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Expansion of highly activated invariant natural killer T cells with altered phenotype in acute dengue infection

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Summary

Invariant natural killer T (iNKT) cells 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 characterize further 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 interferon (IFN)-γ and interleukin (IL)−4 *ex-vivo* enzyme-linked immunospot (ELISPOT) assays following stimulation with alpha-galactosyl-ceramide (αGalCer). We found that circulating iNKT cell proportions were significantly higher ($P = 0{\cdot}03$) 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 correlated significantly with dengue virus-specific immunoglobulin (Ig)G antibody levels. iNKT cells expressing Bcl-6 ($P = 0{\cdot}0003$) and both Bcl-6 and inducible T cell co-stimulator (ICOS) ($P = 0{\cdot}006$) were increased significantly 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 more than 125 countries, causing symptomatic disease in approximately a quarter of individuals infected [2]. Seventy per cent of the estimated total of 390 million dengue infections occur in Asia, and more than 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 countries with the current highest disease burden, while countries which are currently free from dengue still have a potential risk of being affected [2]. Currently, there is no specific treatment for dengue, nor a licensed vaccine, although several dengue vaccines are in the pipeline [4].

Acute dengue infection may be caused by any four of the dengue viruses (DENVs), which are related very closely 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 interferon (IFN)-γ 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 the rapid

activation and production of large quantities of cytokines in the pathogenesis of acute dengue infection.

Invariant natural killer T (iNKT) cells 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 iNKT cells comprises the alpha chain Vα24-Jα18, which is paired with Vβ11 [10]. The role of iNKT cells has been investigated in many viral infections, such as HIV, herpes simplex virus (HSV), hepatitis [10], influenza [11] and more recently in dengue infection [12]. In dengue infection, iNKT cells were shown to be highly activated, and were associated with clinical disease severity [12]. Interestingly, the functionality of iNKT cells in patients with milder forms of dengue such as DF was found to be different to that in patients with DHF, with iNKT cells of patients with DF producing more IFN-γ than interleukin (IL)-4 [12].

iNKT cells 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 $CD8\alpha^+$ are considered predominantly to be cytotoxic and CD4/CD8 double-negative iNKT cells are thought to produce mainly T helper type 1 (Th1) cytokines, $CD4^+$ iNKT cells are thought to produce predominantly Th2-type cytokines [13]. In addition, $CD4^+$ iNKT cells have been shown to play a significant role in the 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, $CD4^+$ iNKT cells 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 mouse models. The inflammatory responses, vascular leak and liver injury were shown to be fewer in mice that were depleted of iNKT cells [17]. Because iNKT cells are known to have diverse functions and are activated rapidly, investigating their function in acute dengue viral infections would be crucial to understand more clearly the role of these cells in the protection or pathogenesis of dengue infection.

In this study, we found that the proportion of $CD4^+$ iNKT cells was increased in those with acute dengue infection and we observed reduced expression of CD161. The iNKT cells were highly activated, and iNKT activation correlated significantly with DENV-specific immunoglobulin (Ig)G 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 cells 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

Forty-nine 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 into the study. Blood samples were collected during days 5–7 of illness (day 1 was considered the 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 patients' discharge from the hospital in order to determine the severity of dengue infection. The severity of acute dengue infection was classified according to the 2011 World Health Organization (WHO) guidelines [18]. All patients who were classified as having DHF either had clinical evidence of fluid leakage or a rise of haematocrit of $>20\%$ of the baseline level. Accordingly, 24 patients had DHF and 25 patients had DF. Five of 24 patients with DHF developed shock, as evidenced by a pulse pressure of ≤ 20 mmHg along with pleural effusions and ascites. Four patients had bleeding manifestations.

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

Ethics statement

The 49 adult patients with clinical features compatible with acute dengue infection were enrolled into the study following informed written consent. Ethical approval was granted by the Ethical Review Committee of the University of Sri Jayawardanapura. All healthy individuals ($n = 22$) also participating in the study gave informed written consent.

Flow cytometry for identification of iNKT cells

Freshly isolated peripheral blood mononuclear cells (PBMCs) were used in all experiments and staining for flow cytometry was performed immediately following PBMC separation. 7-Aminoactinomycin D (7AAD) (Biolegend, San Diego, CA, USA), was used initially to determine the percentage of dead cells. However, as fresh samples were used we found that the percentage of dead cells was negligible. Cells were first washed in cell staining buffer (Biolegend) and blocked with an Fc receptor blocker (Biolegend) prior to surface staining. Anti-Vα24-Jα18 phycoerythrin (PE) mononuclear antibodies (mAbs) (IgG1, clone 6B11) and CD3 antigen-presenting cells (APC) (IgG2a, clone OKT3) (Biolegend) were used to identify the iNKT cell population (defined as $CD3^+$ and $V\alpha24\text{-}J\alpha18^+$ in all patients and controls). Isotype controls for Vα24-Jα18 (Biolegend) were also used. Cells were acquired on a Partec Cyflow Cube 6 and analysed 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 the expression of CD38, human leucocyte antigen D-related (HLA-DR), CD161, CD4 and CD8α on iNKT cells. The following antibodies were used for phenotyping: anti-Vα24-Jα18 mAbs PE (IgG1, clone 6B11), CD38 APC (IgG1, clone HB-7), HLA-DR peridinin chlorophyll (PerCP) (IgG2a, clone L243), CD161 fluorescein isothiocyanate (FITC) (IgG1, clone HP-3G10), CD8α FITC (IgG1, clone HIT8a), CD4 PerCP (IgG2b, clone OKT4), CD3 APC and CD3 FITC (IgG2a, clone OKT3; Biolegend). In order to determine if the iNKT cells were of follicular iNKT cell phenotype, surface staining for inducible T cell co-stimulator (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) staining was performed in 15 patients and 10 healthy individuals. Appropriate conjugated isotype-matched controls were included (Biolegend). The gating strategy of CD161, CD8a, CD161, HLA-DR CD38, ICOS and Bcl-6 is shown in Supporting information, Figs S1–S4.

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 performed in the same samples obtained for flow cytometry and the total lymphocyte counts were available to us, the absolute NK T cell numbers were then calculated after gating on the lymphocytes in the forward- (FSC) and side-scatter (SSC) views.

Functional assays for iNKT cells

Ex-vivo enzyme-linked immunospot (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 described previously [19,20]. ELISPOT plates (Millipore Corporation, Billerica, MA, USA) were coated separately with anti-human IFN-γ antibody and anti-human IL-4 antibody (Mabtech, Nacka Strand, Sweden) overnight. Freshly isolated PBMC (5×10^5/well) were incubated with 100 ng/ml KRN7000 (Cayman Chemicals, Ann Arbor, MI, USA) overnight for IFN-γ detection and for 48 h for IL-4 detection at 37°C and 5% CO_2. All experiments were performed in duplicate. Phytohaemagglutinin (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 antibody to human IFN-γ and washed a further six times. The plates were developed with streptavidin–alkaline phosphatase (Mabtech) and colorimetric substrate, and the spots enumerated using an automated ELISPOT reader. Background (cells plus media) was subtracted and data expressed as the 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 had only 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 of 49 patients had a primary dengue infection (only IgM-positive) and 30 patients had a secondary dengue infection. Early dengue NS1 capture ELISA (Panbio) was also carried out in all patients according to the manufacturer's instructions.

Statistical analyses

Data analysis was performed using GraphPad Prism version 6·0 software. As the data were not distributed normally, differences in means were compared using the Mann–Whitney two-tailed *t*-test. The results were expressed as the median and the interquartile range (IQR). To determine positive and negative associations, the Spearman's two-tailed correlation test was used.

Results

The clinical and laboratory characteristics of the 49 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 were thus classified has having DHF based on the WHO guidelines [18]. Three of the patients classified as having DF had platelet counts of $<25\ 000$ cells/mm^3, but did not have clinical or laboratory evidence of fluid leakage. Twenty of 49 (40·8%) patients were positive for NS1 antigen at the time of recruitment.

High peripheral iNKT cell frequency in dengue patients

Because the monoclonal antibody from clone 6B11 was shown to bind specifically to the invariant loop of the Vα24-Jα18 rearranged T cell receptor (TCR) chain of human NK T cells, and has been used extensively to characterize iNKT cells in humans [22–24], we identified iNKT cells as being positive for both CD3 and 6B11 staining (Fig. 1a). We found that the percentage of the iNKT cell population was significantly higher ($P = 0{\cdot}03$) in patients with acute dengue (median = 0·5%, IQR = 0·31–1·01% 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

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)
Leucopenia	19 (79·2)	23 (92·0)
Platelet counts on admission (cells/mm^3)	119·5 (IQR 87·5–145)	93·5 (IQR 50–130·5)
Lowest platelet count (cells/mm^3)	58,000 (IQR 34·5–104·5)	25,000 (IQR 18·5–38·75
Lowest white cell count (cells/mm^3)	2,454 (IQR 1·6–3·7)	2·2 (IQR 1·7–2·9)
Lowest lymphocyte count (cells/mm^3)	1·62 (IQR 0·97–10·7)	9·3 (IQR 0·88–28·8)

DF = dengue fever; DHF = dengue haemorrhagic fever; IQR = interquartile range.

patients with DHF (median = 0·47%, IQR = 0·27–0·88% of CD3$^+$ cells) when compared to those with DF (median = 0·65%, IQR = 0·34–1·03% 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 (median = 0·46%, IQR = 0·3–1·27%) and those who were NS1 antigen-negative (median = 0·55%, IQR = 0·32–0·84) at the time of recruitment.

As the percentage of iNKT cells would depend upon these patients' lymphocyte counts, we then determined the differences in absolute iNKT numbers in patients with varying severity of dengue infection. The absolute iNKT cell numbers were 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 = 3·04–27·01 cells/mm^3) compared to patients (median = 7·98, IQR = 4·2–14·76 cells/mm^3). However, the median absolute lymphocyte count was almost double in healthy individuals (median = 3115, IQR = 2863–3651 cells/mm^3) than in patients (median = 1410, IQR = 1000–2445 cells/mm^3), 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 = 4·5–14·8 cells/mm^3) and DF (median = 8·3, IQR = 4·8–14·8 cells/mm^3) and also between those with primary (median = 7·8, IQR = 4·5–13·9 cells/mm^3) and secondary dengue infection (median = 11·3, IQR = 3·55–16·7 cells/mm^3) 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 then investigated the iNKT cell subsets in patients with acute dengue. We found that the CD4$^+$ iNKT cell subset was expanded significantly ($P = 0·01$) in patients with acute dengue (median = 55·14, IQR = 38·98–64·71% of iNKT cells) compared to healthy individuals (median = 36·4, IQR = 21·76–50·91% of iNKT cells). The frequency of CD4$^-$CD8$^-$ (DN iNKT cells) was reduced significantly ($P < 0·0001$) in patients with acute dengue (median = 1·5, IQR = 0–9·3% of iNKT cells) when compared to healthy individuals (median = 13·32, IQR = 8·5–28·3% of iNKT cells). The frequency of CD8α$^+$ iNKT cells was also reduced significantly ($P < 0·0001$) in patients with acute dengue (median = 0·49, IQR = 0–3·8% of iNKT cells) when compared to healthy individuals (median = 17·4, IQR = 9·9–22·3% of iNKT cells) (Fig. 2a).

Of the 19 patients in whom we were able to phenotype iNKT cells, seven 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 = 42·0–69·8% of iNKT cells) when compared to those with DHF (median = 47·7, IQR = 17·1–51·3% of iNKT cells) (Fig. 2b). In contrast, the frequency of iNKT cells of the CD8α$^+$ phenotype was lower in patients with DF (median = 0·1, IQR = 0–0·9% iNKT cells) when compared to those with DHF (median = 3·8, IQR = 0–6·3% 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.

iNKT cells are highly activated but were predominantly of the immature phenotype

We then characterized the expression of activation markers on iNKT cells 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 = 4·7–31·5% of iNKT cells) when compared to healthy individuals (median = 4·4, IQR = 3·7–5·6% 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.

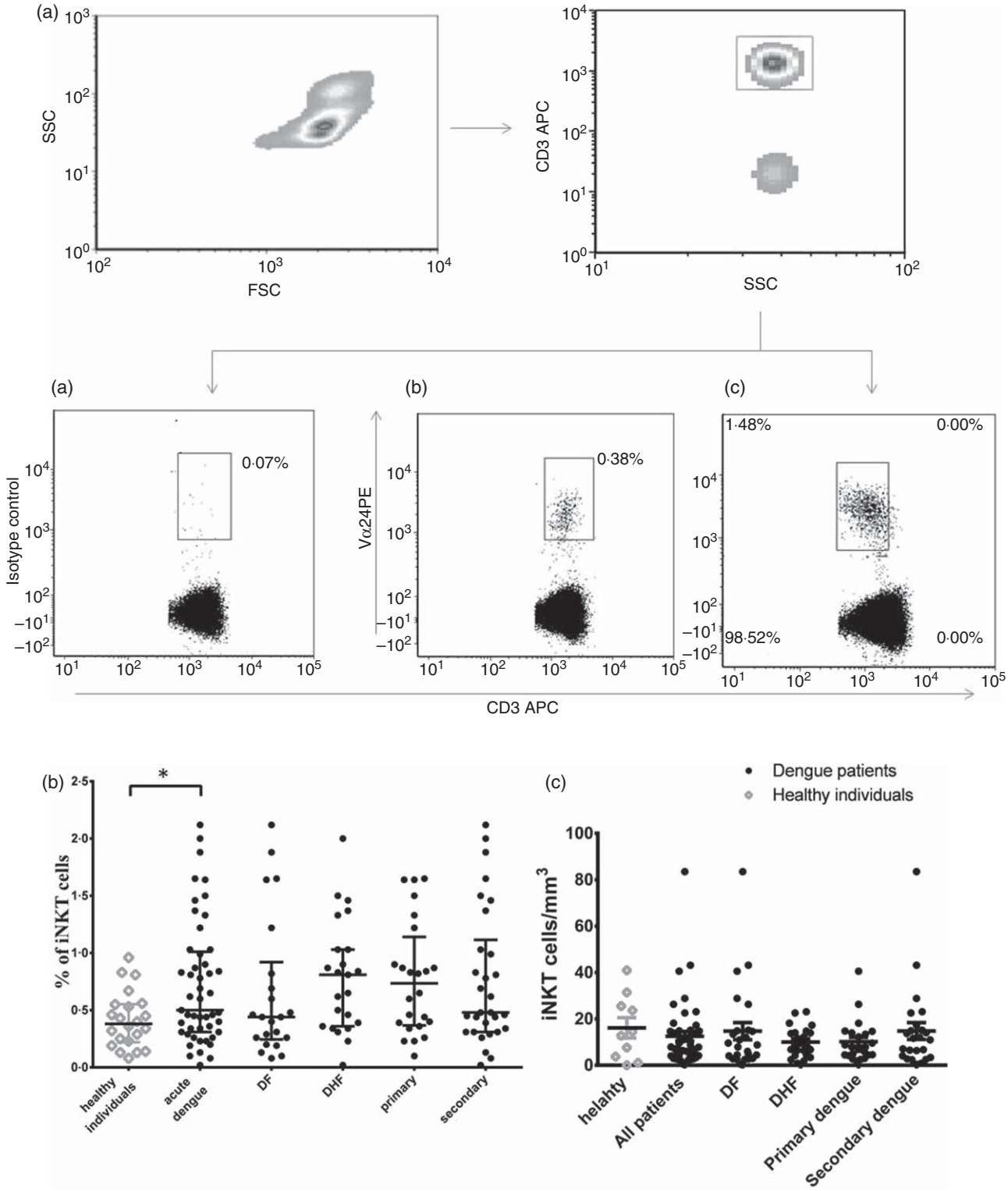

Fig. 1. Percentage of invariant natural killer (iNK) T cells in dengue patients. Peripheral blood mononuclear cells were stained and analysed by flow cytometry. (A) Representative gating strategy for iNKT cells based on CD3 and Vα24-Jα18 expression, showing isotype control (a) healthy individual iNKT (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 dengue haemorrhagic fever (DHF) ($n = 24$) and dengue fever (DF) ($n = 25$) and primary ($n = 19$) and secondary dengue infections ($n = 30$). The lines display the median and the interquartile 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 interquartile ranges. $^*P = 0{\cdot}03$.

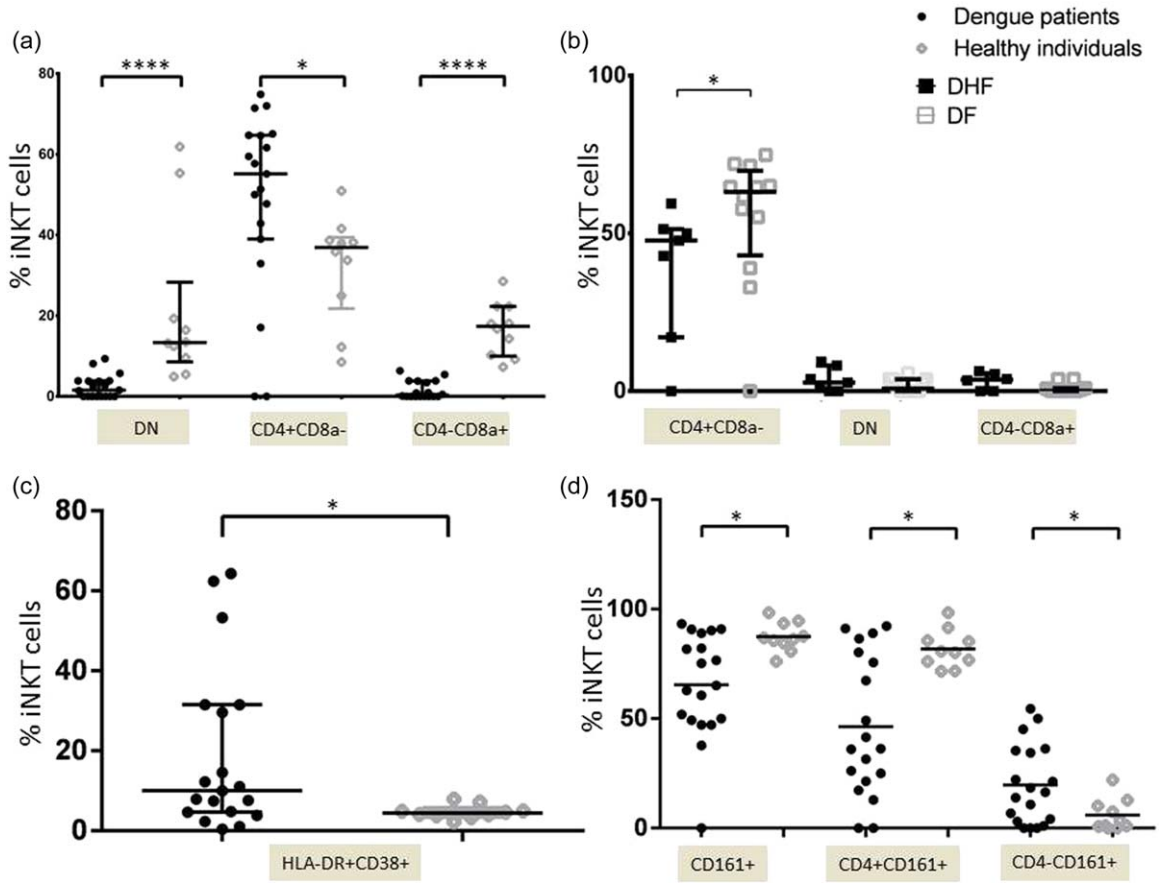

Fig. 2. Phenotyping of invariant natural killer (iNK) T cells in patients with acute dengue. (a). iNKT cells were examined for expression of CD4 and CD8α by flow cytometry in 19 patients with acute dengue infection and 12 healthy individuals; $^{*}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$. (b) iNKT subsets based on CD4 and CD8α were examined in dengue fever (DF) ($n = 12$) and dengue haemorrhagic fever (DHF) ($n = 12$); $P < 0{\cdot}05$. (c) iNKT cells were examined for expression of human leucocyte antigen D-related (HLA-DR) and CD38 by flow cytometry in healthy individuals ($n = 12$) and patients ($n = 19$); $^{*}P < 0{\cdot}05$. The lines display the median and the interquartile ranges. (d) iNKT cells were examined for expression of CD161 by flow cytometry in healthy individuals ($n = 12$) and patients ($n = 19$); $^{*}P < 0{\cdot}05$. The lines display the median and the interquartile ranges.

CD161 is expressed on mature iNKT cells, and in certain infections such as HIV, CD161-expressing iNKT cells are deleted preferentially [14,25]· We found that the percentage of CD161 expression on iNKT cells was significantly lower ($P = 0{\cdot}01$) 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 = 83·68–93·81% of iNKT cells) (Fig. 2d). CD161 expression on $CD4^{+}$ iNKT cells was also significantly ($P = 0{\cdot}01$) lower in patients (median = 36·36%, IQR = 21·39–80·37% of iNKT cells) compared to healthy individuals (median = 80·52%, IQR = 75·07–87·18% 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 be due possibly to a preferential expansion of iNKT cells that do not express this marker.

Association of the frequency of iNKT cells with dengue antibody titres

Different iNKT cells 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 correlated significantly and positively with dengue IgG antibody titres (Spearman's $r = 0{\cdot}5043$, $P = 0{\cdot}03$) (Fig. 3a). However, there was no significant correlation between dengue IgG titres and $CD4^{+}$ CD8α- iNKT cells (Spearman's $r = 0{\cdot}0912$, $P = 0{\cdot}71$). The frequency of activated iNKT cells also correlated significantly with dengue IgG antibody titres (Spearman's $r = 0{\cdot}5018$, $P = 0{\cdot}03$) (Fig. 3b).

iNKT follicular helper cells are a subset of iNKT cells, which are $CD4^{+}$ and induce germinal centre formation

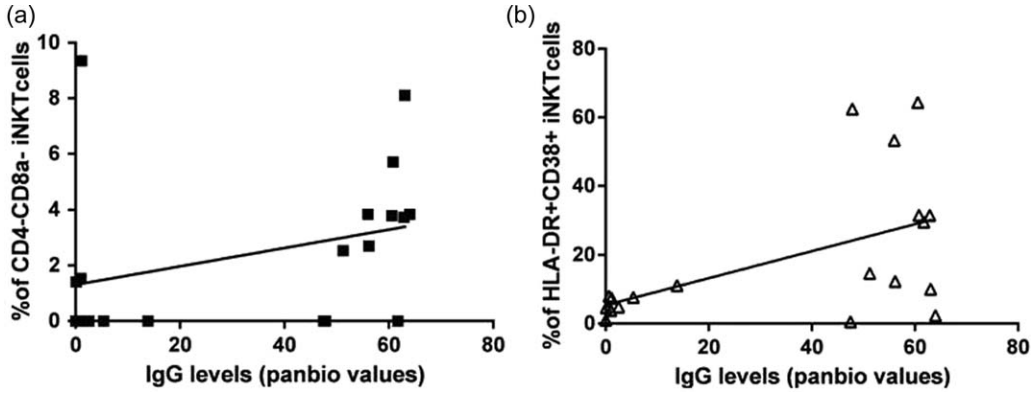

Fig. 3. Correlation of dengue virus (DENV)-specific immunoglobulin (Ig)G levels with the percentage of invariant natural killer (iNK) T cells. (a) Correlation of DENV-specific IgG antibodies (PanBio units) with the percentage of $CD4^{-}CD8^{-}$ iNKT cells as a total proportion of iNKT cells in patients with acute dengue ($n = 19$); $P = 0{\cdot}03$, Spearman's $r = 0{\cdot}5$. (b) Correlation of DENV-specific IgG levels with the percentage of human leucocyte antigen D-related $(HLA\text{-}DR)^{+}CD38^{+}$ iNKT cells as a proportion of the total iNKT cell population ($P = 0{\cdot}03$, Spearman's $r = 0{\cdot}51$).

and provide help to B cells that recognize lipid antigens [27]. This subset of follicular iNKT cells has been shown to express Bcl-6, which was shown to be important in up-regulating ICOS and certain chemokine receptors [27]. As we found that the frequency of iNKT cells co-expressing CD38 and HLA-DR correlated positively with dengue IgG antibody titres in acute infection, we determined 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 ($P = 0{\cdot}003$) and cells co-expressing Bcl-6 and ICOS is increased significantly ($P = 0{\cdot}0006$) 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 ($P > 0{\cdot}05$) correlation between dengue specific IgG antibody titres (Panbio units) in patients with acute dengue. Therefore, although the frequency of iNKT follicular helper cells was increased significantly 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 iNKT cells in patients with acute dengue differed in those with DHF and DF [12]. Using *ex-vivo* IFN-γ and IL-4 ELISPOT assays, we then determined the functionality of iNKT cells in patients with acute dengue when compared to healthy individuals following alpha-galactosylceramide (α-GalCer) stimulation. As reported previously, the mean IFN-γ ELISPOT responses were lower in patients with acute dengue (mean = 78·4, median = 40, IQR = 2·25–93 spot-forming units/PBMCs) when compared

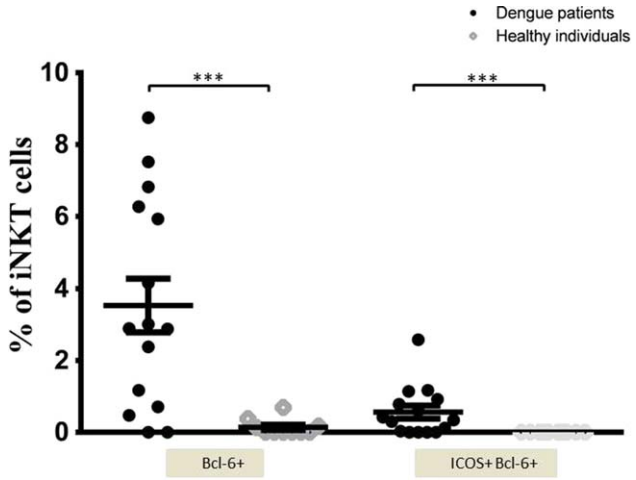

Fig. 4. B cell lymphoma 6 (Bcl6) and inducible T cell co-stimulator (ICOS) expression by invariant natural killer (iNK) T cells. iNKT cells were examined for expression of Bcl-6 and ICOS by flow cytometry in healthy individuals ($n = 10$) and patients ($n = 15$); $^{***}P < 0{\cdot}001$. The lines display the median and interquartile ranges.

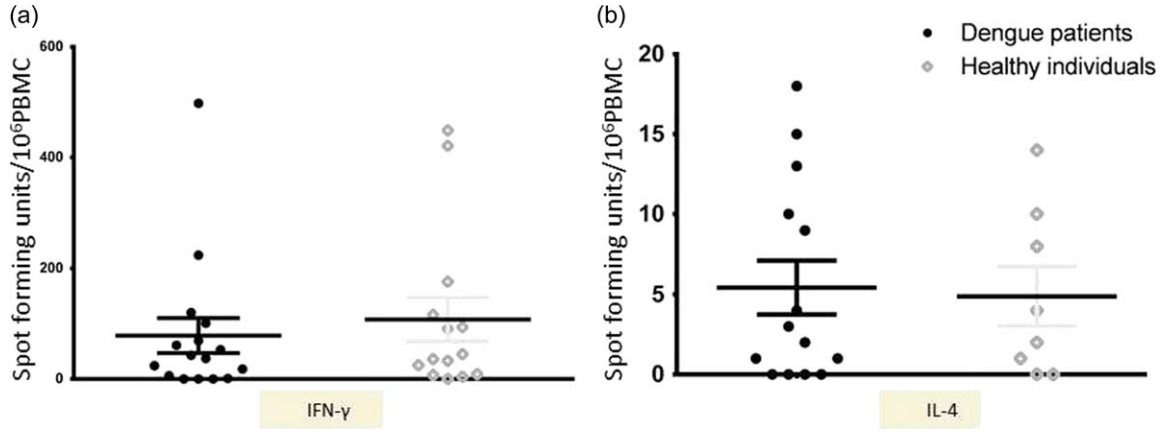

Fig. 5. *Ex-vivo* interferon (IFN)-γ and interleukin (IL)−4 enzyme-linked immunospot (ELISPOT) responses in patients with acute dengue. (a) Production of IFN-γ by invariant natural killer (iNK) T cells was examined by *ex-vivo* IFN-γ ELISPOT assays in response to alpha-galactosyl-ceramide (αGalCer) in healthy donors ($n = 14$) and patients ($n = 16$); $P = 0{\cdot}5$. (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 interquartile ranges.

to healthy individuals (mean = 107·6, median = 40·5, IQR = 8·5–131 SFUs to stimulation with α-GalCer was also not significantly different ($P = 0.98$), in patients (mean-= 5·4, median = 2·5, IQR = 0–10·75 SFUs/1 million PBMCs) when compared to healthy individuals (mean-= 4·7, median = 3·0, IQR = 0·25–9·5 SFUs/1 million PBMCs) (Fig. 5b). Although IFN-γ is thought to be produced by Th1 iNKT cells and IL-4 by Th2 iNKT cells, no association was observed between *ex-vivo* IFN-γ or IL-4 ELISPOT responses (Spearman's $r = 0{\cdot}3899$, $P = 0{\cdot}3024$).

Discussion

In this study we found that the frequency of circulating iNKT cells was significantly higher ($P = 0{\cdot}03$) in adult patients with acute dengue infection, which was evident within the $CD4^+$ subset. This is in contrast to a previous 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, like lymphocytes, innate, able to activate and produce large quantities of cytokines rapidly 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 IFN-γ 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 a role in the background of impaired anti-viral 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 IFN-γ than IL-4 when compared to those with DHF [12]. Therefore, iNKT cells could be playing a significant role in anti-viral defences against the DENV.

iNKT cells are activated by either ligand-dependent mechanisms or independent mechanisms which, for example, can involve expansion and activation of iNKT cells by cytokines such as IL-18, IL-12 and type I IFNs [10,30]. Elevated type I IFNs and cytokines such as IL-12 have been 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 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 determine if natural lipids activate iNKT cells in dengue and also if CD1d up-regulation occurs.

Based on the expression of CD4 and CD8α, different subsets of iNKT cells have been identified [34] and $CD4^+$ iNKT cells have been 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 expanded significantly in acute dengue infection and also expanded significantly 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 depleted preferentially in patients with HIV [36,37] and then restored following anti-retroviral therapy [14]. Therefore, it is possible that the $CD4^+$ subset of iNKT cells is playing a protective role in acute dengue, as 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 IFN-γ 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 and are thought to produce IL-17 [39]. CD161 has been shown to be expressed preferentially 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 fewer 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 activated significantly more in patients with acute dengue than in healthy individuals, as reported previously [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 correlated significantly and positively with DENV-specific IgG antibody titres (Spearman's $r = 0{\cdot}5018$, $P = 0{\cdot}03$). Our subsequent experiments showed that 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]. Conversely, iNKT cells are also known to require B cell help to produce cytokines such as IFN-γ, TNF-α, IL-2 and IL-10 [40]. Although all subsets of iNKT cells have shown to activate B cells to produce antibodies in turn, 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 expanded significantly 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 correlated significantly and positively with DENV-specific IgG antibody titres. Therefore, our results suggest that iNKT cells might play an important role in the pathogenesis of dengue, which should be investigated further.

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Disclosure

The authors have disclosed no disclosures.

Author contributions

A. K., T. N. A. and L. G. performed the experiments, G. N. M., G. S. O., M. S. and V. C. designed the study, N. W., N. L. A. S. were involved in patient recruitment, A. K. and G. N. M. analysed the data, A. K., G. N. M., G. S. O. and M. S. wrote the paper.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Representative plots showing gating pathway of determining the frequency of invariant natural killer (iNK) T cells expressing CD4 and CD8α markers.

Fig. S2. Representative plots showing gating pathway of determining the frequency of invariant natural killer (iNK) T cells co-expressing CD161 and CD4 markers.

Fig. S3. Representative plots showing gating pathway of determining the frequency of invariant natural killer (iNK) T cells co-expressing human leucocyte antigen D-related (HLA-DR) and CD38 markers.

Fig. S4. Representative plots showing gating pathway of determining the frequency of invariant natural killer (iNK) T cells co-expressing Bcl-6 and inducible T cell co-stimulator (ICOS) markers.

Fig. S5. Graph showing percentage of invariant natural killer (iNK) T cells in dengue seropositive ($n = 22$) and seronegative ($n = 3$) healthy individuals