Dengue virus-elicited tryptase induces endothelial permeability and shock

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Abstract

Dengue virus (DENV) infection causes a characteristic pathology in humans involving dysregulation of the vascular system. In some patients with dengue hemorrhagic fever (DHF), vascular pathology can become severe, resulting in extensive microvascular permeability and plasma leakage into tissues and organs. Mast cells (MCs), which line blood vessels and regulate vascular function, are able to detect DENV in vivo and promote vascular leakage. Here, we identified that a MC-derived protease, tryptase, is consequential for promoting vascular permeability during DENV infection, through inducing breakdown of endothelial cell tight junctions. Injected tryptase alone was sufficient to induce plasma loss from the circulation and hypovolemic shock in animals. A potent tryptase inhibitor, nafamostat mesylate, blocked DENV-induced vascular leakage in vivo. Importantly, in two independent human dengue cohorts, tryptase levels correlated with the grade of DHF severity. This study defines an immune mechanism by which DENV can induce vascular pathology and shock.

Introduction

In humans, dengue virus (DENV) causes an acute viral infection that manifests as a broad spectrum of disease: from asymptomatic infection, to a mild febrile illness, dengue fever (DF) that may resolve within 2 weeks, to the most severe form, dengue hemorrhagic fever (DHF), which involves vascular leakage as the primary potentially life-threatening sign. When homeostatic mechanisms fail, the widespread vascular leakage that occurs during DHF can also lead to dengue shock syndrome (DSS) resulting from hypovolemia (1, 2). In the initial classification scheme for DHF/DSS, patients were also graded according to severity of DHF: DHF-I involving fever accompanied with positive tourniquet test and or easy bruising, DHF-II involving spontaneous bleeding or frank hemorrhaging, DHF-III is characteristic of shock such as circulatory failure due to rapid weak pulse and narrowing of pulse pressure and DHF-IV being severe shock without a pulse that is usually fatal(3). The predominant view of the field is that vascular leakage in humans results from immune-mediated pathology rather than infection of endothelial cells themselves (2). One theory is that “cytokine storm” damages the vascular endothelium during infection. These cytokines could be derived from infected cells that contain replicating virus, or other immune cells that respond to infection such as T cells and

mast cells (MCs)(4, 5). TNF, for example, has been shown to promote vascular leakage and death due to dengue in an immunocompromised mouse model (6); however, in human patients there have been less clear associations between specific vasoactive cytokines and vascular leakage during infection and even conflicting associations in various studies (2, 7-9). If cytokine storm is involved, it is also unclear why symptoms of dengue differ from other conditions that involve cytokine storm (2). Others have identified high levels of complement activation in pediatric DSS patients(10), which could be further enhanced in the presence of NS1 protein(11). Reports suggest that NS1 alone can directly induce vascular permeability (12, 13) and there are conflicting reports regarding whether NS1 levels correlate with DENV disease severity in human studies as well as in animal models (14-18). Thus, we do not fully understand the mechanism of DENV-induced vascular leakage in humans. Even in mild dengue cases, usually diagnosed as DF, signs and symptoms of dengue vascular pathogenesis and immune activation can be present, such as bruising, purpura, petechiae, rash, and hemoconcentration (1, 2). It is also possible that the signs of vascular leakage, hemorrhaging and shock are not merely due to an augmentation of the same mechanism, but they may have independent mechanisms that contribute to the spectrum of disease.

Interestingly, some clinical signs similar to those that occur as a result of DENV infection have been associated with the effects of activated MCs in independent clinical contexts, such as during allergic reactions or anaphylactic shock (19). These include signs such as vascular leakage, rash, flushing and abdominal pain(20, 21). In those allergic conditions, MC-derived products can cause urticaria, edema, vasodilation, and blood pressure changes (19). Mature MCs do not circulate in the blood but are found only in connective and mucosal tissues (22). There, they act as sentinels for pathogens, including DENV (23, 24), and also serve an immune regulatory function at tissue sites (25). In addition to their relatively even dispersal throughout the skin and mucosae, a portion of MCs also adopt a perivascular distribution in vivo (26). It has been shown in mice that MCs may even extend processes into the lumen of blood vessels(27). They store vasoactive mediators in their granules, including heparin, tryptase, chymase, additional proteases, and preformed cytokines (e.g. TNF)(26, 28). MC granules remain insoluble for hours after their release as membrane-free particles and they are thought to slowly release their cargo in the extracellular environment (29, 30). Proteases

account for the majority of the granule protein content, of which chymase and tryptase are the most abundant(28). Chymase is understood to impact the renin-angiotensin system as an Angiotensin-I to Angiotensin-II converting enzyme (31), while tryptase has primarily been characterized to play a role in vascular leakage, through its ability to cleave PAR receptors (32).

In vivo studies revealed that MCs play a key role in protective immune response involving cellular recruitment to sites of localized cutaneous DENV infection in mice (24). However, as the infection spreads and becomes systemic, the role of MCs becomes more complex (4). We previously investigated the interactions between MCs and DENV in the mouse model and determined that DENV induces MC degranulation (24). Mature MCs are highly resistant to infection and degranulation occurs independent of virus replication within MCs since UV-inactivated virus triggers a degranulation response similar to live virus(24). MCs also promoted recruitment of T and NK cells to infection sites and viral clearance through de novo chemokine production (24) and activation of cytotoxic T cells, such as γδT cells, through non-classical antigen presentation(33). Contrasting to their protective function in localized infection, widespread activation of MCs during systemic infection can contribute to DENV-induced vascular leakage in mice (34). Antibodies can also enhance the DENV-induced activation of MCs, by crosslinking of activating FcγRs, similar to the mechanism described for MC activation during the reverse arthus reaction (5, 35). In human DENV patients, detection of heightened serum protein levels of the MC-specific and granule-associated product, chymase, during the acute febrile phase of disease, indicated that MCs are activated during DENV infection(34, 36). Chymase was not elevated in the serum of other febrile patients, most of which were experiencing respiratory infections (34). Importantly, chymase levels were higher in the serum of patients diagnosed with DHF than those diagnosed with DF during both the acute febrile (within 3 days of fever onset in that study) and defervescent phases of disease, but highest at the earliest time points, and higher in DHF/DSS patients experiencing secondary infections compared to primary infection (34). Thus, chymase levels were correlated with disease severity prior to diagnosis of severe disease, raising the possibility that chymase could serve as a prognostic biomarker for severe dengue (34, 36). Taken together, the data suggest that systemic DENV infection initiates a cascade of events involving wide-spread MC activation leading to persistent levels of MC-derived products in the serum throughout the acute

phase of disease, which damage the vascular endothelium. Yet, the mechanism by which MCs promote endothelial activation and vascular permeability during DENV infection remains unknown. In this study we sought to identify the MC mediator primarily responsible for endothelial breakdown and vascular leak during DENV disease. We show that DENV-elicited tryptase is a particularly potent inducer of microvascular permeability, which occurs mechanistically through disruption of endothelial tight junctions. Validation of these findings in mice suggest that tryptase promotes substantial increases in plasma loss from the circulation and reduced adhesion molecule expression on vascular endothelial cells, while chymase, in contrast, has a more modest effect. A potent specific inhibitor against tryptase was sufficient to reduce DENV-induced vascular leak in vivo in multiple mouse models. Remarkably, in human patients with DHF, levels of serum tryptase were highest when patients experienced shock (DHF grades III-IV), and were well correlated with the severity of DHF. These findings suggest that high levels of tryptase can cause shock during DHF.

Results

Mast cell proteases, tryptase and chymase, promote endothelial permeability and break tight junctions.

To identify the MC-derived products that promote vascular leakage during DENV infection, we first questioned whether the soluble or particulate (exocytosed MC granules(30)) fractions were primarily responsible for inducing endothelial permeability. The soluble fraction of media after MC activation would contain the de novo synthesized products, such as eicosanoids and most cytokines, as well as pre-stored mediators that are quickly solubilized from the granule after activation, such as histamine. The particulate fraction would contain largely the insoluble pre-stored mediators, including heparin, and the proteases that make up the majority of heparin-associated proteins, including chymase and tryptase (37). These fractions were separated by centrifugation and human microvascular endothelial cells (huMECs) were cultured to form a tight monolayer followed by treatment of the monolayers with total or fractionated portions of DENV-induced MC products. The degree of damage to the endothelial integrity was determined by measuring trans-endothelial

resistance (TER). Our previous studies showed that the TER was dramatically reduced when endothelial monolayers were treated with DENV-elicited MC products compared to the control un-treated groups, treatments with supernatants from mock-infected MCs or with an equivalent amount of DENV in media alone (34). Here, when DENV-elicited MC products were further separated into soluble and particulate fractions, treatment of huMEC monolayers with the particulate fraction significantly reduced the TER compared to the soluble fraction treatment group, while the same concentration of virus alone did not have a significant effect (Figure 1A). This data suggests that it is the MC granule-associated components that are important in breaking endothelial integrity.

The two dominant protein constituents of granules are the MC proteases, chymase and tryptase. We previously had determined that chymase is a biomarker of severe vascular leakage in human DENV patients (34), but whether it also has a functional role in DENV-induced vascular leakage remained unclear. In order to address the direct ability of MC proteases to induce endothelial permeability, we cloned, expressed and purified the two dominant MC proteases, both human tryptase and human chymase, as histidine-tagged recombinant proteins for use in functional assays. The proteins were chromatography purified, tested to be endotoxin free, and tested to verify for functional protease activity. Incubation of tryptase with huMECs resulted in a strong, dose-dependent increase in endothelial permeability, as demonstrated by a drop in the TER (Figure 1B). In contrast, chymase promoted only a moderate decrease in the TER of huMEC monolayers at the highest (1μM) concentration tested (Figure 1C). This data points to the role these proteases could play during DENV disease severity.

Next, we sought to understand the mechanism behind tryptase/chymase-induced endothelial permeability. Since tight junctions between the cells are crucial for maintaining the endothelial barrier function and tryptase, in particular, has been described to cleave the tight junction component PAR-2 (38), we questioned whether loss of tight junctions would underlie the MC-protease induced increases in endothelial permeability. To address this, huMECs were cultured to form a monolayer on glass coverslips, followed by exposing them with two different concentrations (0.1μM and 1μM) of tryptase or chymase for 24h. At 24h post-treatment, cells were fixed and immunostained for tight junction protein ZO-1 and tubulin to reveal each individual cell cytoskeleton, and with DAPI for nuclear

localization. The microscopy images show a uniform continuous staining of ZO-1 in un-treated control cells (Figure 1D), suggestive of intact endothelial tight junctions. However, treatment of huMEC monolayers with tryptase lead to dramatic damage of tight junctions, with ZO-1 staining completely disappearing at the high, 1μM concentration of tryptase (Figure 1E). Individual cells were also observed to lift from the coverslips leaving large gaps between cells (Figure 1E). This effect was substantial even with low dose tryptase treatment (0.1μM), where only punctate staining for ZO-1 at the cell borders remained (Figure 1E). Consistent with the TER data presented in Figure 1C, chymase treatment also caused damage to tight junctions but to a lesser extent compared to that of tryptase treatment (Figure 1F). Collectively these data show that both tryptase and chymase are able to break tight junctions between endothelial cells, causing increases in endothelial permeability.

Tryptase treatment reduces surface expression of adhesion molecule CD31 on vascular endothelium.

To understand in detail how tight junctions are affected in vivo by the MC proteases, chymase and tryptase, we measured the surface expression of cell adhesion molecule CD31 (also called PECAM-1) on vascular endothelial cells after injection of either of these proteases. For this, 100ng of tryptase or chymase was injected in the mouse rear footpad followed by harvest of the tissue from footpad after 6h. Single cell suspensions were prepared from the tissue and endothelial cells were stained using an antibody against CD31, which is both a maker for endothelial cells and a functional component of tight junctions, before being analyzed by flow cytometry. The data demonstrate that the mean fluorescence intensity (MFI) of CD31 staining was significantly decreased upon tryptase treatment compared to that of the saline injection control (Figure 1G-H). Chymase treatment, in contrast, did not influence the surface expression of CD31 (Figure 1H). In the context of DENV infection in vivo, CD31 expression was also found to be significantly reduced on endothelial cells when mice were infected with DENV (Supplemental Figure 1). These data suggest that during DENV infection, tryptase breaks tight junctions between endothelial cells and results in a reduction in the surface expression of cell-cell adhesion molecule, CD31, in vivo. In contrast, the effect of chymase was not significant after injection and did not influence CD31 expression.

MC proteases, tryptase and chymase, promote vascular leakage and induce hypovolemic shock in vivo.

To determine if MC proteases could have a functional role in plasma loss during DENV infection in vivo, we injected them i.v. in mice, aiming for a final serum concentration of proteins around 10ng/mL, approximately equivalent to the concentrations of proteases that were previously reported in the serum of human DHF patients(34), assuming a ~3mL blood volume for a mouse. Mice were injected with chymase or tryptase and at 6h post-injection; hematocrit values were measured from the blood to quantify the degree of plasma loss from circulation in mice after treatments. Both chymase and tryptase were sufficient to induce physiologically significant increases in vascular leakage, which was measured as an increase in hematocrit (Figure 2A). However, tryptase resulted in a ~16% increase in the RBC volume in the blood, compared to a ~3.8% increase in RBC volume after chymase injection (Figure 2A). Injection of a control protein, ovalbumin (OVA) did not significantly influence the hematocrit (Figure 2A). To confirm vascular leakage by a secondary method, we injected Evans blue dye (EBD) 6h post-injection of either chymase or tryptase and, after an additional 30 minutes, quantified the amounts of dye that leaked into the liver tissue (Supplemental Figure 2). EBD leaked significantly into the livers of mice that were tryptase-injected, confirming the induction of vascular leakage, while the increases in EBD in chymase-injected mice were not significant (Supplemental Figure 2). Thus, while both chymase and tryptase can impact vascular homeostasis, tryptase promoted the most substantial vascular leakage.

Next we questioned whether the rise in hematocrit that occurred during tryptase/chymase treatment could result in hypovolemic shock. The standard way to measure infectious or anaphylactic shock in the mouse model is to record a drop in body temperature (39-41). To test this, mice were given 30ng of either tryptase, chymase or OVA i.v., followed by the measurement of temperature at regular intervals. Interestingly, both tryptase and chymase injections resulted in a dramatic drop in the body temperatures of mice, suggestive of a shock (Figure 2B). However, tryptase treatment resulted in a stronger drop in body temperature (~2.5°C below normal) compared to that of chymase treatment (~1.5°C) and persisted longer (Figure 2C). Control animals that were given essentially the same

medium in which proteins were diluted did not undergo any shock and only experienced a moderate and brief temperature decline that is likely attributable to the injection of saline, nor did animals that were injected with an equivalent amount of the exogenous protein, OVA (Figure 2B, C), which was used as a control for protein injection. Overall, these data show that, although both tryptase and chymase are able to cause plasma leakage in vivo, tryptase has a more profound effect on vascular endothelium with regards to inducing vascular leakage and shock, compared to chymase.

Tryptase inhibition protects against DENV-induced hemoconcentration.

Based on the observation that tryptase is highly consequential to vascular leakage in DENV-infected animals and sufficient to induce shock in vivo, we aimed to ameliorate vascular leakage in DENV-infected animals by therapeutically targeting tryptase. For this, we used the drug nafamostat mesylate since this drug is a highly specific inhibitor of tryptase at pico-molar concentrations and also approved for clinical use for intravascular coagulation (42, 43). Animals were treated with nafamostat mesylate after DENV infection and vascular leakage was measured in the animals by obtaining hematocrit readings. We observed that nafamostat mesylate alone did not influence the hematocrit of healthy uninfected control animals. However, in DENV-infected animals where strong hemoconcentration indicating vascular leakage is observed during infection, nafamostat mesylate restored the hematocrit to homeostatic levels (Figure 3A). These data are further solidified by measurements of tryptase and chymase activities in the serum of mice from each experimental group. The enzymatic activities of both tryptase and chymase were significantly higher in the DENV-infected but vehicle-treated animals compared to that of control groups (both mock-infected vehicle-treated and mock infected drug-treated) (Figure 3B, Supplemental Figure 3). Treatment of DENV-infected animals with nafamostat mesylate resulted in functional serum tryptase levels that were at baseline, confirming the specific action of this drug in blocking tryptase activity in vivo (Figure 3B). Together, these data demonstrate that tryptase inhibition is able to therapeutically block DENV-induced plasma loss significantly. The specificity of the drug's action on DENV-induced plasma leakage is further shown by the fact that nafamostat mesylate treatment did not alter chymase activity (Supplemental Figure 3) or ameliorate the drop in platelets that is observed during infection (Figure 3C) and did not

225 significantly influence the titers of virus in vivo (Figure 3D). Since tryptase can theoretically influence
226 the coagulation and complement cascades(44, 45), we measured levels of complement component
227 C3 in the serum of DENV-infected and vehicle- or nafamostat mesylate-treated animals but observed
228 no changes in the levels of C3 with drug treatment, although C3 was reduced in DENV-infected
229 animals compared to controls (Supplemental Figure 4), suggesting DENV induced a tryptase-
230 independent complement activation. These results support that the therapeutic effects of nafamostat
231 mesylate on DENV-vascular leakage are due to its action as a specific inhibitor of tryptase enzymatic
232 activity.

233 We repeated our findings in a severe model of DENV viremia to establish whether vascular
234 leakage could also be reversed in those mice. The AG129 mouse line is deficient in receptors for both
235 Type-I and Type-II interferons and has been used extensively to study DENV infection in vivo.
236 Nafamostat mesylate was effective in reducing hematocrit values in AG129 mice given both high and
237 low inoculating doses of DENV (Figure 4A). Next, we evaluated if tryptase inhibition can also be
238 effective in severe model of dengue disease, characterized by antibody-dependent enhanced
239 infection (ADE) of DENV in AG129 mice. Mice were given an enhancing concentration of antibody
240 4G2, followed by DENV infection, which, as expected, led to increased virus titers through ADE
241 (Supplemental Figure 5). Subsequent to infection, mice were treated daily with the tryptase inhibitor
242 nafamostat mesylate. Nafamostat mesylate treatment significantly reduced the hematocrit values on
243 days 2 and 3 post-infection (Figure 4B), demonstrating that the tryptase-dependent mechanism for
244 reducing vascular leakage is consistent in this second model that involves antibody-enhanced
245 disease. We also measured the serum tryptase and chymase enzymatic activity in serum from each
246 experimental group. Consistent with our data in WT mice (Figure 3B, Supplemental Figure 3) we
247 observed that nafamostat mesylate treatment significantly reduced tryptase activity in AG129 mice on
248 days 2 and 3 post-infection (Supplemental Figure 6A) yet had no effect on serum chymase activity
249 (Supplemental Figure 6B), again confirming the specificity of this tryptase inhibitor. Finally, we
250 examined the efficacy of delayed treatment, where AG129 mice infected with the same antibody-
251 enhanced strategy were treated with nafamostat mesylate daily, but beginning 24h after infection

(Figure 4C). Even with delayed treatment, nafamostat mesylate had a significant therapeutic effect and reduced hematocrit levels following infection (Figure 4C).

To confirm the influence of nafamostat mesylate on vascular leakage by an alternate method, we performed intra-vital multi-photon microscopy. The ears of AG129 mice that had been mock-infected (Supplemental Video 1), infected with DENV in mice that were given vehicle control injections (Supplemental Video 2), or infected with DENV in mice that were given injections of nafamostat mesylate (Supplemental Video 3) were imaged after injection of FITC-dextran dye, which was used to visualize vascular leakage. Still images from various time points beginning 24h post-infection from Supplemental Videos 1-3 are presented in Figure 5A. While dye was confined to the blood vessels of control uninfected tissues for the duration of imaging, it can be visualized leaking into the tissue in DENV-infected animals (Figure 5A, Supplemental Videos 1-2). Nafamostat mesylate treatment reversed the leakiness of the vasculature in DENV-infected animals (Figure 5A, Supplemental Video 3). Quantification of the dye leakage in the videos showed DENV infection allowed dramatic increases in dye detection over the course of visualization while only a slight increase occurred in control uninfected tissues (Figure 5B). In contrast, fluorescence detection remained near baseline in DENV-infected but nafamostat mesylate treated animals (Figure 5B). Additional experiments at 48h post-infection for the same groups as described above confirmed the efficacy for nafamostat mesylate in blocking DENV vascular leakage (Supplemental Videos 4-6). These results further support tryptase as a mechanism of DENV-induced vascular leakage and also suggest this enzyme may be an effective therapeutic target.

Tryptase levels correlate with DENV severity in humans.

After experimentally establishing that both tryptase and chymase could negatively influence the integrity of the vascular endothelium during DENV infection and that they are sufficient to induce plasma leakage in mice, we did a retrospective study of acute human serum samples collected during an epidemic that occurred in Jakarta, Indonesia in 1975-1978(46-48). The 34 samples, from virologically confirmed DENV-infected patients, clinically graded to have a range of DENV pathologies from DF to fatal DHF (Figure 6A) and representing multiple serotypes (18% DENV-1, 24% DENV-3,

3% DENV-4 and 55% serotype undetermined), were blinded as to clinical severity of the patient and virus serotype. Consistent with our earlier published data (34), levels of chymase were significantly elevated in serum of patients with severe DENV outcomes, consistent with the clinical definition of DHF (Figure 6A). Remarkably, the levels of tryptase were also significantly elevated during DHF (Figure 6B). However, chymase levels showed a ~800% increase in DHF patients over DF patients, versus a 75% increase in tryptase for DHF patients over DF patients (Figure 6A-B). These findings are consistent with our previous report that chymase is a robust biomarker for DHF (34). Next, we assessed whether there was a correlation between the levels of tryptase or chymase with the severity of DHF (DHF grades II-IV and fatal DHF). DHF was graded according to the WHO guidelines(3). Interestingly, levels of tryptase followed a strong linear correlation with the severity of DHF in human patients (Figure 6C). However, although the levels of chymase were higher in DHF patients, there was no statistically significant correlation observed with the severity of DHF (R^2=0.5, p=0.3, Supplemental Figure 7A). These data show that increased levels of serum tryptase were correlated with increased disease severity in patients (Figure 6B, C). To confirm this finding, a more recent cohort of serum samples prospectively collected in Sri Lanka in 2012-2013 were tested. These samples were previously shown to have significantly elevated chymase levels in DHF patients compared to DF patients(36). Consistent with findings in the Indonesian cohort, DHF patients compared to DF patients had significantly higher levels of serum tryptase (Figure 6D). Furthermore, tryptase levels (Figure 6E) but not chymase levels (Supplemental Figure 7B) were significantly correlated the grade of DHF. Collectively, these data suggest that tryptase is a mechanistic correlate of vascular leakage during severe dengue that could also potentially be used as a prognostic marker for DHF severity.

Discussion

Multiple theories have been put forward to explain how DENV vascular leakage syndrome is initiated and sustained in vivo. In the absence of strong and consistent evidence that endothelial cells are directly infected by DENV in humans, immune-mediated pathology is presumed to underlie the mechanism of increased endothelial permeability during DHF and DSS (2). In this work, we have

described a novel mechanism of vascular leakage during DENV disease and show that the MC-derived protease, tryptase, is a host protein that is consequential for breaking endothelial tight junctions. Tryptase is a serene protease that functions in vivo to cleave a list of targets including kininogen, complement factor C3, clotting factor XII, fibrinogen, and others(49). Tryptase also regulates the activity of other serine proteases such as pro-urokinase, an important coagulation factor(50). Interestingly, an early report describing fatal DHF in the Philippines reported low levels of circulating fibrinogen accompanying intravascular coagulation (51), which would be consistent with degradation of this product by tryptase. Decreased serum fibrinogen, high levels of fibrinogen split products and intravascular coagulation were also observed subsequently in a cohort of patients from Thailand(52). Although it should be noted that intravascular coagulation is a rare outcome of dengue(52). Indeed, in our study, injection of tryptase alone was sufficient to induce shock in mice. A high affinity tryptase specific inhibitor, nafamostat mesylate, limited DENV-induced vascular leakage in vivo. This drug is currently approved for use to treat disseminated intravascular coagulation (42), which is also a potential clinical sign accompanying DSS (52-54). It is also effective for treatment of conditions where limiting endothelial activation and coagulation are beneficial, such as renal ischemia reperfusion injury(55, 56). While nafamostat mesylate can inhibit some other serine proteases at high concentration, it has been shown to be highly specific for tryptase at the concentrations used in this study(57-59). Some additional pathways that can be influenced directly or indirectly by tryptase include PAR receptors and the fibrinolytic systems(38, 44, 50), as previously discussed, as well as the kallikrien-kinin system and complement activation pathways(45, 60). Tryptase is also a known glycocalyx sheddase(61) and glycocalyx degradation has been observed to occur during human dengue infection(62, 63). Although not tested here, the tryptase-dependent effect of nafamostat mesylate on coagulation factors such as fibrinogen(64, 65), could potentially be an added advantage for the treatment of severe DENV disease since fibrin split products and intravascular coagulation are known to occur during DHF/DSS.

Importantly, nafamostat mesylate was effective in multiple models of DENV infection, including immunocompetent and immunocompromised mice and antibody-enhanced models of severe DENV

disease. We also observed efficacy with delayed treatment. Antibody-enhancement is thought to promote severe disease due to antibody-dependent uptake of virus particles by Fc receptor-bearing cells and we have recently shown that FcR-dependent enhancement of MC degranulation occurs as a second antibody-dependent mechanism leading to enhanced vascular leakage(35). In humans, biomarkers of MC activation such as chymase are detected at higher levels in patients with secondary infection, supporting that enhanced MC degranulation occurs during secondary DENV infection(34), which could be a factor in the efficacy of nafamostat mesylate in the animal model of antibody-enhanced severe disease in addition to the possibility of increased activation of MCs due to higher titer of virus resulting from traditional ADE. Aside from antibody-enhanced degranulation, traditional ADE can allow MCs to become infected, which could augment production of transcriptionally activated inflammatory mediators, such as cytokines, further during secondary infection(66). In human serum samples examined from 2 independent cohorts of patients with severe disease, we show that the levels of tryptase are significantly correlated with the grades of DHF in DENV patients. Our results emphasize the role of tryptase in DENV-induced vascular permeability and raise the possibility of it being a novel therapeutic target in treating severe dengue patients at risk for hemorrhage and shock.

We have previously demonstrated that MCs induce vascular leakage during DENV infection in animal models (34, 35). However, the mechanisms of action of MC products on the vasculature and the mediators involved in vascular permeability in this context were previously unknown. MCs can release a multitude of soluble mediators (lipids, cytokines, chemokines and growth factors) as well as particulate mediators in the form of exocytosed granules. These granule structures are known to contain many vasoactive and immune modulatory products such as proteases, histamine, heparin, TNF, anti-microbial peptides and others (23). To begin to address which of these many mediators contributes most substantially to DENV vascular leakage, we fractionated the products released by DENV-activated MCs in to soluble and particulate fractions and applied them to endothelial monolayers. Serum from DENV patients is also sufficient to induce break down of tight junctions of endothelial monolayers ex vivo (67). Our TER measurements of these huMEC monolayers revealed that the MC granule associated products, and not the soluble mediators, disrupted endothelial monolayer integrity severely. The dominant MC proteases, tryptase and chymase, constitute the

majority of the proteins that are contained within granules. Expression of tryptase and chymase are unique to MCs and their roles in viral pathogenesis are unknown. Chymase is best recognized as an angiotensin-converting enzyme, but it also can influence the vasculature in several ways. For example, chymase is thought to degrade some extracellular matrix components and has been shown to cleave Endothelin-1, promoting vasoconstriction (68). We also recently showed that chymase is important for inducing permeability at the blood brain barrier during JEV infection(69). Tryptase is known to have a more profound and direct influence on peripheral vascular permeability by cleaving PAR receptors at the inter-endothelial junctions(38). Interestingly, using recombinant purified chymase and tryptase we show here that exposure of endothelial monolayers to tryptase breaks endothelial tight junctions in a dose dependent manner. This was further verified by injecting tryptase in vivo, which was sufficient to reduce expression of the adhesion molecule CD31 on vascular endothelium and to induce vascular leakage and shock in mice. Although to a lesser extent at the concentration given, chymase was also able to induce moderately increased hematocrit levels that were not accompanied by significantly increased leakage of EBD into tissues at the time point assessed. The influence of chymase on hematocrit levels here could potentially be through its function to degrade the extracellular matrix(70). The effects of chymase on endothelium could be compounded by the presence of TNF, which is also contained within MC granules(30) and, furthermore, thought to be a possible contributing factor to DENV-induced immune pathology(6).

Notwithstanding these observations, there are other factors that are thought to contribute to vascular permeability during DENV infection. One such host molecule already discussed is TNF, which is not only pre-stored within MC granules, but also produced abundantly by cells that are infected by DENV. Some studies have shown that TNF blockade can limit vascular pathology and promote survival of DENV-infected animals(6). However, most studies implicating TNF experimentally were performed in mice that are immunocompromised and lacking the IFN-response system (6). This experimental system has the potential to over-emphasize the virus replication-dependent aspects of vascular permeability and may result in replication of virus within cell types that would be resistant to infection in IFN-competent animals (2). Recent studies have shown that A129 mice, which are deficient in receptors for Type-I interferons, are susceptible to maternal antibody-enhanced lethal

DENV infection that is TNF-dependent, without showing significantly increased levels of vascular leakage (71). Moreover, in human studies, TNF could not be consistently linked to DENV severity (2, 72). Recently, DENV NS1 protein has also been implicated in vascular leakage in IFN-deficient mouse models (13). Although NS1 may contribute to vascular permeability, further studies are needed to reconcile the observations of some studies that NS1 levels cannot be correlated with severe disease (14-18), including further attempts to control for the potential of antibodies to influence the kinetics of host clearance of NS1 and/or detection of the NS1 protein in the serum. MC degranulation in response to DENV is also dose-dependent; thus, higher levels of DENV should also promote heightened degranulation responses but the reactivity of individual patients to unique amounts of viral antigen could still vary. Identification of MCs as a potential source of vasoactive factors also opens the possibility of additional host-associated factors that influence MC function which might also then influence DENV disease severity, such as the patient's allergic state, commensal microflora (including parasitic infections), exposure to allergy medications, homeostatic mechanisms for protease inhibition and genetic factors governing MC activation and distribution in vivo.

Since MC proteases are pre-synthesized and some of these enzymes are stored within MCs, exclusively, this might also explain why many studies that have attempted to interrogate the pathways involved in DENV-induced vascular leakage through transcriptional profiling or by examining the responses of various infected cell types have not identified increased tryptase or chymase to be associated with DENV infection. We have previously reported that the MC protease, chymase, is a biomarker of disease severity and present in the serum of DHF patients at levels ~10-fold that of DF patients(34). Consistent results were observed in this study, examining cohorts of patients from Indonesia and Sri Lanka. In this case, both chymase and tryptase were elevated in the serum of DHF patients. While chymase may be a more precise biomarker of MC activation during DENV infection, our data shows that tryptase is mechanistically more consequential for leakage and shock. We think that this difference could be partially attributed to the difference in functional stability of these proteins in the circulation. The functional half-life of tryptase in the plasma is ~8min, which is further stabilized in the presence of heparin for ~2h(73). Chymase, in contrast, is very sensitive to the presence of protease inhibitors present in the plasma, such as secretory leukocyte proteinase inhibitor (SLPI)(74),

which could reduce chymase activity in spite of having high circulating protein levels. Furthermore, in contrast to tryptase, SLPI activity is greatly enhanced in the presence of heparin, which was shown to limit the functional half-life of chymase to 0.5sec (74). This is consistent with the observation that injection of high levels tryptase led to a more prolonged drop in temperature in animals, while an equivalent concentration of chymase resulted in only a transient temperature drop. We believe that even though both tryptase and chymase are fairly stable proteins structurally, the differences in their functional enzymatic stability could explain why tryptase is a more potent inducer of endothelial dysfunction during severe DENV disease. Since human MCs display heterogeneity based on their tryptase and chymase content, the types of MCs that are activated might play a role in the severity of disease. While all mature human MCs are thought to contain tryptase within their granules, some MCs have nearly undetectable levels of chymase(75).

Our results also do not suggest that MC proteases must act alone in their ability to induce vascular leakage during DENV infection. We believe that other factors that are produced both by MCs and also by independent lineages of cells (particularly those experiencing viral replication) are likely to contribute to vascular leakage. It is important to note that patients with mild DENV infections display signs of microvascular permeability such as bruising, purpura, and edema (2). This is also the case during the acute phase of disease for those who subsequently develop severe complications. Another unknown is the role of the strain of DENV in activating MCs. It is known that different strains may have variable infectivity for target cells, which may influence transmission dynamics and virulence. Nevertheless, MCs may be key for initiating the substantial vascular leakage that is characterized by severe DENV infection through their ability to degranulate in response to DENV and the ability of their product, tryptase, a dominant MC granule component, to cleave PAR receptors at endothelial junctions and to induce shock.

Materials and Methods

Cell lines, virus strains and culture conditions

The huMEC primary cell line HMVEC-d Ad-Dermal MV Endo Cells (Lonza, CC2543) was maintained in EGM-2MV BulletKit media (Lonza, CC3202). The human clinical isolate of DENV2, strain Eden2, which was obtained from the Early Dengue Infection and Outcomes Study (Eden)(76), was maintained at low passage as previously described (24, 34).

Measurement of endothelial activation and permeability

huMECs were grown in 3μm inserts (BD Biosciences) inside 24-well plates for 4-5 days to form a monolayer. huMECS were then incubated for 24h with different concentrations of either tryptase or chymase (1μM, 0.1μM, 0.01μM, or 0.001μM). Monolayer permeability was measured by acquiring TER readings at baseline (t=0) and 24h after treatment with tryptase, chymase, MC supernatants or appropriate controls. Human ROSA MCs ($3X10^6$ cells)(77), a gift from Michel Arock (Ecole Normale Supérieure de Cachan, Cachan, France) were treated for 1h with either DENV (MOI 1) or control media (untreated), after which, the cellular fraction was removed by two rounds of centrifugation at 500xg for 5 min. The particles were then pelleted from whole supernatant by spinning at 12,000xg for 10 min at 4°C. Soluble fractions were collected and particles were washed and resuspended in maintenance media, followed by exposing them to huMECs monolayers. TER readings were obtained using the Millipore Millicell-ERS (Electrical Resistance System).

Immunofluorescence assay

huMECs were grown on coverslips (Warner Instruments) inside 24-well plates for 2-4 days to form a monolayer. huMECS were then incubated with various concentrations of tryptase or chymase (1μM or 0.1μM). Treated huMECs were incubated for 24h, followed by fixing with paraformaldehyde. Coverslips were then washed with PBS and blocked using 0.1% saponin in 1% BSA in PBS (permeabilizing buffer). Primary antibodies against α-tubulin (GeneTex, GTX11302) and ZO-1 (Invitrogen, 402200) were added to permeabilization buffer and incubated overnight followed by washing using permeabilizing buffer. Next, the secondary antibodies, anti-mouse-conjugated FITC

(Jackson ImmunoResearch, 115-096-006) and anti-rabbit-conjugated AlexaFlour660 (ThermoFisher Scientific, A21073) were added in permeabilizing buffer and incubated for 2-4h. Finally, coverslips were mounted using Pro-Long Gold Anti-fade reagent containing DAPI (Invitrogen, P36931). Cell images were obtained using the LSM710 Carl Zeiss Confocal Microscope using a 63x objective lens.

Cloning and purification of MC proteases

The RNA was isolated from huMCs using RNeasy Mini Kit (Qiagen) and cDNA was made using cDNA synthesis kit (Bio-Rad). For chymase, PCR amplification was performed with the following primers: 5´–CGG CTC ATA TGA TGC TGC TTC TTC CTC TC–3´ and 5´–ATA CTC TCG AGT TAA TTT GCC TGC AGG ATC TG–3´, where the underlined sequence corresponds with *Nde I* and *Xho I* restriction sites. For tryptase, PCR amplification was performed with the following primers: 5´–AGT CTC ATA TGA TGC TGA GCC TGC TGC TGC TGG CG–3´ and 5´–CAA TGA AGC TTT CAC GGC TTT TTG GGG ACT AGT GGT–3´, where the underlined sequence corresponds with *Nde I* and *Hind III* restriction sites. PCR products were subsequently cloned in the pET28a Vector (Novagen) using the restriction sites included within the amplification primers. The recombinant pET28a vectors containing either human tryptase or chymase were sequenced to verify in-frame insertion. Recombinant pET28a plasmids were then transformed into *E. coli* BL-21 (DE3) cells, which were grown in LB broth containing Kanamycin (50µg/ml) at 37°C until an OD-600 of 0.4-0.6 was reached. Protein expression was then induced with 1mM IPTG, overnight at 16°C. Cell lysates were sonicated and clarified supernatant was loaded into HisTrap columns (GE healthcare). HPLC purification was performed according to the manufacturer's instructions under Hybrid conditions using an AKTA machine (GE Healthcare). Recombinant active human chymase (CSB-YP005599HU) and tryptase (CSB-YP024128HU) expressed in yeast were purchased from CUSABIO, USA. Catalytic active sites are highly conserved among human and mouse tryptase and chymase and, therefore, these proteins are functionally active in vivo(78).

Mouse experiments

Wild type mice on a C57BL/6 background were purchased from InVivos and AG129 mice, originally purchased from B&K Universal, UK, were a gift from Sylvie Alonso (NUS, Singapore). For all strains, 6-8 weeks old female mice were used for experiments. All experiments were performed according to protocols approved by the SingHealth Institutional Animal Care and Use Committee. For in vivo studies, due to the large amounts of protein required, yeast-produced MC proteases were purchased from CUSABIO, USA and OVA was purchased from Sigma. Mice were administered chymase, tryptase or OVA in PBS via tail vein injection at the concentrations provided in the figure legends. Blood was collected 6h post-injection and hematocrit values were acquired using an AcT Diff automated hematology analyzer (Beckman Coulter). For experiments involving flow cytometry to assess endothelial cells ex vivo 100ng of chymase or tryptase or $1X10^6$ PFU of DENV was given by subcutaneous injection in the mouse footpads. Shock was measured quantitatively in mice by recording the temperature at regular intervals using a rectal probe after injection of 30ng of chymase or tryptase by tail vein. For drug studies, nafamostat mesylate (Sigma, N0289) was given to mice (0.06 or 0.6 mg/kg) daily i.p. in a 50µl volume of saline and saline alone served as the vehicle for controls, beginning 1h or 24h post-infection, as indicated in figure legends. Hematocrit and platelet readings were obtained from whole blood collect via cheek-vein, using an automatic hematology machine at indicated time points of 24h, 48h or 72h. Virus quantification was performed after RNA isolation from the spleen at 24h, 48h or 72h post-infection. cDNA was synthesized from 1µg of RNA using the iScript cDNA Synthesis Kit (Bio-Rad) with the addition of primer 5′-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3′ to synthesize viral RNA to cDNA. Real-time PCR was performed using SYBR Green reagent (Biorad) and the following DENV2 primers: forward, 5′- TCA ATA TGC TGA AAC GCG CGA GAA ACC G -3′; reverse, 5′- CGC CAC AAG GGC CAT GAA CAG -3′. Systemic infection in AG129 mice was achieved by injecting $1x10^6$ or $5x10^7$ PFU of DENV via the i.p. route. For the severe DENV infection model of antibody-dependent enhancement, AG129 mice were passively given 50µg/mouse of an antibody 4G2, followed by infection with a high dose ($1x10^8$ PFU) of DENV i.p after 24h, according to a published protocol (79). Mice were treated with 0.6 mg/kg of nafamostat mesylate at 24h intervals.

Intra-vital microscopy

AG129 mice were infected with $1X10^6$ PFU DENV by i.p. injection. Mice were either mock treated with vehicle or treated with nafamostat mesylate (0.6 mg/kg), 1h prior to infection or 24h post-infection. Mice were anesthetized and injected with 100µl of 50mg/ml 70KDa FITC-Dextran (Sigma, 46945) (80) and two-photon images of the mouse ears were acquired using a Bergamo® II Series Multiphoton Microscopes from Thorlabs (NJ, USA) 5 minutes post-FITC-dextran injection every 2 sec for 18 minutes (920 nm laser), as described previously(81). For quantifying vascular leakage, 10 areas of equal size were marked near blood vessels and the fluorescence intensity was measured using FIJI(82) and mean fluorescence intensity for each time point were plotted using GraphPad Prism. Images were converted to videos using FIJI (NIH). Videos are representative of 3 experiments.

Measurement of chymase activity, tryptase activity and complement C3 in mouse serum

Functional serum tryptase and chymase activities were measured by using Mast cell degranulation assay kit (Millipore, IMM001) and chymase activity assay kit (Sigma-Aldrich, CS1140) respectively. Following manufacturer instructions, the reaction was carried out in a total volume of 100µl using 5µl of serum and then relative chymase and tryptase activity was calculated by normalizing the values to chymase and tryptase activity in serum of mock-infected mice. Complement C3 concentrations in serum were measured using Mouse Complement C3 ELISA kit (Abcam, ab157711) with a minimum detection range of 3.13ng/ml to 200ng/ml. Serum samples were diluted 1:50,000 times according to the manufacturer's instructions and 100µl of diluted serum was used for the assay.

Detection of MC proteases in clinical samples

Human clinical samples were collected from hospitalized patients as part of surveillance for severe dengue in Jakarta, Indonesia from 1975-1978 (46-48). Other than for an unknown period in the 1990s when a freezer failed and the samples thawed, they were maintained at -60 to -80 C for the four decades of storage by one of us (DJG). The DHF grade was retrospectively classified according to the 1997 WHO classification criteria(3). For the second Sri Lankan cohort, human clinical samples were prospectively obtained by the Ministry of Health in Sri Lanka and provided by the Dengue Tools

Project(83, 84). Dengue Tools recruited patients with undifferentiated febrile illness with a duration less than 7 days, and serum samples were selected for this study based on being confirmed dengue-positive using previously described methods(84). All patients provided blood samples with informed consent. Dengue Tools serum samples collected at the time of patient recruitment were selected for tryptase testing based on having had fever <6 days, a clear discharge diagnosis of either DF or DHF, and having sufficient remaining serum to perform the test. Classification was also determined based on the 1997 WHO criteria(3). Serum was stored at -80°C. Ethical approval was obtained from the Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka and the Institutional Review Board, National University of Singapore, Singapore. All DHF patients met the WHO case classification for Grades I, II, III or IV(3). ELISAs for human chymase (Blue Gene, E01M0368) and human tryptase (Cloud-clone Corp, SEB070Hu) were performed on blinded samples according to manufacturer's instructions and un-blinded only after analysis.

Statistics

Prism 7 and Excel were used to determine statistical significance and SPSS was used to verify that the input-data were normally distributed using the Shapiro-Wilk test prior to analysis using parametric tests. Violin plots were generated using a web-based tool(85). Where appropriate for direct comparisons of two samples, Student's un-paired t-test was used. For multiple groups, 1- or 2-way ANOVAs were performed with post-tests to determine between-groups statistical significance or regression analysis in the case of human biomarker data. Data were considered significant at $p \leq 0.05$. The numbers of biological or technical replicates for each group are indicated in each figure legend.

Study approval

Animal studies were approved by SingHealth Institutional Animal Care and Use Committee, Singapore. The clinical samples were obtained upon approvals from the institutional review board's of National University of Singapore, Singapore and University of Colombo, Sri Lanka.

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584 **Data availability statement**

585 All data needed to evaluate the conclusions in the paper are present in the paper with extended data
586 in a supplementary file.

587

588 **Author Contributions**

589 The project was conceived by ALS and APSR. Experiments were performed by APSR, CKM, SABA,
590 AS, JO, CJJ, and CCG. Data were analyzed and interpreted by ALS, APSR, CKM, and DJG. The
591 manuscript was written by APSR and ALS. Human clinical samples were provided by DJG, HT and
592 AWS. DJG also categorized samples according to dengue classification schemes. Intra-vital
593 microscopy was optimized and analyzed by CKM, CCG, and LGN. All authors contributed to
594 discussions and reviewed the manuscript.

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596 **Competing Interests statement**

597 The authors have declared that no conflict of interest exists.

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Figure 1

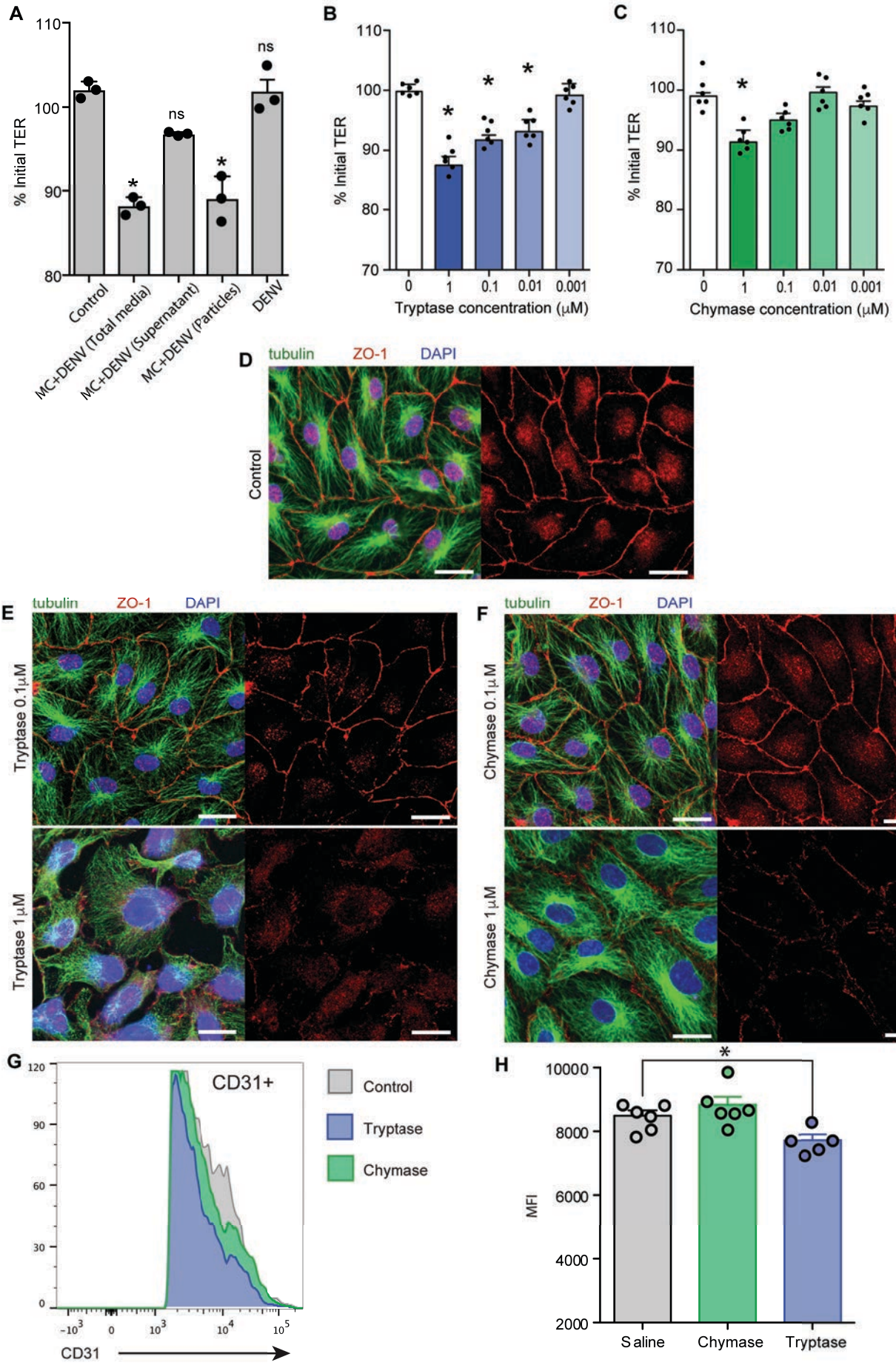
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Figure 1: Tryptase and chymase break tight junctions to disrupt endothelial cell contact sites

(**A**) DENV stimulated MC supernatant was transferred onto huMEC monolayers or separated into soluble and particulate fractions, followed by transfer onto huMEC monolayers. For controls DENV alone or media alone groups were used. TER of huMEC monolayers was measured 24h after treatment. Whole supernatant or isolated MC-particles each significantly reduced the TER of huMEC monolayers ($p<0.05$ by 1-way ANOVA with Dunnett's post-test). (**B-C**) TER of huMEC monolayers after treatment for 24h with either purified, recombinant tryptase (**B**) or chymase (**C**). For **A-C**, * indicates a significant decrease in TER over controls by 1-way ANOVA with Dunnett's post-test ($p<0.05$). (**D-F**) huMECs were treated with low (0.1μM) or high (1μM) concentrations of either tryptase or chymase for 24h, followed by fixation and staining against tubulin, green; nuclei, DAPI and tight junctions (ZO-1, red). (**D**) In control cells tight junctions were intact in between cells, visualized by ZO-1 staining. (**E**) Tryptase induced a concentration-dependent reduction in ZO-1 staining that appeared disjunctive at low concentration and absent at high concentration. Lifting of cells forming gaps was also observed after high concentration tryptase treatment. (**F**) Low concentration chymase had no apparent effect on tight junctions, while staining grew more punctate at high concentration. For A-F, data are representative of 3 independent repeats. For D-F, scale bar=25μm. (**G-H**) Levels of CD31 on endothelial cells were measured by flow cytometry on cells isolated from mouse footpads 6h after injection of 100ng of tryptase, chymase or saline vehicle control. (**G**) $CD31^+$ cells showed reduced levels of its staining after injection of tryptase (representative histogram plots). (**H**) Comparison of mean fluorescence intensity (MFI) of CD31 staining in mouse footpads (n=5-6 each group) showed that tryptase, but not chymase, is sufficient to induce a significant decrease in CD31 staining in vivo (right panel, $p<0.05$ by 1-ANOVA with Dunnett's post-test). For graphs, error bars represent the SEM.

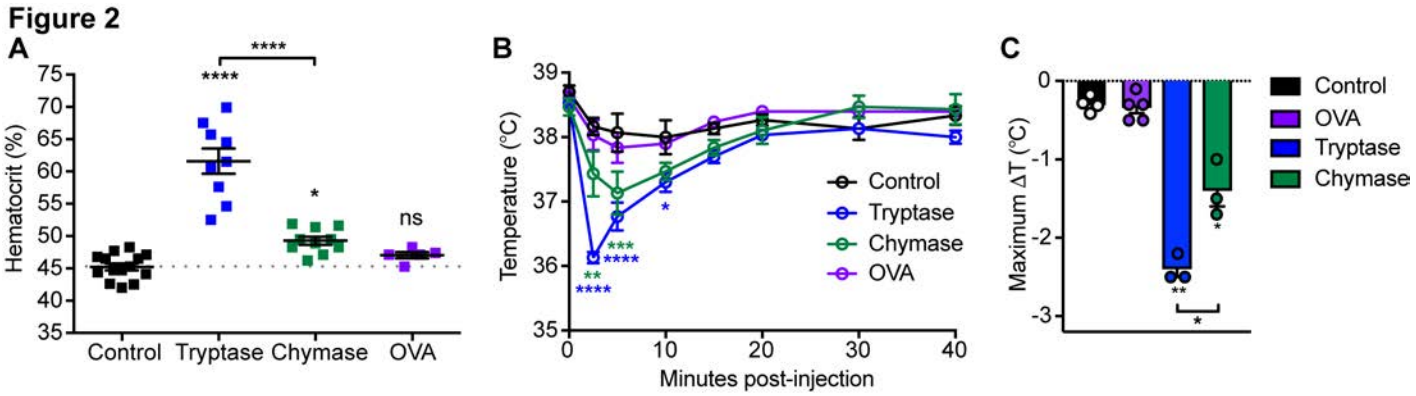

Figure 2: Mast cell proteases promote vascular leakage and shock in vivo

(**A**) Hematocrit values were obtained 6h after injection with saline alone or 30ng of either tryptase, chymase or OVA. Means differ significantly by 1-way ANOVA ($p<0.0001$). Bonferroni's multiple comparison test was used to determine significance amongst groups. For control n=15, for tryptase and chymase n=10 and for OVA n=5. Data were added from two independent experiments. (**B-C**) Mice (n=3-4) were injected with 30ng each of tryptase, chymase, or OVA by the i.v. route or an equivalent volume of saline was injected for controls. To measure shock, the body temperature of animals was recorded at every 5min for the first 15 min and subsequently at 10min intervals. (**B**) Both tryptase and chymase caused sudden drop in body temperature, indicative of shock, compared to both OVA and saline control groups. Data were analyzed by 2-way ANOVA with Holm-Sidak's multiple comparison test to compare temperatures at each time point (**C**) The maximal difference in temperature during the time course is presented, suggesting that tryptase treatment causes significantly higher plasma loss in animals compared to chymase, OVA and saline control groups, determined by 1-way ANOVA with Holm-Sidak's multiple comparison test. For all panels, the mean with error bars representing the SEM are presented and significance is indicated by **** for $p<0.0001$, *** for $p<0.001$, ** for $p<0.01$ and * for $p<0.05$.

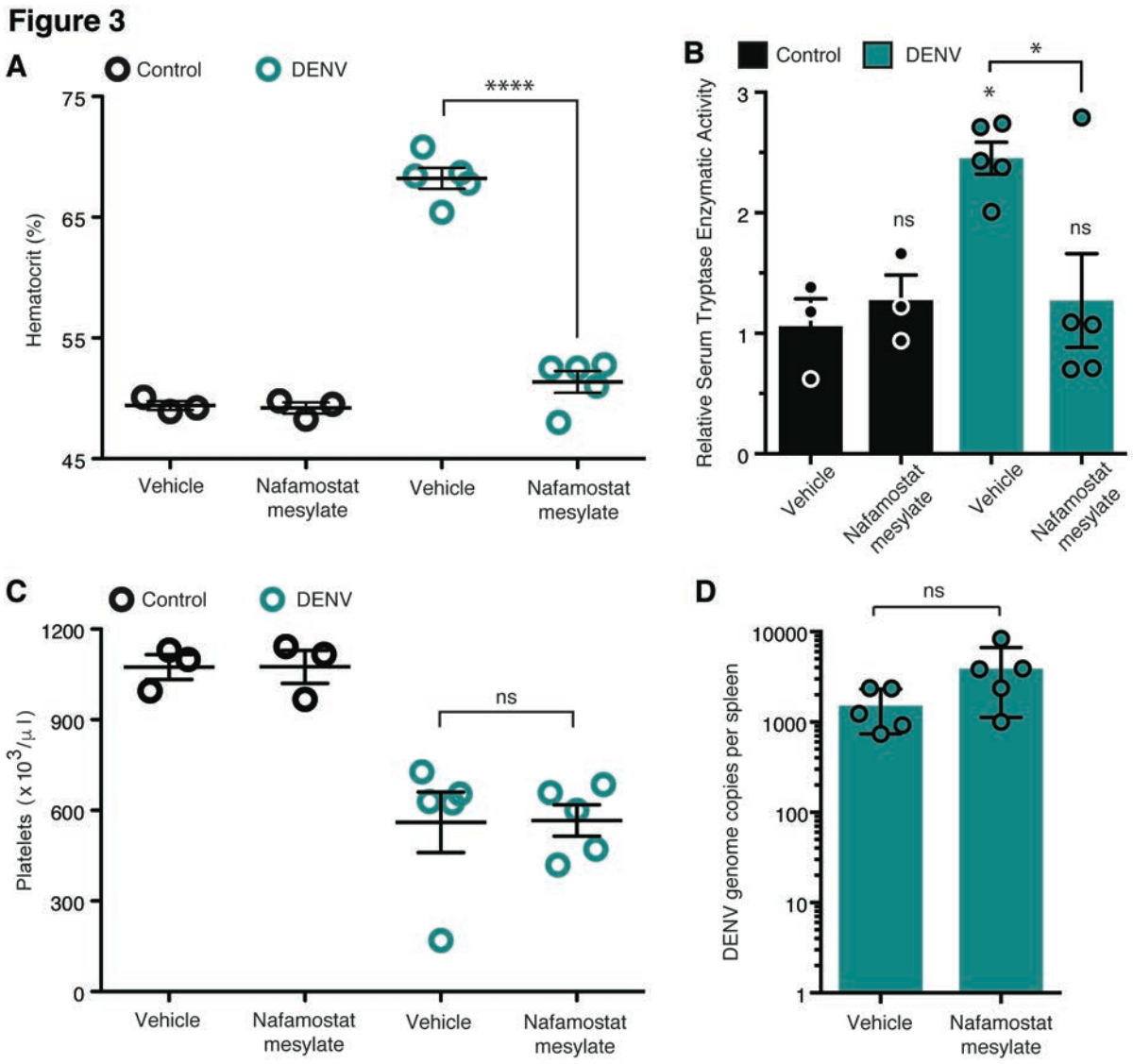

Figure 3: Tryptase inhibition therapeutically blocks vascular leakage during DENV infection

Mice (n=3-5 per group) were either mock-infected or infected with DENV ($1x10^6$ PFU) followed by treatment with vehicle control (saline) or using a specific tryptase inhibitor, nafamostat mesylate at a dose of 0.06mg/kg. (**A**) Hematocrit analysis was performed using an automated hematology analyzer on whole blood at 24h post-treatment. (**B**) Serum was isolated to measure tryptase activity by enzymatic assay. Only DENV-infected mice that were vehicle-treated had elevated tryptase activity over uninfected control group. Nafamostat mesylate treatment reversed tryptase activity to baseline levels. (**C**) Platelet counts at 24h are presented. The data show a strong reduction in DENV-induced vascular leakage upon treatment with tryptase inhibitor, nafamostat mesylate, but no significant (ns) difference in platelet counts compared to DENV-infected and vehicle treated mice. For **A-C** statistical significance was determined using 1-way

ANOVA with Bonferroni's multiple comparison test. (**D**) No difference in the DENV burden in the spleen determined by real time RT-PCR was observed between vehicle and nafamostat mesylate treated animals at 72h post-infection by Student's un-paired t-test. For all panels, the mean with error bars representing the SEM are presented and significance is indicated by **** for $p<0.0001$ and * for $p<0.05$.

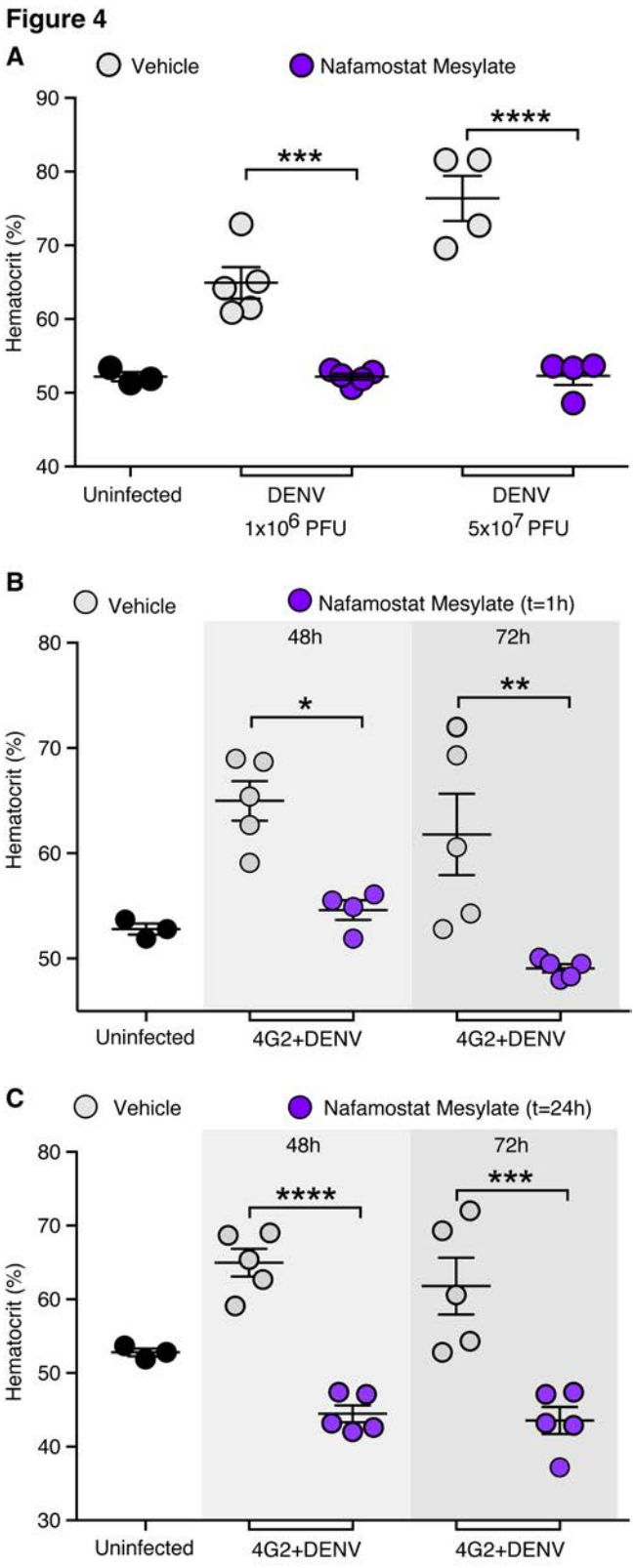

Figure 4. Improvement of vascular leakage in severe DENV infection models

AG129 mice were infected with a low ($1x10^6$ PFU) or a high ($5x10^7$ PFU) dose of DENV and either mock or nafamostat mesylate treated beginning 1h post-infection. Uninfected n=3, DENV infected vehicle treated

n=4-5 and DENV infected nafamostat mesylate treated n=4-5) (**A**) At 24h post-infection, hematocrit was measured. A single treatment of nafamostat mesylate reversed DENV-induced vascular leakage for both infection doses. (**B-C**) Nafamostat mesylate effectively restored hematocrit values to baseline levels at days 2 and 3 post-infection in an antibody-enhanced DENV mouse model. Treatment was initiated (**B**) 1h or delayed (**C**) 24h post-infection and given at 24h intervals thereafter. Statistical significance was calculated using 1-way ANOVA with Bonferroni's multiple comparison test and is indicated by **** for $p<0.0001$, *** for $p<0.001$, ** for $p<0.01$ and * for $p<0.05$. For all graphs, the mean with error bars representing the SEM are presented.

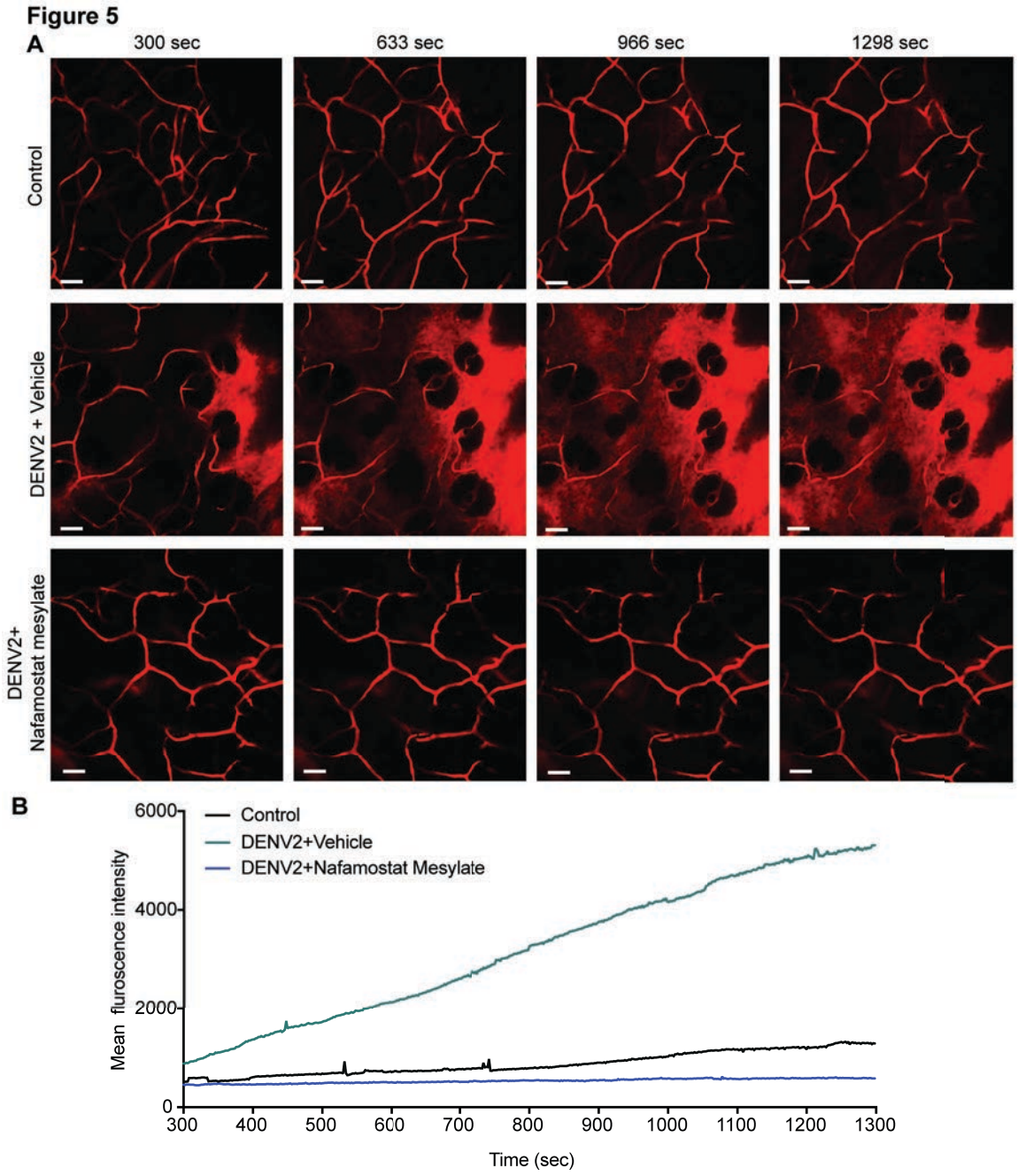

Figure 5. Visualization of inhibition of plasma leakage in DENV-infected mice

AG129 mice were infected with $1x10^6$ PFU of DENV by i.p. and either treated with nafamostat mesylate or with vehicle. Mice (n=3), 24h post-infection, were injected with 70KDa FITC-Dextran. Two-photon images were acquired continuously at 2 sec intervals, beginning 5 min post-injection, for 18 min total. (**A**) Representative images from the indicated time points post-FITC-dextran injection from Supplemental Videos 1-3, showing vascular leakage in the DENV-infected mock-treated ear, while control mice and

DENV-infected mice treated with nafamostat mesylate showed no visually discernible vascular leakage. Scale bar=50μm (**B**) The mean fluorescence intensity in the acquired images over time is presented. Intensity was measured by averaging 10 areas in the interstitial space. Data are representative of 3 independent experiments.

Figure 6

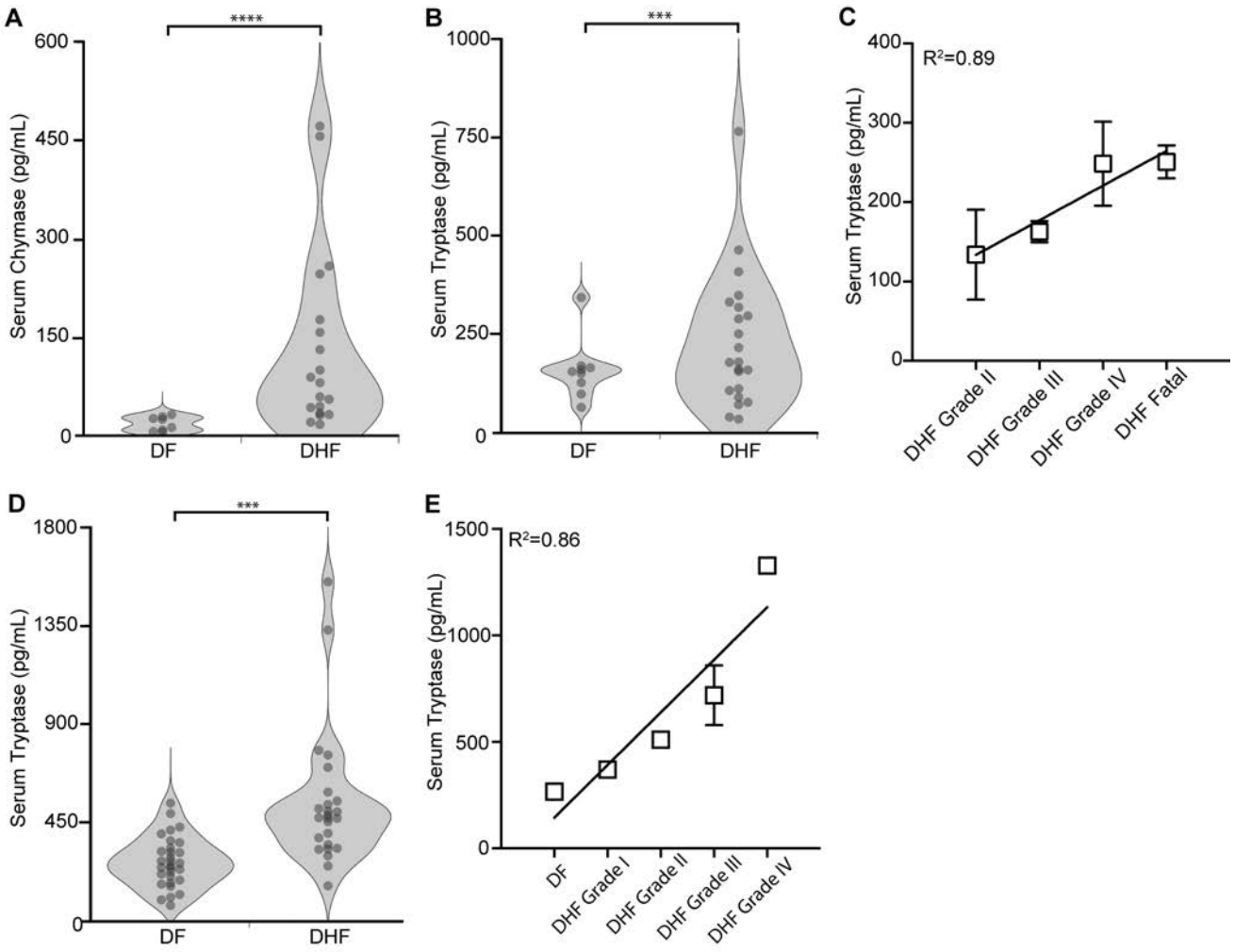

Figure 6. Serum tryptase levels are correlated with DHF/DSS in humans

Serum samples from virologically confirmed hospitalized dengue patients in Jakarta, Indonesia in 1975-78 (n=9 DF and n=25 DHF), were tested retrospectively in a blinded manner for (**A**) chymase and (**B**) tryptase. Both were significantly increased ($p<0.0001$ and $p=0.0004$, respectively, determined by Student's un-paired t-test) in patients with DHF compared to DF. (**C**) Mean serum tryptase levels for each grade of DHF were also strongly correlated with the grade of DHF based on the patient's reported symptoms; $p=0.05$; $R^2=0.89$. (**D-E**) Tryptase was measured in a second cohort of prospectively obtained patient samples from virologically confirmed dengue patients (n=30 DF and n=25 DHF) in Sri Lanka, Colombo in 2012-2013. (**D**) Serum tryptase levels were significantly elevated in serum samples from DHF vs DF patients ($p=0.0005$). (**E**) Mean serum tryptase levels in Sri Lankan samples were strongly correlated with disease severity; $p=0.02$; $R^2=0.86$. For all graphs, the mean with error bars representing the SEM are presented.