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Decreased suppression of $CD8^+$ and $CD4^+$ T cells by peripheral regulatory T cells in generalized vitiligo due to reduced NFATC1 and FOXP3 proteins

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Abstract

Regulatory T cells (Tregs) are involved in the suppression of activated T cells in generalized vitiligo (GV). The study was aimed to investigate Tregs functional defects in Treg:$CD8^+$ and Treg:$CD4^+$ T cells' co-culture systems of 55 GV patients and 45 controls. $CD8^+$ and $CD4^+$ T-cell proliferation was assessed by BrdU assay; production of IL-10, TGF-β and IFN-γ cytokines was assessed by ELISA; and FOXP3, CD25, NFATC1 and CD44 proteins were measured by flow cytometry. Generalized vitiligo patients showed reduced suppression of $CD8^+$ and $CD4^+$ T cells ($P = .0384$, $P = .0084$), increased IFN-γ ($P < .0001$, $P = .0019$), decreased IL-10 and TGF-β ($P < .0001$) and decreased FOXP3, CD25 and NFATC1 proteins ($P < .0001$). Active vitiligo (AV) patients showed reduced suppression of $CD8^+$ & $CD4^+$ T cells ($P = .006$, $P = .015$), increased IFN-γ ($P = .036$, $P = .045$), decreased IL-10 ($P = .009$, $P = .021$), FOXP3 ($P = .0244$) and NFATC1 ($P = .019$). Severe GV (50%-75% VASI) patients showed reduced suppression of $CD8^+$ and $CD4^+$ T cells ($P = .0003$, $P = .001$), increased IFN-γ ($P = .0029$, $P < .0001$), decreased IL-10 ($P = .0057$, $P = .0017$), FOXP3 ($P = .002$) and NFATC1 ($P = .0347$). VASI score was positively correlated with the suppression of $CD8^+$ and $CD4^+$ T cells ($P = .0006$, $P < .0001$), IL-10 ($P = .0096$, $P = .029$), FOXP3 ($P = .0008$) and NFATC1 ($P = .043$), whereas it was negatively correlated with IFN-γ ($P = .0029$, $P = .0017$). Early age of onset patients' Tregs demonstrated decreased suppression of $CD8^+$ and $CD4^+$ T cells ($P = .0156$, $P = .0074$), decreased TGF-β ($P = .0212$, $P = .0083$) and NFATC1 ($P = .0103$). NFATC1 was positively correlated with FOXP3 in Tregs ($P < .0001$). Our results suggest impaired Tregs suppressive function in GV patients due to decreased NFATC1, FOXP3, CD25, IL-10 and TGF-β resulting into increased $CD8^+$ and $CD4^+$ T-cell proliferation and IFN-γ production. For the first time, decreased NFATC1 levels were correlated with decreased FOXP3, thereby altering Treg cell function in GV patients. Additionally, decreased Treg cell function also affected onset, activity and severity of GV.

KEYWORDS

$CD8^+$ and $CD4^+$ T cells, FOXP3, generalized vitiligo, NFAT, regulatory T cells (Tregs), suppressive activity

1 | INTRODUCTION

Generalized vitiligo (GV) is an acquired and polygenic skin depigmenting disease characterized by bilateral, symmetrical depigmented patches over the entire body.[1] GV has been suggested to be caused due to autoimmune-mediated loss of functional melanocytes.[2] About 0.5%-2% population worldwide is affected with vitiligo.[3] The role of autoimmunity in pathogenesis of vitiligo is suggested by $CD8^+$ and $CD4^+$ T cells' infiltration in perilesional skin, presence of melanocyte-derived autoantigens and melanocyte-specific autoantibodies.[4] The involvement of cell-mediated immunity is evident by autoimmune loss of melanocyte in vitiligo patients skin due to melanocyte-specific $CD8^+$ T cells.[5,6] The FAS-FASL-dependent destruction of melanocytes by autoreactive $CD4^+$ T cells also suggests their crucial role in pathogenesis of vitiligo.[7] Therefore, the presence of these autoreactive $CD8^+$ and $CD4^+$ T cells in vitiligo patients' skin and blood samples[8,9] indicates a dysregulation of regulatory T-cell mechanism, which can curb these cells.

Regulatory T cells (Tregs) play an important role in maintaining immune tolerance by such self-reactive $CD8^+$ and $CD4^+$ T cells.[10] Previous studies have reported an altered Treg cell frequency and function in vitiligo patients.[9] Evidence of Tregs' role in vitiligo has been shown previously by adoptive Treg cell transfer that halted vitiligo progression[11] and the fact that Tregs from vitiligo patients fail to suppress cytotoxic T lymphocyte proliferation and activation.[12–15] Nuclear factors of activated T cells (NFATs) are the transcription activator of T cells, whereas FOXP3 is the key transcriptional regulators of Treg cells. NFATs and FOXP3 form co-operative complex resulting into upregulation of Treg suppressive genes (*IL-10*, *TGFβ* and *CTLA4*); however, these genes were found to be reduced in GV.[16] Tregs' CD44 expression has shown to be positively correlated with FOXP3 and Treg suppressive capacity.[17] Thus, any dysfunction in Tregs may lead to failure of Tregs to suppress the self-reactive $CD8^+$ and $CD4^+$ T cells[2] resulting into melanocyte destruction in Vitiligo.[,18]

Given the crucial role of Treg cells, $CD8^+$ and $CD4^+$ T cells in vitiligo pathogenesis, we undertook the current study that aimed to assess the suppressive capacity of Tregs from GV patients and controls by measuring their effects on $CD8^+$ and $CD4^+$ T-cell proliferation, cytokine production (IFN-γ, TGF-β and IL-10), and levels of Treg suppressive proteins (NFATC1, FOXP3, CD25 and CD44) in Treg:$CD8^+$ and Treg:$CD4^+$ T cells' co-culture systems. In addition, the study also investigated the effect of Treg cell function on age of onset of disease, disease activity, disease severity and the gender biasness to develop GV.

2 | MATERIALS AND METHODS

2.1 | Patients and controls

A total of fifty-five GV patients and forty-five unaffected healthy controls from Gujarat participated in the study. The demographic characteristics of patients and controls are mentioned in Table 1. Generalized vitiligo was diagnosed by dermatologists at General hospital, using Wood's lamp as bilateral, symmetrical, depigmented macules or patches occurring in a random distribution over the entire body surface.[1] The extent of vitiligo depigmentation was measured by vitiligo area severity index (VASI) score.[19] As described by Bhor and Pande,[19] the total body VASI score for GV patients was calculated as the product of the vitiligo area and the degree of the depigmentation within each patch determined by the hand units and a hand unit is approximately 1% of the total body surface area. The GV patients were divided into three groups on the basis of VASI score: (a) 10%-25% VASI: Mild GV, (b) 25%-50% VASI: Moderate GV and (c) 50%-75%: Severe GV as mentioned previously.[20] Based on disease activity, the GV patients were divided into two groups: active vitiligo (AV) patients and stable vitiligo (SV) patients. Any increase in size and number of lesions within previous 6 months were defined as AV patients, whereas SV was defined with no such increase in lesions size/number within previous 6 months.[21] Unaffected healthy controls were without any signs of vitiligo. The exclusion criteria include no recruitment of GV patients having other autoimmune conditions, pregnancy and women with newborns. Also, patients undergoing any therapy within the previous month were excluded from the study. There were no patients involved with universal or acrofacial vitiligo in the study. A written consent was obtained from every participant in the study. The Institutional Human Research Ethical Committee, Maliba Pharmacy College, Uka Tarsadia University, India, provided the ethical approval to the study. The ethical standards mentioned in the Helsinki Declaration of 1964 and its subsequent amendments were followed in the study.

2.2 | Isolation of $CD4^+CD25^+$ Tregs and $CD4^+$ T cells

The $CD4^+CD25^+$ Treg cells and $CD4^+$ T cells were isolated from three millilitre blood sample of GV patients and controls in a two-step protocol using Human MACSxpress® Whole Blood Treg Isolation Kit (Miltenyi Biotec) as mentioned previously.[16] At first, by negative selection $CD4^+$ T cells were isolated by immunomagnetic depletion of non-CD4 T cells with MACSxpress Beads. Then, under magnetic field, the isolated $CD4^+$ T cells were subjected to LS column and $CD4^+CD25^+$ T cells were positive selected by using anti-CD25 antibody-coated magnetic microbeads, which resulted in $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T cells, which were immediately processed for in vitro Treg suppression assay. Purity of isolated $CD4^+$ T cells and Tregs was confirmed by flow cytometry (Figure S3a,b).

2.3 | Isolation of $CD8^+$ T cells

Isolation of $CD8^+$ T cells from two millilitre blood sample of GV patients and controls was carried out using Human MACSxpress® Whole Blood CD8 T Cell Isolation Kit (Miltenyi Biotec) as suggested. $CD8^+$ T cells were isolated immunomagnetically using MACSxpress Beads and were immediately processed for in vitro Treg suppression assay.

2.4 | In vitro Treg suppression assay

The co-culture of freshly isolated $CD4^+CD25^+$ Treg cells (5×10^3 cells) was carried out with $CD8^+$ T cells (10×10^3 cells) and $CD4^+$ T cells (10×10^3 cells) at a ratio of 1:2 individually, in triplicates. The cells were stimulated with 200 IU recombinant IL-2 (PeproTech) and anti-CD3/anti-CD28 Dynabeads (Gibco; Thermo Fisher Scientific, Inc) at 1:1 bead to cell ratio. Further, the cells were incubated at final volume of 200 μL RPMI supplemented with 10% foetal bovine serum for 5 days at 37°C and 5% CO_2 in 96-well U-bottom plate. On day 4, the cells were labelled with 10 μM BrdU (Bromodeoxyuridine) (Sigma-Aldrich) for 18 hours and then processed for BrdU cell proliferation assay.

2.5 | BrdU cell proliferation assay

Incorporation of BrdU in proliferating cells was measured by BrdU cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich). Percentage suppression was calculated using the following formula: [(proliferation of $CD4^+/CD8^+$ T cells alone − proliferation of $CD4^+/CD8^+$ T cells treated with Treg)/proliferation of $CD4^+/CD8^+$ T cells alone] × 100.

2.6 | Estimation of IFN-γ, IL-10 and TGF-β proteins levels in cell culture supernatant

On day 5, the levels of IFN-γ, IL-10 and TGF-β proteins were estimated from cell culture supernatant in triplicates using the human IFN-γ, IL-10 and TGF-β ELISA kits (USCN Life Science Inc) as per the manufacturer's protocol (Figure S1a-c).

2.7 | Flow cytometric analysis of FOXP3, NFATC1 and CD44 proteins levels in Tregs

On day 5, the levels of FOXP3, NFATC1, CD25 and CD44 proteins were estimated using flow cytometry. Surface staining of Treg cells was carried out with anti-CD4, anti-CD25 and anti-CD44 antibodies. These cells were permeabilized with Intracellular Staining Permeabilization Wash buffer (Biolegend) and stained with anti-NFATC1 and anti-FOXP3 antibodies. Cells were then fixed in 1% paraformaldehyde solution and immediately acquired using BD Flow Cytometer & Cell sorter (BD Biosciences) followed by analysis using De Novo FCS Express 7 software (DeNovo Software). The FOXP3, NFATC1, CD25 and CD44 protein levels in the Tregs were determined by their mean fluorescence intensity (MFI) (Figure S2d-g; Figure S3d-g). All samples were analysed in triplicates, and following fluorescence-conjugated antibodies were used: CD4 PE (OKT4), CD25 APC (BC96), FOXP3 FITC (206D), CD44 FITC (G44-26) and NFATC1 FITC (7A6) (Biolegend).

TABLE 1 Demographic characteristics of vitiligo patients and unaffected controls

	GV patients (n = 55)	Controls (n = 45)
Average age (mean age ± SD)	35.04 ± 14.24 y	22.76 ± 3.54 y
Gender		
Male	25 (45.45%)	26 (57.78%)
Female	30 (55.54%)	19 (42.22%)
Age of onset (mean age ± SD)	22.69 ± 12.01 y	NA
Duration of disease (mean ± SD)	5.35 ± 7.51 y	NA
Extent of disease		
VASI Score (mean ± SD)	61.91% ± 23.87%	NA
10%-25% VASI (Mild GV)	10 (18%)	
25%-50% VASI (Moderate GV)	06 (11%)	
50%-75% VASI (Severe GV)	39 (71%)	
Disease activity		
Active vitiligo	44 (80.00%)	NA
Stable vitiligo	11 (20.00%)	
Family history	15 (27.27%)	NA

2.8 | Statistical analyses

The non-parametric Mann-Whitney *U* test was used to analyse the comparisons of percentage Treg suppression of $CD8^+$ and $CD4^+$ T cells, cytokine production analysis and MFI values for protein-level analysis, whereas the correlation analysis was carried out using spearman correlation analysis among GV patient and control groups using Prism 8 software (GraphPad software Inc; 2003). The *P* values ≤ .05 were considered statistically significant.

3 | RESULTS

3.1 | Assessment of in vitro Treg suppression of $CD8^+$ and $CD4^+$ T-cell proliferation in GV patients and controls

The suppressive capacity of Tregs on $CD8^+$ and $CD4^+$ T-cell proliferation was analysed in vitro in 55 GV patients and 45 healthy controls by BrdU cell proliferation assay (Table 2). Treg cells of GV, SV and AV patients showed significantly reduced percentage suppression of $CD8^+$ T cells ($P = .0384$, $P = .0468$ and $P = .0169$, respectively; Figure 1A) and $CD4^+$ T cells ($P = .0084$, $P = .0057$ and $P = .0082$, respectively; Figure 1D) when compared with controls. The disease activity-based analysis revealed significantly reduced percentage suppression of $CD8^+$ and $CD4^+$ T cells by AV Treg cells when compared with SV Tregs ($P = .006$ and $P = .015$, respectively; Figure 1A,D). Similarly, analysis based on extent

TABLE 2 In vitro Treg suppression of $CD8^+$ and $CD4^+$ T cells and levels of IFN-γ, IL-10, TGF-β, FOXP3, CD25, NFATC1 and CD44 in generalized vitiligo patients

	CON vs GV	CON vs SV	CON vs AV	SV vs AV
Percentage suppression of $CD8^+$ and $CD4^+$ T cells by Tregs [Mean ± SEM % suppression (*P* value)]				
Percentage suppression of $CD8^+$ T cells by Tregs	46.35 ± 4.08 vs 30.80 ± 3.19; *P* = .0384 ▼	46.35 ± 4.08 vs 35.52 ± 4.01; *P* = .0468 ▼	46.35 ± 4.08 vs 29.62 ± 3.86; *P* = .0169 ▼	35.52 ± 4.01 vs 29.62 ± 3.86; *P* = .006 ▼
Percentage suppression of $CD4^+$ T cells by Tregs	73.90 ± 2.97 vs 61.20 ± 3.69; *P* = .0084 ▼	73.90 ± 2.97 vs 65.11 ± 5.11; *P* = .0057 ▼	73.90 ± 2.97 vs 54.88 ± 4.61; *P* = .0082 ▼	65.11 ± 5.11 vs 54.88 ± 4.61; *P* = .015 ▼
Protein expression level of T cells and Tregs associated cytokines in Treg: $CD8^+$ T cells/Treg: $CD4^+$ T cells [Mean ± SEM pg/mL (*P* value)]				
IFN-γ production by $CD8^+$ T cells in Treg: $CD8^+$ T cells	488.8 ± 25.11 vs 1278 ± 67.12; *P* < .0001 ▲	488.8 ± 25.11 vs 909.2 ± 121.80; *P* < .0001 ▲	488.8 ± 25.11 vs 1274 ± 85.95; *P* < .0001 ▲	909.2 ± 121.80 & 1274 ± 85.95; *P* = .036 ▲
IFN-γ Production by $CD4^+$ T cells in Treg: $CD4^+$ T cells	894.8 ± 74.89 vs 1216 ± 70.61; *P* = .0019 ▲	894.8 ± 74.89 vs 953.2 ± 138.7; *P* = .0053 ▲	894.8 ± 74.89 vs 1169 ± 91.85; *P* = .0143 ▲	953.2 ± 138.7 vs 1169 ± 91.85 *P* = .045 ▲
IL-10 production by Tregs in Treg: $CD8^+$ T cells	30.40 ± 1.50 vs 18.22 ± 0.90; *P* < .0001 ▼	30.40 ± 1.50 vs 21.58 ± 1.19; *P* < .0001 ▼	30.40 ± 1.50 vs 18.03 ± 1.09; *P* < .0001 ▼	21.58 ± 1.19 vs 18.03 ± 1.09; *P* = .009 ▼
IL-10 production by Tregs in Treg: $CD4^+$ T cells	26.66 ± 0.96 vs 16.85 ± 0.95; *P* < .0001 ▼	26.66 ± 0.96 vs 23.66 ± 2.62; *P* < .0001 ▼	26.66 ± 0.96 vs 17.39 ± 1.15; *P* < .0001 ▼	23.66 ± 2.62 vs 17.39 ± 1.15; *P* = .021 ▼
TGF-β production by Tregs in Treg: $CD8^+$ T cells	96.72 ± 2.89 vs 54.74 ± 5.14; *P* < .0001 ▼	96.72 ± 2.89 vs 64.48 ± 15.07; *P* = .0063 ▼	96.72 ± 2.89 vs 51.59 ± 4.817; *P* < .0001 ▼	64.48 ± 15.07 vs 51.59 ± 4.817; *P* = .6064 ■
TGF-β production by Tregs in Treg: $CD4^+$ T cells	98.78 ± 3.01 vs 54.78 ± 2.30; *P* < .0001 ▼	98.78 ± 3.01 vs 52.64 ± 3.473; *P* < .0001 ▼	98.78 ± 3.01 vs 52.06 ± 2.819; *P* < .0001 ▼	52.64 ± 3.473 vs 52.06 ± 2.819; *P* = .7893 ■
Protein expression level of Treg associated genes [Mean ± SEM MFI (*P* value)]				
FOXP3 expression in Tregs	799.5 ± 16.46 vs 390.6 ± 7.26; *P* < .0001 ▼	799.5 ± 16.46 vs 412.1 ± 14.88; *P* < .0001 ▼	799.5 ± 16.46 vs 375.8 ± 7.46; *P* < .0001 ▼	412.1 ± 14.88 vs 375.8 ± 7.46; *P* = .0244 ▼
CD25 expression in Tregs	719.6 ± 12.68 vs 635.1 ± 17.81; *P* < .0001 ▼	719.6 ± 12.68 vs 689.0 ± 38.9; *P* = .0064 ▼	719.6 ± 12.68 vs 621.3 ± 19.70; *P* = .0003 ▼	689.0 ± 38.9 vs 621.3 ± 19.70; *P* = .2590 ■
NFATC1 expression in Tregs	706.1 ± 36.1 vs 513 ± 22.76; *P* < .0001 ▼	706.1 ± 36.1 vs 620.4 ± 60.47; *P* = .0020 ▼	706.1 ± 36.1 vs 488.9 ± 23.14; *P* < .0001 ▼	620.4 ± 60.47 vs 488.9 ± 23.14; *P* = .019 ▼
CD44 expression in Tregs	616.6 ± 43.31 vs 690.9 ± 35.83; *P* = .2924 ■	616.6 ± 43.31 vs 710.3 ± 104.3; *P* = .0891 ■	616.6 ± 43.31 vs 686.5 ± 37.71; *P* = .8565 ■	710.3 ± 104.3 vs 686.5 ± 37.71; *P* = .6727 ■

	Correlation analysis [Spearman's rank correlation coefficient (r value) (*P* value)]		
	% suppression of $CD8^+$ T cells by Tregs	% suppression of $CD4^+$ T cells by Tregs	FOXP3 expression in Tregs
IFN-γ production by $CD8^+$/$CD4^+$ T cells, respectively	*r* = −.62; *P* < .0001 Negative correlation −	*r* = −.64; *P* < .0001 Negative correlation −	NA
IL-10 production by Tregs in Treg: $CD8^+$/Treg: $CD4^+$ T cells	*r* = .64; *P* < .0001 Positive correlation +	*r* = .62; *P* < .0001 Positive correlation +	NA
TGF-β production by Tregs in Treg: $CD8^+$/Treg: $CD4^+$ T cells	*r* = .69; *P* < .0001 Positive correlation +	*r* = .54; *P* < .0001 Positive correlation +	NA

(Continues)

TABLE 2 (Continued)

	Correlation analysis [Spearman's rank correlation coefficient (r value) (P value)]		
	% suppression of $CD8^+$ T cells by Tregs	% suppression of $CD4^+$ T cells by Tregs	FOXP3 expression in Tregs
NFATC1 expression in Tregs	$r = .54$; $P < .0001$ Positive correlation +	$r = .57$; $P < .0001$ Positive correlation +	$r = .41$; $P < .0001$ Positive correlation +
CD25 expression in Tregs	$r = .38$; $P < .0001$ Positive correlation +	$r = .44$; $P < .0001$ Positive correlation +	$r = .41$; $P < .0001$ Positive correlation +

Note: ▾ Significantly decreased levels; ▴ Significantly increased levels; ■ No significant difference; + Positively correlated; − Negatively correlated.

Abbreviations: AV, active vitiligo; CON, Control; GV, generalized vitiligo; SV, stable vitiligo.

of vitiligo as determined by VASI score revealed significantly reduced percentage suppression of $CD8^+$ T cells and $CD4^+$ T cells by Tregs of patients with severe GV (50%-75% VASI) compared to mild GV (25%-50% VASI) ($P = .0003$ and $P = .001$, respectively, Figure 1B,E). Moreover, a negative correlation was observed between Tregs' percentage suppression of $CD8^+$ and $CD4^+$ T cells and VASI score ($r = -.47$, $P = .0006$ and $r = -.66$, $P < .0001$, respectively; Figure 1C,F). However, no significant difference was observed for Tregs' percentage suppression of $CD8^+$ and $CD4^+$ T cells in patients with moderate GV when compared to mild GV ($P = .263$ and $P > .999$, respectively, Figure 1B,E) and severe GV ($P = .967$ and $P = .873$, respectively, Figure 1B,E). In addition, the Treg percentage suppression of $CD8^+$ and $CD4^+$ T cells did not differ between male and female patients (Figure S4a,b). The reduced ability of Tregs to suppress $CD8^+$ and $CD4^+$ T cells suggests an inherent functional defect of Treg cells in GV patients.

3.2 | Increased IFN-γ production by $CD8^+$ and $CD4^+$ T cells in GV patients

Since activated $CD8^+$ and $CD4^+$ T cells produce high levels of IFN-γ, we evaluated IFN-γ production by $CD8^+$ and $CD4^+$ T cells in the presence of Treg cells (in cell culture supernatants of Treg: $CD8^+$ T cells and Treg:$CD4^+$ T cells co-culture systems, respectively) of GV patients and healthy controls (Table 2). We observed significant increased IFN-γ production by $CD8^+$ T cells ($P < .0001$; Figure 2A) and $CD4^+$ T cells ($P = .0019$, $P = .0053$ and $P = .0143$, respectively; Figure 2D) in GV, SV and AV patients as compared to controls. In particular, the IFN-γ production by $CD8^+$ & $CD4^+$ T cells was significantly increased in AV patients compared to SV patients ($P = .036$ and $P = .045$, respectively; Figure 2A,D). Analysis based on VASI score revealed an increase in IFN-γ production by $CD8^+$ & $CD4^+$ T cells in patients with severe GV as compared to mild GV ($P = .0029$ and $P < .0001$, respectively, Figure 2A,D). Also, a positive correlation was observed between IFN-γ production by $CD8^+$ and $CD4^+$ T cells and VASI score ($r = .47$, $P = .0029$ and $r = .51$, $P = .0017$, respectively; Figure S5a,b). However, no significant difference was observed for IFN-γ production by $CD8^+$ and $CD4^+$ T cells in patients with moderate GV as compared to mild GV ($P = .6156$ and $P = .6184$, respectively, Figure 2A,D) and severe GV ($P = .4702$ and $P = .1134$, respectively, Figure 2A,D). Further, we found that IFN-γ production was negatively correlated with percentage suppression of $CD8^+$ and $CD4^+$ T cells in GV patients and controls ($r = -.62$; $P < .0001$ and $r = -.64$, $P < .0001$, respectively; Figure S6a,b). However, the IFN-γ production by $CD8^+$ and $CD4^+$ T cells did not differ between male and female patients (Figure S4g,h). The increased IFN-γ production by $CD8^+$ and $CD4^+$ T cells suggests increased activity of T cells and compromised Treg cell function in GV patients.

3.3 | Decreased IL-10 and TGF-β production by Treg cells in GV patients

The suppressive function of Treg is exerted mainly by cytokines IL-10 and TGF-β. Hence, we further assessed the production of IL-10 and

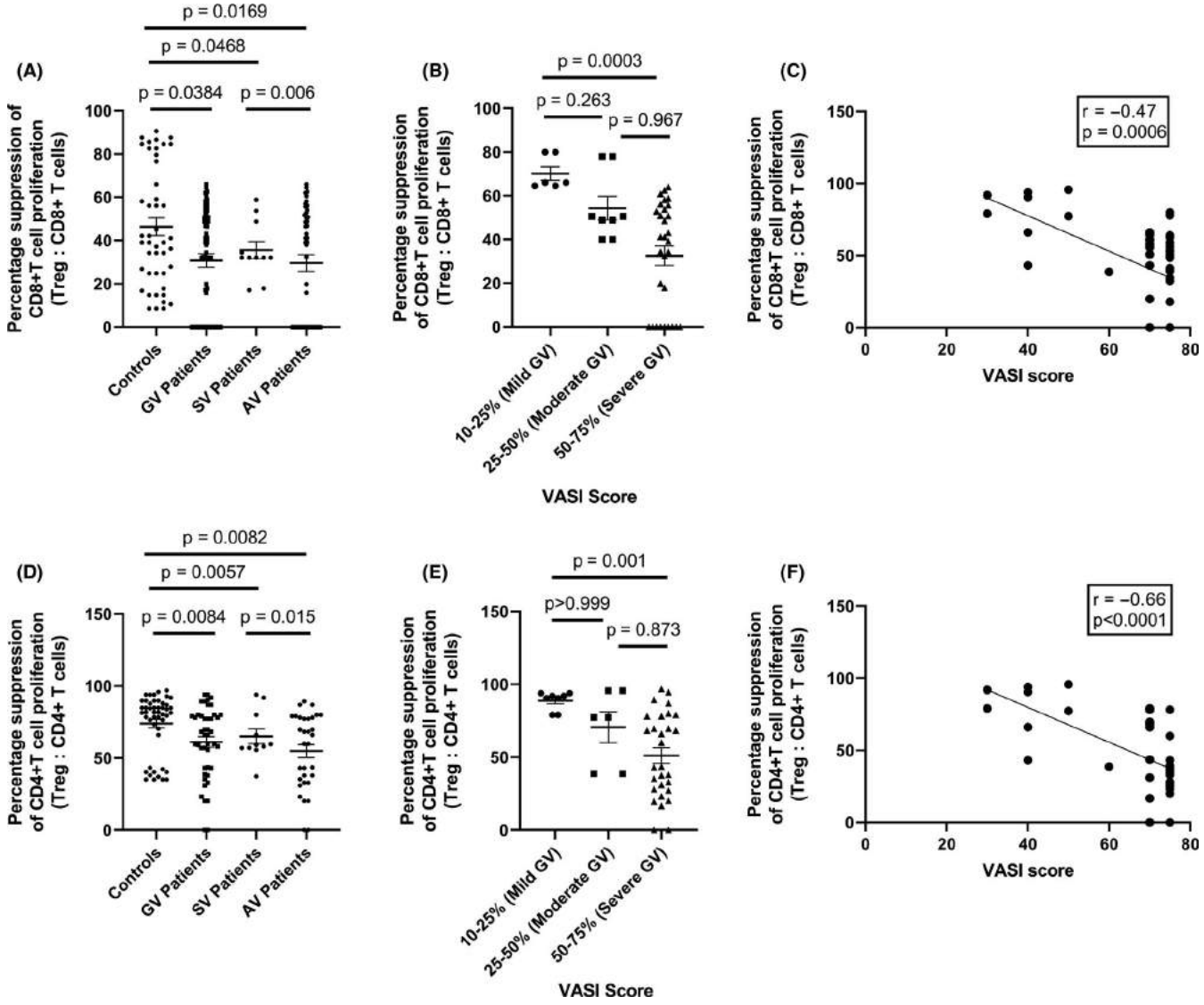

FIGURE 1 In vitro Treg suppression of $CD8^+$ and $CD4^+$ T cells' proliferation in GV patients and controls. In *vitro* Treg suppression of $CD8^+$ and $CD4^+$ T cells proliferation by Treg in 55 generalized vitiligo (GV) patients [44 active vitiligo (AV) patients and 11 stable vitiligo (SV) patients; 10 mild GV patients (10%-25% VASI), 6 moderate GV patients (25%-50% VASI) and 39 severe GV patients (50%-75% VASI)] and 45 healthy controls were analysed by non-parametric Mann-Whitney *U* test. A, Percentage suppression of $CD8^+$ T cells in GV, SV and AV patients vs controls (Mean ± SEM: 30.80 ± 3.19, 35.52 ± 4.01, 29.62 ± 3.86 vs 46.35 ± 4.08; $P = .0384$, $P = .0468$ and $P = .0169$, respectively). Percentage suppression of $CD8^+$ T cells in SV vs AV patients (Mean ± SEM: 35.52 ± 4.01 vs 29.62 ± 3.86; $P = .006$). B, Percentage suppression of $CD8^+$ T cells for 10%-25% VASI (Mild GV) vs 50%-75% VASI (Severe GV) patients (Mean ± SEM: 70.18 ± 3.10 vs 32.56 ± 4.43; $P = .0003$). Percentage suppression of $CD8^+$ T cells for 25%-50% VASI (Moderate GV) vs 10%-25% VASI (Mild GV) and 50%-75% VASI (Severe GV) patients (Mean ± SEM: 54.32 ± 5.37 vs 70.18 ± 3.10 and 32.56 ± 4.43; $P = .263$ and $P = .967$, respectively). C, Correlation of percentage suppression of $CD8^+$ T cells in Treg: $CD8^+$ T cells co-culture system with VASI score for GV patients ($r = -.47$, $P = .0006$). D, Percentage suppression of $CD4^+$ T cells in GV, SV and AV patients vs controls (Mean ± SEM: 61.20 ± 3.69, 65.11 ± 5.11, 54.88 ± 4.61 vs 73.90 ± 2.97; $P = .0084$, $P = .0057$ and $P = .0082$, respectively). Percentage suppression of $CD4^+$ T cells in SV vs AV patients (Mean ± SEM: 65.11 ± 5.11 vs 54.88 ± 4.61; $P = .015$). E, Percentage suppression of $CD4^+$ T cells for 10%-25% VASI (Mild GV) vs 50%-75% VASI (Severe GV) patients (Mean ± SEM: 88.85 ± 2.18 vs 51.10 ± 5.33; $P = .001$). Percentage suppression of $CD4^+$ T cells for 25%-50% VASI (Moderate GV) vs 10%-25% VASI (Mild GV) and 50%-75% VASI (Severe GV) patients (Mean ± SEM: 70.54 ± 10.63 vs 88.85 ± 2.18 & 51.10 ± 5.33; $P > .999$ and $P = .873$, respectively) (SEM, standard error mean). F, Correlation of percentage suppression of $CD4^+$ T cells in Treg: $CD4^+$ T cells co-culture system with VASI score for GV patients ($r = -.66$, $P < .0001$)

TGF-β by Treg cells in cell culture supernatants of Treg:$CD8^+$ T cells and Treg:$CD4^+$ T cells co-culture systems of GV patients and healthy controls (Table 2). We found significantly decreased IL-10 production by Treg cells of GV, SV and AV patients as compared to that of controls in Treg:$CD8^+$ T cells and Treg:$CD4^+$ T cells' co-culture systems ($P < .0001$; Figure 2B,E). Moreover, TGF-β production by Treg cells was significantly reduced in GV, SV and AV patients as compared to controls in Treg:$CD8^+$ T cell co-culture system ($P < .0001$,

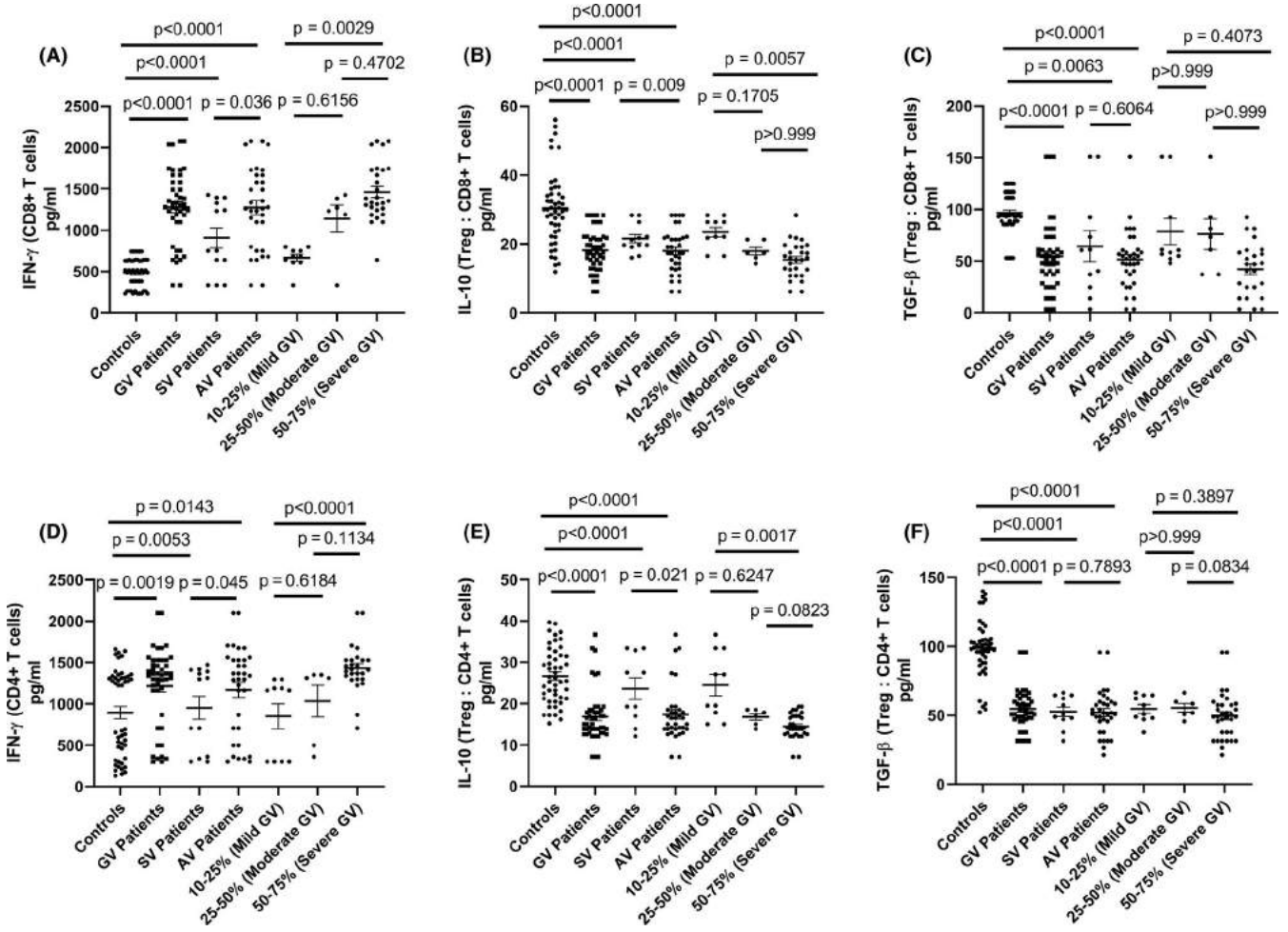

FIGURE 2 Production of IFN-γ by $CD8^+$ and $CD4^+$ T cells and IL-10 and TGF-β by Treg cells in Treg:$CD8^+$ and Treg:$CD4^+$ T cells co-culture systems of GV patients and controls. Total 55 GV patients [44 AV and 11 SV patients; 10 mild GV patients (10%-25% VASI), 6 moderate GV patients (50%-75% VASI) and 39 severe GV patients (50%-75% VASI)] and 45 healthy controls were analysed for production of IFN-γ and IL-10 and TGF-β by non-parametric Mann-Whitney *U* test. A, IFN-γ production by $CD8^+$ T cells in GV, SV and AV patients vs controls (Mean ± SEM: 1278 ± 67.12, 909.2 ± 121.80 and 1274 ± 85.95 vs 488.8 ± 25.11; $P < .0001$); SV vs AV (Mean ± SEM: 909.2 ± 121.80 vs 1274 ± 85.95; $P = .036$); Mild GV vs Severe GV (Mean ± SEM: 667.7 ± 43.19 vs 1462.0 ± 67.32; $P = .0029$); and Moderate GV vs Mild and Severe GV (Mean ± SEM: 1141.0 ± 165.2 vs 667.7 ± 43.19 and 1462.0 ± 67.32; $P = .6156$ and $P = .4702$, respectively). B, IL-10 production by Treg cells in GV, SV and AV patients vs controls (Mean ± SEM: 18.22 ± 0.90, 21.58 ± 1.19 & 18.03 ± 1.09 vs 30.40 ± 1.50; $P < .0001$); SV vs AV (Mean ± SEM: 21.58 ± 1.19 vs 18.03 ± 1.09; $P = .009$); Mild GV vs Severe GV (Mean ± SEM: 23.50 ± 1.39 vs 15.41 ± 0.96; $P = .0057$); and Moderate GV vs Mild and Severe GV (Mean ± SEM: 17.96 ± 1.14 vs 23.50 ± 1.39 & 15.41 ± 0.96; $P = .1705$ and $P > .999$, respectively). C, TGF-β production by Tregs in GV, SV and AV patients vs controls (Mean ± SEM: 54.74 ± 5.14, 64.48 ± 15.07 and 51.59 ± 4.817 vs 96.72 ± 2.89; $P < .0001$, $P = .0063$ and $P < .0001$, respectively); SV vs AV (Mean ± SEM: 64.48 ± 15.07 vs 51.59 ± 4.817; $P = .6064$); Mild GV vs Severe GV (Mean ± SEM: 78.67 ± 12.61 vs 42.22 ± 5.16; $P = .4073$); and Moderate GV vs Mild and Severe GV (Mean ± SEM: 76.25 ± 14.75 vs 78.67 ± 12.61 and 42.22 ± 5.16; $P > .999$, respectively). D, IFN-γ production by $CD4^+$ T cells in GV, SV and AV patients vs controls (Mean ± SEM: 1216 ± 70.61, 953.2 ± 138.7 and 1169 ± 91.85 vs 894.8 ± 74.89; $P = .0019$, $P = .0053$ and $P = .0143$, respectively); SV vs AV (Mean ± SEM: 953.2 ± 138.7 vs 1169 ± 91.85; $P = .045$); Mild GV vs Severe GV (Mean ± SEM: 850.7 ± 150.0 vs 1432.0 ± 56.49; $P < .0001$); and Moderate GV vs Mild and Severe GV (Mean ± SEM: 1033.0 ± 190.8 vs 850.7 ± 150.0 & 1432.0 ± 56.49; $P = .6184$ & $P = .1134$, respectively). E, IL-10 production by Treg cells in GV, SV and AV patients vs controls (Mean ± SEM: 16.85 ± 0.95, 23.66 ± 2.62 and 17.39 ± 1.15 vs 26.66 ± 0.96; $P < .0001$); SV vs AV (Mean ± SEM: 23.66 ± 2.62 vs 17.39 ± 1.15; $P = .021$); Mild GV vs Severe GV (Mean ± SEM: 24.47 ± 2.57 vs 14.51 ± 0.54; $P = .0017$); and Moderate GV vs Mild and Severe GV (Mean ± SEM: 16.81 ± 0.78 vs 24.47 ± 2.57 and 14.51 ± 0.54; $P = .6247$ and $P = .0823$, respectively). F, TGF-β production by Treg cells in GV, SV and AV patients vs controls (Mean ± SEM: 54.78 ± 2.30, 52.64 ± 3.473 and 52.06 ± 2.819 vs 98.78 ± 3.01; $P < .0001$); SV vs AV (Mean ± SEM: 52.64 ± 3.473 vs 52.06 ± 2.81; $P = .7893$); Mild GV vs Severe GV (Mean ± SEM: 54.76 ± 3.04 vs 49.74 ± 2.91; $P = .3897$); and Moderate GV vs Mild and Severe GV (Mean ± SEM: 55.48 ± 3.04 vs 54.76 ± 3.04 vs 49.74 ± 2.91; $P > .999$ and $P = .0834$, respectively)

$P = .0063$ and $P < .0001$, respectively; Figure 2C) and Treg: $CD4^+$ T cell co-culture ($P < .0001$; Figure 2F). In particular, IL-10 production by Treg cells in Treg:$CD8^+$ T cells and Treg:$CD4^+$ T cells co-culture systems was significantly reduced in AV patients when compared to SV patients ($P = .009$ and $P = .021$, respectively; Figure 2B,E). Analysis based on extent of disease revealed significantly reduced

IL-10 production in Treg:CD8$^+$ T cells and Treg: CD4$^+$ T cells' co-culture systems by Tregs of patients with severe GV compared to mild GV ($P = .0057$ and $P = .0017$, respectively, Figure 2B,E). Moreover, a negative correlation was observed between IL-10 production by Tregs in Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells co-culture systems and VASI score ($r = -.41$, $P = .0096$ and $r = -.34$, $P = .029$, respectively; Figure S5c,d). However, IL-10 production by Treg cells in Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells co-culture systems did not differ in patients with moderate GV compared to mild GV ($P = .1705$ and $P = .6247$, respectively, Figure 2B,E) and severe GV ($P > .999$ and $P = .0823$, respectively, Figure 2B,E). There was no significant difference observed in TGF-β production by Treg cells in Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells' co-culture systems between AV and SV patients ($P = .6064$ and $P = .7893$; Figure 2C,F). Similarly, TGF-β production by Treg cells in Treg: CD8$^+$ T cells and Treg: CD4$^+$ T cells co-culture systems did not differ between patients with mild and severe GV ($P = .4073$ and $P = .3897$, respectively; Figure 2C,F), mild and moderate GV ($P > .999$; Figure 2C,F) and moderate and severe GV ($P > .999$, $P = .0834$, respectively; Figure 2C,F). There was no correlation found between TGF-β production by Treg cells and VASI score ($r = -.18$, $P = .27$ and $r = .03$, $P = .8488$, respectively; Figure S5e,f).

Further, we found that IL-10 and TGF-β productions by Tregs were positively correlated with percentage suppression of CD8$^+$ and CD4$^+$ T cells in Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells co-culture systems of GV patients and controls (IL-10: $r = .64$, $P < .0001$; $r = .62$, $P < .0001$ and TGF-β: $r = .69$, $P < .0001$; $r = .54$, $P < .0001$; respectively; Figure S6c-f). However, the IL-10 and TGF-β production by Tregs in Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells co-culture systems did not differ between male and female patients (Figure S4c-f). Thus, the reduced IL-10 and TGF-β production by Tregs indicates the impaired Tregs suppressive function in GV patients.

3.4 | Analysis of IFN-γ:IL-10 and IFN-γ:TGF-β ratio in GV patients and controls

Further, we analysed IFN-γ:IL-10 ratio in cell culture supernatants of Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells co-culture systems of GV patients and healthy controls. We found significantly increased IFN-γ:IL-10 ratio in GV, SV and AV patients of Treg:CD8$^+$ T cells ($P < .0001$) and Treg:CD4$^+$ T cells co-culture system ($P < .0001$, $P = .0012$ and $P < .0001$, respectively) as compared to controls (Figure S7a,b). Similarly, the IFN-γ:TGF-β ratio was significantly increased in GV, SV and AV patients of Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells co-culture systems as compared to controls ($P < .0001$; Figure S7c,d). In particular, the IFN-γ:IL-10 ratio in Treg:CD8$^+$ T cells and Treg:CD4$^+$ T cells co-culture systems was significantly increased in AV as compared to SV patients ($P = .0055$ and $P = .0223$, respectively; Figure S7a,b). However, the IFN-γ:TGF-β ratio did not differ between AV and SV patients in the co-culture systems ($P = .3916$ and $P = .4712$, respectively; Figure S7c,d). The increased IFN-γ:IL-10 and IFN-γ:TGF-β ratio in GV patients clearly indicates an imbalance between pro-inflammatory and anti-inflammatory cytokines leading to defective Tregs suppression function in GV.

3.5 | Assessment of regulatory molecules of Treg cell function (FOXP3, CD25, NFATC1 and CD44) in GV patients and controls

Further, we assessed few important regulatory molecules of Treg cell, which could affect the suppressive function of Treg cell. Through flow cytometry, the protein levels of two intracellular Treg molecules (FOXP3 and NFATC1) and two surface suppressive markers (CD25 and CD44) were analysed in Tregs of GV patients and controls (Table 2). We observed significant reduced protein levels of FOXP3 ($P < .0001$; Figure 3A), CD25 ($P < .0001$, $P = .0064$ and $P = .0003$, respectively; Figure 3B) and NFATC1 ($P < .0001$, $P = .020$ and $P < .0001$, respectively; Figure 3C) in Treg cells of GV, SV and AV patients, respectively, as compared to controls. However, no significant difference for CD44 levels was observed in Tregs of GV, SV and AV patients as compared to controls ($P = .2924$, $P = .0891$ and $P = .8565$, respectively; Figure S8a). The FOXP3 and NFATC1 proteins were significantly reduced in Tregs of AV patients compared to SV ($P = .0244$ and $P = .019$, respectively; Figure 3A,C). Analysis based on extent of vitiligo showed significantly reduced FOXP3 and NFATC1 proteins in Tregs of patients with severe GV compared to mild GV ($P = .002$ and $P = .0347$, respectively; Figure 3D,F). Moreover, a negative correlation was observed between Tregs' FOXP3 and NFATC1 proteins and VASI score ($r = -.55$, $P = .0008$ and $r = -.27$, $P = .043$, respectively; Figure S9a,c). However, the FOXP3 and NFATC1 protein levels did not differ in patients with moderate GV compared to mild GV ($P = .3453$ and $P > .999$, respectively, Figure 3D,F) and severe GV ($P > .999$ and $P = .4297$ respectively, Figure 3D,F). In addition, CD25 and CD44 proteins did not differ between Tregs of AV and SV patients ($P = .2590$ and $P = .6727$, respectively; Figure 3B and Figure S8a, respectively). Similarly, CD25 and CD44 proteins did not differ between patients with mild and severe GV ($P = .7218$ and $P > .999$, respectively; Figure 3E and Figure S8b); mild and moderate GV ($P > .999$; Figure 3E and Figure S8b, respectively) and moderate and severe GV ($P = .3142$, $P > .999$, respectively; Figure 3E and Figure S8b). Also, there was no correlation found between CD25 and CD44 proteins and VASI score ($r = -.28$, $P = .0918$ and $r = -.05$, $P = .7164$, respectively; Figure S9b,d).

Furthermore, NFATC1 and CD25 protein levels in Tregs were positively correlated with percentage suppression of CD8$^+$ and CD4$^+$ T cells by Tregs in Treg:CD8$^+$ T cells (NFATC1: $r = .54$, $P < .0001$; CD25: $r = .38$, $P < .0001$; Figure 4A,C) and Treg:CD4$^+$ T cells co-culture systems (NFATC1: $r = .57$, $P < .0001$; CD25: $r = .44$, $P < .0001$; Figure 4B,D) of GV patients and controls. Interestingly, NFATC1 and CD25 protein levels in Tregs were positively correlated with FOXP3 protein expression in Tregs of GV patients and controls ($r = .41$, $P < .0001$; Figure 4E,F). However, there was no correlation observed for CD44 protein level in Tregs with percentage Treg suppression of CD8$^+$ & CD4$^+$ T cells in Treg: CD8$^+$ & Treg:CD4$^+$ T cells co-culture systems ($r = -.11$, $P = .2925$, and $r = -.04$, $P = .6606$, respectively; Figure S10a,b) and FOXP3 and NFATC1 expression ($r = .10$,

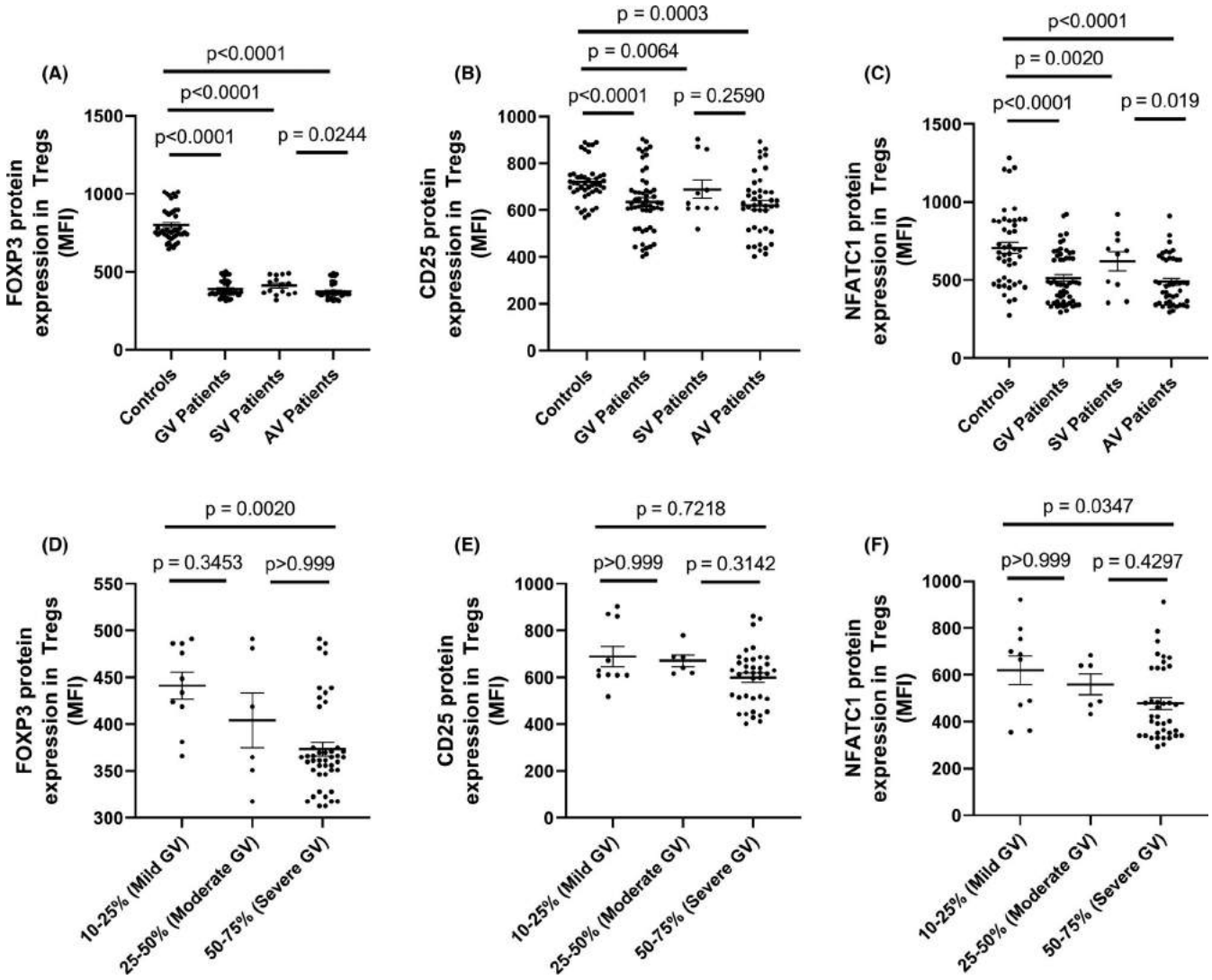

FIGURE 3 FOXP3, CD25, NFATC1 and CD44 protein levels in GV patients. FOXP3, CD25, NFATC1 and CD44 expression levels in 55 Generalized vitiligo (GV) patients [44 Active vitiligo (AV) patients and 11 Stable vitiligo (SV) patients; 10 Mild GV patients (10%-25% VASI), 6 Moderate GV patients (25%-50% VASI) and 39 Severe GV patients (50%-75% VASI)] and 45 healthy controls were analysed by non-parametric Mann-Whitney *U* test. A, FOXP3 expression level in Treg of GV, SV and AV patients vs controls (Mean ± SEM: 390.6 ± 7.26, 412.1 ± 14.88 and 375.8 ± 7.46 vs 799.5 ± 16.46; $P < .0001$). FOXP3 expression level in Treg of SV vs AV patients (Mean ± SEM: 412.1 ± 14.88 and 375.8 ± 7.46; $P = .0244$). B, CD25 expression level in Treg of GV, SV and AV patients vs controls (Mean ± SEM: 635.1 ± 17.81, 689.0 ± 38.9 and 621.3 ± 19.70 vs 719.6 ± 12.68; $P < .0001$, $P = .0064$ and $P = .0003$, respectively). CD25 expression level in Treg of SV vs AV patients (Mean ± SEM: 689.0 ± 38.9 vs 621.3 ± 19.70; $P = .2590$). C, NFATC1 expression level in Treg of GV, SV and AV patients vs controls (Mean ± SEM: 513 ± 22.76, 620.4 ± 60.47 and 488.9 ± 23.14 vs 706.1 ± 36.1; $P < .0001$, $P = .020$ and $P < .0001$, respectively). NFATC1 expression level in Treg of SV and AV patients (Mean ± SEM: 620.4 ± 60.47 vs 488.9 ± 23.14; $P = .019$). D, FOXP3 expression level in Treg for 10%-25% VASI (Mild GV) vs 50%-75% VASI (Severe GV) patients (Mean ± SEM: 441.1 ± 14.15 vs 373.5 ± 7.26; $P = .002$). FOXP3 expression level in Treg for 25%-50% VASI (Moderate GV) vs 10%-25% VASI (Mild GV) and 50%-75% VASI (Severe GV) patients (Mean ± SEM: 404.0 ± 29.23 vs 441.1 ± 14.15 & 373.5 ± 7.26; $P = .3453$ and $P > .999$, respectively). E, CD25 expression level in Treg for 10%-25% VASI (Mild GV) vs 50%-75% VASI (Severe GV) patients (Mean ± SEM: 689.2 ± 43.07 vs 598.9 ± 19.34; $P = .7218$). CD25 expression level in Treg for 25%-50% VASI (Moderate GV) vs 10%-25% VASI (Mild GV) and 50%-75% VASI (Severe GV) patients (Mean ± SEM: 671.5 ± 25.08 vs 689.2 ± 43.07 and 598.9 ± 19.34; $P > .999$ and $P = .3142$, respectively). F, NFATC1 expression level in Treg for 10%-25% VASI (Mild GV) vs 50%-75% VASI (Severe GV) patients (Mean ± SEM: 620.4 ± 60.47 vs 559.3 ± 43.85; $P = .0347$). NFATC1 expression level in Treg for 25%-50% VASI (Moderate GV) vs 10%-25% VASI (Mild GV) and 50%-75% VASI (Severe GV) patients (Mean ± SEM: 559.3 ± 43.85 vs 620.4 ± 60.47 & 559.3 ± 43.85; $P > .999$ and $P = .4297$, respectively). (SEM, standard error mean)

$P = .2551$, and $r = .05$, $P = .5288$, respectively; Figure S10c,d) in Tregs of GV patients and controls. Moreover, the FOXP3, CD25, NFATC1 and CD44 protein expression in Tregs did not differ between male and female patients ($P = .7724$, $P = .6104$, $P = .3875$ and $P = .6707$, respectively; Figure S11a-d). Thus, significantly reduced levels of regulatory proteins FOXP3, NFATC1 and CD25 levels in GV patients suggest that altered levels of these proteins could lead to impaired suppressive function in Tregs.

3.6 | Effect of Treg suppressive function on the age of onset of GV

We also analysed the effect of Treg suppression of $CD8^+$ and $CD4^+$ T cells, cytokine production (IFN-γ, TGF-β and IL-10) and Treg regulatory proteins (FOXP3, NFATC1, CD25 and CD44) on different age of onset groups of GV patients. The Tregs from early age of onset group (1-20 years) of patients exhibited decreased Treg suppression of $CD8^+$ and $CD4^+$ T cells ($P = .0156$ and $P = .0074$; Figure 5A,B), decreased TGF-β production by Tregs in Treg: $CD8^+$ and Treg:$CD4^+$ T cells co-culture systems ($P = .0212$ and $P = .0083$; Figure 5C,D) and decreased NFATC1 protein expression in Tregs ($P = .0103$; Figure 5E) as compared to late age of onset group (41-60 years).

However, when the age of onset group 21-40 years was compared with the age of onset groups 1-20 and 41-60 years, no significant difference was found in Treg suppression of $CD8^+$ T cells ($P = .0802$ and $P = .2535$; Figure 5A) and $CD4^+$ T cells ($P = .1027$ and $P = .2258$; Figure 5B), TGF-β production by Tregs in Treg:$CD8^+$ T cells ($P = .2571$ and $P > .9999$; Figure 5C) and Treg:$CD4^+$ T cells co-culture systems ($P = .7715$ and $P = .3312$; Figure 5D) and NFATC1 protein expression in Treg cells ($P = .1096$ and $P = .2176$; Figure 5E). Similarly, there was no significant difference observed for IL-10 production by Tregs,

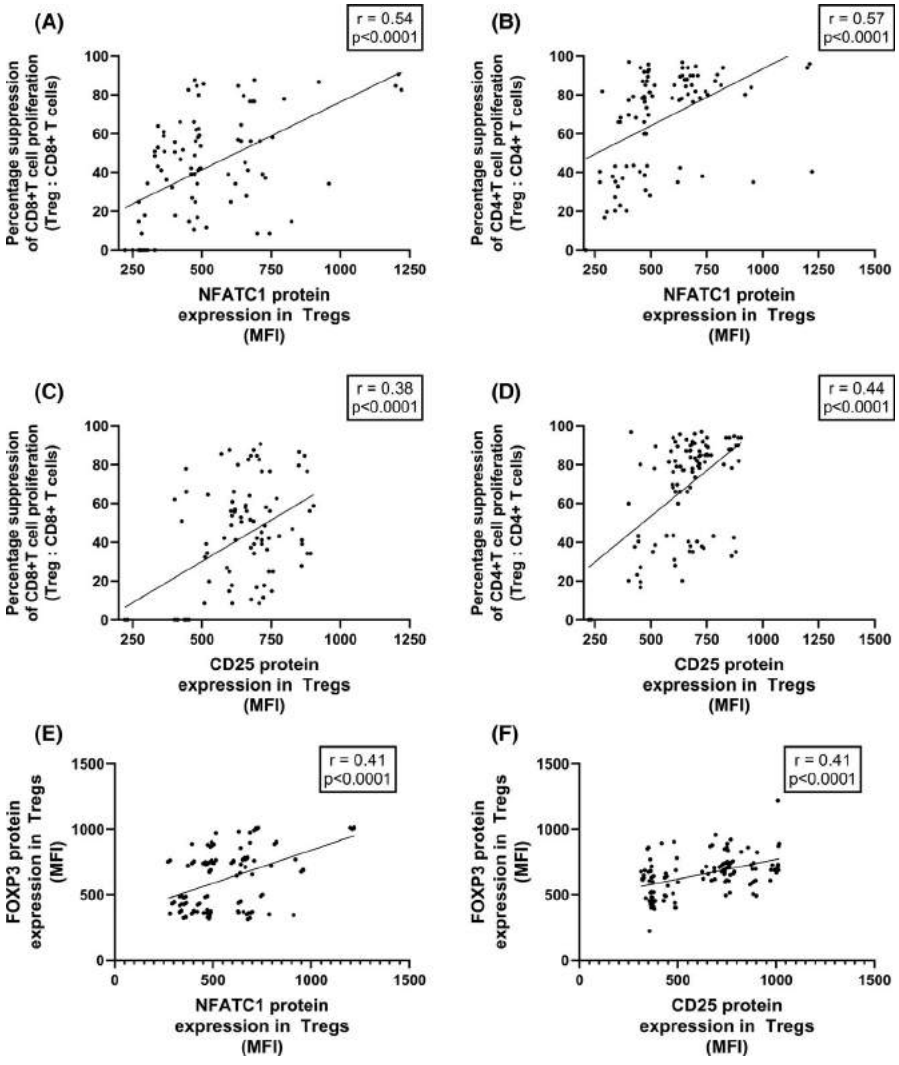

FIGURE 4 Correlation of NFATC1 and CD25 levels with percentage Treg suppression of $CD8^+$ and $CD4^+$ T cells and FOXP3 in Tregs of GV patients and controls. The correlation of NFATC1, CD25 expression with percentage Treg suppression of $CD8^+$ and $CD4^+$ T cells and FOXP3 expression in Tregs in Treg: $CD8^+$ and Treg: $CD4^+$ T cells' co-culture systems of GV patients and controls were analysed by spearman correlation analysis. A, NFATC1 expression in Tregs was positively correlated with percentage suppression of $CD8^+$ T cells in Treg: $CD8^+$ T cells' co-culture system of GV patients and controls ($r = .54$, $P < .0001$). B, NFATC1 expression in Tregs was positively correlated with percentage suppression of $CD4^+$ T cells in Treg: $CD4^+$ T cells' co-culture system of GV patients and controls ($r = .57$, $P < .0001$). C, CD25 expression in Tregs was positively correlated with percentage suppression of $CD8^+$ T cells in Treg: $CD8^+$ T cells' co-culture system of GV patients and controls ($r = .38$, $P < .0001$). D, CD25 expression in Tregs was positively correlated with percentage suppression of $CD4^+$ T cells in Treg: $CD4^+$ T cells' co-culture system of GV patients and controls ($r = .44$, $P < .0001$). E, NFATC1 expression in Tregs was positively correlated with FOXP3 expression in Tregs in GV patients and controls ($r = .41$, $P < .0001$). F, CD25 expression in Tregs was positively correlated with FOXP3 expression in Tregs in GV patients and controls ($r = .41$, $P < .0001$)

IFN-γ production by $CD8^+$ and $CD4^+$ T cells in Treg:$CD8^+$ T cells and Treg:$CD4^+$ T cells' co-culture systems and protein levels of FOXP3, CD25 and CD44 in Tregs among any of the age of onset groups (ie 1-20 vs 21-40; 1-20 vs 41-60; 21-40 vs 41-60; Figure S12a-g). Thus, these results suggest that decreased Treg suppression of $CD8^+$ and $CD4^+$ T cells, reduced TGF-β production by Tregs and reduced NFATC1 protein levels in Tregs might affect the onset of GV in these patients.

4 | DISCUSSION

The cytotoxic $CD8^+$ T cells that are considered as immune systems foot soldiers have emerged to be the important players in promoting several autoimmune diseases including vitiligo.[22] Studies also indicated $CD4^+$ T cells' role in pathogenesis of vitiligo[7] as well as in activation, function and survival of self-reactive $CD8^+$ T cells.[23]

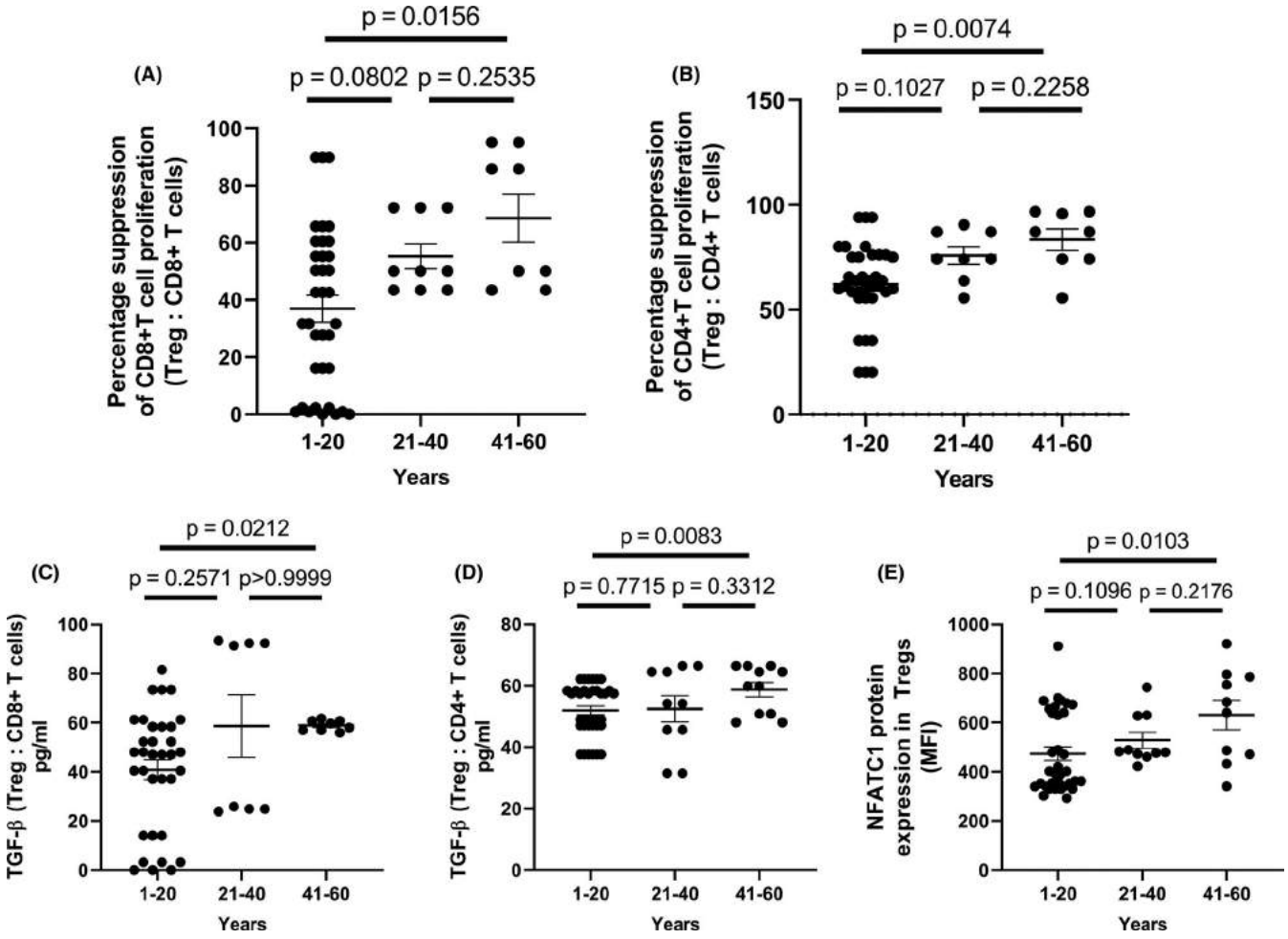

FIGURE 5 Effect of Treg suppressive function on the age of onset of GV. The effect of Treg suppression of $CD8^+$ and $CD4^+$ T cells, TGF-β production and NFATC1 expression in Tregs on age of onset in 55 generalized vitiligo (GV) patients and 45 healthy controls were analysed by non-parametric Mann-Whitney *U* test. A, Treg suppression of $CD8^+$ T cells' proliferation in Treg: $CD8^+$ T cells' co-culture system in GV patients of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 36.93 ± 4.66 vs 68.63 ± 8.39; $P = .0156$). Treg suppression of $CD8^+$ T cells proliferation in Treg: $CD8^+$ T cells' co-culture system in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 55.29 ± 4.35 vs 36.93 ± 4.66 and 68.63 ± 8.39, respectively; $P = .0802$ and $P = .2535$, respectively). B, Treg suppression of $CD4^+$ T cells' proliferation in Treg: $CD4^+$ T cells co-culture system in GV patients of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 62.13 ± 3.18 vs 83.42 ± 5.144; $P = .0074$). Treg suppression of $CD4^+$ T cells' proliferation in Treg: $CD4^+$ T cells co-culture system in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 75.80 ±. 4.287 vs 62.13 ± 3.18 and 83.42 ± 5.144, respectively; $P = .1027$ and $P = .2258$, respectively). C, TGF-β production by Treg cells for Treg: $CD8^+$ T cell co-culture in GV patients of the age of onset group 1-20 y vs 41-60 y (Mean ± SEM: 40.81 ± 4.12 vs 58.86 ± 0.76; $P = .0212$). TGF-β production by Treg cells for Treg: $CD8^+$ T-cell co-culture in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 58.63 ± 12.75 vs 40.81 ± 4.12 and 58.86 ± 0.76, respectively; $P = .2571$ and $P > .9999$, respectively). D, TGF-β production by Treg cells for Treg: $CD4^+$ T cell co-culture in GV patients of the age of onset group 1-20 y vs 41-60 y (Mean ± SEM: 58.69 ± 2.33 vs 51.94 ± 1.40; $P = .0083$). TGF-β production by Treg cells for Treg: $CD4^+$ T-cell co-culture in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 52.45 ± 4.29 vs 58.69 ± 2.33 and 51.94 ± 1.40, respectively; $P = .7715$ and $P = .3312$, respectively). E, NFATC1 protein levels in Tregs of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 473.9 ± 27.86 vs 631.5 ± 59.91; $P = .0103$). NFATC1 protein levels in Tregs of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 528.7 ± 32.34 vs 473.9 ± 27.86 and 631.5 ± 59.91, respectively; $P = .1096$ and $P = .2176$, respectively) (SEM, standard error mean)

Infiltration of $CD8^+$ and $CD4^+$ T cells to the perilesional skin has been observed in vitiligo patients.[8,18] Regulatory T cells (Tregs) control such hyperactive self-reactive T cells[10]; however, decreased Treg cells' frequency and function have been reported in blood and skin biopsies of vitiligo patients.[12-15] However, studies have explored the role of Treg cells, $CD8^+$ and $CD4^+$ T cells in GV pathogenesis, but they are limited to genetic and mRNA level. Previous studies assessing the Treg suppressive function in vitiligo patients had limitations such as smaller sample size, and suppression is checked in either $CD8^+$ or $CD4^+$ T cells and lack of data on regulatory suppressive molecules of Tregs.[12-15] In addition, the functional studies addressing the role of Treg cells, $CD8^+$ and $CD4^+$ T cells in GV pathogenesis are deficient.

In the present study, we found that Treg cells of GV patients fail to suppress activated $CD8^+$ and $CD4^+$ T-cell proliferation in vitro (Figure 1A,D; Table 2). Our findings are in concordance to the previously reported impaired Treg cell suppressive capacity towards $CD8^+$ T cells[12-14] and are in contrast with the previous study where they did not find impaired Treg cell suppression towards $CD4^+$ T cells.[15] The possible explanation for the contrasting results may be due to the smaller sample size used (n = 3) in the previous study, whereas the present study involved a larger sample size of 55 GV patients and 45 controls. Interestingly, the reduced suppression of $CD8^+$ and $CD4^+$ T cells proliferation by AV patients' Tregs suggests the crucial role of Tregs' dysfunction in progression of GV (Figure 1A,D). Moreover, VASI score based analysis of extent of vitiligo showed reduction in percentage suppression of $CD8^+$ and $CD4^+$ T cells by Tregs in patients with severe GV compare to mild GV (Figure 1B,E), indicating the role of impaired Tregs in disease severity as well. This result was further confirmed by correlation analysis in which the percentage suppression of $CD8^+$ and $CD4^+$ T cells were negatively correlated with VASI score (Figure 1C,F). Hence, the study suggests that inherent defective Treg suppressive function in GV patients may lead to widespread activation of melanocyte specific $CD8^+$ and $CD4^+$ T cells. The activated $CD4^+$ T cells may further enhance the activation of $CD8^+$ T cells resulting into $CD8^+$ T cell-mediated GV pathogenesis (Figure S13).

IFN-γ, a pro-inflammatory cytokine secreted by $CD8^+$ and $CD4^+$ T cells,[24] is involved in inflammation and autoimmune diseases.[25] It enhances $CD8^+$ T-cell cytotoxic function by increasing CTL proliferation, perforin expression and FAS/FASL-mediated killing, whereas in skin it increases T-cell migration to the site of inflammation.[24] It has been reported to inhibit melanogenesis and directly induce melanocyte apoptosis[26] (Figure S13). IFN-γ also enhances attachment of T cells to melanocytes by inducing intercellular adhesion molecule-1 (ICAM-1) expression on melanocyte's cell surface resulting in its destruction.[27,28] Earlier, we demonstrated increased serum IFN-γ levels and *ICAM* mRNA in GV patients.[29] Here, we report significant increased levels of IFN-γ production by unchecked $CD8^+$ and $CD4^+$ T cells in GV patients (Figure 2A,D), indicating inability of Tregs to suppress activated $CD8^+$ and $CD4^+$ T cells population. Moreover, the IFN-γ production by these $CD8^+$ and $CD4^+$ T cells is negatively correlated with percentage suppression of $CD8^+$ and $CD4^+$ T cells (Figure S6a,b). Previous studies also reported increased IFN-γ expression by $CD8^+$ T cells and T_{RM} (Tissue resident memory) $CD8^+$ T cells in vitiligo patients.[13,30] In addition, *IFNG* mRNA and serum IFN-γ levels were increased in vitiligo patients.[29,31,32] Interestingly, disease activity-based analysis suggested increase in IFN-γ production by $CD8^+$ and $CD4^+$ T cells in AV patients and it was in concordance with the previously reported increased IFN-γ level by $CD8^+$ and $CD4^+$ T cells in skin samples of AV patients,[30] indicating the crucial role of IFN-γ in disease progression (Figure 2A,D). In addition, VASI score-based analysis also suggested increased IFN-γ production by $CD8^+$ and $CD4^+$ T cells in patients with severe GV (Figure 2A,D). This result was also confirmed by correlation analysis where IFN-γ production by $CD8^+$ and $CD4^+$ T cells was positively correlated with VASI score (Figure S5a,b). These results are in concordance with the previous study,[31] suggesting the crucial role of IFN-γ in the disease severity. Therefore, our results along with the previous studies suggest that due to functionally defective Treg cells, the uncontrolled $CD8^+$ and $CD4^+$ T cells secrete increased IFN-γ, which can lead to melanocyte destruction in GV.

The Treg cell induces suppressive function by secreting key suppressive cytokines such as IL-10 and TGF-β. IL-10, the anti-inflammatory cytokine, regulates hyperactive immune response by suppressing the pro-inflammatory cytokines (IFN-γ and TNF-α) and inhibiting the activation of T cells by downregulating MHC-II expression and co-stimulation.[33] Moreover, IL-10 also induces Type-1 Treg cells.[34] TGF-β plays an important role in inhibition of immune response by suppressing T-cell proliferation and differentiation.[35] TGF-β can induce the expression of CD25 on $CD4^+CD25^-$ cells and transform them into $CD4^+CD25^+$ regulatory cells.[36] It can also induce FOXP3 expression on regulatory T cells.[37] Thus, both IL-10 and TGF-β contribute in Treg cells growth, expansion and suppressive function.[38] In Treg:$CD8^+$ and Treg:$CD4^+$ T cells co-culture assay, we observed significant decrease in IL-10 and TGF-β production by Tregs in GV patients (Figure 2B,C,E,F), indicating the inability of Tregs to suppress $CD4^+$ and $CD8^+$ T cells. Moreover, AV patients' Tregs showed decreased IL-10 production, emphasizing the crucial role of IL-10 in progression of GV (Figure 2B,E). Further, the analysis based on extent of vitiligo suggested reduced IL-10 production by Tregs of patients with severe GV (Figure 2B,E). This result was also confirmed by correlation analysis where IL-10 production was negatively correlated with VASI score (Figure S5c,d), suggesting the crucial role of IL-10 in impaired Treg function leading to increased severity of GV. Moreover, the IL-10 and TGF-β production by Tregs was positively correlated with percentage suppression of $CD8^+$ and $CD4^+$ T cells as well (Figure S6c-f), indicating the crucial immunosuppressive function of these cytokines that was altered in GV patients. The reduced suppressive function of Tregs in GV may be due to decreased immunosuppressive cytokines (IL-10 and TGF-β) production. Previously, significantly reduced IL-10 and TGF-β mRNA and protein levels were reported in serum/skin of GV patients.[16,32,39-44] Moreover, the ratio of pro-inflammatory cytokine to anti-inflammatory cytokine was found to be altered in the in vitro suppression assay. In particular, significantly increased ratio of IFN-γ:IL-10 and IFN-γ:TGF-β was observed in Treg:$CD8^+$ T cells and Treg:$CD4^+$ T

cells' co-culture systems of GV patients (Figure S7a-d), suggesting the crucial role of IL-10 and TGF-β deficiency in compromised suppressive capacity of Treg cells in GV (Figure S13). Interestingly, the IFN-γ:IL-10 ratio was also increased in AV patients indicating the role of altered pro-inflammatory (IFN-γ) and anti-inflammatory (IL-10) cytokines in progression of GV (Figure S7a,b).

Furthermore, the age of onset analysis revealed decreased Treg suppression of $CD8^+$ and $CD4^+$ T cells, TGF-β production by Tregs and expression of NFATC1 in Tregs (Figure 5A-E), suggesting Tregs' role in early onset of the vitiligo. Also, our analysis indicated that there was no effect of Treg suppressive capacity on gender biasness for developing GV.

Our further focus was on to investigate the reason behind reduced Treg suppressive function in GV other than the alteration in immune suppressive cytokines. The key player for the immune suppressive function of Treg is FOXP3, which also governs the growth and development of Tregs.[2] FOXP3 upregulates CD25 and CTLA-4 cell surface immunoregulatory molecules.[45] We found that the FOXP3 protein levels were significantly reduced in GV Tregs (Figure 3A). Studies have shown alterations in FOXP3 expression in $CD4^+CD25^{high}$ Tregs from vitiligo patients compared to controls[9,16] with significantly reduced *FOXP3* transcripts in perilesional and lesional skin.[46] In particular, FOXP3 expression in Tregs was significantly decreased in AV patients, which is in concordance to the previous study[16] suggesting the crucial role of FOXP3 in GV progression (Figure 3A). Further, analysis based on extent of vitiligo suggested decreased FOXP3 expression in patients with severe GV (Figure 3D). This result was also confirmed by correlation analysis where FOXP3 expression was negatively correlated with VASI score (Figure S9a). These results are also in concordance with the previous study,[46] suggesting the crucial role of FOXP3 in severity of GV. Moreover, we found that the protein level of CD25 in GV Tregs was significantly reduced as compared to controls, indicating the altered Tregs suppressive function in GV (Figure 3B). In addition, CD25 was found to be positively correlated with FOXP3 protein expression and $CD8^+$ and $CD4^+$ T cells' suppression in the current study (Figure 4F,C,D). Increased Tregs suppressive capacity and production of IL-10 has been found in $CD44^+$ Tregs.[17] However, we did not find significant difference in Tregs CD44 protein levels between GV patients and controls (Figure S8a).

Furthermore, it became pertinent to find the reason for decreased FOXP3 expression, which gave us insight to look for regulatory molecule of FOXP3 and Treg cell function. One of such molecules is NFAT, which play important role in activation and anergy of T cells. In Treg cells, NFATs form complex with FOXP3 and govern the immunosuppressive function by expression of key immunosuppressive markers like IL-10, TGF-β and CTLA4.[16] NFATC1 plays an important role in iTreg generation by regulating FOXP3 expression through its binding with *FOXP3* CNS1 region[47,48] (Figure S13). FOXP3 peptide-mediated inhibition of NFAT1-FOXP3 interaction has been shown to alter the Treg suppressive function and inhibit Tregs differentiation.[49] Moreover, hyper-activation of NFAT1 demonstrated to increase IL-10 production and Treg accumulation in CNS resulting in resistance of experimental autoimmune encephalomyelitis (EAE).[50] These studies implicated that NFAT1 may be a crucial target molecule in GV as well, where the Treg suppressive function is compromised. Interestingly, the present study shows decreased NFATC1 protein level in GV Tregs (Figure 3C); and this result is in concordance with our previously study in which decreased NFATs transcript levels were reported.[16] Interestingly, the decreased NFATC1 protein in AV patients suggests the role of NFATC1 in GV progression (Figure 3C). In addition, VASI score-based analysis suggested decreased NFATC1 protein in patients with severe GV (Figure 3F). These results were further confirmed by correlation analysis where NFATC1 protein was negatively correlated with VASI score (Figure S9c), indicating the role of NFATC1 in severity of GV. Moreover, the NFATC1 expression was positively correlated with FOXP3 protein expression and $CD8^+$ and $CD4^+$ T cells' suppression (Figure 4E,A,B). Hence, our results suggest that NFATC1 may play a crucial role in decreased FOXP3 expression in Tregs and thereby may affect the functionality of Tregs in GV (Figure S13).

Overall, our findings suggest that the reduced expression of NFATC1 leads to decreased FOXP3 expression in Tregs. The reduced expression of these key Tregs transcription factors (NFATC1 & FOXP3) then results in decreased Treg suppressive function and decreased expression of downstream Treg associated suppressive genes (CD25, IL-10 and TGF-β), thereby leading to unchecked $CD8^+$ and $CD4^+$ T cells proliferation and IFN-γ production resulting in melanocyte death and GV pathogenesis. Additionally, the study also emphasizes the effect of reduced Treg cell suppressive function on disease progression, severity and early age of onset of GV.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTION

PSG and MD performed the research; PSG and MD designed the research study; MD and RB contributed essential reagents and tools; PSG and MD arranged the blood samples; PSG, MD and RB analysed the data; PSG and MD wrote the manuscript; and MD and RB edited it.

ETHICAL APPROVAL

All procedures performed in this study involving human participants were in accordance with the ethical standards of the

Institutional-Human Research Ethical Committee (HREC), Maliba Pharmacy College, Uka Tarsadia University, Bardoli, Gujarat, India, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All patients and healthy control subjects signed informed consent.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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REFERENCES

[1] C. Bergqvist, K. Ezzedine, *Dermatology* **2020**, *2020*, 1.
[2] M. Dwivedi, E. H. Kemp, N. C. Laddha, M. S. Mansuri, A. P. Weetman, R. Begum, *Autoimmun. Rev.* **2015**, *14*, 49.
[3] C. Kruger, K. U. Schallreuter, *Int. J. Dermatol.* **2012**, *51*, 1206.
[4] K. Ongenae, N. Van Geel, J. M. Naeyaert, *Pigment Cell Res.* **2003**, *16*, 90.
[5] R. M. J. G. J. van den Wańkowicz-Kalińska, B. J. Wijngaard, W. Tigges, G. S. Westerhof, V. Ogg, W. J. Cerundolo, P. K. D. Storkus, *Lab. Investig.* **2003**, *83*, 683.
[6] J. Wu, M. Zhou, Y. Wan, A. Xu, *Mol. Med. Rep.* **2013**, *7*, 237.
[7] T. Lambe, J. C. H. Leung, T. Bouriez-Jones, K. Silver, K. Makinen, T. L. Crockford, H. Ferry, J. V. Forrester, R. J. Cornall, *J. Immunol.* **2006**, *177*, 3055.
[8] M. Abdallah, R. Lotfi, W. Othman, R. Galal, *Int. J. Dermatol.* **2014**, *53*, 940.
[9] M. Dwivedi, N. C. Laddha, P. Arora, Y. S. Marfatia, R. Begum, *Pigment Cell Melanoma Res.* **2013**, *26*, 586.
[10] S. Corthay, *J. Immunol.* **2009**, *70*, 326.
[11] S. Chatterjee, J. M. Eby, A. A. Al-Khami, M. Soloshchenko, H.-K. Kang, N. Kaur, O. S. Naga, A. Murali, M. I. Nishimura, I. Caroline Le Poole, S. Mehrotra, *J. Invest. Dermatol.* **2014**, *134*, 1285.
[12] Q. Zhang, T. Cui, Y. Chang, W. Zhang, S. Li, Y. He, B. Li, L. Liu, G. Wang, T. Gao, C. Li, Z. Jian, *J. Cell. Mol. Med.* **2018**, *22*, 4335.
[13] Y. Lili, W. Yi, Y. Ji, S. Yue, S. Weimin, L. Ming, *PLoS One* **2012**, *7*, e37513.
[14] M. Ben Ahmed, I. Zaraa, R. Rekik, A. Elbeldi-Ferchiou, N. Kourda, N. Belhadj Hmida, M. Abdeladhim, O. Karoui, A. Ben Osman, M. Mokni, H. Louzir, *Pigment Cell Melanoma Res.* **2012**, *25*, 99.
[15] J. Klarquist, C. J. Denman, C. Hernandez, D. J. Wainwright, F. M. Strickland, A. Overbeck, S. Mehrotra, M. I. Nishimura, I. C. Le Poole, *Pigment Cell Melanoma Res.* **2010**, *23*, 276.
[16] P. S. Giri, M. Dwivedi, N. C. Laddha, R. Begum, A. H. Bharti, *Pigment Cell Melanoma Res.* **2020**, *33*, 566.
[17] T. Liu, L. Soong, G. Liu, R. König, A. K. Chopra, *Biol. Direct.* **2009**, *4*, 40.
[18] J. G. van den Boorn, D. Konijnenberg, T. A. M. Dellemijn, J. P. W. van der Veen, J. D. Bos, C. J. M. Melief, F. A. Vyth-Dreese, R. M. Luiten, *J. Invest. Dermatol.* **2009**, *129*, 2220.
[19] U. Bhor, S. Pande, *Indian J. Dermatol. Venereol. Leprol.* **2006**, *72*, 315.
[20] M. Sehrawat, T. C. Arora, A. Chauhan, H. K. Kar, A. Poonia, V. Jairath, *ISRN Dermatol.* **2014**, *2014*, e493213.
[21] H. Uda, M. Takei, Y. Mishima, *J. Cutan. Pathol.* **1984**, *11*, 114.
[22] N. Zhang, M. J. Bevan, *Immunity* **2011**, *35*, 161.
[23] P. Novy, M. Quigley, X. Huang, Y. Yang, *J. Immunol.* **2007**, *179*, 8243.
[24] P. Bhat, G. Leggatt, N. Waterhouse, I. H. Frazer, *Cell Death Dis.* **2017**, *8*, e2836.
[25] J. Zhang, *J. Clin. Invest.* **2007**, *117*, 871.
[26] L. Yang, Y. Wei, Y. Sun, W. Shi, J. Yang, L. Zhu, M. Li, *Acta Derm. Venereol.* **2015**, *95*, 664.
[27] J. J. Yohn, M. M. Critelli, B. Lyons, D. A. Norris, *J. Invest. Dermatol.* **1990**, *95*, 233.
[28] A. M. Al Badri, A. K. Foulis, P. M. Todd, J. J. Gariouch, J. E. Gudgeon, D. G. Stewart, J. A. Gracie, R. B. Goudie, *J. Pathol.* **1993**, *169*, 203.
[29] M. Dwivedi, N. C. Laddha, K. Shah, B. J. Shah, R. Begum, *J. Interf. Cytokine Res.* **2013**, *33*, 646.
[30] K. Boniface, C. Jacquemin, A.-S. Darrigade, B. Dessarthe, C. Martins, N. Boukhedouni, C. Vernisse, A. Grasseau, D. Thiolat, J. Rambert, F. Lucchese, A. Bertolotti, K. Ezzedine, A. Taieb, J. Seneschal, *J. Invest. Dermatol.* **2018**, *138*, 355.
[31] S. Ezzedine, E. M. El Rewiny, A. A. Abozied, S. M. Samna, *Egypt. J. Hosp. Med.* **2018**, *73*, 7806.
[32] Y. Ala, M. K. Pasha, R. N. Rao, P. L. Komaravalli, P. Jahan, *Autoimmune Dis.* **2015**, *2015*, 423490.
[33] P. A. Knolle, A. Uhrig, S. Hegenbarth, E. Löser, E. Schmitt, G. Gerken, A. W. Lohse, *Clin. Exp. Immunol.* **1998**, *114*, 427.
[34] P. L. O'Garra, P. Vieira, A. E. G. Vieira, *J. Clin. Invest.* **2004**, *114*, 1372.
[35] A. Dahmani, J.-S. Dahmani, *Cancers* **2018**, *10*, 194.
[36] S. Fu, N. Zhang, A. C. Yopp, D. Chen, M. Mao, D. Chen, H. Zhang, Y. Ding, J. S. Bromberg, *Am. J. Transplant.* **2004**, *4*, 1614.
[37] W. Chen, J. E. Konkel, *J. Mol. Cell Biol.* **2010**, *2*, 30.
[38] P. Hsu, B. Santner-Nanan, M. Hu, K. Skarratt, C. H. Lee, M. Stormon, M. Wong, S. J. Fuller, R. Nanan, *J. Immunol.* **2015**, *195*, 3665.
[39] A. M. Osman, M. M. Mukhtar, K. H. Bakheit, H. Z. Hamdan, *Indian J. Dermatol.* **2015**, *60*, 635.
[40] R. Khan, S. Gupta, A. Sharma, *J. Am. Acad. Dermatol.* **2012**, *66*, 510.
[41] M. H. M. EL-Komy, D. M. Kadry, I. Amin, O. M. Abu-Zeid, D. M. Abdel-Halim, L. A. Rashed, *J. Egypt. Women's Dermatologic Soc.* **2012**, *9*, 151.
[42] M. Kidir, A. A. Karabulut, M. E. Ercin, P. Atasoy, *Int. J. Dermatol.* **2017**, *56*, 581.
[43] M. K. Tembhre, V. K. Sharma, A. Sharma, P. Chattopadhyay, S. Gupta, *Clin. Chim. Acta.* **2013**, *424*, 27.
[44] S. Moretti, A. Spallanzani, L. Amato, G. Hautmann, I. Gallerani, P. Fabbri, *Arch. Dermatol.* **2002**, *138*, 273.
[45] Y. Zheng, A. Y. Rudensky, *Nat. Immunol.* **2007**, *8*, 457.
[46] R. A. Hegazy, M. M. Fawzy, H. I. Gawdat, N. Samir, L. A. Rashed, *Exp. Dermatol.* **2014**, *23*, 283.
[47] M. Vaeth, U. Schliesser, G. Muller, S. Reissig, K. Satoh, A. Tuettenberg, H. Jonuleit, A. Waisman, M. R. Muller, E. Serfling, B. S. Sawitzki, F. Berberich-Siebelt, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 16258.
[48] Y. Tone, K. Furuuchi, Y. Kojima, M. L. Tykocinski, M. I. Greene, M. Tone, *Nat. Immunol.* **2008**, *9*, 194.
[49] T. Lozano, L. Villanueva, M. Durántez, M. Gorraiz, M. Ruiz, V. Belsúe, J. I. Riezu-Boj, S. Hervás-Stubbs, J. Oyarzábal, H. Bandukwala, A. R. Lourenço, P. J. Coffer, P. Sarobe, J. Prieto, N. Casares, J. J. Lasarte, *J. Immunol.* **2015**, *195*, 3180.
[50] S. Ghosh, S. B. Koralov, I. Stevanovic, M. S. Sundrud, Y. Sasaki, K. Rajewsky, A. Rao, M. R. Müller, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 15169.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. Standard curve for estimation of IFN-γ, IL-10 and TGF-β proteins. (a) Expression of IFN-γ protein in human plasma: Standard Curve for IL-10, Human ELISA. (b) Expression of IL-10 protein in human plasma: Standard Curve for IL-10, Human ELISA. (c)

Expression of TGF-β protein in human plasma: Standard Curve for TGF-β, Human ELISA

Figure S2. Gating Strategy (unstained) for $CD4^+CD25^+FOXP3^+$ Treg cells: estimation of protein levels of CD25, FOXP3, NFATC1 & CD44. (a) Lymphocytes were gated on the basis of size and morphology. (b) Treg cells were gated on the basis of CD4 and CD25 expression. (c) Expression of CD4 in Tregs: Representative graphs showing amount of CD4 in the Tregs as mean fluorescence intensity (MFI). (d) Expression of CD25 in Tregs: Representative graphs showing amount of CD25 in the Tregs as mean fluorescence intensity (MFI). (e) Expression of FOXP3 in Tregs: Representative graphs showing amount of intracellular FOXP3 in the Tregs as mean fluorescence intensity (MFI). (f) Expression of NFATC1 in Tregs: Representative graphs showing amount of intracellular NFATC1 in the Tregs as mean fluorescence intensity (MFI). (g) Expression of CD44 in Tregs: Representative graphs showing amount of CD44 in the Tregs as mean fluorescence intensity (MFI)

Figure S3. Gating Strategy (stained): for $CD4^+CD25^+FOXP3^+$ Treg cells: estimation of protein levels of CD25, FOXP3, NFATC1 & CD44. (a) Lymphocytes were gated on the basis of size and morphology. (b) Treg cells were gated on the basis of CD4 and CD25 expression. (c) Expression of CD4 in Tregs: Representative graphs showing amount of CD4 in the Tregs as mean fluorescence intensity (MFI). (d) Expression of CD25 in Tregs: Representative graphs showing amount of CD25 in the Tregs as mean fluorescence intensity (MFI). (e) Expression of FOXP3 in Tregs: Representative graphs showing amount of intracellular FOXP3 in the Tregs as mean fluorescence intensity (MFI). (f) Expression of NFATC1 in Tregs: Representative graphs showing amount of intracellular NFATC1 in the Tregs as mean fluorescence intensity (MFI). (g) Expression of CD44 in Tregs: Representative graphs showing amount of CD44 in the Tregs as mean fluorescence intensity (MFI)

Figure S4. Gender based analysis for in vitro Treg suppression of $CD8^+$ and $CD4^+$ T cells, IL-10 & TGF-β production by Tregs and IFN-γ production by $CD8^+$ and $CD4^+$ T cells in Treg: $CD8^+$ T cells and Treg: $CD4^+$ T cells co-culture systems in generalized vitiligo patients. Treg suppression of $CD8^+$ and $CD4^+$ T cells, IL-10 & TGF-β production by Tregs and IFN-γ production $CD8^+$ and $CD4^+$ T cells in Treg: $CD8^+$ T cells and Treg: $CD4^+$ T cells co-culture system of 25 Male and 30 female Generalized vitiligo (GV) patients were analyzed by non-parametric Mann-Whitney *U* test. (a) Treg suppression of $CD8^+$ T cells proliferation in male vs female GV patients (mean ± SEM: 52.50 ± 6.06 vs 40.99 ± 3.72; $P = .1117$). (b) Treg suppression of $CD4^+$ T cells proliferation in male vs female GV patients (mean ± SEM: 67.89 ± 5.38 vs 61.72 ± 4.13; $P = .1799$). (c) IL-10 production by Tregs in Treg: $CD8^+$ T co-culture systems in male vs female GV patients (mean ± SEM: 20.26 ± 1.38 vs 23.13 ± 0.89; $P = .0736$). (d) IL-10 production by Tregs in Treg: $CD4^+$ T co-culture systems in male vs female GV patients (mean ± SEM: 20.51 ± 1.83 vs 22.54 ± 1.536; $P = .4524$). (e) TGF-β production by Tregs in Treg: $CD8^+$ T co-culture systems in male vs female GV patients (mean ± SEM: 53.27 ± 3.187 vs 63.51 ± 8.07; $P = .3409$). (f) TGF-β production by Tregs in Treg: $CD4^+$ T co-culture systems in male vs female GV patients (mean ± SEM: 57.05 ± 3.534 vs 51.00 ± 1.751; $P = .1991$). (g) IFN-γ production by Tregs in Treg: $CD8^+$ T co-culture systems in male vs female GV patients (mean ± SEM: 619.8 ± 47.72 vs 631.4 ± 42.75; $P = .8047$). (f) IFN-γ production by Tregs in Treg: $CD4^+$ T co-culture systems in male vs female GV patients (mean ± SEM: 577.6 ± 57.52 vs 631.4 ± 42.75; $P = .1994$). (SEM, standard error mean)

Figure S5. Correlation of IFN-γ production by $CD8^+$ & $CD4^+$ T cells and IL-10 & TGF-β production by Treg cells in Treg: $CD8^+$ & Treg: $CD4^+$ T cells co-culture systems with VASI score of generalized vitiligo patients. Correlation of IFN-γ production by $CD8^+$ & $CD4^+$ T cells and IL-10 & TGF-β production by Treg cells in Treg: $CD8^+$ & Treg: $CD4^+$ T cells co-culture systems with VASI score of generalized vitiligo (GV) patients [10 Mild GV patients (10%-25% VASI), 6 Moderate GV patients (25%-50% VASI) & 39 Severe GV patients (50%-75% VASI)] were analyzed by spearman correlation analysis. (a) IFN-γ production by $CD8^+$ T cells in Treg: $CD8^+$ T cells co-culture system was positively correlated with VASI score for GV patients ($r = .47$, $P = .0029$). (b) IFN-γ production by $CD4^+$ T cells in Treg: $CD4^+$ T cells co-culture system was positively correlated with VASI score for GV patients ($r = .51$, $P = .0017$). (c) IL-10 production by Tregs in Treg: $CD8^+$ T cells co-culture system was negatively correlated with VASI score for GV patients ($r = -.41$, $P = .0096$). (d) IL-10 production by Tregs in Treg: $CD4^+$ T cells co-culture system was negatively correlated with VASI score for GV patients ($r = -.34$, $P = .029$). (e) No correlation between TGF-β production by Tregs in Treg: $CD8^+$ T cells co-culture system and VASI score for GV patients ($r = -.18$, $P = .27$). (f) No correlation between TGF-β production by Tregs in Treg: $CD4^+$ T cells co-culture system and VASI score for GV patients ($r = .03$, $P = .8488$)

Figure S6. Correlation of IFN-γ, IL-10 & TGF-β production with percentage Treg suppression of $CD8^+$ & $CD4^+$ T cells in GV patients and controls. The correlation of IFN-γ production by $CD8^+$ & $CD4^+$ T cells and IL-10 & TGF-β production by Tregs with percentage Treg suppression of $CD8^+$ & $CD4^+$ T cells in Treg: $CD8^+$ T & Treg: $CD4^+$ T cells co-culture systems in GV patients and controls were analyzed by spearman correlation analysis. (a) IFN-γ production by $CD8^+$ T cells was negatively correlated with percentage suppression of $CD8^+$ T cells in Treg: $CD8^+$ T cells co-culture system of GV patients and controls ($r = -.62$, $P < .0001$). (b) IFN-γ production by $CD4^+$ T cells was negatively correlated with percentage suppression of $CD4^+$ T cells in Treg: $CD4^+$ T cells co-culture system of GV patients and controls ($r = -.64$, $P < .0001$). (c) IL-10 production by Tregs was positively correlated with percentage suppression of $CD8^+$ T cells in Treg: $CD8^+$ T cells co-culture system of GV patients and controls ($r = .64$, $P < .0001$). (d) IL-10 production by Tregs was positively correlated with percentage suppression of $CD4^+$ T cells in Treg: $CD4^+$ T cells co-culture system of GV patients and controls ($r = .62$, $P < .0001$). (e) TGF-β production by Tregs was positively correlated with percentage suppression of $CD8^+$ T cells in Treg: $CD8^+$ T cells co-culture system of GV patients and controls ($r = .69$, $P < .0001$). (f) TGF-β production by Tregs was positively correlated with percentage suppression of $CD4^+$ T cells in Treg: $CD4^+$ T cells co-culture system of GV patients and controls ($r = .54$, $P < .0001$)

Figure S7. IFN-γ: IL10 and IFN-γ: TGF-β ratio analysis in generalized vitiligo patients. The IFN-γ: IL10 and IFN-γ: TGF-β ratio in Treg: CD8 and Treg: CD4 co-culture system respectively of 55 Generalized vitiligo (GV) patients [44 Active vitiligo (AV) patients & 11 Stable vitiligo (SV) patients] and 45 healthy controls were analyzed by non-parametric Mann-Whitney *U* test. (a) IFN-γ: IL10 ratio in Treg: $CD8^+$ T cells co-culture system of GV,SV & AV patients vs controls (Mean ± SEM: 63.27 ± 4.718, 42.31 ± 4.798 & 70.05 ± 5.597 vs 12.96 ± 1.04; $P < .0001$ respectively). IFN-γ: IL10 ratio in Treg: $CD8^+$ T cells co-culture system of SV vs AV patients (Mean ± SEM: 41.39 ± 5.27 vs 39.83 ± 4.091; $P = .0055$). (b) IFN-γ: IL10 ratio in Treg: $CD4^+$ T cells co-culture system of GV,SV & AV patients vs controls (Mean ± SEM: 61.65 ± 5.994, 43.84 ± 6.55 & 66.10 ± 7.19 vs 13.62 ± 1.55; $P < .0001$, $P = .0012$ & $P < .0001$ respectively). IFN-γ: IL10 ratio in Treg: $CD4^+$ T cells co-culture system of SV vs AV patients vs controls (Mean ± SEM: 28.42 ± 3.46 vs 31.38 ± 2.03; $P = .0233$). (c) IFN-γ: TGF-β ratio in Treg: $CD8^+$ T cells co-culture system of GV,SV & AV patients vs controls (Mean ± SEM: 14.03 ± 1.43, 15.59 ± 2.85 & 13.64 ± 1.65 vs 4.09 ± 0.51; $P < .0001$ respectively). IFN-γ: TGF-β ratio in Treg: $CD8^+$ T cells co-culture system of SV vs AV patients vs controls (Mean ± SEM: 15.59 ± 2.85 vs 13.64 ± 1.65; $P = .3916$). (d) IFN-γ: TGF-β ratio in Treg: $CD4^+$ T cells co-culture system of GV,SV & AV patients vs controls (Mean ± SEM: 11.50 ± 0.83, 12.26 ± 1.82 & 11.31 ± 0.94 vs 4.40 ± 0.73; $P < .0001$ respectively) IFN-γ: TGF-β ratio in Treg: $CD4^+$ T cells co-culture system of SV vs AV patients vs controls (Mean ± SEM: 12.26 ± 1.82 vs 11.31 ± 0.94; $P = .4712$) (SEM, standard error mean)

Figure S8. CD44 protein levels in GV patients and controls. CD44 expression levels in 55 Generalized vitiligo (GV) patients [44 Active vitiligo (AV) patients & 11 Stable vitiligo (SV) patients; 10 Mild GV patients (10%-25% VASI), 6 Moderate GV patients (25%-50% VASI) & 39 Severe GV patients (50%-75% VASI)] and 45 healthy controls were analyzed by non-parametric Mann-Whitney *U* test. (a) CD44 expression level in Treg of GV,SV & AV patients vs controls (Mean ± SEM: 690.9 ± 35.83, 710.3 ± 104.3 & 686.5 ± 37.71 vs 616.6 ± 43.31; $P = .2924$,$P = .0891$ & $P = .8565$ respectively). CD44 expression level in Treg of SV vs AV patients vs controls (Mean ± SEM: 710.3 ± 104.3 vs 686.5 ± 37.71; $P = .6727$). (b) CD44 expression level in Treg for 10%-25% VASI (Mild GV) vs 50%-75% VASI (Severe GV) patients (Mean ± SEM: 710.3 ± 104.3 vs 700.3 ± 40.33; $P > .999$). CD44 expression level in Treg for 25%-50% VASI (Moderate GV) vs 10%-25% VASI (Mild GV) & 50%-75% VASI (Severe GV) patients (Mean ± SEM: 674.2 ± 134.4 vs 710.3 ± 104.3 vs 700.3 ± 40.33 respectively; $P > .999$) (SEM, standard error mean)

Figure S9. Correlation of FOXP3, CD25, NFATC1 & CD44 expression in Treg cells with VASI score of generalized vitiligo patients. Correlation of FOXP3, CD25, NFATC1 & CD44 expression in Treg cells with VASI score of generalized vitiligo (GV) patients [10 Mild GV patients (10%-25% VASI), 6 Moderate GV patients (25%-50% VASI) & 39 Severe GV patients (50%-75% VASI)] were analyzed by spearman correlation analysis. (a) FOXP3 expression in Tregs VASI score was negatively correlated with VASI score for GV patients ($r = -.55$, $P = .0008$). (b) No correlation between CD25 expression and VASI score in Tregs for GV patients ($r = -.28$, $P = .0918$). (c) NFATC1 expression in Tregs was negatively correlated with VASI score for GV patients ($r = -.27$, $P = .043$). (d) No correlation between CD44 expression in Tregs and VASI score for GV patients ($r = -.05$, $P = .7164$)

Figure S10. Correlation of CD44 expression with percent Treg suppression of $CD8^+$ & $CD4^+$ T cells and FOXP3 & NFATC1 expression in Treg: $CD8^+$ & Treg: $CD4^+$ T cells co-culture systems of generalized vitiligo patients and controls. Correlation of CD44 expression with percent Treg suppression of $CD8^+$ & $CD4^+$ T cells and FOXP3 & NFATC1 expression in Treg: $CD8^+$ & Treg: $CD4^+$ T cells co-culture systems of GV patients and controls were analyzed by spearman correlation analysis. (a) No correlation between CD44 expression in Tregs and percent suppression of $CD8^+$ T cells in Treg: $CD8^+$ T cells co culture system of GV patients and controls ($r = -.11$, $P = .2925$). (b) No correlation between CD44 expression in Tregs and percent suppression of $CD4^+$ T cells in Treg: $CD4^+$ T cells co culture system of GV patients and controls ($r = -.04$, $P = .6606$). (c) No correlation between CD44 and FOXP3 expression in Tregs of GV patients and controls ($r = .10$, $P = .2551$). (f) No correlation between CD44 and NFATC1 expression in Tregs of GV patients and controls ($r = .05$, $P = .5288$)

Figure S11. Gender based analysis for protein levels of FOXP3, CD25, NFATC1 and CD44 in generalized vitiligo patients. FOXP3, CD25, NFATC1 and CD44 protein expression levels in Tregs of 25 Male and 30 female Generalized vitiligo (GV) patients were analyzed by non-parametric Mann-Whitney *U* test. (a) FOXP3 expression in Tregs of male vs female GV patients (mean ± SEM: 387.6 ± 9.74 vs 393.1 ± 10.63; $P = .7724$). (b) CD25 expression in Tregs of male vs female GV patients (mean ± SEM: 625.3 ± 27.27 vs 642.9 ± 23.81; $P = .6104$). (c) NFATC1 expression in Tregs of male vs female GV patients (mean ± SEM: 509.5 ± 33.05 vs 481.4 ± 28.28; $P = .3875$). (d) CD44 expression in Tregs of male vs female GV patients (mean ± SEM: 683.0 ± 57.99 vs 701.3 ± 47.45; $P = .6707$). (SEM, standard error mean)

Figure S12. Effect of IL-10 & IFN-γ production by $CD8^+$ & $CD4^+$ T cells, FOXP3, CD25 and CD44 expression in Tregs on age of onset of generalized vitiligo patients. Effect of TGF-β & IL-10 production by Tregs, IFN-γ production by $CD8^+$ & $CD4^+$ T cells, FOXP3, CD25 and CD44 expression in Tregs on age of onset in 55 Generalized vitiligo (GV) patients and 45 healthy controls were analyzed by non-parametric Mann-Whitney *U* test. (a) IL-10 production by Tregs in Treg: $CD8^+$ T cells co-culture system in GV patients of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 17.80 ± 0.91 vs 16.70 ± 0.36; $P = .5639$). IL-10 production by Tregs in Treg: $CD8^+$ T cells co-culture system in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 20.76 ± 1.81 vs 17.80 ± 0.91 and 16.70 ± 0.36, respectively; $P = .2775$ and $P = .0981$, respectively). (b) IL-10 production by Tregs in Treg: $CD4^+$ T cells co-culture system in GV patients of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 21.32 ± 1.453 vs 15.46 ± 0.64; $P = .0908$). IL-10 production by Tregs in Treg: $CD4^+$ T cells co-culture system in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 19.98 ± 2.68 vs 21.32 ± 1.453 and 15.46 ± 0.64,

respectively; $P = .5082$ and $P = .6267$, respectively). (c) IFN-γ production by Tregs in Treg: $CD8^+$ T cells co-culture system in GV patients of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 632.9 ± 41.58 vs 705.6 ± 9.8; $P = .3206$). IFN-γ production by Tregs in Treg: $CD8^+$ T cells co-culture system in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 531.3 ± 105.7 vs 632.9 ± 41.58 and 705.6 ± 9.8, respectively; $P = .1552$ and $P > .9999$, respectively). (d) IFN-γ production by Tregs in Treg: $CD4^+$ T cells co-culture system in GV patients of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 632.9 ± 41.58 vs 705.6 ± 9.8; $P = .3206$). IFN-γ production by Tregs in Treg: $CD4^+$ T cells co-culture system in GV patients of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 515.0 ± 110.0 vs 632.9 ± 41.58 vs 705.6 ± 9.8, respectively; $P = .0916$ and $P > .9999$, respectively). (e) FOXP3 protein levels in Tregs of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 391.4 ± 8.89 vs 383.3 ± 17.99; $P = .7063$). FOXP3 protein levels in Tregs of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 394.2 ± 19.41 vs 391.4 ± 8.89 and 383.3 ± 17.99, respectively; $P = .8926$ and $P = .6996$, respectively). (f) CD25 protein levels in Tregs of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 627.3 ± 20.11 vs 620.6 ± 41.37; $P = .9894$). CD25 protein levels in Tregs of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 677.1 ± 60.65 vs 627.3 ± 20.11 and 620.6 ± 41.37, respectively; $P = .7437$ and $P = .7430$, respectively). (g) CD44 protein levels in Tregs of the age of onset group 1-20 y vs 41-60 y (mean ± SEM: 666.4 ± 43.54 vs 782 ± 111.6; $P = .3739$). CD44 protein levels in Tregs of the age of onset group 21-40 y vs 1-20 and 41-60 y (mean ± SEM: 696.8 ± 63.53 vs 666.4 ± 43.54 and 782 ± 111.6, respectively; $P = .6806$ and $P = .5598$, respectively) (SEM, standard error mean)

Figure S13. Role of altered NFATC1 and FOXP3 expression in Treg cell suppressive function in pathogensis of generalized vitiligo. In Tregs, NFATC1 binds CNS1 regulatory region of FOXP3 gene and stabilizes FOXP3 expression. However, the decreased NFATC1 protein expression leads to decreased FOXP3 protein expression in Tregs. The reduced expression of these key Tregs transcription factors (NFATC1 & FOXP3) results in decreased NFAT:FOXP3 complex formation leading to reduced expression of Treg suppressive cytokines(CD25, IL-10 & TGF-β) and impaired Treg suppressive function. The impaired Tregs thereby leads to unchecked $CD8^+$ and $CD4^+$ T proliferation and IFN-γ production resulting in melanocyte death and GV pathogenesis

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