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Migratory Dendritic Cells Transfer Antigen to a Lymph Node-Resident Dendritic Cell Population for Efficient CTL Priming

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Summary

Skin dendritic cells (DCs) are thought to act as key initiators of local T cell immunity. Here we show that after skin infection with herpes simplex virus (HSV), cytotoxic T lymphocyte (CTL) activation required MHC class I-restricted presentation by nonmigratory $CD8^+$ DCs rather than skin-derived DCs. Despite a lack of direct presentation by migratory DCs, blocking their egress from infected skin substantially inhibited class I-restricted presentation and HSV-specific CTL responses. These results support the argument for initial transport of antigen by migrating DCs, followed by its transfer to the lymphoid-resident DCs for presentation and CTL priming. Given that relatively robust CTL responses were seen with small numbers of skin-emigrant DCs, we propose that this inter-DC antigen transfer functions to amplify presentation across a larger network of lymphoid-resident DCs for efficient T cell activation.

Introduction

Priming of virus-specific cytotoxic T lymphocyte (CTL) responses after infection of the skin is thought to involve the migration of localized dendritic cells (DCs) followed by subsequent presentation of antigen within the draining lymph nodes (Banchereau and Steinman, 1998). The prototypic skin DCs, the Langerhans cells, are found in the outermost epidermal layer and have long been thought to be directly involved in both antigen uptake within infected skin as well as presentation for direct T cell priming (Reis e Sousa et al., 1993; Romani et al., 1989; Schuler and Steinman, 1985). Given this, it was therefore surprising to find that CTL priming subsequent to skin infection with herpes simplex virus (HSV) does not appear to involve direct presentation of class I-restricted antigen by Langerhans cells found within the draining lymph nodes (Allan et al., 2003).

HSV infects both skin and mucosal epithelia. These tissues are rich in Langerhans cells, leading to the reasonable assumption that they, or their progeny, would directly present antigen for T cell priming. However, careful dissection of presentation after these infections excluded this type of presentation (Allan et al., 2003; Zhao et al., 2003). In the case of CTL priming after skin infection, presentation appeared to be dominated by DCs expressing the CD8α homodimer (Allan et al., 2003). These CD8 DCs represent a subset of DCs that have no obvious precursor-product link with peripheral tissue cells. They are sometimes termed "blood-derived" DCs (Itano and Jenkins, 2003; Villadangos and Heath, 2005) to distinguish them from cells that originate from immature tissue-derived DCs such as the epidermal Langerhans cells and the dermal DCs. Given the involvement of CD8 DCs in CTL priming after skin infection, it was proposed that either this subset was also present in the skin and migrated with antigen, or an alternative migratory DC population such as Langerhans cells was involved in antigen transport from the site of virus infection to the draining lymph nodes. In the latter case, however, the trafficking DCs would not be directly involved in priming. Here, we show that appearance of skin-derived DCs in the draining lymph nodes, predominantly of dermal rather than epidermal origin, was intimately tied to the ability to prime a HSV-specific CTL response after infection of the skin and that inhibiting DC migration impaired this facet of antiviral immunity. These results are consistent with a role for migratory DCs in CTL priming despite their exclusion from direct class I-restricted presentation.

Results

Only Lymph Node-Resident DCs Present Class I-Restricted Antigen after Skin Infection with HSV

Skin painting with the dye fluorescein isothiocyanate (FITC) is a simple and reliable means of identifying cutaneous-derived DCs within draining lymph nodes (Macatonia et al., 1987). To this end, we applied FITC to flank skin of C57BL/6 mice prior to infection with HSV. $CD11c^+$ DCs were isolated from the brachial lymph nodes 24 hr after infection and separated on the basis of surface expression of CD8 and FITC fluorescence into $CD8^+FITC^-$, $CD8^-FITC^-$, and $CD8^-FITC^+$ (Figure 1). To examine presentation of viral antigen, these three populations were then mixed with CFSE-labeled lymph node cells from gBT-I mice, which express a TCR transgene specific for the class I-restricted immunodominant determinant from the HSV glycoprotein B (gB) (Mueller et al., 2002a). Antigen presentation was then measured by T cell proliferation, visualized as a progressive dilution of the CFSE fluorescent marker (Lyons and Parish, 1994). Only the $CD8^+$ DCs efficiently stimulated the HSV-specific T cells in the in vitro culture system (Figure 1). These cells did not originate in the skin, as evident by their negative FITC staining. In contrast, those DCs that were clearly FITC positive lacked CD8 expression and did not present antigen at detectable levels. These results reinforce our earlier observation that class I-restricted presentation after skin infection with HSV predominantly resides with the

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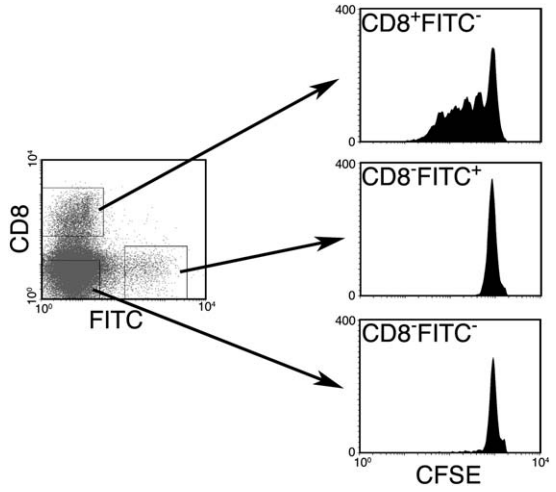

Figure 1. Lymph Node-Resident CD8+ DCs and Not Tissue-Derived DCs Present Class I-Restricted Antigen after Skin Infection with HSV

C57BL/6 mice were painted on the flank skin with a 1% FITC solution prepared in acetone 15 hr prior to inoculation with HSV by flank scarification. 24 hr after infection, the draining brachial lymph nodes were removed from 15 mice, pooled, and depleted of non-DCs. CD11c+ DCs were sorted into CD8+FITC− DCs, CD8−FITC+ DCs, and CD8−FITC− DCs. All sorted DC subsets were then cocultured with CFSE-labeled gBT-I cells in vitro. After 60 hr of culture, gBT-I T cells were analyzed for proliferation by flow cytometry. Data are one representative of three independent experiments.

CD8 DC population (Allan et al., 2003) and further show that these DCs are lymph node resident.

Arrival of Skin-Derived DCs Correlates with the Capacity to Prime a HSV-Specific CTL Response

Previous work in our laboratory has shown that antigen reaches the draining lymph nodes within hours of flank skin inoculation with HSV (Mueller et al., 2002b; Stock et al., 2004). This can be seen in experiments where the inoculation site is removed at various times after infection. Delaying such surgery for 8 hr after infection still gives a robust CD8+ T cell response, albeit at levels less than the maximum seen in control mice not subjected to surgical intervention (Figure 2A). In contrast, T cell priming was absent if surgery was performed 2 hr after infection, showing that between 2 and 8 hr was required for antigen to reach the draining lymph nodes for effective CD8+ T cell priming. In an effort to determine whether this timing corresponded to the arrival of skin-emigrant DCs in the draining lymph nodes, the appearance of FITC+ CD11c+ cells was determined at 2 and 8 hr after infection. Near background percentages of skin-derived DCs were present at 2 hr post infection as detected by FITC labeling, whereas detectable migration was seen at 8 hr (Figure 2B). This result is consistent with previous reports showing the first arrival of skin-derived DCs between 3 and 8 hr after epicutaneous stimulation (Garg et al., 2003; Macatonia et al., 1987; Salomon et al., 1998). We calculate that just over 200 skin-derived DCs have migrated into the draining brachial lymph node within the first 8 hr after infection (Figure 2C). Thus, T cell priming appears to correlate with the arrival of skin-derived DCs into the draining lymph nodes, even though these cells are not themselves responsible for class I-restricted presentation of HSV antigen.

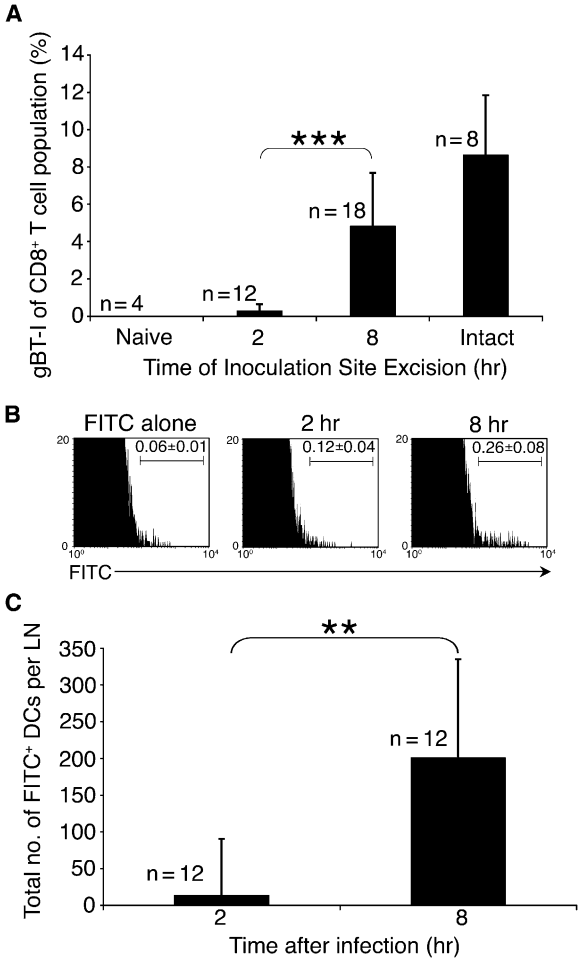

Figure 2. Arrival of Skin-Derived DCs Correlates with the Capacity to Prime a HSV-Specific CTL Response

(A) Between 2 and 8 hr is required for antigen to reach the draining lymph nodes for effective CD8+ T cell priming. 1 × 10^5 gBT-I × B6.Ly5.1 lymph node cells were adoptively transferred into C57BL/6 mice 1 day prior to inoculation with HSV by flank scarification. At 2 and 8 hr after infection, the inoculation site was surgically excised or left intact (Intact). 7 days after infection, the proportion of splenic CD8+ T cells of gBT-I origin was assessed by flow cytometry on the basis of CD8 and CD45.1 expression. Shown is the percentage of CD8+ T cells that are of gBT-I origin from three independent experiments presented as mean ± SEM. n, total number of mice per experimental group. Significance was assessed with the Student's t test, and values are shown: ***p < 0.005.

(B) Skin-derived DCs arrive in the draining lymph nodes between 2 and 8 hr after HSV skin infection. C57BL/6 mice were painted on the flank skin with a 1% FITC solution prepared in acetone 15 hr prior to inoculation with HSV by flank scarification. 2 and 8 hr after infection, single-cell suspensions from two pooled draining brachial lymph nodes were depleted of non-DCs, and the percentage of CD11c+FITC+ DCs was determined by flow cytometry.

(C) The number of skin-derived DCs arriving in the draining lymph node after HSV infection. Shown is the total number of CD11c+FITC+ DCs per lymph node from three independent experiments presented as mean ± SEM. n, total number of mice per experimental group. To obtain the total number of CD11c+FITC+ cells per lymph node, the background CD11c+FITC+ cells obtained from mice painted with FITC alone was subtracted from the total number of CD11c+FITC+ cells. Significance was assessed with the Student's t test, and values are shown: **p < 0.025.

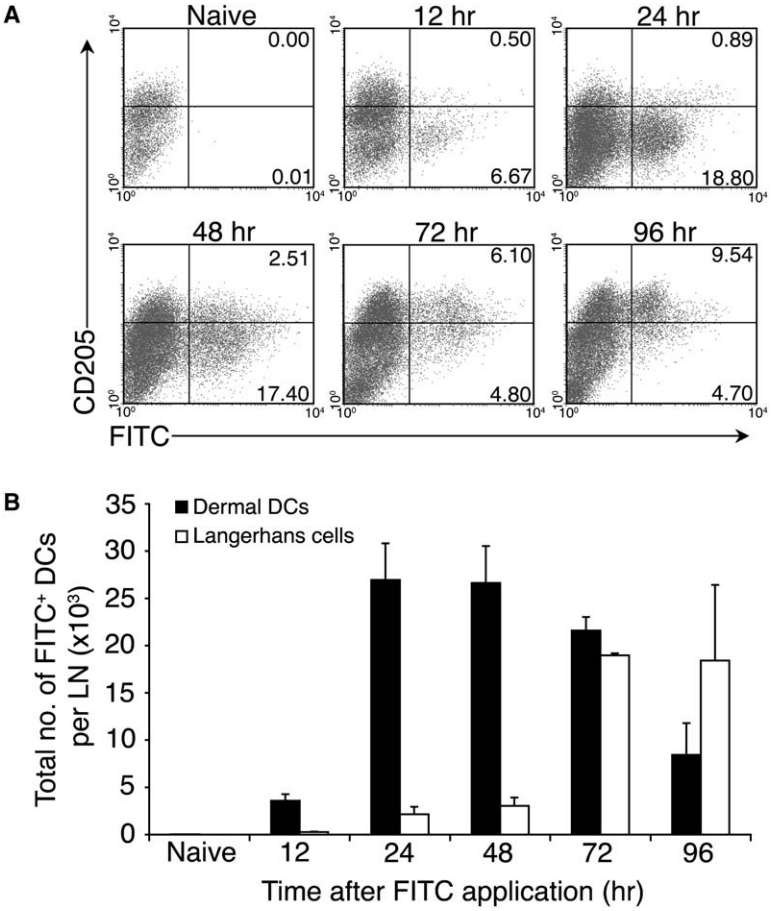

Figure 3. Skin Inflammation Results in Two Distinct Waves of Skin DC Migration

(A) Kinetics of skin DC migration into the draining lymph node after FITC painting. C57BL/6 mice were shaved, depilated, and painted with 12 μl of a 1% FITC solution prepared in a solution of inflammatory stimulating mixture of acetone and dibutyl phthalate (1:1, vol: vol) on the flank skin. At the times indicated, single-cell suspensions from two pooled draining brachial lymph nodes were depleted of non-DCs, and CD11c$^+$ DCs were examined for CD205 and FITC expression by flow cytometry. Shown is the percentage of CD11c$^+$FITC$^+$ DCs in the lower right (dermal DC) and upper right (Langerhans cell) quadrants. Data are one representative of three independent experiments.

(B) The number of skin DCs arriving in the draining lymph node after FITC painting. The total number of CD11c$^+$FITC$^+$ DCs per lymph node in the lower right (dermal DC) or upper right (Langerhans cell) quadrants was assessed by calculation as outlined in Experimental Procedures. Data are one representative of three independent experiments presented as mean ± SD.

CTL Priming Can Be Seen in the Absence of Any Direct Langerhans Cell Involvement

Langerhans cells have been reported to take at least 24 hr to reach draining lymph nodes after treatment of the skin with various inflammatory stimuli. Thus, the correlation between priming and a more rapid arrival of skin DCs argued that these emigrants were unlikely to be Langerhans cells. There are two major populations of DCs in untreated skin that can be separated by expression of the marker CD205 (Henri et al., 2001; Kamath et al., 2002). These are the strongly CD205-expressing Langerhans cells (CD205^{hi}) and the CD205 intermediate dermal DCs (CD205^{int}). Analysis of the brachial lymph nodes after application on the skin of FITC dissolved in the strong irritant dibutyl phthalate (known to rapidly promote DC migration [Macatonia et al., 1987]) shows that it takes more than 24 hr for CD205^{hi} Langerhans cells to reach the draining lymph nodes (Figure 3A). Indeed, skin FITC$^+$ DC migration can be seen in waves, with the first appearance of dermal DCs peaking between 1 and 2 days after skin treatment followed by a later, and more persistent, population of epidermal derived Langerhans cells (Figure 3B).

The kinetics of skin DC subset migration, as determined by differential CD205 expression, largely mirrors that described previously (Kissenpfennig et al., 2005) with EGFP-CD207 (langerin) knockin mice to differentially mark the individual skin DC subsets. We confirmed the identity of these two subsets by using bone-marrow replacement chimeras, taking advantage of the relative radio-resistance of the Langerhans cell precursors (Katz et al., 1979; Merad et al., 2002). It can be seen that the majority of DCs isolated from the epidermis are host-derived CD45.2 cells (Figure 4A) in CD45.1 → CD45.2 bone marrow irradiation chimeras, consistent with the resistance of Langerhans cells to irradiation. These host-derived (CD45.2$^+$) cells are also found in draining lymph nodes and appear FITC positive at 96 hr but not 24 hr after initiation of skin inflammation (Figure 4B), reinforcing that Langerhans cells take longer than 24 hr to arrive in draining lymph nodes after application of an inflammatory stimulus. The FITC$^+$ cells found in draining lymph nodes at 24 hr posttreatment are all donor derived, confirming that the dermal DCs are replaced during irradiation and bone-marrow reconstitution (Figure 4B). These cells are clearly CD205^{int}, in contrast to the host-derived Langerhans cells present 96 hr after treatment, which are CD205^{hi} (Figure 4C). Thus, differential expression of this latter marker is a reasonable means of discriminating between the different skin DC populations. By using CD205 staining, we found that whereas the initiation of T cell priming correlated with the arrival of DCs from infected skin, these early times were biased toward the "first wave" of skin emigrant DCs, which are of dermal rather than epidermal origin. 8 hr after infection, the majority of the FITC-bearing cells are predominantly CD205^{int} dermal DCs, distinct from the FITC$^+$ CD205^{hi} Langerhans cells seen 96 hr after inoculation (Figure 4D). Finally, we confirmed the kinetics of Langerhans cell entry into skin-draining

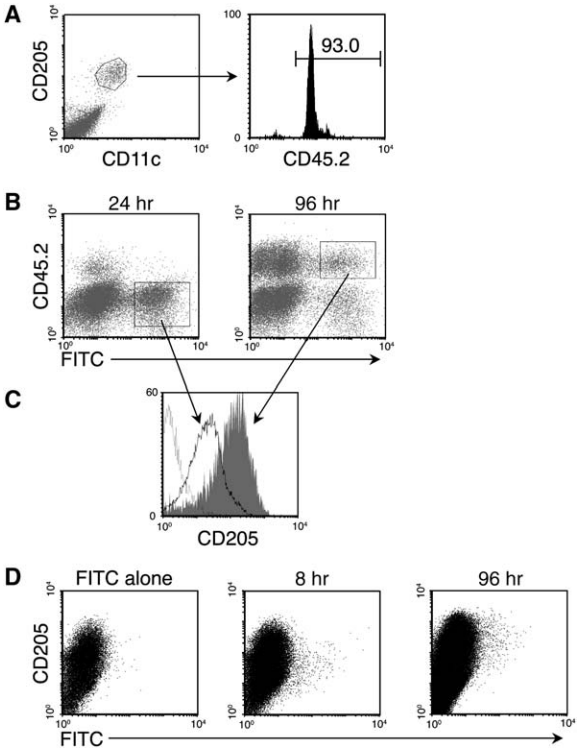

Figure 4. Confirmation of Skin DC Identity by Bone Marrow Replacement Chimeras

(A) Langerhans cells remain host derived in bone marrow replacement chimeras. Epidermal sheets isolated from CD45.1→CD45.2 bone marrow chimeric mice were cultured for 48 hr in medium containing 6Ckine. Cells migrating into the medium were collected in separate 24 hr pools and combined for analysis. Gates were generated on $CD11c^+CD205^+$ Langerhans cells, and these cells were analyzed for expression of CD45.2. The percentage of $CD11c^+CD205^+$ cells that are $CD45.2^+$ is shown in the right histogram.

(B) Skin inflammation causes the initial migration of donor-derived dermal DC into the draining lymph node followed by a second wave of host-derived Langerhans cells. CD45.1→CD45.2 bone-marrow chimeric mice were painted with 12 μl of a 1% FITC solution prepared in acetone:dibutyl phthalate (1:1, vol:vol) on the flank skin. 24 and 96 hr later, single-cell suspensions from two pooled draining brachial lymph nodes were depleted of non-DCs, and $CD11c^+$ DCs were examined for CD45.2 and FITC expression by flow cytometry.

(C) Donor-derived dermal DCs are $CD205^{int}$ and host-derived Langerhans cells are $CD205^{hi}$ in the draining lymph node. CD205 expression was analyzed on gated populations of $FITC^+CD45.2^-$ DCs obtained 24 hr after treatment (dark line) and $FITC^+CD45.2^+$ DCs obtained 96 hr after treatment (gray shaded). CD205 expression was also analyzed on non-$CD11c^+$ cells (dotted line). Data are one representative of three independent experiments.

(D) Skin infection with HSV results in an initial wave of dermal DC migration into the draining lymph node followed by a second wave of Langerhans cells. C57BL/6 mice were painted on the flank skin with a 1% FITC solution prepared in acetone 15 hr before inoculation with HSV by flank scarification. 8 and 96 hr after infection, single-cell suspensions from two pooled draining brachial lymph nodes were depleted of non-DCs, and $CD11c^+$ DCs were examined for the expression of CD205 and FITC by flow cytometry. Data are one representative of three independent experiments.

lymph nodes after FITC skin painting by using direct labeling of DCs with the antibody against the Langerhans cell-specific marker, langerin. We found that $FITC^+$ $langerin^+$ cells made up a substantial proportion of $FITC^+$ DCs in the draining lymph node 96 hr after sensitisation

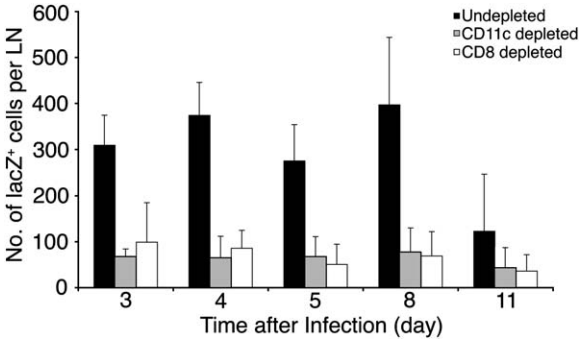

Figure 5. Dominant $CD8^+$ DC Contribution to Class I-Restricted Presentation during Cutaneous HSV Infection

C57BL/6 mice were inoculated with HSV by flank scarification. At different days after infection, the draining brachial lymph nodes were removed from 8 mice and pooled, and single-cell suspensions were either nondepleted or depleted of $CD11c^+$ or $CD8^+$ cells. Depleted preparations were then used to stimulate a *lacZ*-inducible HSV-specific T cell hybridoma. Shown is the total number of *lacZ*-positive cells per lymph node from two independent experiments presented as mean ± SEM with n = 6 for each time examined.

but made only a minimal contribution 24 hr after skin treatment (see Figure S1 in the Supplemental Data available with this article online).

As a consequence of this delayed migration, one could argue that the Langerhans cell contribution to antigen presentation would only become evident at least 3 days after infection. To show that this was not the case, we examined the relative antigen presentation contribution of CD8-negative DCs for 11 days after flank infection, because this subset includes Langerhans cells as well as other DCs of skin origin. Whole lymph node cell digests from HSV-infected mice, treated with anti-CD11c or anti-CD8, were used to stimulate a *lacZ*-inducible HSV-specific T cell hybridoma (Mueller et al., 2002b). The depletion with these antibodies inhibited T cell activation, arguing for a dominant $CD8^+$ DC contribution to class I-restricted presentation from day 3 to day 11 after infection (Figure 5). Finally, quantitative PCR on different DC subsets (purified on the basis of CD205 and CD8 expression) showed minimal virus DNA content across days 1 to 4 after infection in the skin-draining lymph nodes, with values of less than 12 total copies in each subset within a given lymph node (Figure S2). These amounts of viral DNA argue against the contribution of DC infection to direct presentation but still leave open the possibility that it may nonetheless be involved in antigen transfer.

HSV-Specific $CD8^+$ T Cells Are Crossprimed after Flank Skin Infection

$CD8^+$ DC appear to possess a relatively unique ability to crosspresent exogenous, cell-associated antigen (den Haan et al., 2000). In order to determine the importance of crosspresentation in this model of HSV-specific T cell priming, we took advantage of our recent observation that engagement of toll-like receptors (TLRs) via CpG causes $CD8^+$ DC maturation that inhibits crosspresentation of subsequently encountered antigens (Wilson et al., 2006). The CpG-mediated block is transient, disappearing well before cessation of viral replication under normal circumstances (van Lint et al., 2004;

Wilson et al., 2006). As a consequence, CpG administration was used in conjunction with excision of the primary inoculation site to assess the contribution of crosspresentation in T cell priming. Such surgical intervention limits the spread of virus as well as abbreviating the infection while maintaining robust $CD8^+$ T cell priming (Stock et al., 2004). Pretreatment of C57BL/6 animals with CpG prior to flank infection inhibits the expansion of adoptively transferred gBT-I T cells measured 7 days after virus infection (Figure 6A). This inhibition does not appear to be a consequence of the abolition of skin-resident DC migration, because CpG treatment results in only minor changes in $FITC^+$ DC accumulation after skin labeling (Figure 6B), which were statistically insignificant. Therefore, the results indicate that the mechanism underlying the $CD8^+$ DC involvement in T cell priming after skin infection with this virus is tied to the crosspresentation capability of this particular population.

HSV-Specific CTL Priming Can Be Inhibited by Blocking Skin-DC Migration

Given that CTL priming appeared to be tied to DC migration, we reasoned that these cells might traffic antigen to the draining lymph nodes even though they themselves are not directly involved in class I-restricted presentation. If this were the case, then blocking egress of DC from the skin should inhibit class I-restricted presentation in the draining lymph nodes. To this end, we took advantage of the synthetic prostaglandin analog, BW245C, known to inhibit skin DC migration (Angeli et al., 2001). Intradermal administration of BW245C blocks migration of $FITC^+$ DCs into draining lymph nodes after infection with HSV (Figure 7A). BW245C does not alter DC migration when applied to a site adjacent to that labeled with FITC, confirming that the block in migration is not a consequence of an indirect systemic effect by this agent.

We next determined whether skin DC egress was necessary for class I-restricted presentation within the draining lymph nodes. To show this, mice were treated with BW245C prior to HSV infection, and 2 days later these animals received CFSE-labeled gBT-I cells. Proliferation of the transferred T cells was examined 60 hr later in draining brachial lymph nodes in infected mice treated with the prostaglandin analog or vehicle control. Treatment inhibited antigen-specific proliferation of gBT-I cells by more than 75% in terms of overall proliferating cell numbers (Figure 7B). Thus, class I-restricted antigen presentation depended heavily on trafficking of DCs from infected skin to the draining, brachial lymph nodes. Furthermore, inhibition of presentation was mirrored by decreased $CD8^+$ T cell expansion as a consequence of BW245C treatment (Figure 7C). For these experiments, mice were flank-inoculated and the area of skin containing the inoculation site was surgically excised 8 hr later. Treatment with BW245C at the site of inoculation inhibited T cell expansion in these experiments, but had no effect when the site of treatment and inoculation were at separate locations on the mouse flank (Figure 7C). Thus, inhibition of CTL priming results from modification of the local skin environment by BW245C and not from any wider inhibitory effect of this drug.

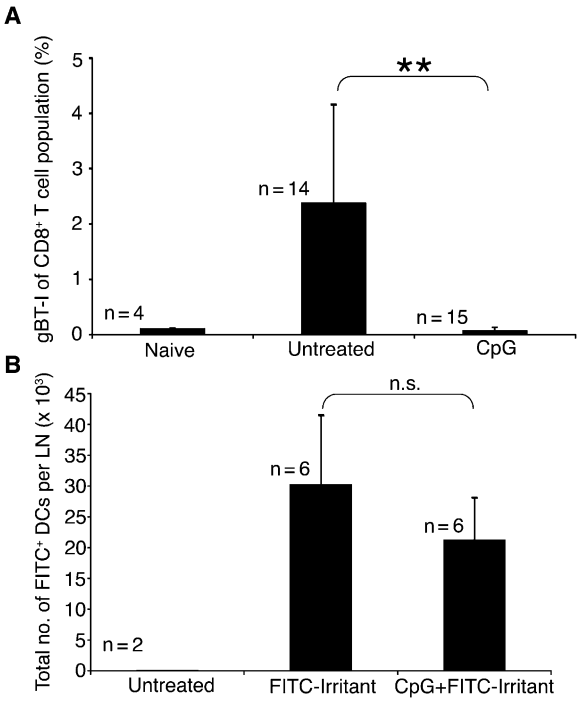

Figure 6. HSV-Specific $CD8^+$ T Cells Are Crossprimed after Cutaneous HSV Infection

(A) Impairment of crosspresentation by pretreatment with CpG results in the abolition of HSV-specific $CD8^+$ T cell priming. 1×10^5 gBT-I × B6.Ly5.1 lymph node cells were adoptively transferred into C57BL/6 mice 1 day before intravenous injection with 20 nmol of CpG. 18 hr later, mice were inoculated with HSV by flank scarification. 8 hr after infection, all animals had their inoculation site surgically removed. After 7 days, the proportion of splenic $CD8^+$ T cells of gBT-I origin was assessed by flow cytometry on the basis of CD8 and CD45.1 expression. Shown is the percentage of $CD8^+$ T cells that are of gBT-I origin from three independent experiments presented as mean ± SEM. n, total number of mice per experimental group. Student's t test and values are shown: $^{**}p < 0.01$.

(B) Skin DC migration is not impaired in CpG-treated mice. C57BL/6 mice were injected intravenously with 20 nmol of CpG. 18 hr later, mice were painted with 12 μl of a 1% FITC solution prepared in acetone:dibutyl phthalate (1:1, vol:vol) on the flank skin. 24 hr after painting, single-cell suspensions from two pooled draining brachial lymph nodes were depleted of non-DCs and the percentage of $CD11c^+FITC^+$ DCs was determined by flow cytometry. Shown is the total number of $CD11c^+FITC^+$ DCs per lymph node from two independent experiments presented as mean ± SEM. n, total number of mice per experimental group. The total number of $CD11c^+FITC^+$ cells was determined as in Figure 2C. Student's t test and values are shown: n.s, not significant.

To further show that alteration of DC migration affects T cell expansion, we pretreated the flank of mice with the contact-sensitizing agent dinitrofluorobenzene (DNFB), which causes transient mobilization of skin DCs. This limits subsequent DC migration (Figure 7D; Bacci et al., 1997). Infection during this period of limited migration inhibits the level of subsequent HSV-specific T cell expansion (Figure 7E). The combined evidence of inhibition of presentation and T cell expansion argue that $CD8^+$ T cell immunity is severely compromised in the absence of DC egress from the skin, consistent with the skin-derived DCs playing a ferrying role independent of actual presentation.

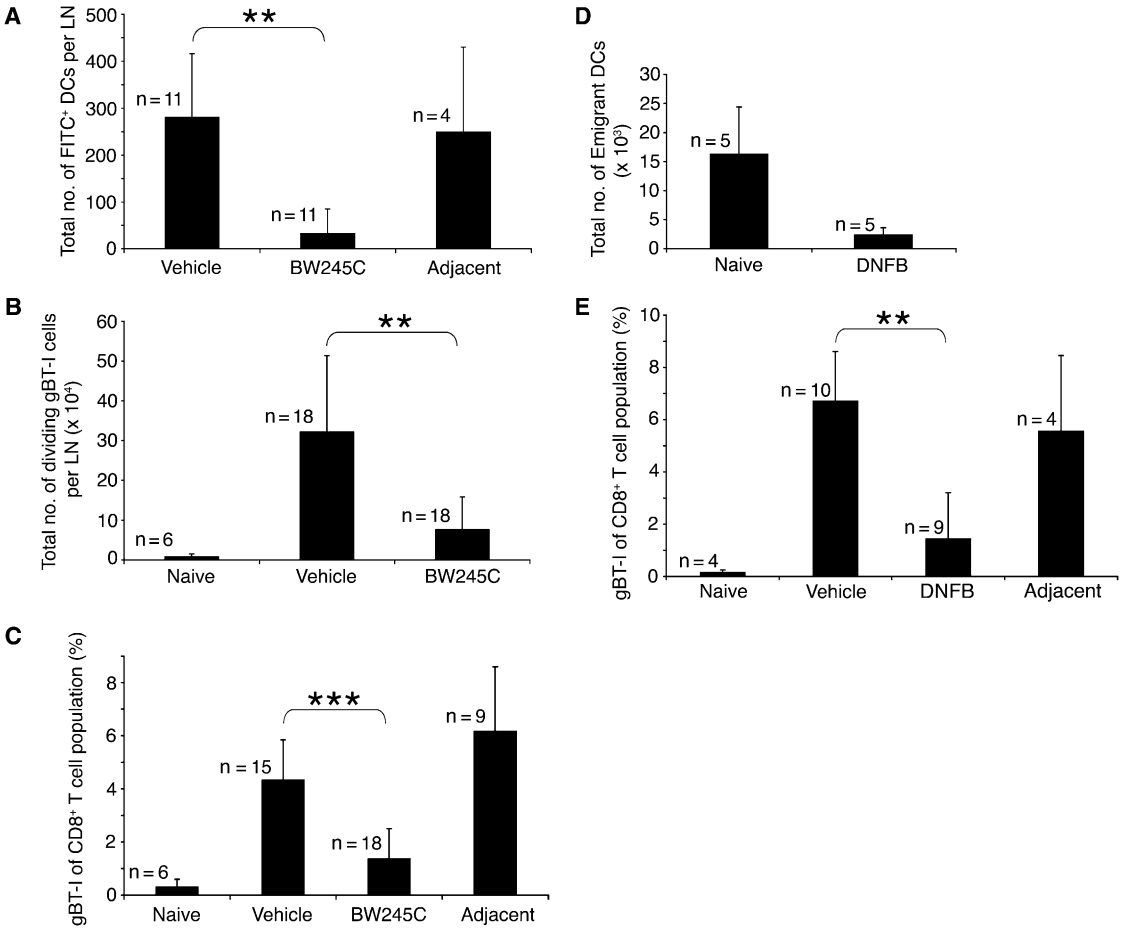

Figure 7. HSV-Specific CTL Priming Can Be Inhibited by Blocking Skin-DC Migration

(A) A prostaglandin D_2 analog blocks skin DC appearance in the skin-draining lymph node. C57BL/6 mice were painted on the flank skin with a 1% FITC solution prepared in acetone. 15 hr later, mice were treated with 20 μl of BW245C (100 nM) or vehicle intradermally on or adjacent to the FITC-painted flank skin and inoculated with HSV by flank scarification on the FITC-treated area. 24 hr after infection, single-cell suspensions from two pooled draining brachial lymph nodes were depleted of non-DCs and the percentage of $CD11c^+FITC^+$ DCs was determined by flow cytometry. Shown is the total number of $CD11c^+FITC^+$ DCs per lymph node from three independent experiments presented as mean ± SEM. n, total number of mice per experimental group. Significance was assessed by the Student's t test and values are shown: **p < 0.01. The total number of $CD11c^+FITC^+$ cells was determined as in Figure 2C.

(B) Skin DC egress is necessary for MHC class I-restricted presentation within the draining lymph node. C57BL/6 mice were treated with BW245C (100 nM) or vehicle intradermally and inoculated with HSV by flank scarification on the treated area (as described in the Experimental Procedures). 8 hr after infection, all animals had their inoculation site surgically removed. 2 days after infection, all mice were injected intravenously with 1×10^6 CFSE-labeled gBT-I × B6.Ly5.1 lymph node cells. 60 hr later, the draining brachial lymph node was removed and the $CD8^+CD45.1^+$ cells were examined for CFSE dilution. Shown is the total number of dividing gBT-I cells per lymph node from three independent experiments presented as mean ± SEM. n, total number of mice per experimental group. Significance was assessed by the Student's t test and values are shown: **p < 0.01.

(C) Skin DC egress is necessary for the expansion of HSV-specific CTL in vivo. C57BL/6 mice were injected intravenously with 1×10^5 gBT-I × B6.Ly5.1 lymph node cells. 1 day later, mice were treated with BW245C or vehicle and infected on the treated area (Vehicle, BW245C) or adjacent to it (Adjacent) with HSV by flank scarification as described in the Experimental Procedures. 8 hr after infection, all animals had their inoculation site surgically removed. 7 days after infection, the proportion of splenic $CD8^+$ T cells of gBT-I origin was assessed by flow cytometry on the basis of CD45.1 and CD8 expression. Shown is the percentage of $CD8^+$ T cells that are of gBT-I origin from four independent experiments presented as mean ± SEM. n, total number of mice per experimental group. Statistical significance was assessed by the Student's t test and values are shown: ***p < 0.005.

(D) Epicutaneous application of DNFB inhibits skin DC egress. C57BL/6 mice were shaved, depilated, and painted on the flank skin with a 0.5% DNFB solution prepared in acetone. 12 hr later, treated full-thickness skin was removed and cultured for 48 hr in medium containing 6Ckine. Cells migrating into the medium were collected in separate 24 hr pools and combined for analysis. Gates were generated on $CD11c^+I\text{-}A^{b+}$ DCs. Shown is the total number of emigrant DCs obtained from two independent experiments presented as mean ± SEM. n, total number of mice per experimental group.

(E) Pretreatment with DNFB inhibits HSV-specific CTL expansion. C57BL/6 mice were injected intravenously with 1×10^5 gBT-I × B6.Ly5.1 lymph node cells. 1 day later, mice were treated with DNFB or vehicle on the flank skin. 12 hr later, mice were infected on the treated area (Vehicle, DNFB) or adjacent to it (Adjacent) with HSV by flank scarification. 8 hr after infection, all animals had their inoculation site surgically removed. 7 days after infection, the proportion of splenic $CD8^+$ T cells of gBT-I origin was assessed by flow cytometry on the basis of CD45.1 and CD8 expression. Shown is the percentage of $CD8^+$ T cells that are of gBT-I origin from three independent experiments presented as mean ± SEM. n, total number of mice per experimental group. Statistical significance was assessed by the Student's t test and values are shown: **p < 0.01.

Discussion

The results presented here build on our previous report showing that Langerhans cells are not involved in direct class I-restricted presentation after HSV infection of the skin (Allan et al., 2003). In that study, Langerhans cells were formally excluded from playing any direct role in CTL priming after HSV infection with bone-marrow replacement chimeras where they survive irradiation. Here, we address the role of the other major subset of DCs originating in the skin, the dermal DCs, and show that these also have little involvement in class I-restricted presentation. Overall, this study highlights the previously observed dominance of the nonmigrating, lymph node-resident $CD8^+$ DCs subset in CTL priming in this virus infection (Belz et al., 2004a; Smith et al., 2003).

Despite this, the egress of DCs from skin appears intimately tied with CTL priming. Priming capacity within lymph nodes required the arrival of skin-derived DCs, and inhibition of this migration severely compromised the ensuing class I-restricted presentation and $CD8^+$ T cell expansion. From these data, it can be concluded that skin DC act as transporters, and once within the draining lymph nodes donate their antigenic cargo to the resident $CD8^+$ DC subset critical to effective presentation. Moreover, such transfer explains the dominant role of the $CD8^+$ DCs in this response, since these cells have a unique ability to crosspresent cell-derived exogenous antigen (den Haan et al., 2000), and crosspresentation is shown here and elsewhere (Orr et al., 2005) to be important in this event.

Formal proof of inter-DC antigen transfer was found in another form of localized infection, namely respiratory infection with influenza virus (Belz et al., 2004b). There, transfer was successfully demonstrated in vitro between lung-derived DCs and the presenting $CD8^+$ DC population. Attempts at reproducing this in the case of skin infection have so far proven futile, even when we resorted to using purified $FITC^+$ DCs as the potential antigen donors in our in vitro culture system (data not shown). This failure could reflect differences between the respective viruses, the progression of each infection, or unique features of the migrating DC populations originating in lung and skin.

A proposal for inter-DC antigen transfer is not without precedent, having been suggested in a number of experimental settings (Belz et al., 2004b; Fleeton et al., 2004; Inaba et al., 1998; Kleindienst and Brocker, 2003; Norbury et al., 2002; Scheinecker et al., 2002; Turley et al., 2003). Most definitively, Inaba et al. (1998) formally showed class II-restricted antigen transfer between migrating DCs and a separate subset within the T cell zones, an area associated with the $CD8^+$ DCs (De Smedt et al., 1996; Pulendran et al., 1997). Strikingly, this was found to be highly efficient, spreading relatively small amounts of input antigen over a large proportion of the lymphoid-resident DC population. Indeed, it has been argued that this is the role of inter-DC transfer, to increase priming efficiency by spreading antigen over the wider DC pool (Lindquist et al., 2004). This efficiency could explain the extreme potency of the migrating cells highlighted by our results. Skin infection with HSV results in very rapid class I-restricted presentation within the draining lymph nodes (Mueller et al., 2002b). Even infection times as short as 8 hr resulted in robust levels of CTL expansion (Figure 2A). FITC labeling showed that only a few hundred skin-derived DCs would have made their way to the draining brachial lymph node by the time infection was stopped by surgical intervention, and yet these few cells provide prolonged presentation and robust levels of CTL expansion (Stock et al., 2004).

Mechanisms for inter-DC antigen transfer remain unclear at this time. Amigorena and colleagues have proposed that exosomes released by migrating DC could serve as antigen spreaders within the lymph nodes (Thery et al., 2002). Others have argued that such transfer occurs within the context of DC apoptosis and provided evidence for transfer of apoptotic bodies (Fleeton et al., 2004; Inaba et al., 1998). While this is attractive, given that HSV infection has been linked with rapid DC apoptosis (Jones et al., 2003) and transfer of antigen from dying cells would be consistent with the importance of crosspresentation in this process (Albert et al., 1998), it remains to be shown that transfer truly requires cell death in this situation. Lindquist et al. (2004) have provided dramatic evidence that newly migrating DCs probe the lymphoid-resident DC network, arguing that this could facilitate antigen spreading. Such extensive probing, however, is difficult to reconcile with a population of incoming cells reported to be functionally compromised by HSV infection (Mikloska et al., 2001; Salio et al., 1999) and marked for rapid cell death (Bosnjak et al., 2005). Clearly, further work is required to clarify this issue.

Possibly one of the most challenging aspects of a proposal for inter-DC antigen transfer is the spatial separation between peripheral infection and effective T cell priming in the lymph nodes. In the context of localized infection, the priming lymphoid environment is removed from the site of virus infection with its potential for direct DC maturation through virus binding to elements like the toll-like receptors (Sato and Iwasaki, 2004; Sporri and Reis e Sousa, 2005). Indeed, many of the discussions of antigen transfer between migratory and lymphoid-resident DC populations have been couched in terms of steady-state presentation and T cell tolerance (Inaba et al., 1998; Steinman and Nussenzweig, 2002). Along these lines, it is easy to envisage CTL priming in the traditional setting where tissue DCs migrate as a consequence of contact with the pathogen and this is then followed by presentation within the draining lymph nodes by these same cells, now mature as a consequence of pathogen encounter at the site of infection. However, DCs can be activated by other mechanisms. Helper T cells can stimulate DCs through CD40 and license them for CTL priming (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). Primary HSV-specific CTL expansion is highly helper T cell dependent (Jennings et al., 1991), and we have shown that $CD4^+$ T cells rapidly license the CD8 DCs after infection (Smith et al., 2004). Migrating antigen-bearing DCs could therefore activate virus-specific helper T cells, which could in turn license the lymph node-resident CD8 DCs. Consistent with this scenario, the mucosal equivalent of the dermal DCs implicated here in antigen transport after skin infection are known to present class II-restricted antigen after mucosal infection with HSV (Zhao et al., 2003).

Overall, our experiments highlight that a simple model where the same DCs migrate and then present antigen is inadequate in the case of skin infection with HSV. While inter-DC transfer of antigen has been formally proposed in other infections (Belz et al., 2004b; Fleeton et al., 2004), its relative contribution has been unclear in the face of presentation by the migrating DC population. Here, the tissue-derived DCs appear to make little contribution to $CD8^+$ T cell priming for reasons unknown. Yet HSV-specific CTL priming as a consequence of skin scarification is extremely rapid and efficient. Therefore, these studies argue that transfer of antigen between migrating DCs to a wider lymph node-resident CD8 DC population provides a powerful means for effective CTL priming.

Experimental Procedures

Mice and Virus

C57BL/6, B6.SJL-$Ptprc^aPep3^b$/BoyJ (B6.Ly5.1), and gBT-I.1 × B6.Ly5.1 were obtained from the Department of Microbiology and Immunology (University of Melbourne). gBT-I.1 ($H\text{-}2^b$) transgenic mice (gBT-I) express a TCR ($V\alpha2V\beta8.1$) specific for the immunodominant epitope of HSV gB ($gB_{498\text{-}505}$) (Mueller et al., 2002a). Mice were housed in SPF conditions and all experimentation was conducted according to institutional ethical guidelines. The KOS strain of herpes simplex 1 virus (HSV) was propagated and titred with VERO cells grown in Minimal Essential Medium containing 10% FCS.

Virus Inoculations and Inoculation Site Excision

Mice were inoculated with 1×10^6 plaque forming units of HSV-KOS via the flank scarification model of HSV infection as previously described (van Lint et al., 2004). In some experiments (Figures 2A, 6B, 7B, 7C, and 7E), the infection site was surgically excised 2 or 8 hr after flank inoculation as described elsewhere (Stock et al., 2004). Briefly, a 0.5 × 0.5 cm area of full-thickness skin encompassing the infection site was excised from anaesthetized mice. The wound was closed with 9 mm surgical wound clips (BD Biosciences).

Generation of Bone Marrow Chimeras

Chimeric mice were generated by irradiation of recipient C57BL/6 mice ($CD45.2^+$) with two doses of 550 cGray, 4 hr apart. 5×10^6 B6.Ly5.1 ($CD45.1^+$) bone marrow cells were injected intravenously. The mice were allowed to reconstitute for 8 weeks before use.

Fluorescein Isothiocyanate Painting

Mice were anesthetized, shaved, and depilated by means of Veet (Reckitt Benckiser, Swindon, UK) as previously described (van Lint et al., 2004). For experiments involving HSV infection, mice were painted on the posterior flank, lateral to the spine and over the spleen with 12 μl of a 1% FITC solution (Isomer 1, Sigma-Aldrich, St. Louis, MO) prepared in acetone prior to infection. 15 hr later, mice were infected with HSV on the painted region via the scarification method. For experiments involving the tracking of skin DC into the lymph node after skin irritation, C57BL/6 and chimeric mice were shaved and depilated as described above. Mice were then painted with 12 μl of a 1% FITC solution prepared in acetone:dibutyl phthalate (1:1, vol/vol; Sigma-Aldrich) as previously described (Macatonia et al., 1987).

Dendritic Cell Isolation from Lymph Nodes

The ipsilateral brachial lymph node draining the site of skin treatment was removed. The lymph nodes were cut into small fragments and digested in collagenase type II (1 mg/ml [Worthington Biochemicals, Lakewood, NJ]) and DNase (1 μg/ml grade II bovine pancreatic DNase [Boehringer-Mannheim, Mannheim, Germany]) and then depleted of plasmacytoid DCs ($CD19^+$) and non-DC populations with antibody and magnetic beads as previously described (Belz et al., 2004a). In brief, single-cell suspensions were incubated for 30 min with predetermined optimal concentrations of purified antibodies: anti-CD3 (KT3), anti-Thy1 (T24/31.7), anti-CD19 (ID3), and anti-erythrocyte (TER-119) prior to the removal of antibody bound cells with anti-rat Ig-coupled magnetic beads (Dynabeads; Dynal, Oslo, Norway). For sorting of DCs into subsets (Figure 1), purified lymph node DCs were labeled with CD11c-PE (HL3; BD PharMingen, CA) and CD8α-APC (53-6.7; BD PharMingen) and sorted by FACS (MoFlo instrument, Cytomation, Fort Collins, CO).

DCs isolated from the draining brachial lymph node of C57BL/6 mice (Figures 2B, 2C, and 3) were stained for CD8α-APC (53-6.7), CD205-PE (NLDC-145), and CD11c-biotin (HL3; BD PharMingen), followed by streptavidin-PerCp (BD PharMingen). Prior to langerin staining (Figure S1), a 30 min CD16/32 blocking step with the 2.4G2 supernatant was performed. Cell-surface langerin staining was carried out by incubation with unconjugated mouse anti-langerin (205C1; AbCys, Paris, France). Antibody bound cells were detected with anti-mouse IgM^a-PE (AF6-78; BD PharMingen). DCs isolated from the lymph nodes of chimeric mice (Figures 4B and 4C) were stained for CD205-PE (NLDC-145), CD45.2-PerCp (104; BD PharMingen), and CD11c-biotin (HL3) and then with streptavidin-APC (BD PharMingen). Analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Quantifying Skin DC Migration into the Lymph Node

To calculate the total number of $CD11c^+FITC^+$ DCs per lymph node (Figures 2C, 3B, 6B, and 7A), the total number of lymph node cells (after DC enrichment) was multiplied by the percentage of $CD11c^+FITC^+$ cells obtained by flow cytometry. To calculate the total number of $FITC^+$ dermal DCs and Langerhans cells per lymph node in Figure 3B, the total number of lymph node cells (after enrichment) was multiplied by the percentage of $CD11c^+FITC^+$ in lower right (dermal DC) or upper right (Langerhans cell) quadrants. In experiments involving FITC application prior to HSV infection (Figures 2C and 7A), the background $CD11c^+FITC^+$ cells obtained from mice painted with FITC alone was subtracted from the total number of $CD11c^+FITC^+$ cells.

Naive $CD8^+$ T Cell Stimulation by Dendritic Cells In Vitro

The gBT-I × B6.Ly5.1 $CD8^+$ T cells were purified by antibody and magnetic bead depletion of lymph node cells and were labeled with 2.5 μM CFSE (Sigma-Aldrich) as previously described (Allan et al., 2003). 2×10^4 purified DCs were cocultured in vitro with 5×10^4 CFSE-labeled gBT-I cells in 200 μl RPMI containing 10% FCS in V-bottom, 96-well tissue culture plates (Costar, Corning Incorporated, NY). Proliferation was measured as the loss of CFSE fluorescence as determined by flow cytometry at 60 hr of culture. Cells were stained with anti-CD8α-APC and anti-CD45.1-PE (A20; BD PharMingen). Dead cells were excluded with propidium iodide (PI; Sigma-Aldrich) staining. $CD8\alpha^+CD45.1^+PI^-$ cells were analyzed for proliferation by a FACSCalibur flow cytometer.

In Vivo gBT-I Stimulation

Lymph nodes from gBT-I × B6.Ly5.1 mice were harvested, made into single-cell suspensions, and labeled with CFSE as described above. 1×10^6 CFSE-labeled lymphocytes were adoptively transferred via i.v. tail vein injection 2 days after infection. 60 hr after transfer, the ipsilateral draining brachial lymph node was harvested and stained with anti-CD8α-APC (53-6.7) and CD45.1-PE (A20), and $CD8\alpha^+CD45.1^+PI^-$ cells were analyzed for proliferation by a FACSCalibur flow cytometer.

In Vivo gBT-I Population Expansion Assays

C57BL/6 mice received 1×10^5 gBT-I × B6.Ly5.1 lymph node cells 1 day prior to flank HSV inoculation. 7 days after infection, mice were sacrificed and their spleens removed. Single-cell suspensions were stained with anti-CD8α-APC (53-6.7) and CD45.1-PE (A20). $CD8\alpha^+CD45.1^+PI^-$ cells were analyzed for expansion by a FACSCalibur flow cytometer.

Prostaglandin D_2, Methylated Oligonucleotide, and 2,4-Dinitrofluorobenzene Treatments

For prostaglandin treatment, mice were shaved and depilated as described above. 20 μl of the prostaglandin D_2 analog, BW245C (100 nM; Cayman Chemical, MI) or DMSO (vehicle control) was injected intradermally into a 0.5 × 0.5 cm area of skin on the posterior

flank. After 20 min, mice were inoculated with HSV by scarification either on, or adjacent to, the BW245C-treated region. Where indicated, the oligonucleotide CpG was administered by the intravenous injection of 20 nmol of synthetic phosphorothioated CpG1668 (Geneworks, SA, Australia) dissolved in PBS 18 hr prior to inoculation with HSV. For 2,4-dinitrofluorobenzene (DNFB) treatment, mice were shaved and depilated prior to the application of 25 μl of 0.5% DNFB (Sigma-Aldrich) or acetone (vehicle) to the skin on the posterior flank, 12 hr prior to inoculation with HSV either on, or adjacent to, the DNFB-treated region.

Dendritic Cell Isolation from Skin Biopsies

Epidermal dendritic cells were isolated from epidermal sheets by culture in the presence of 6Ckine (R&D Systems, MN) as described previously (Allan et al., 2003). The DCs isolated from epidermal sheets of chimeric mice were stained with CD11c-biotin (HL3), CD205-PE (NLDC-145), and CD45.2-FITC (104; BD PharMingen) and then with streptavidin-APC. Analyses were performed with a FACSCalibur flow cytometer. To assess DC emigration after DNFB application, full-thickness skin was excised, removed of subcutaneous tissue by blunt dissection, and cultured in the presence of 6Ckine as previously described (Allan et al., 2003). Isolated cells were stained with CD11c-biotin (HL3) and I-A^b-PE (AF6-120.1) and then with streptavidin-APC prior to flow cytometric analysis. The total number of emigrant DCs was calculated by adding 2×10^4 BD PharMingen Sphero Blank calibration particles (San Diego, CA) to each sample and collecting 1×10^4 beads during analysis.

Depletion of Cell Subsets and the Detection of Lymph Node Antigen Presentation with *lacZ*-Inducible Hybridomas

The draining brachial lymph node was removed from HSV flank-inoculated mice at different times after infection. The lymph nodes were cut into small fragments and digested in collagenase type II (1 mg/ml [Worthington Biochemicals]) and DNase (1 μg/ml grade II bovine pancreatic DNase [Boehringer-Mannheim]) to form a single-cell suspension. Cells were then incubated with either N418 (anti-CD11c) or 53-6.7 (anti-CD8α) for 30 min at 4°C. After washing, the cells were then incubated with sheep anti-rat IgG-coupled magnetic beads (Dynabeads M-450; Dynal Biotech, Oslo, Norway), and bead bound cells were removed with a magnet as previously described (Belz et al., 2002).

The *lac*Z-inducible gB-specific hybridoma HSV-2.3.E2 was cloned and maintained as previously described (Belz et al., 2002; Mueller et al., 2002b). For the detection of antigen presentation, brachial lymph nodes from HSV-infected mice were made into single-cell suspensions, and 2-fold serial dilutions of the CD11c-depleted, CD8-depleted, or nondepleted fractions were prepared in a flat-bottom, 96-well plate beginning at 10^6 cells/well. Hybridoma cells (10^5) were added to each well before overnight culture. Background *lac*Z expression was determined with hybridoma cells cultured in the equivalent cell fractions obtained from naive mice, while the positive expression was measured by hybridoma incubation with the $gB_{498\text{-}505}$ peptide. Detection of activation with X-gal was performed on the cultures as previously described (Belz et al., 2002). Cultures were examined microscopically for the presence of blue cells 12–15 hr after incubation at 37°C. The total number of *lac*Z-positive cells per lymph node was calculated by subtracting the background expression and then multiplying the number of blue events obtained in a well by the total number of cells in the entire lymph node (obtained by standard trypan blue exclusion).

Quantification of HSV Genomic DNA in DC Subsets by Real-Time PCR

Real-time PCR of viral DNA in purified DC subsets was performed as previously described (Wilson et al., 2006).

Statistical Analysis

Normally distributed data was compared by the two-tailed Student's t test, with a p value of <0.05 considered significant. Data are presented as mean ± standard error (SEM).

Supplemental Data

Two Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/1/153/DC1/.

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