

# Dermal CD14<sup>+</sup> Dendritic Cell and Macrophage Infection by Dengue Virus Is Stimulated by Interleukin-4

Evelyn Schaeffer<sup>1</sup>, Vincent Flacher<sup>1</sup>, Vasiliki Papageorgiou<sup>1</sup>, Marion Decossas<sup>1,2</sup>, Jean-Daniel Fauny<sup>1</sup>, Melanie Krämer<sup>1</sup> and Christopher G. Mueller<sup>1</sup>

Dengue virus (DENV) is responsible for the most prevalent arthropod-borne viral infection in humans. Events decisive for disease development occur in the skin after virus inoculation by the mosquito. Yet, the role of human dermis-resident immune cells in dengue infection and disease remains elusive. Here we investigated how dermal dendritic cells (dDCs) and macrophages (dMs) react to DENV and impact on immunopathology. We show that both CD1c<sup>+</sup> and CD14<sup>+</sup> dDC subsets were infected, but viral load greatly increased in CD14<sup>+</sup> dDCs upon IL-4 stimulation, which correlated with upregulation of virus-binding lectins Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN/CD209) and mannose receptor (CD206). IL-4 also enhanced T-cell activation by dDCs, which was further increased upon dengue infection. dMs purified from digested dermis were initially poorly infected but actively replicated the virus and produced TNF- $\alpha$  upon lectin upregulation in response to IL-4. DC-SIGN<sup>+</sup> cells are abundant in inflammatory skin with scabies infection or Th2-type dermatitis, suggesting that skin reactions to mosquito bites heighten the risk of infection and subsequent immunopathology. Our data identify dDCs and dMs as primary arbovirus target cells in humans and suggest that dDCs initiate a potent virus-directed T-cell response, whereas dMs fuel the inflammatory cascade characteristic of dengue fever.

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## INTRODUCTION

The skin is the portal to infectious pathogens, in particular those transmitted by biting insects. Dengue virus (DENV) is a positive-strand RNA virus that belongs to the *Flavivirus* genus of the flaviviridae family and is transmitted by *Aedes* mosquitoes. The virus is responsible for the most prevalent arthropod-borne viral infection in humans, with an estimate of 390 million cases per year worldwide (Bhatt *et al.*, 2013). Infec-

tion with DENV results in a wide spectrum of clinical manifestations ranging from mild, undifferentiated fever to hemorrhage and hypovolemic shock, which can be fatal if untreated (Rigau-Perez *et al.*, 1998). The global health burden of dengue infections is likely to further increase through sustained travel and vector spread; yet, efficient drugs or vaccines are currently unavailable. It is widely believed that the immune response mounted against the virus greatly contributes to pathogenesis (Green and Rothman, 2006; Mongkolsapaya *et al.*, 2003; Pang *et al.*, 2007); yet, our incomplete comprehension of the etiology of DENV-mediated diseases represents a serious hurdle to clinical prognosis and therapeutic action. A level of complexity in the understanding of arbovirus infection is introduced by the insect vector itself. Mosquito salivary proteins trigger Th2 polarization (Cox *et al.*, 2012; Espada-Murao and Morita, 2011; Thangamani *et al.*, 2010) and cause allergic skin reaction (Peng and Simons, 2007). Insect saliva enhances infection of a number of insect-vectorized pathogens including Dengue virus (Cox *et al.*, 2012; Styer *et al.*, 2011). Moreover, inflammatory skin manifestations are observed during an ongoing dengue infection (Saleem and Shaikh, 2008).

DENV, like many other pathogens, enters cells through carbohydrate-binding receptors that normally enable DCs and macrophages (Ms) to sample antigens (Navarro-Sanchez

<sup>1</sup>Laboratory of Immunopathology and Therapeutic Chemistry, CNRS UPR 3572/Laboratory of Excellence MEDALIS, IBMC, University of Strasbourg, Strasbourg, France

<sup>2</sup>Current address: Chemistry and Biology of Membranes and Nanoobjects, CBMN, UMR 5248, CNRS-University of Bordeaux1-IPB, 33600 Pessac, France.

Correspondence: Christopher G. Mueller, Laboratory of Immunopathology and Therapeutic Chemistry, CNRS UPR 3572/Laboratory of Excellence MEDALIS, IBMC, University of Strasbourg, 67084 Strasbourg, France.  
E-mail: c.mueller@ibmc-cnrs.unistra.fr

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; dDC, dermal DC; dM, dermal macrophage; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; DENV, Dengue virus; DHA, 4',6-diamidino-2-phenylindole; LC, Langerhans cell; M, macrophage; MR, mannose receptor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

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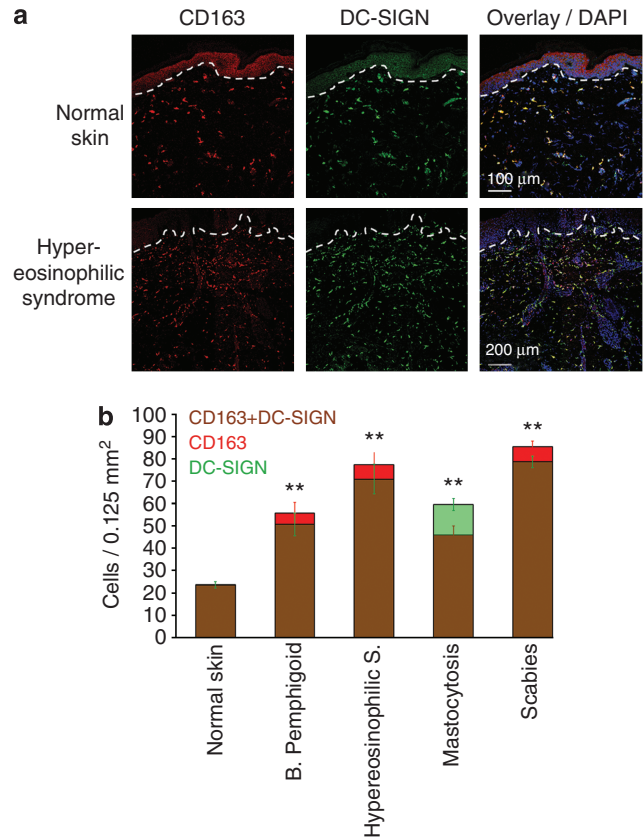
*et al.*, 2003; Tassaneeritthep *et al.*, 2003; van Kooyk and Geijtenbeek, 2003). Thus, monocyte-derived DCs and monocyte-derived Ms that carry the C-type lectins Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN/CD209) and Mannose Receptor (MR/CD206) are productively infected by DENV (Miller *et al.*, 2008; Navarro-Sanchez *et al.*, 2003; Tassaneeritthep *et al.*, 2003). They then release inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which is thought to initiate an inflammatory cascade leading to Dengue (hemorrhagic) fever (Chen *et al.*, 2008; Chen and Wang, 2002; Kwan *et al.*, 2005; Nightingale *et al.*, 2008). Evidence that DC-SIGN has an important role in immunopathology was provided by the study of gene promoter polymorphism, showing that higher DC-SIGN levels increase the risk of developing Dengue fever (Sakuntabhai *et al.*, 2005).

Although the skin is the portal to arboviruses, whether skin-resident immune cells mediate DENV entry and impact on immunopathology remains incompletely understood. The epithelium-resident DCs, known as Langerhans cells (LCs), were shown to be infected (Wu *et al.*, 2000); yet, these cells lack DC-SIGN and MR. Because dermal DCs (dDCs) and dermal Ms (dMs) express these receptors and represent plausible targets of DENV (Angel *et al.*, 2006; Harman *et al.*, 2013; Ochoa *et al.*, 2008; Zaba *et al.*, 2007), elucidation of their role in DENV infection and pathology will help open new ways to disease prediction, vaccines, and treatments.

## RESULTS

### Cutaneous reactions increase the risk of infection by accumulation of DC-SIGN<sup>+</sup> cells

We first assessed the risk of skin infection by determining the presence of cells expressing the C-type lectin DC-SIGN, an important DENV cell receptor (Navarro-Sanchez *et al.*, 2003; Tassaneeritthep *et al.*, 2003). We compared the frequency of DC-SIGN<sup>+</sup> cells in normal skin and, because mosquito-bitten skin is difficult to obtain, in skin lesions that resemble reactions to mosquito bites. Hence, we studied the skin from bullous pemphigoid, hypereosinophilic syndrome, mastocytosis, and scabies. These inflammations share with mosquito bites a number of features such as basophil recruitment and eosinophilia (Ito *et al.*, 2011). DC-SIGN expression was determined in combination with the CD163 marker, which is stably expressed by dMs and CD14<sup>+</sup> dDCs in different inflammatory milieus (Fuentes-Duculan *et al.*, 2010; Haniffa *et al.*, 2009; Pettersen *et al.*, 2011; Zaba *et al.*, 2007). Cross sections were labeled for CD163 and DC-SIGN (Figure 1a), and the cells exhibiting different combinations of markers (CD163<sup>+</sup> DC-SIGN<sup>-</sup>, CD163<sup>-</sup> DC-SIGN<sup>+</sup>, and CD163<sup>+</sup> DC-SIGN<sup>+</sup>) were counted (Figure 1b). DC-SIGN was predominantly expressed by CD163<sup>+</sup> myeloid cells both in normal and in inflamed skin. Only in mastocytosis did we see a population that expressed solely DC-SIGN. The mean number of CD163<sup>+</sup> DC-SIGN<sup>+</sup> cells doubled from 24 to 51 per 0.125 mm<sup>2</sup> in bullous pemphigoid and mastocytosis and tripled to 78 in the hypereosinophilic syndrome and scabies. Therefore, these findings show that normal skin is vulnerable

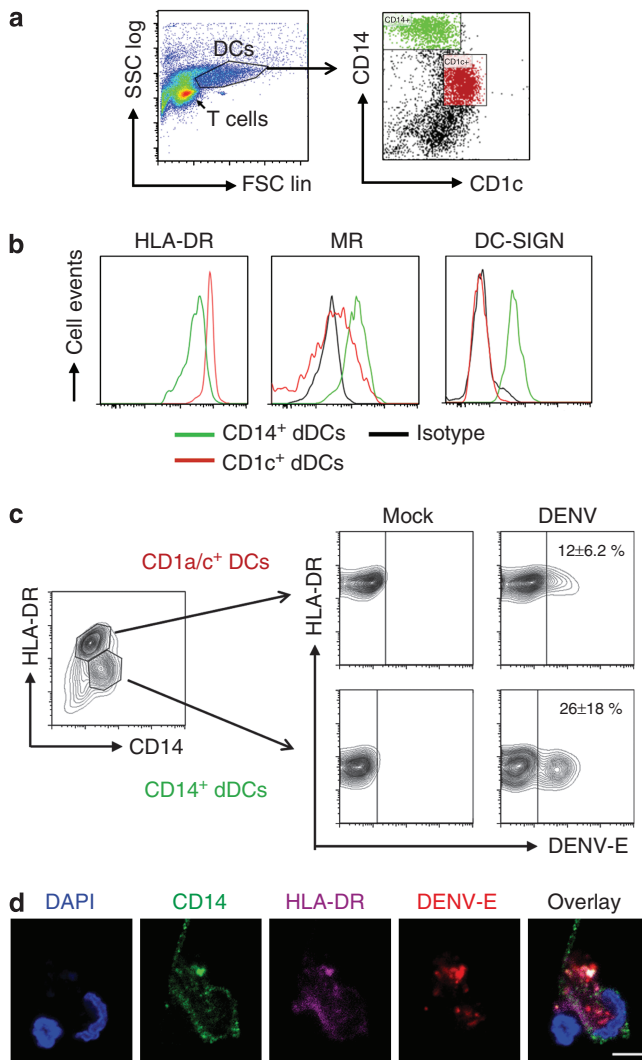


**Figure 1. Increased Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN) expression in dermatitis.** (a) Images of normal and hypereosinophilic syndrome skin stained for CD163 and DC-SIGN with DAPI (4',6-diamidino-2-phenylindole) nuclear counterstain. The dotted line marks the dermo-epidermal junction. Scale bars are shown. (b) Graph depicts the number of CD163<sup>+</sup> (red), DC-SIGN<sup>+</sup> (green), and CD163<sup>+</sup> DC-SIGN<sup>+</sup> (brown) cells per field for each condition. The data present the mean value  $\pm$  SD for five donors. The Wilcoxon test was used for statistical analysis; \*\* $P < 0.01$ .

to DENV infection because it comprises DENV cell targets, and inflammation arising as a cutaneous reaction to insect bites heightens the infectious risk through increased numbers of DC-SIGN<sup>+</sup> dDCs or dMs.

### dDC subsets are permissive to DENV infection

The human dermis contains CD14<sup>+</sup> Ms, CD1a/c<sup>+</sup>, and CD141<sup>Hi</sup> dDCs (Angel *et al.*, 2006; Chu *et al.*, 2012; Haniffa *et al.*, 2009; Haniffa *et al.*, 2012; Nestle *et al.*, 1993). The latter subset was not studied because they constitute a very rare population. In addition, the dermis includes another CD14<sup>+</sup> migratory subset, which we will refer to as CD14<sup>+</sup> dDCs according to their original classification, although recent investigations have demonstrated their proximity to monocytes rather than DCs (McGovern *et al.*, 2014). We isolated a suspension of immune cells from normal skin, comprising dDC subsets, as well as LCs and T cells, following their spontaneous migration from skin explants into culture medium (Nestle *et al.*, 1993; Figure 2a). In comparison with CD1c<sup>+</sup> dDCs, the CD14<sup>+</sup> dDCs expressed lower levels



**Figure 2. Dermal dendritic cells (DCs) are infected by Dengue virus (DENV).** (a) DCs and T cells emigrating from skin explants were identified by FACS. CD14 and CD1c labeling was determined on the gated DC-containing population. (b) HLA-DR, MR, and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin (DC-SIGN) expression was measured on gated CD14<sup>+</sup> dermal DCs (dDCs) (green) and CD1c<sup>+</sup> dDCs (red). Isotype staining is in black. Data are representative of at least five donors. (c) DENV infection of CD1a/c<sup>+</sup> DCs (HLA-DR<sup>hi</sup>CD14<sup>-</sup>) and CD14<sup>+</sup> dDCs (HLA-DR<sup>lo</sup>CD14<sup>+</sup>) was measured by FACS as intracellular staining of DENV-E protein. The mean percentage  $\pm$  SD ( $n=7$ ) of cells positive for DENV-E protein in comparison to mock-infection is indicated. (d) Confocal microscopic visualization of DENV-E protein within a CD14<sup>+</sup> dDC. Scale bar represents 10  $\mu$ m. The image is representative of two donors.

of HLA-DR, more MR, and exclusively DC-SIGN, in accordance with previous findings (Harman *et al.*, 2013; Ochoa *et al.*, 2008; Figure 2b). We assessed DENV infection by exposing unseparated DCs to insect cell-produced DENV (strain 16681, serotype 2) at a multiplicity of infection of 0.5. Viral load was measured after 2 days for each subset by intracellular DENV-E protein labeling and flow cytometry based on HLA-DR and CD14 expression (Figure 2c). Both the HLA-DR<sup>hi</sup> CD14<sup>-</sup> and the HLA-DR<sup>lo</sup> CD14<sup>+</sup> subsets

were infected by the pathogen. We verified viral accumulation by confocal microscopy and observed DENV-E protein in HLA-DR<sup>lo</sup> CD14<sup>+</sup> DCs (Figure 2d). There was a vesicle-like distribution of the protein with partial overlap with CD14 and HLA-DR markers, suggesting an association of the virus with the exocytic pathway. To better distinguish between infection of CD1c<sup>+</sup> dDCs, CD14<sup>+</sup> dDCs, and LCs, we used a complementary gating strategy based on Langerin, DC-SIGN, and differences in HLA-DR expression (Supplementary Fig. 1a). We found that the CD14<sup>+</sup> subset (Langerin<sup>-</sup> DC-SIGN<sup>+</sup> HLA-DR<sup>lo</sup>) carried the highest viral load (mean  $\pm$  SD: 12.91%  $\pm$  7.35), followed by CD1c<sup>+</sup> dDCs (Langerin<sup>-</sup> DC-SIGN<sup>lo</sup> HLA-DR<sup>hi</sup>; 7.75%  $\pm$  4.30) and LCs (Langerin<sup>+</sup> HLA-DR<sup>hi</sup>; 1.76%  $\pm$  2.29; Supplementary Figure 1c). Hence, dDCs are infectious targets for DENV.

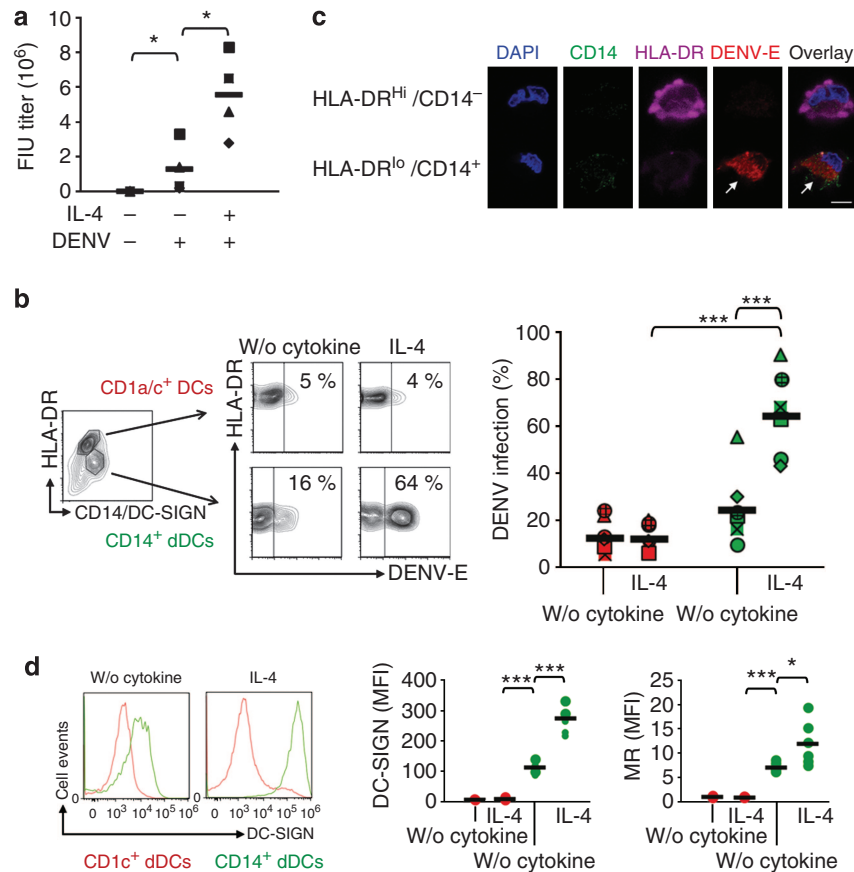
### IL-4 stimulates productive infection of CD14<sup>+</sup> dDCs

Arbovirus infectivity and pathogenicity have been correlated with Th2-type immune reactions provoked by mosquito salivary compounds (Cox *et al.*, 2012; Styer *et al.*, 2011). Th2-type immunity is characterized by the release of IL-4 by basophils, mast cells, and T cells. We therefore investigated the impact of IL-4 on DC infection. To this end, we allowed DCs to migrate into a medium lacking or containing IL-4, harvested the cells, and exposed them to DENV. Two days later, viral titers were determined. As shown in Figure 3a, the viral titer was significantly elevated when DCs were conditioned by IL-4. To determine whether the increased viral production was the result of higher infection of one particular DC subset, we determined the intracellular of DENV-E protein content in each subset by flow cytometry. We found a greatly increased viral load in IL-4-stimulated CD14<sup>+</sup> dDCs but not in CD1c<sup>+</sup> dDCs or LCs (Figure 3b; Supplementary Figure 1b and c). Visual inspection by confocal microscopy showed DENV-E protein evenly distributed throughout the cytoplasm of HLA-DR<sup>lo</sup> CD14<sup>+</sup> dDCs, whereas HLA-DR<sup>hi</sup> CD14<sup>-</sup> DCs were devoid of intracellular E protein (Figure 3c). Because IL-4 stimulates DC-SIGN and MR synthesis (Relloso *et al.*, 2002), we determined expression of these lectins in both dDC subsets. Indeed, IL-4 triggered an upregulation of DC-SIGN and MR on CD14<sup>+</sup> dDCs but not on CD1c<sup>+</sup> dDCs (Figure 3d). Therefore, the presence of IL-4 strongly enhances DENV infection of the CD14<sup>+</sup> dDC subset, reflecting its higher DC-SIGN and MR levels.

### IL-4-activated dMs are infected by DENV

We next examined whether DENV infects dMs. As dMs are non-migratory, enzymatic digestion was necessary to isolate the cells from the tissue (Angel *et al.*, 2006; Zaba *et al.*, 2007). After culturing the dermal cell suspension for 2 days, CD14<sup>+</sup>HLA-DR<sup>+</sup> cells were purified by flow cytometric cell sorting (Supplementary Figure 2). Electron microscopy showed that the purified cells displayed typical dM morphology with melanin-loaded vesicles and few cell membrane protrusions, in contrast to DCs (Figure 4a, left and right; Lenz *et al.*, 1993). Analysis of cell surface markers disclosed that dMs expressed high levels of CD14 and HLA-DR but surprisingly little DC-SIGN and MR (Figure 4b, condition





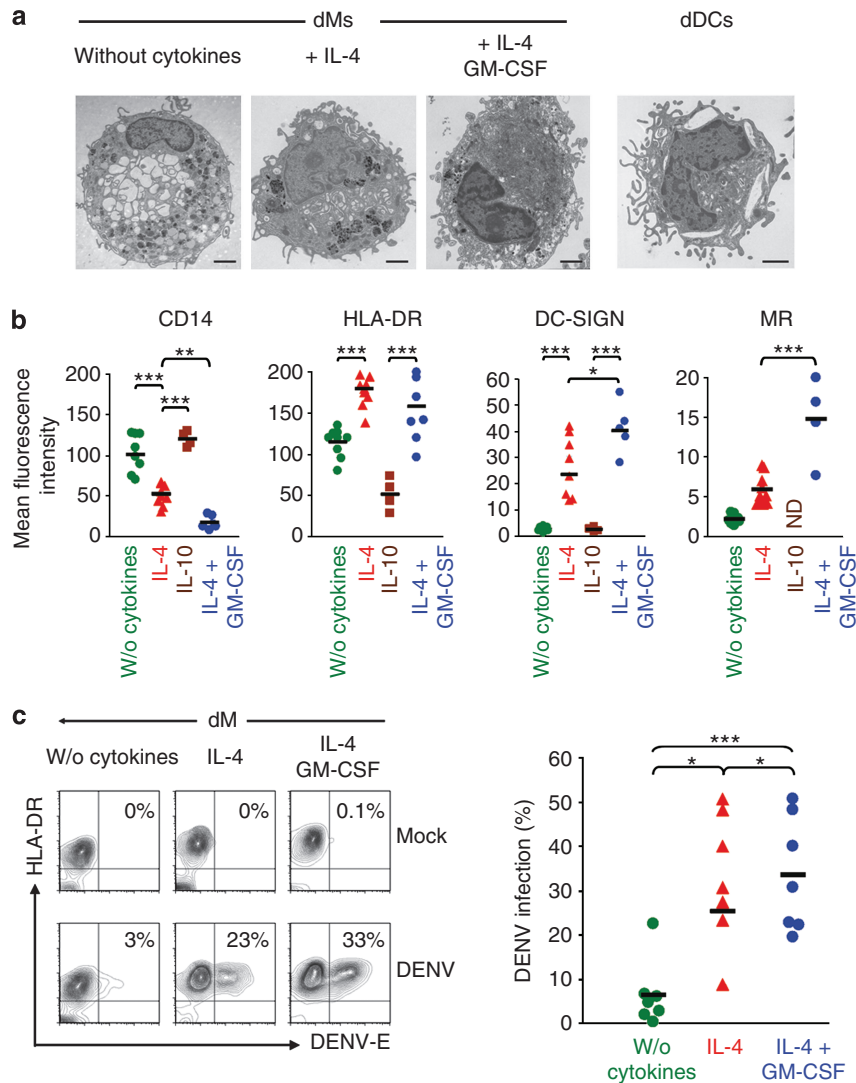
**Figure 3. IL-4 stimulates Dengue Virus (DENV) infection of CD14<sup>+</sup> dermal dendritic cells (dDCs).** (a) Viral titers from mock- or DENV-infected DCs stimulated or not by IL-4 were determined on Vero cells as FACS infectious units (FIU) per ml. Each point represents one donor and the horizontal bar the mean value. (b) DENV infection of untreated or IL-4-stimulated CD1a/c<sup>+</sup> DCs and CD14<sup>+</sup> dDCs was measured by intracellular presence of DENV-E protein. The percentage of DENV-E<sup>+</sup> cells is indicated. The graph depicts the collective data for each donor, represented by a symbol. Horizontal bars are the mean values. (c) Microscopic detection of DENV-E protein in a CD14<sup>+</sup> dDC but lacking from a neighboring CD1a/c<sup>+</sup> DC. Scale bar = 10  $\mu$ m. The image is representative of two donors. (d) Expression of DC-SIGN by CD1c<sup>+</sup> and CD14<sup>+</sup> dDCs emigrating from skin explants with or without IL-4. The graphs depict the mean fluorescence intensity of DC-SIGN and mannose receptor expression for both subsets for each donor. The mean value is shown as horizontal bars. Statistical significance was determined by unpaired Student's *t*-test. \**P* < 0.05; \*\*\**P* < 0.001.

without cytokines). As this may be attributable to enzymatic digestion or to cell dedifferentiation after isolation from the skin, we searched for conditions that would restore DC-SIGN and MR expression. In view of the stimulatory effect of IL-4 on CD14<sup>+</sup> DCs, we cultured the cell suspension for 2 days with IL-4. In addition, because we had previously found that also IL-10 induced DC-SIGN expression on monocytes, we cultured the cells in IL-10 (Kwan *et al.*, 2008). DC-SIGN and MR levels clearly increased in response to IL-4; however, IL-10 had no effect (Figure 4b). To further enhance lectin levels, we exposed dMs to IL-4 and GM-CSF, which resulted in a boost of DC-SIGN and MR levels (Figure 4b). Concomitantly, HLA-DR expression increased, whereas CD14 levels dropped. The cells developed a DC-like morphology with more cytoplasmic extensions but fewer phagosomes reminiscent of DCs (Figure 4a). We then assessed DENV infection of dMs obtained without cytokines, with IL-4 and with IL-4/GM-CSF by exposing the purified cells to DENV, followed after 2 days by intracellular DENV-E protein flow cytometric analysis. Untreated dMs were poorly infected, but IL-4-stimulated dMs became

highly permissive to DENV, which was further enhanced by GM-CSF (Figure 4c). Thus, dMs are infectious targets for DENV when they express the viral-attachment lectins DC-SIGN and MR.

#### DENV-infected dMs release TNF- $\alpha$ , and IL-4 enhances the immunostimulatory function of dDCs

To evaluate the impact of DENV infection of dDCs and dMs on dengue disease development, we determined their potential to stimulate the immune response. Given its cardinal role in dengue pathologies, we first determined the production of TNF- $\alpha$ . Except for one donor, there was very little TNF- $\alpha$  released by virus-infected dDCs. Moreover, IL-4 could not elevate its production (Figure 5a). On the other hand, TNF- $\alpha$  was produced by dMs, in particular when activated with IL-4 or with IL-4/GM-CSF, reflecting increased viral load (Figure 5b). Next, we evaluated whether IL-4 affected the ability of dDCs and dMs to stimulate helper T cells. For this, we performed mixed lymphocyte reactions with allogeneic naive CD4<sup>+</sup> T cells. We found that IL-4 enhanced the T-cell

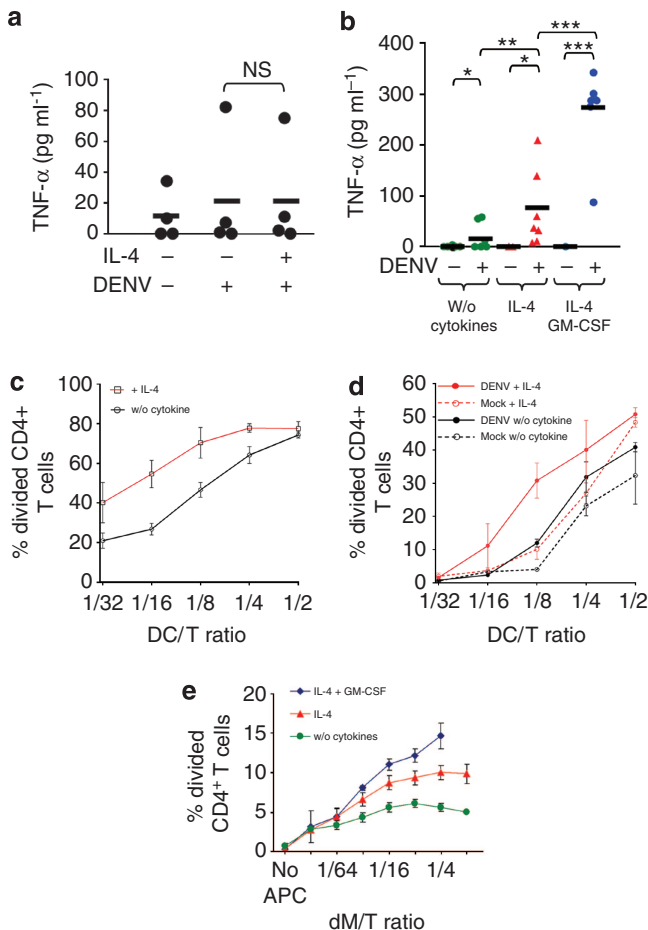


**Figure 4. IL-4-activated dermal macrophages (dMs) are permissive to Dengue virus (DENV) infection.** (a) Transmission electron microscopy of FACS-purified dMs cultured without cytokines, with IL-4 or with IL-4/GM-CSF. In comparison is shown a CD1c<sup>+</sup> dermal dendritic cell (dDC). Bar = 2  $\mu$ m. The data are representative of three donors. (b) Phenotypic characterization of dMs cultured in the absence or presence of the indicated cytokines. The mean fluorescence intensity of the cell surface markers for each skin donor is shown. Horizontal bars represent mean values. (c) FACS analysis of DENV infection of dMs obtained from the indicated conditions. The percentage of DENV-E<sup>+</sup> dMs is indicated. The graph depicts this percentage for each donor. The mean value is shown by a horizontal bar. Statistical significance was determined by unpaired Student's *t*-test. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

stimulatory capacity of dDCs (Figure 5c). To better assess the consequences of DENV infection for the T-cell immune response, we also measured naive T-cell proliferation induced by infected dDCs. We found that infection further stimulates T-cell activation (Figure 5d). As dMs are poor naive T-cell stimulators (Haniiffa *et al.*, 2009) and remain within the tissue, we cultured them together with total blood CD4<sup>+</sup> T cells that include memory T cells. Proliferation induced by untreated dMs was low, but IL-4 and IL-4/GM-CSF converted the cells into better T-cell stimulators (Figure 5e). Taken together, these results predict that the major consequence of DENV infection of dDCs would be a potent virus-directed T-cell response, whereas DENV-infected dMs would principally fuel the local inflammatory reaction.

## DISCUSSION

Although dDCs and dMs reside in the most arbovirus-exposed tissue and are equipped with pattern recognition receptors such as C-type lectins (Harman *et al.*, 2013; Ochoa *et al.*, 2008; Turville *et al.*, 2002), the question of their role in pathogen entry and disease progression has been little explored. We addressed this question using the dengue pathogen, because (i) the virus is inoculated into the skin by mosquitoes, (ii) it recognizes DC-SIGN and MR lectins, and (iii) the early infection events are likely to have a profound effect on progression to life-threatening disease. In this report, we have shown that CD1c<sup>+</sup> and CD14<sup>+</sup> dDCs as well as dMs are primary cell targets for DENV and that IL-4 has an outstanding influence on their viral infection and the immune response.



**Figure 5. Immunostimulatory impact of IL-4-activated dermal dendritic cells (dDCs) and dermal macrophages (dMs).** (a) Measure of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) release by mock or Dengue virus (DENV)-infected dDCs stimulated or not by IL-4. The graph depicts the collective data for each donor, and horizontal bars are the mean values. (b) TNF- $\alpha$  release by mock or DENV-infected dMs cultured in the indicated conditions. Data points of individual donors are shown, and the mean values are horizontal bars. (c) Proliferation of naive CD4<sup>+</sup> T cells by allogeneic DCs obtained from the skin with or without IL-4. T-cell proliferation was measured as the loss of carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye. The data are representative of three donors. (d) Proliferation of naive CD4<sup>+</sup> T cells by allogeneic DCs obtained from the skin with or without IL-4 and exposed to DENV. T-cell proliferation was measured with CFSE, and the data are representative of three donors. (e) Proliferation of CD4<sup>+</sup> T cells by allogeneic dMs cultured in the indicated conditions. T-cell proliferation was measured as the loss of CFSE. The data are representative of three donors. Statistical significance was determined by unpaired Student's *t*-test. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; NS, nonsignificant.

To determine whether DENV infects the two distinct dDC subsets, we adopted an unbiased approach by exposing dermal emigrants to DENV, followed by FACS analysis. Both CD1c<sup>+</sup> and CD14<sup>+</sup> dDCs were infected by DENV and the latter often yielded a higher viral load, probably as a result of increased DC-SIGN and MR expression. The finding that LCs were poorly infected supports previous observations using *in vitro* generated LCs (Lozach *et al.*, 2005) and further highlights the importance of the dermal antigen presenting cells in DENV infection. When stimulated with IL-4, CD14<sup>+</sup>

dDCs infection reached levels as high as 90%, correlating with greatly upregulated DC-SIGN and MR levels. We could not detect a significant production of TNF- $\alpha$  by dDCs, irrespective of activation by IL-4, in line with other reports that failed to observe TNF- $\alpha$  production by either CD1c<sup>+</sup> or CD14<sup>+</sup> dDCs in response to a wide range of stimuli (Haniffa *et al.*, 2009; Haniffa *et al.*, 2012). Instead, the consequences of DENV infection of dDCs are most likely pathogen transport to draining lymph nodes and the priming of virus-specific T cells. This would lead to an anti-viral adaptive immune response, considered a key event in dengue pathologies (Rothman and Ennis, 1999). In view of the findings that CD14<sup>+</sup> dDCs skew the immune response to humoral immunity (Klechevsky *et al.*, 2008; Matthews *et al.*, 2012), a privileged infection of the CD14<sup>+</sup> subset would result in an enhanced antibody-mediated immune response. It is believed that a humoral response heightens the risk of immunopathology during a secondary infection with a heterotypic DENV serotype (Halstead and O'Rourke, 1977). It is therefore tempting to speculate the existence of a positive feedback loop between CD14<sup>+</sup> dDC infection and humoral immunity that becomes relevant with repetitive DENV infections and may provide an understanding into the increased risk of disease development with multiple viral exposures.

Because of an absence of DC-SIGN and MR expression by dMs after their purification from the skin and in keeping with the idea that skin inflammation is likely to affect the susceptibility of dermal immune cells to infection, we found a prominent effect of IL-4, reinforced by GM-CSF, on DC-SIGN and MR expression by dMs. This also demonstrates an unexpected plasticity of dMs. dMs and CD14<sup>+</sup> dDCs share a number of phenotypic markers, and recent findings have shown that CD14<sup>+</sup> dDCs should be considered as an intermediate phenotype between monocytes and Ms (McGovern *et al.*, 2014). Despite this distinction, the CD14<sup>+</sup> cells that we purified from digested dermis formed a homogeneous population with characteristic features of Ms. This suggests that CD14<sup>+</sup> dDCs might have differentiated into Ms during *ex vivo* culture, possibly under the influence of dermal fibroblasts (Chomarat *et al.*, 2000). This plasticity further extends recent views on the ontogenic proximity of CD14<sup>+</sup> dDCs and monocytes (McGovern *et al.*, 2014). IL-4-treated Ms and CD14<sup>+</sup> dDCs show characteristics shared with DCs generated from blood monocytes with GM-CSF and IL-4 *in vitro*—i.e., downregulation of CD14 and increased expression of DC-SIGN—although we did not observe upregulation of CD1a in the dermis or in a culture with IL-4 and GM-CSF. Furthermore, the greater susceptibility to DENV infection upon differentiation of CD14<sup>+</sup> dermal cells is also reminiscent of previous studies comparing monocytes exposed or not to IL-4 (Miller *et al.*, 2008).

As can be expected from the levels of the lectins, together with their active cytoplasm, reflecting intensive endocytosis and biosynthesis, IL-4-activated dMs were clearly permissive to DENV infection. Infected dMs produced high levels of TNF- $\alpha$ , which is thought to be the key cytokine for the development of dengue fever (Green and Rothman, 2006; Pang *et al.*, 2007). Considering their limited ability of T-cell

priming compared with dDCs, even when activated by IL-4, we propose that the principal contribution of dMs in dengue disease lies in inflammation with systemic and local consequences: (i) systemic activation of the immune system promoting dengue fever and (ii) local inflammation resulting in recruitment of immune cells, including lectin-expressing dMs and dDCs, as well as memory T cells. Therefore, dM-released TNF- $\alpha$  would fuel skin inflammation initiated by the response to insect-derived salivary compounds and thus would propel a loop that would render the skin highly vulnerable to infection. Although studies in animal models have revealed a major impact of mosquito saliva and their immune stimulatory action on infectivity and viral pathogenesis (Cox *et al.*, 2012; Styer *et al.*, 2011), so far no correlation has been made in humans between hypersensitivity and pathogenesis of arbovirus. Our finding that skin dermatitis resembling reactions to mosquito bites leads to a massive increase in CD163<sup>+</sup> dDCs or dMs that maintain DC-SIGN expression, taken together with the demonstration that these cells are infectious targets, provides a first experimental basis for such a correlation in man.

By investigating how DENV is handled by resident dermal immune cells, we have uncovered a number of checkpoints that are likely to affect early on the pathogenesis of arboviruses in general and DENV in particular. The results also reveal that therapeutic or prophylactic action, such as interference with lectin binding and IL-4 producing reactions, would be efficacious at the skin level to prevent systemic spread of the virus and immunopathology.

## MATERIALS AND METHODS

### Purification of skin cells

Abdominal skin was obtained with written informed consent and institutional review board approval, in agreement with the Helsinki Declaration and French legislation. DCs were isolated by floating whole skin pieces for 3 days onto complete medium lacking or containing IL-4 (25 ng ml<sup>-1</sup>; Nestle *et al.*, 1993). For dMs purification, the epidermis was removed after trypsin digestion (0.5% in phosphate-buffered saline) and the remaining dermis digested with collagenase and DNase for 18 hours (Angel *et al.*, 2006; Zaba *et al.*, 2007). The cell suspension was cultured in complete medium for 48 hours in the absence or presence of IL-4 (25 ng ml<sup>-1</sup>), and adherent and non-adherent CD14<sup>+</sup> cells were first positively enriched with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) before flow cytometric sorting. Sorted CD14<sup>+</sup> cells were allowed to recover for 24 hours in complete culture medium conditioned by dermal fibroblasts in the absence or presence of IL-4 (25 ng ml<sup>-1</sup>) or GM-CSF (25 ng ml<sup>-1</sup>) before infection.

### Cell phenotype analyses

The phenotypes of dDCs and dMs were analyzed using the following antibodies from BD Biosciences (Franklin Lakes, NJ): HLA-DR-FITC (Tü39 or L243), MR/CD206-FITC (19.2), DC-SIGN/CD209-PerCP-Cy5.5 (DCN46), CD1a-APC (HI149), CD86-FITC (FUN-1), as well as CD14-PE (MEM-15, ImmunoTools, Friesoythe, Germany) and CD1c-APC (AD5-8E7, Miltenyi Biotec). Cells were analyzed on FACSCalibur (BD Biosciences) or Gallios (Beckman-Coulter, Brea, CA) after exclusion of dead cells by Sytox Red (Molecular Probes, Invitrogen,

Grand Island, NY), 7AAD (BD Biosciences) or Fixable Viability Dye-eBio 780 (eBioscience). Data were analyzed using the Cell Quest Pro software (BD Biosciences), Kaluza (Beckman-Coulter), or FlowJo (TreeStar, Ashland, OR).

### DENV production

The pDENV-2 replicon of DENV-2 16681 (5  $\mu$ g; gift of Dr. E. Harris, University of California, Berkeley, CA) was linearized, phenol-chloroform extracted, precipitated, and resuspended in RNase-free water. RNA was synthesized by *in vitro* transcription using the T7 RiboMax Large scale RNA production Systems (Promega, Madison, WI) with additional 7 mG(ppp)A RNA Cap Structure Analog (New England Biolabs, Ipswich, MA). The RNA was transfected into BHK-21 (10<sup>6</sup> cells per well) in a six-well plate using the Lipofectamine RNAiMax kit (Invitrogen): 50  $\mu$ l RNA mixture in 200  $\mu$ l Opti-MEM was added to 50  $\mu$ l Lipofectamine in 200  $\mu$ l Opti-MEM (Life Technologies, Grand Island, NY), incubated for 20 minutes and added to 10<sup>6</sup> cells. After 3 hours, the supernatant was removed and cells were cultured in complete Glasgow MEM medium. Supernatants were collected, spun down to remove cells, and stored in aliquots at -80 °C. DENV-2 was produced in C6/36 *Aedes albopictus* mosquito cells, maintained in Leibovitz L15 medium, by infection with viral supernatants of BHK-21 cells. C6/36 cell supernatants were collected and stored in aliquots at -80 °C.

### DENV infections

Skin-purified cells (0.5  $\times$  10<sup>5</sup> cells) were exposed to DENV serotype 2 (strain 16681) at a multiplicity of infection of 0.5. After incubation for 2 hours at 37 °C in serum-free medium, cells were washed and cultured in complete medium. After 2 days, cells were subjected to intracellular detection of the viral E protein.

### Infection analysis

Cells were fixed with 4% (v/v) paraformaldehyde and permeabilized with 0.1% (v/v) Triton X-100 for 3 minutes at room temperature. After washing, they were labeled with mouse anti-DENV-E protein mAb (IgG1, 3H5-1, Millipore, Molsheim, France), followed by APC-anti-mouse IgG1 (BD Biosciences). We stained with anti-CD14 (AB383) followed by donkey anti-goat IgG-AlexaFluor488 (Molecular Probes, Invitrogen), anti-HLA-DR-PerCP (L243) and, where indicated, anti-DC-SIGN-AlexaFluor488 (111H2 IgG2b, Dendritics, Lyon, France). For analyses of LC infection among total crawlout cell suspensions, we combined anti-DC-SIGN-PerCP-Cy5.5 (DCN46), anti-Langerin/CD207-PE (DCGM4, Dendritics), anti-HLA-DR-AlexaFluor700 (L203, R&D Systems, Minneapolis, MN), and Fixable Viability Dye-eBio780 (eBioscience, San Diego, CA). Fluorescence was measured by flow cytometry (FACSCalibur, BD Biosciences or Gallios, Beckman-Coulter), and the data analyzed using the Cell Quest Pro or FlowJo software. Titers in cell-free supernatants were determined by infection of Vero cells as previously described (Lambeth *et al.*, 2005).

### Cytokine production

Two days after infection, cell supernatants were collected, and TNF- $\alpha$  was measured by ELISA (OptEIA, BD Biosciences).

### T-cell stimulation assay

Graded doses of purified dMs or total skin crawlout cells were incubated with 5  $\times$  10<sup>4</sup> carboxyfluorescein succinimidyl ester (CFSE)-



loaded total blood T cells or  $10^5$  naive  $CD4^+$  T cells purified from peripheral blood by negative selection (Miltenyi Biotec), respectively, in 96-well round-bottom plates in complete medium. After 5-day incubation, the cells were labeled for CD3 and CD4 and analyzed by flow cytometry for CFSE dilution in the  $CD3^+ CD4^+$  gate. Proliferation was determined as the proportion of T cells with decreased intensity of CFSE.

Total skin crawlout cells were infected with DENV-2 for 2 days, and then graded doses were incubated with  $10^5$  CFSE-loaded naive  $CD4^+$  T cells in 96-well plates in complete medium. After 5-day incubation, the cells were labeled with fixable viability dye-eBio780 and anti-CD3-AF700 (BD Biosciences). Live T cells ( $CD3^+$  fixable viability dye-negative) were analyzed by flow cytometry for CFSE dilution.

### Skin sections

Formol-fixed skin sections were cut and prepared for labeling with anti-CD163 mAb 10D6 and anti-DC-SIGN mAb 111H2 (Canard et al., 2011; Dendritics). Counter staining was carried out with DAPI (4',6-diamidino-2-phenylindole). For diseased skins, two areas in the upper dermis and one in the reticular dermis were selected. Labeled cells were counted manually in three non-superimposable optical fields of  $0.125 \text{ mm}^2$  using a computer-assisted image analysis Image J freeware.

### Confocal and electron transmission microscopy

A total of  $5 \times 10^4$  cells per chamber were cultured on polylysine-coated slides (eight chamber Nunc Lab-Tek, Dutscher, Brumath, France) for 2 hours without serum, with or without DENV. Cells were collected and washed three times in complete medium before adding them back to the chamber. After 48 hours, the cells were fixed and labeled for DENV-E protein using Cy3-conjugated mAb 4G2 (kind gift from Dr. Philippe Despres, Institut Pasteur, Paris, France) together with PerCP-anti-HLA-DR (L243) and anti-CD14 (AB383, R&D Systems) followed by Alexa 488-donkey anti-goat (Molecular Probes, Invitrogen). DAPI was used as nuclear counterstaining. Slides were mounted using Fluoromount (Dako, Les Ulis, France). Images were taken on Zeiss LSM 780 (Carl Zeiss, Jena, Germany) with GaAsP detector and Zen acquisition software (Zeiss). Images were further processed using the Image J freeware. Electron microscopy was performed as previously described (Kwan et al., 2008).

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### DISCLAIMER

The funding source was not involved in the study design, analysis, and interpretation of data or in the writing of the manuscript.

### AUTHOR CONTRIBUTIONS

ES, VF, and CGM planned and performed experiments. ES, VF, and CGM wrote the manuscript. VP, MK, MD, and J-DF performed experimental work.

### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

### REFERENCES

- Angel CE, George E, Brooks AE et al. (2006) Cutting edge:  $CD1a^+$  antigen-presenting cells in human dermis respond rapidly to CCR7 ligands. *J Immunol* 176:5730–4
- Bhatt S, Gething PW, Brady OJ et al. (2013) The global distribution and burden of dengue. *Nature* 496:504–7
- Canard B, Vachon H, Fontaine T et al. (2011) Generation of anti-DC-SIGN monoclonal antibodies capable of blocking HIV-1 gp120 binding and reactive on formalin-fixed tissue. *Immunol Lett* 135:165–72
- Chen ST, Lin YL, Huang MT et al. (2008) CLEC5A is critical for dengue-virus-induced lethal disease. *Nature* 453:672–6
- Chen YC, Wang SY (2002) Activation of terminally differentiated human monocytes/macrophages by dengue virus: productive infection, hierarchical production of innate cytokines and chemokines, and the synergistic effect of lipopolysaccharide. *J Virol* 76:9877–987
- Chomarat P, Banchereau J, Davoust J et al. (2000) IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* 1:510–4
- Chu CC, Ali N, Karagiannis P et al. (2012) Resident  $CD141^+$  (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *J Exp Med* 209:935–45
- Cox J, Mota J, Sukupolvi-Petty S et al. (2012) Mosquito bite delivery of dengue virus enhances immunogenicity and pathogenesis in humanized mice. *J Virol* 86:7637–49
- Espada-Murao LA, Morita K (2011) Dengue and soluble mediators of the innate immune system. *Trop Med Health* 39:53–62
- Fuentes-Duculan J, Suarez-Farinas M, Zaba LC et al. (2010) A subpopulation of  $CD163^+$  macrophages is classically activated in psoriasis. *J Invest Dermatol Symp Proc* 130:2412–22
- Green S, Rothman A (2006) Immunopathological mechanisms in dengue and dengue hemorrhagic fever. *Curr Opin Infect Dis* 19:429–36
- Halstead SB, O'Rourke EJ (1977) Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* 265:739–41
- Haniiffa M, Ginhoux F, Wang XN et al. (2009) Differential rates of replacement of human dermal dendritic cells and macrophages during hematopoietic stem cell transplantation. *J Exp Med* 206:371–85
- Haniiffa M, Shin A, Bigley V et al. (2012) Human tissues contain  $CD141^+$  cross-presenting dendritic cells with functional homology to mouse  $CD103^+$  nonlymphoid dendritic cells. *Immunity* 37:60–73
- Harman AN, Bye CR, Nasr N et al. (2013) Identification of lineage relationships and novel markers of blood and skin human dendritic cells. *J Immunol* 190:66–79
- Ito Y, Satoh T, Takayama K et al. (2011) Basophil recruitment and activation in inflammatory skin diseases. *Allergy* 66:1107–13
- Klechevsky E, Morita R, Liu M et al. (2008) Functional specializations of human epidermal Langerhans cells and  $CD14^+$  dermal dendritic cells. *Immunity* 29:497–510
- Kwan WH, Helt AM, Maranon C et al. (2005) Dendritic cell precursors are permissive to dengue virus and human immunodeficiency virus infection. *J Virol* 79:7291–9
- Kwan WH, Navarro-Sanchez E, Dumortier H et al. (2008) Dermal-type macrophages expressing  $CD209/DC-SIGN$  show inherent resistance to dengue virus growth. *PLoS Negl Trop Dis* 2:e311. doi:10.1371/journal.pntd.0000311
- Lambeth CR, White LJ, Johnston RE et al. (2005) Flow cytometry-based assay for titrating dengue virus. *J Clin Invest* 115:3267–72
- Lenz A, Heine M, Schuler G et al. (1993) Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization. *J Clin Invest* 92:2587–96
- Lozach PY, Burleigh L, Staropoli I et al. (2005) Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-



- mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. *J Biol Chem* 280:23698–708
- Matthews K, Chung NP, Klasse PJ *et al.* (2012) Potent induction of antibody-secreting B cells by human dermal-derived CD14+ dendritic cells triggered by dual TLR ligation. *J Immunol* 2012:16
- McGovern N, Schlitzer A, Gunawan M *et al.* (2014) Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. *Immunity* 41:465–77
- Miller JL, Dewet BJ, Martinez-Pomares L *et al.* (2008) The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathog* 4: e17. doi:10.1371/journal.ppat.0040017
- Mongkolsapaya J, Dejnirattisai W, Xu XN *et al.* (2003) Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med* 9:921–7
- Navarro-Sanchez E, Altmeyer R, Amara A *et al.* (2003) Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. *EMBO Rep* 4:723–8
- Nestle FO, Zheng XG, Thompson CB *et al.* (1993) Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J Immunol* 151:6535–45
- Nightingale ZD, Patkar C, Rothman AL (2008) Viral replication and paracrine effects result in distinct, functional responses of dendritic cells following infection with dengue 2 virus. *J Leukoc Biol* 84: 1028–38
- Ochoa MT, Loncaric A, Krutzik SR *et al.* (2008) "Dermal dendritic cells" comprise two distinct populations: CD1+ dendritic cells and CD209+ macrophages. *J Invest Dermatol Symp Proc* 128:2225–31
- Pang T, Cardoso MJ, Guzman MG (2007) Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF/DSS). *Immunol Cell Biol* 85:43–5
- Peng Z, Simons FE (2007) Advances in mosquito allergy. *Curr Opin Allergy Clin Immunol* 7:350–4
- Pettersen JS, Fuentes-Duculan J, Suarez-Farinas M *et al.* (2011) Tumor-associated macrophages in the cutaneous SCC microenvironment are heterogeneously activated. *J Invest Dermatol Symp Proc* 131: 1322–30
- Relloso M, Puig-Kroger A, Pello OM *et al.* (2002) DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. *J Immunol* 168:2634–43
- Rigau-Perez JG, Clark GG, Gubler DJ *et al.* (1998) Dengue and dengue haemorrhagic fever. *Lancet* 352:971–7
- Rothman AL, Ennis FA (1999) Immunopathogenesis of Dengue hemorrhagic fever. *Virology* 257:1–6
- Sakuntabhai A, Turbpaiboon C, Casademont I *et al.* (2005) A variant in the CD209 promoter is associated with severity of dengue disease. *Nat Genet* 37:507–13
- Saleem K, Shaikh I (2008) Skin lesions in hospitalized cases of dengue Fever. *J Coll Physicians Surg Pak* 18:608–11
- Styer LM, Lim PY, Louie KL *et al.* (2011) Mosquito saliva causes enhancement of West Nile virus infection in mice. *J Virol* 85:1517–27
- Tassaneetrithep B, Burgess TH, Granelli-Piperno A *et al.* (2003) DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med* 197:823–9
- Thangamani S, Higgs S, Ziegler S *et al.* (2010) Host immune response to mosquito-transmitted chikungunya virus differs from that elicited by needle inoculated virus. *PLoS One* 5:e12137. doi:10.1371/journal.pone.0012137
- Turville SG, Cameron PU, Handley A *et al.* (2002) Diversity of receptors binding HIV on dendritic cell subsets. *Nat Immunol* 3:975–83
- van Kooyk Y, Geijtenbeek TB (2003) DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol* 3:697–709
- Wu SJ, Grouard-Vogel G, Sun W *et al.* (2000) Human skin Langerhans cells are targets of dengue virus infection. *Nat Med* 6:816–20
- Zaba LC, Fuentes-Duculan J, Steinman RM *et al.* (2007) Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163+FXIIIa+ macrophages. *J Clin Invest* 117:2517–25