Chapter 2

Travelling with Dengue: From the Skin to the Nodes

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Abstract

Dengue virus (DENV) infects humans through the skin. The early infection and encounters between DENV and cutaneous immune and non-immune cells only recently are under investigation. We have reported DENV-infected cutaneous dendritic cells (DCs), also keratinocytes and dermal fibroblasts permissive to DENV infection. Now, upon cutaneously inoculating fluorescently labeled DENV into immune-competent mice, we found DENV mostly in dermis from 1 h post-inoculation. Afterwards, DENV rapidly localized in the subcapsular sinus of draining lymph nodes (DLNs) associated with CD169+ macrophages, suggesting virus travelling through lymph flow. However, DENV association with CD11c+ DCs in the paracortex and T:B border suggests DENV being ferried from the skin to DLNs by DCs too. DENV was not associated with F4/80+ macrophages nor with DEC205+ DCs, but it was inside B cell follicles early after cutaneous inoculation. DENV inside B follicles likely affects the development of humoral responses. Antibody responses deserve very careful scrutiny as neutralizing memory antibodies are crucial to counteract homotypic reinfections whereas non-neutralizing ones might facilitate heterotypic DENV infection or even Zika infection, another flavivirus. Unravelling the DENV journey from skin to lymph into regional nodes and the cellular compartments will aid to understand the disease, its pathology and how to counteract it.

Keywords: dengue virus, skin, lymph nodes, immune tissues, macrophages, dendritic cells, B cell follicles

1. Introduction

Dengue virus (DENV) is an important viral pathogen affecting every year almost 400 million people worldwide [1]. Over the past 50 years, the incidence of dengue has increased 30-fold mainly in tropical and subtropical areas causing serious public health problems [2]. DENV triggers a diversity of clinical manifestations, from an asymptomatic infection in the majority of cases, to a mild febrile illness or a life-threatening hemorrhagic disease such as severe dengue (SD) or dengue shock syndrome (DSS) [3].

DENV is transmitted to humans when a priorly infected *Aedes aegypti* female mosquito bites to take a ~4.2 μl blood meal by probing around 141 s in the skin of a healthy person [4, 5]. By the time that half-length (~0.9 mm) of the proboscis is inserted, around 50,000 DENV plaque-forming units are deposited in the dermis, where a variety of resident immune and non-immune cells are located, including dermal dendritic cells (DCs), macrophages (Mfs), T lymphocytes, mast cells, keratinocytes, fibroblasts, etc., as well as lymphatic and blood vessels through which migrating cells travel to lymph nodes (LNs) and arrive at the skin [6–9].

While the regional (cutaneous) responses to DENV entrance are recently under intense scrutiny [10–14], less much is known about how exactly DENV gets its way into lymphatic vessels, secondary lymphoid tissues (if at all) and to which cells might be associated in each of these compartments. By infecting immune-competent mice through the skin, we recently demonstrated not only a strong germinal center (GC) reaction in the draining lymph nodes (DLNs) but also the presence of viral proteins inside these organs [13]. Others have also reported viral proteins inside LNs (NS1, NS3, PrM and E protein) both in humans and mice, suggesting that at least the interactions of viral antigens (Ags) with LN-immune cells are taking place [13, 15, 16].

There is scarce information of the early events happening in the skin during DENV entrance. Some authors have reported an immunomodulatory environment by the mosquito saliva [17, 18], for instance, downregulation of antiviral molecules such as interferon β (IFN-β), IFN-γ, some pattern recognition receptors, or even sustained viremia, among others, helping to establish the infection [19, 20]. It has also been demonstrated a productive infection of fibroblasts, keratinocytes, DCs and Langerhans cells in the skin, but whether these cells participate in the global pathology of the disease remains unclear [10–12, 14, 21].

The appropriate interactions between Ags and immune system cells are essential to start an efficient adaptive immune response in secondary lymphoid tissues such as LNs. For most Ags—including pathogens—it is not well known how exactly they reach LNs and then the subcompartments within, for instance, the B cell follicles. Experimental evidence suggests that certain free Ags could reach LNs directly by lymph flow and then distribute by subcapsular (SCS) and medullary sinus or by means of conduits inside [22–25]. These Ags could also be ferried to nodes by sentinel cells coming from peripheral tissues such as the skin or mucosae. Of note, these various paths of Ag transport are highly dependent on Ag size [22].

However, there is no information on how DENV reaches the DLNs after cutaneous infection, where exactly DENV might be localized inside DLNs (if it does), and whether the first

contact between DENV and immune cells could influence subsequent immune responses, for instance, neutralizing or facilitating antibodies.

We aimed at assessing the natural *in vivo* route of DENV, from its entrance through the skin until its arrival and distribution inside regional DLN, as well as, its potential association with antigen presenting cells (APCs).

2. Materials and methods

2.1. Mice

Adult male BALB/c mice were used for all the experiments. Mice were fed *ad libitum* and housed in a specific pathogen-free environment at the local animal facilities, UPEAL. The experimental procedures were approved by the ethical committee of the Center for Advanced Research, The National Polytechnic Institute, Cinvestav-IPN. A group of mice was cutaneously inoculated in the shaved inguinal region with a single dosage of 1×10^6 PFU of fluorescently labeled DENV serotype 2, another group with 5 μg of Alexa Fluor 555-Ovalbumin (Life Technologies; Eugene, OR, USA) and the control group with sterile phosphate-buffered saline (PBS) only. After 1, 3, 6, 12 and 24 hours post-inoculation (hpi), small pieces of skin at the inoculation site and DLNs were extracted and processed either to perform flow cytometry or *in situ* fluorescence, as indicated.

2.2. Antibodies and reagents

The following primary antibodies were used: rat anti-F4/80 (Cl:A3-1), rat anti-CD169 (MOMA-1), both were purchased from Serotec-Bio-Rad (Kidlington, UK); hamster anti-CD11c (HL3), rat anti-B220 (RA3-6B2), rat anti-Gr-1 (RB6-8C5), rat anti-I-A/I-E (2G9), all purchased from BD Pharmingen (San Diego, CA, USA) and rat anti-DEC-205 (205yekta) was purchased from eBioscience (San Diego, CA, USA). Primary antibodies were used directly coupled to allophycocyanin, fluorescein isothiocyanate, PerCP/Cy5.5 or were detected by the following secondary reagents: donkey anti-rat Alexa Fluor 488 purchased from Life Technologies (Eugene, OR, USA) and Cy5-streptavidin and 4′,6-diamino-2-fenilindol (DAPI) purchased from Invitrogen (San Francisco, CA, USA).

2.3. Obtaining dengue virus stock

We obtained DENV stock *in vitro* by infecting the insect cell line C6/36 (from *Aedes albopictus* larvae) with brain extracts of infected neonate mice. C6/36 cells were grown in minimum essential medium eagle (MEM) supplemented with 10% Fetal Bovine Serum (Gibco), Amphotericin B, Penicillin, Streptomycin, Pyruvate, Vitamins and L-glutamine, at 34°C in 75-cm^2 culture flask (Corning). Infection was performed when cells reached 95% confluency. After 48 h post-infection, cell supernatant containing DENV was collected and concentrated with Amicon Centrifugal Filter Units (Merk Millipore, Co. Cork, IRL). Quantification

of infectious virions was performed by means of a plaque forming assay in Baby Hamster Kidney cell (BHK-21) and reported as Plaque-Forming Units (PFU)/ml.

2.4. Fluorescent labeling of dengue virus

For *in vivo* DENV tracking in tissues (skin and LNs), we labeled DENV with a strong red fluorescent dye (PKH-26, Sigma). Briefly, DENV was mixed with PKH-26 dye in the appropriate diluent and incubated for 5 min at room temperature. To stop the reaction, we added washing buffer (10% Fetal Bovine Serum in PBS). Because of the virus size, we applied ultrafiltration using centrifugal filter units and centrifuged at 4000 × *g* for 20 min at 4°C. The last step of washing was carried out only with cold sterile PBS.

2.5. Immunofluorescence of LNs and skin

DLN and skin samples were obtained after 1, 3, 6, 12 and 24 hpi from the different groups of experimental animals: PBS-inoculated mice, mice injected with Ovalbumin (OVA) tagged with Alexa Fluor-555 or mice inoculated with fluorescently labeled DENV. Tissues were embedded in optimal cutting temperature (OCT) compound Tissue Tek (Sakura FineTek, Torrance, CA, USA), frozen in liquid nitrogen and cut into 5 μm sections on a cryostat (Leica). Cryosections were put on poly-L-lysine-coated glass slides and fixed with ethanol for 7 min at −20°C. To identify Mfs, we used F4/80 and CD169 antibodies, CD11c and DEC-205 antibodies for DCs and B220 antibody for B lymphocytes; tissue sections were immunolabeled overnight at 4°C with the corresponding primary antibodies. After three washing steps, fluorescent secondary antibodies or streptavidin were incubated during 1 h or 15 min, respectively. Images were scanned with Leica TCS SP8 AOBS Confocal microscopy using objectives with 10× and 40× magnification and analyzed with Leica Software.

2.6. Flow cytometry of lymph node cell suspensions

DLNs were obtained 1, 3, 6, 12 and 24 h after animals were inoculated in the skin with either PBS as a control, or with fluorescently labeled DENV. The cell suspension obtained was blocked with Universal Blocker (BioGenex Laboratories, San Ramón, CA). Then, single-cell suspension was incubated with anti-CD11c, anti-I-A/I-E, anti-Gr-1, anti-F4/80, anti-CD169, anti-B220 and secondary antibodies for 25 min at 4°C in FACS buffer (BSA 1%, NaN_3 0.01% in PBS) to identify APCs and finally fixed with paraformaldehyde 1% for FACS reading. Labeled cell suspensions were acquired with a BD LSR Fortessa III and analyzed with Flowjo X.0.6 for Windows (Ashland, OR).

2.7. Statistical analyses

We performed an ANOVA test for comparing groups using GraphPad Prism v6.0 Software, lines in bars or dots represent ± SEM (standard error of the mean). We considered 95% confidence intervals and significant difference when the *P* value was <0.05.

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3. Results

3.1. Upon cutaneous inoculation DENV is located in the deep dermis and therefore not associated with epidermal DEC-205+ DCs

To assess the distribution of DENV in the skin upon cutaneous (intradermal) inoculation, we used immunocompetent mice and fluorescently labeled DENV. We assessed skin cryosections after 1, 3, 6, 12 and 24 h post-intradermal inoculation of DENV and, as a control we evaluated the distribution of fluorescently labeled OVA at 1 h and 12 hpi. DENV was readily found in the basement of the dermis at all the times evaluated and its presence decreased over time. Putative associations of DENV with epidermal DEC-205+ DCs were not observed, likely because DENV was much deeper than the rather superficial epidermal DEC-205+ cells. At 24 hpi, the skin appears thicker than at early times suggesting recruitment of cells (**Figure 1A**). While no fluorescent signal was detected in skin of mice inoculated only with PBS (**Figure 1A**), fluorescent OVA was clearly seen in the dermis at 1 h but not at 12 hpi (**Figure 1B**).

3.2. Ovalbumin is found in the medullary sinus of the LNs at 1 h but not at 12 h post-cutaneous inoculation

Ovalbumin has been used as model Ag in some experimental approaches to describe the distribution of molecules inside LNs. Because of its molecular size, OVA reaches LNs quickly. We used red fluorescent OVA as a control molecule and evaluated its distribution after 1 h

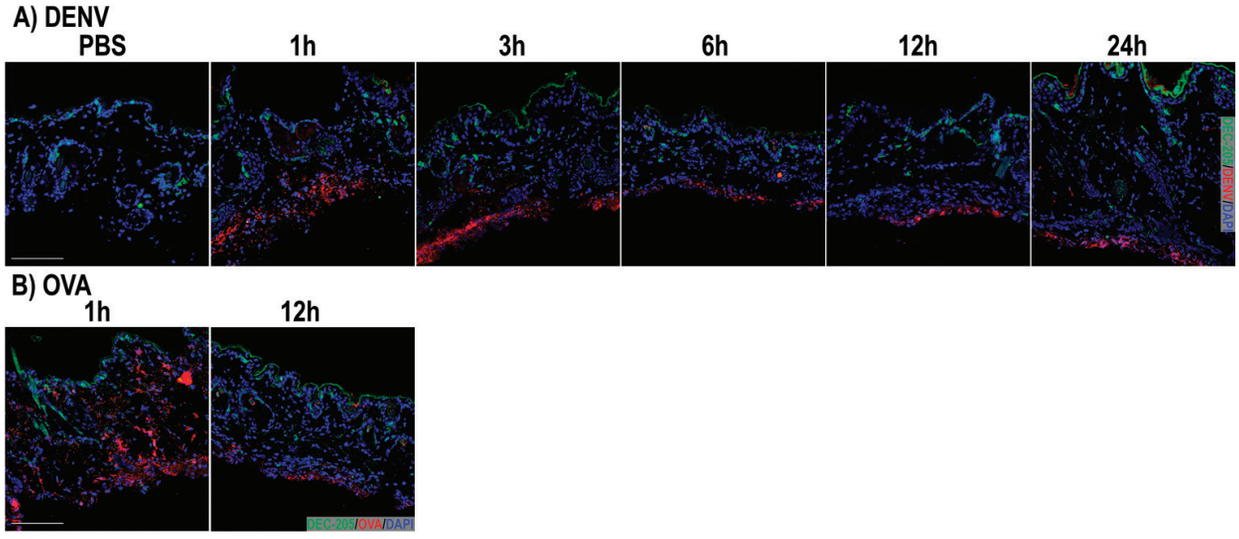

Figure 1. Inoculation of fluorescently labeled DENV allows its identification in the skin of immune-competent mice. Mice were cutaneously inoculated with red fluorescent DENV in the inguinal region and skin cryosections were analyzed after 1, 3, 6, 12 and 24 hpi by confocal microscopy. As controls, mice received either PBS or red fluorescent OVA. DEC-205+ DCs (green) are localized mainly in the epidermal layer in all experimental conditions. (B) Red fluorescent OVA was in the dermis at 1 h but much less was seen at 12 hpi, whereas (A) red fluorescent DENV was located in the deep dermis from 1 hpi decreasing afterwards. In the skin of PBS-inoculated mice, no red fluorescence was detected (left image in (A)). Nuclei were stained with DAPI (blue). Stratum corneum exhibits some autofluorescence at 3 h and 24 hpi (A) and 12 hpi (B). Bar scale = 300 μm. Images are representative of six skin sections per experimental group.

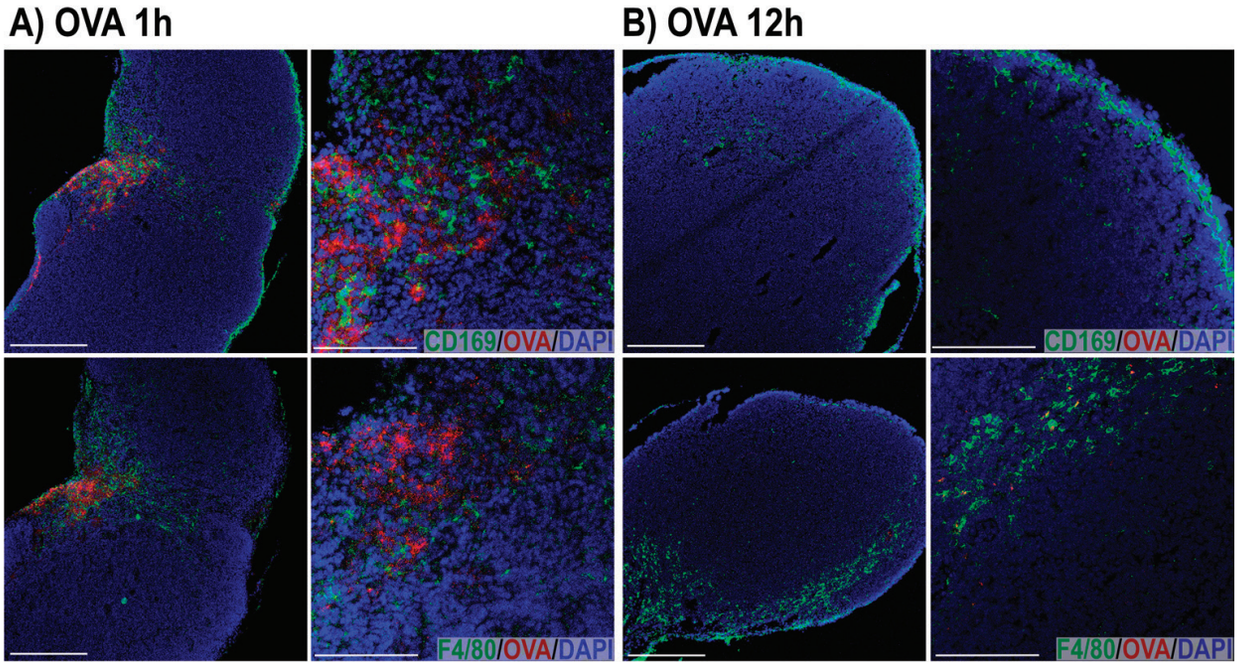

Figure 2. Distribution of red fluorescent OVA in LNs after cutaneous inoculation. DLNs from mice cutaneously inoculated with red fluorescent OVA were harvested at (A) 1 hpi and (B) 12 hpi. CD169+ (top images) and F4/80+ (bottom images) Mfs are seen in green, whereas nuclei are seen in blue. Red fluorescent OVA is clearly observed in the medullary sinus of DLNs at 1 hpi, but not at 12 hpi. The distribution of OVA seems contained by the SCS Mfs but not by F4/80+ Mfs. Bar scale = 300 μm (left images) and 100 μm (right images). Images are representative of six LNs per experimental group.

and 12 hpi. At 1 hpi, OVA was found in the medullary sinus mainly associated with CD169+ Mfs and much less with F4/80+ Mfs (**Figure 2A**). At 12 hpi, only scarce red fluorescence was detected in F4/80+ cells (**Figure 2B**).

3.3. Cutaneous DENV reaches lymph node B follicles since 1 h post-inoculation

To ascertain whether DENV was distributed in B cell follicles at early times post-cutaneous inoculation, we obtained DLNs of mice after 1, 3, 6, 12 and 24 hpi. DLNs cryosections were labeled with B220 antibody to identify B lymphocytes. Red fluorescent DENV was clearly identified in the paracortical areas, the SCS, the medullary sinus and in some B cell follicles (top pictures) at early time points. For a more detailed examination of B cell follicles, we used a higher magnification objective (bottom pictures) showing that at 1 hpi, small clusters of DENV mark were present inside follicles in apparent close association with B cells. At 3 hpi, very few associations were detected in follicles probably because most DENV fluorescence was by then seen at the medullary zone. In contrast, at 6 hpi, we observed some DENV mark inside B cell follicles and even in cells with macrophage-like morphology and the red fluorescence apparently in the cytoplasm. At 12 h and 24 hpi, we did not detect fluorescent DENV signal *in situ* (**Figure 3**).

3.4. Inside lymph nodes DENV appears differentially associated with CD169+ and to F4/80+ macrophages *in situ*

Mfs are crucial for the clearance of pathogens and have been classified according to restricted markers and location inside LNs. Mfs located in the medullary sinus—where highly phagocytic activity is carried out—are called medullary Mfs, whereas those delineating the floor

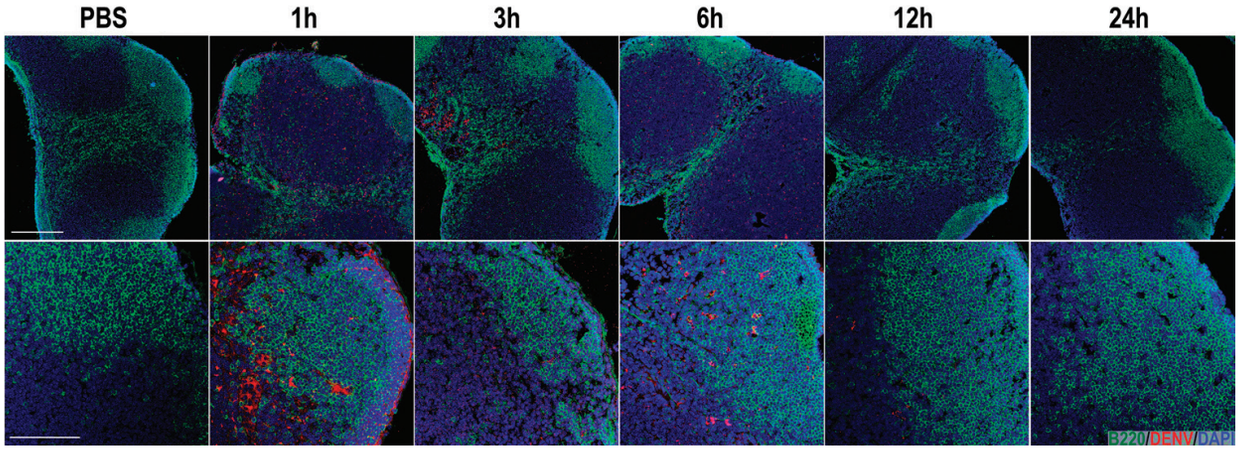

Figure 3. DENV and B cells in DLN cryosections of mice cutaneously inoculated with red fluorescent DENV. LNs cryosections of mice inoculated in the skin with red fluorescent DENV were probed with B220 antibody detected with green fluorescence. Analysis was performed by confocal microscopy at 1, 3, 6, 12 and 24 hpi. We found red fluorescent DENV from 1 h to 6 hpi in B cell follicles (green fluorescence), at 1 hpi DENV was mainly in perifollicular areas. Nuclei were stained with DAPI as observed in blue. As control, DLN sections from PBS-inoculated mice were analyzed, and no red fluorescence was detected (left images). Scale bar = 300 μm (top images) and 100 μm (bottom images). Images are representative of six LNs per experimental group.

of SCS are called SCS Mfs. Medullary Mfs are either CD169+ or F4/80+, whereas SCS Mfs are CD169+ [26].

We assessed *in situ* in DLNs the potential involvement of CD169+ or/and F4/80+ Mfs in capturing DENV after 1, 3, 6, 12 and 24 h post-cutaneous inoculation. Interestingly, since 1 hpi, many CD169+ cells with seemingly cytoplasmic DENV were observed, mainly in SCS and medullary sinus. DENV was also found in paracotical areas in association with CD169- cells (**Figure 4A**). In contrast, at 1 hpi only few F4/80+ medullary Mfs were associated with DENV (**Figure 4B**). After 3 hpi, DENV was mostly restricted to medullary sinus and the CD169+ Mfs (**Figure 4A**). At this time point, DENV was poorly associated with F4/80+ Mfs. Interestingly, many F4/80- cells, most likely CD169+ Mfs, were observed with large amounts of DENV apparently in small phagocytic vesicles, as was observed at high magnification (**Figure 4B**). After 6 hpi, we still detected DENV signal mainly associated with CD169+ Mfs (**Figure 4A**). At the latest times evaluated (12 and 24 hpi), no red fluorescent signal was detected.

3.5. The CD11c+ DCs in regional lymph nodes are DENV+ from early times post-cutaneous inoculation

DCs migrate from peripheral tissues such as the skin (and mucosae) to secondary lymphoid organs like LNs. Because DENV enters through the skin, it is highly likely that skin DCs might participate in delivering DENV into DLNs. We thus probed DLN cryosections with CD11c and DEC-205 antibodies at 1, 3, 6, 12 and 24 h post-cutaneous inoculation of red fluorescent DENV. At 1 hpi, we found DENV in close association with CD11c+ cells but not with DEC-205+ cells in the DLN paracortex. At 3 hpi, we could not find DENV in the paracortex, neither associated with CD11c+ cells nor with DEC-205+ cells; instead, red fluorescent DENV was restricted to medullary areas. At 6 hpi, DENV was associated with some CD11c+ cells, but not with DEC-205+ cells. As mentioned before, DENV was not detected in regional LNs at 12 h nor at 24 hpi (**Figure 5**).

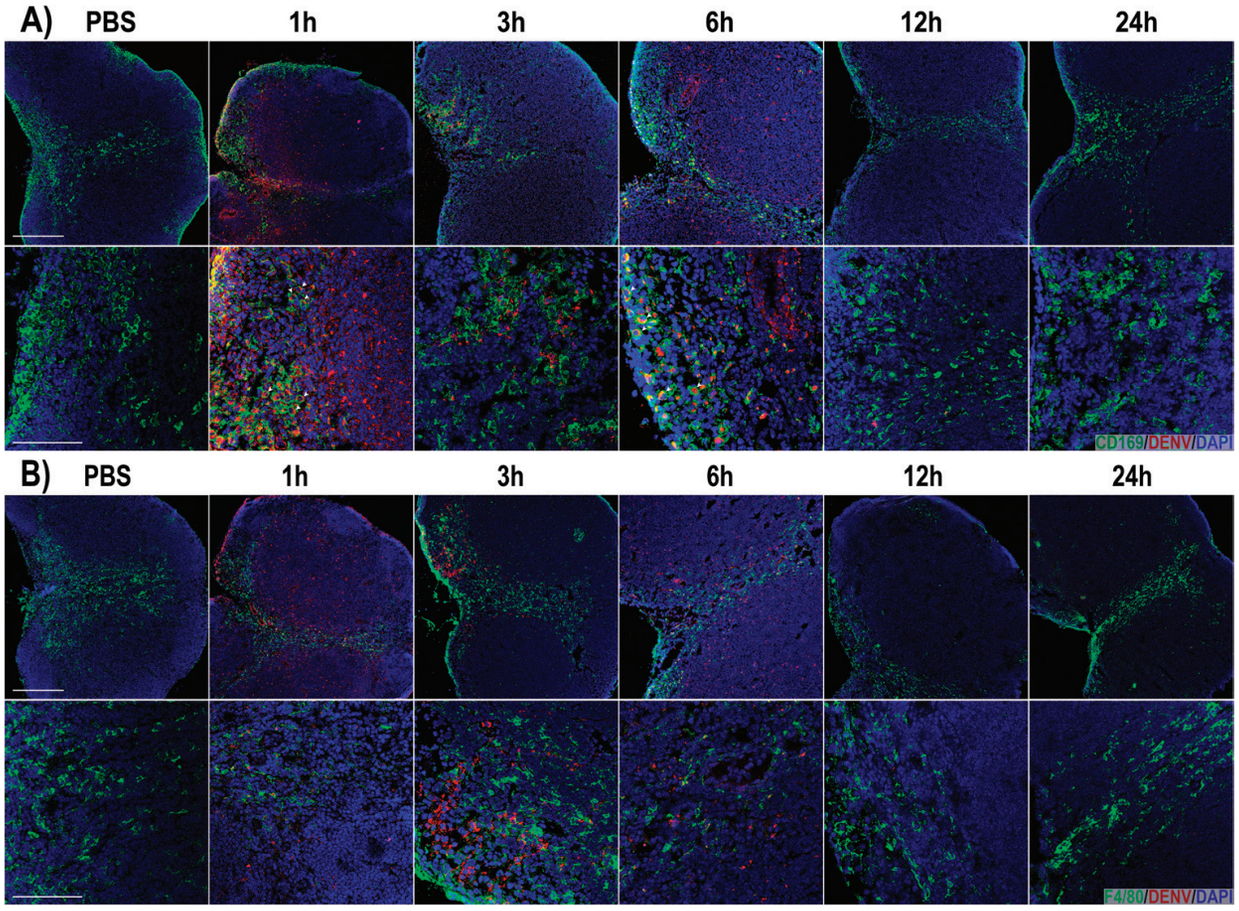

Figure 4. DENV and (CD169+ or F4/80+) macrophages in regional LNs. DLNs from mice inoculated in the skin with PBS or red fluorescent DENV were collected at 1, 3, 6, 12 and 24 hpi, and cryosections were labeled to identify (A) CD169+ or (B) F4/80+ Mfs both in green. Nuclei were stained with DAPI as observed in blue. DLN sections were scanned by confocal microscopy. (A) DENV was distributed in SCS at 1 hpi (yellow color at the edge) and mainly in medullary sinuses apparently in the cytoplasm of CD169+ Mfs from 1 h to 6 hpi (white arrowheads, bottom images). (B) Very few associations of red-fluorescent DENV with F4/80+ Mfs were found in the medullary zones. No red fluorescence was detected in PBS inoculated mice (left images). Bar scale = 300 μm [top images in (A) and (B)] and 100 μm [bottom images in (A) and (B)]. Images are representative of six LNs per experimental group.

3.6. Flow cytometry analysis of Ag presenting cells and DENV in DLNs after cutaneous inoculation

For a more quantitative assessment of APCs associated with DENV, we used DLN cell suspensions from mice inoculated at various times with fluorescent DENV in the skin. A very low proportion (0.06–0.17%) of B cells was positive for DENV at all time points evaluated (1 h-24 hpi). This might be due to the very low number of Ag-specific B cells (**Figure 6A**). CD169+ Mfs were also positive for DENV, with an apparent peak (0.83%) at 3 hpi, which correlates well with the medullary localization observed *in situ* (**Figure 6B**). Regarding F4/80+ Mfs, the proportion of positive cells varied from 0.3 to 0.57% along the experiment, with the maximum at 1 hpi (**Figure 6C**). DCs were identified as Gr-1^{-}, MHC-II^{+} and CD11c^{+} cells and were the most abundant DENV+ population. The highest mean proportion (4%) of DENV+

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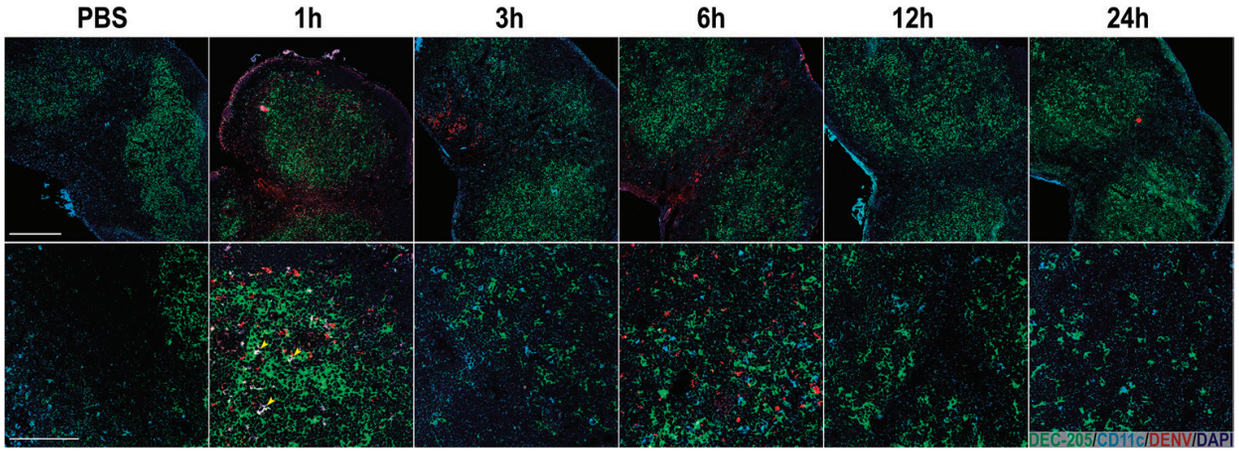

Figure 5. DENV and DCs in regional lymph nodes. Immune-competent mice were intradermally inoculated with red fluorescent DENV, and DLNs were harvested at 1, 3, 6, 12 and 24 hpi. CD11c+ DCs were identified in light blue fluorescence, DEC-205+ DCs in green, whereas nuclei are seen in dark blue. As described, DENV was detected from 1 h to 6 hpi. At 1 hpi, red fluorescent DENV was located in the paracortical area and clearly associated with light blue CD11c+ DCs which then appear white due to merged colors (bottom image, yellow arrowheads indicate white color DCs). Red DENV was not associated with green DEC-205+ DCs. Cryosections of PBS inoculated mice did not show red fluorescence (left images). Bar scale = 300 μm (top images) and 100 μm (bottom images). Images are representative of six LNs per experimental group.

DCs was seen at 1 hpi decreasing afterwards to a minimum (0.85%) at 24 hpi (**Figure 6D**). We integrated all values in a graph (**Figure 6E**) to compare the percentages of all DENV+ APCs assessed. It seems that DCs are the main DENV+ cells, especially at early times post-inoculation, suggesting that either these cells are carrying DENV from skin to the nodes or are the main cells capturing the virus once DENV arrives into the nodes.

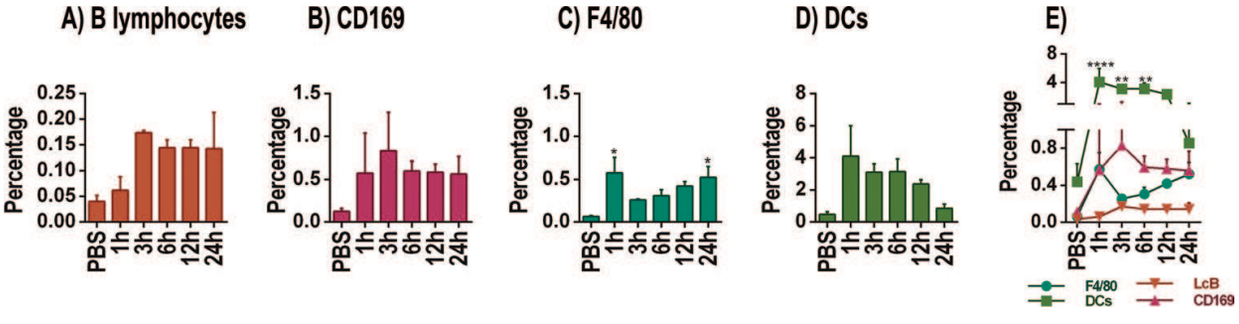

Figure 6. Flow cytometry assessment of DENV+ cells in regional LNs. Cell suspensions of DLNs harvested 1, 3, 6, 12 and 24 h after inoculating red fluorescent DENV were labeled for B cells (B220), Mfs (CD169 and F4/80) and DCs (CD11c). Red fluorescence co-staining was used to determine the percentage of each DENV+ cell population. (A) A very low proportion of DENV+ B cells was observed from 1 h to 24 hpi. (B) The amount of DENV+ CD169+ Mfs was higher than (C) DENV+ F4/80+ Mfs from 1 h to 24 hpi. (D) The proportion of DENV+ CD11c+ DCs was higher than the other populations, reaching a maximum at 1 hpi declining afterwards. Graph in (E) shows the integrated results of all cell subsets during the kinetic follow up. Bars in graphs represent the median ± SEM of percentages, and ANOVA analysis was performed, considering *$p > 0.05$, **$p > 0.01$ and ****$p > 0.0001$.

4. Discussion

While many cells in the skin can make contact with DENV during mosquito biting (a topic under intense investigation recently), very few cells will have the ability to capture DENV and to ferry it into lymphatic vessels and from there to secondary lymphoid tissues. Cutaneous DCs can uptake DENV and migrate into DLNs to the DC area, where the most likely cells to make the first immune contacts with DENV+ DCs will be T lymphocytes [27]. Some B cells could also interact with DENV Ags in this area and follow either an extrafollicular response or get into a GC reaction [28, 29]. Still another possibility inside LNs is the display of DENV Ags by Mfs, either in the SCS or deeper in the paracortical and medullary zones [30]. Thus, LNs are crucial organs to establish effective adaptive immune responses. For this, the efficient interaction between immune cells and Ags is needed. The ensuing events upon arrival of Ags inside LNs might depend on how these Ags are getting there, whether the Ag is arriving alone through the lymph flow or is carried by different cells which could be important to the type of the immune response that follows. Herein, we discuss some of these possibilities regarding DENV infection *in vivo*.

DENV enters the human host through the skin while mosquitoes feed. After locating a suitable host, an infected mosquito probes throughout the dermis introducing the proboscis and is during this process that the salivary glands release the virus [31]. Conceivably, viral particles are likely to interact first with the various cells of epidermal and dermal layers. However, both the very early stages of DENV infection and the initial local encounters between DENV and elements of the local immune system *in situ* remain largely unexplored.

By infecting *ex vivo* healthy human skin explants from non-cadaveric samples, our group demonstrated DENV-infected cutaneous DCs [11]. We have also identified that human keratinocytes and dermal fibroblasts are permissive to DENV infection and that they respond by secreting a wide variety of soluble mediators that contribute to induce an immune-activated microenvironment and an antiviral state [10, 11, 32]. Now, upon cutaneous inoculation of fluorescently labeled DENV into immune-competent mice, we evaluated the presence of DENV as well as its localization in both the skin and the DLNs. We did a kinetic follow-up by *in situ* immunofluorescence as well as in cell suspensions by FACS. In the skin, we found the virus localized mostly through the dermis at 1 h post-inoculation, afterwards DENV was distributed deep in the skin. To the best of our knowledge, this is the first time that fluorescently labeled DENV is used to assess its distribution *in vivo*.

Some reports in humans and mice have shown DENV proteins inside LNs, both at the periphery and inside B cell follicles [13, 16, 33–35]. Where the virus is located inside DLNs could influence the first contact with cells from the immune system and the subsequent immune response. However, how DENV reaches the DLNs after cutaneous infection has not been explored *in vivo*. Using the vesicular stomatitis virus, researchers have investigated how viral particles that enter through peripheral tissues are handled within DLNs, identifying a population of Mfs on the floor of the SCS and in the medulla of LNs that capture viral particles within minutes after subcutaneous injection [30]. Similar to other Ags delivered through the skin, the Bluetongue Virus is transported rapidly through the lymph to the DLNs in its natural host the sheep [36].

After 1 h of skin inoculation and once in DLNs, we found DENV associated with CD169+ Mfs from the subcapsular and medullary sinus and with CD11c+ DCs in the paracortical area. The association with these two cell types was seen especially at early times (1–6 h) after cutaneous inoculation. The rapid localization of DENV in the SCS of the DLNs is consistent with delivery of putative cell-free virus through the lymphatic fluid, besides that DCs might be also carrying the virus [36]. In the immune-deficient murine model (AG129 mice), it has been shown that Mfs from the SCS are important controlling the spreading of DENV. These SCS Mfs contained NS1 protein, likely implying that they are trapping DENV Ags or are being actually infected [37]. In these same immune-deficient mice, after intra-footpad inoculation, it was shown that DENV initially targets Mfs of the DLN [38]. As SIGN-R1, the murine homolog of human DC-SIGN is highly expressed on SCS Mfs [39], it is likely that DENV is infecting these Mfs in the DLN. Indeed, DC-SIGN is one of the molecules used by DENV to enter host cells [40].

After the SCS Mfs trap DENV, these cells could translocate surface-bound viral particles across the SCS floor and make DENV Ags available to other cells, for instance, migrating B cells in the underlying follicles. It seems that these Mfs from the SCS act as vigilants against many different pathogens and are able to discriminate between lymph-borne viruses and other particles of similar size [30]. CD169+ Mfs in LNs could capture lymph-borne viruses preventing their systemic dissemination and could guide captured virions across the SCS floor for the efficient activation of follicular B cells [30, 37].

In addition to this, DENV associated with DCs highly likely implies that DENV is transported from the skin to the DLNs by DCs. We and others have found DENV-infected cutaneous DCs in human cadaveric and non-cadaveric healthy skin explants infected *ex vivo* [11, 14, 21]. Thus, DENV might be reaching DLNs also through cutaneous DCs. Also, migratory DENV-infected DCs have been found in the skin DLNs of mice lacking IFN-α/β receptor ($IFNAR^{-/-}$ mice) infected through the skin, suggesting that dermal DCs might be ferrying DENV to regional nodes and likely triggering the adaptive immune response [14].

DCs are specialized sentinel cells that uptake Ags at peripheral tissues and travel to DLNs ferrying Ags to paracortical areas, where B cells migrating toward follicles are likely to encounter these Ags [23, 27, 41]. A hallmark of specific B lymphocyte activation is BCR-mediated capturing/acquisition of Ags. This will facilitate the subsequent Ag presentation from B cells to T cells in order to develop efficient T cell-dependent antibody responses [42]. DCs may provide B cells with broader access to Ags, particularly those of large sizes or associated with particulate materials. Lastly, through Ag presentation to T cells, DCs could subserve functions such as cellular platforms facilitating activation, colocalization and mutual communication of rare Ag-specific T and B cells, whose interaction may ultimately lead to optimal T and B cell responses [41].

Tracking *in vivo* fluorescently labeled DENV from the skin permitted us to know the rapid (1 hpi) localization of DENV in subcapsular and medullary Mfs of DLNs, its association with CD11c+ DCs in the T:B border and the presence of DENV inside B cell follicles. All these events make highly plausible that Ag-specific B cells would be recognizing DENV and makes feasible that they would be receiving T cell help, these cellular interactions are needed to lead

to full antibody responses. To this respect, it is worth mentioning that antibody responses to DENV need very careful examination; ideally, these responses should be neutralizing [43, 44]. However, the potentially detrimental impact of non-neutralizing antibodies enhancing the infection *in vivo* is only beginning to be elucidated, not only in heterotypic reinfections with DENV but also in infections with other flaviviruses such as Zika [44–48].

Altogether, these data suggest that after being inoculated into skin, DENV is reaching the regional lymph nodes in at least two ways, through the lymph fluids but also being carried by cells. Travelling with DENV from the skin to the regional LNs will allow us to better understand how the immune system is being alerted or affected and which might be the cells responding at each stage. We believe that it is important to decipher the *in vivo* biology of these cellular responses to DENV entrance, not only for making better models to study early interactions among DENV, skin-resident and LN cells but also to better understand the pathology of DENV.

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