Original Article
Cellular immune response of dengue virus infection at different phases

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Abstract: Dengue fever is one of the major human infectious diseases in tropical and subtropical areas every year. In the present article, blood samples were collected from patients in hospital of Guangzhou city. Dengue patients were determined at the IgM⁻IgG⁻, IgM⁺, IgM⁺IgG⁺ and IgG⁺ phases according to the secretion of IgM and IgG antibodies. The cellular characteristics of patients at these four phases were compared. Results showed that the counts of leukocyte and platelet at all four phases of illness were significantly lower than that in health control, with IgM⁺ phase showing the lowest counts among four phases. Moreover, white blood cell profile at each phase had different degrees of change. The percentage of CD3⁺ cells decreased significantly at four phases in comparison with healthy controls, and IgG⁺ phase exhibited the lowest among them. These patients at IgG⁺ phase exhibited enhanced production of Th1 cells compared with the other patients ($P<0.01$). The numbers of Th17 and Tregs cells were elevated at different phase in patients. Th17 cell appears to be associated with dengue fever.

Keywords: Dengue fever, peripheral blood, T cells, Th17 cells

Introduction

Dengue fever, an important and growing public health problem, is a mosquito-borne viral caused disease that spread very quickly, with a 30 fold increase in global incidence in the past 50 years [1, 2]. Tropical and subtropical regions are major sites of transmission with 70% of cases appearing in Asia, 14% in Americas and 16% in Africa [3]. Dengue virus has already presented in more than 120 countries and there is a fear for potential spread to non-endemic areas in near future [4]. Some of the postulated hypotheses on dengue immunopathogenesis include (i) the antibody enhancement theory, (ii) cross-reactive memory T cells activation and (iii) the original antigenic sin [5]. Various mechanisms have been proposed to explain the severe clinical presentations of dengue, the cellular and molecular mechanisms seem to be the most reliable hypotheses. Nevertheless, the research about cellular characteristics of dengue fever at different phases remains unclear till now.

Both T and B cell response can play a protective or pathogenic role in dengue virus infection. When dengue virus (DENV) enters into the circulation, it will encounter natural antibodies produced by B-1 cells [6], which produce polyspecific IgM. This IgM is present in the circulation and can bind to and neutralize the virus at very early stages, thereby controlling the infection [7]. Moreover, IgG response production following the presence of IgM antibodies are mainly IgG1 and IgG3 [8]. Thus, we divide the dengue fever infection into four phases depending on the antibodies that present in the whole phase of illness: IgM⁻IgG⁻ phase, IgM⁺ phase, IgM⁺IgG⁺ phase and IgG⁺ phase. Furthermore, the clinical parameters (age, white blood cells, neutrophils, lymphocytes, monocytes, and platelet count) were observed to study illness.

T cell activation is a critical event for an effective immune response against infection, including the production of cytokines. Several lines of evidence suggest that both CD4+ and CD8+T cells may contribute to protection against homologous re-infection or heterologous dengue infection [9]. Recently, another subset of T cells, called Th17 cells, are distinguished from Th1 or Th2 cells by their expression of IL-17A, IL-17F, IL-21, and IL-22 [10, 11]. They have an important role in host defense against infection, by recruiting neutrophils and macrophages to infected tissues [12, 13]. Moreover, in some diseases, Th17 cells can cooperate with Th1 cells for interferon-gamma production (IFN-γ), further contributing to the pro-inflammatory state [14]. Regulatory T (Treg) cells, defined by the expressions of CD4, CD25, and the transcription factor forkhead box P3 (FoxP3), can release anti-inflammatory cytokines - interleukin (IL)-10 and transforming growth factor [15]. They have anti-inflammatory effect and maintain the tolerance to self-components [16]. Some studies suggest that the balance between Th17 and Treg cells is important in the development/prevention of inflammatory and autoimmune diseases [17, 18].

In the present article, dengue patients were determined at the IgM-IgG-, IgM+, IgM+IgG+ and IgG+ phases according to the secretion of IgM and IgG antibodies. The cellular characteristics of patients in these four phages were investigated.

Materials and methods

Patients and samples

All patients had a clinical diagnosis of dengue fever according to the criteria of the World Health Organization/WHO/TDR-2009. Blood samples were obtained from 211 DENV infected patients at the Third Affiliated Hospital of Guangzhou Medical University and Traditional Chinese Medicine Hospital of Guangdong province. The characteristics and clinical features of patients were recorded. Thirty control samples were randomly selected from healthy individuals. Experimental work was approved by the ethical committee of the Institution and University Hospital, and informed consent was obtained from patients and controls.

Detection of dengue antigen

DV NS-1 antigen was detected by Diagnostic Kit for Dengue Virus Antigen (ELISA) (WANTAI Biological Pharmacy Enterprise, Beijing, China) as per manufacturer's instruction. 50 µl of sample serum and dilution were added to each well and the plate was incubated at 37° for 1 h. After five washes, 50 µl enzyme solution was added and incubated at 37° for 30 min. The plate was washed as mentioned above and the reaction was visualized by adding 100 µl TMB Substrate Reagent to each well for 10 min in the dark at room temperature. The reaction was stopped by adding 50 µl Stop Solution, and the absorbance of each well were measured at 450 nm using an ELISA plate reader (Model ELX-800; BioTeas).

Classification of infection by IgM and IgG antibodies

Anti DV IgM and IgG antibodies were detected by diagnostic kit for dengue IgG/IgM antibody (Colloidal Gold) (WONDFO, Guangzhou, China) as per manufacturer's instruction. In brief, add 10 µl serum in the round hole and 2 drops of buffer solution at the bottom of the hole in the kit. Read the result after 15 minutes. If the positive line appeared in the detection area, the result is determined as antibody positive, otherwise negative.

Antibodies

PerCP conjugated anti-human CD3 (SK7), FITC conjugated anti-human CD4 (SK3), PE conjugated anti-human CD8 (SK1), PerCP-cy5.5 conjugated anti-human CD4 (SK3), PE conjugated anti-human CD25 (2A3), Alexa Fluor® 647 conjugated anti-human Foxp3 (259D/C7), Alexa Fluor® 488 conjugated anti- human IL-17 (N49-653), APC conjugated anti-huaman IFN-γ (B27), and isotype-matched control monoclonal antibodies (X40) were purchased from BD/Pharmingen (San Diego, CA).

Isolation of lymphocytes

Blood samples from Dengue patients and healthy controls were collected in tubes containing 14.4 units of heparin per ml of blood (BD Biosciences), and then washed thrice in RPMI 1640. Lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation. Isolated cells were washed twice in Hanks' balanced salt solution and re-suspended at 2×10^6 cells/ml in complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 g/ml strepto-

Table 1. Hematological characteristics and clinical parameters of dengue patients and healthy controls

Characteristics	Health	Dengue infection
Gender (F:M)	18:12	97:87
Age in years	44.26±17.80	46.34±20.67
White blood cells (10^9/L)	7.27± 2.04	3.72±1.94**
Neutrophils (10^9/L))	4.25±1.56	2.05±1.68**
Neutrophils%	58.57±8.95	51.92±16.11*
Lymphocytes (10^9/L))	2.36±0.91	1.19±0.68**
Lymphocytes%	32.86±9.04	34.62±14.47
Monocyte (10^9/L))	0.42±0.15	0.41±0.19
Monocyte%	5.96±1.42	11.61±3.8**
Platelet (10^9/L)	249.79± 38.18	114.60±60.84**
P-LCR%	25.97±6.81	31.86±8.71**

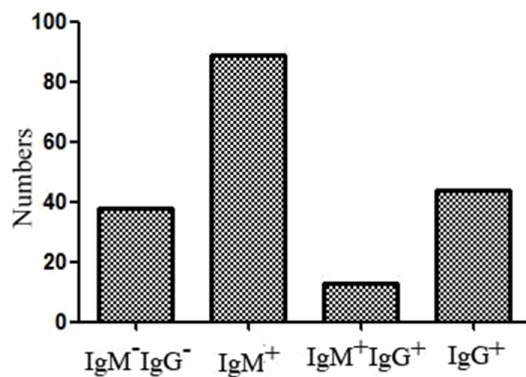Note: *$P<0.05$, **$P<0.01$, compared with healthy controls.

Figure 1. Different phases of disease determined by detection of IgM and IgG antibodies. Anti-dengue-virus IgM and IgG antibodies in serum of patients with positive DV NS-1 antigen were detected by immune colloidal gold technique. The phases of dengue virus infection were divided into four phases: IgM⁻IgG⁻ (38), IgM⁺ (89), IgM⁺IgG⁺ (13) and IgG⁺ (44).

mycin, 2 mM glutamine and 50 lM 2-mercaptoethanol and 50 lM 2-mercaptoethanol.

Surface and intracellular staining for T cells and subsets

Cells were stained for 30 min at 4°C in dark environment with conjugated antibodies that specific to the cell surface antigens CD3, CD4 and CD8. The expression phenotypes of antibody-labeled lymphocytes (200 000-300 000 cells per run) were analyzed by flow cytometry (BD Calibur and Aria II) and results were analyzed with FLOWJO version 6.0 (Tree Star Inc., Ashland, OR). Isotype-matched controls for cytokines were included in each staining protocol.

Detection of cell surface markers and intracellular cytokine expression

For detection of Th17 and Th1 cells, the peripheral blood mononuclear cells (PBMC) from patients and healthy controls were stimulated with 20 ng/ml PMA plus 1 lg/ml ionomycin for 5 h at 37° under a 5% CO_2 atmosphere. Brefeldin A (10 lg/ml; Sigma-Aldrich, St. Louis, MO) was added at 1 h after the incubation. Cells were washed twice in PBS, fixed with 4% paraformaldehyde and permeabilized overnight at 4°C in PBS buffer containing 0.1% saponin (SigmaAldrich), 0.1% BSA and 0.05% NaN_3. Cells were then stained for 30 min at 4°C in the dark with conjugated antibodies that specific to CD3, CD4, IFN-γ and IL-17 for detection of Th1 and Th17 cells, respectively, according to the manufacturer's protocol and analyzed with a FACS Calibur flow cytometer.

For detection of Treg cells, the Human Regulatory T Cell Staining Kit (eBioscience) was used. A single cell suspension was prepared, and 1×10^6 cells were surface stained with anti-CD3-PerCP mAbs (eBioscience), antiCD4-FITC mAbs and anti-CD25-APC mAbs, followed by fixation and permeabilization with Cytofix/Cytoperm and intracellular staining with anti-Foxp3-PE or IgG2a-PE human immunoglobulin control antibody, according to the manufacturer's protocol. Cells were gated on the CD3⁺CD4⁺CD25⁺FoxP3⁺ population for analysis of Treg cells.

Statistics

Data were analyzed using SPSS (v11.0). Statistical evaluation of difference between means was performed by unpaired, two-tailed Student's t-tests. $P<0.05$ was considered to be statistically significant.

Results

Characteristics of study population

Hematological characteristics and clinical parameters (gender, age, white blood cells, neutrophils, lymphocytes, monocyte and platelet count) of dengue patients and healthy controls are described in **Table 1**. The mean age and the male/female gender ratio were not statistically different between the two groups ($P>0.05$). The
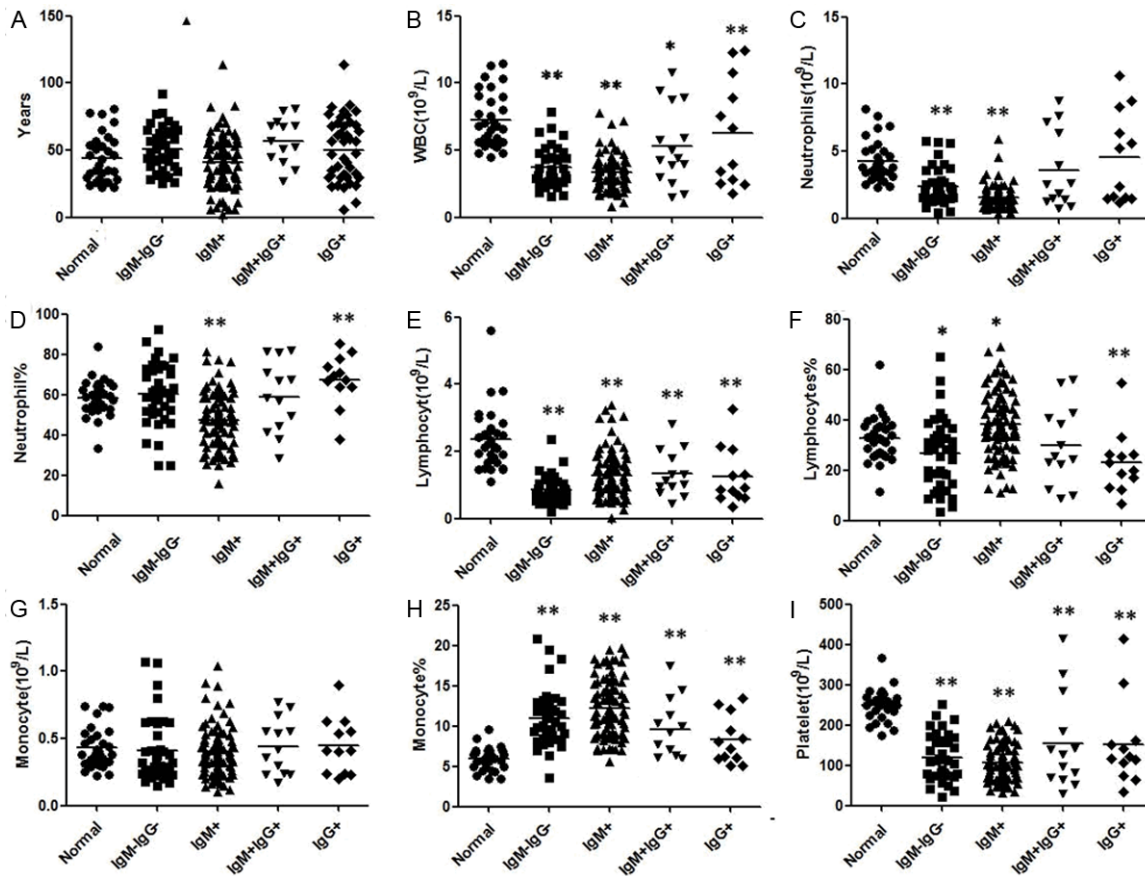
Figure 2. Clinical characteristics at different phases of dengue fever. The years (A), white blood cells (B), the absolute counts of neutrophils (C), the percentage of neutrophils (D), the absolute counts of lymphocytes (E), the percentage of lymphocytes (F), the absolute counts of monocyte (G), the percentage of monocyte (H) and count of platelet (I) in dengue at different phase of illness (*, $P<0.05$, **, $P<0.01$, compared with normal controls).

number of white blood cells in dengue patients was significantly lower than that in healthy controls (7.27±2.04 versus 3.72±1.94, $P<0.01$). Additionally, decreased numbers of neutrophils and lymphocytes were observed in dengue patients compared to healthy controls. There was no significant difference between the count of monocyte in the healthy control and dengue patients, whereas the proportion of that in dengue patients was increased compared to control group. Moreover, in parallel with the result obtained with white cells, the number of platelet decreased after infection (114.60±60.84 versus 249.79±38.18, $P<0.01$, **Table 1**).

Different phases of disease determined by detection of IgM and IgG antibodies

184 patients were diagnosed positive for DV NS-1 antigen by ELISA. Anti-dengue-virus IgM

and IgG antibodies in the serum of patients with positive DV NS-1 antigen were detected in our study cohort. Serologically, 102 patients were found to be IgM positive, and amongst them, 89 was IgG negative and the others were IgG positive. Moreover, 44 patients presented dengue specific IgG but no IgM. The phases of dengue virus infection were divided into four groups according to the presence of dengue specific IgM and IgG: IgM⁻IgG⁻, IgM⁺, IgM⁺IgG⁺ and IgG⁺. **Figure 1** shows dengue patient distribution at four phases. We opted to gain preliminary insights on differences in the characteristics at different phases of dengue virus infection.

Clinical characteristics at different phases of dengue fever

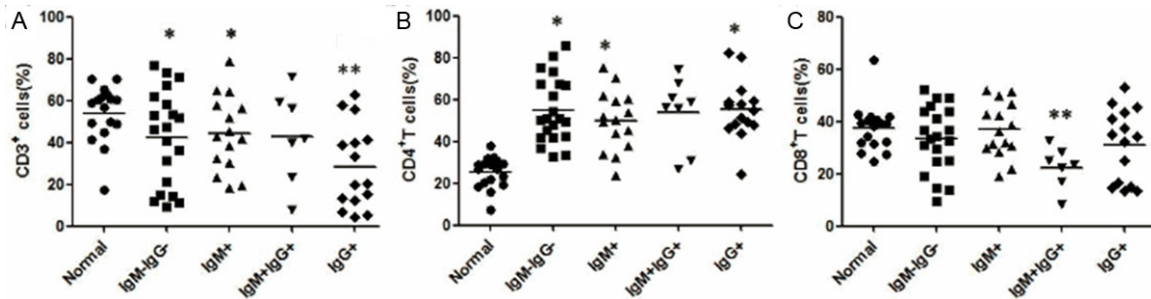To enable comparison of dengue responses at different phases, the levels of white blood cell

Figure 3. Detection of lymphocyte subpopulations at different phases of dengue fever. PBMC from 57 patients and 17 health controls were stained with anti-CD3, anti-CD4 mAbs and anti-CD8 mAbs and were analyzed by FCM. A. Percentage of the CD3+ cells; B. The percentage of CD3+CD4+T cells; C. The percentage of CD3+CD8+T cells (*, $P<0.05$, **, $P<0.01$, compared with normal controls).

profile and platelet were tested. Meanwhile, the white blood cell (WBC) profile (**Figure 2**) of this study population, including WBC, neutrophils, lymphocytes, monocytes, was tested. The count of white blood cells was lower in all phases during infection status (**Figure 2B**), with lowest level at IgM+ phase (IgM-IgG-: $3.72\pm1.47\times10^9$/L; IgM+: $3.31\pm1.24\times10^9$/L; IgM+IgG+: $5.07\pm3.07\times10^9$/L; IgG+: $6.30\pm4.02\times10^9$/L; IgM+ versus IgM+IgG+, $P<0.01$; IgM+ versus IgG+, $P<0.01$). The number and percentage of neutrophils at the IgM+ phase were significantly lower than that of normal controls, IgM-IgG-, IgM+IgG+ and IgG+ ($P<0.01$). Counts of lymphocytes were decreased in all four phases, whereas its percentage at IgM+ was significantly increased compared to normal control and other three phases (IgM+ versus control; $P<0.05$, IgM+ versus IgM-IgG-, $P<0.01$; IgM+ versus IgM+IgG+, $P<0.05$; IgM+ versus IgG+, $P<0.01$) (**Figure 2E** and **2F**). There was no significant difference in the counts of monocytes between health controls and infected patients, whereas the proportion of monocytes was significantly higher in the four subsets compared to the controls (**Figure 2H**, $P<0.01$). Furthermore, platelet count was decreased at all four phases of the disease ($P<0.01$). Hence, we conclude that white blood cell (WBC) profile was markedly influenced by infection.

Detection of lymphocyte subpopulations

PBMC from 57 patients (IgM-IgG-, 20; IgM+IgG-, 15; IgM+IgG+, 7; IgM-IgG+, 15) and 17 health controls were studied for T cell surface markers (**Figure 3**) by FACS. Positive gating for lymphocytes based on forward and side scatter was followed by CD3+, CD3+CD4+ and CD3+CD8+

gating (**Figure 3**). The percentage of CD3+T cells was lower at all four phases in the patients than that in the controls (*$P<0.05$, **$P<0.01$), although not statistically significant at IgM+IgG+ phase ($P>0.05$). The percentage of CD3+CD4+T cells was significantly higher at IgM-IgG- and IgG+ phases in the patients than that in the controls ($55.02\pm15.65\%$ versus $25.25\pm7.40\%$, $P<0.05$; $55.10\pm14.09\%$ versus $25.25\pm7.40\%$, $P<0.05$) while the percentages of CD3+CD8+ lymphocytes were stable at these two phases. Importantly, percentage of CD3+CD8+ were lower at IgM+IgG+ phase in patients than that in the controls (**$P<0.01$), whereas there were no significant differences between patients at the IgM-IgG-, IgM+, and IgG+ phases and controls ($P>0.05$).

Percentage of Th1, Th2 and Treg cells

To further explore the expression of different T subsets at different phases, lymphocytes were isolated from PBMC of normal controls and patients, and stimulated by PMA and Ionomycin. ICS (intracellular cytokines staining) was done and cells were detected by FACS. The proportions of IFN-γ+CD4+T cells was significantly higher (*$P<0.05$, **$P<0.01$) in overall dengue patients when compared to the controls (**Figure 4**), indicating a more vigorous inflammatory response in infections. Moreover, the proportion of IFN-γ+CD4+T cells during IgG+ phase was the highest among the four phases. Consistent with IFN-γ release (**Figure 4A**), a significantly higher percentage of IL-17+CD4+T cells during overall infection process was observed, whereas there was no significant difference between four phases (**Figure 4B**). As shown in **Figure 4C**, the frequency of Treg cells (CD4+CD25+FoxP3+T
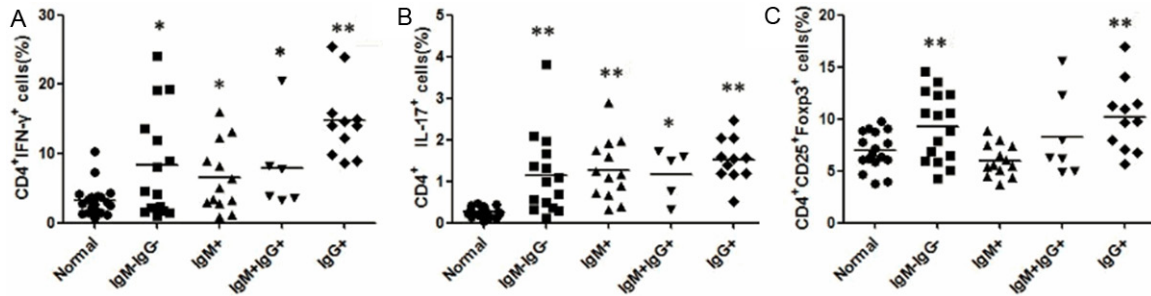
Figure 4. Expression of IFN-γ+CD4+T cells IL-17+CD4+T cells and CD4+CD25+FoxP3+T cells. PBMC from 57 patients and 17 health controls were stimulated with PMA, ionomycin and BFA. Cells were stained with anti-CD3-FITC, anti-CD4-Percp5.5, anti-CD25-APC and then intracellularly stained with PE-conjugated antibodies against IFN-γ, IL-17, FoxP3 for FACS analysis. Intracellular IFN-γ and IL-17 expression by gated populations of CD3+CD4+T cells during the course of infection. A-C. The percentages of Th1 (IFN-γ+CD4+T) cells, Th17 (IL-17+CD4+T) cells and Treg (CD4+CD25+FoxP3+T) cells (*, *P*<0.05; **, *P*<0.01).

cells) was significantly increased in the peripheral blood of patients at IgM-IgG- (9.28±3.29%) phase and IgG+ (10.16±3.52%) phase compared with those of healthy controls (6.92± 1.81%, *P*<0.01). Importantly, patients at IgM+ phase showed the lowest frequency of Treg cells among four phases (*P*<0.05, **P*<0.01).

Discussion

Dengue fever is one of the major human infectious diseases in tropical and subtropical areas every year. In the summer and autumn of year 2014, a severe outbreak of dengue fever emerged in Guangdong, china. Thousands were infected, and some people even lost life. NS-1 antigen is a special antigen for dengue-virus, which is usually used as a marker for DV detection, especially in PCR [19]. In the present article, samples with positive DV NS-1 antigen were collected to investigate the cellular characteristics.

It is reported that anti-dengue-virus IgM is detected typically about 5 days after fever onset and lasts for 2-3 months in a primary infection, while anti-dengue virus IgG with low titres was observed in 8-10 days after fever onset [20]. However, in secondary infections, anti-dengue-virus IgG evolves rapidly, with high titres soon after fever onset and anti-dengue-virus IgM can be undetectable in some cases. The dengue virus infection process was divided into four phases by the secretion of anti-dengue-virus IgM and IgG in our test - IgM-IgG-, IgM+, IgM+IgG+ and IgG+. IgM and IgG was undetectable for roughly 0-5 days after fever onset, which was the first phase during the infection

and was named IgM-IgG- phase in our test. The second period was the presence of IgM at about 5 days after fever onset, whereas anti-dengue virus IgG with very low titres. At the third phase, the IgM and IgG were both presented in the serum at about 10 days after fever onset. However, IgM was undetectable for 3 month after fever onset, whereas IgG was detectable. We measured the level of IgM and IgG in the serum of patients by immune colloidal gold technique to divide infection into four phases and analyzed the characteristic of pathogenesis and immunology. As shown in **Figure 1**, most patients investigated were at the second stage.

During this study, white blood cells and platelet were found to be decreased in patients with dengue, suggesting a complex regulation, consistent with previous reports [21]. Notably, dengue infection induced a much greater decrease in the counts of lymphocytes compared to the healthy controls (**Table 1**). The present statistics provided direct evidence that the counts of white blood cells and platelet were significantly lower in patients with IgM-IgG- and IgM+ when compared to patients with IgM+IgG+ and IgG+ (**Figure 2B**, **2I**), which reveals the decrease of white cells and platelet is one of the early and immediate events following viral infection and they have a relative recovery trend in the following periods. Meanwhile, we found out that patients at IgM+ exhibited a significant increase in frequencies of lymphocytes and monocyte when compared to patients with IgM-IgG-, IgM+IgG+ or IgG+; however, by comparing with healthy controls, the absolute counts of lymphocytes were still lower (**Figure 2E**, **2H**). These

results demonstrated that different characteristic of pathogenesis and immunology involved at different phases of dengue virus infection.

Some scholars have speculated that T cells contribute to disease pathogenesis by producing high level of pro-inflammatory cytokines [22, 23], which may have a dual role, both helping to clear the virus and causing bystander tissue damage [24]. The results showed that the frequency of CD3$^+$T cells was weakened significantly in PBMCs at IgM$^+$, IgM$^-$IgG$^-$, and IgG$^+$, and also there was a decrease in IgM$^+$IgG$^+$, although not statistically significant. Moreover, the IgG$^+$ phase showed a lowest percentage in the CD3$^+$T cells. The percentage of CD3$^+$CD4$^+$T cells was significantly higher in patients at the phases of IgM$^-$IgG$^-$, IgM$^+$, and IgG$^+$ than that in the controls, while the percentage of CD3$^+$CD8$^+$ lymphocytes showed a slight decrease during these phases by comparing with healthy controls. Notably, there is a significant decrease of CD3$^+$CD8$^+$ in the patients at IgM$^+$IgG$^+$ phase compared to the healthy controls and even the other three periods (**Figure 3**). These results indicated T cells especially CD4$^+$T cells might play important roles in the development of dengue fever. The decreasing in the percentage of CD3$^+$T cells might correlated with the increasing of B cells in PBMC, as reported that B cells might be the principal cells containing DENV RNA in peripheral blood [25].

Besides Th1 cells, other reports and researches demonstrated Th17 cells, preferentially secreting cytokine IL-17, might play an important role in mediating inflammation of some diseases, while there is little about dengue. IFN-γ and IL-17 were the classic cytokines secreted by T cell subsets -Th1 and Th17 cells, respectively. Role of IL-17 in infection of hepatitis C virus and Herpes Simplex Virus-1 has been suggested [26-28]. Human immunodeficiency virus type 1 (HIV1) and cytomegalo virus (CMV) specific IL-17 produced by CD4$^+$T cells was detected during HIV infection [29]. There is very little published information on the role of Th17 in dengue infections. IFN-γ and IL-17 were the classic cytokines secreted by Th1 and Th17 subset T cells [30, 31], respectively. In the current study, we compared IFN-γ and IL-17 expression in CD4$^+$T cells from the PMBCs of patients at different phases in response to nonspecific stimulation with PMA and ionomycin. As shown in **Figure 4**, secretions of IFN-γ and IL-17 by CD4$^+$T lymphocytes were significantly enhanced by infection at different phases, and the proportion of IFN-γ$^+$CD4$^+$T cells was the highest at IgG$^+$ phase among the four phases ($P<0.05$). Consistent with the IFN-γ expression in CD4$^+$Th cells, the percentages of IL-17$^+$CD4$^+$T in four phases were higher than that of the healthy controls. The result demonstrated that the proportion of Th1 and Th17 cells in the PBMCs increased continuously as over the course of infection, indicating that the infection induced the simultaneous generation of Th1 and Th17 cells. Importantly, Th1-polarized response reached a peak at the IgG$^+$ phase, which indicated that the ability of cellular immunity was increasing in this stage of infection, and meant the recovery of disease.

Tregs play a crucial role in the maintenance of immune homeostasis and it expresses the surface marker CD25 and the transcription factor Foxp3 [32]. By analyzing the fraction of Tregs cells, we did observe a significant up-regulation in patients at IgM$^-$IgG$^-$ phase but no significant up-regulation in IgM$^+$ and IgM$^+$IgG$^+$ phases. There was also a significant higher increase at IgG$^+$ phase (**Figure 4**). It is known that Th17 and Treg cells have opposite roles in the development of inflammatory diseases. While Th17 cells promote autoimmunity, Treg cells serve to control it and therefore play a very important role in autoimmune pathogenesis by maintaining self tolerance and controlling expansion and activation of auto-reactive CD4$^+$T effector cells [33]. However, this phenomenon was not found in our investigation, the mechanism needs more researches to clarify.

In summary, our data suggest that Th17 and Tregs cells appear to be associated with dengue fever, which were elevated at different phase (IgM$^-$IgG$^-$, IgM$^+$, IgM$^+$IgG$^+$ and IgG$^+$ phases) in patients.

Acknowledgements

Disclosure of conflict of interest

None.

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