

Role of dengue NS1 antigen in the pathogenesis of acute dengue infection

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Summary

Both dengue NS1 antigen and serum IL-10 levels have been shown to associate with severe clinical disease in acute dengue infection and IL-10 has also been shown to suppress dengue specific T cell responses. Therefore, we proceeded to investigate the mechanisms by which dengue NS1 contributes to disease pathogenesis and if it is associated with altered IL-10 production.

Serum IL-10 and dengue NS1 antigen levels were assessed serially in 36 adult Sri Lankan individuals with acute dengue infection. We found the serum IL-10 levels positively correlated with dengue NS1 antigen levels (Spearman's $R=0.47$, $p<0.0001$), and NS1 also correlated with annexin V expression by T cells in acute dengue (Spearman's $R=0.63$, $p=0.001$). However, NS1 levels did not associate with the functionality of T cell responses or with expression of co-stimulatory molecules. Therefore, we further assessed the effect of dengue NS1 on monocytes and T cells by co-culturing primary monocytes and peripheral blood mononuclear cells (PBMC), with varying concentrations of NS1 up to 96 hours. Apoptosis of T cells was determined by annexin V expression. Monocytes co-cultured with NS1 produced high levels of IL-10 with the highest levels seen at 24 hours, and then gradually declined. Recombinant NS1 also induced annexin V expression by both CD4⁺ and CD8⁺ T cells in a dose dependent manner although a wide individual variation was seen.

Therefore, our data show that dengue NS1 appears to contribute to pathogenesis of dengue infection by inducing IL-10 production by monocytes and possibly inducing apoptosis of T cells.

Introduction

Dengue viral infections are one of the most rapidly emerging mosquito borne viral infections in the world, resulting in a huge economic burden in affected countries (1). It is estimated that 390 million individuals are infected by the virus each year, resulting in 96 million apparent infections (2). Approximately, 70% of these infections occur in Asian countries, which have inadequate resources to handle such vast patient numbers (2, 3). Currently there is no licensed vaccine to prevent dengue, nor an effective specific drug for its treatment.

Infection with the dengue virus (DENV) results in asymptomatic infection or causes an undifferentiated febrile illness in the majority of infected individuals. However, some individuals develop dengue fever or severe clinical disease which manifests as dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) (4). The hallmark of severe clinical disease is vascular leak, which results in reduced blood pressure, pleural effusions, ascites and if severe, impaired organ perfusion and shock (5). Although the exact mechanisms that lead to severe clinical disease are not clear, it appears to occur due to the complex interaction between the DENV and the host immune response (5, 6). Our previous studies showed that serum IL-10 levels were significantly elevated in patients with severe clinical disease and IL-10 suppressed DENV-specific T cell responses, possibly contributing to disease pathogenesis (7, 8). It has also been shown that dengue NS1 protein was associated with severe clinical disease and that a level of >600ng/ml in the first 72 hours of illness was associated with development of DHF (9). We too found that dengue NS1 antigen persisted in patients with DHF and that it could be possibly used as a marker of severe dengue (10).

Dengue NS1 is a 50kDa nonstructural protein, which is a lipoprotein that also exists in the secreted form (11, 12). Since NS1 carries lipids in its secretory form it is thought to have many implications in disease pathogenesis as lipoproteins are important in coagulation pathways and are associated with vascular inflammation (11). Indeed it was recently shown that NS1 of all four DENV serotypes, induced vascular leak in dengue mice models by inducing endothelial barrier dysfunction(13). It was also shown that dengue NS1 stimulated cytokine production from innate immune cells by acting through the TLR4 (14). The immune complexes formed by secretory NS1 have been shown to activate complement, which is also thought to contribute to disease pathogenesis (15). Therefore, although the main effects of NS1 in the pathogenesis of dengue infections are believed to occur due to immune complexes and anti-NS1 antibodies (16-19), more recent studies have shown that NS1 is pathogenic by itself (13, 14). However, a recent study of 13 autopsy cases showed that neither dengue NS1 nor dengue NS1 antibodies were detected in the endothelium of patients who died due to dengue infection, questioning the hypothesis of the role of NS1 antibodies in the pathogenesis of acute dengue (20).

Since dengue NS1 circulates in patients with acute dengue in the secretory form and binds to both infected and non-infected cells, and also from the data originating from the dengue mice models (13), it is likely that NS1 could have direct effects on the immune system apart from binding to anti-NS1 antibodies. As studies have shown that higher NS1 levels are found in patients with more severe forms of clinical disease (9), in this study we proceeded to investigate the potential effects of NS1 on monocytes and also on the functionality of the T cell responses and T cell apoptosis. We found that in acute infection, NS1 levels correlated with serum IL-10 levels and that dengue NS1 induces production of IL-10 from primary monocytes

in a dose dependent manner. In addition, we found that dengue NS1 also correlated with annexin V expression of T cells in acute dengue infection and that NS1 resulted in annexin V expression in both CD4⁺ and CD8⁺ T cells in a dose dependent manner. Therefore, dengue NS1 appears to contribute to the pathogenesis of dengue infection in possibly by causing T cell apoptosis and inducing production of immunosuppressive cytokines.

Materials and Methods

Patients

As our initial aim was to determine if dengue NS1 was associated with severe clinical disease, we first determined the kinetics of dengue NS1 levels and serum IL-10 levels in 36 adult individuals with acute dengue infection, from the Colombo South Teaching Hospital, Sri Lanka following informed written consent.

In order to determine changes in the kinetics of dengue NS1 antigen levels and serum IL-10 levels, serial blood samples were taken in the morning (6 a.m.) and again at 1.00p.m., from the time of admission to the time of discharge from hospital. The duration of illness at the time of admission was based on the number of days the patient was febrile before admission to hospital. For instance, if the patient had fever for 4 days prior to admission, the patients' duration of illness was considered as 96 hours. All clinical features such as presence of fever, abdominal pain, vomiting, bleeding manifestations, hepatomegaly, blood pressure, pulse pressure and evidence of fluid leakage were recorded several times each day. The severity of dengue infection was classified according to the 2011 WHO dengue diagnostic criteria (4). Accordingly, patients with a rise in haematocrit $\geq 20\%$ of the baseline haematocrit or clinical or ultrasound scan evidence of plasma leakage in a patient was classified as having DHF(4). Shock was defined as having cold clammy skin, along with a narrowing of pulse pressure of ≤ 20 mmHg. According to this definition 25 had DHF and 11 DF.

After determining the kinetics of dengue NS1 antigen levels and serum IL-10 levels, in order to investigate the functionality of T cell responses we recruited an additional 24 adult individuals

with acute dengue infection. The first blood samples were collected during day 3–5 of illness (day 1 was considered as the first day of fever) in all patients and the second samples were collected 2 days later. Blood was also collected from 13 healthy dengue seropositive volunteers. In order to determine the severity of dengue infection, serial recordings of clinical features and laboratory investigations (platelet counts, haematocrits, white cell counts) were made in this cohort as well. Clinical disease severity was classified according to the WHO 2011 dengue guidelines(4). Of these 24 patients, 18 patients had DHF based on the 2011 WHO criteria and 6 patients had DF.

Ethics statement

Ethics approval was obtained by the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayawardenapura. All patients were recruited following informed written consent.

Confirmation of dengue infection and determining dengue NS1 antigen levels

Acute dengue infection was confirmed in the serum samples using the NS1 early dengue ELISA (Panbio, Australia) or with the commercial capture-IgM and IgG enzyme-linked immunosorbent assay (ELISA) (Panbio, Brisbane, Australia). The ELISA was performed and the results were interpreted according to the manufacturers' instructions. This ELISA assay has been validated as both sensitive and specific for primary and secondary dengue virus infections (21, 22). In order to determine the kinetics of dengue NS1 antigen levels, the NS1 antigen levels were determined in serial blood samples and the levels expressed as Panbio units. Those

with clinical features of a suggestive dengue infection who were either positive for dengue NS1 or were positive for dengue IgM antibody were considered to be having an acute dengue infection.

Quantification of cytokines

IL-10 quantitative cytokine assays (Mabtech, Sweden) were done in duplicate on serum samples of patients with acute infection, healthy volunteers and also in serum samples obtained during the convalescent phase according to manufacturer's instructions. It was also done in cultured monocyte supernatants stimulated with different concentrations of DENV1- NS1 recombinant full length protein. All reactions were carried out in duplicate.

Determining the effect of dengue NS1 antigen on monocytes

Monocytes were isolated from fresh PBMCs in four dengue seronegative donors, by using MACS columns with anti-human CD14 microbeads (Miltenyi Biotec, Auburn, CA), followed by magnetic separation. The purity of positively selected monocytes was again assessed by flowcytometry using CD14FITC (Biolegend, USA) and CD3 APC (Biolegend, USA). The purity was found to be >98%. 2×10^6 /ml monocytes were co-cultured with varying concentrations of dengue NS1 full length recombinant protein which was certified as free from Lipopolysaccharide (Abcam, UK) for up to 96 hours. The monocytes were cultured in RPMI 1640 with 5% autologous serum and then dispensed in a volume of 200 μ L per well into individual wells of 96-well round-bottom plates (Corning, Acton, MA). Recombinant DENV1- NS1 full length protein (Abcam, UK) was used at concentrations of 250ng/ml, 500ng/ml and 1000ng/ml. A well containing only media was added as the control for each time

point. Cells were centrifuged and the supernatants harvested at 24, 48, 72 and 96 hours to determine IL-10 levels the supernatant.

Although we used a NS1 full length recombinant protein which was certified as free from Lipopolysaccharide, from DENV1, we repeated the above experiments using a NS1 full length recombinant protein from DENV3 expressed from a mammalian cell line (Abcam, UK) in order to compare the results obtained with DENV-1 NS1 protein on monocytes. Since possible bacterial contaminants in NS1 full length recombinant protein derived from E.coli could stimulate IL-10 production from monocyte, we used a mock protein (PRF full length protein) generated by the same method, which is of equal molecular weight from the same manufacturer as the E.coli and mammalian derived dengue NS1 protein (Abcam, UK).

Determining the effects of dengue immune sera on NS1 antigen

Monocytes were isolated from fresh PBMCs in four dengue seronegative donors, by using MACS columns with anti-human CD14 microbeads (Miltenyi Biotec, Auburn, CA), followed by magnetic separation. As both E.coli derived NS1 full length protein from DENV1 and mammalian derived NS1 from DEN3 showed comparable results, the E.coli derived DENV1 NS1 was used in these experiments. E-coli derived DENV1 NS1 (Abcam, UK), was used at a final concentration of 250 ng/ml per well.

The DENV immune serum pool for all four DENV serotypes was prepared for experiments for determining if antibodies causes enhancement (ADE) of the effects of NS1 on monocytes. The ADE experiments were done as previously described (23). In order to determine the effects of

DENV specific antibodies serum from healthy volunteers with known serotype of past dengue infection was collected and pooling together (23-25). Pooled serum was heat inactivated before used in ADE experiments. The sera were incubated in dilutions of 1:5, 1:10 and 1:100 in RPMI media (Gibco, Life technologies, USA) containing DENV1 NS1 at 37⁰ C for 1 hour prior to incubation with the purified monocytes.. A well containing only media, only serum and only NS1 at the same concentration (250ng/ml) was used as the controls for each time point. Cells were centrifuged and the supernatants harvested at 24, 48, 72 and 96 hours to determine IL-10 levels the supernatant.

Determining Annexin V expression levels in T cells in acute dengue infection

In order to investigate if Annexin V expression in T cells in patients with acute dengue was associated with dengue NS1 antigen, Annexin V expression was determined in 22 adult patients with confirmed acute dengue infection. PBMCs were stained with Annexin V FITC, IL-10R PE, 7-AAD, CD3 APC and acquired on a Partec Cyflow Cube 6 and analyzed with De novo FCS Express version 4.

Determining effect of dengue NS1 antigen on Annexin V expression on T cells

These experiments were carried out in 10 healthy dengue seropositive individuals. PBMCs were incubated for 72 hours with DENV1- NS1 glycoprotein full length protein (Abcam) at 37°C in 5% CO₂. Dengue NS1 antigen was used at concentrations ranging from 250ng/ml to 1000ng/ml. Annexin V expression was determined at 24, 48 and 72 hours. Every 24 hours cells were washed and stained for surface staining using Annexin V FITC (Biolegend, USA), CD8

PE (Biolegend, USA), CD4 PerCP (Biolegend, USA) and CD3 APC (Biolegend, USA). Cells were acquired on a Partec Cyflow Cube 6 and analyzed with De novo FCS Express version 4.

Peptides

The DENV- NS3 peptides were 20 mer peptides overlapping by 10 amino acids, which spanned the whole length of the DENV-3 NS3 protein. These were 20mer peptides which were synthesized in house in an automated synthesizer using F-MOC chemistry. The purity of the peptides was determined to be greater than 90% by high-pressure liquid chromatography analysis and mass spectrometry. The synthetic NS3 20mer peptides were pooled together to represent the whole NS3 protein. The FEC peptides (synthesized in house) that were used contained a panel of 23, 8–11 amino acid CD8+ T cell epitopes of Epstein Barr virus (EBV), Flu and CMV viruses and have been used as quality control in ELISpot assays.

Surface staining and Intracellular cytokine assays

In order to determine IFN γ production by T cells, PBMCs were isolated from whole blood and these *ex vivo*- PBMCs were stimulated at 1×10^6 to 2×10^6 /ml in RPMI 1640 plus 10% FCS with DENV-NS3 overlapping peptides and PMA and ionomycin for 16 hours according to manufacturer's instructions in the presence of Brefeldin A (Biolegend, USA). To determine CD107a expression, staining for CD107a was carried out prior to adding peptides. Prior to permeabilization of cells, a surface stain was carried out to determine the expression of CTLA-4 APC (Biolegend, USA), TIM-3 APC (Biolegend, USA), PD-1 FITC (Biolegend, USA), and CD28 (FITC). For intracellular staining, cells were then permeabilized and fixed with

Cytofix/Cytoperm (Biolegend, USA) and then stained with IFN γ APC. Propidium Iodide (PI) was used in the CD107a detection assays to gate out dead cells. Isotype-matched controls were included in each experiment. Cells were acquired on a Partec Cyflow Cube 6 and analyzed with De novo FCS Express version 4.

Statistical analysis

Statistical analysis was performed using Graph pad PRISM version 6. As the data were not normally distributed, differences in means were compared using the Mann-Whitney U test (two tailed). Degree of association between serum NS1 antigen levels and serum IL-10 levels were analysed using Spearman's correlation. Power calculations to determine if NS1 antigen levels were higher in patients with DHF when compared to DF, were calculated by the mean and standard deviation of the population being assessed. The means and standard deviations of NS1 antigen levels (Panbio units) for this study were derived from the values obtained from a previous large study of 186 patients (10).

Results

Dengue NS1 antigen has previously been shown to associate with DHF and indeed we found that it could also be considered as a marker of severe clinical disease (10). In addition, our previous studies showed that serum IL-10 levels were also associated with severe dengue (7, 8). Therefore, we proceeded to investigate the kinetics of both dengue NS1 antigen and serum IL-10 levels in 36 patients with acute dengue infection throughout the course of their illness. We found that although dengue NS1 antigen levels were not different between those with DHF (n=25) and those with DF (n=11) during early infection, dengue NS1 levels remained high in those with DHF, (Fig 1). By 144 hours of illness, 11/25(44%) of the patients with DHF and 3/11 (27.2%) patients with DF were positive for dengue NS1. Of these 36 patients, 6/25 patients with DHF had a primary dengue infection and 3/11 patients with DF had a primary dengue infection. All of the 3 patients with DF who were still positive for dengue NS1 at 144 hours had a primary dengue infection, while 6/11 of the patients with DHF who were positive for dengue NS1 at 144 hours had a primary dengue infection.

Relationship between dengue NS1 antigen and serum IL-10 levels

We then proceeded to determine the relationship between dengue NS1 antigen levels and serum IL-10 levels in this cohort of patients throughout the course of the illness. In measuring dengue NS1 antigen levels, we used the NS1 early dengue ELISA (Panbio, Australia) and the NS1 antigen levels were expressed as Panbio units. Serum IL-10 levels significantly ($p<0.0001$) correlated with dengue NS1 antigen levels (Spearman's $R=0.47$). Furthermore, the

patterns of variation in serum IL-10 levels and dengue NS1 antigen levels were very similar in patients with DHF and DF. For instance, the IL-10 levels decreased at 132-144 hours, closely following the patterns of dengue NS1 in patients with DF (Fig 2A). However, in patients with DHF, similar to the observations with dengue NS1, serum IL-10 levels persisted for longer periods (Fig 2B).

Dengue NS1 antigen stimulated IL-10 production by monocytes

Our previous studies showed that monocytes were an important source of IL-10 in acute dengue infection (8). Since we found that serum IL-10 concentrations correlated well with dengue NS1 levels, we investigated if NS1 stimulated IL-10 production by monocytes. Freshly isolated monocytes from four dengue seronegative individuals were co-cultured with varying concentrations of dengue NS1, which were comparable to concentrations reported in vivo in patients in previous studies (9). We found that monocytes co-cultured with dengue NS1 antigen (E. coli derived, certified as LPS free), produced high levels of IL-10 at 24 hours which gradually decreased by 96 hours (Fig 3A). No IL-10 production was seen in monocytes cultured in media alone. Although production of IL-10 was higher when monocytes were cultured with 1000ng/ml of dengue NS1 when compared to lower concentrations, this was not significant. However, although the E.coli derived NS1 full length recombinant protein was certified as LPS free, as other bacterial contaminants could induce IL-10 production from monocytes, we used a mock protein (PRF full length protein), which was purchased from the same manufacturer, and was produced in the same manner and was of similar molecular weight. We found that primary monocytes of 4 individuals, did not produce any IL-10 in the

presence of PRF 250 ng/ml, while monocyte that we co-cultured with dengue NS1 at a similar concentration (250ng.ml), produced IL-10.

Although the E. coli expressed recombinant NS1 full length protein from DENV1 was certified as LPS free, in order to further exclude possible effects of E.coli contamination, we repeated these experiments in 4 individuals using full length NS1 recombinant protein from DENV3 which was expressed in a mammalian cell line. We found mammalian expressed DENV3 NS1 protein also induced IL-10 from monocytes (Fig 3B).

DENV specific antibodies and their effect on NS1 on monocytes

DENV specific antibodies have been shown to enhance disease severity which is thought to be mediated by antibody dependent enhancement (ADE) (23, 26, 27). NS1 specific antibodies are believed to contribute to disease pathogenesis by activating complement and also leading to endothelial dysfunction (15, 17). Therefore, in order to investigate if DENV NS1 specific antibodies could potentiate the effects of NS1, we used DENV immune sera in ADE experiments as previously described. We found that there was a trend towards DENV immune sera potentiating the effects of NS1 on monocytes and the production of IL-10 was higher especially at 48 hours (Fig 4).

Dengue NS1 and annexin V expression by T cells

Massive apoptosis of T cells is known to occur in acute dengue infection (28-30). Our previous studies have shown that serum IL-10 levels were associated with annexin V expression on T cells (29). However, subsequent studies showed that IL-10 did not cause T cell apoptosis of T

cells in culture. Since in this study we found that serum IL-10 correlates with dengue NS1, we investigated if dengue NS1 caused apoptosis of T cells. Initially, we determined annexin V expression and dengue NS1 levels in 22 patients with acute dengue infection. We found that dengue NS1 antigen levels (Panbio units) positively correlated with annexin V expression on T cells (Spearman's $R = 0.63$, $p = 0.001$). Since annexin V expression on T cells in patients with acute dengue correlated with dengue NS1 antigen levels, we then investigated if NS1 caused apoptosis of T cells. PBMCs of 10 healthy individuals were co-cultured with varying concentrations of dengue NS1 for 24 to 72 hours and annexin V expression was assessed. Annexin V expression was up-regulated in both CD4⁺ and CD8⁺ T cells in a dose dependent manner (Fig 5). However, the increase in annexin V expressing CD8⁺ and CD4⁺ T cells varied widely between individuals. Although not significant, the mean annexin V expression in CD8⁺ T cells at NS1 antigen concentrations of 250ng/ml (mean 18.09, $SD \pm 8.6$ percentage of expression) was higher than CD8⁺ T cells cultured with media alone (mean 13.6, $SD \pm 3.8$ percentage of expression). Annexin V expression was even higher in CD8⁺ T cells at NS1 antigen at 500ng/ml concentrations (mean 22.9, $SD \pm 13.1$ percentage of expression) (Fig 5).

Dengue NS1 and functionality of the T cell responses in acute dengue

Since dengue NS1 appears to cause apoptosis of T cells, we went on to determine if dengue NS1 had any other effects on the functionality of T cells. In addition, since the functionality of T cell responses and the expression of co-stimulatory markers have not been studied in detail before, we also investigated the expression of PD-1, CTLA-4, CD28 and also TIM-3 in 24 patients with acute dengue and 13 healthy individuals. In order to assess the functionality of T cell responses intracellular cytokine assays were performed for IFN γ and CD107a expression

following stimulation by dengue NS3 overlapping peptides. We found that dengue NS1 antigen levels (Panbio units) had no correlation with the frequency of NS3 specific IFN γ producing T cells, or NS3 specific CD107a expressing T cells. Dengue NS1 antigen levels also did not show any correlation with expression of PD-1, CTLA-4, TIM-3 or CD28 levels.

Although PD-1 is known to be associated with T cell dysfunction and is thought to be a negative regulator of T cell function, its expression has shown to be up regulated in acute viral infections, driven by responses to viral antigens (31-33). We found that, although not significant ($p=0.12$), patients with acute dengue had a higher percentage of PD-1 expressing T cells (mean 65.2, SD \pm 17.6 MFI), when compared to healthy individuals (mean 57.6, SD \pm 10.2 MFI) (Fig 6A). There was no difference in expression of PD-1 in the general T cell population (mean 65.2, SD \pm 17.6 median fluorescent intensity) when compared to DENV-NS3 specific T cells (mean 65.3, SD \pm 17.3 median fluorescent intensity). There was no difference in the expression of PD-1 in the first sample taken at day 3-5 of illness and the second sample taken at day 6-8 of illness. There was again no difference in the expression of CTLA-4 or TIM-3 in patients with acute dengue infection and healthy individuals. The expression levels were not different between the first blood sample and the second blood sample.

CD28 is essential for antiviral responses (34). We found that in patients with acute dengue, CD28 expression by DENV-NS3 specific T cells positively correlated with CD107a expressing DENV-NS3 specific T cells (Spearman's $R=0.59$, $p=0.007$) (Fig 6B). However, no such correlation was observed between CD28 expression by DENV-NS3 specific T cells and IFN γ production. Although, CD28 has been shown to be required for development of early antiviral

responses, we found that CD28 expression was significantly less ($p=0.01$) in T cells of patients with acute dengue (mean 56.8, $SD\pm 19.04$ percentage of CD3+ T cells) when compared to healthy individuals (mean 73.48, $SD\pm 10.94$ percentage of CD3+ T cells) (Fig 6C).

Discussion

In this study our initial observation that serum IL-10 levels correlated with dengue NS1 antigen levels, led to the experiments that showed that dengue NS1 induced IL-10 production by monocytes and was associated with apoptosis of T cells. It has been shown previously that a dengue NS1 antigen level of over 600ng/ml in the first 72 hours was associated with development of DHF (9). In addition, patients with DHF have been shown to have higher viral loads and a delay in clearance of the virus (35, 36). Although secretory NS1 circulates independent of the virus, it has been shown that dengue NS1 antigen levels do correlate with viral loads in acute infection (37). Therefore, persistent NS1 antigenaemia could be a reflection of delayed clearance of the virus. We too found that dengue NS1 antigen positivity at day 5 to 6 of the illness was associated with development of DHF and shock (10). However, since NS1 circulates independent of the virus, and has shown to be pathogenic by itself (13), we proceeded to investigate the changes in NS1 levels and clinical disease severity. In this study, as NS1 antigen levels were measured serially in patients with acute dengue infection, we found that by 144 hours (day 6 of illness) 11/25 patients with DHF and 3/11 patients were positive. However, all the 3 patients with DF and 6/11 patients with DHF who were positive had a primary dengue infection. This is consistent with the finding that dengue NS1 persists for a longer time in primary dengue infection (38).

Our previous studies and studies done by others have shown that serum IL-10 was associated with severe disease (7, 39, 40). In addition, we previously reported that IL-10 inhibits DENV-specific T cell responses (8). Since dengue NS1 was also shown to associate with severity, we

determined the association of serum IL-10 and dengue NS1 antigen levels (Panbio units) in serial blood samples collected throughout the course of the illness. We found that serum IL-10 levels significantly correlated with dengue NS1 antigen levels. In our subsequent experiments we also found that dengue NS1 stimulates production of IL-10. The highest levels of IL-10 were observed when monocytes were co-cultured with NS1 of 1000ng/ml. Therefore, these experiments show that dengue NS1 may contribute to disease pathogenesis by stimulating IL-10 production by monocytes. Although we carried out our experiments using both recombinant dengue NS1 derived from E.coli and also mammalian expressed protein, since other bacterial contaminants may stimulate IL-10 production, we also used a mock protein generated by E.coli which was of similar molecular weight. No IL-10 was detected in monocytes, co-cultured with this mock protein. It was recently shown that NS1 is recognized via TLR4 (14). Although this group did not assess IL-10 production, they have shown that dengue NS1 acts through TLR-4 and induced transcription of many proinflammatory cytokine genes (14). It has been shown that lipopolysaccharide induces production of IL-10 from macrophages through TLR-4 activation (41). Since NS1 was shown to have LPS like activity and act through TLR-4, it is likely that production of IL-10 by monocytes could also occur due to similar mechanisms. Therefore, it would be crucial to determine if the virulence of the DENVs are also dependent on the LPS like activity of NS1

Although dengue NS1 was previously thought to contribute to disease pathogenesis by forming immune complexes with anti-NS1 antibody and thus leading to complement activation (15, 18), other effects of NS1 such as stimulation of immunosuppressive cytokine production have not been reported until recently. However, apart from anti-NS1 antibody immune complexes

resulting in complement activation, there is a possibility of NS1 specific antibodies contributing to disease pathogenesis by increased IL-10 production as suggested in our experiments. However, although we observed higher levels of IL-10 production when NS1 was incubated with varying concentrations of DENV-specific antibodies, it is possible that other components in human serum apart from DENV antibodies could potentiate the effects of NS1. However, we did not observe any IL-10 production by monocytes co-cultured with non-dengue immune serum alone, suggesting that serum alone did not induce IL-10 production.

Due to the association of dengue NS1 levels with serum IL-10 levels, we carried out further experiments to determine possible other roles of NS1 in acute dengue. Massive apoptosis of T cells is known to occur in acute dengue infection and many genes associated with apoptosis are up-regulated (28, 29, 42). Although serum IL-10 was associated with apoptosis of T cells in acute illness, *in vitro* experiments showed that IL-10 did not induce apoptosis in T cells in healthy individuals or in patients (29). In this study we found that dengue NS1 also correlated with annexin V expression by T cells and our *in vitro* studies on PBMCs of healthy individuals showed that dengue NS1 did result in increased expression of annexin V in both CD4+ and CD8+ T cells in a dose dependent manner. Therefore, these experiments suggest that NS1 could be contributing to disease pathogenesis by causing apoptosis of T cells. However, the increase in annexin V expression in CD4+ and CD8+ T cells in PBMCs co-cultured with dengue NS1 varied markedly between individuals. Therefore, it would now be important to further investigate the mechanisms by which NS1 causes T cell apoptosis and the reasons for marked individual variations. Since the majority of dengue infections result in asymptomatic

infection, it would be interesting to investigate whether the susceptibility of T cells to apoptosis by dengue NS1 was associated with clinical disease severity and higher viremia.

Since NS1 was associated with T cell apoptosis, we further proceeded to investigate if it had any effects on the function of DENV specific T cells or on expression of co-stimulatory molecules. We found that dengue NS1 was not associated with the functionality of the DENV-specific T cell response and did not associate with expression of co-stimulatory molecules such as CTLA-4, CD28 or PD-1. However, although not significant, an increased expression of PD-1 was observed in patients with acute dengue when compared to healthy individuals. Although PD-1 is a negative regulator of T cell responses and is associated with T cell dysfunction, it is up-regulated in activated T cells (33, 43, 44). Therefore, increased expression of PD-1 in patients with acute dengue is most likely to be due to viral antigen induced activation.

CD28 is potent inducer of antiviral T cell responses (34), and we too found that CD28 expression by DENV-NS3 specific T cells significantly correlated with T cell degranulation. However, we also found that CD28 expression was significantly less in T cells in patients with acute dengue infection. It has been shown that during T cell differentiation and proliferation, T cells reduce expression of CD28 and CD27 in viral infections. In H1N1 infection too it was shown that CD8⁺ T cells expressing CD28 were low during acute illness (45, 46). Therefore, it would be important to determine if lower expression of CD28 in T cells in patients with acute dengue infection, was due to proliferation and differentiation of DENV- specific T cells in acute dengue

In summary, we found that dengue NS1 antigen levels correlated with serum IL-10 levels and were higher in patients with DHF. Dengue NS1 appears to contribute to disease pathogenesis by inducing IL-10 production by monocytes and also possibly by inducing apoptosis of T cells. Therefore, it would be crucial to further investigate the role of NS1 in the pathogenesis of dengue by determining mechanisms by which NS1 induces IL-10 production by monocytes and also the mechanisms by which it possibly induces apoptosis of T cells. Since persistence of dengue NS1 was associated with severe clinical disease and also with primary dengue infection, NS1 targeted treatments could be useful in reducing disease pathogenesis.

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TNA, LG, AK, MS and NW were involved in carrying out experiments. TNA was involved in writing the manuscript and analyzing the data.

NLAS and NW were involved in patient recruitment and obtaining data from patients.

GSO and GNM were involved in the conceptual design, writing the manuscript, analyzing the data and funding.

Conflicts of interest

None

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Figure Legends

Fig 1: Kinetics of dengue NS1 levels in acute dengue: Levels of serum dengue NS1 antigen were measured twice a day by ELISA in 25 patients with DHF and 11 patients with DF from the time of admission until discharge. Error bars indicate the standard error of the mean. (---) indicates the titre above in which the NS1 ELISA gives a positive result.

Fig 2: Relationship between dengue NS1 and serum IL-10 levels

2A: Dengue NS1 antigen levels and serum IL-10 levels were measured by ELISA in 25 patients with DHF from the time of admission to discharge. Error bars indicate the standard error of the mean. The dengue NS1 antigens levels are expressed in PanBio ELISA units and IL-10 levels are expressed as pg/ml.

2B: Dengue NS1 antigen levels and serum IL-10 levels were measured by ELISA in 11 patients with DF from the time of admission to discharge. Error bars indicate the standard error of the mean. The dengue NS1 antigens levels are expressed in PanBio ELISA units and IL-10 levels are expressed as pg/ml.

Fig 3: Production of IL-10 by monocytes co-cultured with varying concentrations of dengue NS1.

3A: Monocytes isolated from 4 dengue seronegative individuals were incubated with media or E.coli expressed (certified as LPS free) dengue NS1 protein of DENV serotype 1 at concentrations of 250ng/ml, 500ng/ml or 1000ng/ml for 96 hours. All experiments were done in duplicate. The IL-10 levels in the supernatants were measured every 24 hours by ELISA.

3B: Monocytes isolated from 4 dengue seronegative individuals were incubated with media or mammalian expressed dengue NS1 protein of DENV serotype 3 at concentrations of 250ng/ml, 500ng/ml or 1000ng/ml for 96 hours. All experiments were done in duplicate. The IL-10 levels in the supernatants were measured every 24 hours by ELISA.

Fig 4: Effect of dengue immune sera on effects of NS1 on monocytes

Monocytes isolated from 5 individuals were incubated with media or E.coli expressed (certified as LPS free) dengue NS1 protein of DENV serotype 1 at concentrations of 250ng/ml and dengue immune sera of 1:5, 1:10 and 1: 100 dilutions for 96 hours. All experiments were done in duplicate. The IL-10 levels in the supernatants were measured every 24 hours by ELISA.

Fig 5: Effect of dengue NS1 on annexin V expression by T cells: Annexin V expression of CD4+ and CD8+ T cells co-cultured with dengue NS1. Annexin V expression on CD4+ and CD8+ T cells of 10 healthy individuals was determined by flow cytometry following co-culture with media alone or dengue NS1 concentrations of 250ng/ml, 500ng/ml or 1000ng/ml for 24 hours.

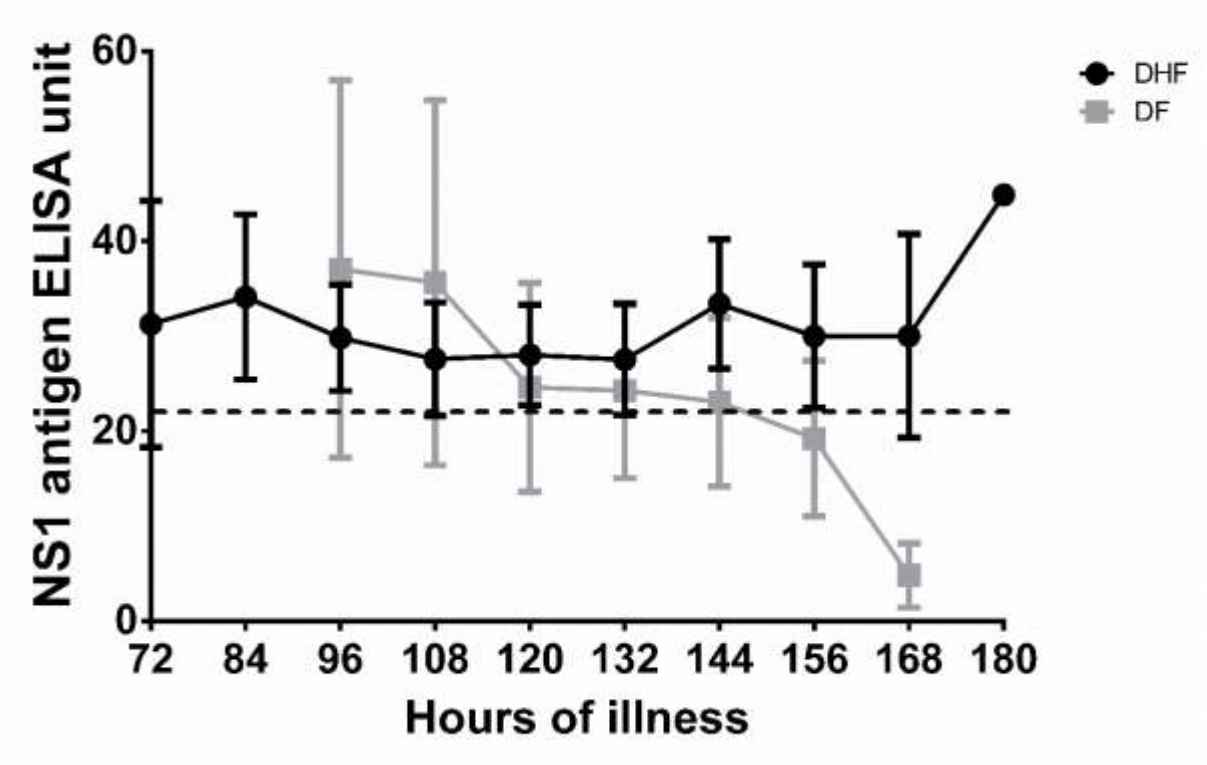
Fig 6: Expression of co-stimulatory molecules in T cells in patients with acute dengue.

Fig 6A: Expression of PD-1 in patients with acute dengue (n=24) and healthy individuals (n=12). PD-1 expression in DENV-NS3 specific T cells was determined by flow cytometric-guided gating on IFN γ producing T cells following stimulation with DENV-NS3 overlapping peptides.

6B: Correlation of DENV-NS3 specific CD28 expressing T cells with DEN-NS3 specific CD107a expressing T cells with acute dengue infection (n=20). DENV-NS3 specific T cells were determined by flow cytometric-guided gating on CD107a producing T cells following stimulation with DENV-NS3 overlapping peptides. Spearman's $R=0.59$, $p=0.007$

6C: Expression of CD28 by DENV-NS3 specific T and total T cells was determined by flow cytometry in patients with acute dengue (n=20) and healthy individuals (n=12)

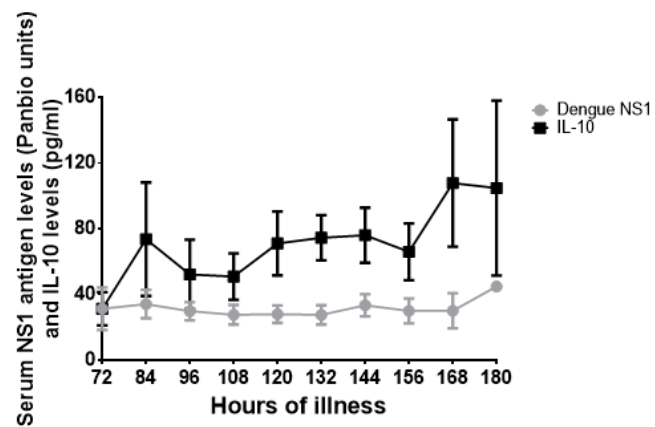
1 **Figures**



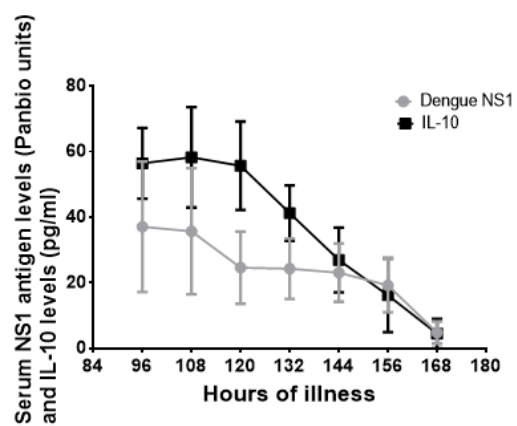
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3 Fig 1

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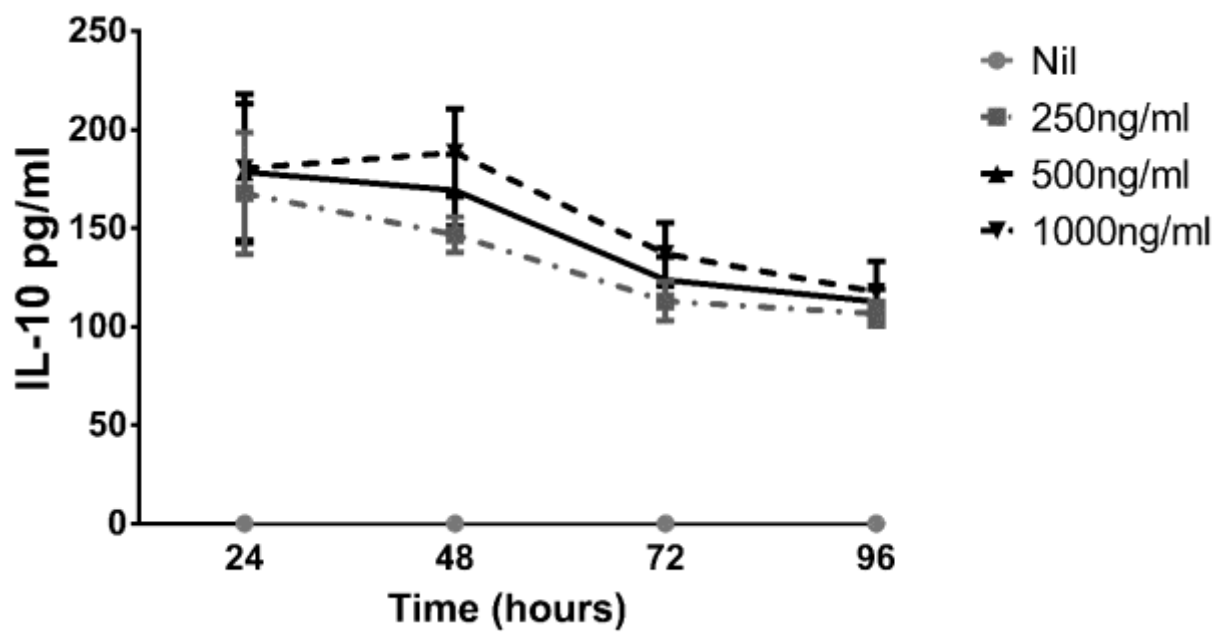
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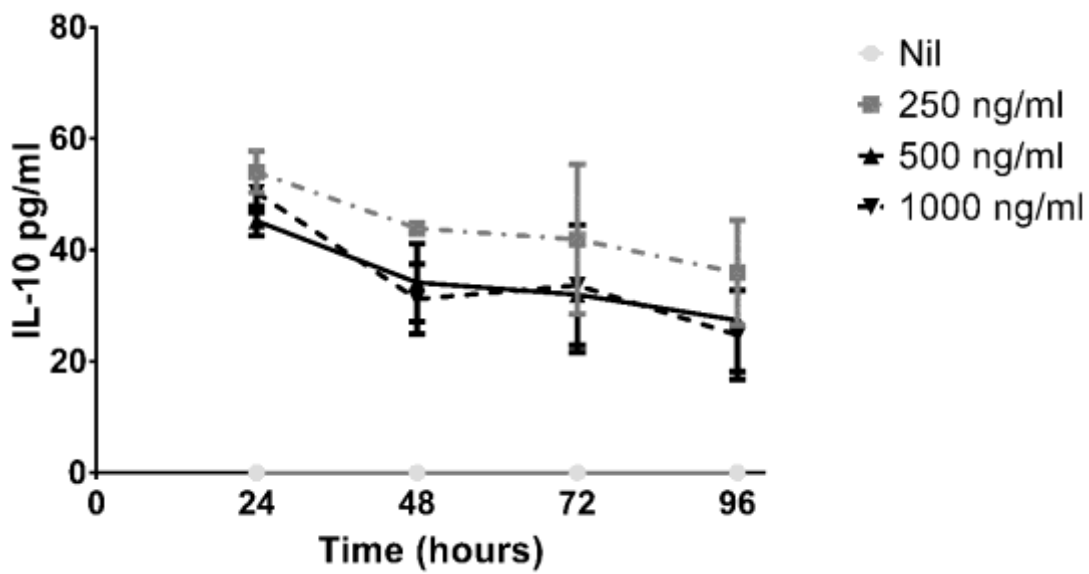
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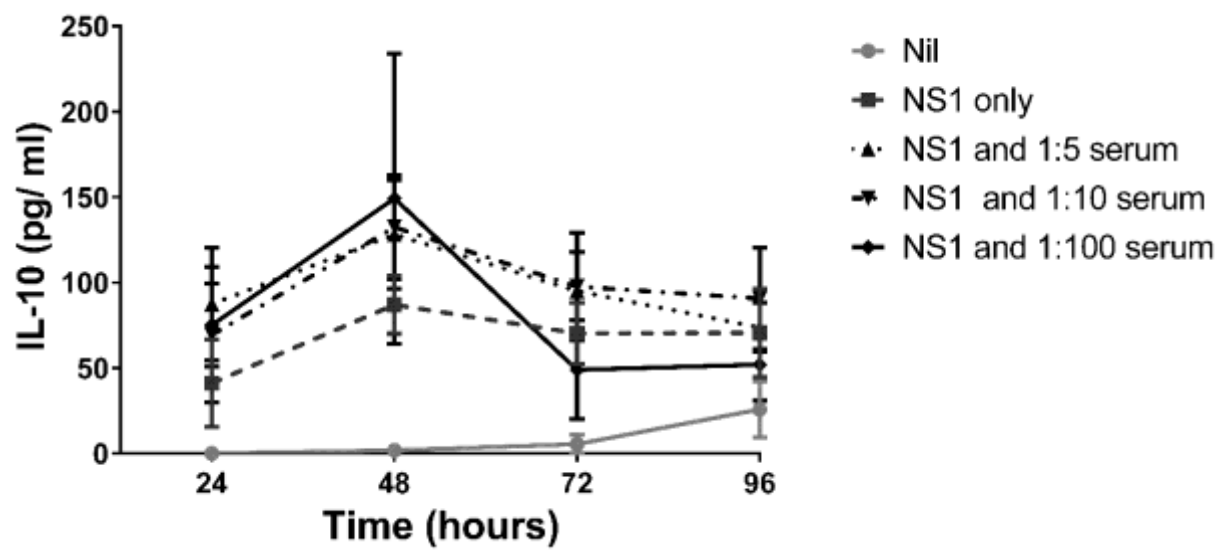


Fig 4

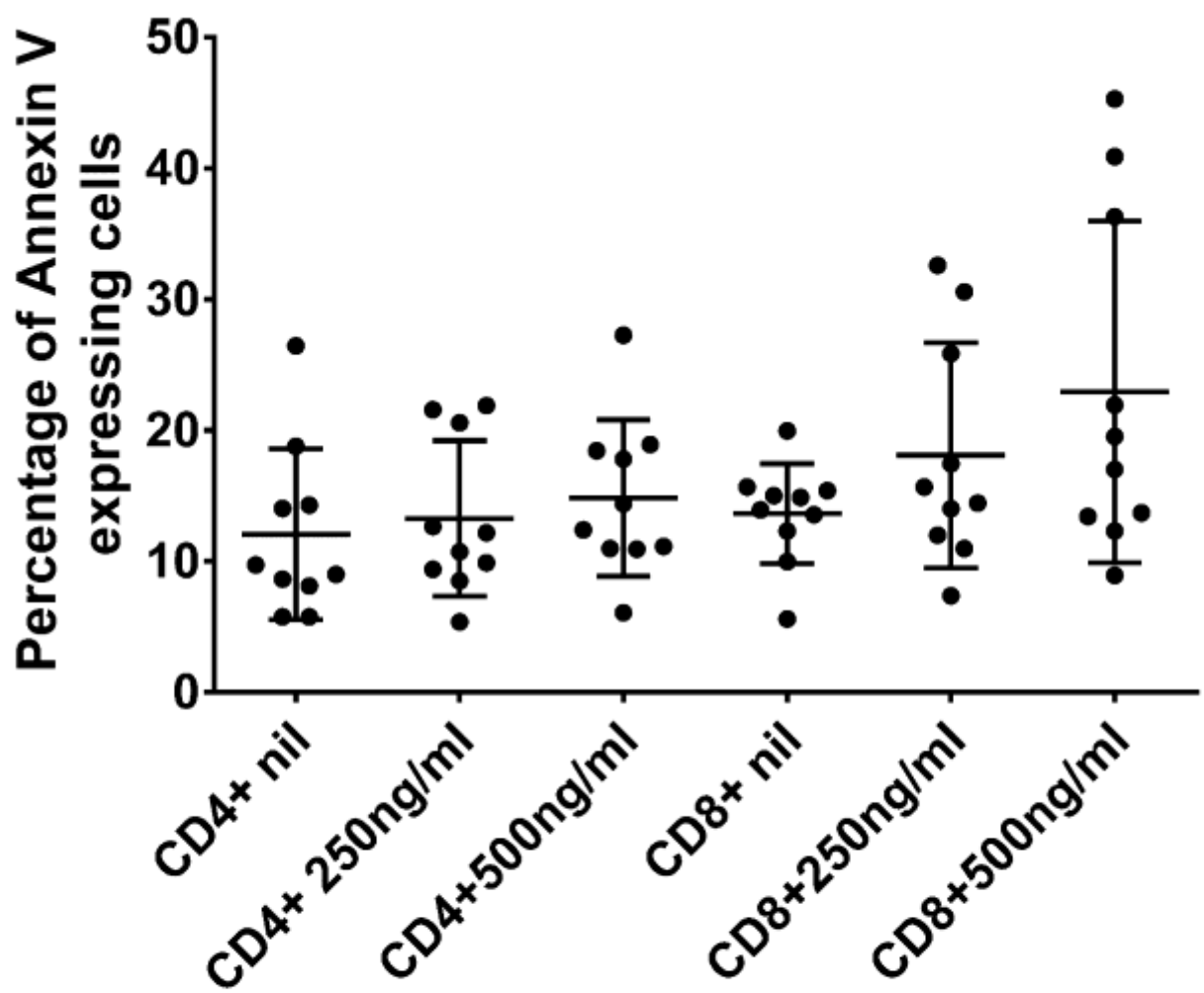


Fig 5

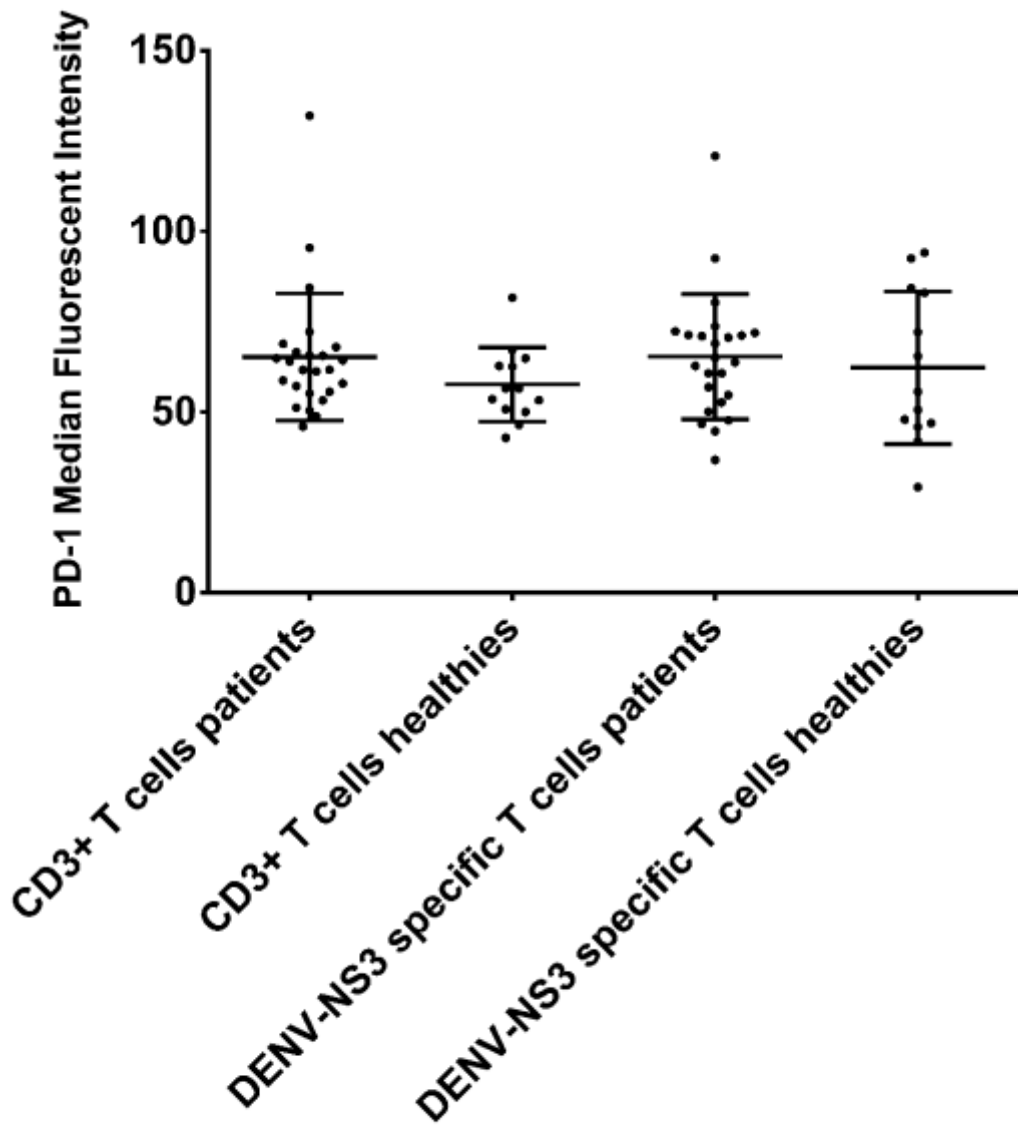


Fig 6A

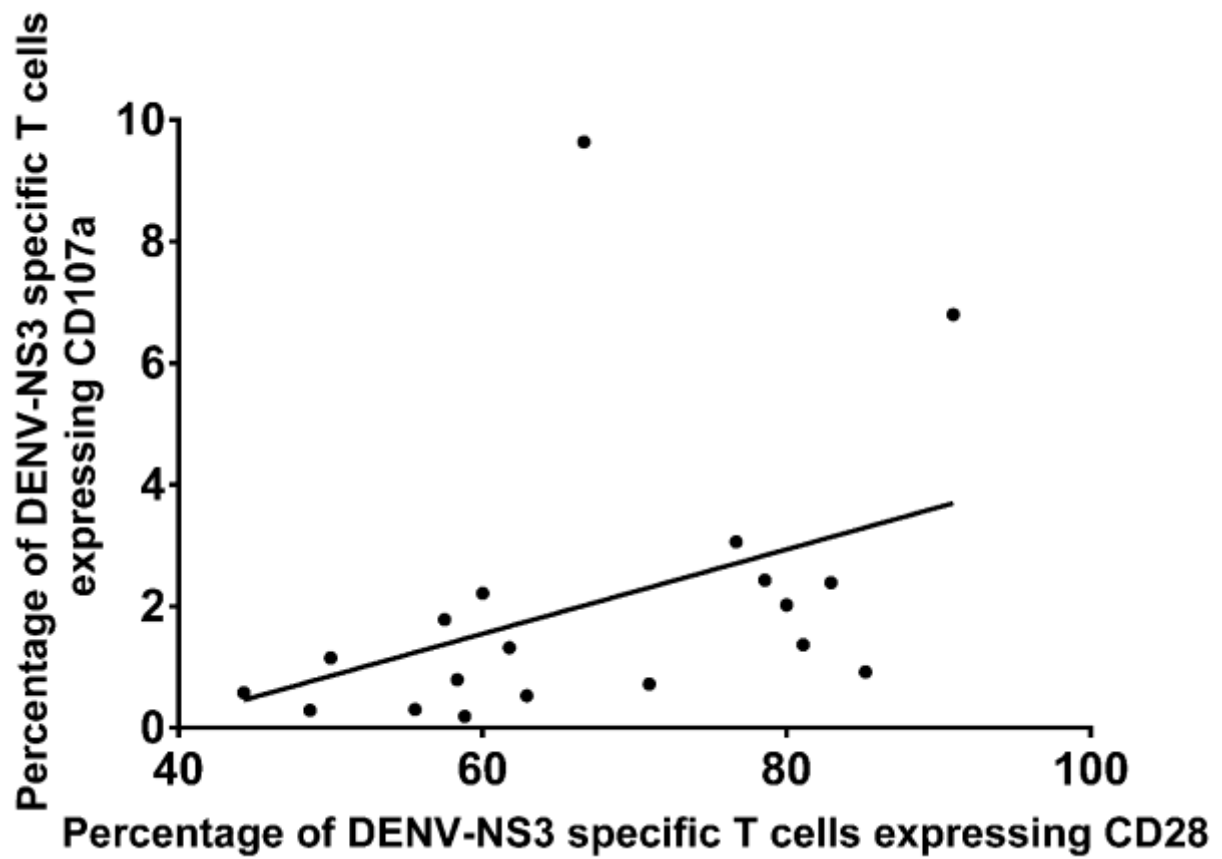


Fig 6B

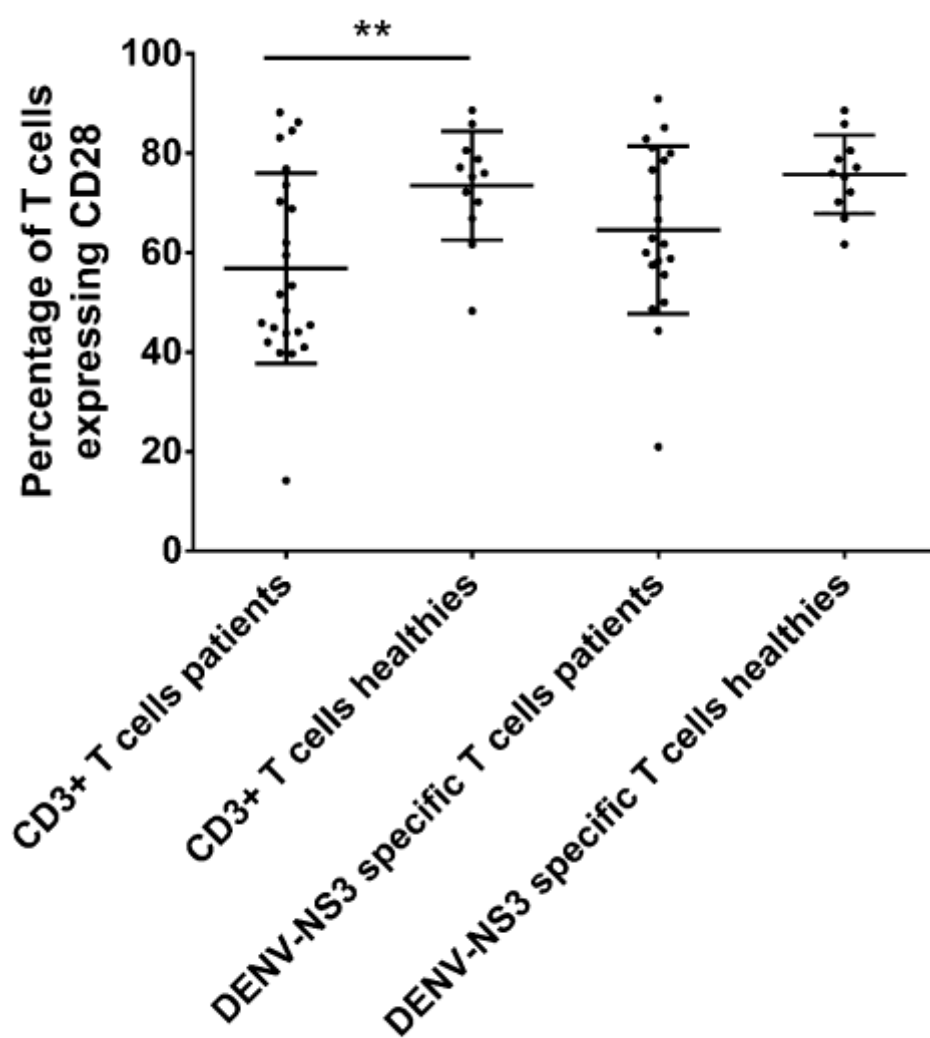


Fig 6C