DTR mice that have the diptheria toxin receptor driven by the langerin promoter, even after ablation of Langerhans cells by diptheria toxin treatment (data not shown), exclude Langerhans cells as sole contributors to skin-derived Ag presentation.

Radio-resistant APCs can drive OVA-specific T cell deletion in the K5-mOVA transgenic mice

Skin-derived DCs, such as the Langerhans cells that have migrated to the LN, are considered the prototypic mature DC subset (25),

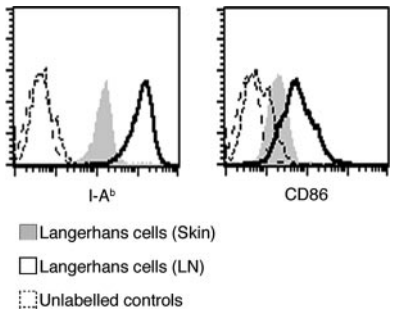

FIGURE 5. Flow cytometric analysis of $CD11c^+CD207^+$ Langerhans cell surface expression of MHC II ($I\text{-}A^b$) and CD86, freshly isolated from either epidermal sheets (skin) or the s.c. LN.

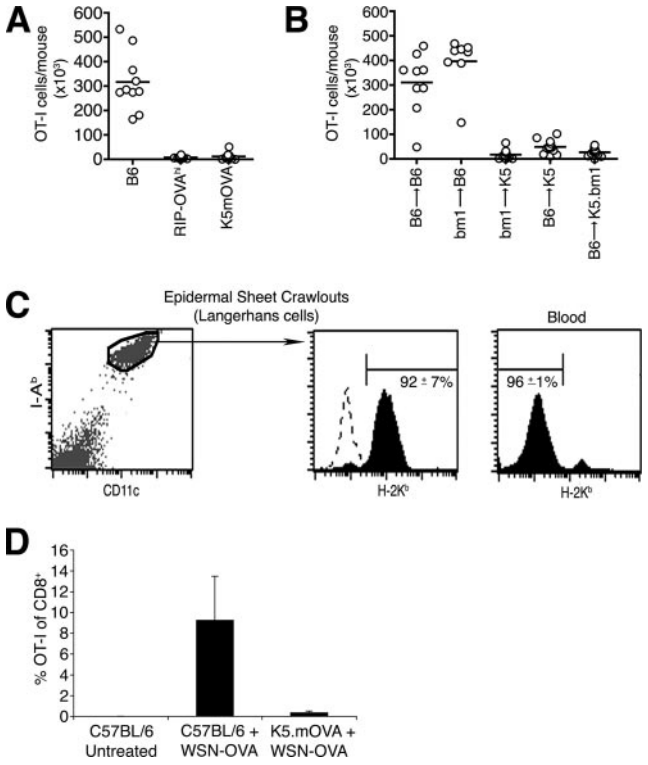

FIGURE 6. Presentation of skin-derived self-Ag by DC of skin origin induces deletion of OVA-specific $CD8^+$ T cells. *A*, A total of 5×10^6 OT-I T cells were adoptively transferred into C57BL/6 (B6) mice (10 animals), RIP-OVA^{high} transgenic mice (10 animals), K5mOVA transgenic mice (10 animals), or the following bone marrow chimeric mice: *B*, B6→B6 (9 animals), bm1→B6 (8 animals), bm1→K5mOVA (13 animals), B6→K5mOVA (13 animals), and B6→K5mOVA.bm1 (11 animals). After 6 wk, the number of OT-I T cells in the LNs and spleen was determined by flow cytometry. Individuals (○) and means (—) for each group are shown. *C*, Epidermal sheets isolated from bm1→K5mOVA chimeric mice were cultured for 48 h in complete medium containing 6Ckine. Cells migrating into the medium were collected in separate 24-h pools and combined for staining and analysis. Gates were generated on $CD11c^+$MHC class II^+ ($I\text{-}A^b$) Langerhans cells, and these cells were analyzed for expression of $H\text{-}2K^b$ (black). The percent ± SD of Langerhans cells that are $H\text{-}2K^{b+}$ is shown in the *left histogram*. To show chimerism, blood lymphocytes from these mice were also analyzed for their expression of $H\text{-}2K^b$ (black) and the percent ± SD of lymphocytes that were $H\text{-}2K^{b-}$ is shown in the *right histogram.* The dotted line indicates unlabeled controls. *D*, A total of 1×10^5 OT-I T cells was adoptively transferred into either C57BL/6 or K5.mOVA recipient mice. Four weeks later, recipients were immunized with 500 PFU of recombinant influenza-OVA virus (WSN-OVA) to permit the clonal expansion and detection of the OT-I T cells. Seven days after immunization, splenocytes were stained with Abs specific for CD8, Vα2, and Ly5.1 and analyzed by flow cytometry.

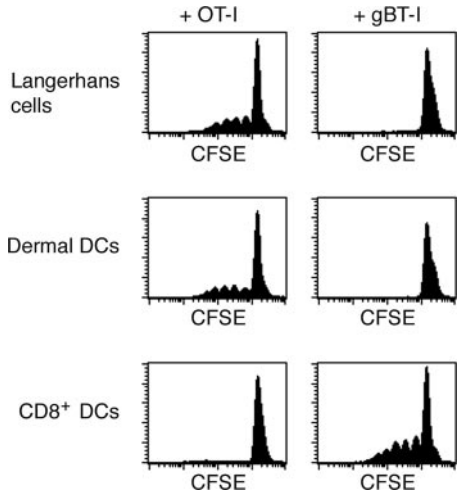

FIGURE 7. Cutaneous HSV infection does not alter the pattern of DC subset presentation of skin-derived self-Ag. DC subsets were isolated from the brachial LNs of K5mOVA mice 2 days post-flank inoculation with HSV. The $CD11c^+$ DCs were flow cytometrically sorted into $CD8^-CD205^{high}$ Langerhans cells, $CD8^-CD205^{int}$ dermal DCs, and $CD8^+CD205^{int}$ DCs. Two-fold serial dilutions of purified DC subsets (6.25×10^4 DC shown) were cocultured with either 5×10^4 CFSE-labeled OT-I T cells or gBT-I T cells in vitro at 37°C. After 60 h of culture, both $CD8^+$ OT-I T cells and gBT-I T cells were analyzed by flow cytometry for proliferation. This experiment was performed twice with similar results.

especially in contrast to their direct immature predecessors in peripheral tissues (26). This difference in maturation status was confirmed by showing that skin-derived $CD11c^+CD207^+$ cells (Langerhans cells) in the LN expressed high levels of maturation markers MHC class II and CD86 (Fig. 5), whereas their tissue-derived counterparts, the $CD11c^+CD207^+$ cells in the epidermis, exhibited low-level expression of these maturation markers. It should be noted that while low, this staining was nonetheless clearly above background. Finally, $CD205^{int}CD8^-$ dermal DCs in skin-draining LNs also show a mature status when assessed using these costimulatory markers (data not shown).

Steady state presentation of peripheral self-Ag can lead to deletional tolerance (27). This can also be seen in the K5.mOVA transgenic mice where transferred OT-I T cells are largely deleted over a 6-wk period (Fig. 6*A*). This is similar to the elimination seen in RIP.OVA^{high} mice previously shown to support deletional tolerance (28), and contrasts to the extended survival of these T cells in non-Ag-expressing C57BL/6 (B6) controls. T cell elimination is also seen in the B6→K5.mOVA.bm1 mice (Fig. 6*B*), consistent with the replacement of most of the host K^{bm1}-bearing dermal DCs with those of B6 origin, capable of presenting the tolerogenic peptide. Most importantly, deletion is evident in the bm1→K5.mOVA chimeric animals (Fig. 6*B*) where the epidermal Langerhans cells are clearly of host origin (Fig. 6*C*) and therefore capable of presenting the class I-restricted OVA peptide. Finally, transferred OT-I T cells are functionally tolerant in the K5.mOVA mice because their expansion is severely impaired on subsequent challenge with a recombinant influenza virus carrying the OVA peptide determinant (Fig. 6*D*). Combined with the data above on migratory DC maturation once they reach the LNs, these results suggest that the mature descendants of DCs that originate in the skin can drive deletional tolerance of self-reactive T cells.

Skin infection does not alter the pattern of DC subset presentation of skin-derived self-Ag

Skin infection with cytopathic viruses such as HSV and vaccinia virus is associated with $CD8^+$ DC presentation of class I-restricted Ag (6–9). We were interested whether such infection would modify DC subset presentation of skin-derived self-Ag. To this end, we infected the K5.mOVA mice with HSV and examined DC subset presentation of both the self-Ag to the OT-I T cells and the foreign HSV gB peptide to the corresponding virus-specific T cells from the gBT-I transgenic mouse (29). Fig. 7 shows no alteration in the dominance of skin-derived DC presentation in the case of the OVA peptide. Conversely, all presentation of the HSV Ag was confined to the $CD8^+$ DC subset, as found in previous studies. Thus, infection does not alter the pattern of self-Ag presentation, which is intrinsically distinct from the presentation of the virus-derived peptide.

Discussion

Questions regarding the role of Langerhans cells in immunosurveillance have followed the observation that these cells do not appear to operate in some skin infections (7, 10, 30, 31), as well as being dispensable in contact sensitization (32, 33). The results here and elsewhere argue that these skin-derived DCs can acquire Ag derived from peripheral tissues for ultimate cross-presentation to class I-restricted T cells. The location of this acquisition remains undefined, although the immature status of the cells in the skin in combination with studies showing that Langerhans cells can take up Ag for cross-presentation (34) suggest that this steady state presentation reflects Ag transfer between keratinocytes and skin-resident DCs. Dermal DCs may also directly contact the keratinocytes or, more likely, may acquire Ag from the migrating Langerhans cells as they traverse the dermis on their way to the draining LNs (25).

Interestingly, class I-restricted presentation of skin-expressed Ag clearly does not occur in the case of skin infection with viruses such as HSV, where an exclusion of skin-derived DC presentation is observed. This could indicate that this type of infectious agent inactivates the cross-presentation pathway, for example, by maturing these cells (35), or that it inactivates the DCs themselves, for example by directly killing infected cells (36, 37). Both these scenarios are still compatible with steady state presentation of self-Ag in the face of infection, which presumably reflects Ag acquisition and migration before infection.

We show that skin-derived DC presentation of self-Ag leads to deletional tolerance. Propositions for a skin-derived DC role in T cell down-modulation are not without precedent. Ablation of Langerhans cells has been shown to lead to exacerbation of contact sensitization in some (12) but not other (32) (33) transgenic mouse systems, and individuals with psoriasis have been found to have defects in Langerhans cell migration (11), suggesting that these cells may under some circumstances play a regulatory role dampening immune responses. However, skin-derived DCs have also been linked to certain types of antiviral immunity, especially those associated with noncytopathic infections (8). Thus, a purely tolerogenic role for this population may prove unlikely. Regardless, skin DC-mediated tolerance in the steady state is surprising given that these cells appear mature within the draining LNs as defined by markers such as MHC class II and accessory molecule expression. This is most striking in the case of Langerhans cells. These are the prototypic migrating DCs and it has been assumed that the concomitant phenotypic maturation acts as a key driver of T cell immunity. Although it is tempting to speculate that the phenotypic changes such as the up-regulation of costimulatory molecules are intimately tied to continuous exposure to environmental agents within the skin, it may simply form part of the constitutive migrational pathway and, as such, be independent of direct DC activation (38). As a consequence, migration may be associated with more indirect maturation that would, in turn, not convert DCs to their immunogenic form. It has been shown that while DC activation resulting from engagement of TLRs directly translates to effective immunity, indirect cytokine-mediated maturation is deficient in this respect (39). Thus, Langerhans cell migration in the absence of some form of additional immunogenic stimulation appears to result in an apparently mature DC population, which is nonetheless capable of driving deletional T cell tolerance.

Given the tolerance observed here, our results appear to contradict earlier work by Mayerova et al. (14) and Shibaki et al. (40) who showed that presentation of self-Ag by skin-derived DCs led to autoimmune tissue destruction. We were unable to explain these differences, although it should be noted that while here we show the elimination of the transferred OT-I T cells, these T cells also exhibited autoaggressive behavior at the microscopic level (17). Indeed, deletion and autoimmunity are not necessarily mutually exclusive. It is known that presentation of self-Ag by tolerogenic APCs can lead to varying levels of T cell activation before their functional elimination, ranging from fairly muted responses (41) to full-blown induction of effector function (42). What controls this apparent continuum that ranges from the largely proliferative T cell response, as seen here, to an overtly autoaggressive response, as seen in Kurts et al. (27), remains undefined.

In summary, we have focused on the difference in the presentation mediators that operate during virus infection of the skin and the establishment of tolerance to skin-derived Ag. In our studies, tolerance is mediated by the migratory DCs, including the Langerhans cells derived from the epidermal layer harboring Ag expression. Moreover, this tolerance is achieved despite these cells appearing phenotypically mature once they reach the skin-draining LNs.

Acknowledgments

We thank Ken Field of the University of Melbourne Flow Cytometric Facility for technical assistance and Dr. Claerwen Jones for critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

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