Immunity 24, 191–201, February 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.01.005

# Dendritic Cells Rapidly Recruited into Epithelial Tissues via CCR6/CCL20 Are Responsible for CD8$^+$ T Cell Crosspriming In Vivo

**Marie Le Borgne,[1,2] Nathalie Etchart,[1,2] Anne Goubier,[1,2] Sergio A. Lira,[3] Jean Claude Sirard,[4,5] Nico van Rooijen,[6] Christophe Caux,[7] Smina Aït-Yahia,[7] Alain Vicari,[7] Dominique Kaiserlian,[1,2,*] and Bertrand Dubois[1,2,*]**

[1] INSERM U404 "Immunité et Vaccination"
[2] Université Claude Bernard Lyon 1
IFR128 BioSciences Lyon-Gerland
F-69365 Lyon
France
[3] Immunobiology Center
Mount Sinai School of Medicine
1425 Madison Ave.
12-20C Box 1630
New York, New York 10029
[4] INSERM E0364
[5] Institut de Biologie
Université de Lille 2
F-59021 Lille
France
[6] Department of Molecular Cell Biology
Free University Medical Center
Amsterdam 1081 BT
Netherlands
[7] Schering-Plough Laboratory for Immunological Research
F-69570 Dardilly
France

## Summary

**The nature of dendritic cell(s) (DC[s]) that conditions efficient in vivo priming of CD8$^+$ CTL after immunization via epithelial tissues remains largely unknown. Here, we show that myeloid DCs rapidly recruited by adjuvants into the buccal mucosa or skin are essential for CD8$^+$ T cell crosspriming. Recruitment of circulating DC precursors, including Gr1$^+$ monocytes, precedes the sequential accumulation of CD11c$^+$ MHC class II$^+$ DCs in dermis and epithelium via a CCR6/CCL20-dependent mechanism. Remarkably, a defect in CCR6, local neutralization of CCL20, or depletion of monocytes prevents in vivo priming of CD8$^+$ CTL against an innocuous protein antigen administered with adjuvant. In addition, transfer of CCR6-sufficient Gr1$^+$ monocytes restores CD8$^+$ T cell priming in CCR6$^{-/-}$ mice via a direct Ag presentation mechanism. Thus, newly recruited DCs likely derived from circulating monocytes are responsible for efficient crosspriming of CD8$^+$ CTL after mucosal or skin immunization.**

## Introduction

DCs are professional APCs with the unique property of inducing priming and differentiation of naive CD4$^+$ and CD8$^+$ T cells into helper T cells and cytotoxic effectors, respectively. Langerhans cells (LCs), the prototype of immature DCs residing in pluri-stratified epithelia of skin, buccal mucosa, and vagina, are sentinels specialized in the uptake and sampling of tissue antigens (Ag), which they transport via afferent lymph to draining lymph nodes (LN) for presentation to T cells (Banchereau and Steinman, 1998). Under steady-state conditions, resident LCs migrate continuously at a low rate to draining LN, presumably to induce or maintain tolerance to self or innocuous antigens. Danger signals provided by a variety of stimuli, such as microbial compounds activating Toll-like receptors and contact-sensitizing haptens that induce local release of inflammatory cytokines and chemokines, trigger DC maturation. This complex DC maturation process is characterized by an increased expression of cell-surface MHC/peptide complexes and costimulatory molecules associated with an enhanced rate of migration to the T cell area of draining lymph nodes, where DCs become mature antigen-presenting DCs able to activate naive T cells and initiate their differentiation into functional effectors. It is becoming increasingly clear that DCs of lymphoid organs comprise various subsets that likely differ in their relative ability to prime functionally distinct CD4$^+$ or CD8$^+$ effector T cells (reviewed in Heath et al., 2004; Itano and Jenkins, 2003).

However, the contribution of DCs residing in mucosae and skin in priming of specific effector T cells remains largely debated. Two studies with in vivo models of epithelial HSV infection argued against a role for LC in direct priming of protective antiviral-specific T cells (Allan et al., 2003; Zhao et al., 2003). In addition, LCs were recently shown to be dispensable for priming effector cells of contact sensitivity, a prototype of Ag-specific T cell-mediated skin inflammatory response (Bennett et al., 2005; Kaplan et al., 2005; Kissenpfennig et al., 2005). The prevailing correlate of these findings—that dermal or submucosal DC might be instrumental for inducing T cell priming after mucosal or cutaneous immunization—has received some support (Belz et al., 2004; Itano et al., 2003; Zhao et al., 2003). Besides these epithelial tissue resident DCs, we and others have observed that DCs are rapidly recruited into muco-cutaneous tissues by proinflammatory stimuli from bacteria and virus (McWilliam et al., 1996; Zhao et al., 2003), soluble Ag, such as haptens (Desvignes et al., 1998) and viral proteins (Etchart et al., 2001), or mucosal adjuvants such as Cholera toxin (CT) (Anjuere et al., 2004). Remarkably, delivery of the hapten 2-4 dinitrofluorobenzene (DNFB) (Desvignes et al., 1998) or measles virus nucleoprotein (NP) (Etchart et al., 2001) through the skin or buccal mucosa generated in vivo priming of specific CD8$^+$ CTL. This suggested that these two proinflammatory immunogens had intrinsic adjuvant properties that may be mediated by their ability to induce DC recruitment. Along these lines, in vivo studies revealed that monocytes can be recruited from peripheral blood at sites of inflammation and could differentiate locally into MHC class II$^{high}$ CD11c$^{low}$ DCs (Randolph et al., 1999). However, how these cells are recruited and whether they play a role

*Correspondence: dubois@cervi-lyon.inserm.fr (B.D.); kaiserlian@cervi-lyon.inserm.fr (D.K.)

in the induction of specific effector T cells, as compared to resident DC, remains largely unknown.

Monocyte chemotactic proteins, CCL20/MIP-3α, and other chemokines have been reported to attract immature DCs in vitro (reviewed in Dieu-Nosjean et al., 1999). CCL20, produced by epithelial cells of inflamed epithelial tissues (reviewed in Schutyser et al., 2003), is the most potent chemokine for selective attraction of epidermal LC in vitro via interaction with the CCR6 receptor (Charbonnier et al., 1999; Dieu-Nosjean et al., 2000) and might be responsible for DC accumulation in psoriatic lesional skin (Dieu-Nosjean et al., 2000). Besides, CCR6$^{-/-}$ mice exhibit impaired humoral immune response to orally administered Ag and to enteropathic rotavirus infection (Cook et al., 2000). However, in vivo evidence in relevant patho-physiological situations for a role for CCR6/CCL20 chemokine receptor/ligand interaction in DC recruitment in skin or mucosal tissues has been scarcely documented (Merad et al., 2004).

In this study, we investigated the mechanism and functional outcome of adjuvant-dependent DC recruitment into pluri-stratified epithelial tissues. Our findings demonstrate that freshly recruited DCs derived from circulating monocytes are responsible for efficient priming of CD8$^+$ CTL effectors after mucosal or skin immunization with a soluble protein Ag.

## Results

### DCs Transiently Accumulate in Dermis and Epithelium at the Site of Immunization with DNFB or NP

We previously documented that the hapten DNFB and measles virus NP are two potent immunogens that share the ability to induce specific CD8$^+$ CTL without CD4$^+$ T cell help, after transepithelial delivery via the skin or buccal mucosa, two pluristratified epithelial tissues that exhibit a similar anatomical distribution of DC, including LC (Desvignes et al., 1998; Etchart et al., 2001). Topical DNFB delivery onto the buccal mucosa generated, within 2 to 6 hr, a transient local inflammatory reaction characterized by enlarged blood vessels and edema of the lamina propria (i.e., dermis) with mononuclear cell infiltrates extending to the underlying muscle layer (Figures 1A and 1B, left panel). Buccal NP immunization generated a milder local inflammatory reaction with only focal mononuclear cell infiltration of dermal papillae (Figures 1C and 1D, left panel). DNFB or NP delivery onto the buccal mucosa (Figures 1A–1D) or skin (data not shown) induced, within 6 hr, a transient accumulation of MHC class II$^+$ cells at the site of immunization, confirming our previous results (Desvignes et al., 1998; Etchart et al., 2001). Staining with anti-CD11c or anti-CD11b Abs revealed that part of the MHC II$^+$ cells that accumulate after immunization represented bona fide myeloid CD11c$^+$ DCs (Figures 1A–1D). An immunohistochemical analysis of buccal mucosa at various times after DNFB painting revealed a rapid accumulation of MHC II$^+$ cells in the lamina propria with a subsequent increase in the number of epithelial LCs as assessed by a staining for Langerin (Valladeau et al., 2002, 2000) (Figures 1E and 1F). Kinetics analysis of MHC class II$^+$ DCs and of Langerin$^+$ cells, accumulating in the dermis and the epithelium, respectively, showed that the density of MHC class II$^+$ DCs at the dermo-epithelial junction was twice higher between 2 to 6 hr after immunization, whereas the number of LCs gradually increased between 2 and 6 hr (Figure 1G). Both dermal DC and epithelial LC numbers returned to steady-state levels by 24 hr, reminiscent to the kinetics of DC migration to draining LN (Ruedl et al., 2000). These data demonstrate that transepithelial delivery of proinflammatory Ag, such as DNFB and NP, promotes sequential accumulation of bona fide DCs into the dermis and the epithelium.

### Recruitment of Circulating DC Precursors Contributes to DNFB-Induced Local LC Accumulation

Accumulation of DC at the site of immunization may result from the attraction of resident cells migrating from adjacent areas and/or recruitment of circulating DCs or DC precursors. Monocytes constitute the most abundant source of blood DC precursors and, in response to inflammatory stimuli, can migrate into the skin where they differentiate into DCs (Qu et al., 2004; Randolph et al., 1999). Analysis of skin epithelial cell suspensions revealed that topical delivery of DNFB induced the recruitment of CD11b$^+$Gr1$^{high}$ cells and CD11b$^+$Gr1$^{+/low}$ cells (Figure 2A), corresponding to neutrophils and monocytes, respectively (Lagasse and Weissman, 1996). Thus, DNFB delivery rapidly mobilizes circulating Gr1$^+$ monocytes to the skin.

We next analyzed the contribution of circulating DC precursors to the accumulation of LCs. To this end, we exploited a model of bone marrow chimeras in which, at steady-state, tissue resident (radioresistant) LCs remain of recipient origin, whereas other DCs and DC precursors are replaced by donor bone marrow cells (Merad et al., 2002). This offers a unique opportunity to determine whether DNFB-recruited LCs derive from circulating donor DC precursors. CD45.1 mice were lethally irradiated and reconstituted with bone marrow cells from congenic CD45.2 mice (CD45.2→CD45.1 bone marrow chimeras). Eight weeks after the bone marrow transfer, the vast majority of MHC II$^+$ LC in skin epithelial sheets were still of recipient origin (e.g., CD45.1$^+$, Figure 2B), in accordance with previous reports (Allan et al., 2003; Merad et al., 2002). In contrast, blood myeloid cells and most dermal DCs were derived from the bone marrow transplant (e.g., CD45.2$^+$). Interestingly, the proportion of skin LC expressing the donor CD45.2 marker increased by more than 2-fold 6 hr after DNFB painting (Figure 2B). In light of the sequential accumulation of MCH-II$^+$ cells into the dermis and epithelium, these results demonstrate that recruitment of circulating DC precursors contributes to LC accumulation at the site of DNFB immunization.

### Critical Role of CCR6/CCL20 in DC Accumulation into Epithelial Tissues

CCL20 is the major chemokine that attracts LC and LC-precursors and has been proposed to induce LC recruitment at sites of inflammation (Dieu-Nosjean et al., 2000; Merad et al., 2004). Thus, we examined whether buccal delivery of DNFB or NP triggered local CCL20 production. Reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that CCL20 transcripts,

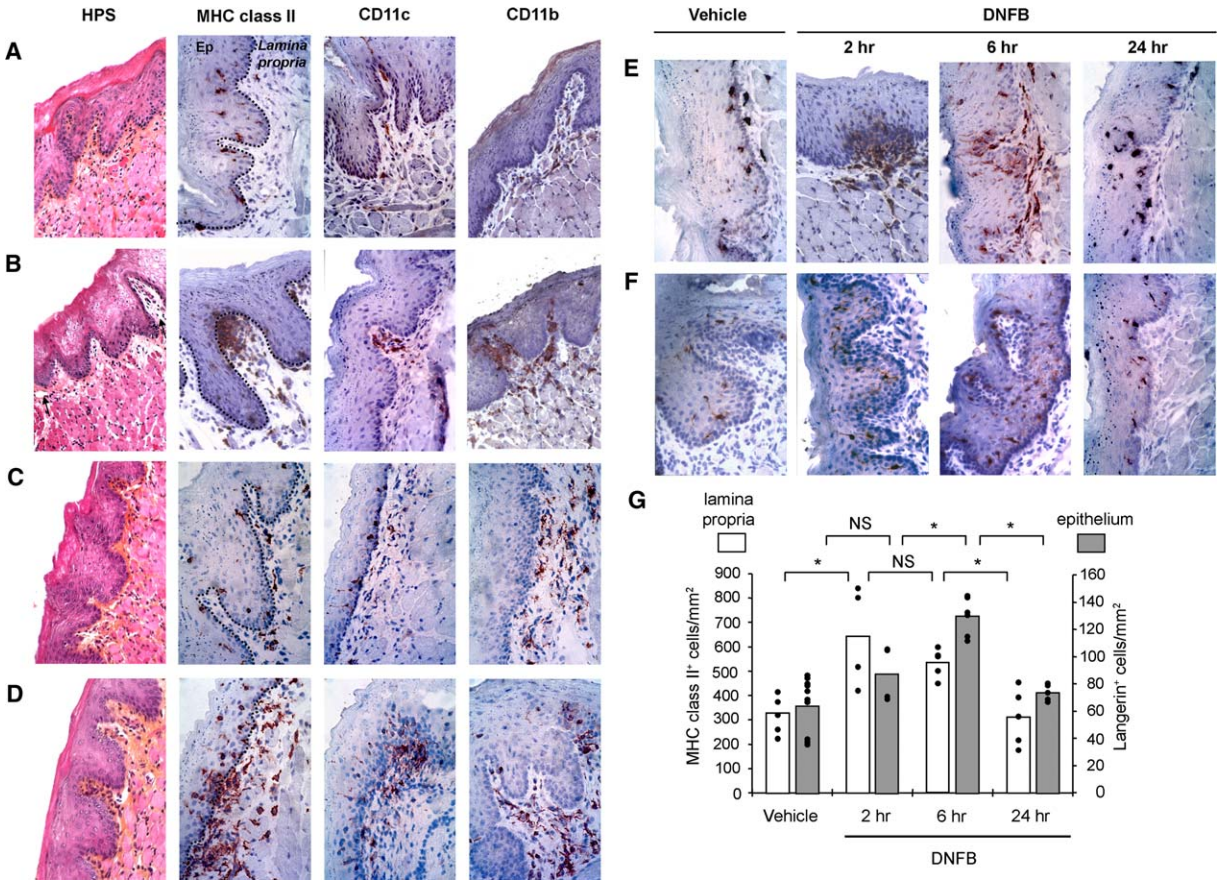


Figure 1. Immunohistochemical Analysis of DC Accumulation Induced by Buccal Immunization with DNFB or NP

(A–D) HPS staining and staining for MHC class II, CD11c, CD11b, or Langerin molecules were conducted on cryostat sections of buccal mucosa two hr after mucosal delivery of either vehicle (A), DNFB (B), PBS (C), or NP (D). Final magnification is ×400. Ep, epithelium; dotted line, basement membrane; arrow, blood vessel.

(E–G) Staining for MHC class II (E) or Langerin molecules (F) was carried out at 2, 6, and 24 hr after topical application of DNFB or vehicle alone (2 hr) onto the buccal mucosa.

(G) The density of MHC class II$^+$ cells in the lamina propria and of Langerin$^+$ cells in the epithelium is expressed as the number of positive cells per square millimeter of tissue. Each dot represents the mean of four fields in four sections of buccal mucosa; each histogram bar represents the mean values obtained for three mice per group. The asterisk indicates $p < 0.01$; NS, not significant.

expressed constitutively at variable interindividual levels, were dramatically upregulated as early as 30 min to 2 hr after NP (Figure 3A) or DNFB (Figure 3B) immunization. Skin painting with DNFB similarly upregulated local CCL20 production (not shown).

Because CCR6 is the only receptor for CCL20 (Cook et al., 2000), its role in DC accumulation was addressed by using CCR6-deficient (CCR6$^{o/o}$) mice. In agreement with a previous report (Cook et al., 2000), we observed that CCR6 is not involved in maintenance or self renewal of epithelial tissue DC at the steady-state, as CCR6$^{o/o}$ and wild-type (wt) mice harbored similar densities of MHC class II$^+$ DC in dermis or epithelium that were not affected by injection of PBS (data not shown) or topical application of acetone and oil that were used as control vehicles (Figure 3C). More importantly, DNFB or NP immunization via buccal mucosa (Figures 3C–3E) or skin (data not shown) was totally unable to promote DC accumulation in CCR6$^{o/o}$ mice. Thus, Ags triggering of local CCL20 production in epithelial tissues promotes local accumulation of DCs via a CCR6-dependent mechanism.

## CCR6/CCL20 Is Required for CD8$^+$ T Cell Crosspriming Induced by DNFB or NP

We previously documented that DNFB-specific cytotoxic CD8$^+$ T cells, induced by transepithelial skin or buccal immunization, mediate contact hypersensitivity (CHS) responses (Desvignes et al., 1998; Kehren et al., 1999). Hapten-specific IFN$\gamma$-producing CD8$^+$ T cells can be efficiently primed in C57BL/6 mice after cutaneous immunization with doses of DNFB down to 0.1% (Figure 4A). Interestingly, CCR6 deficiency impaired hapten-specific response, especially at suboptimal doses of DNFB, and only CCR6-sufficient mice were primed at the lowest dose of 0.1% DNFB. These findings demonstrate that CCR6 is critical for priming of hapten-specific CD8$^+$ T cells with infra-optimal doses of Ag, which most likely requires its local uptake by DCs.

We reasoned that CCR6-mediated DC recruitment into epithelial tissues might be a prerequisite for priming of CD8$^+$ CTL against innocuous protein Ag. To test this hypothesis, we used OVA as a prototype of poorly immunogenic protein and exploited the adjuvant properties of DNFB and NP, which are both capable of

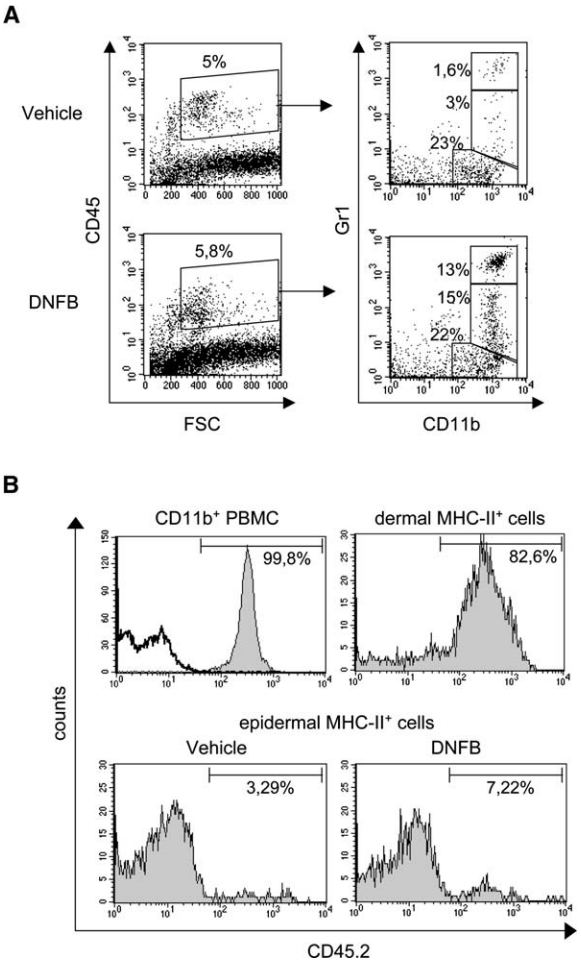


Figure 2. Circulating DC Precursors Are Recruited to the Skin and Contribute to LC Accumulation

(A) Epidermal cell suspensions from ear skin were prepared six hours after painting with DNFB or vehicle alone, and hematopoietic cells ($CD45^+$) were analyzed for the expression of CD11b and Gr1. Results are representative of three independent experiments.

(B) The ears of CD45.2→CD45.1 chimeric mice were painted with DNFB or vehicle alone and analyzed 6 hr later. Histogram plots show expression of CD45.2 (donor) on gated blood $CD11b^+$ PBMCs, MHC $II^+$ dermal cells, and MHC $II^+$ epidermal DCs. The percentage of $CD45.2^+$ cells is indicated. These data are representative of two independent experiments.

inducing OVA-specific $CD8^+$ T cell effectors via the buccal mucosa or skin (Figure 4 and N.E., M.L.B., and D.K., unpublished data). As expected, immunization with 1 mg of OVA alone was poorly efficient at generating OVA-specific IFN$\gamma$-producing $CD8^+$ T cells (Figure 4B). Alternatively, OVA+DNFB immunization via either buccal mucosa (Figure 4B) or skin (Figure 4C) generated a significant frequency of functional OVA-specific $CD8^+$ CTL effectors as demonstrated both by IFN$\gamma$ ELISPOT and in vivo CTL assays (Figure 4D). Interestingly, DNFB can adjuvant OVA-specific $CD8^+$ CTL responses in the absence of $CD4^+$ T cell help, as demonstrated by using I-A$\beta$ deficient mice (see Figure S1 in the Supplemental Data available with this article online). Comparable results were obtained with doses of OVA down to 100 $\mu$g per mouse (data not shown). Likewise, buccal immunization with NP+OVA generated OVA-specific IFN$\gamma$-producing $CD8^+$ effector T cells (Figure 4E). Remarkably, the adjuvant-dependent, OVA-specific $CD8^+$ T cell priming was critically dependent on CCR6, insomuch as DNFB+OVA or NP+OVA immunization was unable to induce significant OVA-specific class I-restricted $CD8^+$ T cell priming in $CCR6^{-/-}$ mice. In addition, immunization with OVA+NP in the presence of a neutralizing anti-CCL20 Ab strongly reduced the frequency of SIINFEKL-specific IFN$\gamma$-producing $CD8^+$ T cells, to a level similar to that observed in $CCR6^{-/-}$ mice (Figure 4E). These findings demonstrate that the CCR6/CCL20 pathway dictates successful crosspriming of $CD8^+$ CTL after transdermal immunization.

### Most Adjuvants Require CCR6 to Induce $CD8^+$ T Cell Crosspriming

We then examined whether the need for CCR6 could be generalized to other $CD8^+$ T cell adjuvants. Cholera toxin (CT) and Flagellin (FliC) from Salmonella, two classical mucosal adjuvants (Anjuere et al., 2004; Didierlaurent et al., 2004) that similarly to DNFB and NP induce DC recruitment into epithelia by triggering CCL20 production (Anjuere et al., 2004; Sierro et al., 2001), also promoted generation of OVA-specific $CD8^+$ T cell effectors less efficiently in $CCR6^{-/-}$ mice than in wt mice (Figures 5A and 5B). Likewise, poly(I:C) (especially at low doses) exhibited a CCR6-dependent CD8 adjuvant effect (Figure 5C). In contrast, CpG ODN did not require CCR6 to promote $CD8^+$ T cell priming (Figure 5D). Thus, efficient $CD8^+$ T cell priming with most adjuvants, but not CpG ODN, critically depends on CCR6 expression.

### Transfer of $Gr1^+$ Monocytes Restores $CD8^+$ T Cell Crosspriming in CCR6-Deficient Mice

The above results suggest that epithelial delivery of adjuvants, by promoting the recruitment of circulating DC precursors, allows for $CD8^+$ T cell crosspriming against the soluble coadministrated protein Ag and that this process requires a functional CCR6/CCL20 pathway. Thus, we tested whether adoptive transfer of blood DC precursors at the time of OVA+DNFB cutaneous immunization was sufficient to restore efficient OVA-specific $CD8^+$ T cell response in CCR6-deficient mice. Remarkably, $CCR6^+$, but not $CCR6^-$, blood myeloid cells fully restored the functional defect in $CCR6^{-/-}$ mice, resulting in a frequency of OVA-specific IFN$\gamma$-producing $CD8^+$ T cells comparable to that of wt mice (Figure 6A). Myeloid mononuclear cells contain $CD115^+$ monocytes and $CD11c^+$ cells (Figures S2 and S3A), which can both differentiate into DCs in vivo (del Hoyo et al., 2002; Geissmann et al., 2003; Randolph et al., 1999). Importantly, the rescuing effect was restricted to $CD115^+$ blood monocytes (Figure S3B) and appeared as a hallmark of $Gr1^+$ monocytes (Figure 6B), a subset of monocytes that has been shown to preferentially home to inflamed tissues (Geissmann et al., 2003; Sunderkotter et al., 2004), including DNFB-painted skin (Figure 2A). Interestingly, transfer of monocytes by intravenous injection or intradermal injection at the site of immunization similarly restored $CD8^+$ T cell priming (Figure 6C). Altogether, these data demonstrate that CCR6-sufficient $Gr1^+$ monocytes, which are rapidly mobilized to the site of

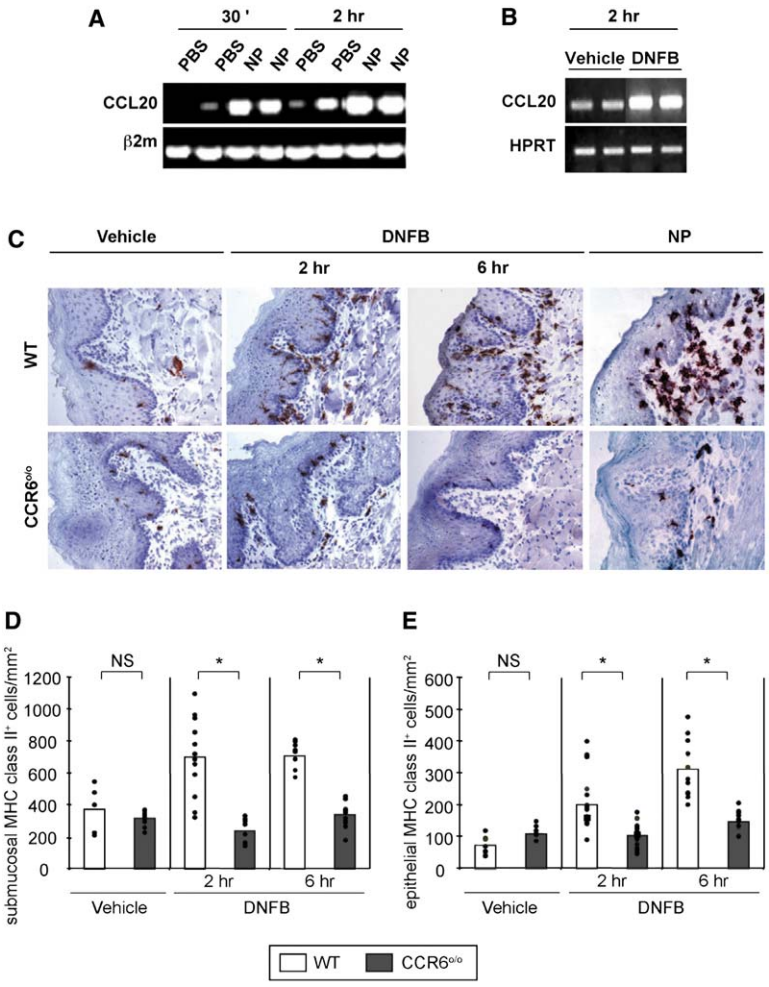


Figure 3. DC Accumulation Is Associated with Increased CCL20 Production and Is Dependent on CCR6

(A and B) RT-PCR analysis of CCL20 expression in the buccal mucosa 30 min or 2 hr after NP injection (A) or topical application of DNFB (B). Primers for β2m (A) and HPRT (B) were used as controls.

(C) Staining for MHC class II was conducted on sections of buccal mucosa of B6 ([C], upper) and CCR6$^{o/o}$ ([C], lower) mice after DNFB (2 and 6 hr) or NP (2 hr) immunization. Final magnification was ×400.

(D and E) Semiquantitative analysis of MHC class II$^+$ cells in the lamina propria (D) and epithelium (E) of B6 (white bars) and CCR6$^{o/o}$ (black bars) mice. Results are expressed as the number of class II$^+$ cells per square millimeter of tissue (*$p < 0.01$; NS, not significant). Each dot represents the mean of four fields in four sections in one buccal mucosa; each histogram bar represents mean values of three to six mice per group.

cutaneous immunization, play a critical role in CD8$^+$ T cell crosspriming.

### H2-K$^{bm1}$ Monocytes Failed to Restore CD8$^+$ T Cell Crosspriming in CCR6-Deficient Mice

To determine whether monocytes need to present the Ag to CD8$^+$ T cells to allow efficient in vivo priming, similar transfer experiments were performed with monocytes purified from H2-K$^{bm1}$ mice. These mice have mutated K$^b$ MHC class I molecules that alter the presentation of the dominant SIINFEKL OVA peptide and thus prevent CD8$^+$ T cell priming (Nikolic-Zugic and Carbone, 1990). As shown in Figure 6D, adoptive transfer of blood myeloid cells from H2-K$^{bm1}$ mice into CCR6$^{o/o}$ H2-K$^b$ recipients poorly ameliorated the efficacy of OVA+DNFB immunization as compared to the transfer of monocytes expressing functional MHC class I molecules. This demonstrates that presentation of the Ag to T cells by the progeny of monocytes represents a critical step for CD8$^+$ T cell crosspriming.

### Clodronate Liposomes Impair CD8$^+$ T Cell Crosspriming

To further confirm the contribution of monocytes to the induction of CD8$^+$ T cell crosspriming, we analyzed the consequences of clodronate (clo-lipo) injection 24 hr prior to OVA+DNFB immunization in B6 mice. Such treatment resulted in the transient depletion of >96% of circulating monocytes for 48 hr (Sunderkotter et al., 2004 and Figure 7A). As depicted in Figure 7B, clo-lipo treatment strongly impaired OVA-specific CD8$^+$ T priming. Thus, the absence of circulating monocytes at the time of immunization correlates with a reduced capacity to initiate a CD8 response.

## Discussion

Priming of specific CD8$^+$ effector T cells after immunization with soluble proteins via mucosa or skin may critically depend on the number, origin and function of DC subsets available at the site of immunization, their access to sufficient amounts of Ag, and their migratory capacity. This study demonstrates that DCs, newly recruited into epithelial tissues by adjuvants, are responsible for efficient in vivo crosspriming of CD8$^+$ cytotoxic T cells to a coadministered protein Ag and highlights the crucial role played by the CCR6/CCL20 pathway in this process.

We previously documented that the proinflammatory hapten DNFB and measles virus NP are two powerful CD8$^+$ T cell immunogens (Etchart et al., 2001; Kehren et al., 1999) that share the remarkable property of triggering rapid, transient, and local increase of MHC II$^+$ cells when administrated via pluristratified epithelia

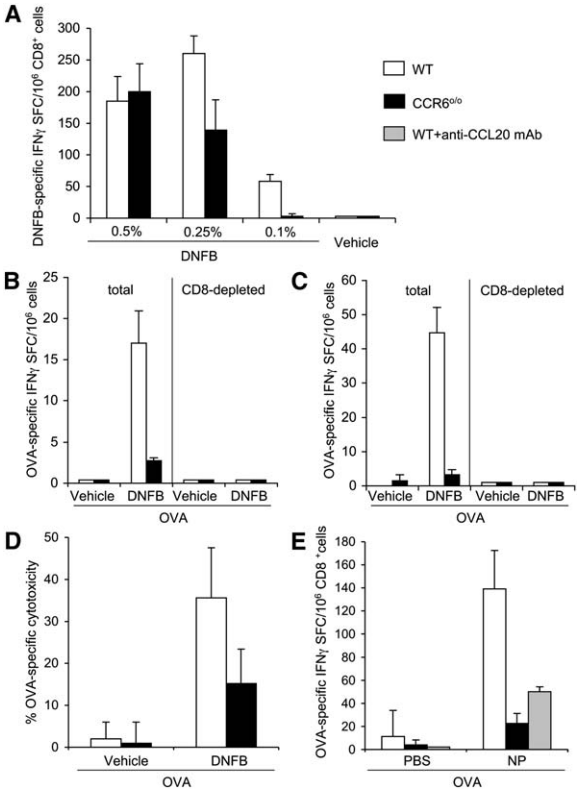


Figure 4. Critical Role of CCR6/CCL20 Interaction for Induction of Ag-Specific IFNγ-Producing CD8+ CTL

(A) B6 (white bars) or CCR6$^{o/o}$ mice (black bars) were painted on the shaved abdominal skin with various doses of DNFB or vehicle alone. The frequency of hapten-specific IFNγ-producing CD8+ T cells was determined 5 days later in skin draining lymph nodes.

(B–E) B6 (white bars), CCR6$^{o/o}$ (black bars), or anti-CCL20-treated B6 mice (gray bars) were immunized with either OVA (1 mg) + DNFB (0.5%) administered via the buccal mucosa (B and D) or the ear skin (C), or OVA (1 mg) +NP (30 μg) delivered via the buccal mucosa (E). (B and C) Mean frequency of OVA-specific IFNγ SFC in unfractionated or CD8+-depleted draining lymph node cells. No IFNγ-SFC was observed when cells were incubated without SIINFEKL.

(D) In vivo OVA-specific CTL response was determined in draining LN as described in the Experimental Procedures. Results are expressed as the mean percentage of OVA-specific cytotoxicity ±SD.

(E) Frequency of OVA-specific IFNγ CD8+ SFC was determined in the spleen. Data are representative of five experiments with three mice per group.

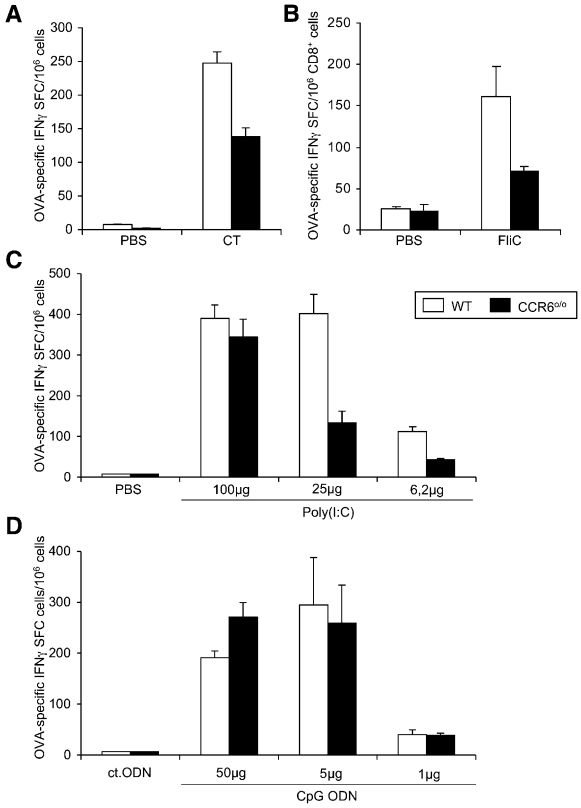


Figure 5. Most Adjuvants Require CCR6 Expression to Induce CD8+ T Cell Priming

B6 (white bars) or CCR6$^{o/o}$ (black bars) mice were immunized via the buccal mucosa with OVA+PBS (A–C), OVA+CT (A), OVA+FliC (B), OVA+poly(I:C) (C), OVA+CpG ODN1668, or OVA+control-ODN1720 (D). Frequency of OVA-specific IFNγ-SFC in unfractionated draining lymph nodes (A, C, and D) and in purified spleen CD8+ T cells (B). Results are representative of two (CT, FliC and CpG ODN) to three (poly[I:C]) experiments with three mice per group.

(Desvignes et al., 1998; Etchart et al., 2001). Here, we demonstrate that most of these cells, which accumulate at the dermo-epithelial junction and in the suprabasal layer of the epithelium, are MHC class II+CD11c+ myeloid DCs and include both dermal DCs and epithelial Langerin+ DCs. By using bone marrow chimeras, Merad et al. (2002) demonstrated that under steady-state conditions, LCs are constantly renewed in the skin independently of circulating precursors, whereas, after UV-induced inflammation, LCs are replaced by blood-borne LC progenitors. By using the same approach, we demonstrated that rapid mobilization of circulating DC precursors into epithelial tissues significantly contributes to LC accumulation at the site of DNFB immunization. DC accumulation was first detected in dermal papillas, a highly vascularized area, further arguing for a contribution of cells recruited from blood. Indeed, Gr1+ monocytes, the most abundant blood DC precursors (Geissmann et al., 2003), are rapidly mobilized to the skin after DNFB immunization. This is consistent with other studies demonstrating that Gr1+ blood monocytes rapidly extravasate into inflamed tissues and subsequently differentiate into cells with features of DC (Geissmann et al., 2003; Qu et al., 2004; Sunderkotter et al., 2004). Thus, although recruitment of tissue-resident DCs from adjacent skin areas likely occurs after immunization, extravasation of blood precursors, including Gr1+ monocytes, in the dermis or lamina propria significantly contributes to DC accumulation at the site of immunization.

Extravasation of blood leukocytes into epithelial tissues and their further migration within the dermal and epidermal compartments is governed by local gradients of chemokines. The epithelial tissue chemokine CCL20 has the capacity to induce in vitro chemotaxis of immature DCs, including monocyte-derived DCs, LCs, and LC-committed progenitors (Dieu-Nosjean et al., 2000; Yang et al., 1999), via binding to its unique receptor CCR6 (Cook et al., 2000). At the steady-state, CCR6/CCL20 is dispensable for homing and renewal of DCs into pluristratified epithelial tissues such as skin (Cook et al., 2000) and buccal mucosa (this study). Alternatively, CCL20 could recruit immature DCs into epithelia

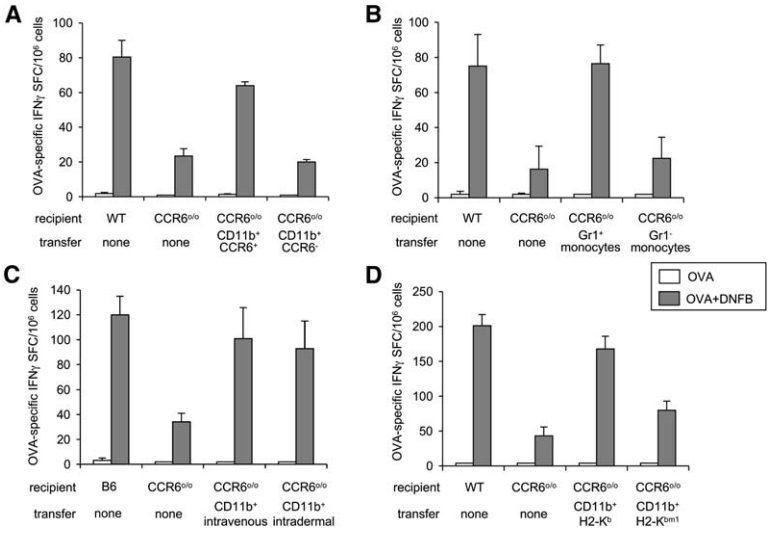


Figure 6. Transfer of CCR6$^+$ Blood Gr1$^+$ Monocytes Restores OVA-Specific CD8$^+$ T Cell Priming in CCR6$^{°/°}$ Recipients

CCR6$^{°/°}$ mice received an intravenous (A–D) or intradermal (C) transfer of enriched blood monocytes from B6 (A, C, and D), CCR6$^{°/°}$ (A), or H-2K$^{bm1}$ mice (D) or of purified Gr1$^+$ or Gr1$^-$ CD115$^+$ monocytes from B6 mice (B). Mice were immunized with OVA alone (white bars) or OVA+DNFB (gray bars) via the ear skin, and the frequency of OVA-specific IFN$\gamma$ SFC in draining lymph nodes was determined. No IFN$\gamma$-SFC was observed when cells were incubated without SIINFEKL. All experiments, which included three mice per group, were performed at least twice with similar results.

during inflammatory conditions such as in the cases of psoriasis (Dieu-Nosjean et al., 2000) and graft-versus-host disease (Merad et al., 2004). Our finding that adjuvant-induced DC recruitment is observed in wt, but not in CCR6$^{°/°}$ mice, provides in vivo evidence in a physiological setting that inflammation caused by transepithelial (DNFB) or transdermal (NP) immunization induces local CCL20 secretion and promotes CCR6-mediated DC chemotaxis into epithelial tissues. The fact that neither circulating DCs nor monocytes respond to CCL20 in vitro (Vanbervliet et al., 2002) and that monocytes do not express significant levels of CCR6 (Geissmann et al., 2003) makes it unlikely that CCR6-mediated chemotaxis dictates extravasation of blood DC or DC-precursors into the dermis or lamina propria. Most likely, a first set of chemokines, such as MCPs, promotes extravasation of DC precursors into the dermis, and CCL20 subsequently allows recruited cells to further navigate within the tissue and possibly reach the epidermis (Vanbervliet et al., 2002). In this scenario, CCR6 expression by neo-recruited DC precursors might be induced by locally produced factors and/or by the transendothelial migration process. This hypothesis is supported by studies showing that although transendothelial migration initiates monocyte differentiation into DCs, full acquisition of DC markers and functions requires additional signals (Randolph et al., 1998). We propose from our data that CCR6-mediated chemotaxis allows the nascent monocyte-derived DCs to gain access to cytokines, including GM-CSF, IL-4, TNF$\alpha$, IL-15, and TGF-$\beta$, produced by activated dermal mast cells and epithelial cells, which favor their differentiation into bona fide DCs (Chomarat et al., 2003; Mohamadzadeh et al., 2001). It is also possible that CCR6 engagement by CCL20 might directly contribute to DC differentiation, as was recently demonstrated for CCR8 (Qu et al., 2004).

DC accumulation in epithelial tissues has been reported after mucosal or skin delivery of microbial agents (McWilliam et al., 1996; Zhao et al., 2003), components thereof (Anjuere et al., 2004; Etchart et al., 2001), and proinflammatory haptens (Desvignes et al., 1998), yet contribution of this process to the induction of efficient T cell priming after transepithelial or transmucosal immunization has not so far been recognized. Our findings clearly demonstrate that local DC recruitment plays a critical role for CD8$^+$ T cell crosspriming either against DNFB used as an immunogen or against the innocuous OVA protein Ag combined with DNFB or NP as adjuvants. This conclusion is supported by the observation that CCR6-deficient mice, which are unable to recruit DC at the site of immunization, failed to generate hapten-specific CD8$^+$ effectors at a suboptimal dose of Ag. In addition, CD8$^+$ T cell priming in response to local immunization with the innocuous protein OVA, with either DNFB or NP as an adjuvant, critically depended on CCR6. Finally, generation of OVA-specific IFN$\gamma$-producing CD8$^+$ CTL effectors was dramatically impaired both in CCR6$^{°/°}$ mice and in wt mice that received a neutralizing anti-CCL20 Ab together with the immunogen.

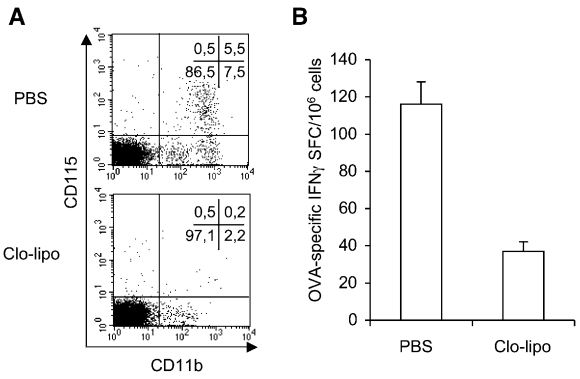


Figure 7. Injection of Clodronate Liposomes Impairs OVA-Specific CD8$^+$ T Cell Priming in B6 Mice

B6 mice were injected intravenously with clo-lipo 24 hr prior to immunization with OVA+DNFB via the ear skin.

(A) Analysis of CD11b and CD115 expression on PBMCs in unteated or clo-lipo-injected mice reveals nearly complete absence of monocytes in blood 24 hr after injection.

(B) Frequency of OVA-specific IFN$\gamma$ SFC in draining lymph nodes. No IFN$\gamma$-SFC was observed in mice immunized with OVA alone. These data are representative of two independent experiments with three mice per group.

Several observations argue for an essential role of circulating monocytes and their progeny in CCR6-dependent $CD8^+$ T cell priming. First, adoptive or local transfer of blood monocytes from wt (but not $CCR6^{o/o}$) mice was sufficient to fully rescue the OVA-specific CD8 response in $CCR6^{o/o}$ mice. Second, in vivo depletion of circulating monocytes before immunization strongly impaired $CD8^+$ T cell crosspriming in normal mice. Third, the capacity of monocytes to restore $CD8^+$ T cell priming was confined to the subset of $Gr1^+$ monocytes previously reported to home to inflamed tissues (Geissmann et al., 2003; Sunderkotter et al., 2004), including skin (Qu et al., 2004), and to differentiate into DCs (Randolph et al., 1999). Finally, $Gr1^+$ monocytes are rapidly recruited into the skin at the site of (DNFB) immunization. Thus, although we cannot formally exclude that circulating monocytes may directly differentiate into lymph node DCs, our findings strongly support that monocytes recruited by adjuvants at the site of immunization give rise to immunostimulatory DCs that are required for successful $CD8^+$ T cell crosspriming.

An important issue with respect to vaccine development is whether the need for newly recruited DCs for efficient $CD8^+$ T cell priming can be generalized to conventional adjuvants. Interestingly, we found that both CT and FliC, which share with DNFB and NP the ability to recruit DCs into epithelia via CCR6 (Anjuere et al., 2004; Sierro et al., 2001), induce priming of OVA-specific $CD8^+$ T cells by a mechanism that is at least partly dependent on CCR6. Titrating poly(I:C), but not CpG ODN, also revealed a window of doses at which CCR6/CCL20 is required for $CD8^+$ T cell crosspriming. That CpG acts independently of CCR6 indicates that this type of adjuvant either bypasses the need for monocyte recruitment at the immunization site or triggers alternative pathways of DC recruitment. It is thus tempting to speculate that depending on the nature and dose of the adjuvant used, distinct subsets of DCs may be involved in the induction of CD8 responses (Figure S4). The finding that B6, but not $H\text{-}2K^{bm1}$, monocytes harboring nonfunctional mutated MHC class I molecules restored $CD8^+$ T cell crosspriming in CCR6-deficient mice strongly supports the hypothesis that direct Ag presentation by newly recruited monocyte-derived DCs to naive $CD8^+$ T cells is instrumental for CCR6-dependent $CD8^+$ T cell crosspriming. Alternatively, CpG, by inducing functional activation of LN DCs (Shah et al., 2003), may promote presentation to $CD8^+$ T cells of even small amounts of free Ag that drain from epithelial tissues into the draining lymph nodes (Figure S4).

There is increasing evidence against a prominent role of LC in T cell priming induced by mucosal or cutaneous immunization or infection (Allan et al., 2003; Kamath et al., 2002; Kaplan et al., 2005; Kissenpfennig et al., 2005; Ritter et al., 2004; Zhao et al., 2003), and some authors propose that dermal or interstitial DCs might indeed constitute the key immunostimulatory APC (Itano et al., 2003; Kissenpfennig et al., 2005; Zhao et al., 2003). The present study identifies the progeny of recruited monocytes as the critical APC for inducing T cell priming, at least in the case of $CD8^+$ T cells. It is unlikely that recruited cells simply add to the pool of resident DCs leading to an increased number of APC in the draining lymph nodes. Alternatively, it may be postulated that monocytes recruited from blood provide a source of freshly differentiating DCs able to respond better to danger signals than terminally differentiated resident DCs that have experienced the immunosuppressive tissue microenvironment (rich in IL-10 and TGF$\beta$). In addition, as demonstrated for CCR5 (Aliberti et al., 2000), CCR6 engagement may not solely be involved in DC chemotaxis but could also possibly favor their subsequent maturation and/or immunolostimulatory function.

The present study demonstrates that blood-derived myeloid DCs freshly recruited into epithelial tissues are essential for successful priming of $CD8^+$ CTL against an inert protein. This finding may have important implications both for anti-infectious and antitumor vaccine development. We propose that selection of molecules with the dual capacity to induce local proinflammatory cytokines and CCL20 production in epithelial tissues in vivo should be most relevant to the design of mucosal or skin vaccines.

## Experimental Procedures

### Mice

C57BL/6 female mice were purchased from Charles River Laboratories (L'Arbresle, France). $CCR6^{o/o}$ mice (Cook et al., 2000) on the B6 background, C57BL/6-Ly5a, and $H\text{-}2K^{bm1}$ mice were bred at the institute's animal facility (Plateau de Biologie Expérimentale de la Souris-Ecole Normale Supérieure de Lyon) under pathogen-free conditions. For all experiments, 4- to 10-week-old mice were used. Experiments have been approved by the Comité Regional d'Ethique pour l'Expérimentation Animale.

### Transplantation of Congenic Bone Marrow Cells

Seven- to eight-week-old recipient $CD45.1^+$ C57Bl/6 mice were lethally irradiated (10 Gray) and were reconstituted by intravenous injection of $8 \times 10^6$ bone marrow cells isolated from $CD45.2^+$ mice. Eight weeks after transplantation, mice were ear sensitized with DNFB (Sigma-Aldrich, St. Quentin Fallavier, France). Six hr after immunization, ears were split into dorsal and ventral halves and incubated for 45 min at 37°C with 0.25% trypsin in PBS. Epidermal and dermal sheets were separated and cut into small pieces. Dermal fragments were further incubated for 60 min at 37°C with 1000 U/ml Collagenase IA (Sigma), 1000 U/ml Hyaluronidase (Sigma), and 200 U/ml DNase I (Roche Diagnostics, Mannheim, Germany). Peripheral blood was harvested by cardiac puncture. PBMCs were isolated by Lympholyte M gradient, and residual red blood cells were lysed with 0.83% $NH_4Cl$. Cells were incubated with 2.4G2 hybridoma supernatant to block Fc$\gamma$ receptors and then stained with various combinations of the following anti-mouse antibodies (BD Pharmingen): FITC anti-$I\text{-}A^b$ (2G9), PE anti-$I\text{-}A^b$ (M5/114.15.2), PE anti-CD45.1 (A20), PerCP-Cy5.5 anti-CD11b Ab (M1/70), and biotinylated anti-CD45.2 (104). Immunofluorescence was analyzed on a FACScalibur flow cytometer with CellQuest software (Becton Dickinson).

### Immunizations and Ab Treatment

Mice under pentobarbital anesthesia were injected intradermally, either into the ear pinnae or the buccal mucosa (Etchart et al., 2001), with 15 $\mu$l of PBS containing 0.1–1 mg of LPS-free ovalbumin (OVA, Seikagaku corporation, Tokyo, Japan) alone or mixed with either 30 $\mu$g recombinant NP produced in baculovirus (Etchart et al., 2001), 1 $\mu$g CT (Sigma-Aldrich), 10 $\mu$g flagellin from Salmonella *enterica serovar typhimurium* (FliC) prepared as previously described (Sierro et al., 2001), 100 $\mu$g poly(I:C) (Sigma-Aldrich), or 50 $\mu$g of CpG ODN (5′-TCCATGACGTTCCTGATGCT-3′) or of control ODN (5′-TCCATGAGCTTCCTGATGCT-3′) (MWG Biotech, France) as adjuvants. Endotoxin levels in OVA and recombinant NP preparations were <0.1 U/mg as determined by the Limulus assay (Bio Whittaker, Walkersville, USA). Coimmunization with OVA+DNFB was carried out the same way as OVA+NP immunization except that DNFB (diluted at 0.5% in acetone:olive oil, 4:1 v/v) was painted onto the

ears or buccal mucosa (Desvignes et al., 1998) immediately after OVA administration. For certain experiments, mice received an intradermal injection of 30 μg of neutralizing anti-mouse CCL20 polyclonal goat IgG Ab (R&D Systems) into the buccal mucosa just before coimmunization with OVA+NP. For certain experiments, 200 μl of dichloromethylene-bisphosphonate liposomes (clo-lipo) were injected i.v. 24 hr before immunization. Clodronate was a gift from Roche (Mannheim, Germany) and was incorporated into liposomes as described elsewhere (Van Rooijen and Sanders, 1994).

### Histology and Immunohistochemistry

Buccal mucosae were deep frozen in Cryomount (Histolab, Göteborg, Sweden) for immunohistochemical analysis or paraffin-embedded for histological staining with hematoxylin phloxin safran (HPS). Cryostat sections (5 μm thick) were fixed in acetone for 20 min, rehydrated in PBS, and incubated for 1 hr at room temperature with specific antibodies including anti-MHC class II mAb (CD311) (Etchart et al., 2001), anti-Langerin mAb (929 F3) kindly provided by Sem Saeland (Schering-Plough, Dardilly, France), anti-CD11b mAb (M1/70), or overnight at 4ºC with anti-CD11c mAb (N418). Specific binding was revealed by 30 min incubation with biotinylated mouse adsorbed F(ab')$_2$ rabbit anti-rat IgG or goat anti-hamster IgG antibodies (Vector Laboratories, Burlingame, CA) followed by 30 min incubation with either streptavidin biotinylated horse raddish peroxidase (HRP) (Amersham Biosciences, UK) or with StreptABCComplex/HRP (Dako, Carpenteria, CA). Sections were incubated with 3-amino-9-ethylcarbazole (Dako) and counterstained with hematoxylin (Dako). Cell counting was performed by using an Axioscope microscope equipped with Zeiss Axiovision software (Zeiss, Germany). Statistical analysis was performed by using the Mann-Whitney-Wilcoxon nonparametric U test.

### RT-PCR Analysis of CCL20 Gene Expression

Tissue samples were snap-frozen in liquid nitrogen and then crushed into powder in a cold mortar over dry ice. For each sample, the powder was resuspended in 700 μl 4 M guanidium thyocyanate, 25 mM Na citrate (pH 7.0), 0.5% Sarcosyl, and 0.1 M β-mercaptoethanol solution (all from Sigma-Aldrich), and RNA was extracted according to standard procedures (Chomczynski and Sacchi, 1987). Total RNA (5 μg) was pretreated with DNase I in the presence of RNase inhibitor before reverse transcription into cDNAs by using oligo(dT) primers (Pharmacia, Uppsala, Sweden) and the SuperScrip kit. RT-PCR was performed with the AmpliTaq enzyme and buffer (Perkin-Elmer, Paris, France), dNTPs at 0.8 mM, and 5% DMSO (final concentration). Cycle conditions were 92ºC for 1 min, 60ºC for 2 min, and 72ºC for 3 min for 28 to 35 cycles. The following primers were used in this study: Beta-2 microglobulin, 3′-TAGTCTTCCTGGTGC TTGTC-5′, 5′-TGCTATTTCTTTCTGCGTGC-3′; CCL20, 3′-TGGGTA CTGCTGGCTCAC-5′, 5′-CATCTTCTTGACTCTTAGGCT-3′; HPRT, 3′-GTAATGATCAGTCAACGGGGGAC-5′, 5′-CCAGCAAGCTTGCAA CCTTAACCA-3′.

### IFNγ ELISPOT Assay

Seven to ten days after immunization, graded numbers of unfractionated or CD8$^+$ T cells were incubated for 36 hr at 37ºC in nitrocellulose 96 well plates (MAHA 45, Millipore, Bedford, MA) coated with the anti-IFNγ R46A2 mAb (Pharmingen). Purified spleen CD8$^+$ T cells were incubated with irradiated splenocytes and 2 μM SIINFEKL or 0.4 mM DNBS. Total or CD8$^+$ T cell-depleted lymph node cells were stimulated with either 2 μM SIINFEKL or with 0.4 mM DNBS. IFNγ-producing cells were detected by using biotinylated anti-IFNγ Ab (XMG1.2, Pharmingen), streptavidin-alkaline phosphatase (Roche Diagnostics), and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich). The number of IFNγ spot forming cells (SFC) was counted in each well by using a binocular, and the results were expressed as the number of IFNγ SFC per $10^6$ cells.

### In Vivo Cytotoxic Assay

CD8$^+$ T cell cytotoxicity was assessed in vivo as previously described (Aichele et al., 1997). Seven to ten days after immunization, mice were injected i.v. with a mixture of $10 \times 10^6$ SIINFEKL-pulsed (2 μM) and $10 \times 10^6$ unpulsed spleen cells previously stained with a low (0.5 μM) or high dose (5 μM) of CSFE (Molecular Probes, PooetGebouw, The Netherlands), respectively. Twenty hours later, spleen cells were collected, and 10,000 CFSE$^+$ cells were analyzed on the FACSCalibur (BD Biosciences). In vivo cytotoxicity was calculated by determining the ratio of control targets/pulsed targets.

### Adoptive Transfer of Monocytes

PBMCs were isolated by Lympholyte M (Cedarlane) gradient, and residual red blood cells were lysed with 0.83% $NH_4Cl$. For transfer of unfractionated myeloid PBMCs, CD4$^+$, CD8$^+$, and CD19$^+$ cells were depleted with a mix of anti-CD4, anti-CD8, and anti-CD19 mAb-coated microbeads and LD columns (Miltenyi Biotec). Purified cells contained 80%–90% CD11b$^+$ cells including 40%–60% of monocytes as determined by CD115 expression (Figure S2). Blood CD115$^+$Gr1$^+$ and CD115$^-$Gr1$^-$ monocytes were purified from CD3-deficient mice by cell sorting with a FacsVantage (Becton Dickinson). $10^5$ cells were injected either i.v. or i.d. at the site of immunization into CCR6$^{o/o}$ mice 10 min before ear-skin immunization with OVA+DNFB.

### Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.immunity.com/cgi/content/full/24/2/191/DC1/.

### Acknowledgments

The authors would like to thank G. Trinchieri and S. Amigorena for helpful discussions, and the flow cytometry facility and the animal facility (PBES) for continuous help. This work was supported by an institutional grant from INSERM and a specific financial support from Schering-Plough. A.G. benefited from a doctoral fellowship from Fondation pour la Recherche Médicale.

Received: January 18, 2005
Revised: December 9, 2005
Accepted: January 12, 2006
Published: February 14, 2006

### References

Aichele, P., Brduscha-Riem, K., Oehen, S., Odermatt, B., Zinkernagel, R.M., Hengartner, H., and Pircher, H. (1997). Peptide antigen treatment of naive and virus-immune mice: antigen-specific tolerance versus immunopathology. Immunity *6*, 519–529.

Aliberti, J., Reis e Sousa, C., Schito, M., Hieny, S., Wells, T., Huffnagle, G.B., and Sher, A. (2000). CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha+ dendritic cells. Nat. Immunol. *1*, 83–87.

Allan, R.S., Smith, C.M., Belz, G.T., van Lint, A.L., Wakim, L.M., Heath, W.R., and Carbone, F.R. (2003). Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. Science *301*, 1925–1928.

Anjuere, F., Luci, C., Lebens, M., Rousseau, D., Hervouet, C., Milon, G., Holmgren, J., Ardavin, C., and Czerkinsky, C. (2004). In vivo adjuvant-induced mobilization and maturation of gut dendritic cells after oral administration of cholera toxin. J. Immunol. *173*, 5103–5111.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245–252.

Belz, G.T., Smith, C.M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F.R., and Heath, W.R. (2004). Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. Proc. Natl. Acad. Sci. USA *101*, 8670–8675.

Bennett, C.L., van Rijn, E., Jung, S., Inaba, K., Steinman, R.M., Kapsenberg, M.L., and Clausen, B.E. (2005). Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. J. Cell Biol. *169*, 569–576.

Charbonnier, A.S., Kohrgruber, N., Kriehuber, E., Stingl, G., Rot, A., and Maurer, D. (1999). Macrophage inflammatory protein 3alpha is involved in the constitutive trafficking of epidermal langerhans cells. J. Exp. Med. *190*, 1755–1768.

Chomarat, P., Dantin, C., Bennett, L., Banchereau, J., and Palucka, A.K. (2003). TNF skews monocyte differentiation from macrophages to dendritic cells. J. Immunol. *171*, 2262–2269.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Cook, D.N., Prosser, D.M., Forster, R., Zhang, J., Kuklin, N.A., Abbondanzo, S.J., Niu, X.D., Chen, S.C., Manfra, D.J., Wiekowski, M.T., et al. (2000). CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. Immunity *12*, 495–503.

del Hoyo, G.M., Martin, P., Vargas, H.H., Ruiz, S., Arias, C.F., and Ardavin, C. (2002). Characterization of a common precursor population for dendritic cells. Nature *415*, 1043–1047.

Desvignes, C., Esteves, F., Etchart, N., Bella, C., Czerkinsky, C., and Kaiserlian, D. (1998). The murine buccal mucosa is an inductive site for priming class I-restricted CD8+ effector T cells in vivo. Clin. Exp. Immunol. *113*, 386–393.

Didierlaurent, A., Ferrero, I., Otten, L.A., Dubois, B., Reinhardt, M., Carlsen, H., Blomhoff, R., Akira, S., Kraehenbuhl, J.P., and Sirard, J.C. (2004). Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. J. Immunol. *172*, 6922–6930.

Dieu-Nosjean, M.C., Vicari, A., Lebecque, S., and Caux, C. (1999). Regulation of dendritic cell trafficking: a process that involves the participation of selective chemokines. J. Leukoc. Biol. *66*, 252–262.

Dieu-Nosjean, M.C., Massacrier, C., Homey, B., Vanbervliet, B., Pin, J.J., Vicari, A., Lebecque, S., Dezutter-Dambuyant, C., Schmitt, D., Zlotnik, A., and Caux, C. (2000). Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. J. Exp. Med. *192*, 705–718.

Etchart, N., Desmoulins, P.O., Chemin, K., Maliszewski, C., Dubois, B., Wild, F., and Kaiserlian, D. (2001). Dendritic cells recruitment and in vivo priming of CD8(+) CTL induced by a single topical or transepithelial immunization via the buccal mucosa with measles virus nucleoprotein. J. Immunol. *167*, 384–391.

Geissmann, F., Jung, S., and Littman, D.R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity *19*, 71–82.

Heath, W.R., Belz, G.T., Behrens, G.M., Smith, C.M., Forehan, S.P., Parish, I.A., Davey, G.M., Wilson, N.S., Carbone, F.R., and Villadangos, J.A. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. Immunol. Rev. *199*, 9–26.

Itano, A.A., and Jenkins, M.K. (2003). Antigen presentation to naive CD4 T cells in the lymph node. Nat. Immunol. *4*, 733–739.

Itano, A.A., McSorley, S.J., Reinhardt, R.L., Ehst, B.D., Ingulli, E., Rudensky, A.Y., and Jenkins, M.K. (2003). Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. Immunity *19*, 47–57.

Kamath, A.T., Henri, S., Battye, F., Tough, D.F., and Shortman, K. (2002). Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. Blood *100*, 1734–1741.

Kaplan, D.H., Jenison, M.C., Saeland, S., Shlomchik, W.D., and Shlomchik, M.J. (2005). Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. Immunity *23*, 611–620.

Kehren, J., Desvignes, C., Krasteva, M., Ducluzeau, M.T., Assossou, O., Horand, F., Hahne, M., Kagi, D., Kaiserlian, D., and Nicolas, J.F. (1999). Cytotoxicity is mandatory for CD8(+) T cell-mediated contact hypersensitivity. J. Exp. Med. *189*, 779–786.

Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhe, C., Perrin, P., Romani, N., Tripp, C.H., Douillard, P., Leserman, L., Kaiserlian, D., et al. (2005). Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity *22*, 643–654.

Lagasse, E., and Weissman, I.L. (1996). Flow cytometric identification of murine neutrophils and monocytes. J. Immunol. Methods *197*, 139–150.

McWilliam, A.S., Napoli, S., Marsh, A.M., Pemper, F.L., Nelson, D.J., Pimm, C.L., Stumbles, P.A., Wells, T.N., and Holt, P.G. (1996). Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. J. Exp. Med. *184*, 2429–2432.

Merad, M., Manz, M.G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I.L., Cyster, J.G., and Engleman, E.G. (2002). Langerhans cells renew in the skin throughout life under steady-state conditions. Nat. Immunol. *3*, 1135–1141.

Merad, M., Hoffmann, P., Ranheim, E., Slaymaker, S., Manz, M.G., Lira, S.A., Charo, I., Cook, D.N., Weissman, I.L., Strober, S., and Engleman, E.G. (2004). Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. Nat. Med. *10*, 510–517.

Mohamadzadeh, M., Berard, F., Essert, G., Chalouni, C., Pulendran, B., Davoust, J., Bridges, G., Palucka, A.K., and Banchereau, J. (2001). Interleukin 15 skews monocyte differentiation into dendritic cells with features of Langerhans cells. J. Exp. Med. *194*, 1013–1020.

Nikolic-Zugic, J., and Carbone, F.R. (1990). The effect of mutations in the MHC class I peptide binding groove on the cytotoxic T lymphocyte recognition of the Kb-restricted ovalbumin determinant. Eur. J. Immunol. *20*, 2431–2437.

Qu, C., Edwards, E.W., Tacke, F., Angeli, V., Llodra, J., Sanchez-Schmitz, G., Garin, A., Haque, N.S., Peters, W., van Rooijen, N., et al. (2004). Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. J. Exp. Med. *200*, 1231–1241.

Randolph, G.J., Beaulieu, S., Lebecque, S., Steinman, R.M., and Muller, W. (1998). Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. Science *282*, 480–483.

Randolph, G.J., Inaba, K., Robbiani, D.F., Steinman, R.M., and Muller, W.A. (1999). Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. Immunity *11*, 753–761.

Ritter, U., Meissner, A., Scheidig, C., and Korner, H. (2004). CD8 alpha- and Langerin-negative dendritic cells, but not Langerhans cells, act as principal antigen-presenting cells in leishmaniasis. Eur. J. Immunol. *34*, 1542–1550.

Ruedl, C., Koebel, P., Bachmann, M., Hess, M., and Karjalainen, K. (2000). Anatomical origin of dendritic cells determines their life span in peripheral lymph nodes. J. Immunol. *165*, 4910–4916.

Schutyser, E., Struyf, S., and Van Damme, J. (2003). The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev. *14*, 409–426.

Shah, J.A., Darrah, P.A., Ambrozak, D.R., Turon, T.N., Mendez, S., Kirman, J., Wu, C.Y., Glaichenhaus, N., and Seder, R.A. (2003). Dendritic cells are responsible for the capacity of CpG oligodeoxynucleotides to act as an adjuvant for protective vaccine immunity against Leishmania major in mice. J. Exp. Med. *198*, 281–291.

Sierro, F., Dubois, B., Coste, A., Kaiserlian, D., Kraehenbuhl, J.P., and Sirard, J.C. (2001). Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. Proc. Natl. Acad. Sci. USA *98*, 13722–13727.

Sunderkotter, C., Nikolic, T., Dillon, M.J., Van Rooijen, N., Stehling, M., Drevets, D.A., and Leenen, P.J. (2004). Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J. Immunol. *172*, 4410–4417.

Valladeau, J., Ravel, O., Dezutter-Dambuyant, C., Moore, K., Kleijmeer, M., Liu, Y., Duvert-Frances, V., Vincent, C., Schmitt, D., Davoust, J., et al. (2000). Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. Immunity *12*, 71–81.

Valladeau, J., Clair-Moninot, V., Dezutter-Dambuyant, C., Pin, J.J., Kissenpfennig, A., Mattei, M.G., Ait-Yahia, S., Bates, E.E., Malissen, B., Koch, F., et al. (2002). Identification of mouse langerin/CD207 in Langerhans cells and some dendritic cells of lymphoid tissues. J. Immunol. *168*, 782–792.

Van Rooijen, N., and Sanders, A. (1994). Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Methods *174*, 83–93.

Vanbervliet, B., Homey, B., Durand, I., Massacrier, C., Ait-Yahia, S., de Bouteiller, O., Vicari, A., and Caux, C. (2002). Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. Eur. J. Immunol. *32*, 231–242.

Yang, D., Howard, O.M., Chen, Q., and Oppenheim, J.J. (1999). Cutting edge: immature dendritic cells generated from monocytes in the presence of TGF-beta 1 express functional C-C chemokine receptor 6. J. Immunol. *163*, 1737–1741.

Zhao, X., Deak, E., Soderberg, K., Linehan, M., Spezzano, D., Zhu, J., Knipe, D.M., and Iwasaki, A. (2003). Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. J. Exp. Med. *197*, 153–162.