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

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Skin-draining lymph nodes contain a number of dendritic cell (DC) 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 autoreactive CD8+ T cell response as a direct consequence of the maturation 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-PtprcPep3/BoyJ (B6.Ly5.1), B6.C-H-2bm1 (bm1) (15), RIP.OVA (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 (RB6-8C5), anti-I-A/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-CDS 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 10⁶ CFSE-labeled OT-I T cells (Ly5.1+, CD8+,

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and Voα2β) 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 CD8α 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 x 10^6 PFU 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. After 42 h, pooled brachial and inguinal LNs from individual mice were analyzed by flow cytometry with PI used to exclude dead cells.

**Flow cytometric analysis of OT-I T cell expansion**
K5.mOVA or C57BL/6 mice that received 1 x 10^6 OT-I x B6.Ly5.1 LN cells 4 wk earlier were inoculated with a recombinant influenza virus carrying the MHC class I-restricted OVA via footback infection. Seven days after infection, mice were sacrificed and their spleens were removed. Single-cell suspensions were stained with anti-Ly5.1-IF-TTC (A20), anti-Voα2-PE (B20.1), and anti-CD8α-allophycocyanin (53-67). 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 2-2ME, 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 (HL3; BD Pharmingen), and anti-CD8α-allophycocyanin (53-67; 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; 20S1C1; AbCys). Ab-bound cells were detected using anti-mouse IgM-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 x 10^4 each of DC population were cocultured in vitro with 5 x 10^5 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 x 10^5 OT-I T cells (Ly5.1, CD8α, Voα2β) 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-IF-TTC (A20) and anti-CD8α-allophycocyanin (53-67), together with either anti-Voα2-PE (B20.1) or H-2Kβ- OVA552-564-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 x 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 x (number beads in sample/number beads collected/sample volume (19).
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. 1C 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 CD205high Langerhans cells) with those from non-skin-draining LN s such as the mesenteric LNs (where Langerhans cells are absent) (Fig. 2A). In contrast, interstitial DCs, such as the dermal DCs, give rise to a population of CD205low cells found in skin-draining and non-skin-draining LN s. Fig. 2B 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⁻ CD205low CD8⁻ cells could drive OT-I proliferation (Fig. 2C). 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 LN s.

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 CD205high Langerhans cells migrating from the epidermal sheets were almost exclusively of host (CD45.2) origin (Fig. 3A), confirming their resistance to irradiation. In contrast, a large proportion of whole skin migrants were of donor (CD45.1) origin (Fig. 3B). The CD205low dermal DCs in these emigrating populations had undergone ~90% replacement, while most of the CD205high cells were of host origin reflecting their Langerhans cell classification. These are slightly lower levels of dermal DC replacement than recently reported by...
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 T cells (Fig. 4A) than the K5mOVA→K5mOVA chimeric animals (Fig. 4B). 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 K8-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. 4E), 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 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.
Presentation of skin-derived self-Ag by DC of skin origin induces deletion of CD8\(^+\) T cells. A, A total of 5 \times 10^5 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-A\(^b\)) Langerhans cells, and these cells were analyzed for expression of H-2K\(^b\) (black). The percent \pm SD of Langerhans cells that are H-2K\(^b\)\(^+\) is shown in the left histogram. To show chimerism, blood lymphocytes from these mice were also analyzed for their expression of H-2K\(^b\) (black) and the percent \pm SD of lymphocytes that were H-2K\(^b\)\(^+\) is shown in the right histogram. The dotted line indicates unlabeled controls. D. A total of 1 \times 10^3 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-11001). T cell elimination is also seen in the B6→K5.mOVA.bm1 mice (Fig. 6B), consistent with the replacement of most of the host K\(^b\)\(^{bm1}\)-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. 6B) where the epidermal Langerhans cells are clearly of host origin (Fig. 6C) 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. 6D). 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 maturating 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 migratory 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 Kürts 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.

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Disclosures

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