**Title: 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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## Introduction

Generalized vitiligo (GV) is an acquired, non-contagious skin disease which is characterized by appearance of symmetrical milky white patches due to autoimmune loss of melanocytes and thus loss of pigmentation from the involved areas (Nordlund et al., 2006). Based on a few dermatological outpatients records the worldwide prevalence of vitiligo in children/adolescents and adults has found to be 0.5–2% (Kruger and Schallreuter, 2012). The highest prevalence of vitiligo was reported in Gujarat & Rajasthan states of India i.e ~8.8% (Valia et al., 1996); however, the hospital-based point prevalence of vitiligo in India was reported to be 9.98% (Kumar et al., 2014).

The key role of autoimmunity in the pathogenesis of the disease has been suggested (Le Poole and Luiten, 2008) by the involvement of both humoral and cellular immunity due to presence of both circulating melanocyte autoantibodies (Cui et al., 1995; Laddha et al., 2014) and autoreactive CD8+cytotoxic T (Ogg et al., 1998; Dwivedi et al., 2013). These autoantibodies and autoreactive CD8+T cells that recognize melanocyte specific antigens are more abundant in active vitiligo patients compared to stable vitiligo (Naughton et al., 1986; Dwivedi et al., 2013). In addition, studies on skin samples from vitiligo patients have demonstrated the presence of both CD4+ and CD8+ T cells in vitiligo lesions (Abdallah et al., 2014). These activated cytotoxic T cells if remained unchecked may lead to melanocytes destruction developing lesions in vitiligo patients.

Regulatory T cells (Tregs) plays a crucial role in curbing such self-reactive $CD8^+$ T-cells response through their active suppression and expansion and thus maintain peripheral tolerance (Dwivedi et al., 2015). Previous studies demonstrated significant decrease in Tregs number and their impaired function in patients suggesting that Tregs fail to control the widespread activation of CD8+ T-cells, which may lead to the destruction of melanocytes in GV (Klarquist et al., 2010; Lili et al., 2012; Dwivedi et al., 2013; Dwivedi et al., 2015). Moreover, a significantly lower Treg cells have been reported in active cases in comparison to stable cases of vitiligo (Abdallah et al., 2014). The role of Tregs in vitiligo has been begged by few important studies including an adoptive transfer of Treg cells which halts vitiligo progression in H3TA2 mouse model (Chatterjee et al., 2014; Eby et al., 2014). Moreover, studies of murine melanoma suggested that the depletion of Tregs caused the activation of anti-melanoma cytotoxic T cells that destroyed melanoma tumours but also generated vitiligo as a side-effect (Zhang et al., 2007). Thus, Tregs potentially represent a

therapeutic target in GV, so a detailed study of their involvement in pathogenesis of GV is essential.

One of the not so far studied regulatory molecules of Tregs in GV is nuclear factor of activated T cells (NFATs). NFAT family comprises of four classical NFAT proteins (NFATC1, NFATC2, NFATC3 and NFATC4*)* and tonicity enhancer binding protein NFAT5. The antigen stimulation of immune cells activates the transcription factors NFAT1-5; which are key regulators of T cell activation and anergy. NFATs form co-operative complexes with the adaptor protein complex 1 (AP-1) family of transcription factors and regulate T cell activation-associated genes (Wu et al., 2006). In Tregs, NFATs form analogous co-operative complexes with FOXP3, which then mediate Treg cell function by repressing the expression of cytokines (IL-2 & IL-4) and up-regulating the expression of Treg cell markers CTLA-4 and CD25 (Wu et al., 2006).

Another regulatory molecule for development and function of Treg cells is Forkhead Box P3 **(**FOXP3) which belongs to the forkhead–winged-helix family. Treg cells are defined by the constitutive expression of *FOXP3* and are considered indispensable for the Tregs suppressive functions (Fontenot et al., 2003, Hori et al., 2003). *FOXP3* activates several genes including *CD25*, *CTLA4*, GITR, *IL10* and *TGFB*, which are crucial for suppressive activity of Treg cells (Corthay, 2009) and downregulates *IL2* and *IL4*, which are crucial for T cell function (Nedoszytko et al., 2017). Previous studies have shown decreased *FOXP3* expression in skin and blood of vitiligo patients. The FOXP3 expression was significantly decreased in CD4+CD25high Tregs from vitiligo patients compared to controls (Dwivedi et al., 2013). The *FOXP3* mRNA levels in lesional and perilesional skin were significantly reduced in vitiligo patients when compared with skin from healthy individuals (Hegazy et al., 2014). Thus, the reduced expression of *FOXP3* may lead to decreased levels of immune suppressive genes such as *CTLA4, IL10, TGFB* and *CD25*.

Given the role of Tregs in GV, it becomes pertinent to study the immunosuppressive mechanisms and molecules involved in Tregs; so as to delineate the Tregs specific defects in GV patients. Therefore, the present study for the first time aimed: to assess the *NFATs*(1 to 5) and *FOXP3* transcript expression in Tregs isolated from GV patients and unaffected controls; to assess transcripts of Tregs immune suppressive genes (*sCTLA-4, flCTLA4, IL10, TGFB)* and associated gene*s (CD25, IL2, IL4)*; to assess the suppressive capacity of Tregs from GV patients and controls by measuring their effects on $CD8^+$ and $CD4^+$ T cells proliferation,

cytokine production (IFN-γ, TGF-β, & IL-10), and levels of Treg suppressive proteins (NFATC1, FOXP3, CD25 & CD44) in Treg: CD8+ & Treg : CD4+ T cells co-culture systems.

**Objectives**

1) To evaluate the expression of *NFATs*(1-5) and *FOXP3* (Tregs associated transcription factor) in generalized vitiligo patient Tregs.
2) To evaluate the expression of *FOXP3/NFAT*-regulated immune modulatory genes(*sCTLA-4, flCTLA4, IL10, TGFB*) in generalized vitiligo patient Tregs.
3) To analyze the suppressive capacity of Tregs isolated from generalized vitiligo patients in in Treg : $CD8^+$ & Treg : $CD4^+$ T cells co-culture systems by:

    a) Assessing the effects of Tregs on $CD8^+$ and $CD4^+$ T cell proliferation by BrdU cell proliferation assay.
    b) Measuring the T cells associated cytokine production (IFN-γ) and Treg associated cytokine production (TGF-β, & IL-10).
    c) Evaluating the levels of Treg suppressive proteins (NFATC1, FOXP3, CD25 & CD44)

**Materials and methods**

**Patients and controls:**

Total 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 dermatologist at General hospital, using Wood's lamp as bilateral, symmetrical, depigmented macules or patches occurring in a random distribution over the entire body surface. The Extent of vitiligo depigmentation was measured by Vitiligo area severity index (VASI) score(Bhor & Pande, 2006). VASI score for GV patients was calculated as described by Bhor and Pande (2006), on the basis of 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 i) 10 - 25% : GV patients (Mild), ii) 25% - 50% : GV patients (Moderate) and iii) 50% - 75% : GV patients (severe) as mentioned previously(Sehrawat el al., 2014). 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 were defined with no such increase in lesions size/number within previous 6 months(uda et al., 1984). 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. 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.

**Isolation of $CD4^+CD25^+$ Tregs and $CD4^+$ T cells:**

The CD4+CD25+ Treg cells and $CD4^+$ T cells and were isolated from three milliliter blood sample of GV patients and controls in a twostep protocol using MACSxpress® Whole Blood Treg Isolation Kit human (Miltenyi Biotec, Auburn, CA) as mentioned in the previous study(Giri et al.,2020). In the first step, by negative selection CD4+ T cells were isolated by immunomagnetic depletion of non-CD4 T cells with MACSxpress Beads. Subsequently in the second step, these $CD4^+$ T cells were then subjected to LS columns under magnetic field 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. Flow cytometry was carried out to confirm the purity of isolated Treg cells and CD4+ T cells (Figure 1).

**Isolation of $CD8^+$ T cells:**

$CD8^+$ T cells were isolated from two milliliter blood sample of GV patients and controls using MACSxpress® Whole Blood CD8 T Cell Isolation Kit human (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. All the non-CD8 T cells were immunomagnetically depleted with MACSxpress Beads. The isolated $CD8^+$ T cells were immediately processed for *in vitro* Treg suppression assay.

***In vitro* Treg suppression assay:**

The freshly isolated $CD4^+CD25^+$ Treg cells ($5 \times 10^3$ cells) were co-cultured with $CD8^+$ T cells ($10 \times 10^3$ cells) and $CD4^+$ T cells ($10 \times 10^3$ cells) at a ratio of 1:2 individually. The cells were stimulated with 200 IU recombinant IL-2 (PeproTech, NJ, USA) and antiCD3/anti-CD28 Dynabeads (Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA) at 1:1 (bead : cell) ratio. Further, the cells were incubated at final volume of 200µl RPMI supplemented with 10% fetal bovine serum for 5 days at 37˚C and 5% $CO_2$ in 96 well U-bottom plate. On day 4, the cells were labeled with 10µM BrdU (Sigma-Aldrich, Missouri, USA) for 18 hrs and then processed for BrdU cell proliferation assay. All experiments were performed in triplicates.

**BrdU cell proliferation assay:**

Incorporation of BrdU in proliferating cells was measured by BrdU cell proliferation enzyme-linked immunosorbent assay kit (Sigma-Aldrich, Missouri, USA) according to the manufacturer's instructions. Percentage suppression was calculated using the following formula: [(proliferation of Tconv cells alone – proliferation of Tconv cells treated with Treg)/proliferation of Tconv cells alone] x 100.

**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 & TGF-β proteins were estimated from cell culture supernatant using the Enzyme-Linked Immunosorbent Assay Kits for Human IFN-γ, IL-10 and TGF-β (USCN Life Science Inc., Wuhan, China) as per the manufacturer's protocol. All samples were analyzed in triplicates.

**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. The cells were surface stained with antibodies for anti-CD4 and anti-CD25 and anti-CD44. Surface stained cells were fixed and permeabilized using Intracellular Staining Permeabilization Wash buffer (Biolegend, CA, USA) according to the manufacturer's instructions. Briefly, after washing, the cells were stained with anti-NFATC1

and anti-FOXP3 antibodies and incubated for 30 minutes at room temperature in the dark. After staining, the cells were fixed in PBS containing 1% paraformaldehyde, and acquired the same day on a BD Flow Cytometer & Cell sorter (BD Biosciences, San Jose, CA) followed by analysis using De Novo FCS Express 7 software (DeNovo Software, LA, CA, USA). The amount of FOXP3, NFATC1, CD25, CD44 present in the Tregs were measured as mean fluorescence intensity (MFI) (Figure 1). All samples were analyzed 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).

### 2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from purified Treg cells using Trizol reagent (Invitrogen, Carlsbad, California, United States) according to the manufacturer's instructions. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. cDNA synthesis was performed using 1 µg of total RNA by RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in Eppendorf Mastercycler Gradient Thermocycler (Eppendorf$^{TM}$, Germany).

### 2.4. Quantitative realtime PCR (qPCR)

The expression of *NFATs (NFAT C1-5), FOXP3, CD25, CTLA4, IL10, TGFB, IL2, IL4* and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcripts were measured by qPCR using gene specific primers (Integrated DNA technology, Coralville, Iowa, USA). Expression of the *GAPDH* gene was used as a reference. Gene expression levels were investigated using PowerUp™ SYBR® Green Master Mix (Waltham, Massachusetts, United States), qPCR was performed in duplicates in 20 µl volume using Rotor-Gene Q (Qiagen, CA) following the manufacturer's instructions. The fluorescence data collection was performed during the extension step. At the end of the amplification phase, a melt curve analysis was carried out to check the specificity of the products formed. The PCR cycle at which PCR amplification begins its exponential phase and product fluorescence intensity finally rises above the background and becomes visible was considered as the cycle threshold ($C_T$). The $\Delta C_T$ value was determined as the difference between the cycle threshold of target genes and reference

gene (*GAPDH*). The difference between the two Δ $C_T$ values (Δ $C_T$ Controls and Δ $C_T$ patients) was considered as ΔΔ $C_T$ to obtain the value of fold expression ($2^{-\Delta\Delta CT}$).

**Statistical analyses:**

The comparisons of percentage suppression, proteins level analysis, MFI values, Δ $C_T$ values among patients and controls were carried out by non-parametric Mann-Whitney U test whereas the correlation analysis was carried out using spearman correlation analysis with using Prism 8 software (GraphPad software Inc; 2003). The p-values ≤ 0.05 were interpreted as statistically significant.

**RESULTS:**

**Assessment of *In vitro* Treg suppression of $CD8^+$ and $CD4^+$ T cells proliferation in GV patients and controls**

The suppressive capacity of Tregs on $CD8^+$ and $CD4^+$T cells proliferation were analysed *in vitro* in 55 GV patients [44 AV & 11 SV patients; 10 GV patients (Mild), 6 GV patients (Moderate) & 39 GV patients (Severe)] and 45 healthy controls by BrdU cell proliferation assay. Treg cells of GV, SV & AV patients showed significantly reduced percentage suppression of $CD8^+$T cells (p=0.0384, p=0.0468 & p=0.0169 respectively; Figure 2a) and $CD4^+$T cells (p=0.0084, p=0.0057 & p=0.0082 respectively; Figure 2b) when compared with controls. In addition, analysis based on disease activity revealed Treg cells of AV patients showed significantly reduced percentage suppression of $CD8^+$ & $CD4^+$T cells when compared with SV patients (p=0.006 & p=0.015 respectively; Figure 2a,b). Moreover, analysis based on extent of vitligo depigmentation (VASI Score) revealed signficantly reduced percentage suppression of $CD8^+$T cells and $CD4^+$T cells by Tregs of GV patients (Severe) group compared to GV patients (Mild) group (p=0.0003 & p=0.001 respectively, Figure 2c,d). Correlation analysis also revealed a negative correlation between percentage suppression of $CD8^+$T cells & $CD4^+$T cells by Tregs and VASI score (r=-0.47, p=0.0006 & r=-0.66, p<0.0001 respectively). However, no significant difference was observed for Tregs percentage suppression of $CD8^+$T cells and $CD4^+$T cells for GV patients (Moderate) group compared to GV patients (Mild) group (p=0.263 & p>0.999 respectively, Figure 2c,d) and GV patients (Severe) group (p=0.967 & p=0.873 respectively, Figure 2c,d). The reduced

ability of Tregs to suppress $CD8^+$ and $CD4^+$T cells suggests an inherent functional defect of Treg cells in GV patients.

**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. We observed significant increased IFN-γ production by $CD8^+$T cells ($p<0.0001$; Figure 3a) and $CD4^+$T cells ($p=0.0019$, $p=0.0053$ & $p=0.0143$ respectively; Figure 3b) in GV,SV & AV patients as compared to controls. In particular, the IFN-γ production by $CD8^+$ & $CD4^+$T were significantly increased in AV patients compared to SV patients ($p=0.036$ & $p=0.045$ respectively; Figure 3a,b). In addition, the IFN-γ production by $CD8^+$ & $CD4^+$T were significantly increased in GV patients (Severe) group compared to GV patients (Mild) group ($p=0.0029$ & $p<0.0001$ respectively, Figure 3a,b). Also, the IFN-γ production by $CD8^+$ & $CD4^+$T cells were positively correlated with VASI score ($r= 0.47$, $p=0.0029$ & $r= 0.51$, $p=0.0017$ respectively). However, no significant difference was observed for IFN-γ production by $CD8^+$ & $CD4^+$T for GV patients (Moderate) group compared to GV patients (Mild) group ($p=0.6156$ & $p=0.6184$ respectively, Figure 3a,b) and GV patients (Severe) group ($p=0.4702$ & $p=0.1134$ respectively, Figure 3a,b). Further, we found that IFN-γ production was negatively correlated with percentage suppression of $CD8^+$ and $CD4^+$T cells in GV patients and controls ($r = -0.62$; $p<0.0001$ & $r = -0.64$, $p<0.0001$, respectively). 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.

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

The suppressive function of Treg is exterted mainly by cytokines IL-10 and TGF-β. Hence, we further assessed the production of IL-10 and 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. We found significantly decreased IL-10 production by Treg cells of GV, SV & AV patients as compared to that of controls in Treg:$CD8^+$T cells and Treg:$CD4^+$ T cells co-culture systems ($p<0.0001$; Figure 3c,d). Moreover, TGF-β production by Treg cells

was significantly reduced in GV,SV & AV patients as compared to controls in Treg: $CD8^+$T cell co-culture system ($p<0.0001$, $p=0.0063$ & $p<0.0001$ respectively; Figure 3e) and Treg: $CD4^+$T cell co-culture ($p<0.0001$; Figure 3f). 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 ($p=0.009$ & $p=0.021$; Figure 3c,d respectively). In addition, IL-10 production by Treg cells in Treg: $CD8^+$T cells and Treg: $CD4^+$T cells co-culture systems was significantly reduced in GV patients (Severe) group compared to GV patients (Mild) group ($p=0.0057$ & $p=0.0017$ respectively, Figure 3c,d). Moreover, the IL-10 production by Treg cells in Treg: $CD8^+$T cells and Treg: $CD4^+$T cells co-culture systems were negatively correlated with VASI score ($r= -0.41$, $p=0.0096$ & $r= -0.34$, $p=0.029$ respectively). However, IL-10 production by Treg cells in Treg: $CD8^+$T cells and Treg: $CD4^+$T cells co-culture systems did not differ for GV patients (Moderate) group compared to GV patients (Mild) group ($p=0.1705$ & $p=0.6247$ respectively, Figure 3c,d) and GV patients (Severe) group ($p>0.999$ & $p=0.0823$ respectively, Figure 3c,d). There was no significant difference 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 ($p=0.6064$ & $p=0.7893$; Figure 3e,f). Moreover, there was no significant difference in TGF-β production by Treg cells in Treg: $CD8^+$T cells and Treg: $CD4^+$T cells co-culture systems between GV patients (Mild) group, GV patients (Moderate) group and GV patients (Severe) group (Figure 3e,f).

Further, we found that IL-10 and TGF-β production 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: $r = 0.64$, $p<0.0001$; $r=0.62$, $p<0.0001$ & TGF-β: $r=0.69$, $p<0.0001$; $r= 0.54$, $p<0.0001$; respectively) Thus, the reduced IL-10 and TGF-β production by Tregs further indicates the impaired Tregs suppressive function in GV patients.

**Assessment of regulatory molecules of Treg cell function (FOXP3, CD25 & NFATC1) in GV patients and controls**

Further, we assessed few important regulatory molecules of Treg cell, which could affect the Treg cell suppressive function. Through flow cytometry, the protein levels of two intracellular Treg molecules (i.e. FOXP3 & NFATC1) and two surface suppressive markers were analyzed in Tregs of GV patients and controls. We observed significant reduced protein levels of FOXP3 ($p<0.0001$; Figure 4a), CD25 ($p<0.0001$, $p=0.0064$ & $p=0.0003$

respectively; Figure 4c) & NFATC1 ($p<0.0001$, $p=0.020$ & $p<0.0001$ respectively; Figure 4e) in Treg cells of GV, SV & AV patients respectively as compared to controls. In particular, protein levels of FOXP3 and NFATC1 were significantly reduced in Tregs of AV compared to SV patients ($p=0.0244$ & $p=0.019$ respectively; Figure 4a,e). Moreover, FOXP3 and NFATC1 expression in Tregs were significantly reduced in GV patients (Severe) group compared to GV patients (Mild) group ($p=0.002$ & $p=0.0347$ respectively, Figure 4b,f). Correlation analysis also revealed a negative correlation for FOXP3 and NFATC1 expression in Tregs with VASI score ($r= -0.55$, $p=0.0008$ & $r= -0.27$, $p=0.043$ respectively). However, FOXP3 and NFATC1 expression by Treg cells did not differ for GV patients (Moderate) group compared to GV patients (Mild) group ($p=0.3453$ & $p>0.999$ respectively, Figure 4b,f) and GV patients (Severe) group ($p>0.999$ & $p=0.4297$ respectively, Figure 4b,f).

Furthermore, NFATC1 & CD25 protein levels in Tregs were positively correlated with percentage suppression of $CD8^+$ & $CD4^+$T cells by Tregs in Treg:$CD8^+$T cells (NFATC1: $r=0.54$, $p<0.0001$; CD25: $r=0.38$, $p<0.0001$; Figure 5a,c) and Treg:$CD4^+$ T cells co-culture systems (NFATC1: $r=0.57$, $p<0.0001$; CD25: $r=0.44$, $p<0.0001$; Figure 5b,d) of GV patients and controls. Interestingly, NFATC1 & CD25 protein levels in Tregs were positively correlated with FOXP3 protein expression in Tregs of GV patients and controls ($r=0.41$, $p<0.0001$; Figure 5e,f). 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.

### *NFATs* transcripts levels in Tregs of Generalized Vitiligo patients

The analysis of *NFATs* transcript levels in Tregs isolated from GV patients and controls was carried out after normalization with *GAPDH* expression. Comparison of the findings showed significant decreased expression of all five *NFATs* i.e. *NFATC1, NFATC2, NFATC3, NFATC4* and *NFAT5* in GV patients compared to controls ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.0001$ & $p=0.0003$ respectively; figure 6). The $2^{-\Delta\Delta CT}$ analysis showed approximately 0.19, 0.14, 0.21, 0.48 and 0.31 - fold changes in expression of *NFATC1, NFATC2, NFATC3, NFATC4* and *NFAT5* transcripts respectively in GV patients as compared to controls. Further, the analysis of *NFATs* transcript levels was carried out based on the disease progression. The active vitiligo (AV) patients showed significantly decreased transcript levels of *NFATC3, NFATC4* and *NFAT5* in Tregs when compared to stable vitiligo (SV) ($p=0.0005$, $p=0.0003$ & $p=0.0002$; Figure 6c,d,e respectively). However, there was no significant difference in

transcript levels of *NFATC1* and *NFATC2* in AV as compared SV (p=0.487 & p=0.101; Figure 6a,b respectively). The $2^{-\Delta\Delta CT}$ analysis showed approximately 0.72, 0.28, 0.25, 0.44 and 0.12 - fold changes in expression of *NFATC1, NFATC2, NFATC3, NFATC4* and *NFAT5* transcripts respectively in AV patients as compared to SV. In addition, SV patients showed significantly decreased levels of *NFATC1, NFATC2* and *NFATC3* as compared to controls (p<0.0001, p<0.0001 & p=0.0005; Figure 6a,b,c respectively). There was no significant difference in transcript levels of *NFATC4* and *NFATC5* in SV as compared controls (p=0.164 & p=0.944; Figure 6d,e respectively). The $2^{-\Delta\Delta CT}$ analysis showed approximately 0.23, 0.28, 0.45, 0.76 and 1.03- fold changes in expression of *NFATC1, NFATC2, NFATC3, NFATC4* and *NFAT5* transcripts respectively in SV patients as compared to controls. The AV patients showed significantly decreased levels of *NFATC1, NFATC2, NFATC3, NFATC4* and *NFAT5* when compared to controls (p<0.0001; Figure 6). The $2^{-\Delta\Delta CT}$ analysis showed approximately 0.16, 0.082, 0.11, 0.33 and 0.13 - fold changes in expression of *NFATC1, NFATC2, NFATC3, NFATC4* and *NFAT5* transcripts respectively in AV patients as compared to controls.

### ***FOXP3*** **transcripts levels in Generalized Vitiligo**

Since, *NFAT*s controls the expression of *FOXP3* in Treg cells we further analyzed the expression of *FOXP3* in isolated Treg cells from GV patients and controls. The analysis of *FOXP3* transcript levels was carried out after normalization with *GAPDH* expression. The *FOXP3* transcript levels were significantly decreased in GV, SV and AV patients compared to controls (p<0.0001; figure 6f). The $2^{-\Delta\Delta CT}$ analysis showed approximately 0.40, 0.50 and 0.30 fold changes in *FOXP3* transcripts respectively in GV, SV and AV patients as compared to controls. The analysis based on type of the disease activity suggested significantly reduced *FOXP3* transcript levels in AV as compared to SV (p=0.020; figure 6f). There was approximately 0.65 fold change in expression of *FOXP3* transcripts in AV as compared to SV patients.

### **Transcript levels of Treg associated suppressive genes** ***(IL10, TGFB, sCTLA4, flCTLA4)*** **in Generalized Vitiligo**

The NFAT:FOXP3 complex of Tregs is crucial in expression of downstream suppressive genes; hence, we analyzed the expression of *IL10, TGFB, sCTLA4, flCTLA4* in Treg cells isolated from GV patients and controls. The analysis of *IL10, TGFB, sCTLA4, flCTLA4* transcript levels was carried out after normalization with *GAPDH* expression. Interestingly,

*IL10, flCTLA4* and *sCTLA4* transcript levels were significantly decreased in GV patients as compared to controls ($p<0.0001$, $p<0.0001$, $p=0.001$; figure 7a,c,d respectively). However, there was no significant difference in transcript levels of *TGFB* between GV patient and control groups ($p=0.753$; figure 7b). The $2^{-\Delta\Delta CT}$ analysis showed approximately 0.49, 0.32, 0.42 and 0.19- fold changes in expression of *IL10, TGFB, sCTLA4* and *flCTLA4* transcripts respectively in GV patients as compared to controls. Analysis based on activity of the disease revealed that *TGFB* and *flCTLA4* transcript levels were significantly decreased in AV patients in comparison to SV ($p<0.0001$ and $p=0.006$, Figure 7b,c respectively). There was no significant difference found in transcript levels of *IL10* and *sCTLA4* in AV as compared to SV ($p=0.487$ and $p=0.079$; Figure 7a,d, respectively). The AV Tregs showed approximately 0.67, 0.025, 0.39 and 0.18 fold change expression of *IL10, TGFB, sCTLA4* and *flCTLA4* transcripts respectively as compared to SV. Moreover, the SV patients showed significantly decreased levels of *IL10* and *flCTLA4* when compared to controls ($p=0.002$ & $p=0.007$; Figure 7a,c respectively). There was no significant difference in transcript levels of *TGFB* and *sCTLA4* in SV as compared controls ($p>0.999$ and $p=0.143$; Figure 7b,d respectively). The $2^{-\Delta\Delta CT}$ analysis showed approximately 0.63, 0.95, 0.73 and 0.52- fold changes in expression of *IL10, TGFB, sCTLA4* and *flCTLA4* transcripts respectively in SV Tregs as compared to controls. The AV Tregs showed significantly decreased levels of *IL10, TGFB, flCTLA4* and *sCTLA4* when compared to controls ($p<0.0001$, $p<0.0001$, $p<0.0001$ & $p=0.0002$ respectively; figure 7). The AV Tregs showed approximately 0.43, 0.023, 0.28 and 0.09- fold change in expression of *IL10, TGFB, sCTLA4* and *flCTLA4* transcripts respectively as compared to controls.

**Discussion**

The cytotoxic $CD8^+$T cells which are considered as immune systems foot soldiers have emerged to be the important players in promoting several autoimmune diseases including vitiligo(Ahmed et al., 2012). Studies also indicated $CD4^+$T cells' role in pathogenesis of vitiligo(Wu et al., 2013) as well as in activation, function and survival of self-reactive $CD8^+$T Cells(Klaurist et al., 2010). Infiltration of $CD8^+$ and $CD4^+$T cells to the perilesional skin has been observed in vitiligo patients(lambe et al., 2006; Zhang et al., 2011). Regulatory T cells (Tregs) control such hyperactive self-reactive T cells(Dwivedi et al., 2013) however, decreased Treg cells frequency and function have been reported in blood and skin biopsies of vitiligo patients(Chatterjee et al., 2014; Giri et al., 2020; Liu et al., 2009). Though, 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, suppression is checked in either $CD8^+$ or $CD4^+$T cells and lack of data on regulatory suppressive molecules of Tregs(Brunkow et al., 2001; Chatterjee et al., 2014; Giri et al., 2020; Liu et al., 2009). 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 cells proliferation *in vitro* (Figure 2a,b; Table 2). Our findings are in concordance to the previously reported impaired Treg cell suppressive capacity towards $CD8^+$T cells(Brunkow et al., 2001; Chatterjee et al., 2014; Giri et al., 2020) and are in contrast with the previous study where they did not find impaired Treg cell suppression towards $CD4^+$T cells(Liu et al., 2009). The possible explanation for the contrasting results may be due to the smaller sample size used (n=3) in the previous study; whereas in the present study involved a larger sample size of 55 GV patients and 45 controls. In particular, Treg cells from AV showed significantly reduced suppression of $CD8^+$ and $CD4^+$T cells proliferation in comparison to SV, suggesting the crucial role of Tregs dysfunction in GV progression (Figure 2a,b). In addition, we observed reduced percentage suppression of $CD8^+$T cells and $CD4^+$T cells by Tregs in GV patients (Severe) group compared to GV patients (Mild) group (Figure 2c,d). Moreover percentage suppression of $CD8^+$T cells and $CD4^+$T cells were negatively correlated with VASI score, indicaing the role of imparied Tregs in increased severity of GV. Hence, the study suggests impaired inherent Treg suppressive function in vitiligo patients may lead to widespread activation of melanocyte specific $CD8^+$ and $CD4^+$T cells. These activated $CD4^+$T cells may further enhance the activation of $CD8^+$T cells leading to $CD8^+$T cell mediated GV pathogenesis (Figure 8 & 9).

IFN-γ a pro-inflammatory cytokine secreted by $CD8^+$T cells and $CD4^+$T cells(Zhang et al., 2007) is involved in inflammation and autoimmune diseases(Bhat et al., 2017). 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 site of inflammation(Zhang et al., 2007). It inhibits melanogenesis and directly induces melanocyte apoptosis(Yang et al., 2015) (Figure 8 & 9). It 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(Yohn et al., 1990; Badri et al, 1993). Earlier, we demonstrated increased serum IFN-γ levels and *ICAM* mRNA in GV patients(Dwivedi et al., 2013). Here, we also report significant increased levels of IFN-γ production by unchecked $CD8^+$ and $CD4^+$T cells in GV patients (Figure 3a,b); indicating inability of Tregs to suppress activated $CD8^+$ and $CD4^+$T cells population. Moreover, correlation analysis suggested that IFN-γ production by these $CD8^+$ and $CD4^+$T is negatively correlated with percentage suppression of $CD8^+$ and $CD4^+$T cells. Previous studies also reported increased IFN-γ expression by $CD8^+$T cells and $T_{RM}$ (Tissue resident memory) $CD8^+$T cells in vitiligo patients(Boniface et al., 2018). In addition, *IFNG* mRNA and serum IFN-γ levels were increased in vitiligo patients(Ala et al., 2015; ezzzedine et al., 2018). In particular, IFN-γ production by $CD8^+$ and $CD4^+$T cells were significantly increased in AV compared to SV which is in concordance to the previously reported increased IFN-γ expression by $CD8^+$ and $CD4^+$T in skin samples of AV compared to SV patients(Boniface et al., 2018) suggesting the crucial role of IFN-γ in disease progression (Figure 3a,b). In addition, IFN-γ production by $CD8^+$ and $CD4^+$T cells was found to be increased in GV patients (Severe) group compared to GV patients (Mild) group (Figure 3a,b). Moreover, IFN-γ production by $CD8^+$ and $CD4^+$T cells were positively correlated with VASI score, suggesting the crucial role of IFN-γ in increased 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 & TNF-α) and inhibiting the activation of T cells by downregulating MHC-II expression and co-stimulation(knolle et al., 1998). Moreover, IL-10 also induces Type-1 Treg cells(O'garra et al., 2004). TGF-β plays an important role in inhibition of immune response by suppressing T cell proliferation and differentiation(Dahmani et al., 2018). TGF-β can induce the expression of CD25 on $CD4^+CD25^-$ cells and transform them into $CD4^+CD25^+$ regulatory cells(Fu et al., 2004). It can also induce FOXP3 expression on regulatory T-cells(Chen et al., 2010). Thus, both IL-10 and TGF-β contribute in Treg cells growth, expansion and suppressive function(Hsu et al., 2015). In Treg:$CD8^+$ and Treg:$CD4^+$T cells co-culture assay, we observed significant decreased IL-10 and TGF-β production by Tregs in GV patients (Figure 3c,d,e,f). In

particular, IL-10 production by Tregs was significantly decreased in AV compared to SV emphasizing the crucial role of IL-10 in disease progression (Figure 3c,d). In addition, IL-10 production by Tregs was significantly reduced for GV patients (Severe) group compared to GV patients (Mild) group (Figure 3c,d). We also found a negative correlation for IL-10 production by Tregs with VASI score suggesting the crucial role of IL-10 in impaired Treg function leading to increased GV severity. Moreover, correlation analysis suggested that IL-10 and TGF-β production by Tregs is positively correlated with percentage suppression of $CD8^+$ and $CD4^+$T cells. The reduced suppressive function of Tregs in GV may be due to decreased immune suppressive cytokine production. Previously, significantly reduced IL-10 and TGF-β mRNA and protein levels were reported in serum/skin of GV patients(Van der boon et al., 2009; Tembhre et al., 2013). Moreover, the ratio of pro-inflammatory cytokine to anti-inflammatory cytokine was found to be altered in the *in vitro* suppression assay. In particular, a significantly increased IFN-γ : IL10 and IFN-γ : TGF-β ratio was observed in Treg:$CD8^+$T cells and Treg:$CD4^+$ T cells co-culture systems of GV patients. These results indicate the crucial role of IL-10 and TGF-β deficiency leading to compromised suppressive capacity of Treg cells in GV (Figure 8 & 9).

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. FOXP3 upregulates CD25 & CTLA-4 cell-surface immunoregulatory molecules(Zheng et al.., 2007). We found that the FOXP3 protein and mRNA levels were significantly reduced in GV Tregs (Figure 4a). Studies have shown alterations in FOXP3 expression in $CD4^+CD25^{high}$ Tregs from vitiligo patients compared to controls(Dwivedi et al., 2013). with significantly reduced *FOXP3* transcripts in perilesional and lesional skin(Hegazy et al., 2014. In particular, FOXP3 protein and mRNA levels expression in Tregs was significantly decreased in AV compared to SV, which is in concordance to the previous study(Dwived et al., 2013) suggesting the crucial role of FOXP3 in GV progression (Figure 4a). Moreover, FOXP3 protein and mRNA levels expression in Tregs was significantly decreased for GV patients (Severe) group compared to GV patients (Mild) group (Figure 4b). FOXP3 expression in Tregs was also found to be in negative correlation with VASI score suggesting the crucial role of FOXP3 in increased GV severity. Moreover, we found that the protein level of CD25 – a Treg surface suppressive marker, in GV Tregs was significantly reduced as compared to controls, indicating the altered Tregs suppressive function in GV

(Figure 4c). 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 5f,c,d). Increased Tregs suppressive capacity and production of IL-10 has been found in $CD44^+$Tregs. However, we did not find significant difference in Tregs CD44 protein levels between GV patients and controls.

Now it became pertinent to find the reason for decreased FOXP3, 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(Giri et al., 2020). NFATC1 plays an important role in iTreg generation by regulating FOXP3 expression through its binding with *FOXP3* CNS1 region(Vaeth et al., 2012) (Figure 8 & 9). FOXP3 peptide mediated inhibition of NFAT1-FOXP3 interaction has been shown to alter the Treg suppressive function and inhibit Tregs differentiation(Lozano et al., 2015). 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 show significant decreased NFATC1 protein levels in GV Tregs (Figure 4e); in concordance to this we also found decreased NFATs mRNA levels in GV Tregs(Dwivedi et al., 2013). In particular, NFATC1 expression in Tregs was significantly decreased in AV compared to SV suggesting the role of NFATC1 in GV progression (Figure 4e). In addition, NFATC1 expression in Tregs was significantly decreased for GV patients (Severe) group compared to GV patients (Mild) group (Figure 4f). NFATC1 expression in Tregs was also found to be in negative correlation with VASI score indicating the role of NFATC1 in increased severity of GV. Moreover, the correlation analysis also suggested that NFATC1 is positively correlated with FOXP3 protein expression and $CD8^+$ and $CD4^+$T cells suppression (Figure 5e,a,b). Hence, our results suggest that NFAT may play a crucial role in decreased FOXP3 expression in Tregs and thereby may affect the functionality of Tregs in GV (Figure 8 & 9).

In Treg cells, the NFAT:FOXP3 complex controls the regulatory T cells function by upregulating the expression of suppressive markers like *CD25, CTLA4, IL10, TGFB* and downregulating the expression of *IL2* and *IL4 (*Giri et al., 2020*)*. The downstream immunoregulatory genes of Tregs such as *CD25*, *CTLA4*, *IL10*, *TGFB*, *IL2* and *IL4* play a crucial role in maintaining immune tolerance. Disruptive mutations and the altered levels of

*NFAT*:*FOXP3* complex leads to non-functional Tregs (Vaeth and Feske, 2018). Since, we found decreased expression of both *NFATs* and *FOXP3,* we further assessed their effect on downstream immunosuppressive genes in Tregs of GV patients. Interestingly, we found that the suppressive capacity of Tregs was compromised in GV patients as the Tregs of GV patients showed significantly reduced expression of immune suppressive genes *CTLA4 (sCTLA4 & flCTLA4)* and *IL10* compared to controls. Previously, studies detected systemic levels of these genes in vitiligo patients; however, the present study has shown expression of the immune suppressive genes in Tregs isolated from GV patients and controls. In addition, a significant decreased expression of *flCTLA4* transcripts were observed in Tregs of AV patients compared to SV patients, indicating the role of *flCTLA4* in disease progression.

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 & TGF-β, *sCTLA4 & flCTLA4*), thereby leading to unchecked $CD8^+$ and $CD4^+$T proliferation and IFN-γ production resulting in melanocyte death and GV pathogenesis.

**Impact of the research in the advancement of knowledge or benefit to mankind**

- The study for the first time has revealed decreased mRNA and protein expression of FOXP3 and NFATs in Tregs of generalized vitiligo patients suggesting the crucial role of FOXP3 and NFATs in GV pathogenesis and progression.
- Moreover, the study suggested the decreased *NFATs(1-5)* and *FOXP3* mRNA expression leads to reduced downstream immunosuppressive genes leading to GV pathogenesis and progression.
- Additionally, Treg suppression assay revealed decreased Treg cells capacity to suppress $CD4^+$ and $CD8^+$ T cells, which was evident by increased production of IFN-γ by $CD4^+$ and $CD8^+$ T cells, decreased production of Treg suppressive cytokine's IL-10 & TGF-β by GV Tregs suggesting the compromised suppressive capacity of Tregs leading to GV pathogenesis.

- Previous studies had suggested decreased Treg number and function in vitiligo patients, however the current study has provided an in-detail mechanisms for the Tregs defects in GV patients, which could lead to Treg based therapeutics for GV.
- Overall, the long-term impact of the current study could be developing safe and effective Treg based therapeutics targeting NFATs and FOXP3 regulatory molecules for treatment of GV.

**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 years | 22.76 ± 3.54 years |
| **Sex: Male :**<br>**Female :** | 25 (45.45%)<br>30 (55.54%) | 26 (57.78%)<br>19 (42.22%) |
| **Age of onset (mean age ± SD)** | 22.69 ± 12.01 years | NA |
| **Duration of disease (mean ± SD)** | 5.35 ± 7.51 years | NA |
| **VASI Score (mean ± SD)** | 61.91% ± 23.87% | NA |
| **Active vitiligo**<br>**Stable vitiligo** | 44 (80.00%)<br>11 (20.00%) | NA |
| **Family history** | 15 (27.27%) | NA |

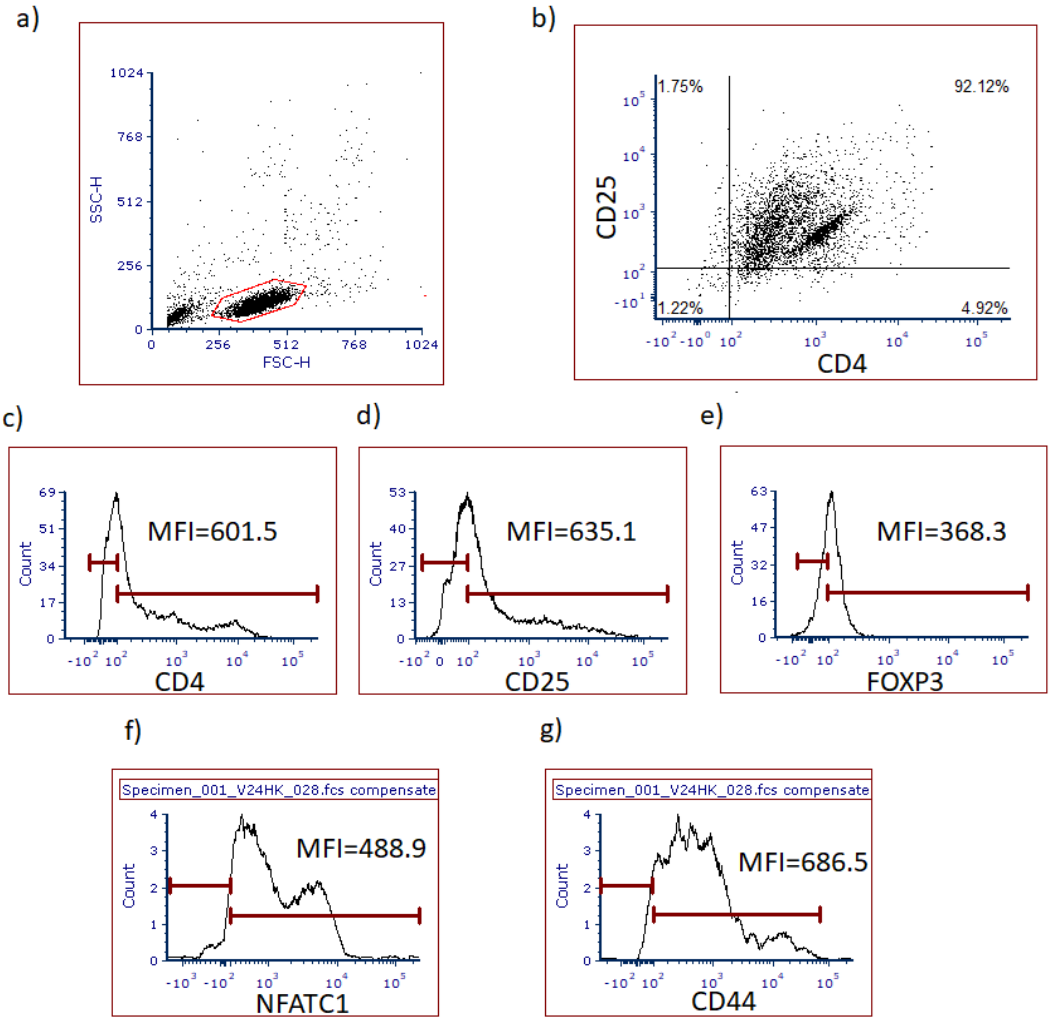


**Figure 1. Gating Strategy (stained): for CD4+CD25+FOXP3+ Treg cells: estimation of protein levels of CD25, FOXP3, NFATC1 & CD44.**

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).

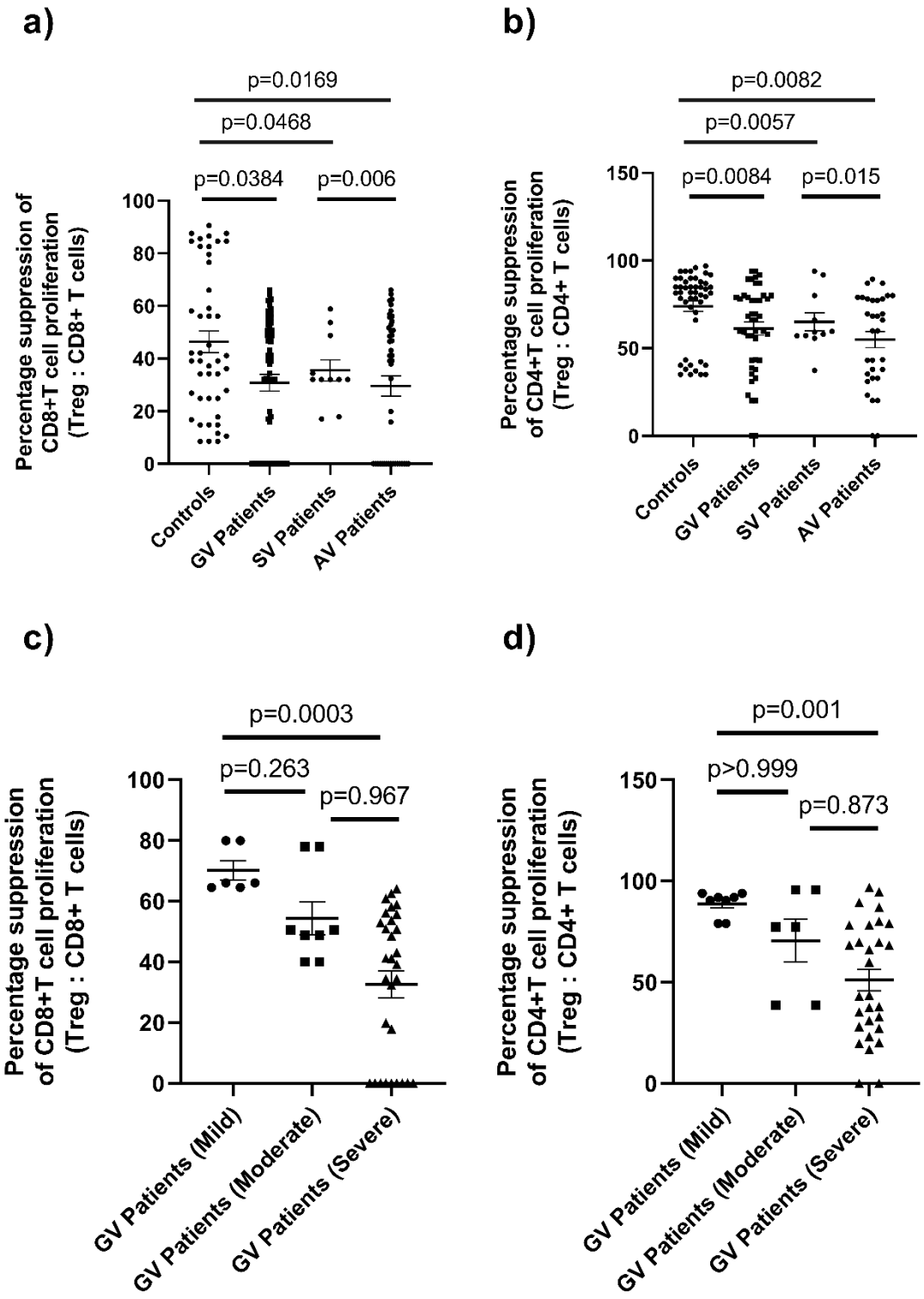


**Figure 2.** ***In vitro*** **Treg suppression of CD8$^+$ and CD4$^+$ T Cells proliferation in GV patients and controls.**

In *vitro* Treg suppression of CD8$^+$ and CD4$^+$ T cells Proliferation by Treg in 55 Generalized vitiligo (GV) patients [44 Active vitiligo (AV) patients & 11 Stable vitiligo (SV) patients; 10

GV patients (Mild), 6 GV patients (Moderate) & 39 GV patients (Severe)] and 45 healthy controls were analyzed by non-parametric Mann-Whitney U test.

a) Percentage suppression of $CD8^+$ T cells in GV,SV & AV patients vs Controls (Mean ± SEM: 30.80 ± 3.19,35.52 ± 4.01, 29.62 ± 3.86 vs 46.35 ± 4.08; p=0.0384, p=0.0468 & p=0.0169 respectively). Percentage suppression of $CD8^+$ T cells in SV vs AV patients (Mean ± SEM: 35.52 ± 4.01 vs 29.62 ± 3.86; p=0.006).

b) Percentage suppression of $CD4^+$ T cells in GV SV & AV patients vs Controls (Mean ± SEM: 61.20 ± 3.69, 65.11 ± 5.11, 54.88 ± 4.61 vs 73.90 ± 2.97; p=0.0084,p=0.0057 & p=0.0082 respectively). Percentage suppression of $CD4^+$ T cells in SV vs AV patients (Mean ± SEM: 65.11 ± 5.11 vs 54.88 ± 4.61;p=0.015)

c) Percentage suppression of CD8+ T cells for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 70.18 ± 3.10 vs 32.56 ± 4.43; p=0.0003). Percentage suppression of CD8+ T cells for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 54.32 ± 5.37 vs 70.18 ± 3.10 & 32.56 ± 4.43; p=0.263 & p=0.967 respectively)

d) Percentage suppression of CD4+ T cells for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 88.85 ± 2.18 vs 51.10 ± 5.33; p=0.001). Percentage suppression of CD4+ T cells for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 70.54 ± 10.63 vs 88.85 ± 2.18 & 51.10 ± 5.33; p>0.999 & p=0.873 respectively) (SEM, standard error mean).

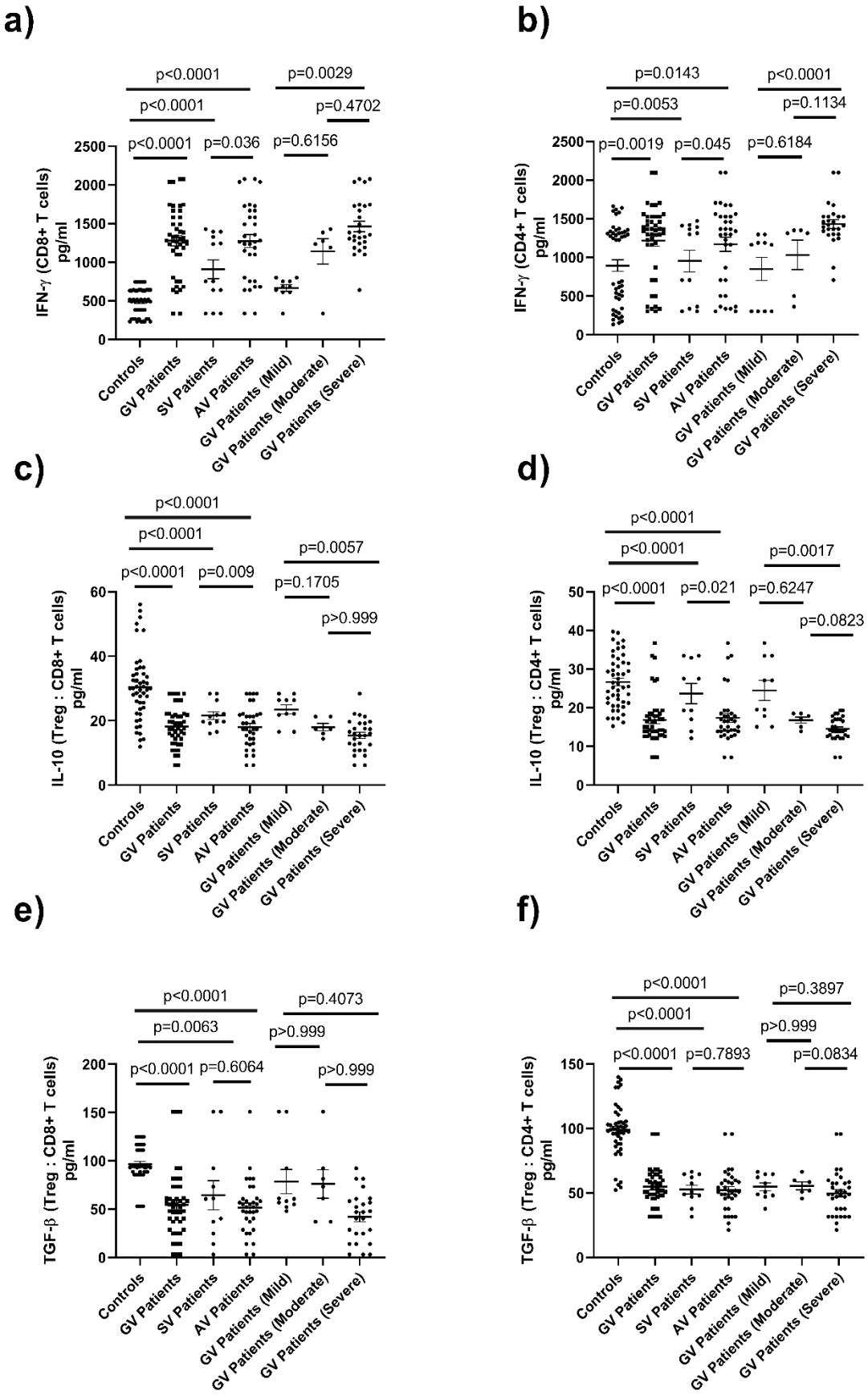
a)
p<0.0001
p<0.0001
p=0.0029
p=0.4702
p<0.0001
p=0.036
p=0.6156
IFN-γ (CD8+ T cells) pg/ml
2500
2000
1500
1000
500
0
Controls
GV Patients
SV Patients
AV Patients
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)
b)
p=0.0143
p=0.0053
p<0.0001
p=0.1134
p=0.0019
p=0.045
p=0.6184
IFN-γ (CD4+ T cells) pg/ml
2500
2000
1500
1000
500
0
Controls
GV Patients
SV Patients
AV Patients
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)
c)
p<0.0001
p<0.0001
p=0.0057
p<0.0001
p=0.009
p=0.1705
p>0.999
IL-10 (Treg : CD8+ T cells) pg/ml
60
40
20
0
Controls
GV Patients
SV Patients
AV Patients
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)
d)
p<0.0001
p<0.0001
p=0.0017
p<0.0001
p=0.021
p=0.6247
p=0.0823
IL-10 (Treg : CD4+ T cells) pg/ml
50
40
30
20
10
0
Controls
GV Patients
SV Patients
AV Patients
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)
e)
p<0.0001
p=0.0063
p=0.4073
p>0.999
p<0.0001
p=0.6064
p>0.999
TGF-β (Treg : CD8+ T cells) pg/ml
200
150
100
50
0
Controls
GV Patients
SV Patients
AV Patients
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)
f)
p<0.0001
p<0.0001
p=0.3897
p>0.999
p<0.0001
p=0.7893
p=0.0834
TGF-β (Treg : CD4+ T cells) pg/ml
150
100
50
0
Controls
GV Patients
SV Patients
AV Patients
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)

**Figure 3. IFN-γ production by $CD8^+$ & $CD4^+$ T cells and IL-10 & TGF-β production by Treg cells in GV patients and controls.**

IFN-γ production by $CD8^+$ and $CD4^+$ T cells and IL-10 & TGF-β production by Treg cells in Treg : $CD8^+$ T cells and Treg : $CD4^+$ T cells co-culture systems respectively, in 55 Generalized vitiligo (GV) patients [44 Active vitiligo (AV) patients & 11 Stable vitiligo (SV) patients; 10 GV patients (Mild), 6 GV patients (Moderate) & 39 GV patients (Severe)] and 45 healthy controls were analyzed by non-parametric Mann-Whitney U test.

a) IFN-γ production by $CD8^+$ T cells in Treg : $CD8^+$ T cells co-culture system of GV,SV & AV patients versus controls (Mean ± SEM: 1278 ± 67.12, 909.2 ± 121.80 & 1274 ± 85.95 vs 488.8 ± 25.11; $p < 0.0001$). IFN-γ production by CD8+ T cells in Treg : CD8+ T cells co-culture system of SV vs AV patients(Mean ± SEM: 909.2 ± 121.80 vs 1274 ± 85.95; $p=0.036$). IFN-γ production by $CD8^+$ T cells in Treg : $CD8^+$ T cells co-culture system for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 667.7 ± 43.19 vs 1462.0 ± 67.32; $p=0.0029$). IFN-γ production by $CD8^+$ T cells in Treg : $CD8^+$ T cells co-culture system for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 1141.0 ± 165.2 vs 667.7 ± 43.19 & 1462.0 ± 67.32; $p=0.6156$ & $p=0.4702$ respectively).

b) IFN-γ production by $CD4^+$ T cells in Treg : $CD4^+$ T cells co-culture system GV,SV & AV patients versus controls (Mean ± SEM: 1216 ± 70.61, 953.2 ± 138.7 & 1169 ± 91.85 vs 894.8 ± 74.89; $p=0.0019$,$p=0.0053$ & $p=0.0143$ respectively). IFN-γ production by $CD4^+$ T cells in Treg : $CD4^+$ T cells co-culture system of SV vs AV patients (Mean ± SEM: 953.2 ± 138.7 vs 1169 ± 91.85; $p=0.045$). IFN-γ production by $CD4^+$ T cells in Treg : $CD4^+$ T cells co-culture system for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 850.7 ± 150.0 vs 1432.0 ± 56.49; $p<0.0001$). IFN-γ production by $CD4^+$ T cells in Treg : $CD4^+$ T cells co-culture system for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 1033.0 ± 190.8 vs 850.7 ± 150.0 & 1432.0 ± 56.49; $p=0.6184$ & $p=0.1134$ respectively).

c) IL-10 production by Treg cells for Treg : $CD8^+$ T cells co-culture system in GV,SV & AV patients vs controls (Mean ± SEM: 18.22 ± 0.90, 21.58 ± 1.19 & 18.03 ± 1.09 vs 30.40 ± 1.50; $p<0.0001$). IL-10 production by Tregs in Treg : $CD8^+$ T cells co-culture system of SV vs AV patients(Mean ± SEM: 21.58 ± 1.19 vs 18.03 ± 1.09; $p=0.009$). IL-10 production by Treg cells for Treg : $CD8^+$ T cells co-culture system for GV patients (Mild) vs GV patients

(Severe) (Mean ± SEM: 23.50 ± 1.39 vs 15.41 ± 0.96; p=0.0057). IL-10 production by Treg cells for Treg : $CD8^+$ T cells co-culture system for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 17.96 ± 1.14 vs 23.50 ± 1.39 & 15.41 ± 0.96; p=0.1705 & p>0.999 respectively).

d) IL-10 production by Treg cells for Treg : $CD4^+$ T cells co-culture system in GV,SV & AV patients vs controls (Mean ± SEM: 16.85 ± 0.95, 23.66 ± 2.62 & 17.39 ± 1.15 vs 26.66 ± 0.96; p <0.0001). IL-10 production by Tregs in Treg : $CD4^+$ T cells co-culture system of SV vs AV patients(Mean ± SEM: 23.66 ± 2.62 vs 17.39 ± 1.15; p =0.021). IL-10 production by Treg cells for Treg : $CD4^+$ T cells co-culture system GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 24.47 ± 2.57 vs 14.51 ± 0.54; p=0.0017). IL-10 production by Treg cells for Treg : $CD4^+$ T cells co-culture system for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 16.81 ± 0.78 vs 24.47 ± 2.57 & 14.51 ± 0.54; p=0.6247 & p=0.0823 respectively).

e) TGF-β production by Tregs for Treg: $CD8^+$ T cells co-culture system in GV,SV & AV patients vs controls (Mean ± SEM: 54.74 ± 5.14, 64.48 ± 15.07 & 51.59 ± 4.817 vs 96.72 ± 2.89; p<0.0001, p=0.0063 & p<0.0001 respectively). TGF-β production by Tregs in Treg : CD8+ T cells co-culture system of SV vs AV patients (Mean ± SEM: 64.48 ± 15.07 vs 51.59 ± 4.817; p=0.6064). TGF-β production by Tregs for Treg: $CD8^+$ T cells co-culture system for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 78.67 ± 12.61 vs 42.22 ± 5.16; p=0.4073). TGF-β production by Tregs for Treg: $CD8^+$ T cells co-culture system for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 76.25 ± 14.75 vs 78.67 ± 12.61 & 42.22 ± 5.16; p>0.999 & p>0.999 respectively).

f) TGF-β production by Treg cells for Treg: $CD4^+$ T cells co-culture system in GV,SV & AV patients vs controls (Mean ± SEM: 54.78 ± 2.30, 52.64 ± 3.473 & 52.06 ± 2.819 vs 98.78 ± 3.01; p<0.0001). TGF-β production by Tregs in Treg : $CD4^+$ T cells co-culture system of SV vs AV patients(Mean ± SEM: 52.64 ± 3.473 vs 52.06 ± 2.; p =0.7893). TGF-β production by Tregs for Treg: $CD4^+$ T cells co-culture system for VASI score GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 54.76 ± 3.04 vs 49.74 ± 2.91; p=0.3897). TGF-β production by Tregs for Treg: $CD4^+$ T cells co-culture system for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 55.48 ± 3.04 vs 54.76 ± 3.04 vs 49.74 ± 2.91; p>0.999 & p=0.083 respectively) (SEM, standard error mean).

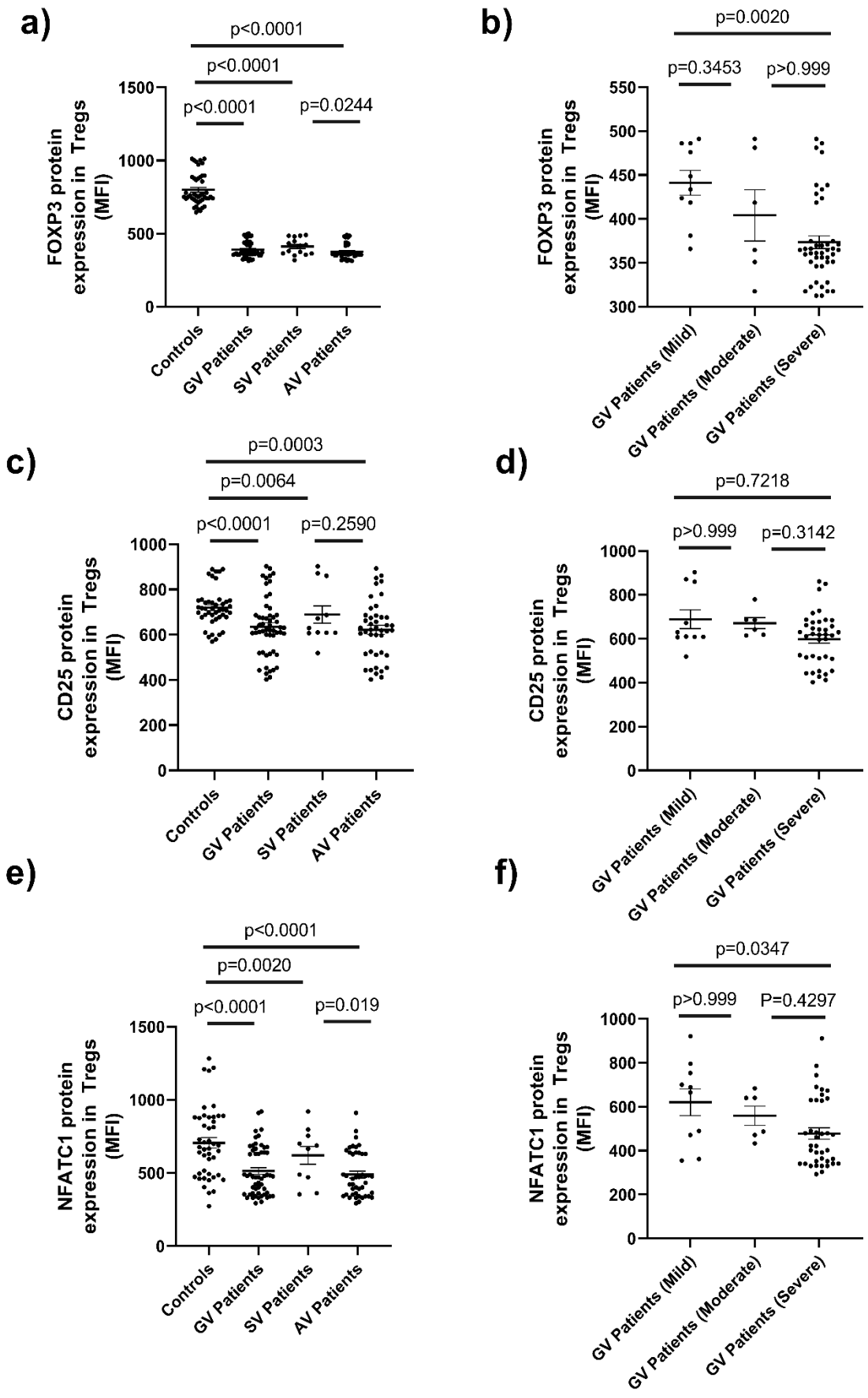
a)
p<0.0001
p<0.0001
p<0.0001
p=0.0244
FOXP3 protein expression in Tregs (MFI)
1500
1000
500
0
Controls
GV Patients
SV Patients
AV Patients
b)
p=0.0020
p=0.3453
p>0.999
FOXP3 protein expression in Tregs (MFI)
550
500
450
400
350
300
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)
c)
p=0.0003
p=0.0064
p<0.0001
p=0.2590
CD25 protein expression in Tregs (MFI)
1000
800
600
400
200
0
Controls
GV Patients
SV Patients
AV Patients
d)
p=0.7218
p>0.999
p=0.3142
CD25 protein expression in Tregs (MFI)
1000
800
600
400
200
0
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)
e)
p<0.0001
p=0.0020
p<0.0001
p=0.019
NFATC1 protein expression in Tregs (MFI)
1500
1000
500
0
Controls
GV Patients
SV Patients
AV Patients
f)
p=0.0347
p>0.999
P=0.4297
NFATC1 protein expression in Tregs (MFI)
1000
800
600
400
200
0
GV Patients (Mild)
GV Patients (Moderate)
GV Patients (Severe)

**Figure 4. FOXP3, CD25, NFATC1 protein levels in GV patients.**

FOXP3, CD25, NFATC1 and CD44 expression levels in 55 Generalized vitiligo (GV) patients [44 Active vitiligo (AV) patients & 11 Stable vitiligo (SV) patients; 10 GV patients (Mild), 6 GV patients (Moderate) & 39 GV patients (Severe)] and 45 healthy controls were analyzed by non-parametric Mann-Whitney U test.

a) FOXP3 expression level in Treg of GV,SV & AV patients vs controls (Mean ± SEM: 390.6 ± 7.26, 412.1 ± 14.88 & 375.8 ± 7.46 vs 799.5 ± 16.46; $p<0.0001$). FOXP3 expression level in Treg of SV vs AV patients (Mean ± SEM: 412.1 ± 14.88 & 375.8 ± 7.46; $p=0.0244$).

b) FOXP3 expression level in Treg for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 441.1 ± 14.15 vs 373.5 ± 7.26; $p=0.002$). FOXP3 expression level in Treg for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 404.0 ± 29.23 vs 441.1 ± 14.15 & 373.5 ± 7.26; $p=0.3453$ & $p>0.999$ respectively).

c) CD25 expression level in Treg of GV,SV & AV patients vs controls (Mean ± SEM: 635.1 ± 17.81, 689.0 ± 38.9 & 621.3 ± 19.70 vs 719.6 ± 12.68; $p<0.0001$, $p=0.0064$ & $p=0.0003$ respectively). CD25 expression level in Treg of SV vs AV patients (Mean ± SEM: 689.0 ± 38.9 vs 621.3 ± 19.70; $p=0.2590$).

d) CD25 expression level in Treg for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 689.2 ± 43.07 vs 598.9 ± 19.34; $p=0.7218$). CD25 expression level in Treg for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 671.5 ± 25.08 vs 689.2 ± 43.07 & 598.9 ± 19.34; $p>0.999$ & $p=0.3142$ respectively).

e) NFATC1 expression level in Treg of GV,SV & AV patients vs controls (Mean ± SEM: 513 ± 22.76, 620.4 ± 60.47 & 488.9 ± 23.14 vs 706.1 ± 36.1; $p<0.0001$, $p=0.020$ & $p<0.0001$ respectively). NFATC1 expression level in Treg of SV & AV patients (Mean ± SEM: 620.4 ± 60.47 vs 488.9 ± 23.14; $p=0.019$).

f) NFATC1 expression level in Treg for GV patients (Mild) vs GV patients (Severe) (Mean ± SEM: 620.4 ± 60.47 vs 559.3 ± 43.85; $p=0.0347$). NFATC1 expression level in Treg for GV patients (Moderate) vs GV patients (Mild) & GV patients (Severe) (Mean ± SEM: 559.3 ± 43.85 vs 620.4 ± 60.47 & 559.3 ± 43.85; $p>0.999$ & $p=0.4297$ respectively). (SEM, standard error mean).

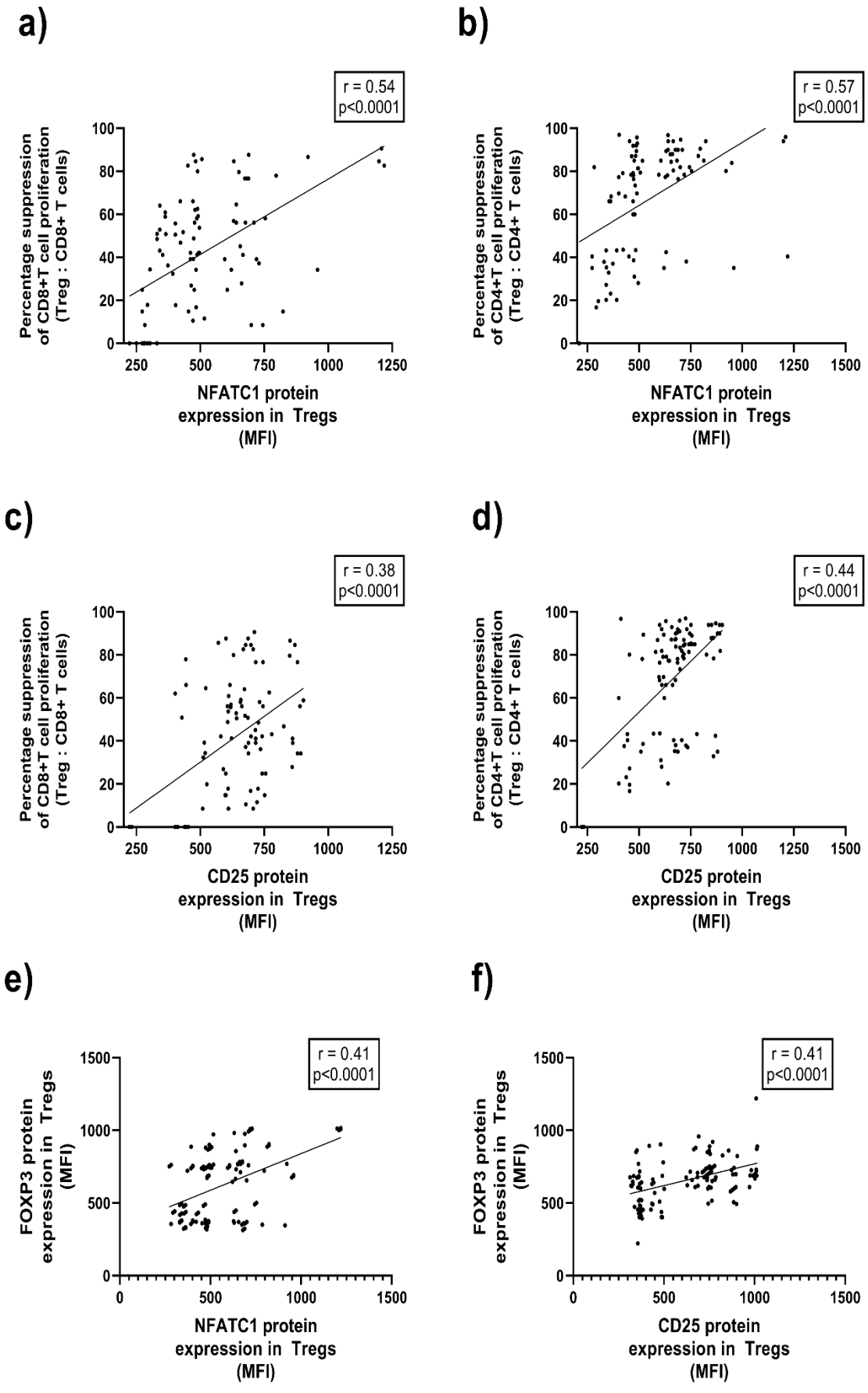
a)
r = 0.54
p<0.0001
Percentage suppression of CD8+T cell proliferation (Treg : CD8+ T cells)
NFATC1 protein expression in Tregs (MFI)
b)
r = 0.57
p<0.0001
Percentage suppression of CD4+T cell proliferation (Treg : CD4+ T cells)
NFATC1 protein expression in Tregs (MFI)
c)
r = 0.38
p<0.0001
Percentage suppression of CD8+T cell proliferation (Treg : CD8+ T cells)
CD25 protein expression in Tregs (MFI)
d)
r = 0.44
p<0.0001
Percentage suppression of CD4+T cell proliferation (Treg : CD4+ T cells)
CD25 protein expression in Tregs (MFI)
e)
r = 0.41
p<0.0001
FOXP3 protein expression in Tregs (MFI)
NFATC1 protein expression in Tregs (MFI)
f)
r = 0.41
p<0.0001
FOXP3 protein expression in Tregs (MFI)
CD25 protein expression in Tregs (MFI)

**Figure 5. Correlation of NFATC1 & CD25 levels with percentage Treg suppression of $CD8^+$ & $CD4^+$ T cells and FOXP3 in Tregs of GV patients and controls.**

The correlation of NFATC1, CD25 expression with percentage Treg suppression of $CD8^+$ & $CD4^+$ T cells and FOXP3 expression in Tregs in Treg : $CD8^+$ & Treg : $CD4^+$ T cells co-culture systems of GV patients and controls were analyzed 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 ($r = 0.54$, $p<0.0001$).

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 ($r = 0.57$, $p<0.0001$).

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 ($r = 0.38$, $p<0.0001$).

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 ($r = 0.44$, $p<0.0001$).

e) NFATC1 expression in Tregs was positively correlated with FOXP3 expression in Tregs in GV patients and controls ($r = 0.41$, $p<0.0001$).

f) CD25 expression in Tregs was positively correlated with FOXP3 expression in Tregs in GV patients and controls ($r = 0.41$, $p<0.0001$).

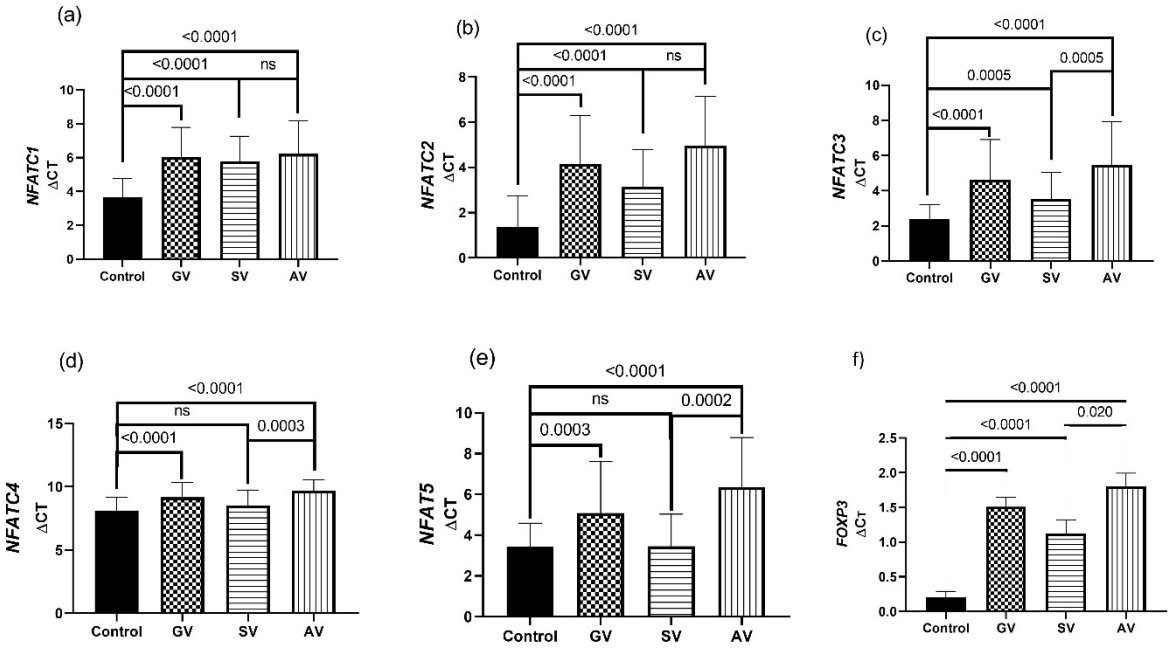


**Figure 6: *NFATs* and *FOXP3* transcript level in generalized vitiligo**

(a) GV, SV and AV showed a significantly decreased transcript levels of *NFATC1* compared to controls. (mean ± SEM: 6.02±0.24, 5.76±0.31, 6.22±0.36 respectively vs 3.66±0.15; p<0.0001). The transcript levels of *NFATC1* in AV was comparable to that of SV patients (mean ± SEM: 6.22±0.36 vs 5.76±0.31; p=0.487).

(b) GV, SV and AV showed a significantly decreased transcript levels of *NFATC2* compared to controls. (mean ± SEM: 4.15±0.30, 3.13±0.34, 4.95±0.41 respectively vs 1.35±0.19; p<0.0001). The transcript levels of *NFATC2* in AV was comparable to that of SV patients (mean ± SEM: 4.95±0.41 vs 3.13±0.34; p=0.101).

(c) GV, SV and AV showed a significantly decreased transcript levels of *NFATC3* compared to controls. (mean ± SEM: 4.61±0.32, 3.52±0.32, 5.48±0.46 respectively vs 2.37±0.11; p<0.0001, p=0.0005 and p<0.0001 respectively). Active patients showed a significant decrease in transcript levels of *NFATC3* compared to stable patients. (mean ± SEM: 5.48±0.46 vs 3.52±0.32; p=0.0005).

(d) GV and AV showed a significantly decreased transcript levels of *NFATC4* compared to controls. (mean ± SEM: 9.17±0.16, 9.68±0.16 respectively vs 8.12±0.14; $p<0.0001$). The transcript levels of *NFATC4* in SV was comparable to that of controls (mean ± SEM: 8.52±0.25 vs 8.12±0.14; $p=0.164$).Active patients showed a significant decrease in transcript levels of *NFATC4* compared to stable patients. (mean ± SEM: 9.68±0.16 vs 8.52±0.25 vs; $p=0.0003$).

(e) GV and AV showed a significantly decreased transcript levels of *NFAT5* compared to controls. (mean ± SEM: 5.07±0.35 and 6.35±0.46 respectively vs 3.43±0.16; $p=0.0003$ and $p<0.0001$ respectively). The transcript levels of *NFAT5* in SV was comparable to that of controls (mean ± SEM: 3.45±0.33 vs 3.43±0.16; $p=0.944$).Active patients showed a significant decrease in transcript levels of *NFAT5* compared to stable patients. (mean ± SEM: 6.35±0.46 vs 3.45±0.33; $p=0.0002$).

f) GV,SV and AV showed a significant decrease in transcript levels of *FOXP3* compared to controls (mean ± SEM: 1.50±0.14, 1.12±0.19, 1.80±0.18 respectively vs 0.19±0.09; $p<0.0001$). AV showed a significantly decreased transcript levels of *FOXP3* when compared to SV (mean ± SEM: 1.12±0.19 vs 1.80±0.18; $p=0.020$).

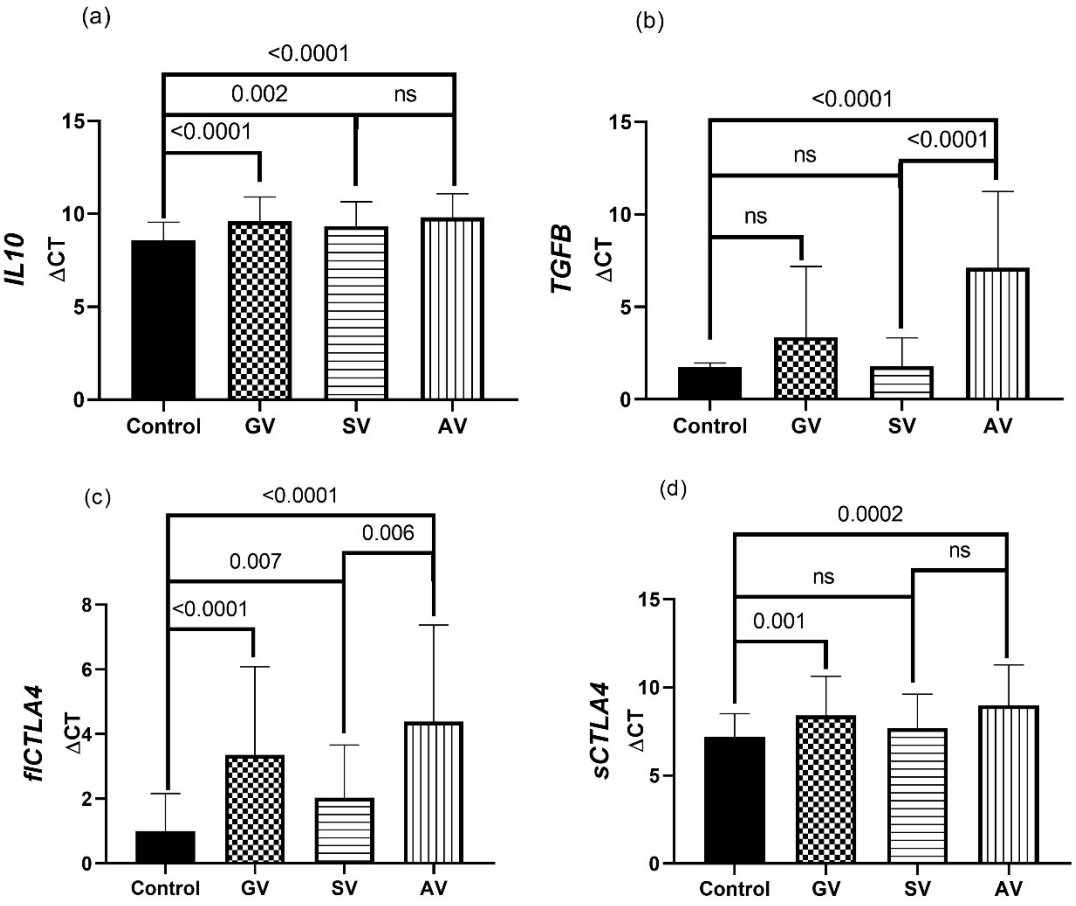


**Figure 7: Transcript levels of Treg associated suppressive genes *(IL10, TGFB, flCTLA4, sCTLA4)* in generalized vitiligo**

Expression of *IL10, TGFB, sCTLA4, flCTLA4* transcripts in 45 controls, 48 generalized vitiligo (GV) [21 stable vitiligo(SV) and 27 Active vitiligo (AV)] was analyzed by non-parametric Mann-Whitney U test.

(a) GV,SV and AV showed a significant decrease in transcript levels of *IL10* compared to controls (mean ± SEM: 9.60±0.18, 9.34±0.28 and 9.81±0.23 respectively vs 8.60±0.13; $p<0.0001$, $p=0.002$ and $p<0.0001$ respectively). The transcript levels of *IL10* in AV was comparable to that of SV patients (mean ± SEM: vs 9.81±0.23 vs 9.34±0.28; $p=0.487$).

(b) AV showed a significant decrease in transcript levels of *TGFB* compared to controls and SV (mean ± SEM: 7.12±0.77 vs 1.73±0.03 and 1.80±0.32 respectively; $p<0.0001$). The transcript levels of *TGFB* in GV and SV was comparable to that of controls (mean ± SEM: 3.374±0.53, 1.80±0.32 vs 1.73±0.03 respectively; $p=0.753$ and $p>0.999$ respectively).

(c) GV,SV and AV showed a significant decrease in transcript levels of *flCTLA4* compared to controls (mean ± SEM: 3.35±0.38, 2.03±0.34 and 4.39±0.56 respectively vs 1.00±0.16; $p<0.0001$, $p=0.007$ and $p<0.0001$ respectively). The transcript levels of *flCTLA4* in AV was significantly decreased to that of SV patients (mean ± SEM: 4.39±0.56 vs 2.03±0.34; $p=0.006$).

(d) GV and AV showed a significant decrease in transcript levels of *sCTLA4 c*ompared to controls (mean ± SEM: 8.41±0.31 and 8.98±0.43 respectively vs 7.19±0.18; $p=0.001$, $p=0.0002$ respectively). The transcript levels of *sCTLA4* in SV was comparable to that of controls and AV patients (mean ± SEM: 7.69±0.40 vs 7.19±0.18 and 8.98±0.43 respectively; $p=0.143$ and $p=0.079$).

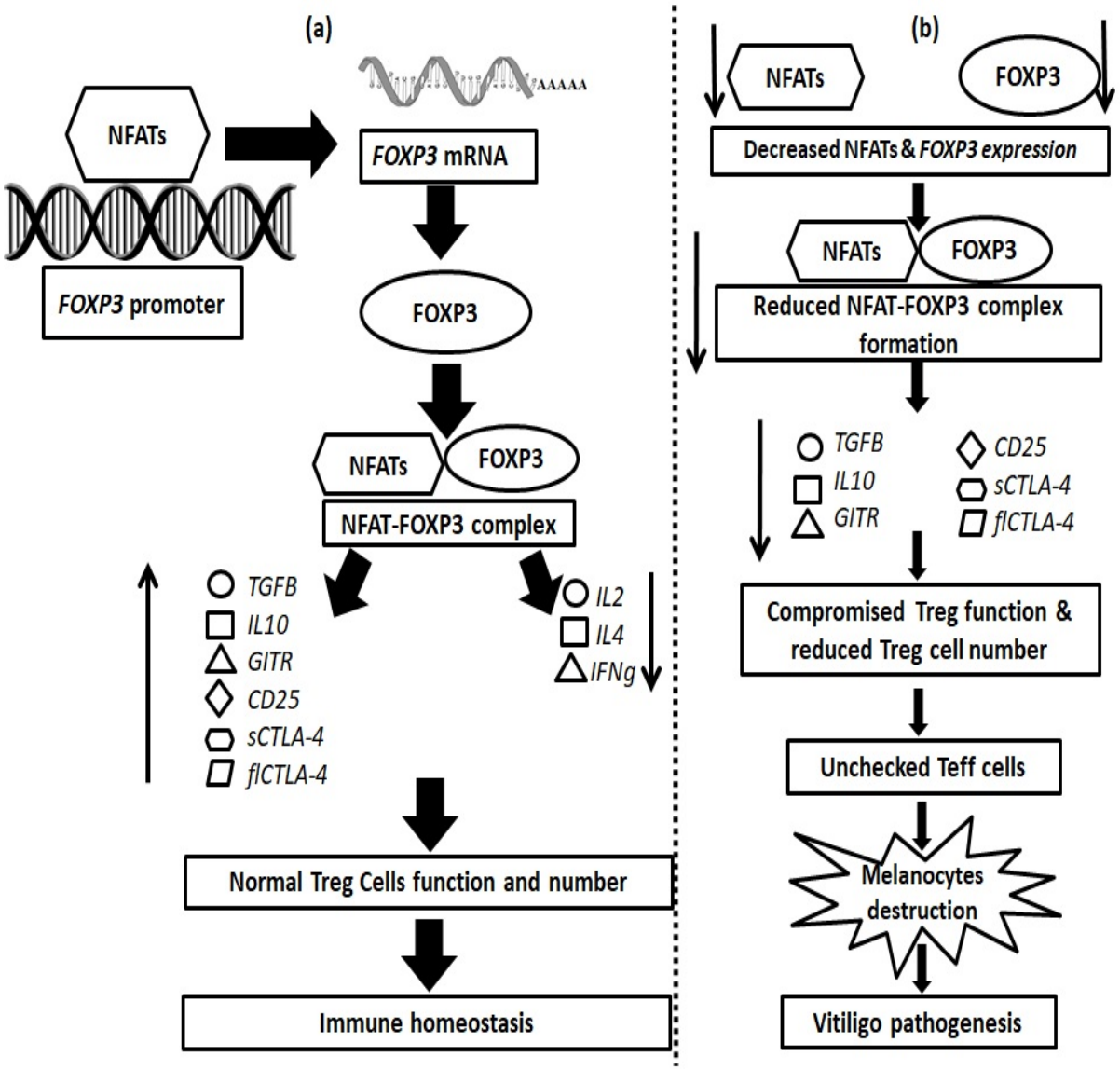


**Figure 8 Mechanism of regulation of Treg cells by NFAT and FOXP3 in vitiligo pathogenesis.**

(a) NFATs are responsible to govern the expression of transcription factor FOXP3 by binding to the CNS1 promoter region of *FOXP3*. These NFATs and FOXP3 form a complex which has a crucial role in regulating expression of downstream genes. It upregulates suppressive genes such as *CTLA4*, *CD25, IL10, and TGFB* and on the other hand is responsible to downregulate T effector cell-associated genes such as *IL2, IL4,* and *IFNG*. Thus, NFAT:FOXP3 complex maintains normal Treg cell function and numbers and hence the immune homeostasis. (b) Altered expression of *NFATs* and *FOXP3* leads to inhibition of NFAT:FOXP3 complex which is responsible for downregulation of the immune-suppressive genes such as *CTLA4, CD25, IL10,* and *TGFB* eventually leading to dysfunctional Treg cells.

These compromised Treg cells are unable to suppress melanocyte-specific T effector cells (CD8+T cells) and hence lead to CD8+ T-cell-mediated death of melanocytes in vitiligo patients.

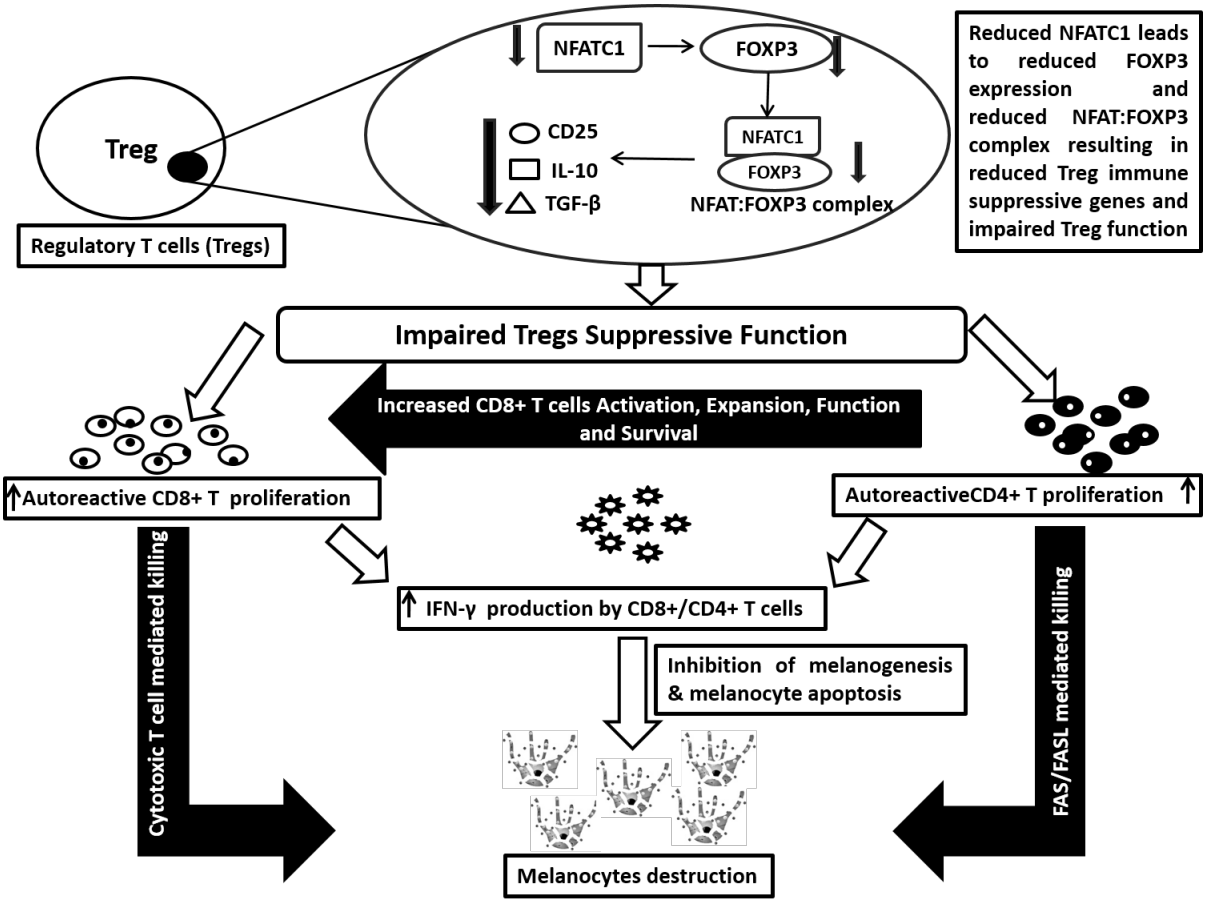


**Figure 9. Role of altered NFATC1 and FOXP3 expression in Treg cell suppressive function in pathogensis of generalized vitiligo.**

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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