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Th2-predominant immune response underlies the pathogenesis of Dengue

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ABSTRACT

Background: Dengue is a rapidly emerging pandemic-prone disease, whose manifestations range from asymptomatic infection to life-threatening complications like Dengue Hemorrhagic Fever and Dengue Shock Syndrome. This study investigates and compares the immune response in clinically defined cohorts of Dengue with and without warning signs, with the aim of identifying immunological correlates of clinical disease and potential markers of disease severity.

Methods: Blood samples, collected from study participants fulfilling the WHO definition of Dengue with and without warning signs and healthy volunteers, were analyzed using flow cell-based fluorometric methods for cytokines and chemokines. Gene expression analysis, using RT-PCR, was conducted on T helper cell subset-specific transcription factors and cytokines. Demographic details, virological markers, serotype distribution, and hematological parameters were also investigated in all the subjects.

Results: The 35 participants recruited in the study, included 11 healthy volunteers and 12 patients each fulfilling the WHO criteria of Dengue with and without warning signs. While the demographic characteristics and serotype distribution was similar in Dengue with and without warning signs cohorts of the disease, platelet counts and Aspartate Aminotransferase (AST) levels changed significantly between Dengue with and without warning signs patients. Plasma cytokine analysis showed up-regulation of IL-4, IL-10, IP-10, and MCP-1 in Dengue patients compared to healthy volunteers. Disease severity was associated with elevated levels of IL-10, IP-10, IL-4, MCP-1, and MIP-1α. IL-8 and MIP-1α were significantly up-regulated in Dengue with warning sign compared to Dengue without warning signs cases. Transcription factor analysis indicated increased expression of RORα, FoxP3, and GATA3 in Dengue patients. mRNA expression of TGFβ and IL-4 was also elevated in Dengue patients. A positive correlation between mRNA expression of IL-4 and plasma IL-4 was observed.

Conclusion: The study reveals a Th2-predominant immune response in all Dengue patients, regardless of disease severity, with overexpression of IL-8 and MIP-1α being observed in patients with warning signs.

1. Introduction

The WHO has described Dengue as a "fast-emerging pandemic-prone" disease worldwide. In the last 50 years, its global incidence has increased 30-fold [1]. Up to 50–100 million infections are estimated to occur annually in over 100 countries, putting half of the world's population at risk [1]. India too has witnessed a recent increase in the burden of Dengue; with the number of cases rising by 133 % and 160 % in 2021 and 2022 respectively, compared to the annual average of the previous 5 years [2].

Abbreviations: DENV, Dengue Virus; DHF, Dengue Hemorrhagic Fever; DSS, Dengue Shock Syndrome; Th, T Helper; IFNγ, Interferon-gamma; TNF, Tumor Necrosis Factor; Treg, T Regulatory; Tfh, T follicular helper; IL, Interleukin; ROR-γt, Retinoic acid receptor-related orphan nuclear receptor-γt; T-bet, T-box expressed in T cells; GATA3, GATA binding protein 3; FoxP3, Forkhead Box P3; BCL6, B-cell lymphoma 6; MCP-1, macrophage chemo-attractant protein 1; MIP, Macrophage Inflammatory Proteins; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; PBMCs, Peripheral Blood Mononuclear Cells; DF, Dengue without warning signs; DW, Dengue with warning signs; TGFβ1, Transforming Growth Factor beta.

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The Dengue Virus (DENV) circulates as four distinct serotypes, DENV-1 through DENV-4, and causes a spectrum of manifestations of varying severity. While in the majority of cases the infection remains subclinical or presents with mild Dengue fever, approximately a quarter of the infected individuals experience severe forms of Dengue, viz. Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). Though the determinants influencing the disease progression and severity of Dengue are unclear, a growing body of evidence suggests that a dysregulated host response contributes to the immunopathogenesis of Dengue through impaired viral clearance and triggering of severe inflammation [3].

Notwithstanding several recent papers, in ex vivo systems and animal models, on the characteristics of immune dysregulation in specific compartments of the innate and adaptive immune system [4–7]; there is a dearth of studies undertaking a comparison of the immunological correlates of mild and severe Dengue in clinical samples derived from patients fulfilling the diagnostic criteria of Dengue without warning signs (DF) and Dengue with warning signs (DW) and severe Dengue. We hypothesized that a relative analysis of immune parameters associated with these two cohorts of Dengue patients would not only offer insights into the mechanisms of immune dysregulation that underlie these two diverse disease forms but would also reveal potential diagnostic and prognostic biomarkers that are currently unavailable in clinical practice.

In this study, using flow cell-based fluorometric estimation of key cytokines and chemokines in blood samples of Dengue patients and healthy volunteers and validating the differentially expressed titers through gene expression analysis of Th cell transcription factors and the cytokines, we observe a Th2 predominance in all Dengue patients, irrespective of their clinical severity, and a relative over-expression of IL-8 and MIP-1α in patients with DW.

2. Material Methods

2.1. Recruitment of participants

We recruited two groups of Dengue patients, viz. DF (n = 12) and DW (n = 12), categorized as per the classification scheme provided by the WHO (WHO 2009). The former group consisted of patients exhibiting symptoms such as nausea, vomiting, rash, aches, and pains, while the latter group had features like leukopenia, abdominal pain or tenderness, mucosal bleeding, lethargy, liver enlargement, elevated hematocrit (HCT), and thrombocytopenia. The diagnosis of dengue was based on reactive status in the Dengue NS1 Ag Microlisa kit (manufactured by J. Mitra & Co. Pvt. Ltd) and NIV Dengue IgM capture ELISA (manufactured by National Institute of Virology, Pune), which were performed as per the manufacturer's instructions. Additionally, 11 age- and gender-matched healthy volunteers were also recruited into the study. The study protocol was approved by the institutional human ethics committee. Informed consent was obtained from each participant and 6 ml of blood was collected from each of the study participants.

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMC was isolated from EDTA blood using the density gradient centrifugation method. Briefly, the plasma was separated by centrifugation at 400 xg for 20 min at 25 °C and either used immediately or stored at −80 °C for later use. The remaining blood was mixed with an equal volume of phosphate-buffered saline (PBS), and the mixture was layered on top of the Histopaque-1077® (Sigma) solution. The samples were then centrifuged at 400 xg for 30 min. The interface containing PBMCs was carefully collected and washed twice with PBS. The purified cells were resuspended in a freezing medium consisting of 90 % FBS and 10 % DMSO and immediately stored using a pre-chilled Mr. Frosty container (Sigma) at −80 °C overnight before being transferred to liquid nitrogen for long-term storage.

2.3. Cytokine assays

In the study, plasma samples of the recruited participants were used for determining the concentration of 21 cytokines using the Human Cytokine Storm 21-Plex Human ProcartaPlex™ Panel (Thermo Fisher, Waltham, MA). The cytokines measured included G-CSF (CSF-3), GM-CSF, IFN α, IFN γ, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-13, IL-17A (CTLA-8), IL-18, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNF α and TNF β. Data acquisition was performed using the Luminex 200™ system and analyzed with Bioplex Manager software. The final concentration of the cytokines was determined using 5-parameter logistic regression with the provided standard curve for each experimental batch. In addition, TGFβ levels were estimated using a commercial ELISA kit (Human TGF-Beta1 GENLISA™ ELISA) as per the manufacturer's instruction. Data was acquired using the ELISA reader (Multiskan FC, Thermo Scientific). Each sample was analyzed in duplicate, and the mean concentration was expressed in pg/mL (Table 2).

For the data visualization and interactive analysis, cytokine data is presented as a heat map chart (Fig. 1A). A heat map clustering and distance matrix were generated using the ward D algorithm using Metaboanalyst 5.0 [8].

2.4. Viral RNA isolation and dengue serotyping

Viral RNA was extracted from 140 μL of plasma sample using the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany), and the RNA concentration was quantitated using the NanoDrop OneC micro nucleic acid concentration analyzer (Thermo Scientific). Real-time DENV serotyping was conducted using the RealStar Dengue RT-PCR kit (Altona Diagnostics, Germany) and the CFX96™ Real-Time PCR Detection System (Bio-Rad, Germany), following the manufacturer's instructions.

2.5. Expression analysis of T cell subset-specific transcript in PBMCs

Total RNA was extracted from the PBMCs using the Qiagen RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. Briefly, the frozen PBMC samples were thawed and suspended in RPMI-1640 medium (Gibco, USA). They were then centrifuged at 400 x g for 10 min, washed twice with PBS, and the pellet was used for RNA isolation. The quantity of RNA was checked using the NanoDrop OneC micro nucleic acid concentration analyzer (Thermo Scientific). All quantified RNA was further treated with an "RNase-free DNase Set" kit (Qiagen) to eliminate DNA contamination. The primers used in the study are listed in Supplementary Table 1. Reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen) as per the manufacturer's instructions. The prepared cDNA was then treated with 2 U of RNaseH for 20 min at 37 °C to eliminate the RNA from the RNA-cDNA duplex.

To measure the expression of transcription factors (T-bet, GATA3, RORα, BCL6, and FoxP3) and cytokines (IL-4, IL-10, and TGFβ), reverse transcriptase PCR was performed with 1 μL of template cDNA. The protocol for the Real-Time Polymerase Chain Reaction (RT-PCR) is depicted in Supplementary Table 1. The relative quantification of the target gene was calculated using the delta-delta Ct method [9] with a reference gene (GAPDH).

2.6. Statistical analysis

Clinical parameters and group comparisons were analyzed using GraphPad Prism (version 8.0.1). The non-parametric Kruskal-Wallis test and Mann-Whitney *U* test were employed for multiple comparisons of bead array and mRNA expression data. In the case of a significant result in the Kruskal-Wallis test, non-parametric pairwise multiple comparisons were conducted using Dunn's test. The correlation between mRNA expression and plasma cytokine levels was assessed using Spearman's

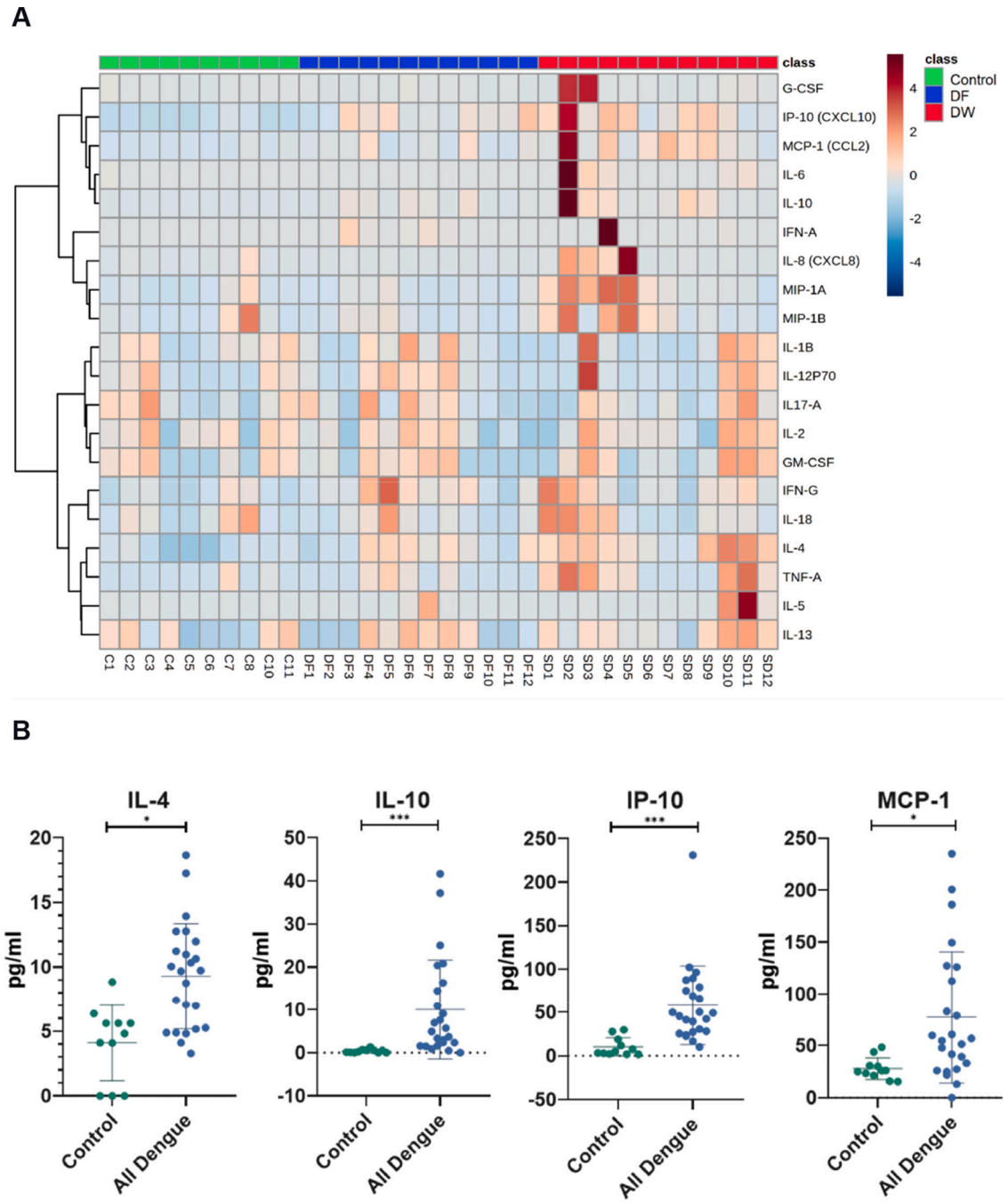

Fig. 1. (A) Clustering visualization of the cytokine concentrations in individual patients: The results are displayed as a heatmap (B) Comparison of cytokine levels between healthy volunteers versus Dengue patients using the Mann-Whitney *U* test. *-p < 0.05, **-p < 0.01, ***-p < 0.001.

correlation. p-value < 0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics of study participants

This study was conducted on 35 participants, which included 11 healthy volunteers and 12 patients each with DF and DW. The characteristics of the three study groups, including their age and gender distribution, results of diagnostic assays (NS1 antigen and IgM ELISA assays), serotype distribution, and general hematological parameters are depicted in Table 1. While the demographic distribution was similar in all three study groups, a significant difference was observed in the platelet counts and Aspartate Aminotransferase (AST) levels between the patients with mild and DW ($p = 0.001$ and $p = 0.03$ respectively). Apart from one patient with DW, whose serotype could not be determined by multiplex RT-PCR assay, the serotype distribution between the patients with DF and DW was found to be similar. Serotypes 2 and 3 were the prevalent serotypes in both cohorts (Table 1).

3.2. Plasma cytokine levels in the study cohorts

To gain an overview of the differential immune response in study cohorts, we first compared the levels of 22 cytokines in plasma samples of the study participants. To compare the plasma cytokine levels between the three cohorts, the cytokine levels of each participant were plotted on a heatmap (Fig. 1A). Comparing the cytokine titers between Dengue patients and healthy volunteers, we observed significant up-

Table 1
Demographic, virological, haematological and biochemical characteristics of study participants.

Clinical parameters		Dengue without warning signs patients (n = 12)	Dengue with warning signs patients (n = 12)	Healthy volunteers (n = 11)	p Value
Age (years) (Mean ± SD)		13 ± 8	13 ± 7	13 ± 4	0.95
Gender (M:F)		6:6	5:7	7:4	0.57
Day of illness (Mean ± SD)		5 ± 1	6 ± 1	–	0.19
Lymphocyte		40 ± 16.89	39.8 ± 12.98	–	0.98
Hb		13.34 ± 1.39	13.20 ± 2.24	–	0.87
Platelets		113500 ± 62645.4	32644.44 ± 28894.55	–	**0.001**
AST		83.36 ± 61.41	415.4 ± 275.2	–	**0.05**
ALT		76.82 ± 91.23	276.2 ± 136.24	–	**0.03**
Neutrophil		51.9 ± 19.80	46.97 ± 24.09	–	0.66
Eosinophil		1.30 ± 1.43	2.42 ± 1.51	–	0.14
WBC		4864.19 ± 3728.39	6186.55 ± 3669.89	–	0.43
HCT		37.94 ± 3.98	38.23 ± 8.53	–	0.94
Serological Test					
Dengue NS1 ELISA Positive		12	12		–
Dengue IgM ELISA Positive		9	12		0.028
Dengue Serotyping	Serotype 1	–	–		–
	Serotype 2	5	4		0.67
	Serotype 3	5	4		0.67
	Serotype 4	–	–		–
	Mixed Infection (Serotype 2 and Serotype 3	2	3		–
	Undetermined	–	1		–

Table 2
Distribution of cytokine concentration in plasma (pg/mL) across study groups.

Sr.	Cytokine	Healthy volunteers (HV) (Mean ± SD)	DF (Mean ± SD)	DW (Mean ± SD)	p value		
					HV vs DF	HV vs DW	DF vs DW
1	G-CSF (CSF-3)	7.80 ± 4.99	5.88 ± 5.27	61.89 ± 125.7	0.99	0.99	0.28
2	GM-CSF	23.44 ± 19.90	22.51 ± 22.06	31.88 ± 26.36	0.99	0.99	0.99
3	IFN γ	2.78 ± 2.69	3.74 ± 2.54	3.90 ± 2.49	0.47	0.31	0.99
4	IL-1β	1.38 ± 0.98	1.196 ± 1.354	1.466 ± 1.84	0.99	0.99	0.99
5	IL-2	8.01 ± 4.75	7.11 ± 5.08	9.11 ± 5.92	0.99	0.99	0.99
6	IL-4	4.10 ± 2.92	7.39 ± 2.95	11.16 ± 4.29	0.23	**<0.001**	0.13
7	IL-8 (CXCL8)	12.57 ± 21.20	2.21 ± 2.12	44.85 ± 72.74	0.99	0.09	**0.01**
8	IL-10	0.37 ± 0.40	6.84 ± 7.06	14.51 ± 14.34	**0.001**	**<0.001**	0.63
9	IL-12p70	2.05 ± 2.26	2.64 ± 2.54	3.18 ± 4.53	0.99	0.99	0.99
10	IL-13	14.64 ± 9.74	14.97 ± 11.09	19.60 ± 9.17	0.99	0.89	0.99
11	IL-17A (CTLA-8)	1.77 ± 1.40	1.82 ± 1.52	1.73 ± 1.43	0.99	0.99	0.99
12	IL-18	27.86 ± 31.96	18.15 ± 15.04	27.74 ± 23.15	0.99	0.99	0.99
13	IP-10 (CXCL10)	10.36 ± 10.60	44.46 ± 25.03	72 ± 57.00	**0.01**	**<0.001**	0.99
14	MCP-1 (CCL2)	27.89 ± 10.34	53.90 ± 38.33	110.2 ± 71.71	0.26	**<0.001**	0.97
15	MIP-1 α (CCL3)	3.37 ± 2.59	2.59 ± 1.86	12.35 ± 11.33	0.99	**0.03**	**0.03**
16	MIP-1 β (CCL4)	28.41 ± 44.88	10.66 ± 10.63	43.23 ± 47.44	0.99	0.42	0.28
17	TNF α	0.85 ± 1.63	0.90 ± 1.45	3.67 ± 3.54	0.99	0.08	0.09

regulation of IL-4 ($p < 0.016$), IL-10 ($p < 0.001$), IP-10 ($p < 0.001$), and MCP-1 ($p < 0.041$) in Dengue patients.

(Fig. 1B). For all the study participants, the TGFβ, TNF β, IFN α, IFN β, and IL-5 levels were found to be below the detection limit.

We next explored if the plasma level of any cytokine(s) was associated with disease severity. We observed that, compared to healthy volunteers, patients with DF demonstrated up-regulation of IL-10 ($p <$ 0.001) and IP-10 (p 0.01), and patients with DW demonstrated up-regulation of IL-10 ($p < 0.001$), IP-10($p < 0.001$), IL-4 ($p < 0.001$), MCP-1 ($p < 0.001$), and MIP-1α (p 0.03). Compared to DF patients, IL-8 ($p = 0.01$) and MIP-1α ($p = 0.03$) cytokines were significantly up-regulated in DW (Fig. 2).

3.3. mRNA expression of transcription factors in dengue with and without warning signs patients

After identifying elevated levels of multiple cytokines that might potentially contribute to the pathogenesis of Dengue and its severity, we focused on analyzing the T-cell subsets and their corresponding transcription factors. The transcription factors analyzed included T-bet, GATA3, RORα, FoxP3, and BCL6, which are known to be associated with the differentiation of Th1, Th2, Th17, Tregs, and Tfh cells, respectively. Compared to healthy volunteers, mRNA expression of RORα ($p <$ 0.0001), FoxP3 ($p < 0.0001$), and GATA3 ($p < 0.0001$) was significantly increased in Dengue patients (Fig. 3). However, the expression of these transcription factors was not significantly different between DF and DW patients.

3.4. mRNA expression of cytokines associated with specific T cell subsets

Having observed significant upregulation in the expression of GATA3 and FoxP3 transcription factors in Dengue patients, we further investigated the relative mRNA expression levels of the cytokines IL-4, TGFβ, and IL-10 in the study cohorts. Significant upregulation was observed in the mRNA expression of TGFβ and IL-4 in the Dengue patients, compared to healthy volunteers ($p < 0.001$ and $p < 0.001$, respectively). The mRNA expression levels of TGFβ and IL-4 were significantly upregulated in both DF ($p = 0.007$ and $p < 0.001$) and DW ($p < 0.001$ and $p = 0.008$) Dengue patients, compared to healthy volunteers (Fig. 4). IL-10 expression levels were found to be similar in

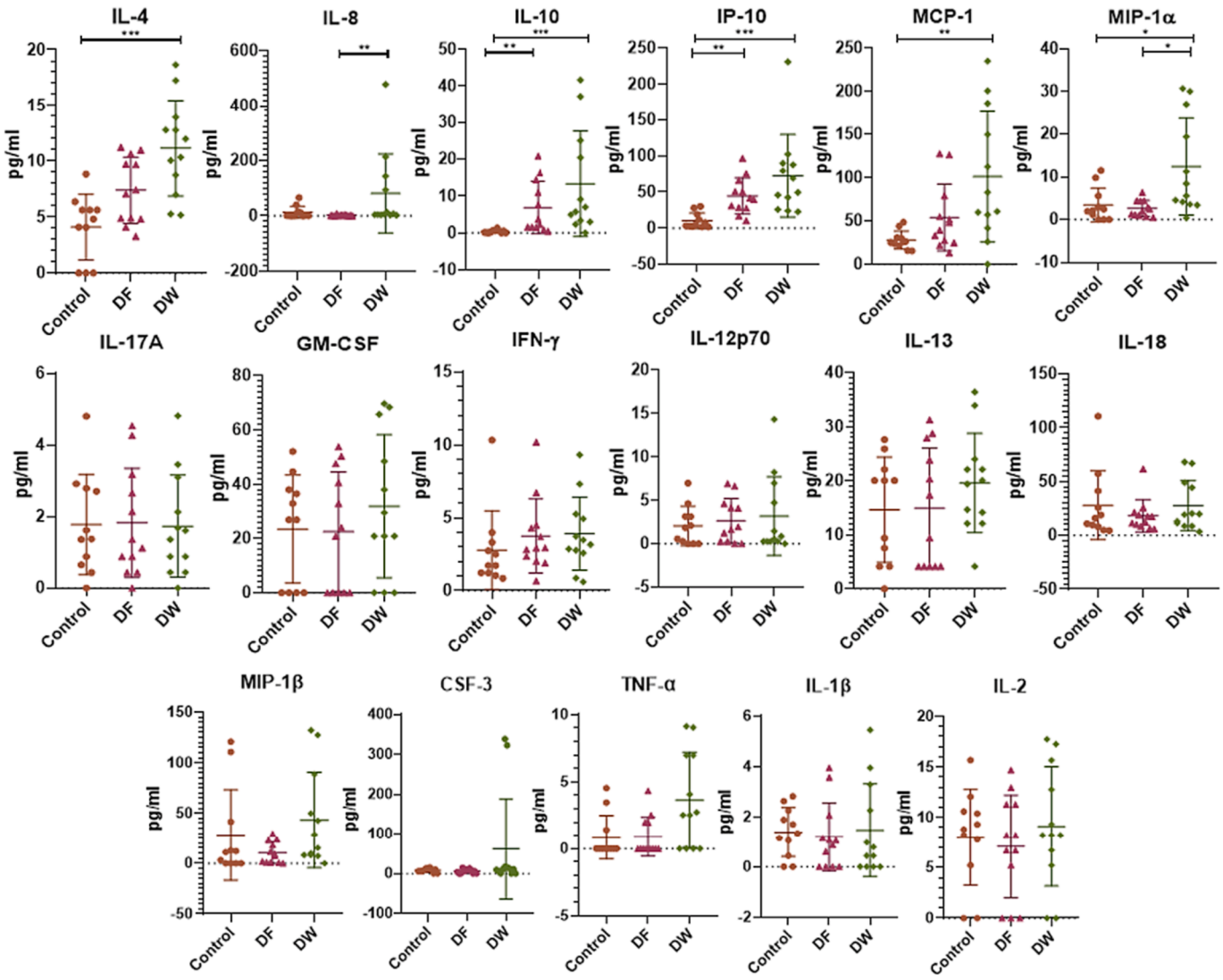

Fig. 2. Cytokine levels in healthy volunteers (control), DF, and DW subjects. The intergroup comparison was done between the three cohorts using the non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. *-$p < 0.05$, **-$p < 0.01$, ***-$p < 0.001$.

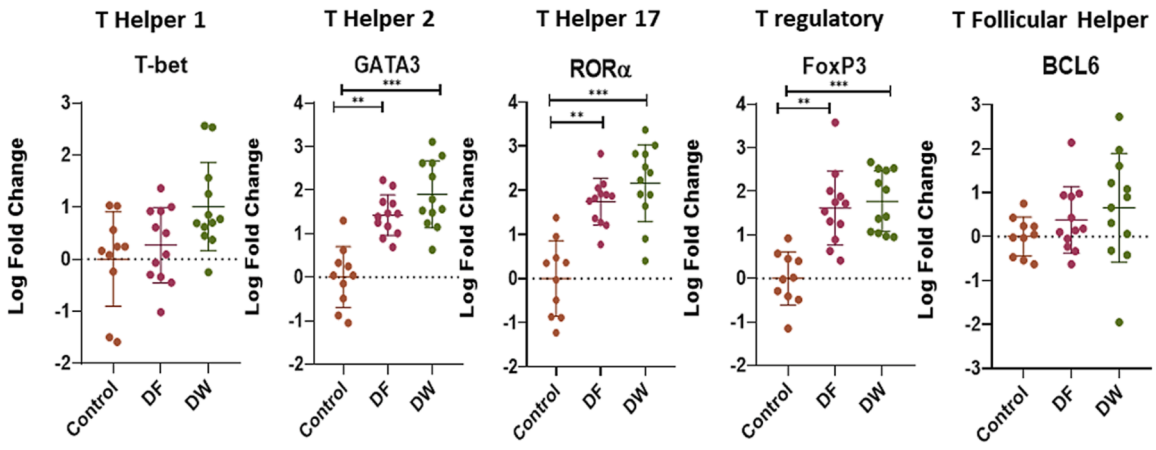

Fig. 3. Comparison of expression levels of transcription factors. mRNA expression of transcription factors was compared between healthy volunteers (control), Dengue without warning signs (DF), and Dengue with warning signs (DW) cohorts. The comparison was performed using the Kruskal-Wallis test and Dunn's multiple comparison test. *-$p < 0.05$, **-$p < 0.01$, ***-$p < 0.001$.

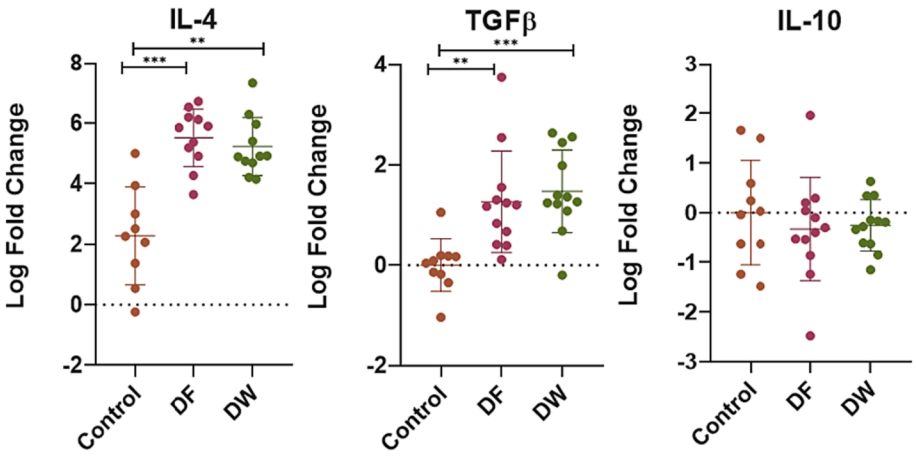

Fig. 4. Relative expression levels of IL-4, TGFβ, IL-10. Cytokine mRNA expression in healthy volunteers (control), and Dengue without warning signs (DF) and Dengue with warning signs (DW) patients using the Kruskal-Wallis test and Dunn's multiple comparison test. *-p < 0.05, **-p < 0.01, ***-p < 0.001.

healthy volunteers and Dengue patients.

3.5. Correlation between cytokine levels of IL-4, IL-10 and their corresponding mRNA

We evaluated the correlation between mRNA expression levels of IL-4 and IL-10 in PBMC samples and the titers of the corresponding cytokines in plasma samples (Fig. 5). Our results indicated a positive correlation between the two variables for IL-4, with Spearman's rank correlation coefficient of 0.22. However, for IL-10 the correlation coefficient was 0.01 only.

4. Discussion

In this manuscript, we report skewed T-cell differentiation towards the Th2 lineage in Dengue patients, which is consistently reflected in the levels of the transcription factor GATA-3, and gene expression and protein levels of the signature Th2 cytokine, viz. IL-4. We also observed upregulation of the Treg transcription factor, FoxP3, in Dengue patients. In addition, compared to patients with DF, DW is shown to be associated with elevated levels of IL-8 and MIP-1α.

Similar to our findings, previous authors have also reported higher expression of GATA-3 and FoxP3 transcription factors in Dengue patients. Identifying immunological markers associated with severe Dengue in Mexican patients, Estrada-Jiménez et al observed a predominance of Th2 response accompanied by low expression of the Th1 transcription factor, T-bet, and reduced functional cytotoxic T cells in patients with Dengue Hemorrhagic Fever. The observed immune dysregulation in severe Dengue, characterized by low activation of Th1 cells and downregulation of the antiviral cytotoxic activity, was suggested by these authors to be induced by regulatory T cells [10]. Similarly, Khanam et al ascribed skewed immune activation favoring Th2 response to influence increased viral production in severe Dengue through the phenomenon of intrinsic antibody-dependent enhancement (ADE) [3]. In another study reported from Kuwait, a shift from Th1 to Th2-type response was observed in the pathogenesis of severe Dengue [11]. Mechanistically these observations highlighting the up-regulated Th2 response in Dengue, as reported in our study and these previous studies, are in sync with the mutually antagonistic differentiation of naïve Th cells along Th1 and Th2 lineages and the protective antiviral immune response associated with Th1 activation [12]. Furthermore, this is also in sync with the reported adoption of multiple strategies by the Dengue virus to evade host immune defenses through the interaction between the viral non-structural proteins and pattern recognition receptors (PRRs) [13] and through the inhibition of RIG-I-directed antiviral immune responses [14].

Apart from the increased viral burden associated with disrupted antiviral immunity, the severity of Dengue is known to be a function of the dysregulated inflammatory response resulting from the cytokine storm observed in this condition [3]. Among other factors, the Dengue virus has been reported to increase the expression of TREM-1 on human neutrophils and thereby lead to their activation through the generation of neutrophil extracellular traps (NETs) and increased production of inflammatory mediators [15]. Our observation on the association of DW

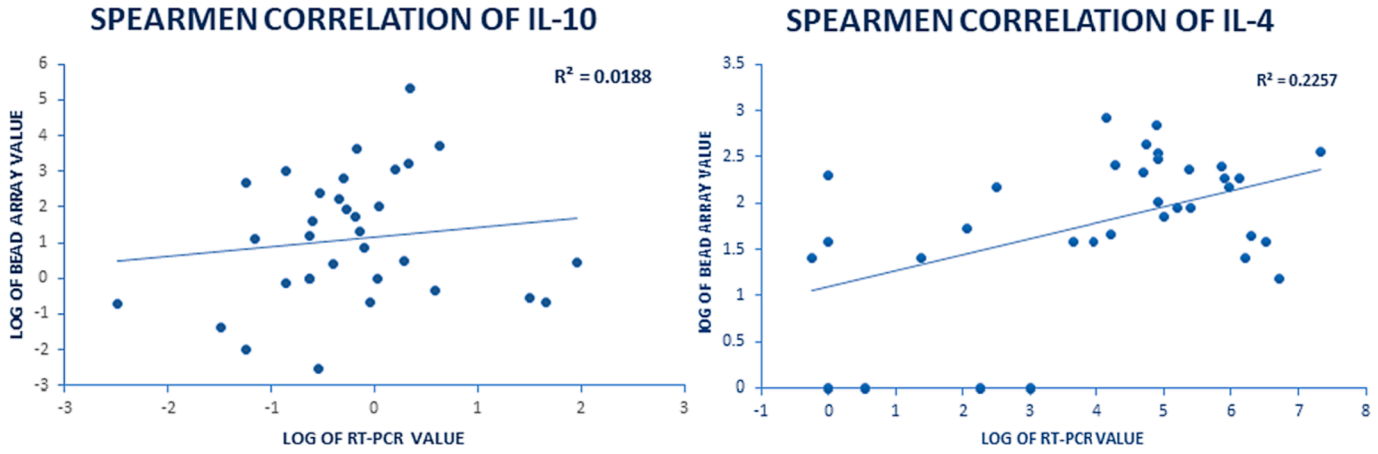

Fig. 5. Spearman's rank correlation between plasma concentrations and mRNA expression of IL-4 and IL-10 in PBMCs.

with raised titers of IL-8 and MIP-1α, which are secreted from neutrophils and macrophages respectively, is in line with the activation of these cells in the inflammatory milieu that characterizes severe Dengue. Similar findings on increased levels of proinflammatory cytokines and chemokines like IL-8 and MIP-1α have been reported previously, as well [16,17].

The present study analyses the immune markers that distinguish Dengue patients from healthy volunteers and secondarily attempts to identify the ones that discriminate between DF and DW. Our findings featuring the up-regulation of Th2 and Treg response in all Dengue patients irrespective of their disease severity, and the ones demonstrating the association of pro-inflammatory cytokines, IL-8 and MIP-1α, with DW hint at the potential immunopathogenesis pathways that underlie the manifestations of Dengue. Despite the limitations of sample size and the inability to compare relative frequencies of various Th cell subsets in the study groups, we speculate that a skewed Th2 response is critical to the subversion of protective antiviral host response in Dengue, and is instrumental in driving the progression of an inapparent, subclinical infection to clinically manifest disease. Advancement of the disease from DF to DW is orchestrated by the unregulated inflammatory response, wherein the pro-inflammatory cytokines secreted from the acute inflammatory cells, neutrophils, and macrophages, are more significantly up-regulated than the ones derived from Th cells like TNFα and IL-6. We understand that this also calls for future studies on the role of the unregulated activation of neutrophils and macrophages in influencing the deleterious consequences of other viral hemorrhagic fevers with similar pathogenic mechanisms like Ebola, Marburg, CCHF, and Hanta.

Though we observed elevated expression of Th17 transcription factor in Dengue patients, the same was not reflected in elevated levels of IL-17. While elucidating the reason for this apparent discrepancy was beyond the scope of this study, we understand that a multitude of factors including post-transcriptional regulation (instability or degradation or miRNA-mediated suppression of the transcription factor mRNA); translational or post- translational modification (phosphorylation, glycosylation or other modifications affecting IL-17 secretion and stability); alternative splicing isoforms of IL-17 and operational negative feedback loops might explain this observation. Specifically, previous papers have reported alternative splicing of IL-17 mRNA and presence of multiple miRNA binding sites at the 3′ UTR of IL-17 mRNA [18]. Post-translational modifications like O- GlcNAcylation have also been observed in the IL-17 protein [19]. We believe that further investigation will shed light on the intricate mechanisms underlying the interaction between Th17 transcription factors and IL-17 production in Dengue and thus enable deeper understanding of the disease pathogenesis.

The observed correlation between IL-4 transcript and protein levels, but not IL-10, is intriguing. While exploring the underlying mechanism is beyond the scope of this study, a potential explanation lies in the differing stability of their mRNA molecules. IL-10 mRNA bears a longer 3′ untranslated region (UTR) (1033 bp in humans) that is linked to mRNA instability [20]. Additionally, its 3′ UTR contains six AUUUA pentamers [21] that serve as hallmark “AU-rich elements” (AREs) recognized by RNA-binding proteins like Tristetraprolin (TTP) that promote mRNA destabilization. Consequently, IL-10 mRNA exhibits a shorter half-life. In contrast, the IL-4 mRNA is considerably stabler; as it has a shorter 3′ UTR (221 bp in humans) that contains fewer and weaker ARE motifs. Though this relative instability of IL-10 mRNA could explain the observed lack of correlation, interconnected regulatory networks and feedback loops governing cytokine levels make definitive mechanistic speculations challenging. Supporting this, it has been observed that levels of fewer than 40 % of the cellular proteins can be predicted from mRNA measurements [22] owing to multiple processes beyond transcript concentration, such as translation rate; ncRNA modulation of translation rate; protein synthesis delay and protein half-life regulation through ubiquitin- proteasome pathway or autophagy [23].

Apart from offering an insight into the immunopathogenesis of Dengue, our study also identifies immune markers that can be further evaluated for clinical applicability as diagnostic and prognostic biomarkers for Dengue. Considering the occurrence of this disease as sudden outbreaks in resource-constrained settings and the consequent mortality and morbidity, the availability of such biomarkers can be invaluable in identifying patients who are more prone to develop life-threatening complications associated with severe Dengue and thus triage them for institutional care and closer monitoring. It would be prudent to explore the performance and time kinetics of IL-4, IL-8, and MIP-1α in predicting the onset of severe Dengue and monitoring the clinical course of this condition.

Apart from the limitations mentioned above, a third limitation of our study was the inability to characterize the effects of primary and secondary Dengue infection on the immune parameters included in this study. Though of academic interest, we understand that such differentiation into primary and secondary infection has minimal bearing on the clinical classification and management of this condition. Since clinical decisions in Dengue are primarily influenced by the nature and severity of manifestations, we were inclined to compare the immunological correlates of Dengue in cohorts of DF and DW and thus obtain clinically relevant insights.

In conclusion, we surmise that a Th2-predominant immune response underlies the pathogenesis of symptomatic Dengue; with the progression of disease severity being associated with raised titers of acute inflammatory mediators like IL-8 and MIP-1α.

5. Formatting of funding sources

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CRediT authorship contribution statement

Dipesh Kale: Writing – original draft, Methodology, Formal analysis. **Ashish Kumar Vyas:** Formal analysis, Conceptualization. **Girish Chandra Bhatt:** Investigation, Data curation. **Ashvini Kumar Yadav:** Methodology, Investigation, Formal analysis, Data curation. **Anirudh K. Singh:** Investigation, Data curation. **Shashwati Nema:** Writing – review & editing, Investigation, Data curation. **Debasis Biswas:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2024.156562.

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