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A Role for Human Skin Mast Cells in Dengue Virus Infection and Systemic Spread

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Dengue virus (DENV) is a mosquito-borne flavivirus that causes serious global human disease and mortality. Skin immune cells are an important component of initial DENV infection and systemic spread. Here, we show that mast cells are a target of DENV in human skin and that DENV infection of skin mast cells induces degranulation and alters cytokine and growth factor expression profiles. Importantly, to our knowledge, we also demonstrate for the first time that DENV localizes within secretory granules in infected skin mast cells. In addition, DENV within extracellular granules was infectious in vitro and in vivo, trafficking through lymph to draining lymph nodes in mice. We demonstrate an important role for human skin mast cells in DENV infection and identify a novel mechanism for systemic spread of DENV infection from the initial peripheral mosquito injection site. ***The Journal of Immunology*****, 2016, 197: 4382–4391.**

Dengue virus (DENV) causes serious human disease and mortality worldwide. In recent years, there has been increased epidemic activity and geographic expansion of DENV infection along with its mosquito vector, primarily *Aedes aegypti*, and it is considered a serious emerging global health problem (1–4). The disease has an enormous impact on the health and economies of tropical and subtropical regions, with dengue infections occurring in Asia, the Americas, Africa, Pacific, and Mediterranean regions (4–8). In addition, there have been recent outbreaks in Texas and Florida where transmission occurred on American soil (9, 10). There are no vaccines or specific therapeutic agents approved for DENV, aside from the Sanofi Pasteur vaccine (Dengvaxia) that is recommended by the World Health Organization for limited use in highly endemic regions (11). The development of a safe and effective vaccine has been hindered by the phenomenon of Ab-dependent enhancement, where Abs against one serotype can lead to severe disease, such as dengue hemorrhagic fever and dengue shock syndrome, upon infection with a second serotype (8, 12, 13). The initial introduction of DENV into a person is through the bite of an infected *Aedes* spp. mosquito. The virus is deposited along with mosquito saliva during feeding and probing in the skin. Although there has been some limited investigation into which skin cells are involved in this initial infection, and dendritic cells have long been considered the main target of DENV following skin inoculation (14), a comprehensive picture remains to be formed.

Mast cells, tissue resident cells that are the main effector cell type of allergic reactions, are highly prevalent in the skin (15–17) and may be among the first immune cells infected with DENV after mosquito injection. This prevalence, along with the localization of mast cells to areas near blood vessels, suggests that they could be a primary target of initial DENV infection (18). There has been limited research on the infection dynamics and pathogenesis of DENV in mast cells. DENV was shown to infect and promote cytokine production in the human mast cell lines KU812 and HMC-1 (19), but studies using human primary mast cells have not been reported. It has also been shown that DENV can replicate in cells from the rat-derived mast cell-like rat basophilic leukemia (RBL) cell line, and cause mast cell degranulation in the LAD2 human tumor-derived mast cell line as well as in mast cells from DENV-infected monkey skin explants (20). There is evidence of an innate response to DENV infection in RBLs that includes chemokines, cytokines, and the RIG-I pathway. DENV infection of and interaction with mast cells has also been associated with vascular leakage and disease severity (21). In addition, mast cell–deficient mice have a reduced ability to clear DENV infection, which is thought to be a result of a reduced ability to recruit NKT cells to the infected skin (20). Mast cell–deficient mice also exhibited enhanced DENV infectivity and infiltration of macrophages to the skin inoculation site, and increased DENV-associated bleeding time compared with wild-type mice (22).

The primary mechanism by which mast cells exert their immunological influence is through the release of preformed inflammatory mediators that are stored in cytoplasmic secretory granules, and released immediately after mast cell activation. Mast cell activation and degranulation are generally associated with crosslinking of the high affinity receptor for IgE, FcεRI, but can also be activated by other factors like complement anaphylatoxins,

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Abbreviations used in this article: DENV, dengue virus; DLN, draining lymph nodes; HMVDEC, human microvascular dermal endothelial cell; MOI, multiplicity of infection; p.i., postinfection; qRT-PCR, quantitative real-time PCR; RT, room temperature; VEGF, vascular endothelial growth factor.

neuropeptides, and certain toxins. Mast cell secretory granules contain many different inflammatory mediators including β-hexosaminidase, histamine, serine proteases tryptase and/or chymase, and cytokine TNF, which are the causative agents of allergic reactions (23). Importantly, intact extracellular mast cell granules released during degranulation are capable of entering lymphatic vesicles and trafficking through the circulatory system, thus demonstrating an ability to deliver their inflammatory cargo to distant physiological sites (24). It is likely that DENV particles may be able to use this extracellular chaperone function of mast cell granular particles, as they contain heparan proteoglycans, which have been shown to bind flavivirions (24, 25).

In this study we investigate the potential for DENV to infect primary human skin mast cells and evaluate cytokine, chemokine, and growth factor expression profiles. We also examine the effects of DENV infection in mast cells on neighboring endothelial cell activation and proliferation. Finally, we investigate the role of mast cell degranulation and extracellular granules in DENV infection. Together, these findings demonstrate a critical and, to our knowledge, previously unrecognized role for skin mast cells in the infection and propagation of DENV in humans.

Materials and Methods

Isolation and purification of human skin mast cells

Human skin mast cells were isolated and purified from fresh surgical specimens of human skin obtained from the Cooperative Human Tissue Network (CHTN) of the National Cancer Institute, as approved by the human studies Internal Review Board at University of South Carolina. Briefly, the tissue was mechanically disrupted with surgical scissors, digested with collagenase type II, hyaluronidase, and DNase I in HBSS wash buffer containing amphotericin B and antibiotic/antimycotic solution, and filtered through 40 μm nylon cell strainers. This was repeated for a total of three digestions. The filtered cells were collected by centrifugation, washed and resuspended with wash buffer, and separated on Percoll by density centrifugation. The cells at the interface of buffer and Percoll layers were collected, washed and resuspended at 5×10^5 cells/ml in serum-free X-VIVO 15 media containing recombinant human stem cell factor (SCF, 100 ng/ml). The cells were maintained under standard culture conditions (37°C, 5% CO_2) with weekly media changes. Purity was assessed cytochemically by metachromatic staining with acidic toluidine blue. Greater than 95% purity is typically achieved through 6 wk of culturing.

Mast cell activation

IgE-sensitized human skin mast cells (10^6/ml) were activated by cross-linking FcεRI with the hapten NP-BSA (4-hydroxy-3-nitrophenylacetyl conjugated to BSA at a 16:1 molar ratio) at 37°C. For sensitization, 10^6 cells/ml were incubated in X-VIVO 15 media or Tyrode's buffer (135 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes, 5 mM KCl, 1.8 mM $CaCl_2$, 5.6 mM glucose; pH 7.4, 0.05% BSA) containing 1 μg/ml chimeric human anti-NP IgE (human Fc + mouse Fab) (clone JW8/1; AbD Serotec, Raleigh, NC) for 3 h at 37°C. After washing to remove unbound IgE, the mast cells were resuspended at 10^6 cells/ml in X-VIVO 15 media or Tyrode's buffer, and then activated with 100 ng/ml NP-BSA.

β-Hexosaminidase assays

Mast cell degranulation was assessed by β-hexosaminidase release assay. Human skin mast cells were activated with 100 ng/ml NP-BSA for 30 min at 37°C in Tyrode's buffer. Calcium ionophore was used as a positive control for degranulation. After the incubation period, the mast cells and buffer were separated by centrifugation (2000 rpm for 5 min), and the pelleted cells lysed with an equal volume of 1% Triton X-100. β-Hexosaminidase activity in supernatant and cell lysate was assayed by measuring the release of p-nitrophenol from substrate p-nitrophenyl N-acetyl-β-D-glucosaminide (pNAG; Sigma-Aldrich, St. Louis, MO) (26, 27). In a 96-well plate, 5 μl of supernatant or lysate was mixed with 45 μl of 4 mM p-Nitrophenyl N-acetyl-β-D-Glucosaminide (pNAG) in citric acid buffer (pH 4.5) and incubated for 1 h at 37°C. The reaction was stopped by adding 150 μl of 0.2 M glycine, pH 10.7. Absorbance values at 405 nm were acquired with a BioTek Synergy HT microplate reader. Percent degranulation was calculated as percentage release of β-hexosaminidase using the formula: % β-hex release = ((supernatant)/(supernatant + lysate)) × 100.

DENV infection

We used both high-passage (NGC) and low-passage (K0048) strains of DENV-2 virus for our initial studies. Virus was propagated in C6/36 *Ae. albopictus* mosquito cells and titered using the Vero monkey kidney cell line. Infections were done at a multiplicity of infection (MOI) of 1.0 unless otherwise indicated in the figure legends. Briefly, virus was added to cell cultures and allowed to infect for 1 h at 37°C. The cells were washed, and infection allowed to continue for indicated times.

Mast cell granule experiments

Mature mast cells obtained from human skin tissue were infected with DENV, which induces degranulation within 24 h in our studies. At the 24-h time point, the exocytosed extracellular granules were collected from cell supernatant by centrifugation (12,000 × *g* for 10 min at 4°C) and washed 10 times with PBS and media. The isolated granules were resuspended in media and placed on naive cells. Cells were analyzed for infection 24 h post infection (p.i.) by quantitative real-time PCR (qRT-PCR) on isolated RNA and immunofluorescence staining. Alternatively, isolated granules were injected into footpads of 5-week-old AGB6 mice (200 μl/footpad) and popliteal draining lymph nodes (DLN) and spleen were dissected at the 24-h time point. Tissues were analyzed for DENV infection by qRT-PCR. Animals were maintained and procedures were performed at the University of Pittsburgh in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. Mice deficient in receptors for type I and type II IFNs, ($IFNAGR^{-/-}$, AGB6) were bred under specific pathogen-free conditions.

Gene expression analysis

RNA was isolated from infected cells or tissues and purified using RNeasy kit (Qiagen, California) according to the manufacturer's instructions. For analysis of DENV infection, the qRT-PCR analysis was done using the QuantiFast kit according to the manufacturer's instructions (Qiagen) (28, 29). This kit directly uses RNA in reactions.

Oligonucleotides for the DENV qRT-PCR reactions were: DENV envelope, forward, 5′-CATTCCAAGTGAGAATCTCTTTGTCA-3′, reverse, 5′-CAGATCTCTGATGAATAACCAACG-3′. Gene expression analysis was performed using RT2 Profiler Human PCR Arrays (Qiagen) according to the manufacturer's protocol and qRT-PCR.

Transwell assay

Uninfected (naive) human microvascular dermal endothelial cells (HMVDECs) (CC-2543; Lonza) were grown according to the manufacturer's instructions and plated onto 24-well plates. Cells were allowed to grow to 50% confluence overnight at 37°C, 5% CO_2. Mature mast cells obtained from human skin tissue were infected with DENV and 125,000 cells per well were plated on Transwell permeable inserts (Corning), which were inserted into the medium above the HMVDECs. At 24 h after initiation of coculture, both cell supernatants were removed for ELISA analysis, as indicated in figure legends. A subset of HMVDECs was removed and counted in a cell proliferation assay. RNA was isolated from a subset of HMVDECs and used in qRT-PCR analysis to quantify ICAM and VCAM gene expression.

In-cell ELISAs

An additional subset of HMVDECs from the Transwell assay was used for an in-cell ELISA assay. Briefly, cells were washed twice with 1× PBS, fixed with 4% paraformaldehyde and then treated with 0.1% Triton X-100 for 5 min at room temperature (RT). Cells were incubated with 1:500 dilutions of Abs recognizing VCAM (#NBP2-33182H; Novus) and ICAM (#ab195528; Abcam) at 4°C overnight. Plates were washed three times and incubated for 1 h at RT with 1:2000 dilutions of HRP secondary Abs (Santa Cruz Biotechnology, Texas). Plates were then incubated with the substrate 3,3,5,5-tetramethylbenzidine (TMB), 1-StepTM Turbo TMB-ELISA Substrate Solution (Life Technologies, New York), for 3 min at RT. The reaction was stopped with 2 N sulfuric acid and plates were read at 450 nm in a BioTek Synergy HT microplate reader (Biotek Instruments, Vermont).

ELISAs

ELISAs were performed to measure cytokines and other immune mediators released from mast cells. The following kits were used—CCL5/RANTES: DuoSet ELISA kit (#DY278-05; R&D Systems); and vascular endothelial growth factor (VEGF): Human VEGF ELISA kit (#KHG0111; Novex/Life Technologies). All kits were used according to the manufacturer's instructions. To measure IL-6 and IL-8, we used our in-house ELISA protocol, as previously described (30). Briefly, supernatant was diluted and coated onto

plates in a 384-well format. Capture (purified) and detection (biotinylated) Abs (BD Biosciences/Pharmigen) used were: IL-6 (MQ2-13A5 and MQ2-39C3), IL-8 (554716 and 554718). Serially diluted recombinant cytokine standards (BD Biosciences) were used to generate standard curves. After developing with the substrate for peroxidase 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma-Aldrich), absorbance values at 405 nm were obtained and cytokine concentrations in experimental samples determined with a BioTek Synergy HT microplate reader (Biotek Instruments) and Gen5 Data Analysis Software.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 20 min at RT, washed with PBS(−) and then stained with appropriate Abs. Slides of skin tissue were deparaffinized using standard protocols, treated with Sudan Black (Fisher Scientific) for 1 h at RT to remove background fluorescence, and incubated with 1% BSA blocking buffer for 1 h at RT. Both cells and skin slides were stained with Abs against mast cell chymase (#orb4912; Biorbyt) and/or DENV-2 (# MAB10226; Millipore) and/or DENV capsid protein (made in-house in mice), and counterstained with DAPI and/or phalloidin-594 (Molecular Probes), as indicated in figure legends. The Abs were diluted in 1% BSA at 1/250 and cells were incubated for 20 min at RT. All secondary Abs used were standard (anti-mouse or anti-rabbit tetramethylrhodamine isothiocyanate (TRITC) and FITC), and were diluted according to manufacturer's instructions. Infection was visualized using the EVOS FL Cell Imaging system (Thermo Fisher). More specifics can be found in figure legends.

Mosquito infection and skin feeding

The Rockefeller strain of *Ae. aegypti* was infected by blood feeding, using 400 μl of DENV-infected C6/36 cell supernatant added to 1 ml of serum-inactivated human donor blood (The Blood Center, New Orleans, LA). Mosquitoes were fed for 20 min at RT using a hemotek feeder and maintained in groups of 10 at 30°C, 80% humidity. Mosquitoes were supplied sucrose water as a source of dietary sugar. On day 14 p.i., mosquitoes were allowed to feed on biopsy punches of human skin tissue (CHTN). Uninfected mosquitoes fed on separate skin punches for controls. Skin was maintained in full cell culture media for 24 h, then fixed in 4% paraformaldehyde for 1 h at RT. The skin tissue was processed and sectioned in paraffin by the Instrumentation Resource Facility (IRF) at the University of South Carolina School of Medicine.

Results

DENV infects human skin mast cells

Mature mast cells were isolated and purified from human skin tissue as previously described (30) (Supplemental Fig. 1). We infected the human skin mast cells with a low-passage (K0048) or high-passage (NGC-2) strain of DENV serotype 2, and collected the cells at 24 h p.i. Human skin mast cells were readily infected with DENV as demonstrated by qRT-PCR (Fig. 1A). By immunofluorescence, we also demonstrated colocalization of DENV and chymase within the secretory granules of skin mast cells (Fig. 1B). We also examined the effect of DENV infection on degranulation. Previous reports indicate that DENV infection alone can induce mast cell degranulation (19, 20). We found that DENV infection induced degranulation at levels comparable to that induced by FcεRI cross-linking as measured by the release of β-hexosaminidase (Fig. 1C). Additionally, we demonstrated that IgE sensitization in the absence of Ag increased the degranulation induced by DENV infection (Fig. 1D).

Mast cells are a target of initial DENV infection in human skin

We found that ex vivo human skin mast cells were readily infected by DENV. The next step was to determine whether mast cells were an actual target for natural DENV infection in human skin. To this end, we infected *Ae. aegypti* mosquitoes with DENV by blood feeding as described (29). At day 14 p.i., we fed the mosquitoes on samples of human skin tissue. For control samples, noninfected mosquitoes were injected with either saliva alone or saliva and DENV on separate skin samples. We isolated RNA from the skin tissue and performed qRT-PCR analysis to quantify DENV infection (Fig. 2A). We then analyzed the skin tissues using immunohistochemistry. Skin sections were stained with fluorescent Abs specific for chymase to visualize mast cells and for DENV capsid protein to identify infected cells. We were able to detect mast cells in skin tissue exposed to DENV-infected mosquitoes that stained positively for DENV (Fig. 2B). Thus we demonstrated that mast cells in human skin were targets of DENV following exposure to infected mosquitoes.

DENV infection of human skin mast cells alters immune gene expression

Next, we examined the effect of DENV infection on the expression profile of various immune-related genes in purified human skin mast cells. To do this, we used the RT^2-Profiler PCR Array Human Allergy & Asthma panel (Qiagen). Human skin mast cells were infected with DENV NGC-2 at an MOI of 1.0, RNA was isolated 48 h p.i. and used in qRT-PCR according to the manufacturer's instructions. We found 21 genes to be either up- or down- regulated >9-fold compared with noninfected mast cells (Fig. 3). The full results can be found in Supplemental Fig. 2A. Remarkably, expression of the chemokine CCL5 (RANTES) was up-regulated almost 10,000 fold during DENV infection. Previous studies have demonstrated a selective induction of CCL5/RANTES as well as MIP-1α, and MIP-1β from DENV-infected human mast cell lines (19). CCL5 is known to attract monocytes, T helper cells and eosinophils that could contribute to DENV-induced inflammation. Additionally, DENV infection is known to increase the release of several cytokines and chemokines, which can contribute to the development of severe disease (31–33). Therefore, we measured the release of CCL5 as well as cytokines known to play a role in DENV infection. We found that IL-6, IL-8 and CCL5 protein levels were significantly increased in the supernatant of DENV-infected human skin mast cells as compared with noninfected controls, with or without IgE sensitization (Fig. 4). Levels of TNF and IL-10 were below the level of detection.

DENV-infected human skin mast cells secrete high levels of endothelial growth factors

Vascular endothelial cells have been implicated in the development of severe disease due to DENV infection. As these cells are prevalent in the skin, we examined the effects of DENV-infected skin mast cells on human endothelial cells. To begin, we measured the expression of VEGFs in DENV-infected mast cells using the RT^2-Profiler PCR Array Human VEGF Signaling panel (Qiagen). Our analysis revealed significant increases in expression of 34 genes during DENV infection of human skin mast cells compared with noninfected controls (Fig. 5). The full results can be found in Supplemental Fig. 2B. Particularly impressive was a >100-fold increase in expression of members of the NF of activated T cells (NFAT) family of transcription factors, neurophilin, PIK3CB, PIK3RB, and paxillin. Noteworthily, the expression of VEGF-A was increased >81-fold during DENV infection compared with control cells. These data provide evidence to suggest that DENV-infected mast cells might have an impact on endothelial cell activation and/or proliferation. To explore this possibility, we used HMVDECs in a Transwell assay. We placed DENV-infected human skin mast cells in the top layer and noninfected HMVDECs in the bottom chamber. The HMVDECs were removed at 24 h p.i. and used in several assays. First, VEGF levels in the mast cell supernatants were measured by ELISA analysis. We found that levels increased during DENV infection as compared with uninfected cells (Fig. 6A). Next, we performed in-cell ELISAs to measure the expression of ICAM and VCAM by the HMVDECs. We found increased levels of both ICAM and VCAM

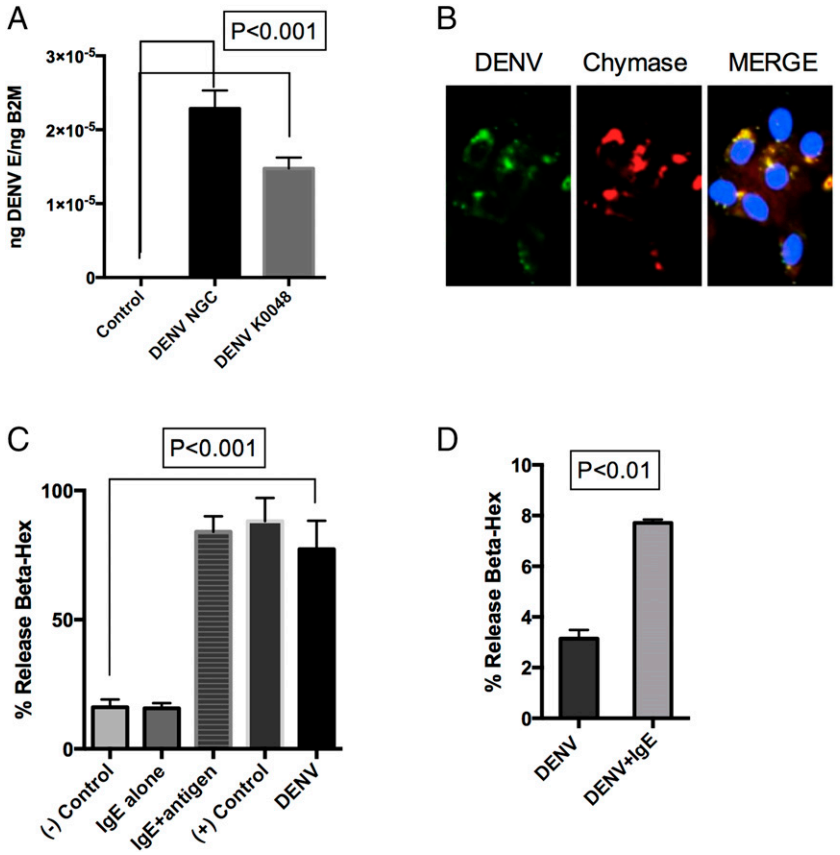

FIGURE 1. DENV infects human skin mast cells and causes degranulation. Primary mast cells were isolated from human skin tissue as described in the methods. (**A**) Cells were infected with DENV-2, either strain NGC or K0048, at an MOI of 1.0 and infection was measured using qRT-PCR analysis at 24 h p.i. Control cells were uninfected. Both DENV infection and qRT-PCR analysis were done in triplicate. Data is pooled and error bars indicate SD. Student *t* test was used for statistical analysis. Results were $p < 0.001$ for both comparisons. (**B**) Primary human skin mast cells were infected with DENV-2 NGC at an MOI of 1.0. Cells were fixed in 4% paraformaldehyde 24 h p.i. and stained with Abs against chymase and DENV. Cells were counterstained with DAPI to visualize nuclei. A representative image is shown at original magnification ×20. (**C**) Human skin mast cells were uninfected and unsensitized (negative control), uninfected and sensitized with IgE for 24 h (IgE alone), uninfected, sensitized with IgE for 24 h and then activated with specific Ag (IgE+antigen), used in a degranulation assay with positive controls as described in the methods (positive control) or infected with DENV-2 NGC at an MOI of 1.0 for 24 h (DENV). Cells were lysed and degranulation was measured using a β-hexosaminidase release assay as described. Scientific and technical replicates were both done in triplicate. Student *t* test was used for statistical analysis. Results were $p < 0.001$. (**D**) Human skin mast cells were infected with DENV-2 NGC at an MOI of 0.1 and either unsensitized (DENV) or sensitized with IgE for 24 h. At 24 h p.i., cells were lysed and degranulation was measured by β-hexosaminidase release assay. Student *t* test was used for statistical analysis. Results were $p < 0.01$.

in HMVDECs plated with DENV-infected skin mast cells as compared with cells plated with noninfected control mast cells (Fig. 6B). Sensitization with IgE did not significantly alter either ICAM or VCAM levels. RNA was isolated from a subset of HMVDECs and expression levels of VCAM and ICAM were quantified by qRT-PCR to determine the activation status of the cells. We found that both VCAM and ICAM expression levels were significantly increased in the HMVDECs plated adjacent to DENV-infected skin mast cells as compared with those plated next to noninfected mast cells (Fig. 6C). Lastly, HMVDECs plated with DENV-infected skin mast cells exhibited increased proliferation over the 24-h period compared with those plated with noninfected control mast cells as demonstrated by a basic proliferation assay (Fig. 6D). Together, these data indicate that DENV-infected human skin mast cells were able to signal neighboring endothelial cells for activation and proliferation.

DENV travels from infected skin mast cells in intact granules

We next used immunofluorescence analysis to examine the location and abundance of DENV in infected human skin mast cells. Mast cells were stained for DENV envelope protein and counterstained with DAPI to visualize the nucleus. Interestingly, we found that DENV localized in the mast cell cytoplasmic granules (green circles, Fig. 7A) and even in extracellular granules released during degranulation (red arrows, Fig. 7A). It was recently shown that extracellular granules are able to travel via lymph to DLN and function as chaperones for mast cell mediators in vivo (24). Therefore, we hypothesized that localization to mast cell granules could be a novel dissemination mechanism by which DENV propagates infection from the periphery. First, we sought to determine if the virus carried in or by extracellular granules was infectious. To this end, we purified the extracellular granules from DENV-infected human skin mast cells. To confirm the presence of granules, a portion of the purified extracellular granule solution was labeled with AlexaFluor 488–conjugated avidin, a probe which selectively binds heparin and has been shown to specifically label isolated extracellular mast cell granules in solution (24) (Shown in Supplemental Fig. 3A). To confirm that there was DENV in the granule solution, we used qRT-PCR to quantify DENV nucleic acid in RNA isolated from the purified granules (Supplemental Fig. 3B). The purified granules were then added to cultures of naive human dermal endothelial cells, Huh7 cells, Vero cells, and the LAD2 human mast cell line. As hypothesized, purified granules isolated from DENV-infected skin mast cells caused

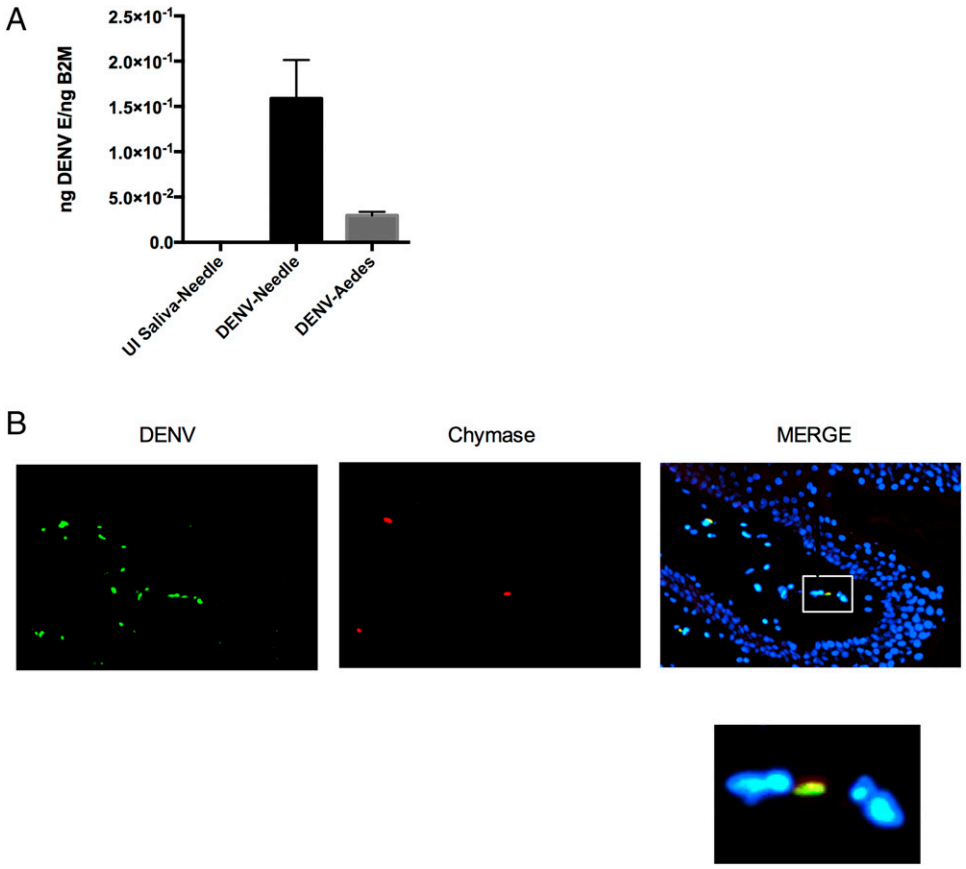

FIGURE 2. Mast cells are targets for DENV mosquito transmission and infection in human skin. *Ae. aegypti* were fed a 1:1 mixture of human blood and DENV-2 NGC. At 14 d p.i., mosquitoes were allowed to feed on biopsy punches of human skin tissue samples (DENV-*Aedes*). As controls, we injected mosquito saliva (uninfected [UI] Saliva-Needle) for the negative control or mosquito saliva plus DENV-2 NGC (DENV-Needle) for the positive control. Skin was maintained in full cell culture media for 24 h. (**A**) RNA was isolated from the skin and DENV infection was quantified by qRT-PCR analysis. (**B**). Skin samples were fixed in 4% paraformaldehyde and sectioned in paraffin for immunohistochemistry analysis. Slides of section samples were fluorescently labeled with an Ab that recognizes chymase (red) and one that recognizes DENV capsid protein (green). Representative image of DENV-*Aedes* are shown at original magnification ×20.

productive DENV infection in all cell lines as assessed by qRT-PCR and immunofluorescence (Fig. 7B–D). Importantly, isolated granules were vigorously washed 10 times and only washes 1–3 were able to cause detectable infection when added to naive cells. This indicated that washes 4–10 did not contain infectious DENV in solution and that the purified granules were the source of infectious DENV in the above experiments. To examine if this phenomenon held true in vivo, we injected granules purified from DENV-infected mast cells into mouse footpads and dissected both DLN and spleen at 24 h p.i. Because granules were previously shown to travel through the lymph to the DLN in mice (24), we anticipated that the DENV-containing granules would travel to the DLN and cause productive infection. We found that two out of six mice had detectable DENV infection in DLN and spleen as measured by qRT-PCR analysis (Fig. 7F) and none of the granules from uninfected mast cells caused infection. We next looked at how this granule infection compared with natural infection with pure DENV virus. To do this, we injected pure DENV (from unadulterated stocks) and granules purified from DENV-infected mast cells into separate mouse footpads and dissected both DLN and spleen from the mice 24 h p.i. In this second experiment, we found that 6 out of 10 mice injected with granules had DENV infection in both DLN and spleen (Fig. 7G). All six out of six mice had infection with pure DENV, as expected (Fig. 7G). None of the mice injected with granules purified from unexpected mast cells had any DENV infection.

In summary, we show here that 1) human skin mast cells are readily infected with DENV and are likely targets for mosquito transmission 2), there is a significant up-regulation of specific immune-related genes and growth factors during DENV infection 3), infectious DENV can travel in extracellular mast cell granules and is able to infect naive cells, and 4) DENV-containing granules are able to travel from footpads to DLN in mice and cause productive infection. These findings reveal the effects of DENV infection in human skin mast cells, and identify a novel role for mast cells in the dissemination and propagation of

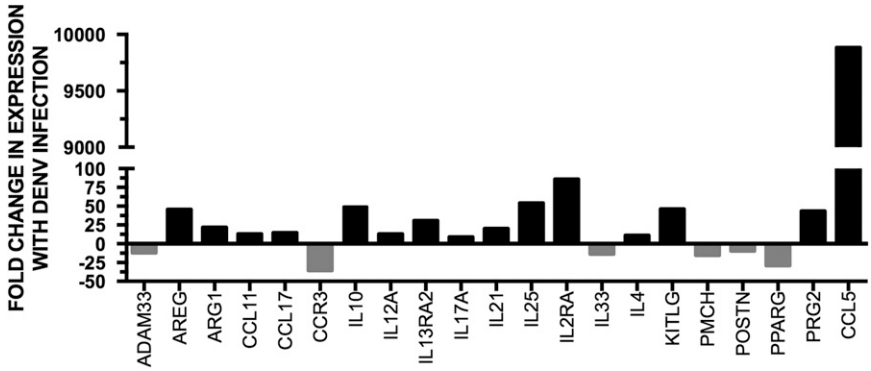

FIGURE 3. DENV infection of human skin mast cells alters immune-related gene expression profile. Primary mast cells were isolated from human skin tissue as described and infected with DENV-2 NGC at an MOI of 1.0. RNA was isolated from lysed cells and used in qRT-PCR analysis to quantify gene expression. The 21 genes shown had over 10-fold alteration in expression during DENV infection as compared with uninfected control cells. Black bars indicate an up-regulation in expression and gray bars indicate a down regulation in expression.

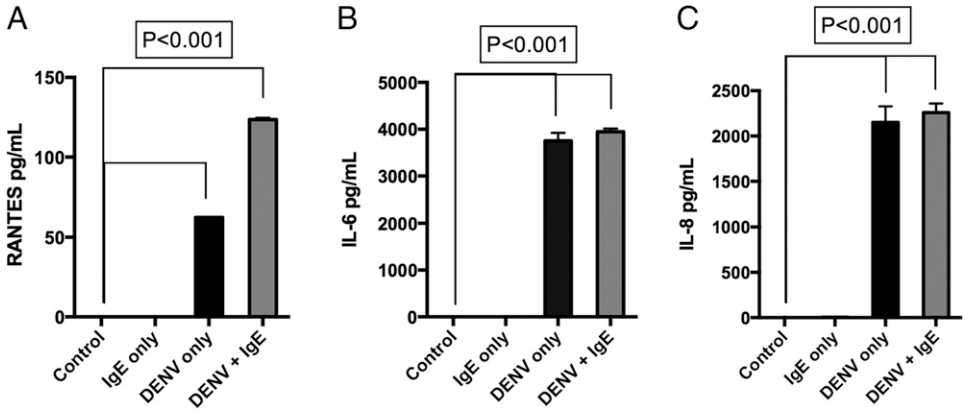

FIGURE 4. DENV infection of human skin mast cells increases cytokine production. ELISA analysis was done with supernatants of uninfected/unsensitized (control), uninfected/IgE sensitized (IgE only), DENV-2 infected/unsensitized (DENV only) or DENV-2 infected/IgE sensitized (DENV+IgE). Graphs show pg/ml concentrations of RANTES/CCL5 (**A**), IL-6 (**B**), and IL-8 (**C**). Experiments were done in triplicate. One-way ANOVA was used for statistical analysis. Results were $p < 0.001$ for all panels, all comparisons.

DENV in humans. A schematic diagram in Fig. 8 summarizes these results.

Discussion

Human skin is the site of initial infection by DENV following a mosquito bite; and thus a critical organ in the early immune response to DENV infection. Resident skin mast cells are likely early targets of DENV infection as they are prevalent in skin, localize near blood vessels, and would accumulate at the site of a mosquito bite. In this study, we analyzed the effect of DENV on human skin mast cells, which express both tryptase and chymase in granules (34), and have completed their differentiation and maturation in vivo. Previous studies have used in vitro–derived mast cells, which are often immature and are not identical to those that develop in vivo. For example, the mast cell leukemia cell line, HMC-1, and two mast cell leukemia/sarcoma cell lines, LAD1 and LAD2, differ significantly from tissue-derived mast cells (35, 36). HMC-1 cells lack surface FcεRI receptors, are stem cell factor–independent and express levels of histamine and tryptase that are only 1% of those found with tissue-derived mast cells. LAD cells exhibit a variety of chromosomal abnormalities by karyotypic analysis. Thus, in vitro–derived mast cells are functionally distinct from the human skin tissue–derived mast cells that were used in the current study (37).

Cells are infected with DENV through receptor-mediated endocytosis. Fusion occurs at the endocytic membrane, RNA is released, and replication occurs on membranous formations at the endoplasmic reticulum in the cytoplasm. Usually, budding of newly formed DENV is at the endoplasmic reticulum membrane and then virus is exocytosed to the cell surface for release (38, 39). We show that in infected skin mast cells, DENV is often located both within the cytoplasmic granules and in granules found in extracellular space following degranulation. Importantly, we demonstrate that the extracellular granules purified from the supernatant of infected skin mast cells are able to cause productive DENV infection in naive cells. As we extensively washed the isolated granules, and the latter washes did not contain infectious virus, we hypothesize that the DENV is contained within the purified granules. It was recently shown that extracellular mast cell granules are able to travel via lymph to DLN, trafficking immune molecules such as TNF over significant distances (24). It was also shown that these extracellular granules contain heparin, and both heparin and heparin-like glycosaminoglycans are known binding partners for DENV envelope protein (25, 40). In addition, we demonstrate that purified DENV-containing granular particles are able to cause productive infection in lymph nodes and spleen of mice after footpad injection. We saw detectable infection in these organs in 33–50% of animals examined. The levels of DENV in the granules may be extremely low in comparison with the amount of free virus we usually use in animal studies. This could be one explanation for why some of the animals were not infected, though we saw significant DENV infection in all in vitro experiments. Our findings suggest that DENV may be able to travel in these extracellular granules from the initial site of infection in the skin to DLN. This indicates that granule transport in lymph could be an important, to our knowledge, previously unrecognized mechanism for system spread of DENV.

Supporting this hypothesis is our finding that DENV-infected human skin mast cells expressed significantly increased levels

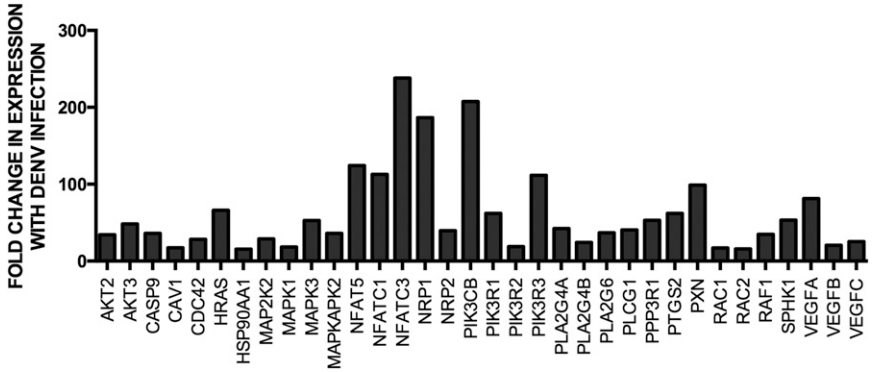

FIGURE 5. DENV infection of human skin mast cells alters growth factor gene expression profile. Primary mast cells were isolated from human skin tissue as described and infected with DENV-2 NGC at an MOI of 1.0. RNA was isolated from lysed cells and used in qRT-PCR analysis to quantify gene expression. The 34 genes shown had over 10-fold alteration in expression during DENV infection as compared with uninfected control cells.

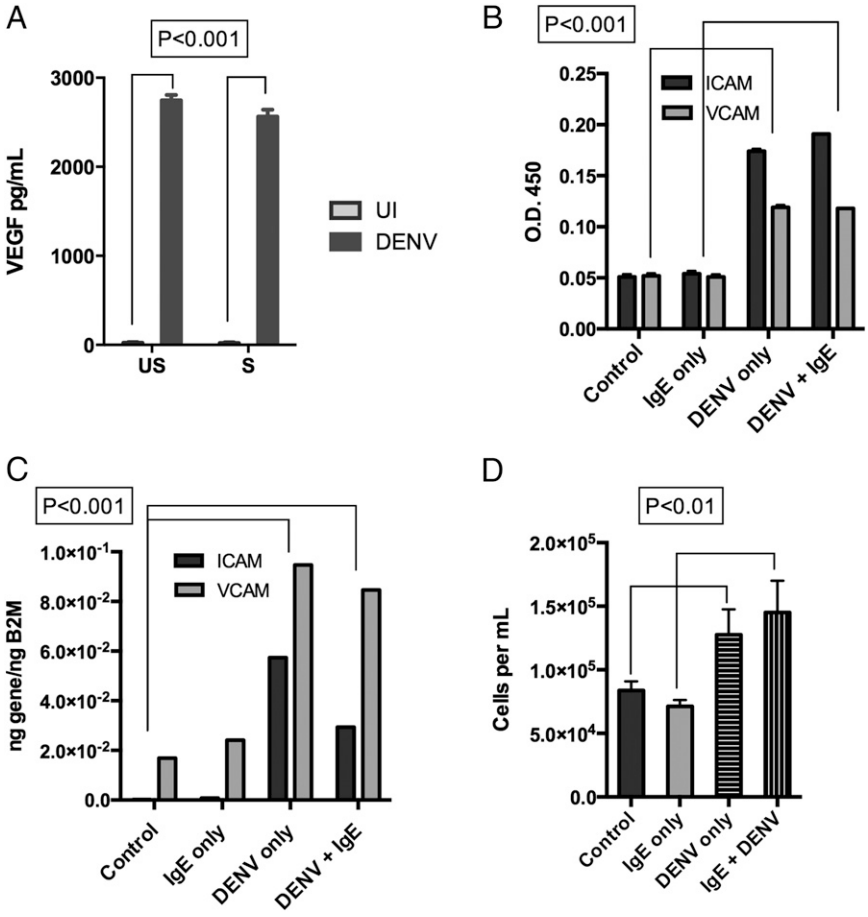

FIGURE 6. DENV infection of human skin mast cells induces endothelial cell activation and proliferation. Primary mast cells were isolated from human skin tissue as described. Cells were then: 1) uninfected/unsensitized (control), 2) uninfected/IgE sensitized (IgE only), 3) DENV-2 infected/unsensitized (DENV only), or 4) DENV-2 infected/IgE sensitized (DENV +IgE). Infections were done with DENV-2 NGC at an MOI of 1.0. At 24 h p.i., the mast cells were plated onto the top layer of a Transwell culture system. Uninfected (naive) HMVDECs were plated on the lower wells of each plate. At 24 h after initiation of coculture, the HMVDECs were collected. (**A**) The mast cell supernatants were used in ELISA analysis to measure levels of VEGF. US is unstimulated and S is IgE sensitized. (**B**) In-cell ELISA analysis was done to measure the expression of ICAM and VCAM by the HMVDECs. Student *t* test was used for statistical analysis. Results were $p < 0.001$ for all comparisons. (**C**) RNA was isolated from lysed HMVDECs and used in qRT-PCR analysis to quantify ICAM and VCAM gene expression. Student *t* test was used for statistical analysis. Results were $p < 0.001$ for all comparisons. (**D**) HMVDECs from each coculture group were analyzed using a simple proliferation assay. Cells counts per milliliter are indicated on the graph. Student *t* test was used for statistical analysis. Results were $p < 0.01$ for all comparisons. All experiments were done in triplicate.

of VEGF-A and neuropilin (NRP1), and were able to signal neighboring endothelial cells for activation and proliferation. VEGF is a well-known angiogenic and lymphangiogenic factor that is produced by human mast cells (41–43). NRP1, a coreceptor for VEGF, interacts with the most proangiogenic VEGF isoform VEGF-A_{165} thereby increasing its affinity for VEGFR-2 and facilitating its downstream signaling (44–46). A recent study observed associated increased plasma levels of VEGF and the mast cell proteases tryptase and chymase with severe forms of dengue disease (47). Our novel finding that DENV localizes within mast cell granules suggests that the induced production of VEGF following DENV infection could be a self-serving mechanism that serves to facilitate viral transmission by providing a lymphatic route by which DENV in extracellular granules can travel to DLN for systemic spread. As mentioned, extracellular mast cell granules have been shown to traffic from the periphery to DLN in mice (24). Thus, the induction of VEGF secretion could promote lymphangiogenesis for the transport of DENV within granules that have been released through degranulation, which itself was induced by DENV infection.

Inflammation is a main component of the host immune response to DENV infection, and can have both protective and pathogenic roles. IFN-γ, IL-12, and IL-18 are essential for an effective host immune response (48, 49). Other proinflammatory cytokines can play pathogenic roles. Several studies have shown that TNF-α levels are higher in patients with severe dengue versus mild dengue fever (50, 51). Various chemokines have also been associated with dengue severity and clinical outcomes in humans. Levels of the CCL2, CCL3, and CCL4 have been correlated with severe dengue disease in humans in several studies (52, 53), and mice lacking these chemokines have reduced lethality and milder disease during dengue infection (54). Thus, excessive inflammation and increased levels of certain cytokines and chemokines can contribute to vascular leakage and endothelial permeability that may lead to severe forms of dengue disease (31–33). It is important to note that mast cell degranulation and activation, in the absence of viral infection, has also been shown to drive the gene expression of certain cytokines and growth factors (55–57). The role of degranulation as a driver of gene expression during virus infection is not entirely clear.

In this study, we show that the chemokine CCL5 (RANTES) is highly up-regulated during DENV infection in human skin mast cells. CCL5/RANTES has been previously associated with DENV infection in several cell types and model systems. The NS5 protein of DENV has been shown to activate CCL5/RANTES production by increasing the binding of NF-κB to its binding sites on the RANTES promoter (58). When the nuclear localization signal is removed from DENV NS5, the production of RANTES is no longer increased (59). Infection with DENV-2 was previously shown to significantly increase both RANTES and IL-6 in lung cancer cell lines, and RANTES was correlated with the development of dengue hemorrhagic fever and dengue shock syndrome in human patients (60). The activation of RANTES by DENV was shown to involve JNK, Erk, and p38 signaling pathways. Finally, it has been shown that up-regulation of RANTES gene expression by DENV infection in liver cells is caused by both oxidant-dependent and oxidant-independent pathways (61). In mast cells, RANTES acts as a mediator of acute inflammatory responses. One study also describes a fundamental role for RANTES in histamine and serotonin generation and cell function in mast cells (62). RANTES is thought to

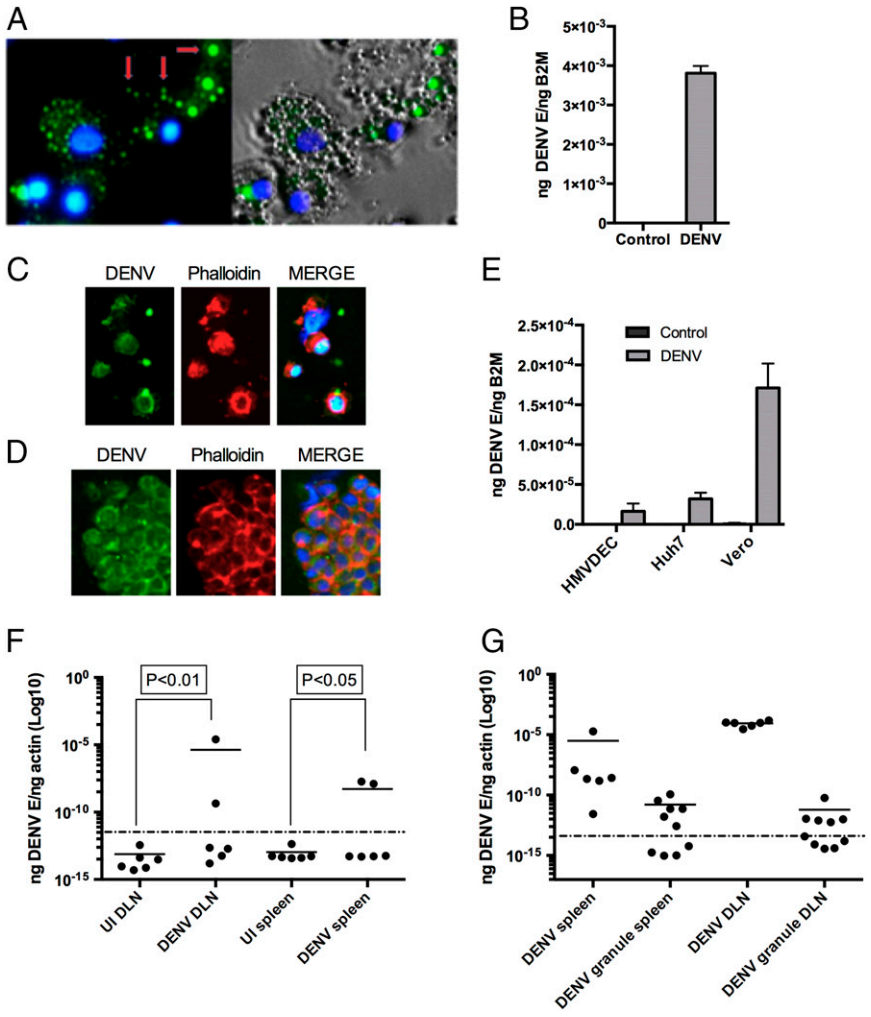

FIGURE 7. Cytoplasmic granules released from DENV-infected human skin mast cells are infectious in vitro and travel from the periphery to infect DLN in vivo. Primary mast cells were isolated from human skin tissue as described and infected with DENV-2 NGC at an MOI of 1.0. Control cells were uninfected. (**A**) At 48 h p.i., cells were fixed in 4% paraformaldehyde and labeled for DENV infection. Cells were counterstained with DAPI to visualize nuclei. Both darkfield and phase contrast overlay images are shown to visualize granules. (**B**–**F**). Purified granules from DENV-infected mast cells were added to naive LAD-2 (B), Vero (C and E), Huh7 (D and E), and HMVDE (E) cells, and cells were analyzed by immunofluorescence at 24 h p.i. (C and D) using Abs recognizing DENV (green). Cells were counterstained with phalloidin (red) to visualize cellular structure and DAPI to visualize nuclei. (B and F) DENV infection was quantified by qRT-PCR analysis. Control cells are uninfected. (F and **G**) The purified DENV granules (F) or pure DENV and purified DENV granules (G) were injected into the footpads of AGB6 mice. At 24 h p.i., RNA was isolated from the spleen and the popliteal DLN and used in qRT-PCR analysis to quantify infection. The dotted line represents infection threshold. Both *p* values shown were calculated using Student *t* tests. UI, uninfected.

contribute to IL-13 production through a CCR5 and Akt cell signaling pathway-dependent mechanism, which would indicate RANTES as a participant in mast cell–related inflammation (63). Increased levels of RANTES have been seen during DENV infection of the mast cell–like cell line, HMC-1, and a role for mast cells in the chemokine-dependent host responses to DENV infection was proposed (19). Therefore, CCL5/RANTES appears to be an important player in the very early immune response to DENV infection in human skin mast cells.

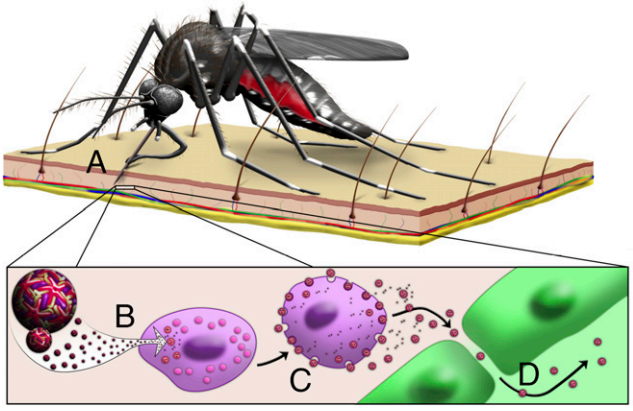

FIGURE 8. Schematic diagram of the putative role that mast cell granules play in DENV systemic spread from the initial infection in the skin. After (**A**) deposit of DENV in skin by an infected mosquito, (**B**) mast cells are likely targets of initial infection. (**C**) When granules are released by infected skin mast cells, they contain infectious DENV virions. (**D**) These granules could potentially travel through lymph, systemically spreading DENV infection from the periphery.

In addition to CCL5/RANTES, DENV-infected human skin mast cells also expressed notable increases (25–100 fold) in cytokines and receptors involved in Th2 immune responses including amphiregulin (AREG), IL-10, IL-25, IL-2Ra, and IL-13RA2. Interestingly, several members of the NFAT transcription factors NFAT5, NFATC1, and NFATC3, which are known to be involved in Th2 cytokine production in FcεRI-activated mast cells (64–66), were also strikingly up-regulated in DENV-infected human skin mast cells. Together, these findings reinforce our current knowledge of the ability of DENV to mediate inflammation, and further suggest an important role for the NFAT family of transcription factors in the production of Th2 cytokines and inflammation following DENV infection of skin mast cells.

Our study demonstrates an important role for human skin mast cells in initial DENV infection and immune response. We present novel data, to our knowledge, showing that extracellular mast cell granules play a critical role in the DENV viral life cycle, infection, and spread from the periphery. Overall, our data establish a previously unrecognized role, to our knowledge, for human skin mast cells in infection and systemic spread of DENV.

Disclosures

The authors have no financial conflicts of interest.

References

1. Beatty, M. E., A. Stone, D. W. Fitzsimons, J. N. Hanna, S. K. Lam, S. Vong, M. G. Guzman, J. F. Mendez-Galvan, S. B. Halstead, G. W. Letson, et al; Asia-Pacific and Americas Dengue Prevention Boards Surveillance Working Group. 2010. Best practices in dengue surveillance: a report from the Asia-Pacific and Americas Dengue Prevention Boards. *PLoS Negl. Trop. Dis.* 4: e890.
2. Mangold, K. A., and S. L. Reynolds. 2013. A review of dengue fever: a resurging tropical disease. *Pediatr. Emerg. Care* 29: 665–669; quiz 670–661.
3. Gibbons, R. V., and D. W. Vaughn. 2002. Dengue: an escalating problem. *BMJ* 324: 1563–1566.
4. Gubler, D. J. 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol.* 10: 100–103.
5. Gubler, D. J., and G. G. Clark. 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerg. Infect. Dis.* 1: 55–57.
6. Burke, D. S., and S. Kliks. 2006. Antibody-dependent enhancement in dengue virus infections. *J. Infect. Dis.* 193: 601–603, author reply 603–604.
7. Mackenzie, J. S., D. J. Gubler, and L. R. Petersen. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.* 10(Suppl.): S98–S109.
8. Guzman, M. G., S. B. Halstead, H. Artsob, P. Buchy, J. Farrar, D. J. Gubler, E. Hunsperger, A. Kroeger, H. S. Margolis, E. Martínez, et al. 2010. Dengue: a continuing global threat. *Nat. Rev. Microbiol.* 8(Suppl.): S7–S16.
9. Murray, K. O., L. F. Rodriguez, E. Herrington, V. Kharat, N. Vasilakis, C. Walker, C. Turner, S. Khuwaja, R. Arafat, S. C. Weaver, et al. 2013. Identification of dengue fever cases in Houston, Texas, with evidence of autochthonous transmission between 2003 and 2005. *Vector Borne Zoonotic Dis.* 13: 835–845.
10. Muñoz-Jordán, J. L., G. A. Santiago, H. Margolis, and L. Stark. 2013. Genetic relatedness of dengue viruses in Key West, Florida, USA, 2009-2010. *Emerg. Infect. Dis.* 19: 652–654.
11. Govindarajan, D., L. Guan, S. Meschino, A. Fridman, A. Bagchi, I. Pak, J. ter Meulen, D. R. Casimiro, and A. J. Bett. 2016. A rapid and improved method to generate recombinant dengue virus vaccine candidates. *PLoS One* 11: e0152209.
12. Guzman, M. G., M. Alvarez, and S. B. Halstead. 2013. Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: an historical perspective and role of antibody-dependent enhancement of infection. *Arch. Virol.* 158: 1445–1459.
13. Halstead, S. B. 2003. Neutralization and antibody-dependent enhancement of dengue viruses. *Adv. Virus Res.* 60: 421–467.
14. Marovich, M., G. Grouard-Vogel, M. Louder, M. Eller, W. Sun, S. J. Wu, R. Putvatana, G. Murphy, B. Tassaneetrithep, T. Burgess, et al. 2001. Human dendritic cells as targets of dengue virus infection. *J. Investig. Dermatol. Symp. Proc.* 6: 219–224.
15. Babina, M., S. Guhl, M. Artuc, N. N. Trivedi, and T. Zuberbier. 2016. Phenotypic variability in human skin mast cells. *Exp. Dermatol.* 25: 434–439.
16. Muto, Y., Z. Wang, M. Vanderberghe, A. Two, R. L. Gallo, and A. Di Nardo. 2014. Mast cells are key mediators of cathelicidin-initiated skin inflammation in rosacea. *J. Invest. Dermatol.* 134: 2728–2736.
17. Le, Q. T., G. Gomez, W. Zhao, J. Hu, H. Z. Xia, Y. Fukuoka, N. Katunuma, and L. B. Schwartz. 2011. Processing of human protryptase in mast cells involves cathepsins L, B, and C. *J. Immunol.* 187: 1912–1918.
18. Eady, R. A., T. Cowen, T. F. Marshall, V. Plummer, and M. W. Greaves. 1979. Mast cell population density, blood vessel density and histamine content in normal human skin. *Br. J. Dermatol.* 100: 623–633.
19. King, C. A., R. Anderson, and J. S. Marshall. 2002. Dengue virus selectively induces human mast cell chemokine production. *J. Virol.* 76: 8408–8419.
20. St John, A. L., A. P. Rathore, H. Yap, M. L. Ng, D. D. Metcalfe, S. G. Vasudevan, and S. N. Abraham. 2011. Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. *Proc. Natl. Acad. Sci. USA* 108: 9190–9195.
21. St John, A. L., A. P. Rathore, B. Raghavan, M. L. Ng, and S. N. Abraham. 2013. Contributions of mast cells and vasoactive products, leukotrienes and chymase, to dengue virus-induced vascular leakage. *eLife* 2: e00481.
22. Chu, Y. T., S. W. Wan, R. Anderson, and Y. S. Lin. 2015. Mast cell-macrophage dynamics in modulation of dengue virus infection in skin. *Immunology* 146: 163–172.
23. Azouz, N. P., I. Hammel, and R. Sagi-Eisenberg. 2014. Characterization of mast cell secretory granules and their cell biology. *DNA Cell Biol.* 33: 647–651.
24. Kunder, C. A., A. L. St John, G. Li, K. W. Leong, B. Berwin, H. F. Staats, and S. N. Abraham. 2009. Mast cell-derived particles deliver peripheral signals to remote lymph nodes. *J. Exp. Med.* 206: 2455–2467.
25. Marks, R. M., H. Lu, R. Sundaresan, T. Toida, A. Suzuki, T. Imanari, M. J. Hernáiz, and R. J. Linhardt. 2001. Probing the interaction of dengue virus envelope protein with heparin: assessment of glycosaminoglycan-derived inhibitors. *J. Med. Chem.* 44: 2178–2187.
26. Schwartz, L. B., K. F. Austen, and S. I. Wasserman. 1979. Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells. *J. Immunol.* 123: 1445–1450.
27. Schwartz, L. B., R. A. Lewis, D. Seldin, and K. F. Austen. 1981. Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells. *J. Immunol.* 126: 1290–1294.
28. Conway, M. J., B. Londono-Renteria, A. Troupin, A. M. Watson, W. B. Klimstra, E. Fikrig, and T. M. Colpitts. 2016. *Aedes* aegypti D7 saliva protein inhibits dengue virus infection. *PLoS Negl. Trop. Dis.* 10: e0004941.
29. Londono-Renteria, B., A. Troupin, M. J. Conway, D. Vesely, M. Ledizet, C. M. Roundy, E. Cloherty, S. Jameson, D. Vanlandingham, S. Higgs, et al. 2015. Dengue virus infection of *Aedes aegypti* requires a putative cysteine rich venom protein. *PLoS Pathog.* 11: e1005202.
30. Gomez, G., S. Jogie-Brahim, M. Shima, and L. B. Schwartz. 2007. Omalizumab reverses the phenotypic and functional effects of IgE-enhanced Fc epsilonRI on human skin mast cells. *J. Immunol.* 179: 1353–1361.
31. Nielsen, D. G. 2009. The relationship of interacting immunological components in dengue pathogenesis. *Virol. J.* 6: 211.
32. Srikiatkhachorn, A. 2009. Plasma leakage in dengue haemorrhagic fever. *Thromb. Haemost.* 102: 1042–1049.
33. Navarro-Sánchez, E., P. Desprès, and L. Cedillo-Barrón. 2005. Innate immune responses to dengue virus. *Arch. Med. Res.* 36: 425–435.
34. Irani, A. A., N. M. Schechter, S. S. Craig, G. DeBlois, and L. B. Schwartz. 1986. Two types of human mast cells that have distinct neutral protease compositions. *Proc. Natl. Acad. Sci. USA* 83: 4464–4468.
35. Butterfield, J. H., D. Weiler, G. Dewald, and G. J. Gleich. 1988. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk. Res.* 12: 345–355.
36. Kirshenbaum, A. S., C. Akin, Y. Wu, M. Rottem, J. P. Goff, M. A. Beaven, V. K. Rao, and D. D. Metcalfe. 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk. Res.* 27: 677–682.
37. Furitsu, T., H. Saito, A. M. Dvorak, L. B. Schwartz, A. M. Irani, J. F. Burdick, K. Ishizaka, and T. Ishizaka. 1989. Development of human mast cells in vitro. *Proc. Natl. Acad. Sci. USA* 86: 10039–10043.
38. Mukhopadhyay, S., R. J. Kuhn, and M. G. Rossmann. 2005. A structural perspective of the flavivirus life cycle. *Nat. Rev. Microbiol.* 3: 13–22.
39. Fernandez-Garcia, M. D., M. Mazzon, M. Jacobs, and A. Amara. 2009. Pathogenesis of flavivirus infections: using and abusing the host cell. *Cell Host Microbe* 5: 318–328.
40. Lin, Y. L., H. Y. Lei, Y. S. Lin, T. M. Yeh, S. H. Chen, and H. S. Liu. 2002. Heparin inhibits dengue-2 virus infection of five human liver cell lines. *Antiviral Res.* 56: 93–96.
41. Detoraki, A., R. I. Staiano, F. Granata, G. Giannattasio, N. Prevete, A. de Paulis, D. Ribatti, A. Genovese, M. Triggiani, and G. Marone. 2009. Vascular endothelial growth factors synthesized by human lung mast cells exert angiogenic effects. *J. Allergy Clin. Immunol.* 123: 1142–1149, 1149. e1141–1145.
42. Marone, G., G. Varricchi, S. Loffredo, and F. Granata. 2016. Mast cells and basophils in inflammatory and tumor angiogenesis and lymphangiogenesis. *Eur. J. Pharmacol.* 778: 146–151.
43. Boesiger, J., M. Tsai, M. Maurer, M. Yamaguchi, L. F. Brown, K. P. Claffey, H. F. Dvorak, and S. J. Galli. 1998. Mast cells can secrete vascular permeability factor/ vascular endothelial cell growth factor and exhibit enhanced release after immunoglobulin E-dependent upregulation of fc epsilon receptor I expression. *J. Exp. Med.* 188: 1135–1145.
44. Zachary, I. 2014. Neuropilins: role in signalling, angiogenesis and disease. *Chem. Immunol. Allergy* 99: 37–70.
45. Varricchi, G., F. Granata, S. Loffredo, A. Genovese, and G. Marone. 2015. Angiogenesis and lymphangiogenesis in inflammatory skin disorders. *J. Am. Acad. Dermatol.* 73: 144–153.
46. Marone, G., and F. Granata. 2014. Angiogenesis, lymphangiogenesis and clinical implications. Preface. *Chem. Immunol. Allergy* 99: XI–XII.
47. Furuta, T., L. A. Murao, N. T. Lan, N. T. Huy, V. T. Huong, T. T. Thuy, V. D. Tham, C. T. Nga, T. T. Ha, Y. Ohmoto, et al. 2012. Association of mast cell-derived VEGF and proteases in Dengue shock syndrome. *PLoS Negl. Trop. Dis.* 6: e1505.
48. Fagundes, C. T., V. V. Costa, D. Cisalpino, F. A. Amaral, P. R. Souza, R. S. Souza, B. Ryffel, L. Q. Vieira, T. A. Silva, A. Atrasheuskaya, et al. 2011. IFN-γ production depends on IL-12 and IL-18 combined action and mediates host resistance to dengue virus infection in a nitric oxide-dependent manner. *PLoS Negl. Trop. Dis.* 5: e1449.
49. Costa, V. V., C. T. Fagundes, D. F. Valadão, D. Cisalpino, A. C. Dias, K. D. Silveira, L. M. Kangussu, T. V. Ávila, M. R. Bonfim, D. Bonaventura, et al. 2012. A model of DENV-3 infection that recapitulates severe disease and highlights the importance of IFN-γ in host resistance to infection. *PLoS Negl. Trop. Dis.* 6: e1663.
50. Chaturvedi, U. C., R. Agarwal, E. A. Elbishbishi, and A. S. Mustafa. 2000. Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. *FEMS Immunol. Med. Microbiol.* 28: 183–188.
51. Mangada, M. M., T. P. Endy, A. Nisalak, S. Chunsuttiwat, D. W. Vaughn, D. H. Libraty, S. Green, F. A. Ennis, and A. L. Rothman. 2002. Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. *J. Infect. Dis.* 185: 1697–1703.
52. Rothman, A. L. 2011. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat. Rev. Immunol.* 11: 532–543.
53. Bozza, F. A., O. G. Cruz, S. M. Zagne, E. L. Azeredo, R. M. Nogueira, E. F. Assis, P. T. Bozza, and C. F. Kubelka. 2008. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. *BMC Infect. Dis.* 8: 86.
54. Guabiraba, R., R. E. Marques, A. G. Besnard, C. T. Fagundes, D. G. Souza, B. Ryffel, and M. M. Teixeira. 2010. Role of the chemokine receptors CCR1, CCR2 and CCR4 in the pathogenesis of experimental dengue infection in mice. *PLoS One* 5: e15680.
55. Moon, T. C., E. Lee, S. H. Baek, M. Murakami, I. Kudo, N. S. Kim, J. M. Lee, H. K. Min, N. Kambe, and H. W. Chang. 2003. Degranulation and cytokine expression in human cord blood-derived mast cells cultured in serum-free medium with recombinant human stem cell factor. *Mol. Cells* 16: 154–160.

56. Urb, M., and D. C. Sheppard. 2012. The role of mast cells in the defence against pathogens. *PLoS Pathog.* 8: e1002619.
57. Marshall, J. S., and D. M. Jawdat. 2004. Mast cells in innate immunity. *J. Allergy Clin. Immunol.* 114: 21–27.
58. Khunchai, S., M. Junking, A. Suttitheptumrong, S. Kooptiwut, G. Haegeman, T. Limjindaporn, and P. T. Yenchitsomanus. 2015. NF-κB is required for dengue virus NS5-induced RANTES expression. *Virus Res.* 197: 92–100.
59. Khunchai, S., M. Junking, A. Suttitheptumrong, U. Yasamut, N. Sawasdee, J. Netsawang, A. Morchang, P. Chaowalit, S. Noisakran, P. T. Yenchitsomanus, and T. Limjindaporn. 2012. Interaction of dengue virus nonstructural protein 5 with Daxx modulates RANTES production. *Biochem. Biophys. Res. Commun.* 423: 398–403.
60. Lee, Y. R., C. Y. Su, N. H. Chow, W. W. Lai, H. Y. Lei, C. L. Chang, T. Y. Chang, S. H. Chen, Y. S. Lin, T. M. Yeh, and H. S. Liu. 2007. Dengue viruses can infect human primary lung epithelia as well as lung carcinoma cells, and can also induce the secretion of IL-6 and RANTES. *Virus Res.* 126: 216–225.
61. Lin, Y. L., C. C. Liu, J. I. Chuang, H. Y. Lei, T. M. Yeh, Y. S. Lin, Y. H. Huang, and H. S. Liu. 2000. Involvement of oxidative stress, NF-IL-6, and RANTES expression in dengue-2-virus-infected human liver cells. *Virology* 276: 114–126.
62. Castellani, M. L., M. A. De Lutiis, E. Toniato, F. Conti, P. Felaco, M. Fulcheri, T. C. Theoharides, A. Caraffa, P. Antinolfi, P. Conti, et al. 2010. Impact of RANTES, MCP-1 and IL-8 in mast cells. *J. Biol. Regul. Homeost. Agents* 24: 1–6.
63. Zhang, H., H. Yang, W. Ma, and S. He. 2011. Induction of IL-13 production and upregulated expression of protease activated receptor-1 by RANTES in a mast cell line. *Cytokine* 53: 231–238.
64. Klein, M., S. Klein-Hessling, A. Palmetshofer, E. Serfling, C. Tertilt, T. Bopp, V. Heib, M. Becker, C. Taube, H. Schild, et al. 2006. Specific and redundant roles for NFAT transcription factors in the expression of mast cell-derived cytokines. *J. Immunol.* 177: 6667–6674.
65. Hock, M. B., and M. A. Brown. 2003. Nuclear factor of activated T cells 2 transactivation in mast cells: a novel isoform-specific transactivation domain confers unique FcepsilonRI responsiveness. *J. Biol. Chem.* 278: 26695–26703.
66. Turner, H., M. Gomez, E. McKenzie, A. Kirchem, A. Lennard, and D. A. Cantrell. 1998. Rac-1 regulates nuclear factor of activated T cells (NFAT) C1 nuclear translocation in response to Fcepsilon receptor type 1 stimulation of mast cells. *J. Exp. Med.* 188: 527–537.