CHAPTER III- CHARACTERIZATION OF CYTOKINES AND CHEMOKINES LEVELS IN SERUM OF DENGUE INFECTED PATIENTS.

3.1 Introduction

Infection caused by any of the DENV serotypes can cause a variety of clinical symptoms, including mild Dengue fever (DF), Dengue Hemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS) . The later two groups are severe types of Dengue fever caused by altered microvascular permeability. Fever, arthralgia, retro-orbital pain ,myalgia, and rash are symptoms of classic dengue fever. In severe dengue cases symptoms like vascular permeability, plasma leakage, thrombocytopenia, severe bleeding or organ impairment are reported[1]. In addition to the typical classical appearance, dengue infection can cause a variety of unusual clinical symptoms that are together referred to as expanded dengue syndrome (EDS). WHO introduced the term "EDS" in 2011 to describe the rare forms of dengue that cause significant harm to the kidneys, liver, heart, bone marrow, or brain. They could be connected to co-infections that go hand in hand, underlying comorbidities, or protracted shock. EDS is more common in high-risk populations, including young children, pregnant women, the elderly, people with hemoglobinopathies, coronary artery disease, and people with impaired immune systems[2, 3]. Uncertainties exist regarding the pathogenesis of DENV infections and the variables that lead to severe clinical illness. It is believed that a complicated interaction between the virus, host genetics, and host immunological mechanisms causes DHF/DSS[4]. The pathology of severe dengue, including DHF and DSS with hemorrhagic symptoms and enhanced capillary permeability, is directly impacted by platelet function. In the meantime, an increase in platelet count was found to be a sign of recovery whereas a decrease in platelets number is a predictive factor for severe dengue[5].

Several theories have been proposed regarding the immunopathogenesis of dengue, such as cross-reactive memory cell activation, the antibody enhancement theory, and the original antigenic sin. These theories all contribute to either an excess or an uneven cytokine profile which is why the term "cytokine storm" or "cytokine tsunami" is used[6, 7]. This cytokine storm increases capillary permeability and causes leakage, which directly affects the vascular endothelial cells[8].

Cytokines are small signaling proteins secreted by the cells that participate in interactions and communications between cells and help control inflammation. Since cytokines are unable to penetrate the lipid bilayer of cells and reach the cytoplasm, they usually connect

with certain cytokine receptors present on the surface of the cell to carry out their intended actions. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors. Chemokines are a specific subset of structurally similar cytokines. This is typically referred to as chemotactic cytokines or CHEMOtactic CytoKINES[9]. These proteins belong to a family of secreted low molecular weight proteins, some of which have many activities, but most of which are involved in leukocyte activation and migration[10]. Pro-inflammatory cytokines released in excess may increase vascular permeability, which in turn increases the severity of the illness. Increased vascular permeability can lead to shock, haemorrhage, and organ failure in addition to plasma leakage. Studies has shown that alongside pro-inflammatory cytokines, there was an increased in anti-inflammatory cytokines TGF-β and IL-10 [11, 12]. Uncontrolled inflammation aggravates pathogenesis, causes damage to tissues and organs, and ultimately results in death. Determining key pro- and anti-inflammatory cytokines from the onset of symptoms can predict the severity and outcome of the disease.

Considering that platelet count was one of the predictive markers of Dengue infection. We focused our study in analysis the correlation of serum cytokine and chemokine profile with platelet count. Given the genetic makeup of individuals from North eastern part of India is distinct [13, 14, 15] it was pertinent to study the severity of dengue in relation to key cytokine and chemokines in it.

As platelet count is one of the predictive markers of Dengue infection. We have focused our study in analysis the correlation of serum cytokine and chemokine profile with platelet count.

3.2 Materials and methods

3.2.1 Study design

- **Ethical approval-** Ethics approval of the study was obtained from both the Institutional Ethics Committee of Tezpur Medical College and Hospital and Gauhati Medical College and Hospital, India vide letter no. 18/IEC/TMCH, 127/2022/TMC&H and 190/2007/pt-11/MAR-19 respectively. Samples and clinical data were collected from collaborating hospitals after obtaining written informed consent and following SOPs /ICMR guidelines for sample collection.

- **Inclusion criteria-** Patients with clinically suspected dengue**,** confirmed by laboratory tests for DENV infection, patient with a fever history or current fever

- **Exclusion criteria-**

a. Dengue infection along with other flavivirus infections.

b. Other vulnerable patients like terminally ill/mentally challenged.

3.2.2 Study site and population

A hospital-based study was carried out with confirmed cases of dengue and non-dengue cases in the population of Assam. Samples were collected at collaborating hospitals GMC&H and TMC&H.

3.2.3 Sample collection and preparation

Sample collection-. Patient with 2 to 3 days post hospitalization were enrolled in the study. 2 ml of blood was collected after getting written consent. The serum was separated by centrifugation. The serum was aliquoted and stored at -20˚C for use in the study. In addition, Clinical data of Dengue-positive patients were collected.

We have assessed the cytokine profiles, both pro- and anti-inflammatory (IL1β, IL6, IL12p70, IL10, and TNF) and chemokines (CXCL10, IL8, CCL2, CXCL9and CCL5) in 61 NS1/IgM confirmed Dengue patients and 12 control participants.

3.2.4 Cytokine and Chemokine protein expression study by Cytometric bead array assay (CBA)

CBA is a bead-based captured array method in which a particular protein-capturing antibody is coupled onto the surface of each bead. This assay was used in bench top Flow cytometric platform for evaluating the profile of both inflammatory and anti-inflammatory cytokines (IL1β, IL6, IL12p70, IL10, and TNF) and chemokines (CXCL10, IL8, CCL2, CXCL9 and CCL5) in 61 hospitalized NS1/IgM confirmed Dengue patients and 12 control participants.

3.2.5 Determination of Monocyte and Dendritic cells population by Flow cytometry

Analysis of differential Monocyte (Classical, Intermediate and Non-Classical) and Dendritic (Classical and Plasmacytoid) cell (DC) population in DENV infected patients' blood has been studied using monocyte-specific cell surface marker such as CD14 and CD16 and Dendritic cell specific surface marker like CD123, CD11c and CD1c. Cell population were analysed using the Tabletop Flow Cytometer, BD Accuri C6 plus table-top Flow-Cytometer (BD Biosciences). 200µl of freshly collected whole blood samples were analysed in triplicate tubes. After proper RBC lysis, incubation with respective cell markers were performed.

3.2.6 Statistical analysis

Statistical analysis was conducted to compare Flow cytometric CBA protein level estimates of inflammatory cytokines and chemokines, alongside Flow cytometric percentage estimates of various monocyte and dendritic cell (DC) subsets, between Dengue and Control patients. Unpaired t-tests were performed using GraphPad Prism 10.1.0 to determine significance, with a threshold p-value of < 0.05. Co-relation and Principal Component Analysis were performed using XLSTAT.

3.3 Results

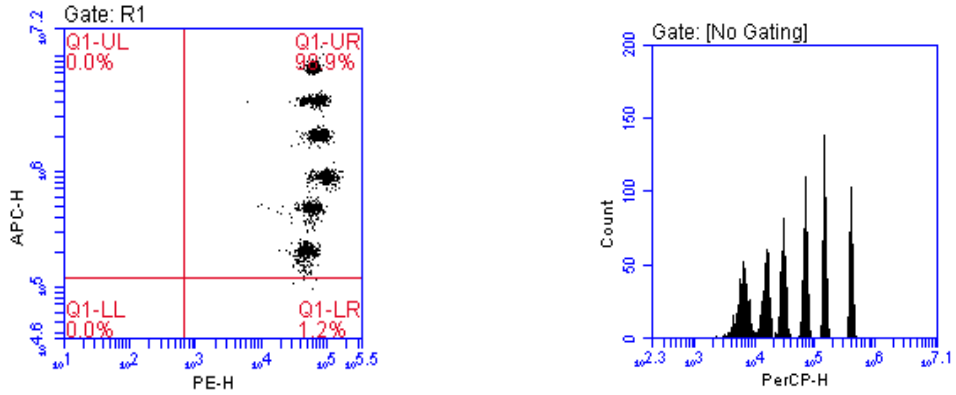
3.3.1 Demographic and clinical profiles of the study participants

The study participants comprised individuals from three linguistic groups namely Austro-Asiatic (AA), Indo-European (IE), and Tibeto-Burman (TB) populations with a median age of 28 years as shown in Table 3.1. The demographic profile and clinical profile of participants have been shown in Table 3.1. Male: Female gender ratio of our study population is 1.5:1. Every disease participant presented with a fever and symptoms like head ache and body ache were found to be common in our study population. Mean platelet count in disease was 1,06,900.

Characteristics	
Disease Profile	
Dengue positive	71.83%
Non-Dengue control	17.6%
Gender & ethnicity	
Male: Female gender ratio	1.5:1
Median Age	28
AA	9.21
TB	10.53
IE	80.26
Clinical profile	
Fever	100%
Headache	52%
Body ache	68%
Haemorrhagic manifestations (skin,oral,gut)	20%
Other symptoms (nausea, vomiting, loose stool dizziness, cough, heavy breathing)	44%
Mean Platelet count in Dengue patient	1,06,900

Table 3.1 Demographic and clinical profile of participants.

3.3.2 Cytokine expression



(A) (B)

Figure 3.1- A) Quantification of inflammatory cytokine proteins in healthy participant. Flow cytometry image showing results of bead-based capture of all the six cytokine proteins (IL-10, IL-12p70, TNF, IL-1β, IL-6, and IL-8) in an individual sample (B) and Fluorescence intensity of the proteins in that sample.

Expression of inflammatory cytokines were studied with the help of Human Inflammatory Cytokine Cytometric Bead Array Kit (CBA, BD Bioscience) using the bench top flowcytometry system, BD Accuri C6 Plus (BD Biosciences) (Figure 3.1). Mean levels of cytokines in case (n=61) and control serum (n=12) were compared (Figure 3.2). In comparison to healthy control we observed an increase in the levels of interleukin 10 (IL10), interleukin 6 (IL6) and interleukin 1β (IL1β) in DENV-positive patients (statistically not significant) which is suggestive of monocyte- neutrophil based activation of the innate immune system. A significant difference in mean expression level of IL12p70 was observed with a p-value < 0.0001 (Figure 3.2 A).

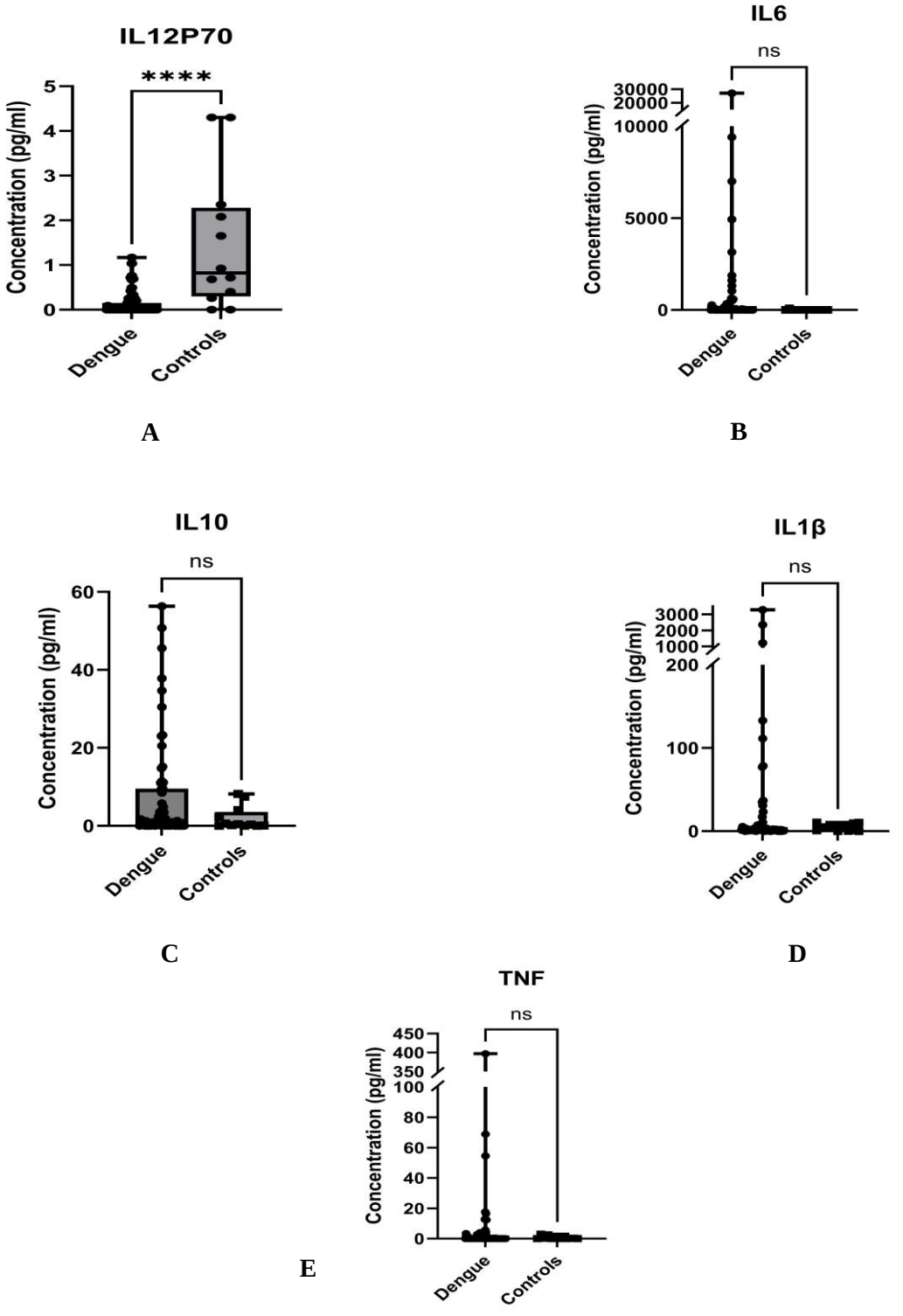
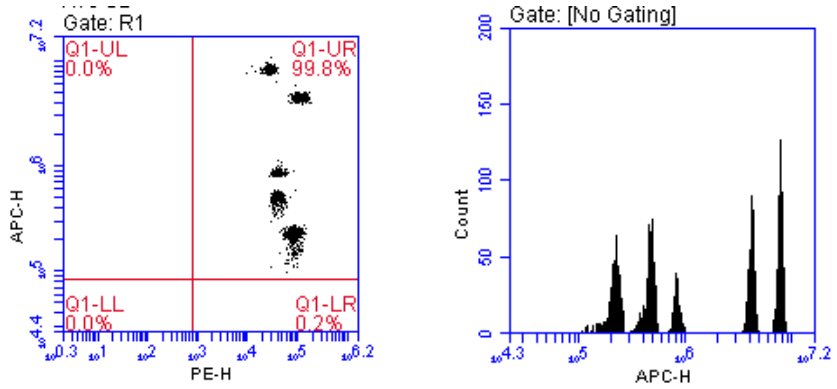


Figure 3.2 Expression of cytokines in healthy controls and dengue-positive subjects. Values indicate protein expression in pg/mL and error bars represent the standard deviation from the mean. Unpaired t-tests were performed using GraphPad Prism 10.1.0 to determine significance, with a threshold p-value of < 0.05.

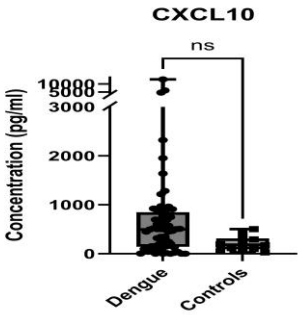
3.3.3 Chemokine expression



(a) (b)

Figure 3.3 a : **Quantification of chemokine proteins in healthy participant**. Flow cytometry image showing results of bead-based capture of all the five chemokines (CCL2, CCL5, IL8, CXCL0, and CXCL9) in an individual sample (b) and Fluorescence intensity of the proteins in that sample.

Expression of chemokines were studied with the help of Human Chemokine Cytometric Bead Array Kit (CBA, BD Bioscience) using the bench top flow cytometry system, BD Accuri C6 Plus (BD Biosciences) (Figure 3.3). Mean levels of chemokine in case (n=61) and control serum (n=12) were compared (Figure 3.4). High expression levels of CXCL10, CXCL9, CCL2 and IL8 were seen in the dengue patients (statistically not significant), whereas expression levels CCL5 were significantly lower in dengue patients with a p-value of <0.0001. High levels of CXCL10, CXCL9, CCL2 and IL8 suggests activation of the inflammatory axis as well as recruitment and activation of NK and T cells of adaptive immune response.



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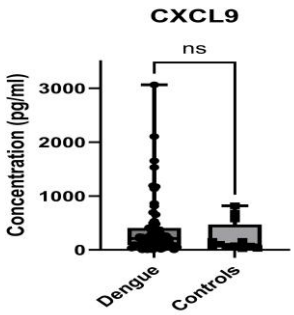

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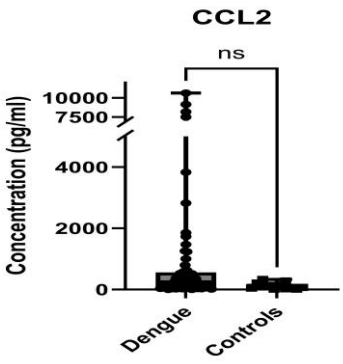

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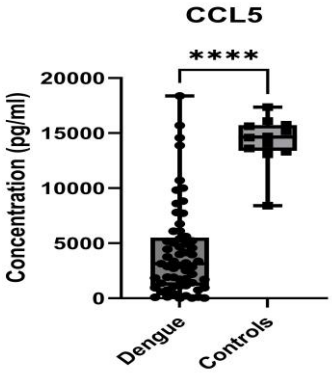

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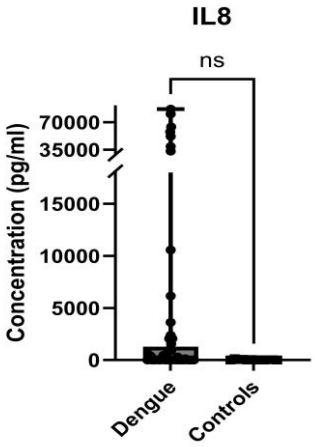


E

Figure 3.4. Expression of chemokines in healthy controls and dengue-positive subjects. Values indicate protein expression in pg/mL and error bars represent standard deviation from the mean. Unpaired t-tests were performed using GraphPad Prism 10.1.0 to determine significance, with a threshold p-value of < 0.05.

3.3.4 Correlation of cytokine and chemokine

Correlation analysis between the levels of inflammatory cytokines in patient serum revealed a significant positive correlation between IL10 ,IL6, IL1β and whereas no correlation was seen between IL12p70 and any of the studied cytokines (**Table 3.2)** A significant correlation between Platelet count and IL12p70 was observed with a p-value **0.002.**

Correlation analysis between the levels of chemokines in patient serum revealed a significant positive correlation between CXCL10 and CXCL9, CCL2 and IL8 whereas no correlation was seen between CCL5 and any of the studied chemokine (Table 3.2). A significant correlation between Platelet count and CCL5 was observed with a p-value **0.005.** A heat map representing the correlation between the studied cytokines, chemokines and platelet count is shown in Figure 3.5.

Variables	IL10	IL6	IL1β	TNF	CCL2	IL8	Platelet	IL12P70	CCL5	CXCL9	CXCL10
IL10	**1**	**0.349**	0.266	0.162	0.149	**0.366**	-0.039	-0.141	0.045	-0.080	0.084
IL6	**0.349**	**1**	**0.988**	**0.965**	**0.713**	**0.459**	-0.201	-0.109	-0.049	0.081	0.171
IL1β	0.266	**0.988**	**1**	**0.974**	**0.708**	**0.427**	-0.200	-0.096	-0.052	0.051	0.105
TNF	0.162	**0.965**	**0.974**	**1**	**0.760**	**0.423**	-0.223	-0.086	-0.119	0.063	0.093
CCL2	0.149	**0.713**	**0.708**	**0.760**	**1**	**0.804**	-0.266	0.009	0.100	0.028	0.033
IL8	**0.366**	**0.459**	**0.427**	**0.423**	**0.804**	**1**	-0.184	0.169	0.333	0.013	-0.083
Platelet	-0.039	-0.201	-0.200	-0.223	-0.266	-0.184	**1**	**0.510**	**0.461**	-0.081	-0.192
IL12P70	-0.141	-0.109	-0.096	-0.086	0.009	0.169	**0.510**	**1**	**0.563**	-0.187	-0.151
CCL5	0.045	-0.049	-0.052	-0.119	0.100	0.333	**0.461**	**0.563**	**1**	0.006	0.083
CXCL9	-0.080	0.081	0.051	0.063	0.028	0.013	-0.081	-0.187	0.006	**1**	**0.599**
CXCL10	0.084	0.171	0.105	0.093	0.033	-0.083	-0.192	-0.151	0.083	**0.599**	**1**

Table 3.2: Correlation matrix (Pearson) between cytokines, chemokines and Platelet count

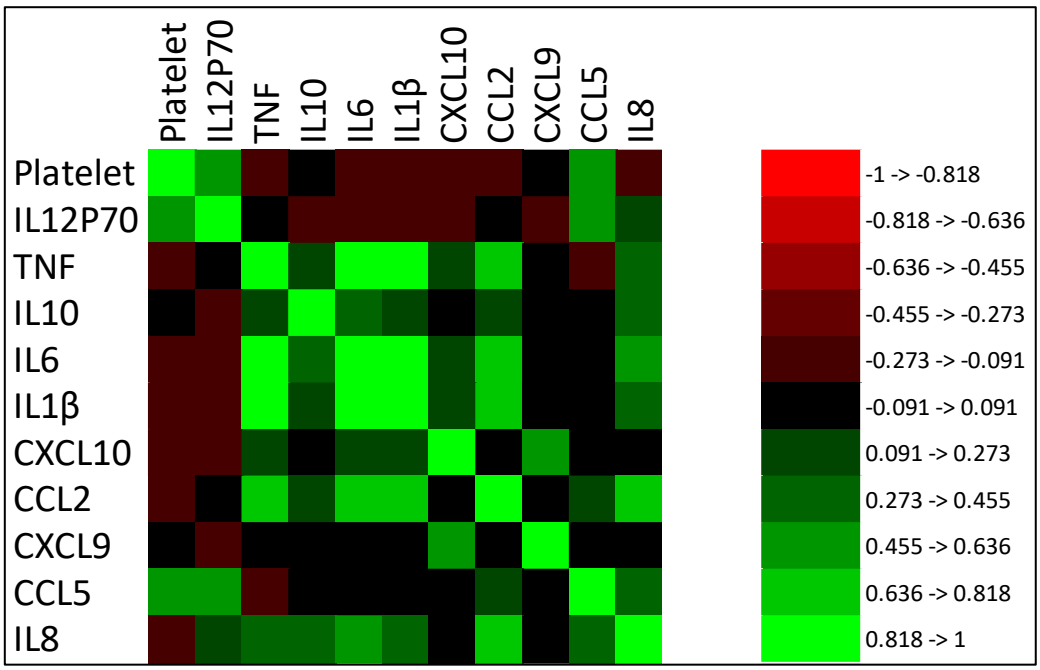
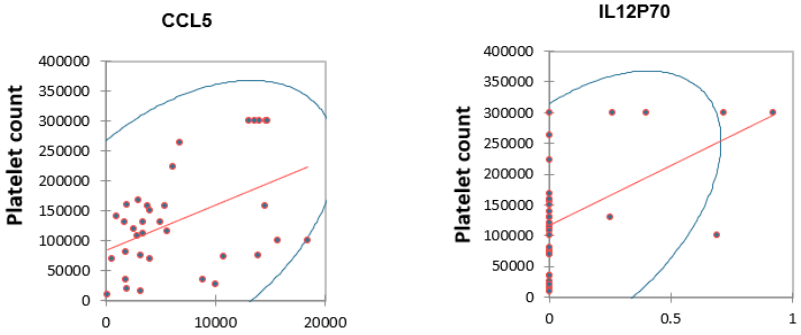


Figure 3.5- Heatmap showing expression of key cytokines and chemokines in dengue patients . Light green color represents very high expression, dark green color represents intermediate expression and red color represents low expression. Expression profile similarities are depicted by the branch lengths of the dendrogram

3.3.5 Correlation analysis of platelet count with CCL5 and IL12p70

Positive correlation of platelet count with CCL5 and IL12p70 was observed in the studied participants



A B

Figure 3.6- Correlation analysis revealed a positive correlation of expression of A) CCL5 and B) IL12p70 with platelet count in dengue.

3.3.6 Principal Component Analysis

The results showed that chemokines and cytokines tended to cluster closely together,

indicating a strong relationship or similarity between these two types of molecules. However, certain variables, specifically platelets, IL12p70 (Interleukin-12), and CCL5 (a chemokine also known as RANTES), formed a separate cluster. This suggests that while platelets, IL12p70, and CCL5 are related to each other, they exhibit different patterns compared to the other cytokines and chemokines in the dataset.

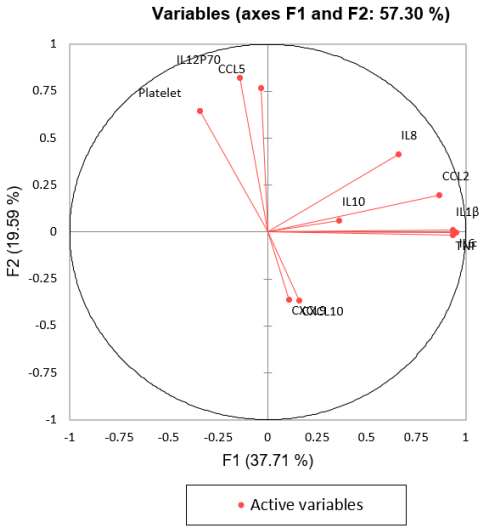


Figure 3.7: Principal Component Analysis (PCA), platelet count, cytokines, and chemokines were the active variables under consideration.

3.3.7 Monocyte and Dendritic cells population by Flow cytometry

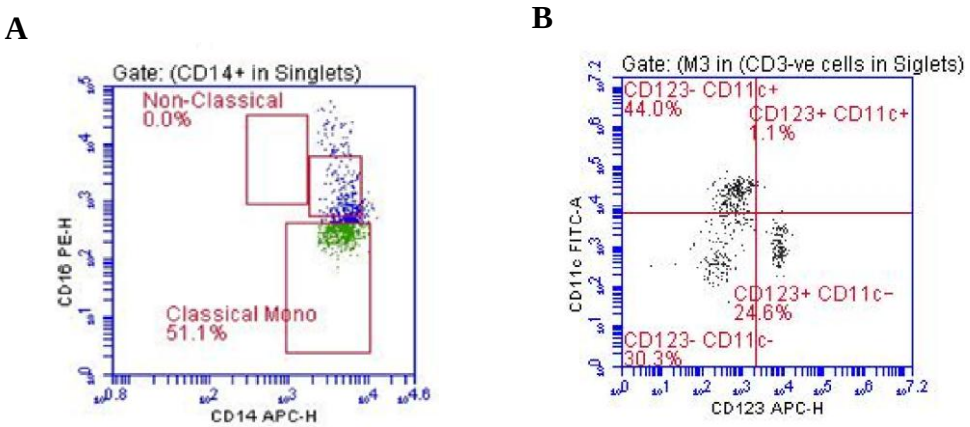


Figure 3.8 : Flow Cytometric Analysis showing gating strategy A) Differential Monocyte population in blood sample of dengue patient B) Differential Dendritic Cell population in blood sample of dengue patient

Differential cell population has been studied in 16 dengue patient samples. Gating strategy of phenotyping study has been shown in Figure 3.8. Monocyte population was predominantly CD14++ classical monocytes and the intermediate CD16+ CD14+ (Figure 3.9). The classical monocyte population exhibits phagocytic function and secretion of proinflammatory cytokines, while intermediate population is reported to have a role in antigen presentation and secretion of cytokines, suggesting activation of inflammatory responses and probable priming of T cells for adaptive immune responses. Predominance of plasmacytoid DC population was seen in in dengue patients with p value < 0.01 (Figure 3.10).

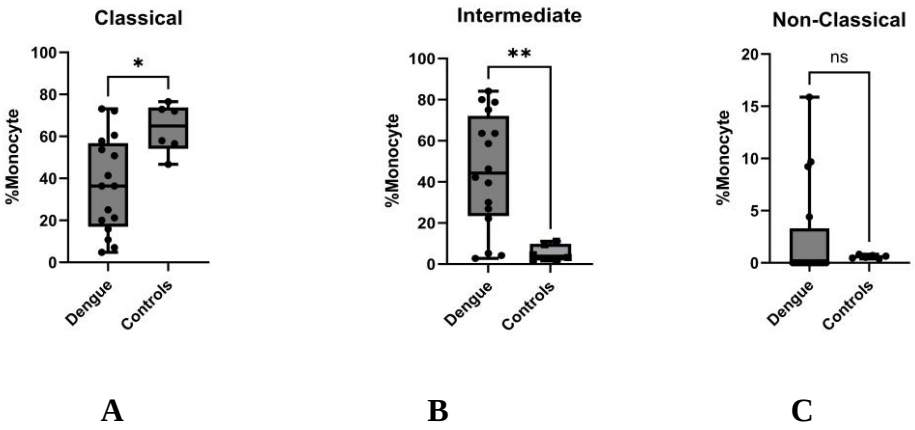


Figure 3.9: Percentage Frequency of A) Classical B) Intermediate C) Non-classical monocytes in Dengue patients and in controls. Unpaired t-tests were performed using GraphPad Prism 10.1.0 to determine significance, with a threshold p-value of < 0.05.

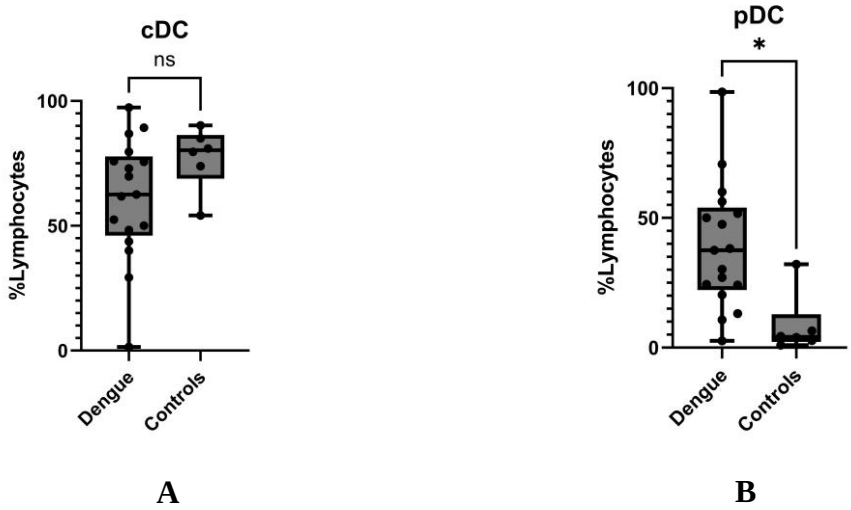


Figure 3.10: Percentage Frequency of A) Conventional Dendritic Cell and B) Plasmacytoid Dendritic Cell in Dengue patients and in controls. Unpaired t-tests were performed using GraphPad Prism 10.1.0 to determine significance, with a threshold p-value of < 0.05.

3.4 Discussion

Our findings reveal a predominance of CD14++ classical monocytes and intermediate CD16+ CD14+ monocytes in Dengue patients, which aligns with previous studies that reported increased activation of monocytes during Dengue fever [16, 17]. Classical monocytes are known for their phagocytic activity and secretion of proinflammatory cytokines. In contrast, intermediate monocytes play a role in antigen presentation and cytokine secretion, indicating activation of inflammatory responses and the priming of T cells for adaptive immunity [18,19]. Additionally, a shift towards an increased population of plasmacytoid dendritic cells, along with higher CXCL10, suggests CXCL10-driven migration of dendritic cells, which in turn secrete interferons, proinflammatory cytokines, and chemokines during viral infections [20].

Our data also show elevated levels of TNF, IL-1β, and IL-10 in Dengue patients, suggesting a monocyte-driven proinflammatory environment that targets endothelial cells and leads to thrombocytopenia . Patro et.al also reported elevated levels of IL-10 and its significant correlation with disease severity [21]. Interestingly, the observed low levels of CCL5, despite the predominance of monocytes, suggest that CCL5 expression might be a combined contribution from platelets and other immune cells. Another study from Kolkata ,India also reported the upregulation of cytokines (IL6, IL10) and chemokines (IL8 and CXCL10) along with downregulation of Chemokine CCL5 at early stage of dengue infection [22]. This finding in concordance with our study. Hence, elevated IL-10, CXCL10 and low level of CCL5 could serve as a signature marker for dengue severity. Futhermore , we have observed a positive correlation of platelet count with CCL5.

In summary, our findings of higher levels of proinflammatory cytokines, alongside increased levels of monocyte-neutrophil recruiting chemokines such as CXCL10, CCL2, and CXCL9, and the proinflammatory phenotype of peripheral monocytes and dendritic cells, highlight the significant role of the monocyte-neutrophil axis in mediating inflammation during Dengue virus infection.

3.5 References

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