Expansion of highly activated iNKT cells with altered phenotype in acute dengue infection

Achala Kamaladasa[1], Nilanka Wickramasinghe[1], Thiruni N. Adikari[1], Laksiri Gomes[1], N.L.A. Shyamali[2], Mariolina Salio[3], Vincenzo Cerundolo[3], Graham S. Ogg *[3,4,5] and Gathsaurie Neelika Malavige *[1,3]

[1]Centre for Dengue Research, Department of Microbiology, [2]Department of Medicine, Faculty of Medical Sciences, University of Sri Jayawardanapura, Sri Lanka, [3]MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, OX3 9DS, UK, [4]Oxford NIHR Biomedical Research Centre and University of Oxford, OX3 9DS, UK, [5]Department of Dermatology, Churchill Hospital, Oxford, OX3 7LJ, UK.

Correspondence should be addressed to:

Dr. Neelika Malavige DPhil, FRCP, FRCPath

Centre for Dengue Research, Faculty of Medical Sciences,

University of Sri Jayawardanapura, Sri Lanka

Tel +94 (0) 772443193; Fax: +94 (0) 112802026

*Authors contributed equally

Summary

Invariant natural killer T cells (iNKTs) are capable of rapid activation and production of cytokines upon recognition of antigenic lipids presented by CD1d molecules. They have been shown to play a significant role in many viral infections and were observed to be highly activated in patients with acute dengue infection. In order to further characterize their role in dengue infection, we investigated the proportion of iNKT cells and their phenotype in adult patients with acute dengue infection. The functionality of iNKT cells in patients was investigated by both IFNγ and IL-4 *ex vivo* ELISpot assays following stimulation with alpha-galactosyl-ceramide (αGalCer).

We found that circulating iNKT cells proportions were significantly higher (p=0.03) in patients with acute dengue when compared to healthy individuals and were predominantly of the CD4+ subset. iNKT cells of patients with acute dengue had reduced proportions expressing CD8α and CD161 when compared to healthy individuals. The iNKT cells of patients were highly activated and iNKT activation significantly correlated with dengue virus-specific IgG antibody levels. iNKT cells expressing Bcl-6 (p=0.0003) and both Bcl-6 and ICOS (p=0.006) were significantly increased in patients when compared to healthy individuals.

Therefore, our data suggest that in acute dengue infection there is an expansion of highly activated CD4+ iNKT cells, with reduced expression of CD161 markers.

Introduction

Dengue viral infections are a leading public health problem, predominantly in developing resource-poor countries, resulting in a huge burden to their economies (1). Dengue infection occurs in epidemic proportions in over 125 countries, causing symptomatic disease in approximately a quarter of individuals infected (2). 70% of the estimated total of 390 million dengue infections occur in Asia and over 40% of the world population is at risk of being infected with dengue virus (3). It has been projected that the future transmission of dengue infections will be more intense in the countries with the current highest disease burden, while countries which currently are free from dengue still have a potential risk of been affected (2). Currently, there is no specific treatment for dengue, nor a licensed vaccine, although there are several dengue vaccines in the pipeline (4).

Acute dengue infection may be caused by any four of the dengue viruses (DENVs) which are very closely related and share 60-70% homology (5). Although the majority of dengue infections manifest as asymptomatic infection or undifferentiated fever, a significant proportion of infections manifest as dengue fever (DF), dengue haemorrhagic fever (DHF) or dengue infection with organ dysfunction (6). However, many questions regarding factors that lead to severe disease and the pathophysiology of dengue infection itself remain unanswered. Our previous studies on T cell responses in acute dengue infection have shown that DENV-specific T cells have an impaired ability to produce cytokines such as IFNγ and to degranulate (7). In addition, T cells have been shown to undergo dramatic apoptosis in dengue infection (8, 9). Therefore, it

would be crucial to examine the role of other immune cells that are capable of rapid activation and production of large quantities of cytokines in the pathogenesis of acute dengue infection.

Invariant natural killer T cells (iNKTs) are a subset of lymphocytes, some of which express an invariant T cell receptor that recognizes both endogenous and microbial lipid antigens displayed on CD1d molecules. In humans, the T cell receptor of iNKTs comprises the alpha chain Vα24-Jα18 which is paired with Vβ11 (10). The role of iNKTs has been investigated in many viral infections such as HIV, HSV, hepatitis(10), influenza (11) and more recently in dengue infection (12). In dengue infection, iNKTs were shown to be highly activated and this was associated with clinical disease severity (12). Interestingly, the functionality of iNKTs in patients with milder forms of dengue such as DF was found to be different to that in patients with DHF, with iNKTs of patients with DF producing more IFNγ than IL-4 (12).

iNKTs are thought to display a wide functional diversity based on their expression of CD4 and CD8α, and also markers such as CD161(13, 14). While the CD8α+ are predominantly considered to be cytotoxic and CD4/CD8 double negative iNKTs are thought to mainly produce Th1 type cytokines, CD4+ iNKTs are thought to produce predominantly Th2 type of cytokines(13). In addition, CD4+ iNKTs have been shown to play a significant role in proliferation of B cells and in stimulating B cells to produce antibodies in a CD1d dependent manner (15, 16). In certain chronic viral infections such as in HIV, CD4+ iNKTs were shown to be depleted and numbers were restored during anti-retroviral therapy. iNKT cells have been shown to associate with severe clinical disease in dengue mice models. The inflammatory responses, vascular leak and liver injury were shown to be less in mice that were depleted of iNKTs (17). Since iNKTs are

known to have diverse functions and are rapidly activated, investigating their function in acute dengue viral infections would be crucial to better understand the role of these cells in the protection or pathogenesis of dengue infection.

In this study, we have found that the proportion of CD4+ iNKTs was increased in those with acute dengue infection and we observed reduced expression of CD161. The iNKTs were highly activated and iNKT activation significantly correlated with DENV-specific IgG antibody levels. However, the functionality of iNKT cells in patients with acute dengue did not appear to be significantly different from healthy individuals. Therefore, our data suggest that iNKT of predominantly the CD4+ subset are likely to play a role in the pathogenesis of dengue infection and may contribute to activation of B cells and DENV-specific antibody production.

Materials and methods

Study population

49 adult patients with clinical features compatible with acute dengue infection who were admitted to a general medical ward in a tertiary care hospital in Colombo were enrolled in the study. Blood samples were collected during day 5-7 of illness (day 1 was considered as first day of fever) in all patients. Serial recordings of their clinical features and laboratory investigations (platelet counts, haematocrits, and white cell counts) were made from time of admission to until they were discharged from the hospital in order to determine the severity of dengue infection. The severity of acute dengue infection was classified according to the 2011 WHO guidelines (18). All patients who were classified as having DHF, either had clinical evidence of fluid leakage or a rise of the haematocrit of >20% of the baseline level. Accordingly, 24 patients had DHF and 25 patients had DF. 5/24 patients with DHF developed shock as evidenced by a pulse pressure of ≤20 mmHg along with pleural effusions and ascites. 4 patients had bleeding manifestations.

22 healthy dengue seropositive individuals were also recruited as healthy individuals for determining the frequency of iNKT cells and their phenotype.

Ethics statement

49 adult patients with clinical features compatible with acute dengue infection were enrolled in the study following informed written consent. The ethical approval was granted from the Ethical

Review Committee of the University of Sri Jayawardanapura. All healthy individuals (n=22) who also participated in the study gave informed written consent.

Flow cytometry for identification of iNKT cells

Freshly isolated peripheral blood mononucleocytes (PBMCs) were used in all experiments and staining for flowcytometry was done immediately following PBMC separation. 7AAD (Biolegend, USA), was initially used to determine the percentage of dead cells. However, since fresh samples were used, we found that the percentage of dead cells were negligible. Cells were first washed in cell staining buffer (Biolegend, USA) and blocked with an Fc receptor blocker (Biolegend, USA) prior to surface staining. Anti-Vα24-Jα18 PE mAbs (IgG1, clone 6B11) and CD3 APC (IgG2a, clone OKT3), (Biolegend, USA) were used to identify the iNKT cell population (defined as CD3+ and Vα24-Jα18+ in all patients and controls). Isotype controls for Vα24-Jα18 (Biolegend, USA) were also used. Cells were acquired on a Partec Cyflow Cube 6 and analyzed with De novo FCS Express version 4.

Phenotyping of iNKT cells

These experiments were carried out in 19 patients with acute dengue infection and 12 healthy individuals due to limited cell numbers. Freshly isolated PBMCs were used and staining was carried out immediately following separation of PBMCs. Four colour flow cytometry was used to determine expression of CD38, HLA-DR, CD161, CD4 and CD8α on iNKTs. The following antibodies were used for phenotyping: anti-Vα24-Jα18 mAbs PE (IgG1, clone 6B11), CD38 APC (IgG1, clone HB-7), HLA-DR PerCP (IgG2a, clone L243), CD161 FITC (IgG1, clone HP-3G10), CD8α FITC (IgG1, clone HIT8a), CD4 PerCP (IgG2b, clone OKT4), CD3 APC and CD3

FITC (IgG2a, clone OKT3), (Biolegend, USA). In order to determine if the iNKTs were of follicular iNKT cell phenotype, surface staining for ICOS FITC (IgG, clone 398.4A), anti-Vα24-Jα18 mAbs PE and CD3 PerCP (IgG2a, clone OKT3) and intracellular staining for Bcl-6 APC (IgG2a, clone 7D1) (Biolegend, USA) staining was done in 15 patients and 10 healthy individuals. Appropriate conjugated-isotype-matched controls were included which were obtained from Biolegend, USA. The gating strategy of CD161, CD8a, CD161, HLA-DR CD38, ICOS and Bcl-6 is shown in supplementary figures 1, 2, 3 and 4.

The absolute iNKT cell numbers were determined in 49 acute dengue patients and 10 healthy individuals by a crude method of calculating the iNKT cell numbers from the total lymphocyte counts. As the total white cell counts were done in the same samples obtained for flowcytometry and the total lymphocyte counts were available to us, the absolute NKT cell were then calculated after gating on the lymphocytes in the FSC and SSC view.

Functional assays for iNKT cells

Ex vivo ELISpot assays were performed in 16 patients with acute dengue and 14 healthy dengue seropositive individuals due to limited cell numbers. *Ex vivo* ELISpot assays were performed as previously described (19, 20). ELISpot plates (Milliopre Corp, USA) were separately coated with anti-human IFN-γ antibody and anti-human IL-4 antibody (Mabtech, Sweden) overnight. Freshly isolated PBMC (5×10^5/well) were incubated with 100ng/ml KRN7000 (Cayman Chemicals, USA) overnight for IFNγ detection and for 48 hours for IL-4 detection at 37°C and 5% CO_2. All experiments were done in duplicate. PHA was always included as a positive control, and media alone with the PBMCs was included as a negative control. The cells were

removed and the plates developed with a second biotinylated Ab to human IFNγ and washed a further six times. The plates were developed with streptavidin-alkaline phosphatase (Mabtech AB) and colorimetric substrate, and the spots enumerated using an automated ELISpot reader. Background (cells plus media) was subtracted and data expressed as number of spot-forming units (SFU) per 10^6 PBMC.

Serology

Acute dengue infection was confirmed by testing the serum samples which were collected after day 6 of illness with a 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 manufacturer's instructions. Patients who only had dengue virus specific IgM were classified as having a primary dengue infection while those who had a positive result for both IgM and IgG were classified as having a secondary dengue infection(21). Accordingly, 19/49 patients had a primary dengue infection (only IgM positive) and 30 patients had a secondary dengue infection. Early dengue NS1 capture ELISA (Panbio, Brisbane, Australia) was also carried out in all patients according to manufacturer's instructions.

Statistical analyses

Data analysis was performed using GraphPad Prism 6.0 software. As the data was not normally distributed, differences in means were compared using the Mann-Whitney t test (two tailed). The results were expression as the median and the inter quartile range (IQR). To determine positive and negative associations, the Spearman's correlation test was used (two tailed).

Results

The clinical and laboratory characteristics of the 49 patients with acute dengue infection patients are shown in Table 1. All patients with DHF either had clinical evidence of fluid leakage or had a rise in the haematocrit of >20% of the baseline value and thus were classified has having DHF based on the WHO guidelines (18). Three of the patients who were classified as having DF, had platelet counts of <25,000 cells/mm3, but did not have clinical or laboratory evidence of fluid leakage. 20/49 (40.8%) of the patients were positive for NS1 antigen at the time of recruitment.

<u>High peripheral iNKT cell frequency in dengue patients</u>

Since the monoclonal antibody from clone 6B11 was shown to specifically bind to the invariant loop of the Vα24-Jα18 rearranged TCR chain of human NKT cells and has been extensively used to characterize iNKT cells in humans(22-24), we identified iNKT cells as cells being positive for both CD3 and 6B11 staining (Fig. 1A). We found that the percentage of the iNKT cell population was significantly higher (p=0.03) in the patients with acute dengue (median 0.5%, IQR 0.31 to 1.01% of CD3+ cells) compared to healthy individuals (median 0.38%, IQR 0.22-0.55% of CD3+ cells) (Fig. 1B). Although the percentage of iNKTs was lower in patients with DHF (median 0.47%, IQR 0.27 to 0.88 % of CD3+ cells) when compared to those with DF (median 0.65 %, IQR 0.34 to 1.03 % of CD3+ cells) this was not significant (p=0.49) (Fig 1B). There was no difference in iNKT cell percentages among patients with primary (median 0.65%, IQR 0.3-1.12%) and secondary (median 0.46%, IQR 0.31-0.95%) dengue infections (Fig.1B).

The iNKT cell percentages were also similar in those who were NS1 antigen positive at the time of recruitment (median 0.46%, IQR 0.3 to 1.27%) and those who were NS1 antigen negative (median 0.55%, IQR 0.32 to 0.84).

As the percentage of iNKTs would depend on the lymphocyte counts of these patients, we then proceeded to determine the differences in absolute iNKT numbers in patients with varying severity of dengue infection. The absolute iNKT cell number was determined in both patients and healthy individuals in the same blood sample that was used to determine the proportion of iNKT cells. Although not statistically significant (p=0.46) the absolute iNKT cell numbers were higher in healthy individuals (median 13.67, IQR 3.04 to 27.01 cells/mm3) compared to patients (median 7.98, IQR 4.2 to 14.76 cells/mm3). However, the median absolute lymphocyte count was almost double in healthy individuals (median 3115, IQR 2863 to 3651 cells/mm3) than in patients (median 1410, IQR 1000 to 2445 cells/mm3) which could reflect the slight increase in absolute iNKT cell numbers. Further there was no significant difference in absolute iNKT cell numbers between patients with DHF (median 7.8, IQR 4.5 to 14.8 cells/mm3) and DF (median 8.3, IQR 4.8 to 14.8 cells/mm3) and also between those with primary (median 7.8, IQR 4.5 -13.9 cells/mm3) and secondary dengue infection (median 11.3, IQR 3.55-16.7 cells/mm3) infections (Fig 1C).

Expansion of the CD4+ iNKT subset in patients with acute dengue

As different subsets of iNKT cells have been defined based on expression of CD4 and CD8α (13), we proceeded to investigate the iNKT subsets in patients with acute dengue. We found that the CD4+ iNKT subset was significantly expanded (p=0.01) in patients with acute dengue (median 55.14, IQR 38.98 to 64.71% of iNKT cells) compared to healthy individuals (median

36.4, IQR 21.76 to 50.91% of iNKT cells). The frequency of CD4-CD8- (DN iNKT cells) was significantly reduced ($p<0.0001$) in patients with acute dengue (median 1.5, IQR O TO 9.3% of iNKT cells) when compared to healthy individuals (median 13.32, IQR 8.5 to 28.3% of iNKT cells). The frequency of CD8α+ iNKT cells was also significantly reduced ($p<0.0001$) in patients with acute dengue (median 0.49, IQR 0 TO 3.8% of iNKT cells) when compared to healthy individuals (median 17.4, IQR 9.9 to 22.3% of iNKT cells) (Fig 2A).

Of the 19 patients in which we were able to phenotype iNKT cells, 7 patients had DHF while 12 had DF. The frequency of CD4+ iNKT cells was significantly higher ($p=0.04$) in patients with DF (median 63.1, IQR 42.0 to 69.8 % of iNKT cells) when compared to those with DHF (median 47.7, IQR 17.1 to 51.3 % of iNKT cells) (Fig2B). In contrast, the frequency of iNKT cells of the CD8α+ phenotype was lower in patients with DF (median 0.1, IQR 0 to 0.9% iNKT cells) when compared to those with DHF (median 3.8, IQR 0 to 6.3% of iNKT cells) although this was not significant ($p=0.06$). There was no difference in the frequency of DN iNKT phenotype in patients with DHF and DF.

<u>iNKTs are highly activated but were predominantly of the immature phenotype</u>

We proceeded to characterize the expression of activation markers on iNKT cells by using HLA-DR and CD38. We found that iNKT cells of patients with acute dengue had significantly higher ($p=0.02$) co-expression of HLA-DR and CD38 (median 10, IQR 4.7 to 31.5% of iNKT cells) when compared to healthy individuals (median 4.4, IQR 3.7 to 5.6% of iNKT cells) (Fig 2C). However, we did not observe any difference in expression of HLA-DR and CD38 in patients with DF when compared to those with DHF.

CD161 is expressed on mature iNKT cells; and in certain infections such as HIV, CD161 expressing iNKT cells are preferentially deleted (14, 25). We found that the percentage of CD161 expression on iNKT cells was significantly lower (p=0.01) in patients with acute dengue patients (median 65.23%, IQR 49 to 35-89% of iNKT cells) compared to healthy individuals (median 86.64%, IQR 83.68 to 93.81% of iNKT cells) (Fig.2D). CD161 expression on CD4+ iNKT cells was also significantly (p=0.01) lower in patients (median 36.36%, IQR 21.39 to 80.37% of iNKTs) compared to healthy individuals (median 80.52%, IQR 75.07 to 87.18% of iNKTs). This suggests that although the CD4+ iNKT subset was expanded in patients with acute dengue, they were of the immature phenotype. We did not observe any difference in CD161 expression on CD4+ iNKT cells in patients with DF and DHF. The reduction in CD161 expression in iNKT cells could possibly be due to a preferential expansion of iNKT cells that do not express this marker.

Association of the frequency of iNKT with dengue antibody titres

Different iNKTs are thought to activate B cells to produce antibodies and promote proliferation and differentiation of B cells (16, 26). Therefore, using dengue Panbio ELISA units as an indicator of dengue IgG antibody titre, we investigated if certain iNKT subsets were associated with dengue IgG antibodies. We found that the frequency of DN iNKT cells significantly and positively correlated with dengue IgG antibody titres (Spearmans' r=0.5043, p=0.03) (Fig 3A). However, there was no significant correlation between dengue IgG titres and CD4+CD8α- iNKT cells (Spearmans' r=0.0912, p=0.71). The frequency of activated iNKT cells also significantly correlated with dengue IgG antibody titres (Spearmans' r=0.5018, P=0.03) (Fig 3B).

iNKT follicular helper cells are a subset of iNKT cells, which are CD4+ and induce germinal centre formation and provide help to B cells that recognise lipid antigens (27). This subset of follicular iNKT cells have shown to express Bcl-6, which was shown to be important in up regulating ICOS and certain chemokine receptors (27). Since we found that the frequency of iNKT cells co-expressing CD38 and HLA-DR positively correlated with dengue IgG antibody titres in acute infection, we proceeded to determine if the follicular iNKT cell population was expanded in acute dengue infection. We found that in patients with acute dengue the frequency of iNKT cells expressing Bcl-6 ($p=0.003$) and cells co-expressing Bcl-6 and ICOS is significantly increased ($p=0.0006$) compared to healthy individuals, who showed negligible expression of both these markers on iNKT cells (Fig 4). However, iNKT cells expressing Bcl-6 or those co-expressing ICOS and Bcl-6 did not show a significant ($p>0.05$) correlation between dengue specific IgG antibody titres (Panbio units) in patients with acute dengue. Therefore, although the frequency of iNKT follicular helper cells was significantly increased in the patients, they did not appear to associate with dengue antibody titres.

Functionality of iNKTs in acute dengue

In previous studies it was shown that the type of cytokines produced by iNKTs in patients with acute dengue differed in those with DHF and DF (12). Using ex vivo IFNγ and IL-4 ELISpot assays, we proceeded to determine the functionality of iNKT cells in patients with acute dengue when compared to healthy individuals following α-GalCer stimulation. As reported previously,

the mean IFNγ ELISpot responses were lower in patients with acute dengue (mean 78.4, median 40, IQR 2.25 to 93 Spot forming units/PBMCs) when compared to healthy individuals (mean 107.6, median 40.5, IQR 8.5 to 131 Spot forming units/1 million PBMCs), although this was not significant (p=0.56) (Fig 5A). IL-4 production in response to stimulation with α-GalCer was also not significantly different (p=0.98), in patients (mean 5.4, median 2.5, IQR 0 to 10.75 spot forming units/1 million PBMCs) when compared to healthy individuals (mean 4.7, median 3.0, IQR 0.25 to 9.5 Spot forming units/1 million PBMCs) (Fig 5B). Although IFNγ is thought to be produced by TH1 type iNKT cells and IL-4 by TH2 type iNKT cells, no association was observed between ex vivo IFNγ or IL-4 ELISpot responses (Spearmans' r=0.3899, p=0.3024).

Discussion

In this study we found that the frequency of circulating iNKT cells was significantly higher (p=0.03) in adult patients with acute dengue infection, which was evident within the CD4+ subset. This is in contrast to a previously study in children which showed that the frequency of iNKT cells in patients was similar to healthy donors (12). Although the reasons for this

difference in not clear, it is possible that the iNKT cell frequencies are different in adults and children. Furthermore, we included more patients in our study than in the previously published work. iNKT cells are innate like lymphocytes which are able to rapidly activate and produce large quantities of cytokines and have also been shown to have cytotoxic properties (10). They are known to play an important role in both acute (11) and chronic viral infections (14, 28) including a recent report on their role in dengue infection (12). Acute dengue is known to associate with a marked lymphopenia (29), especially with reduction of T cells due to massive T cell apoptosis (8, 9). We have also observed that DENV-specific IFNγ T cell responses are impaired during acute dengue, especially in those with more severe forms of dengue (7). Therefore, it is possible that innate immune responses are likely to play role in the background of impaired antiviral T cell responses. Although not significant, we observed that patients with DF had a higher frequency of iNKT cells and also higher iNKT cell numbers. In a previous study, it was shown that patients with DF produced more IFNγ than IL-4 when compared to those with DHF (12). Therefore, iNKT cells could be playing a significant role in antiviral defenses against the DENV.

iNKT cells are activated by either ligand dependent mechanisms or by independent mechanisms, which for example can involve expansion and activation of iNKTs by cytokines such as IL-18, IL-12 and type I interferons (10, 30). Elevated type I interferons and cytokines such as IL-12 have shown to associate with milder clinical disease in acute dengue infection (31, 32). Therefore, although not significant, cytokines could have contributed to the expansion of iNKT cells in DF patients as compared with DHF patients. Apart from activation of iNKT cells by cytokines, it is also possible that CD1d molecules could be presenting natural glycolipid ligands

in acute dengue thereby resulting in their activation. For instance, levels of glucosylceramide, which is known to be a potential activator of iNKT cells through CD1d, were shown to be elevated in patients with acute dengue (33). Therefore, it would be important to find out if natural lipids do activate iNKT cells in dengue and also if CD1d upregulation occurs.

Based on the expression of CD4 and CD8α, different subsets of iNKT cells have been identified (34) and the CD4+ iNKT cells have shown to produce both TH1 and TH2 cytokines and are less cytotoxic than the other subsets(13, 26, 35). We found that the CD4+ iNKT cells were significantly expanded in acute dengue infection and also significantly expanded in those with DF when compared to those with DHF. The CD4+ subset of iNKT cells is thought to produce both TH1 and TH2 cytokines and is thought to be less cytotoxic (13). This subset of iNKT cells has been shown to be preferentially depleted in patients with HIV (36, 37) and then restored following antiretroviral therapy (14). Therefore, it is possible that the CD4+ subset of iNKT cells is playing a protective role in acute dengue, since they were more expanded in patients with DF when compared to those with DHF. However, when investigating their functionality we found that although not significant, PBMCs of patients with acute dengue tend to produce lower amounts of IFNγ in response to α-GalCer stimulation when compared to healthy individuals, in agreement with a previous report (12). The production of IL-4 was similar. Therefore, although there appears to be an expansion of iNKT cells which are likely to be less cytotoxic, they also appear to have a reduced ability to produce cytokines. Preliminary data suggest no difference in PD-1 expression in iNKT cells in Dengue patients, but the mechanism underlying their poor reactivity remains to be investigated.

IL-17 levels have also been shown to be elevated in patients with acute dengue (38) and CD4- iNKT cells, which express CD161 are thought to produce IL-17(39). CD161 has been shown to be preferentially expressed on mature iNKT cells and CD4+CD161+ cells have been shown to be deleted in certain infections such as HIV (14, 25). We found that CD4- CD161+ expressing iNKT cells were significantly less in patients with acute dengue when compared to healthy individuals. Therefore, although we did not assess IL-17 production by iNKT cells in response to α-GalCer stimulation, iNKT cells in acute dengue are unlikely to be a source of IL-17 due to the reduction of this particular subset of iNKT cells in the peripheral blood.

We found that the iNKT cells were significantly more activated in patients with acute dengue than in healthy individuals, as previously reported (12). However, no difference was observed in the frequency of iNKT cell activation in patients with DF and DHF. Interestingly, we found that iNKT cell activation significantly and positively correlated with DENV-specific IgG antibody titres (Spearmans' r=0.5018, P=0.03). Our subsequent experiments showed this could be due to expansion of iNKT follicular helper cells (expressing ICOS and Bcl-6). iNKT follicular helper cells are a subset of iNKT cells, which are CD4+ and induce germinal centre formation and provide help to B cells that recognize lipid antigens (27). On the other hand, iNKT cells are also known to require B cell help to produce cytokines such as IFNγ, TNFα, IL-2 and IL-10 (40). Although all subsets of iNKT cells in turn have shown to activate B cells to produce antibodies, and also cause B cell proliferation and plasma cell differentiation (16, 26), follicular iNKT cells specifically provide help to B cells that recognize lipid antigen.

In summary, we have found that iNKT cells are significantly expanded in acute dengue infection and are highly activated. We also found that these iNKT cells are predominantly of the CD4+ subset which may account for an overall reduced expression of CD161. In addition, activation of iNKT cells significantly and positively correlated with DENV specific IgG antibody titres. Therefore, our results suggest that iNKTs might play an important role in the pathogenesis of dengue, which should be further investigated.

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Conflicts of interest

None

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Tables

Table 1: Clinical and laboratory characteristics of patients with acute dengue infections

Clinical features	DF N=24 (%)	DHF N=25 (%)
Abdominal pain	6 (26.1)	18 (69.2)
Hepatomegaly	1 (4.3)	15 (57.7)
Vomiting	8 (34.8)	13 (50)
Pleural effusions or ascites	0 (0)	9 (36.0)
Bleeding manifestations	0 (0)	4 (1.6)
Leukopenia	19 (79.2)	23 (92.0)
Platelet counts on admission (cells/mm3)	119.5 (IQR 87.5 to 145)	93.5 (IQR 50 to 130.5)
Lowest platelet count (cells/mm3)	58,000 (IQR 34.5 to 104.5)	25,000(IQR 18.5 to 38.75
Lowest white cell count (cells/mm3)	2,454 (IQR 1.6 to 3.7)	2.2 (IQR 1.7 to 2.9)
Lowest lymphocyte count (cells/mm3)	1.62 (IQR 0.97 to 10.7)	9.3 (IQR 0.88 to 28.8)

Figure Legends

Fig.1 Percentage of iNKT cells in dengue patients

Peripheral blood mononuclear cells were stained and analyzed by flow cytometry.

A: Representative gating strategy for iNKT cells based on CD3 and Vα24-Jα18 expression, showing isotype control (a) healthy individual iNKT (b) and dengue patient iNKT (c).

B: Percentage of iNKT cells in proportion of the total T cells in healthy individuals (n=22), all patients with acute dengue infection (n=49), patients with DHF (n= 24) and DF (n=25) and primary (n=19) and secondary dengue infections (n=30). The lines display the median and the inter quartile ranges.

C: Absolute iNKT cell count in healthy individuals (n=10) patients with DHF (n= 24) and DF (n=25) and primary (n=19) and secondary infections (n=30). The lines display the median and the inter quartile ranges. *p=0.03.

Fig. 2 Phenotyping of iNKT cells in patients with acute dengue.

A. iNKT cells were examined for expression of CD4 and CD8α by flow cytometry in 19 patients with acute dengue infection and 12 healthy individuals. *p<0.01 ****p<0.0001..

B: iNKT subsets based on CD4 and CD8α were examined in DF (n=12) and DHF (n-12). P<0.05

C : iNKT cells were examined for expression of HLA-DR and CD38 by flow cytometry in healthy individuals (n=12) and patients (n=19). *p<0.05. The lines display the median and the inter quartile ranges.

D : iNKT cells were examined for expression of CD161 by flow cytometry in healty individuals (n=12) and in patients (n=19). *p<0.05 The lines display the median and the inter quartile ranges.

Fig.3 Correlation of DENV specific IgG levels with the percentage of iNKT cells

A: Correlation of DENV-specific IgG antibodies (PanBio units) with the percentage of CD4-CD8- (DN) iNKT cells as a total proportion of iNKT cells in patients with acute dengue (n=19). P=0.03, Spearmans' R=0.5

B: Correlation of DENV-specific IgG levels with the percentage of HLA-DR+CD38+ iNKT cells as a proportion of the total iNKT cell population (, p=0.03 Spearman r=0.51).

Fig.4: iNKT cells were examined for expression of Bcl-6 and ICOS by flow cytometry in healthy individuals (n=10) and patients (n=15). ***p<0.001. The lines display the median and the inter quartile ranges.

Fig. 5: *Ex vivo* IFNγ and IL-4 ELISpot responses in patients with acute dengue

A: Production of IFNγ by iNKT was examined by *ex vivo* IFNγ ELISpot assays in response to αGalCer in healthy donors (n= 14) and patients (n=16). p=0.5.

B: Production of IL-4 by iNKT was examined by *ex vivo* IL-4 ELISpot assays in response to αGalCer in healthy donors (n=8) and patients (n=16). The lines display the median and the inter quartile ranges.

Supplementary figures

Figure S1: Representative plots showing gating pathway of determining the frequency of iNKT cells expressing CD4 and CD8α markers.

Figure S2: Representative plots showing gating pathway of determining the frequency of iNKT cells co-expressing CD161 and CD4 markers.

Figure S3: Representative plots showing gating pathway of determining the frequency of iNKT cells co-expressing HLA-DR and CD38 markers.

Figure S4: Representative plots showing gating pathway of determining the frequency of iNKT cells co-expressing Bcl-6 and ICOS markers.

Figure S5: graph showing percentage of iNKT cells in dengue seropositive (n=22) and seronegative (n=3) healthy individuals

Figures

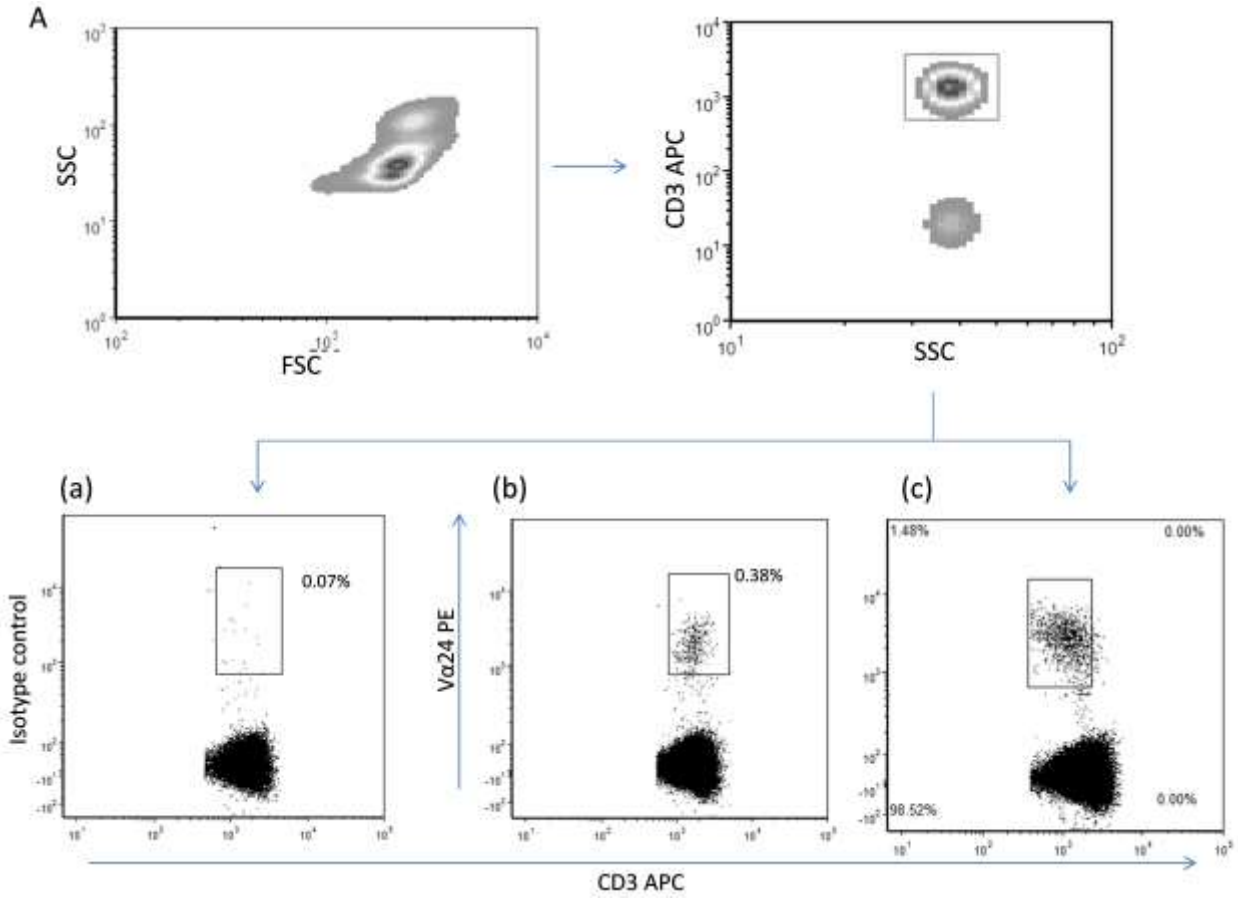


Fig 1A

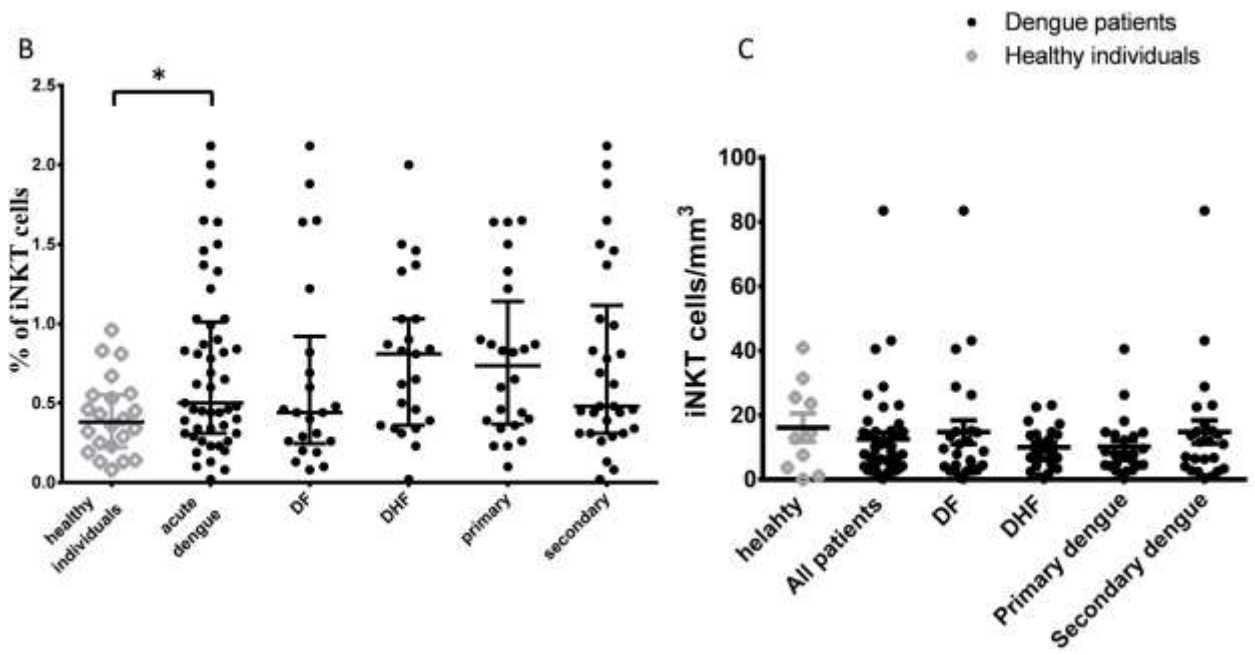

B
*
% of iNKT cells
2.5
2.0
1.5
1.0
0.5
0.0
healthy individuals
acute dengue
DF
DHF
primary
secondary
C
Dengue patients
Healthy individuals
iNKT cells/mm3
100
80
60
40
20
0
helahty
All patients
DF
DHF
Primary dengue
Secondary dengue

Fig 1 B and C

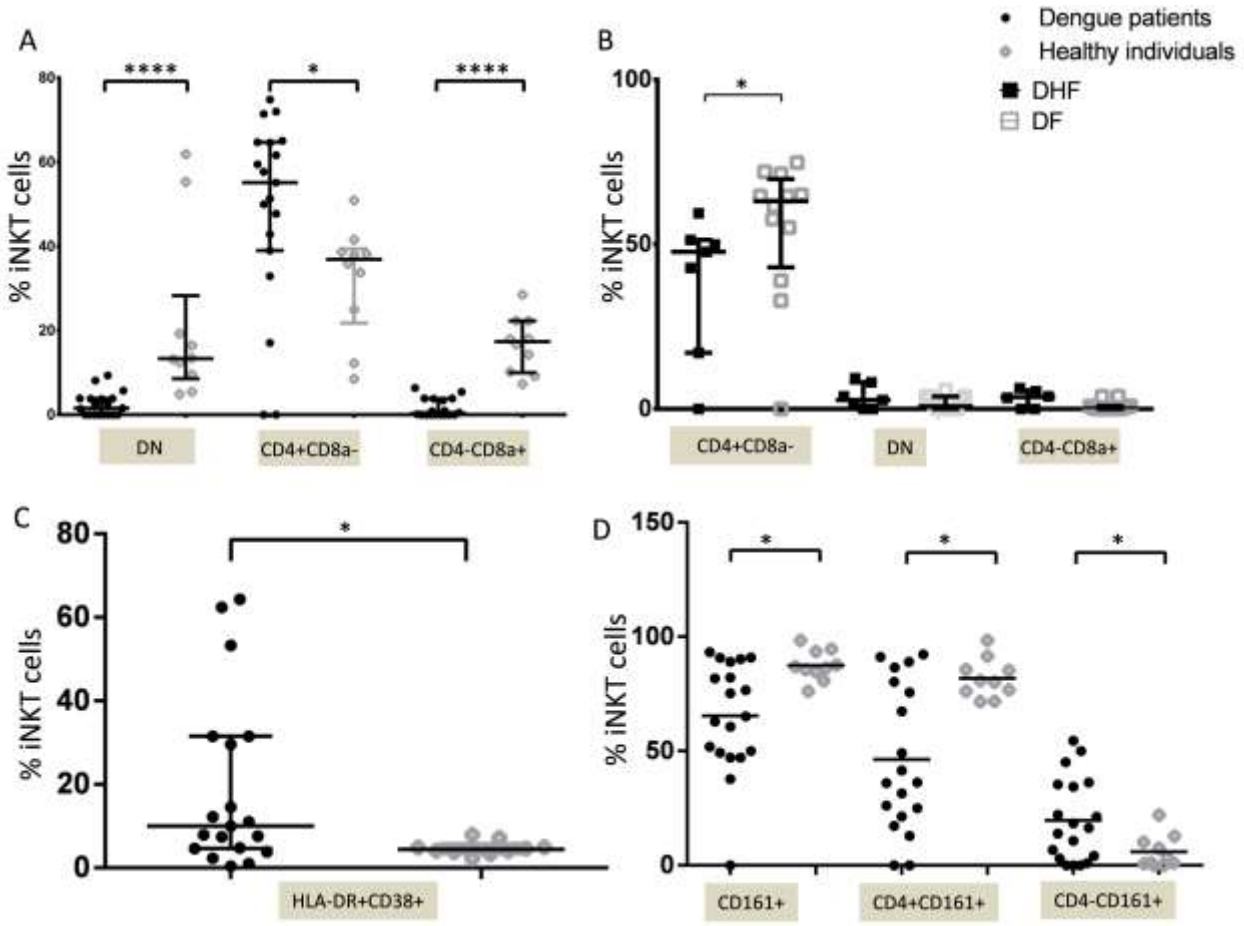

A
B
C
D
Dengue patients
Healthy individuals
DHF
DF
% iNKT cells
DN
CD4+CD8a-
CD4-CD8a+
HLA-DR+CD38+
CD161+
CD4+CD161+
CD4-CD161+

Fig 2

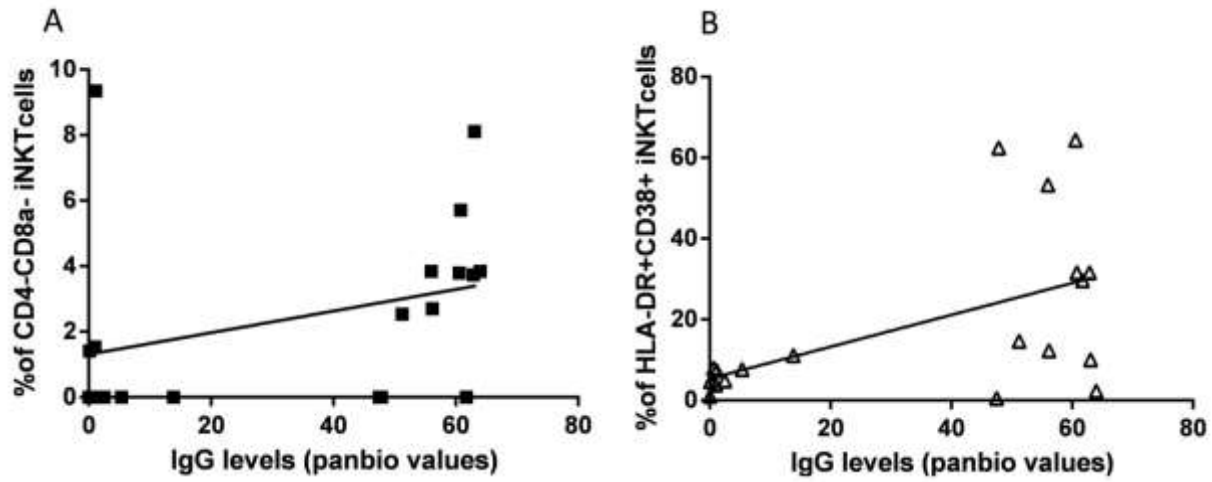

A
%of CD4-CD8a- iNKTcells
10
8
6
4
2
0
0
20
40
60
80
IgG levels (panbio values)
B
%of HLA-DR+CD38+ iNKTcells
80
60
40
20
0
0
20
40
60
80
IgG levels (panbio values)

Fig 3

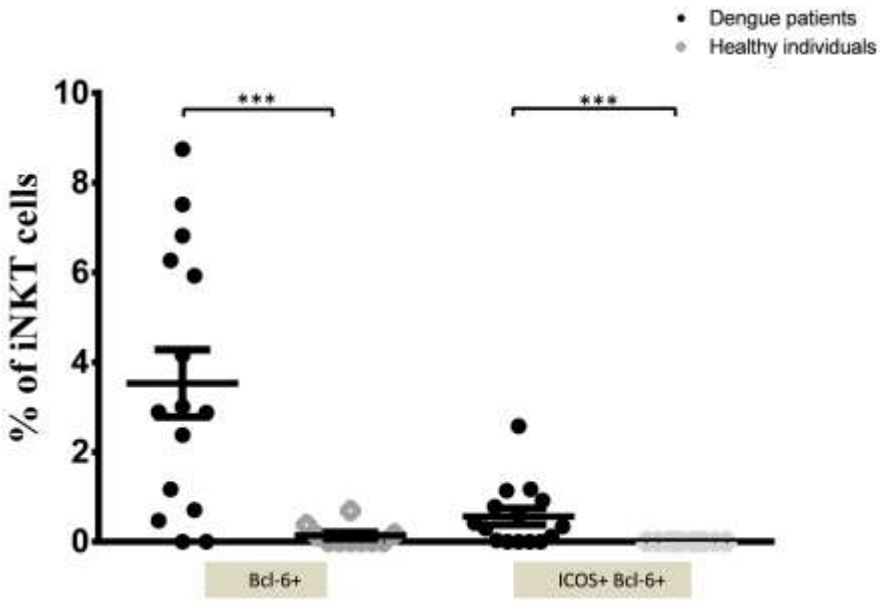

Dengue patients
Healthy individuals
% of iNKT cells
10
8
6
4
2
0
***
***
Bcl-6+
ICOS+ Bcl-6+

Fig 4

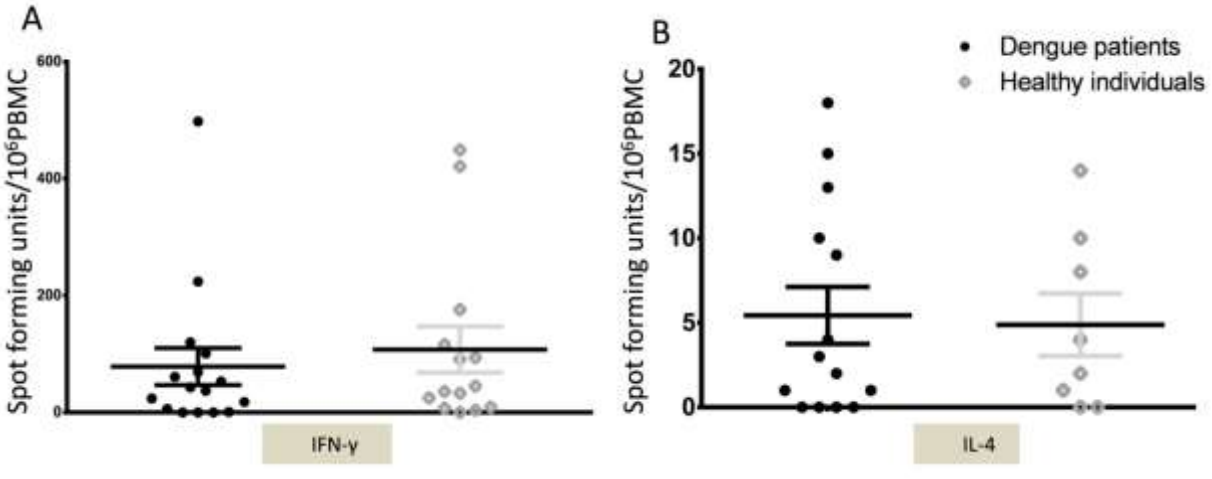

A
Spot forming units/10⁶PBMC
600
400
200
0
IFN-γ
B
Spot forming units/10⁶PBMC
20
15
10
5
0
Dengue patients
Healthy individuals
IL-4

Fig 5

Supplementary figures

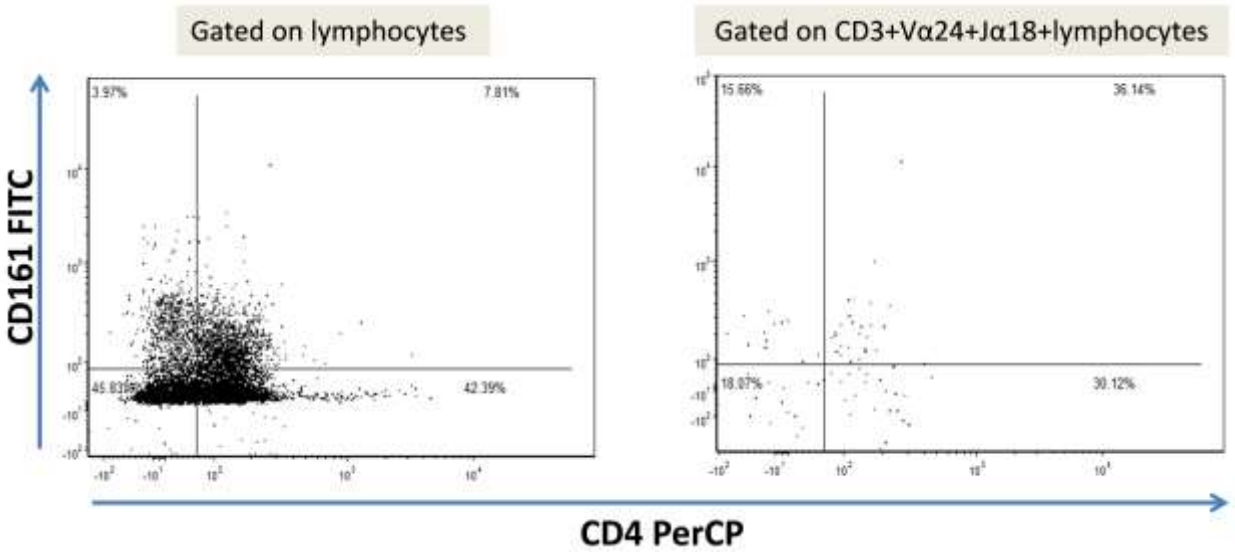


Fig S1

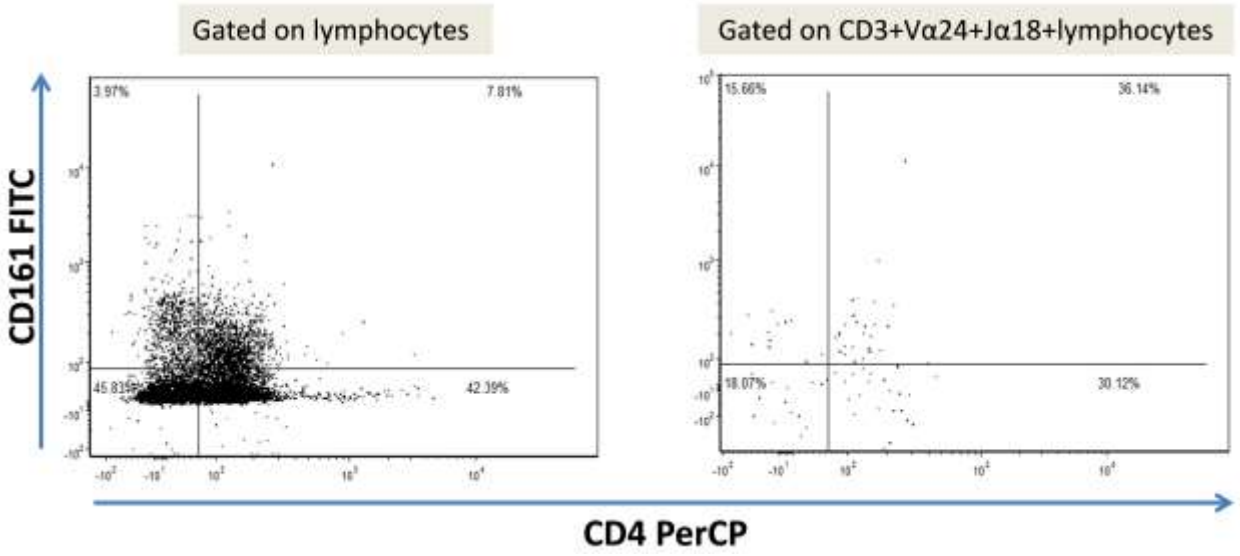
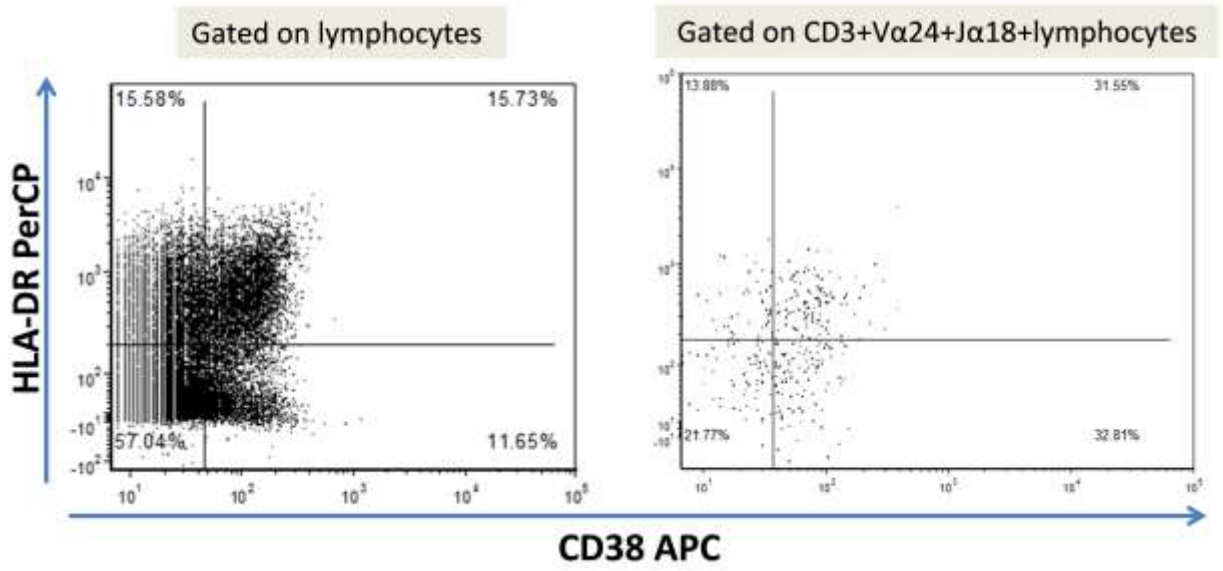

Gated on lymphocytes
Gated on CD3+Vα24+Jα18+lymphocytes
CD161 FITC
CD4 PerCP
3.97%
7.81%
45.83%
42.39%
15.66%
36.14%
18.07%
30.12%

Fig S2

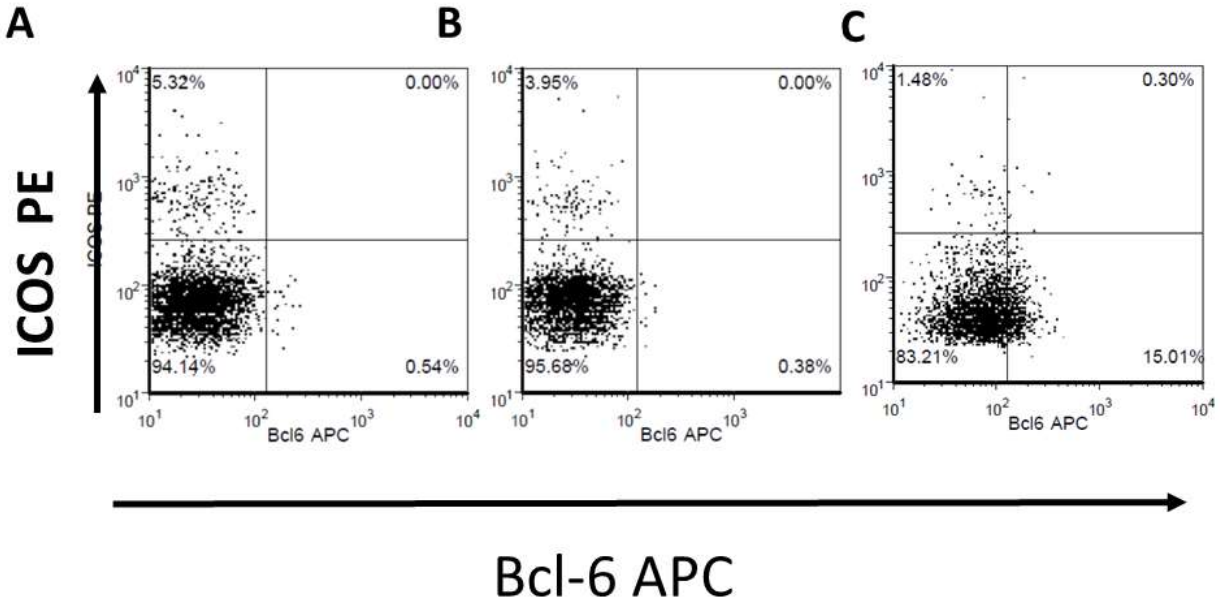


1

2 Fig S3

3

4



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6 Fig S4

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8

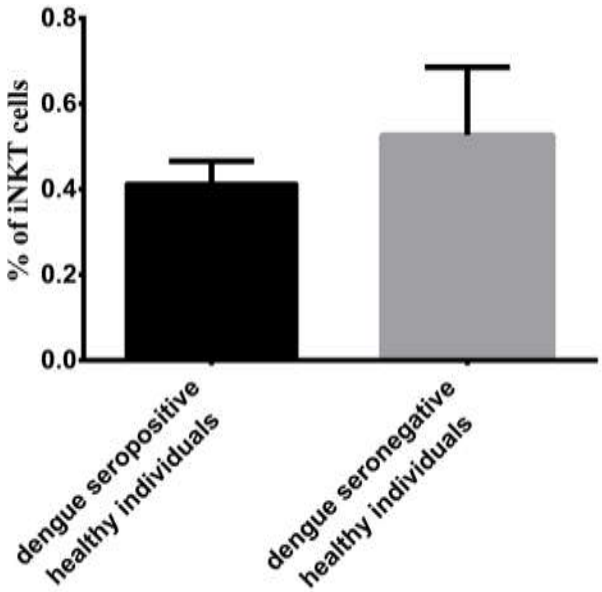
0.8
0.6
0.4
0.2
0.0
% of iNKT cells
dengue seropositive healthy individuals
dengue seronegative healthy individuals

Fig S5