Host cell responses to dengue virus infection
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DENGUE TROPISM FOR MACROPHAGES AND DENDRITIC CELLS: THE HOST CELL EFFECT

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Abstract

Dengue virus infects immune cells, including monocytes, macrophages and dendritic cells (DC). We compared virus infectivity in macrophages and DC, and found that the virus-origin determined the cell tropism of progeny virus. The highest efficiency of re-infection was seen for macrophage-derived dengue virus. Furthermore, in the presence of enhancing antibodies, macrophage-derived virus gave higher enhancement of infection compared to immature DC-derived virus. Taken together, our results highlight the importance of macrophages in dengue infection.
Dengue is currently the most prevalent mosquito-borne viral disease worldwide. There are four distinct dengue virus (DENV) serotypes (DENV1–DENV4) and each of them can cause clinical manifestations ranging from mild flu-like illness to severe, potentially fatal, dengue haemorrhagic fever or dengue shock syndrome. Two groups of individuals are at risk for severe disease: individuals experiencing a heterologous secondary infection, and infants with waning titers of maternal antibodies towards DENV. These epidemiological observations led to the theory of antibody-dependent enhancement (ADE) of DENV infection, where antibodies enhance rather than neutralize infection.

In humans, DENV replicates predominantly in cells of the innate immune system, including immature dendritic cells (iDC), monocytes, macrophages (MΦ) and mature dendritic cells (mDC). iDC were found to be highly susceptible to DENV in the absence of antibodies. Multiple studies have shown that iDC do not support ADE. However, this appears to depend on the virus origin as iDC-derived DENV was described to facilitate ADE in iDC. Monocytes, MΦ, and mDC are less susceptible to DENV than are iDC in the absence of antibodies, but facilitate ADE. Thus, the literature suggests that distinct subsets of cells are important for DENV viraemia in the absence and in the presence of enhancing concentrations of DENV-specific antibodies.

Although there is much evidence that the abovementioned cell types are important during DENV infection, no studies have reported a systematic paired analysis on the infectious properties of DENV in primary MΦ, iDC and mDC and determined how susceptibility relates to the production of progeny virions in these cells. Hence, that was the aim of this study. Furthermore, we assessed if and how the origin of the virus influences its tropism, both in both the absence and presence of DENV antibodies.

Following a mosquito bite, DENV is deposited into the skin and bloodstream, where it will encounter potential target cells. Therefore, we first investigated the susceptibility of MΦ, iDC and mDC to DENV2 derived from the mosquito cell line C6/36. Human monocytes were isolated from buffy coats (Sanquin Bloodbank, the Netherlands) and differentiated into MΦ and iDC, as previously described. Maturation of iDC into mDC was performed according to a published protocol. Fig. S1 (Supplementary Material) shows the surface marker expression pattern of the resulting cells and confirms the expected phenotype.

Primary human iDC, mDC and MΦ were infected with DENV2 strain 16681 at a m.o.i. of 1 and 10, unless indicated otherwise. DENV infectivity was assessed on the basis of the percentage of infected cells (flow cytometric analysis using the 3H5 antibody) and the number of DENV2 produced in the supernatant in terms of genome-equivalent copies [GEc; as determined by quantitative reverse-transcription PCR (qRT-PCR).
and infectious particles (p.f.u. assay). Virus infectivity was determined at 24 h post-infection (p.i.) Fig. 1 (a) shows a representative dot blot of the percentage of infected MF, iDC and mDC. In line with previous publications, we observed that iDCs are highly susceptible to DENV infection. The fraction of infected iDC did not increase between m.o.i. 1 and 10, while the number of infected MΦ and mDC further increased between m.o.i. 1 and 10 (Fig. 1a). In agreement with these results, m.o.i.-dependent virus particle production was seen up to at least m.o.i. 10 for MΦ (Fig. S2). For iDC, m.o.i.-dependent growth was only observed below m.o.i. 1 (Fig. S2).

**FIGURE 1** | Susceptibility and productivity of MΦ, iDC and mDC towards DENV2. (a) Representative dot plots of the number of DENV2-infected primary human MΦ, iDC and mDC at 24 h p.i. following infection at m.o.i. 1 and 10. Mock-infected cells are shown as control. DENV infection was detected by flow cytometry using the DENV2-specific 3H5 antibody. (b) Number of secreted infectious virus at 24 h p.i. as determined by plaque assay (p.f.u.) on BHK-21 clone 15 cells. (c) Number of GEc produced as determined by qRT-PCR at 24 h p.i. MΦ and iDC were infected with DENV2 at m.o.i. 1 and 10. mDC were only infected with DENV at m.o.i. 1. Shown are N=12, 8, 9 and 4 data points and the SEM, respectively. Human MΦ, iDC and mDC were infected at m.o.i. 10 (MΦ and iDC) or m.o.i. 1 (MΦ, iDC and mDC). Shown are N=12, 9, 9, 9 and 4 data points and SEM, respectively. (d) The GEc per p.f.u. ratio of DENV2 produced on MΦ, iDC and mDC after infection at m.o.i. 1 and m.o.i. 10. Shown are SEM of N=12, 7, 9, 13 and 4 data points, respectively. Values connected by lines are derived from cells of the same donor and the same experiment. The p.f.u. and the GEc titre were not both determined in all samples, which explains the differences in N between (b), (c) and (d). Statistical analysis was done by 2-tailed ANOVA analysis: P 0.05 (*) or 0.01 (**).
Next, we evaluated the number of GEc and p.f.u. secreted from infected cells (Fig. 1b, c). The data are summarized in Table S1, which shows that, at m.o.i. 10, iDC produced 10-fold more GEc than did MΦ, yet comparable numbers of infectious particles were detected (Table S1, based on paired samples from cells of the same donor). At m.o.i. 1, iDC produced close to 100-fold more GEc than did Mf and mDC. Interestingly, comparable numbers of infectious virus particles were secreted by iDC and mDC. MΦ produced 10-fold lower numbers of infectious particles than did iDC and mDC. The discord between the GEc and p.f.u. titres of iDC suggests that these cells produce low-infectious virus particles. Indeed, the GEc/p.f.u. ratio calculated for individual donors revealed that the infectivity of iDC-derived DENV was lower (~3500) compared with that of MΦ-derived DENV (~400) after infection at m.o.i. 10. At m.o.i. 1, the GEc/p.f.u. ratio was approximately 1700 for iDC-derived DENV and 250 for MΦ-derived DENV. DENV particles secreted from mDC had the highest infectivity (~25, Table S1).

Boonnak et al\textsuperscript{15} also determined the number of physical and infectious particles from infected iDC, MΦ and mDC, yet did not report the infectivity (GEc/p.f.u.). For comparison, we calculated the GEc/p.f.u. ratio based on the information in their report, and found a ratio of ~100 for MΦ, ~1 for iDC and below 1 for mDC. A GEc/p.f.u. ratio below 1 is not possible, so caution is required as we cannot deduce whether the data points are derived from paired samples. However, it is clear that our ratios are different from those extracted from the report of Boonnak et al\textsuperscript{15}. These authors used Vero cells for initial virus production and plaque titration whilst we used C6/36 cells and BHK-21 clone 15 cells, respectively. To rule out that the cell type used for plaque titration was responsible for the differences in the results, we attempted to perform DENV plaque assays on Vero cells. However, the three Vero cell lineages (Vero-ATCC, Vero-WHO and Vero-E6) available in our lab did not support DENV plaque formation. As an alternative approach, we applied flow cytometry to assess the infectivity of the viruses on Vero cells. Fig. S2(f) clearly shows that MΦ-derived DENV is more infectious than iDC-derived DENV in Vero-WHO cells, which is in line with the infectivity observed in BHK-21-clone 15 cells. Furthermore, when comparing the infectivity of DENV2 produced by C6/36 cells and by Vero-WHO cells, the infectivity did not differ between these two producer cell lines (Fig. S2g). Hence, our observation that iDC-derived DENV has a lower infectivity than MΦ-derived DENV2 is not influenced by the cell lines used to produce virus stocks or to quantify infectious titres.

Collectively, we conclude that during the first round of infection with mosquito-derived virus, iDC are the most susceptible cell type. iDC produce more particles compared with DENV-infected MΦ and mDC but these iDC-derived particles exhibit relatively low-grade infectivity.
An earlier report showed that iDC-derived DENV cannot infect new iDC\textsuperscript{16}. This finding prompted us to further investigate the role of virus origin in cell tropism of progeny DENV2. To this end, we tested the capacity of iDC- and MΦ-derived DENV2 to infect MΦ and iDC. The technical limitations of culturing a large batch of mDCs prevented us from producing sufficient mDC-derived DENV2 stocks and subsequent testing of this virus origin. We decided to infect MΦ and iDC on the basis of an equal multiplicity of genomes (m.o.g.) of 500. We did so as we (unpublished) and others\textsuperscript{22,23} observed a virus dose-dependent activation of inflammatory responses in primary cells. Thus implying that infectivity experiments based on equal m.o.i. but different m.o.g. may give biased results. In accordance with previously published data\textsuperscript{16}, we found that iDC are least susceptible towards iDC-derived virions, followed by MΦ-derived and C6/36-derived DENV (Fig. 2a). MΦ, however, were equally susceptible to iDC- and MΦ-derived DENV albeit the infectivity was very low (~0.5% at m.o. g. 500). Again, MΦ were infected more readily by mosquito-derived DENV2 than by mammalian cell-derived DENV2 in MΦ (Fig. 2a).

The differences in infectivity for MΦ- and iDC-derived virions might be caused by soluble factors, e.g. cytokines, present in the supernatants of infected cells. Given the known antiviral properties of IFNα\textsuperscript{24}, we first determined the IFNα concentration by ELISA (IBL International). Surprisingly, MΦ supernatants contained higher concentrations of IFNα than did iDC supernatants: in two donors we found 475.3 pg ml\(^{-1}\) versus 24.1 pg ml\(^{-1}\), and 166.9 pg ml\(^{-1}\) versus 20.8 pg ml\(^{-1}\), respectively. Thus, the lower infectivity of iDC-derived DENV2 cannot be explained by IFNα. We next tested whether other soluble factors present within DENV-infected cell supernatants are important for infectivity. To this end, cell supernatants were first subjected to UV treatment to impair the infectious properties of DENV present within the supernatant. Indeed, no viral plaques were observed in the UV-inactivated cell supernatants. Then, a fixed number of C6/36- derived DENV GEc was incubated with several dilutions of UV-treated cell supernatants for 1 h at 37°C and viral infectivity was measured by plaque assay. Fig. 2(b) shows that both iDC and MΦ supernatants possess antiviral properties (<100% of expected C6/36-titres), yet no differences were seen between the two supernatant origins. This indicates that the differences in infectivity of progeny virions derived from MΦ and iDC cannot be explained by the concentration of IFNα or other soluble factors in the medium. Another possibility is that iDC secrete a large number of defective interfering particles. Indeed, defective interfering particles are seen in dengue patients\textsuperscript{25}. Secretion of defective interfering particles is often related to the m.o.i. used to infect cells: the higher the m.o.i. the lower the infectivity\textsuperscript{26,27}. In line with this, we observed that the infectivity of DENV was lower when the virus had been produced in cells infected with a high m.o.i. (Fig. 1d and S3b).
A. B.

FIGURE 2 | Infectious properties of MΦ, iDC or mosquito cell-derived DENV in cells. (a) Infection of primary MΦ and iDC by virions of mammalian (MΦ and iDC) or mosquito origin (C6/36): MΦ and iDC were incubated with a m.o.g. of 500 of MΦ-derived, iDC-derived and C6/36-derived DENV2. Infectivity was determined at 24 h p.i. by flow cytometry (3H5 antibody). N=2 donors (MΦ) or 4 donors (iDC), with each condition in duplicate. Fill-in denotes the same donor. (b) UV-inactivated supernatants of infected MΦ- and iDC-cultures were diluted with DMEM medium (Gibco) and subsequently a fixed number of C6/36-derived DENV2 GEc were added and incubation was continued for 1 h. Thereafter, a plaque assay was performed. The percentage of supernatant dilution indicated was maintained throughout the titration. Titres are expressed as percentages of controls (DMEM with equal percentage of RPMI medium (Gibco), 20% FBS). N=4 paired donors.

We thus far showed that the origin of the virus determines its tropism in the absence of antibodies, but we did not know whether the origin of the virus also influences infection under conditions of ADE. We focused on MΦ and mDC, as these experiments have already been performed for iDC\textsuperscript{16}. First, we determined the infectivity of mosquito cell-derived DENV2 in MΦ and mDC after incubating the virus with serial dilutions of human DENV-immune sera. The sera were obtained from Nicaraguan patients with an acute secondary DENV infection\textsuperscript{28} (kind gift Dr Eva Harris, University of California, Berkeley). At 26 h p.i., the fraction of infected cells was determined by flow cytometry (Fig. 3a). MΦ showed peak enhancement of infection at high concentrations of serum antibodies, whereas mDC demonstrated peak enhancement at very low concentrations (Fig. 3a). Similar results were obtained when sera was used from two other DENV-infected patients (data not shown). These results suggest that serum antibodies co-determine the host cell tropism of DENV2 and point towards a MΦ-oriented tropism under conditions with high DENV antibody titres. Contrary to infection of MΦ, infection of mDC and iDC will be suppressed under conditions of high levels of serum antibodies (Fig. 3a and\textsuperscript{14,15}).

Next, we tested whether the ADE-effect observed was dependent on the origin of the virus. Given the large number of conditions involved, we were not able to
perform paired analyses in primary cells from the same donor. Therefore, we decided to use murine MΦ-like P338D1 cells for these experiments. P338D1 cells express Fcγ receptors I–III and can be infected with DENV2 in the absence and the presence of antibodies. To avoid possible epitope- and isotype-biased results, two murine antibodies targeting two different epitopes were used: a flavivirus-reactive antibody against the envelope protein fusion loop (4G2, IgG2a; Fig. 3b) and a DENV2-specific antibody against the envelope protein domain III (3H5, IgG1; Fig. S3a). P338D1 cells were infected with iDC-derived, MΦ-derived and C6/36-derived DENV2 at m.o.g. 500 in the presence of a range of antibody concentrations (Figs 3b and S3a). Virus production was scored at 48 h p.i. by plaque assay. Comparable baseline titres and ADE profiles were obtained for MΦ-derived, iDC-derived and C6/36-derived DENV2 in P338D1 cells. Yet, the highest fold enhancement of infection was seen for MΦ-derived DENV2 virions. This effect was independent of the epitope (Fig. 3b vs Fig. S3a).

We conclude that mosquito-derived DENV particles are prone to infect iDC (Fig. 1). Subsequently, infected iDC produce progeny DENV2. However, this iDC-derived DENV-production is hallmarked by a low ratio of infectious virus to total virus production (Fig. 1d and Table S1). Hence, there is low efficacious re-infection of other iDC and MΦ (Fig. 2a and ). MΦ-derived DENV is more infectious in iDC than in MΦ (Fig. 2a). The lower susceptibility of MΦ and mDC is compensated for by the higher infectivity of progeny virus. Therefore, we postulate that DC and MΦ contribute equally to DENV dissemination.

The observation that iDC produce virions with low-infectious properties is intriguing. It is not known why iDC produce large numbers of low-quality virions, yet it is tempting to speculate that this serves to facilitate efficient priming of the adaptive immune system as described for other viruses. Future research is required to address this observation in more detail.

Our results imply that during secondary infection, when anti-DENV antibodies are present, the cell tropism of DENV changes. Early in infection, the serum antibody concentration is low and therefore mosquito-derived virus likely infects primarily iDC through interaction with the virus receptor. During subsequent rounds of infection, both iDC and MΦ (Fig. 3b) contribute to viraemia and support ADE of DENV infection. The relative importance of iDC and MΦ in DENV ADE warrants further study. In MΦ the highest fold enhancement of DENV production is seen for virions derived from MΦ (Fig. 3b), which suggests that MΦ play an important role in virus dissemination during secondary infection.
A.

![Graph showing Fold Enhancement of DENV2 infection](image)

**FIGURE 3** | Effect of serum concentrations and virus origin on antibody-dependent DENV2 infection. (a) Antibody-dependent enhancement of DENV2-infected cell mass in primary human Mϕ and mDC. DENV2 was opsonized with serially diluted sera from Nicaraguan patients experiencing an acute secondary DENV infection. Cells were infected at m.o.i. 1. Infectivity was scored at 26 h p.i., and normalized to infection in the absence of serum. (b) ADE-profile of C6/36-derived, iDC-derived and Mϕ-derived DENV2 in P338D1 cells. P338D1 cells were infected at m.o.g. 500 with DENV2 opsonized for 1 h at 37 C with increasing concentrations of the anti-flavivirus antibody 4G2. Virus titres were quantified at 48 h p.i. by plaque assay and expressed as fold change over 'No Ab'. Shown are the SEM of at least three experiments with viruses derived from two donors, and all conditions in duplicate.
Supplementary Material

SUPPLEMENTARY TABLE S1 | Characteristics of DENV2 production by primary human MΦ, immature dendritic cells and mature dendritic cells at 24 h p.i. The values presented in this table are averages of all individual yet paired samples. The reported GEc/p.f.u ratio is the average of the GEc/p.f.u calculated per donor.

<table>
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<th>MOI 10</th>
<th>MOI 1</th>
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<tr>
<td>MΦ iDC</td>
<td>MΦ iDC mDC</td>
</tr>
<tr>
<td>Produced</td>
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<tr>
<td>GEc prod (GEc/mL)</td>
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<tr>
<td>PFU prod (PFU/mL)</td>
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<td>GEc/PFU ratio ±SEM</td>
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SUPPLEMENTARY FIGURE S1 | Phenotype and morphology of the cell types used in this study.
(a) Representative examples of the surface marker expression (left) and morphology (right) of primary human macrophages and immature dendritic cells (iDC). The phenotypes were determined after 6 days of differentiation (No LPS), or 6 days in culture followed by 48h stimulation with 1mg/mL of ultrapure LPS (Sigma Aldrich). After six days of culture, ≥95% of the cells had typical macrophage morphology and expression pattern: CD14+, CD80low, CD86+, CD206low, and MHC-II+. Similar for immature dendritic cells: CD14-, CD80low, CD86+/++, CD206low, CD11c++, and CD83-. Upon LPS stimulation, cells changed their expression pattern by upregulation of CD80, and CD86. (b) Phenotypic analysis of mature dendritic cells (mDC), derived from the immature dendritic cells (iDC) described in A). After 24h of maturation, the expression of CD40, CD83 and CD86 were upregulated in mDC relative to iDC. All cells are derived from healthy, anonymous blood donors with informed consent (Sanquin Bloodbank, Groningen, NL).
SUPPLEMENTARY FIGURE S2 | Virus particle production. (a) Growth curves of DENV2 on primary human MΦ showing an m.o.i-dependent curve for m.o.i 1 to 10. Statistics as per Wang, G.P. & Bushman, F.D. (2006), resulted in a 2-tailed p-value of 0.04 (m.o.i 1 vs m.o.i 10). N=5 donors; see A2C. Curves were normalized to be able to study the kinetics by compensating for donor variations. All curves were normalized relative to the latest time point at m.o.i 10 (set at 100%). (b) Growth curves of DENV2 on
primary human iDC showing m.o.i-independent curves for M.O.Is 1 to 10, but m.o.i-dependent at m.o.i 0.01 and 0.1. N = 5 donors, see A2D. (c) Growth curves of DENV2 on primary human mDC showing m.o.i-dependent curves for m.o.i 0.01 to 10. N = 3 donors. (d) and (e) Growth curve of 5 individual donors for MΦ (c) and iDC (d) showing the effect of the m.o.i on the growth curve, and the variability among the donors (~10-fold difference in maximum titer within the same cell type). (f) Percentage of infected cells as measured with flow cytometry. Vero-WHO cells were infected with 500 physical particles per cell of DENV2 derived from MΦ, iDC, or C6/36- mosquito cells. At 24hpi, flow cytometric analysis was performed to quantify the infectivity of the virus particles. N= 2 independent experiments, each in duplicate. (g) The GEc/p.f.u ratio of C6/36-derived DENV2 and Vero WHO-derived DENV2. C6/36-derived DENV2 (N=6), Vero WHO-derived DENV2 (N=3). Shown are SEM of the ratios based on qRT-PCR (GEc) and plaque assay on BHK-21 clone 15 (p.f.u).


SUPPLEMENTARY FIGURE S3 | 3H5 ADE-profile on P338D1 cells and DENV infectivity on iDC (a) as in Fig. 3B, yet using the DENV2-specific E domain III antibody. Shown is the SEM of 3 experiments with virus derived from one donor, all conditions in duplicate. Baseline titres of 'No Ab' did not differ between the three origins. (b) GEc/p.f.u ratio of DENV produced in iDC. N = 2 donors with each condition in duplicate. Fill-in denotes the same donor.

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