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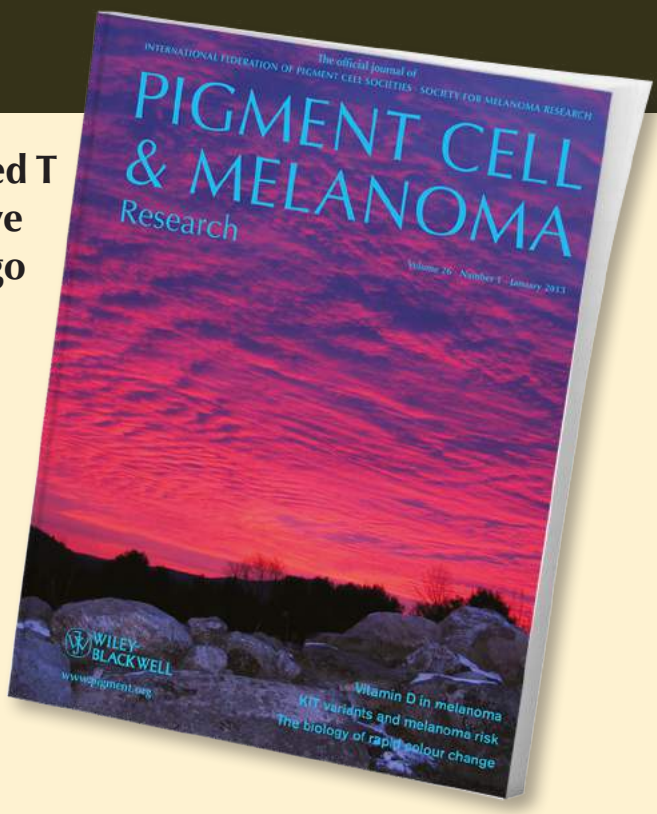

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DOI: 10.1111/pcmr.12862

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Received: 31 July 2019 | Revised: 15 December 2019 | Accepted: 6 January 2020

DOI: 10.1111/pcmr.12862

ORIGINAL ARTICLE

WILEY

Altered expression of nuclear factor of activated T cells, forkhead box P3, and immune-suppressive genes in regulatory T cells of generalized vitiligo patients

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Funding information
This work was supported by grant to MD {ECR/2017/000858} Science & Engineering Research Board, Department of Science & Technology (SERB-DST), New Delhi, India.

Abstract

The study was aimed to analyze expression of nuclear factor of activated T cells (*NFATs*), forkhead box P3 (*FOXP3*), and their associated genes (s*CTLA4*, *flCTLA4*, *IL10*, *TGFB*, *IL2*, *IL4*, *CD25)* in regulatory T cells (Tregs) of 48 generalized vitiligo (GV) patients and 45 unaffected controls. The transcripts of *NFATC1* to *NFATC4*, *FOXP3*, *IL10*, *flCTLA4* ($p < .0001$), *NFAT5* ($p = .0003$), *sCTLA4* ($p = .001$), and FOXP3 protein in Tregs and plasma IL-10 levels were reduced significantly ($p < .0001$) in GV Tregs compared to controls. The *FOXP3* promoter polymorphisms [rs3761548(C > A), rs3761547(A > G), and rs2232365(A > G)] revealed significantly decreased FOXP3 protein levels in patients' Tregs with susceptible AA, GG, and GG genotypes ($p < .0001$, $p = .028$, $p = .022$, respectively). The active vitiligo Tregs showed reduced levels of *NFATC3*, *NFATC4*, *NFAT5*, *FOXP3*, *TGFB*, *and flCTLA4* transcripts ($p = .0005$, $p = .0003$, $p = .0002$, $p = .020$, $p < .0001$, $p = .006$, respectively) and FOXP3 and TGF-β proteins ($p = .0394$ and $p = .0013$) compared to stable vitiligo. Early-onset patients (1–20 years) demonstrated decreased *IL-10*, *sCTLA-4*, *flCTLA-4*, *TGFB*, *and FOXP3* transcripts and FOXP3 protein as compared to late-onset patients (41–60 years) ($p = .001$, $p = .003$, $p = .009$, $p = .005$, $p = .038$, $p = .0226$, respectively). Overall, our results for the first time suggest a likely role of *NFATs* and *FOXP3* together with Treg immune-suppressive genes in GV pathogenesis and disease progression, warranting additional investigations.

KEYWORDS

active vitiligo, forkhead box P3, generalized vitiligo, induced tregs, mRNA expression, nuclear factor of activated T cells, promoter polymorphisms, regulatory T cells, stable vitiligo

1 | INTRODUCTION

Generalized vitiligo (GV) is a non-contagious, acquired skin disease which is characterized by symmetrical milky white patches resulting due to autoimmune destruction of melanocytes and thus loss of pigmentation from the involved areas (Nordlund, Ortonne, & Le Poole, 2006). The prevalence of vitiligo was found to be 0.5%–2% worldwide (Krüger & Schallreuter, 2012). The key role of autoimmunity in the pathogenesis of the disease has been suggested by the involvement of both humoral and cellular immunities due to the presence of both circulating melanocyte autoantibodies and autoreactive cytotoxic ($CD8^+$) T cells (Laddha et al., 2013), specifically in patients with active vitiligo (AV) compared to stable vitiligo (SV) (Dwivedi, Laddha, Arora, Marfatia, & Begum, 2013). Moreover, lesional skin of vitiligo patients showed abundance of both $CD4^+$ and $CD8^+$ T cells (Abdallah, Lotfi, Othman, &

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Galal, 2014). These activated $CD8^+$ T cells, if remained unchecked, may lead to melanocyte destruction resulting into white patches in vitiligo patients. Regulatory T cells (Tregs) plays a critical role in curbing such self-reactive $CD8^+$ T-cell response through their active suppression and expansion and thus maintains peripheral tolerance (Dwivedi et al., 2015). The role of Tregs in vitiligo has been evidenced as reduction in Tregs in murine melanoma model led to the development of vitiligo symptoms as side effects (Zhang, Côté, Vries, Usherwood, & Turk, 2007), and in H3TA2 mouse model, vitiligo progression was curbed by Treg cells adoptive transfer (Chatterjee et al., 2014). Previous studies demonstrated significant decrease in Tregs number and impaired Treg function in vitiligo patients which were unable to suppress the activated $CD8^+$ T cells (Dwivedi et al., 2015). Moreover, a significantly lower Treg cells have been reported in AV in comparison with SV patients (Abdallah et al., 2014; Dwivedi et al., 2013). However, the detailed studies exploring the regulatory molecules of Tregs in vitiligo are lacking.

One of the not so far studied regulatory molecules of Tregs in GV is nuclear factor of activated T cells (NFATs). The NFAT transcription factors (NFATC1, NFATC2, NFATC3, NFATC4, and NFAT5) are the key regulators of T-cell activation and anergy. In Tregs, NFATs form analogous co-operative complexes with FOXP3, which then mediate Treg cell function by repressing the expression of cytokines (IL-2 and IL-4) and upregulating the Treg cell markers (CTLA-4 and CD25) (Wu et al., 2006), thereby activating the Treg suppressor program. The role of NFAT1, NFAT2, and NFAT5 has been explored in animal models of autoimmune diseases, suggesting that their deficiency could impair the function of Tregs and lead to autoimmune diseases (Boland et al., 2015; Kwon et al., 2016; Shin et al., 2014; Vaeth et al., 2014). These studies indicate that NFATs could be important target molecules for exploring Treg-mediated pathogenesis of GV.

Treg cells are characterized by the constitutive expression of forkhead box P3 (FOXP3) and are considered indispensable regulatory molecule for the development and suppressive functions of Treg cells. Forkhead Box P3 activates several genes including *CTLA4*, *GITR*, *IL10*, and *TGFB*, which are crucial for suppressive activity of Treg cells (Corthay, 2009). Previous studies have shown decreased *FOXP3* mRNA and protein expression in skin and blood of vitiligo patients which could impair the Treg function (Table S1). The reduced expression of *FOXP3* may lead to decreased levels of immune-suppressive genes in Tregs such as *CTLA4*, *IL10*, and *TGFB*. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a T-cell downregulator molecule and plays a crucial role in Treg function. Indeed, both soluble (*sCTLA4*) and full-length (*flCTLA4*) *CTLA4* mRNAs were found to be decreased in vitiligo patients (Dwivedi, Laddha, Imran, Shah, & Begum, 2011). Additionally, Treg cells maintain their suppressive function by production of cytokines such as IL10 and TGF-β. Several studies suggested a role for decreased IL-10 and TGF-β in the pathogenesis of vitiligo (Khan, Gupta, & Sharma, 2012; Tembhre, Sharma, Sharma, Chattopadhyay, & Gupta, 2013). The deficiency in *CD25* expression was also reported in various autoimmune diseases such as diabetes mellitus, IPEX syndrome, systemic lupus erythematosus, and psoriatic arthritis (Wing & Sakaguchi, 2008).

Significance

The molecular mechanisms of deregulation of regulatory T cells (Tregs) have not been explored fully in generalized vitiligo (GV). The study is first of kinds to reveal the altered expression of NFATs and FOXP3 in Tregs which may further lead to decreased expression of downstream immunosuppressive genes, causing defective Treg functions and reduced numbers as observed earlier in GV patients. This knowledge for the first time adds the plausible role of NFATs in Treg-mediated GV pathogenesis and progression. The study suggests NFATs to be new target molecules to be explored further to develop effective Treg-based therapy for GV.

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 Treg-specific defects in GV patients. Therefore, the present study for the first time aimed to assess the expression of *NFATs* (1–5), *FOXP3* transcripts, and FOXP3 protein in Tregs isolated from GV patients and unaffected controls; to assess transcripts of Treg immune-suppressive and associated genes (*sCTLA-4*, *flCTLA4*, *IL10*, *TGFB*, *CD25*, *IL2, and IL4)*; to establish the genotype–phenotype correlation of *FOXP3* promoter polymorphisms [rs3761547 (A > G promoter), rs3761548 (C > A promoter), and rs2232365 (A > G promoter)]; and to assess the effect of *NFATs*, *FOXP3*, and their immune-suppressive genes on the age of onset of GV.

2 | MATERIALS AND METHODS

2.1 | Patients and controls

A total of 48 generalized vitiligo (GV) patients and 45 healthy controls were recruited from Gujarat population. The demographic details of patients and controls are shown in Table 1. Control subjects were healthy with no symptoms of vitiligo. The disease was diagnosed by dermatologist at General hospital, Vyara, through observation of depigmented skin lesions under Wood's lamp. Prior to recruitment for the study, these patients did not undergo any therapy for at least 4 weeks. The GV patients with other autoimmune diseases and pregnant or lactating women were excluded from the study. Generalized vitiligo is the most common type of vitiligo which is characterized by multiple scattered lesions symmetrically distributed over the entire body. The GV patients were divided into two groups based on whether the existing lesions were spreading and/or new lesions had appeared within

the previous 6 months: An affirmative answer to one or both of those questions led to inclusion of the patient in the active vitiligo (AV) group, whereas patients with no increase in lesion size or number within the previous 6 months were included in the stable vitiligo (SV) group (Uda, Takei, & Mishima, 1984). All participants gave their written consent for the study. The ethical clearance was obtained for the work plan from Institutional-Human Research Ethical Committee, Maliba Pharmacy College, Uka Tarsadia University, India. The study was carried out in accordance with ethical standards of 1964 Helsinki Declaration and its later amendments.

2.2 | Isolation of $CD4^+CD25^+$ Tregs

Treg cells were isolated from GV patients and controls using MACSxpress® Whole Blood Treg Isolation Kit human (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions and protocol followed by previous study (Richter et al., 2016). The kit had an isolation efficiency of 3.5×10^4 cells per ml of whole blood with a purity of 90% for $CD4^+CD25^+$ Treg cells. Flow cytometry was carried out to confirm the purity of isolated Treg cells. Three milliliter whole blood was subjected to a two-step protocol. In the first step, all the non-$CD4^+$ T cells were immunomagnetically depleted with MACSxpress Beads. Subsequently in the second step, these $CD4^+$ cells were applied on the LS columns under magnetic field to enrich $CD25^+$ cells. The eluted fraction represented $CD4^+$ $CD25^+$ Tregs, which were immediately processed for total RNA isolation.

2.3 | Total RNA extraction and cDNA synthesis

The total RNA was isolated from purified Treg cells using TRIzol reagent (Invitrogen). The RNA integrity, yield, and purity were determined. One microgram of total RNA was used for cDNA synthesis by RevertAid First Strand cDNA Synthesis Kit (Fermentas) in Eppendorf Mastercycler Gradient Thermocycler (Eppendorf™).

TABLE 1 Demographic characteristics of generalized vitiligo (GV) patients and unaffected controls

	GV patients	Controls
	(*n* = 48)	(*n* = 45)
Average age (mean age ± *SD*)	36.5 ± 17.80 years	22 ± 6.07 years
Sex		
Male	22 (45.83%)	21 (46.67%)
Female	26 (54.17%)	24 (53.33%)
Age of onset (mean age ± *SD*)	23.46 ± 16.97 years	NA
Duration of disease (mean ± *SD*)	13.10 ± 12.51 years	NA
Active vitiligo	27 (56.25%)	NA
Stable vitiligo	21 (43.75%)	NA

2.4 | Relative gene expression by qPCR

The transcript expression of *NFATs (1–5)*, *FOXP3*, *CD25*, *CTLA4*, *IL10*, *TGFB*, *IL2*, *IL4*, and *GAPDH* (reference gene) was determined by qPCR using specific primers (Integrated DNA technology) as shown in Table S2. The gene expression levels were measured using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific). All samples were analyzed in duplicate. The qPCR was carried out in duplicates in 20 µl volume using Rotor-Gene Q (Qiagen), and cycle threshold (C_T) was determined for each gene. PCR conditions are shown in Table S2. For checking the specificity of the products, melt curve analysis was also performed. The difference between the cycle threshold of target gene and reference gene (*GAPDH*) was designated as ΔC_T value, whereas the difference between two ΔC_T values (i.e., ΔC_T controls and ΔC_T patients) was termed as $\Delta\Delta C_T$. The fold expression of gene was determined by $2^{-\Delta\Delta CT}$.

2.5 | Expression of FOXP3 protein in Tregs and estimation of plasma IL-10 and TGF-β proteins

The FOXP3 protein in isolated Tregs was determined by intracellular staining of FOXP3. All samples were analyzed in duplicate. The FoxP3-PE cells were fixed and permeabilized using Human FoxP3 Buffer Set (BD Biosciences). Briefly, after washing, the cell pellet was resuspended in 0.5 ml of freshly prepared 1X fixation/permeabilization solution and incubated for 30 min. at room temperature in the dark. After staining, cells were washed twice in FACS staining buffer [phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide], fixed in PBS containing 1% paraformaldehyde, and acquired the same day on a FACS AriaIII system (BD Biosciences) followed by analysis using FlowJo software version 7.6.5 (Tree Star). The amount of FOXP3 present in the Tregs was measured as mean fluorescence intensity (MFI) (Figure S1a). The plasma levels of IL-10 and TGF-β in GV patients and controls were measured using the Enzyme-Linked Immunosorbent Assay Kits for Human IL-10 and TGF-β (USCN Life Science Inc.) as per the manufacturer's protocol (Figure S1b,c). All samples were analyzed in duplicate.

2.6 | Genotyping of *FOXP3* promoter region polymorphisms

Three hundred microliter venous blood was used for genomic DNA isolation using HiPurA™ Blood Genomic DNA Miniprep Purification Kit (Himedia). The *FOXP3* promoter polymorphisms (rs3761548, rs3761547, and rs2232365) were genotyped using PCR-RFLP

technique (Figure S2). The primer sequences and restriction enzymes used are mentioned in Table S3.

2.7 | Statistical analyses

The comparisons of mean ΔC_T values, fold change, and MFI values among patients (GV, SV, and AV) and controls for various analyses [relative gene expression analysis, proteins (FOXP3, IL-10, and TGF-β) analysis, gender-based analysis, age of onset analysis, and genotype–phenotype correlations] were carried out by non-parametric Mann–Whitney *U* test using Prism 8 software (GraphPad software Inc; 2003). The *p*-values < .05 were interpreted as statistically significant.

3 | RESULTS

3.1 | *NFAT* transcript levels in Regulatory T cells (Tregs)

The *NFAT* transcript levels were analyzed in Tregs isolated from GV patients and controls. The Tregs of GV patients showed significantly reduced expression of all five *NFATs*, that is, *NFAT (C1–C4)* ($p < .0001$) and *NFAT5* ($p = .0003$) as compared to controls (Figure 1). The $2^{-\Delta\Delta CT}$ analysis revealed 0.19-, 0.14-, 0.21-, 0.48-, and 0.31-fold changes in the expression of *NFAT (1–5)* transcripts in GV Tregs as compared to controls ($p < .0001$; Table 2; Figure S3a–e). Further, the analysis based on disease progression suggested significantly decreased transcript levels of *NFATC3*, *NFATC4*, and *NFAT5* in active vitiligo (AV) patients' Tregs as compared to stable vitiligo (SV) Tregs ($p = .0005$, $p = .0003$, and $p = .0002$, respectively) (Figure 1c–e). However, no significant difference was observed in *NFATC1* and *NFATC2* transcript levels between AV and SV Tregs ($p = .487$ and $p = .101$, respectively) (Figure 1a,b). The $2^{-\Delta\Delta CT}$ analysis revealed 0.72-, 0.28-, 0.25-, 0.44-, and 0.12-fold changes in the expression of *NFATC1* ($p = .8726$), *NFATC2* ($p = .1011$), *NFATC3* ($p = .0005$), *NFATC4* ($p = .0003$), and *NFAT5* ($p = .0038$) transcripts, respectively, in AV Tregs as compared to SV (Table 2; Figure S3g–i). In addition, SV Tregs showed significantly decreased levels of *NFATC1*, *NFATC2* ($p < .0001$), and *NFATC3* ($p = .0005$) as compared to controls (Figure 1a–c). There was no significant difference found in *NFATC4* and *NFATC5* transcript levels between SV and control Tregs ($p = .164$ and $p = .944$, respectively) (Figure 1d,e). There were 0.23-, 0.28-, 0.45-, 0.76-, and 1.03-fold changes in the expression of *NFATC1*, *NFATC2*, *NFATC3* ($p < .0001$), *NFATC4* ($p = .246$), and *NFAT5* ($p = .449$) transcripts, respectively, in SV patients' Tregs as compared to controls (Table 2; Figure S3a–e). The AV patients' Tregs showed significantly decreased levels of all five *NFATs (1–5)* when compared to controls ($p < .0001$) (Figure 1). There were 0.16-, 0.082-, 0.11-, 0.33-, and 0.13-fold changes in the expression of *NFAT (1–5)* transcripts in AV Tregs as compared to controls ($p < .0001$; Table 2; Figure S3a–e). Nevertheless, the gender-based analysis did not show significant difference in *NFAT* transcript levels between male and female GV patients' Tregs (Figure S4a–e). Therefore, the reduced *NFAT* transcripts in patients' Treg suggest for their important role in GV pathogenesis irrespective of the gender and decreased transcripts of *NFATC3*, *NFATC4*, and *NFAT5* suggest for their likely role in the disease progression.

3.2 | Expression of *FOXP3* transcript and protein in Tregs

Since *NFAT*s control the expression of *FOXP3* in Treg cells, we further analyzed the expression of *FOXP3* in Treg cells isolated from GV patients and controls. The expression of *FOXP3* transcript was significantly reduced ($p < .0001$) in GV, SV, and AV patients' Tregs compared to controls (Figure 2a). There were 0.40-, 0.50-, and 0.30-fold changes in *FOXP3* transcript expression, respectively, in GV, SV, and AV patients' Tregs as compared to controls ($p < .0001$; Table 2; Figure S3f). Moreover, the FOXP3 protein expression was also found to be reduced in GV, SV, and AV patients' Tregs ($p < .0001$, $p = .0383$, and $p < .0001$, respectively) compared to controls (Figure 2b). Further, the analysis based on disease activity suggested a significant reduction in *FOXP3* transcript ($p = .020$) as well as FOXP3 protein ($p = .0394$) in Tregs of AV as compared to SV (Figure 2a,b). There was 0.65-fold change in *FOXP3* transcript expression in AV Tregs as compared to SV ($p = .0202$; Table 2; Figure S3i). However, there was no significant difference found in *FOXP3* transcript as well as FOXP3 protein levels between Tregs of male and female GV patients (Figure S4f,g). Hence, reduction in both transcript and protein expression of *FOXP3* indicates that FOXP3 is one of the crucial factors in deregulating the Tregs in GV patients and its altered levels may also aid in the progression of GV.

3.3 | Genotype–phenotype correlation of *FOXP3* promoter polymorphisms

Three well-documented *FOXP3* promoter polymorphisms [rs3761547 (A > G), rs3761548 (C > A), and rs2232365 (A > G)] were also genotyped in GV patients and controls by PCR-RFLP method (Figure S2) to correlate them with *FOXP3* expression in Tregs. Interestingly, the genotype–phenotype analysis for the rs3761548 (C > A) polymorphism showed a significant decrease in *FOXP3* transcript ($p < .0001$) (Figure S5a) and protein expression ($p < .0001$) (Figure 2c) in patients with susceptible AA genotype when compared with controls. The significant decrease in FOXP3 transcript ($p < .0001$) and protein ($p = .032$) was also observed in patients with heterozygous CA genotypes when compared with controls (Figure S5a and Figure 2c). The rs3761547 (A > G) polymorphism showed a significantly decreased *FOXP3* transcript ($p = .019$) and protein expression ($p = .028$) in patients with susceptible GG genotype when compared with controls (Figure S5b and Figure 2d). The rs2232365 (A > G) SNP also showed a significant decreased *FOXP3* transcript ($p < .0001$) and protein expression ($p = .022$) in patients with susceptible GG genotype

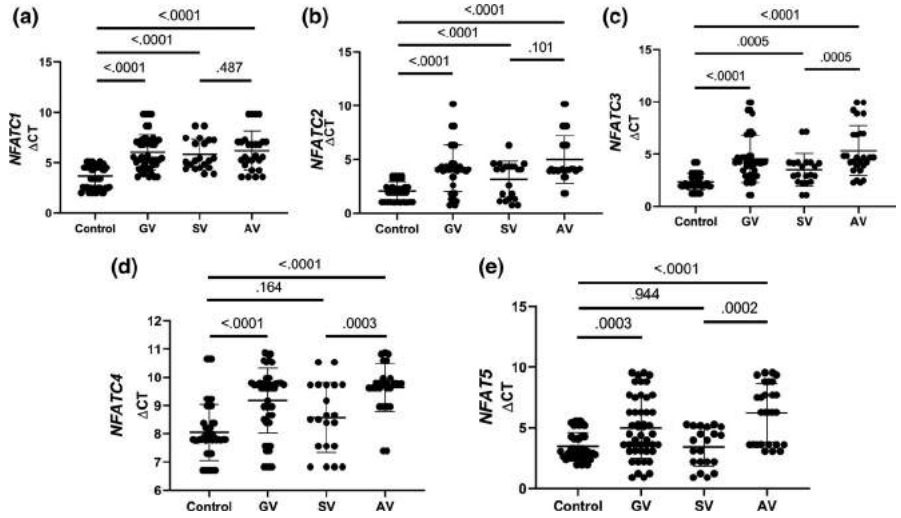

FIGURE 1 *NFAT* transcript levels in Tregs of generalized vitiligo patients and controls. The transcript levels of *NFATC1*, *NFATC2*, *NFATC3*, *NFATC4*, and *NFAT5* were analyzed in 45 controls and 48 GV patients [21 stable vitiligo (SV) and 27 active vitiligo (AV)] by non-parametric Mann–Whitney *U* test. (a) *NFATC1* transcript levels in GV, SV, and AV patients' Tregs versus controls' Tregs (mean ± *SEM*: 6.02 ± 0.24, 5.76 ± 0.31, and 6.22 ± 0.36, respectively, vs. 3.66 ± 0.15; $p < .0001$). *NFATC1* transcript levels in AV patients' Tregs versus SV patients' Tregs (mean ± *SEM*: 6.22 ± 0.36 vs. 5.76 ± 0.31; $p = .487$). (b) *NFATC2* transcript levels in GV, SV, and AV patients' Tregs versus controls' Tregs (mean ± *SEM*: 4.15 ± 0.30, 3.13 ± 0.34, and 4.95 ± 0.41, respectively, vs. 1.35 ± 0.19; $p < .0001$). *NFATC2* transcript levels in AV patients' Tregs versus SV patients' Tregs (mean ± *SEM*: 4.95 ± 0.41 vs. 3.13 ± 0.34; $p = .101$). (c) *NFATC3* transcript levels in GV, SV, and AV patients' Tregs versus controls' Tregs (mean ± *SEM*: 4.61 ± 0.32, 3.52 ± 0.32, 5.48 ± 0.46, respectively, vs. 2.37 ± 0.11; $p < .0001$, $p = .0005$, and $p < .0001$ respectively). *NFATC3* transcript levels in AV patients' Tregs versus SV patient Tregs (mean ± *SEM*: 5.48 ± 0.46 vs. 3.52 ± 0.32; $p = .0005$). (d) *NFATC4* transcript levels in GV and AV patients Tregs' versus controls' Tregs (mean ± *SEM*: 9.17 ± 0.16 and 9.68 ± 0.16, respectively, vs. 8.12 ± 0.14; $p < .0001$). *NFATC4* transcript levels in SV patients' Tregs versus controls' Tregs (mean ± *SEM*: 8.52 ± 0.25 vs. 8.12 ± 0.14; $p = .164$). *NFATC4* transcript levels in AV patients' Tregs versus SV patient Tregs (mean ± *SEM*: 9.68 ± 0.16 vs. 8.52 ± 0.25 vs.; $p = .0003$). (e) *NFAT5* transcript levels in GV and AV patients' Tregs versus controls' Tregs (mean ± *SEM*: 5.07 ± 0.35 and 6.35 ± 0.46, respectively, vs. 3.43 ± 0.16; $p = .0003$ and $p < .0001$, respectively). *NFAT5* transcript levels in SV patients' Tregs versus controls' Tregs (mean ± *SEM*: 3.45 ± 0.33 vs. 3.43 ± 0.16; $p = .944$). *NFAT5* transcript levels in AV patient Tregs versus SV patient Tregs (mean ± *SEM*: 6.35 ± 0.46 vs. 3.45 ± 0.33; $p = .0002$). [*SEM*: standard error mean]

when compared with controls (Figure S5c and Figure 2e). These results suggest for the genetic susceptibility of GV patients in terms of *FOXP3* SNPs by correlating decreased transcript expression of *FOXP3* with susceptible genotypes. These susceptible genotypes may be responsible for decreased FOXP3 expression in GV patients.

3.4 | Expression of Treg suppressive genes *(IL10, TGFB, sCTLA4, and fICTLA4)*

The NFAT:FOXP3 complex in Tregs is crucial for the expression of downstream suppressive genes; hence, we further analyzed the expression of *IL10*, *TGFB*, *sCTLA4*, and *fICTLA4* in Treg cells isolated from GV patients and controls. The transcript levels of *IL10*, *fICTLA4*, and *sCTLA4* genes decreased significantly in GV patients' Tregs as compared to controls ($p < .0001$, $p < .0001$, and $p = .001$, respectively) (Figure 3a,e,f). Interestingly, the plasma IL-10 protein level was also decreased ($p < .0001$) in GV patients' Tregs as compared to controls (Figure 3b). However, *TGFB* transcript and protein levels did not differ ($p = .753$ and $p = .2698$) between Tregs of GV patient and control groups (Figure 3c,d). There were 0.49-, 0.32-, 0.42-, and 0.19-fold changes in the expression of *IL10* ($p < .0001$), *TGFB* ($p = .0654$), *sCTLA4* ($p < .0001$), and *fICTLA4* ($p < .0001$) transcripts, respectively, in GV patients' Tregs as compared to controls (Table 2; Figure S6a,b,d,e). The analysis based on disease activity showed that *TGFB* and *fICTLA4* transcripts and TGF-β protein levels decreased significantly ($p < .0001$, $p = .006$, and $p = .0013$, respectively) in AV Tregs when compared to SV (Figure 3c,e,d). However, *IL10 and sCTLA4* transcripts in Tregs and plasma IL-10 protein levels did not differ ($p = .487$, $p = .079$, and $p = .526$, respectively) between AV and SV patients (Figure 3a,f,b). The AV Tregs showed 0.67-, 0.025-, 0.39-, and 0.18-fold changes in the expression of *IL10* ($p = .4818$), *TGFB* ($p = .011$), *sCTLA4* ($p = .014$), and *fICTLA4* ($p = .0063$) transcripts, respectively, as compared to SV (Table 2; Figure S6c,f). Moreover, the SV patients' Tregs showed decreased levels of *IL10 and fICTLA4* transcripts and plasma IL-10 protein ($p = .002$, $p = .007$, and $p < .0001$, respectively) when compared to controls (Figure 3a,e,b). There was no significant difference found in *TGFB* and *sCTLA4* transcripts and TGF-β protein levels between SV and control Tregs ($p = .999$, $p = .143$, and $p = .1192$, respectively) (Figure 3c,f,d). The SV Tregs showed 0.63-, 0.95-, 0.73-, and 0.52-fold changes in the expression of *IL10* ($p < .0001$), *TGFB* ($p = .999$), *sCTLA4* ($p = .1983$), and *fICTLA4* ($p < .0001$) transcripts, respectively, as compared to controls (Table 2; Figure S6a,b,d,e). The AV patients showed significantly decreased levels of *IL10*, *TGFB*, *fICTLA4* ($p < .0001$), *and sCTLA4* ($p = .0002$) transcripts in Tregs (Figure 3a,c,e,f) and plasma IL-10 and TGF-β proteins ($p < .0001$) (Figure 3b,d) as compared to controls. The

TABLE 2 Expression of *NFATs*, *FOXP3*, and immune-suppressive genes in Regulatory T cells (Tregs) and Plasma of generalized vitiligo patients and unaffected controls

Gene	Controls versus GV	Controls versus SV	Controls versus AV	SV versus AV
mRNA expression in Tregs as fold change (*p* value)				
NFATC1	0.19 ($p < .0001$)↓	0.23 ($p < .0001$)↓	0.16 ($p < .0001$)↓	0.72 ($p = .8726$)•
NFATC2	0.14 ($p < .0001$)↓	0.28 ($p < .0001$)↓	0.082 ($p < .0001$)↓	0.28 ($p = .1011$)•
NFATC3	0.21 ($p < .0001$)↓	0.45 ($p < .0001$)↓	0.11 ($p < .0001$)↓	0.25 ($p = .0005$)↓
NFATC4	0.48 ($p < .0001$)↓	0.76 ($p = .246$)•	0.33 ($p < .0001$)↓	0.44 ($p = .0003$)↓
NFAT5	0.31 ($p < .0001$)↓	1.03 ($p = .449$)•	0.13 ($p < .0001$)↓	0.12 ($p = .0038$)↓
FOXP3	0.40 ($p < .0001$)↓	0.50 ($p < .0001$)↓	0.30 ($p < .0001$)↓	0.65 ($p = .0202$)↓
IL10	0.49 ($p < .0001$)↓	0.63 ($p < .0001$)↓	0.43 ($p < .0001$)↓	0.67 ($p = .4818$)•
TGFB	0.32 ($p = .0654$)•	0.95 ($p = .999$)•	0.023 ($p = .002$)↓	0.025 ($p = .011$)↓
fICTLA4	0.19 ($p < .0001$)↓	0.52 ($p < .0001$)↓	0.09 ($p < .0001$)↓	0.18 ($p = .0063$)↓
sCTLA4	0.42 ($p < .0001$)↓	0.73 ($p = .1983$)•	0.28 ($p < .0001$)↓	0.39 ($p = .014$)↓
Protein expression in Tregs as mean ± *SEM* MFI (*p* value)				
FOXP3	967.6 ± 35.44 versus 624.1 ± 67.61 ($p < .0001$)↓	967.6 ± 35.44 versus 765.3 ± 113.7 ($p = .0383$)↓	967.6 ± 35.44 versus 488.6 ± 66.86 ($p < .0001$)↓	765.3 ± 113.7 versus 488.6 ± 66.86 ($p = .0394$)↓
Protein expression in plasma as mean ± *SEM* pg/ml (*p* value)				
IL-10	58.54 ± 7.04 versus 17.69 ± 2.47 ($p < .0001$)↓	58.54 ± 7.04 versus 21.69 ± 4.73 ($p < .0001$)↓	58.54 ± 7.04 versus 14.58 ± 2.33 ($p < .0001$)↓	21.69 ± 4.73 versus 14.58 ± 2.33 versus ($p = .526$)•
TGF-β	39.90 ± 1.07 versus 39.13 ± 1.53 ($p = .2698$)•	39.90 ± 1.07 versus 43.66 ± 2.49 ($p = .1192$)•	39.90 ± 1.07 versus 33.43 ± 1.21 ($p < .0001$)↓	43.66 ± 2.49 versus 33.43 ± 1.21 ($p = .0013$)↓

Note: pg/ml: picogram/milliliter; ↓: significantly decreased levels; •: no significant difference.

Abbreviations: AV, active vitiligo; GV, generalized vitiligo; MFI, mean fluorescence intensity; SV, stable vitiligo.

AV Tregs showed 0.43-, 0.023-, 0.28-, and 0.09-fold changes in the expression of *IL10* ($p < .0001$), *TGFB* ($p = .002$), *sCTLA4* ($p < .0001$), and *fICTLA4* ($p < .0001$) transcripts, respectively, as compared to controls (Table 2; Figure S6a,b,d,e). However, the gender-based analysis did not show significant difference in *IL10*, *TGFB*, *sCTLA4*, *and fICTLA4* transcripts in Tregs and plasma IL-10 protein between male and female GV patients (Figure S7a,b,c,d,h). These results suggest that decreased *IL10*, *fICTLA4*, and *sCTLA4* expression in GV Tregs may be crucial for decreased suppressive capacity of Tregs and reduced *TGFB* and *fICTLA4* levels in AV patients indicate their likely role in progression of GV.

3.5 | Expression of Treg-associated genes' transcripts *(CD25*, *IL2*, and *IL4)*

The analyses of Treg-associated genes such as *CD25*, *IL2*, and *IL4* were also performed in GV patients and controls. There was no significant difference observed for transcript levels of *CD25*, *IL4*, and *IL2* in GV ($p = .111$, $p = .055$, and $p = .71$, respectively), SV ($p = .45$, $p = .122$, and $p = .226$, respectively), and AV ($p = .18$, $p = .115$, and $p = .521$, respectively) patients' Tregs as compared to controls (Figure S8a–c). However, the GV patients' Tregs showed 0.45-, 0.39-, and 0.93-fold changes ($p = .255$, $p = .065$, and $p = .151$, respectively), whereas SV Tregs showed 0.48-, 0.59-, and 0.82-fold changes ($p = .230$, $p = .140$, and $p = .95$, respectively) and AV Tregs showed 0.43-, 0.28-, and 1.03-fold changes ($p = .550$, $p = .151$, and $p = .98$, respectively) in the expression of *CD25*, *IL4*, and *IL2* transcripts, respectively, as compared to controls (Figure S9a–c). There was no significant difference found in transcript levels of *CD25*, *IL4*, and *IL2* between AV and SV Tregs ($p = .92$, $p = .988$, and $p = .525$) (Figure S8a–c). However, the AV patients' Tregs showed 0.90-, 0.48-, and 1.26-fold changes in the expression of *CD25* ($p = .555$), *IL4* ($p = .980$), and *IL2* ($p = .20$) transcripts compared to SV (Figure S9d–f). The gender-based analysis did not show significant difference in the expression of *CD25*, *IL2*, and *IL4* between Tregs of male and female GV patients (Figure S7e–g). The similar expression of *CD25*, *IL2*, and *IL4* genes in GV patients and control Tregs suggests that there was normal constitutive expression of *CD25*, *IL2*, and *IL4* in Tregs and these genes might not be involved in GV pathogenesis.

3.6 | Effect of *NFATs*, *FOXP3*, and Treg suppressive genes' expression on age of onset of generalized vitiligo

Further, the expressions of *NFATs*, *FOXP3*, and other Treg-associated genes were determined in Tregs of different age of onset groups of GV patients. The Tregs from early age of onset group (1–20 years) of patients exhibited decreased expression of *FOXP3*, *IL10*, *sCTLA4*, *fICTLA4*, and *TGFB* transcripts as compared to late age of onset group

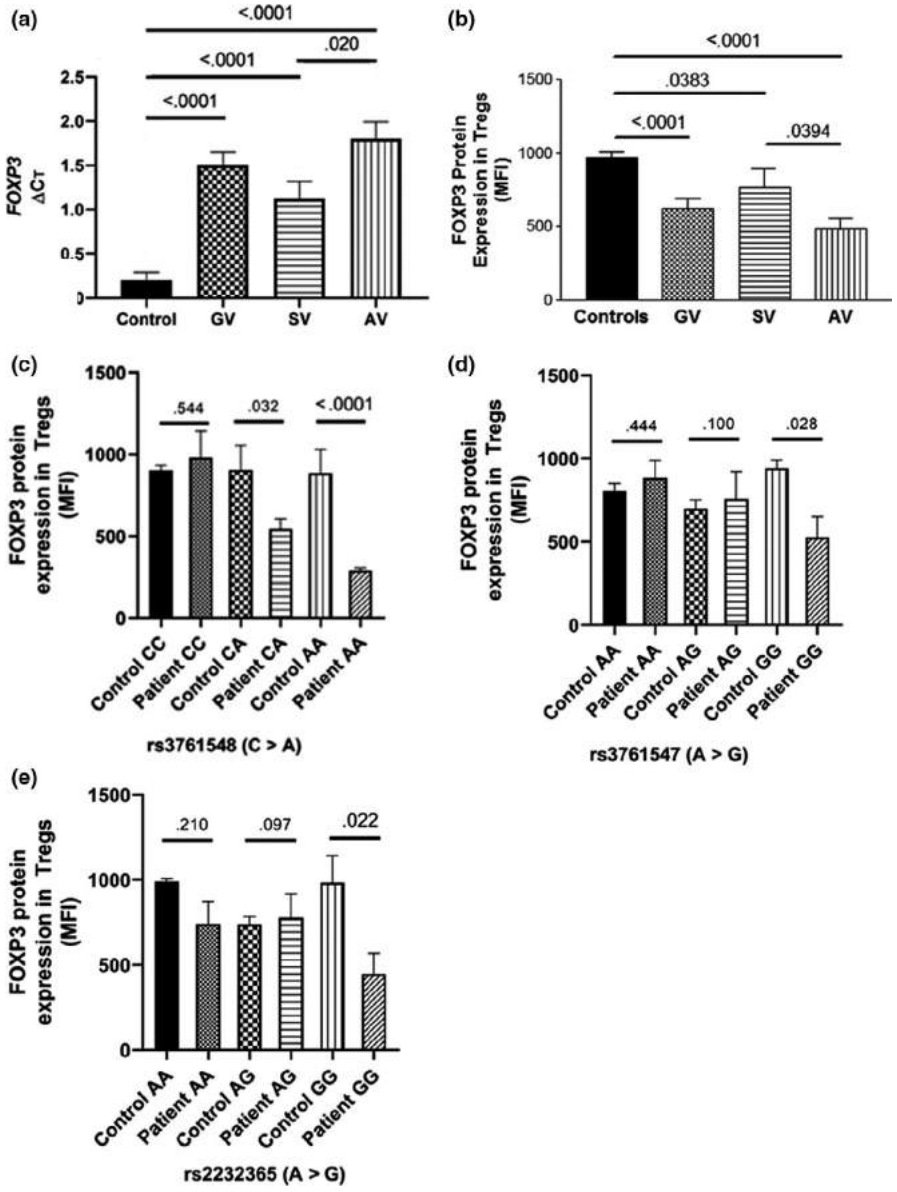

FIGURE 2 *FOXP3* transcript and protein levels in Tregs of generalized vitiligo patients and controls and genotype–phenotype correlation of *FOXP3* polymorphisms. The expression of *FOXP3* transcript and protein levels in 45 controls and 48 GV patients [21 stable Vitiligo (SV) and 27 active vitiligo (AV)] and genotype–phenotype correlation for *FOXP3* rs3761548 (C > A), rs3761547 (A > G), and rs2232365 (A > G) SNPs were analyzed by non-parametric Mann–Whitney *U* test. (a) *FOXP3* transcripts levels in GV, SV, and AV patient Tregs versus control Tregs (mean ± *SEM*: 1.50 ± 0.14, 1.12 ± 0.19, and 1.80 ± 0.18, respectively, vs. 0.19 ± 0.09; $p < .0001$). *FOXP3* transcript levels in AV patient Tregs versus SV patient Tregs (mean ± *SEM*: 1.12 ± 0.19 vs. 1.80 ± 0.18; $p = .020$) (*SEM*, standard error mean). (b) FOXP3 protein levels in GV, SV, and AV patient Tregs versus control Tregs (mean ± *SEM*: 624.1 ± 67.61, 765.3 ± 113.7, and 488.6 ± 66.86, respectively, vs. 967.6 ± 35.44; $p < .0001$; $p = .0383$; $p < .0001$, respectively). FOXP3 protein levels in AV patient Tregs versus SV patient Tregs (mean ± *SEM*: 488.6 ± 66.86 vs. 765.3 ± 113.7; $p = .0394$) (MFI, mean fluorescence intensity). (c) FOXP3 protein levels in patients with AA and CA genotypes versus controls with AA and CA genotypes (mean ± *SEM*: 289.40 ± 17.06 ($n = 13$) versus 885.00 ± 144.70 ($n = 6$); $p < .0001$ and 544.80 ± 62.39 ($n = 24$) versus 906.50 ± 147.90 ($n = 21$); $p = .032$, respectively). FOXP3 protein levels in patients with CC genotype versus controls with CC genotype (mean ± *SEM*: 980.20 ± 161.90 ($n = 11$) versus 902.30 ± 31.39 ($n = 18$), respectively; $p = .544$). (d) FOXP3 protein levels in patients with GG genotype versus controls with GG genotype (mean ± *SEM*: 526.70 ± 125.30 ($n = 9$) versus 943.40 ± 45.46 ($n = 7$); $p = .028$). FOXP3 protein levels in patients with AA and AG genotypes versus controls with AA and AG genotypes (mean ± *SEM*: 883.80 ± 103.40 ($n = 26$) versus 802.70 ± 48.58 ($n = 25$); $p = .444$ and 759.10 ± 162.20 ($n = 13$) versus 697.90 ± 53.97 ($n = 13$); $p = .100$, respectively). (e) FOXP3 protein levels in patients with GG genotype versus controls with GG genotype (mean ± *SEM*: 447.70 ± 121.30 ($n = 9$) versus 983.50 ± 158.90 ($n = 6$); $p = .022$). FOXP3 protein levels in patients with AA and AG genotype versus controls with AA and AG genotype (mean ± *SEM*: 740.90 ± 130.80 ($n = 21$) versus 988.90 ± 18.78 ($n = 24$); $p = .210$ and 778.00 ± 141.10 ($n = 18$) versus 738.70 ± 45.92 ($n = 15$); $p = .097$, respectively)

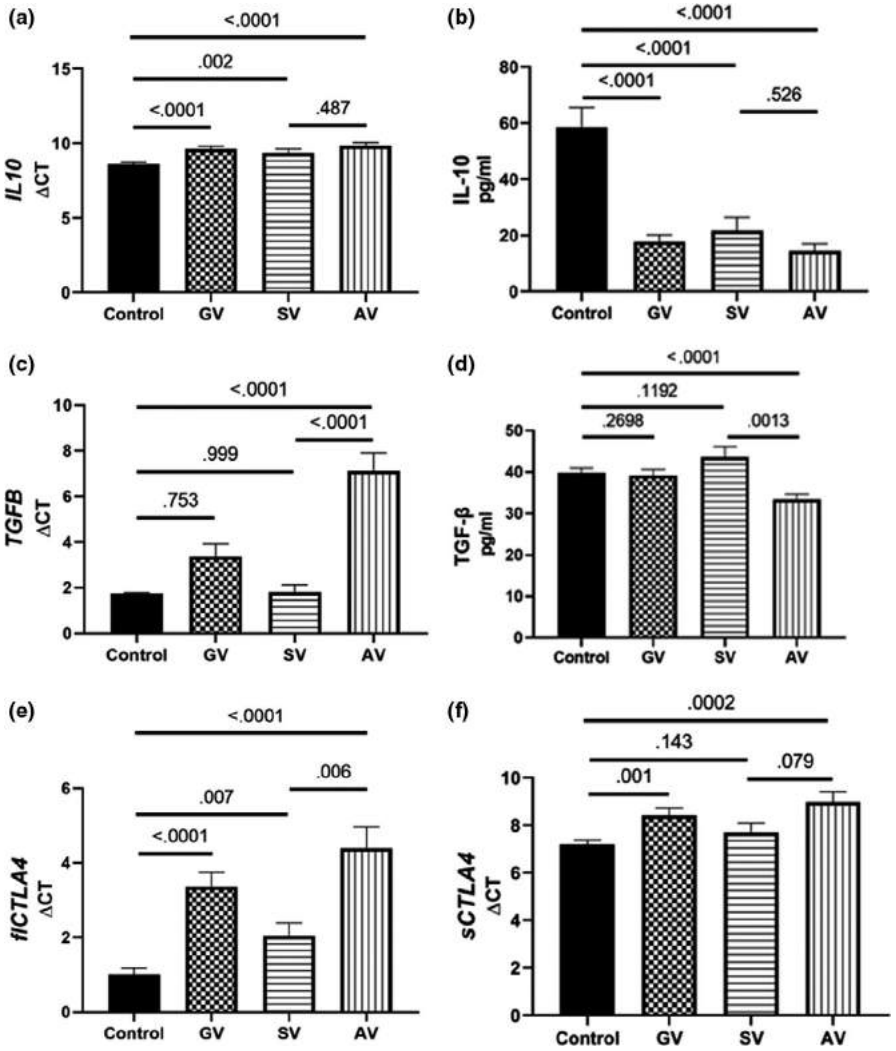

FIGURE 3 Expression of *IL10*, *TGFB*, *flCTLA4*, *and sCTLA4* transcripts in Tregs and IL-10 and TGF-β protein levels in generalized vitiligo patients and controls. The expression of *IL10*, *TGFB*, *flCTLA4*, *and sCTLA4* transcripts in Tregs and plasma IL-10 and TGF-β proteins in 45 controls and 48 GV patients [21 stable vitiligo (SV) and 27 active vitiligo (AV)] was analyzed by non-parametric Mann–Whitney *U* test. (a) *IL10* transcript levels in GV, SV, and AV patients' Tregs versus controls' Tregs (mean ± *SEM*: 9.60 ± 0.18, 9.34 ± 0.28, and 9.81 ± 0.23, respectively, vs. 8.60 ± 0.13; $p < .0001$, $p = .002$, and $p < .0001$, respectively). *IL10* transcript levels in AV patients' Tregs versus SV patients' Tregs (mean ± *SEM*: 9.81 ± 0.23 vs. 9.34 ± 0.28; $p = .487$). (b) IL-10 protein levels in GV, SV, and AV patients versus controls (mean ± *SEM*: 17.69 ± 2.47, 21.69 ± 4.73, and 14.58 ± 2.33, respectively, vs. 58.54 ± 7.04; $p < .0001$). IL-10 protein levels in AV versus SV (mean ± *SEM*: 14.58 ± 2.33 vs. 21.69 ± 4.73; $p = .526$). (c) *TGFB* transcript levels in AV patients' Tregs versus controls' Tregs and SV Tregs (mean ± *SEM*: 7.12 ± 0.77 vs. 1.73 ± 0.03 and 1.80 ± 0.32, respectively; $p < .0001$). *TGFB* transcript levels in GV and SV patients' Tregs versus controls' Tregs (mean ± *SEM*: 3.374 ± 0.53, 1.80 ± 0.32, vs. 1.73 ± 0.03, respectively; $p = .753$ and $p = .999$, respectively). (d) TGF-β protein levels in AV versus controls and SV (mean ± *SEM*: 33.43 ± 1.21 vs. 39.90 ± 1.07 and 43.66 ± 2.49; $p < .0001$ and $p = .0013$, respectively). TGF-β protein levels in GV and SV patients versus controls (mean ± *SEM*: 39.13 ± 1.53 and 43.66 ± 2.49, respectively, vs. 39.90 ± 1.07; $p = .2698$ and $p = .1192$, respectively). (e) *flCTLA4* transcript levels in GV, SV, and AV patients' Tregs versus controls' Tregs (mean ± *SEM*: 3.35 ± 0.38, 2.03 ± 0.34, and 4.39 ± 0.56, respectively, vs. 1.00 ± 0.16; $p < .0001$, $p = .007$, and $p < .0001$, respectively). *flCTLA4* transcript levels in AV patients' Tregs versus SV patients' Tregs (mean ± *SEM*: 4.39 ± 0.56 vs. 2.03 ± 0.34; $p = .006$). (f) *sCTLA4* transcript levels in GV and AV patients' Tregs versus controls (mean ± *SEM*: 8.41 ± 0.31 and 8.98 ± 0.43, respectively, vs. 7.19 ± 0.18; $p = .001$, $p = .0002$, respectively). *sCTLA4* transcript levels in SV patients' Tregs versus controls and AV patients' Tregs (mean ± *SEM*: 7.69 ± 0.40 vs. 7.19 ± 0.18 and 8.98 ± 0.43, respectively; $p = .143$ and $p = .079$)

(41–60 years) ($p = .038$, $p = .001$, $p = .003$, $p = .009$, and $p = .005$, respectively) (Figure 4a,c,d,e,f). Interestingly, FOXP3 protein expression was also found to be reduced ($p = .0226$) in the age of onset group 1–20 years as compared to the age of onset group 41–60 years (Figure 4b).

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 transcripts levels of *FOXP3* ($p = .145$ and $p = .124$), *IL10* ($p = .124$ and $p = .349$), *sCTLA4* ($p = .242$ and $p = .426$), *flCTLA4* ($p = .122$ and $p = .203$), and *TGFB* ($p = .141$ and $p = .098$)

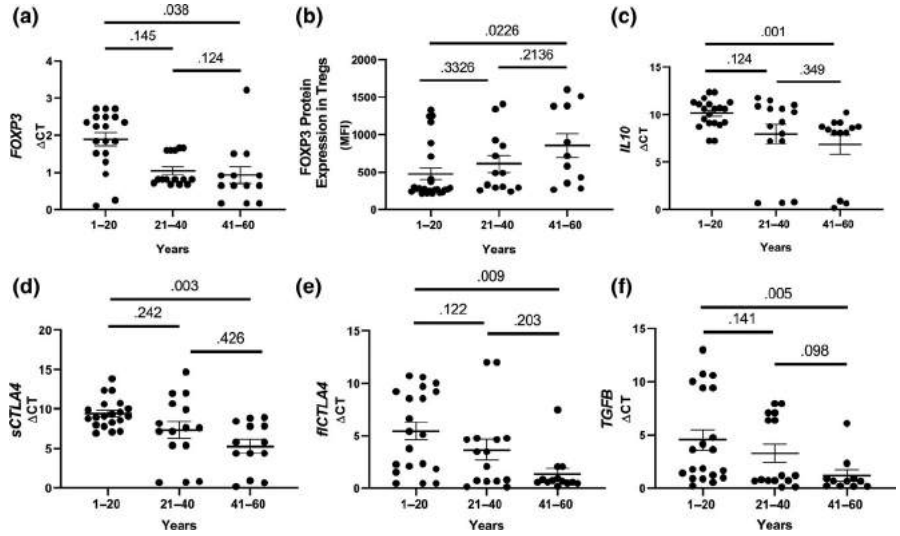

FIGURE 4 Effect of *FOXP3*, *IL10*, *sCTLA4*, *flCTLA4*, and *TGFB* transcripts and FOXP3 protein on age of onset of generalized vitiligo patients. The expression of *FOXP3*, *IL10*, *sCTLA4*, *flCTLA4*, and *TGFB* transcripts with respect to different age of onset groups [1–20 years (n = 24); 21–40 years (n = 13); 41–60 years (n = 11)] was analyzed in 48 GV patients by using non-parametric Mann–Whitney *U* test. (a) *FOXP3* transcript levels in Tregs of the age of onset group 1–20 years versus 41–60 years (mean ± *SEM*: 1.60 ± 0.26 vs. 0.81 ± 0.31; p = .038). *FOXP3* transcript levels in Tregs of the age of onset group 21–40 years versus 1–20 and 41–60 years (mean ± *SEM*: 1.13 ± 0.15 vs. 1.60 ± 0.26 and 0.81 ± 0.31, respectively; p = .145 and p = .124, respectively) (*SEM*, standard error mean). (b) FOXP3 protein levels in Tregs of the age of onset group 1–20 years versus 41–60 years (mean ± *SEM*: 477.6 ± 78.77 vs. 854.4 ± 158.1; p = .0226). FOXP3 protein levels in Tregs of the age of onset group 21–40 years versus 1–20 and 41–60 years (mean ± *SEM*: 610.7 ± 113.0 vs. 477.6 ± 78.77 and 854.4 ± 158.1, respectively; p = .3326 and p = .2136, respectively) (MFI, mean fluorescence intensity). (c) *IL10* transcript levels in Tregs of the age of onset group 1–20 years versus 41–60 years (mean ± *SEM*: 10.02 ± 0.34 vs. 6.30 ± 1.26; p = .001). *IL10* transcripts in the age of onset group 21–40 versus 1–20 and 41–60 years (mean ± *SEM*: 7.23 ± 1.34 vs. 10.02 ± 0.34 and 6.30 ± 1.26, respectively; p = .124 and p = .349, respectively). (d) *sCTLA4* transcript levels in Tregs of the age of onset group 1–20 years versus 41–60 years (mean ± *SEM*: 9.28 ± 0.42 vs. 5.37 ± 1.13; p = .003). *sCTLA4* transcripts in the age of onset group 21–40 versus 1–20 and 41–60 years (mean ± *SEM*: 7.07 ± 1.43 vs. 9.28 ± 0.42 and 5.37 ± 1.13, respectively; p = .242 and p = .426, respectively). (e) *flCTLA4* transcript levels in Tregs of the age of onset group 1–20 years versus 41–60 years (mean ± *SEM*: 4.96 ± 0.94 vs. 1.08 ± 0.81; p = .009). *flCTLA4* transcript levels in the age of onset group 21–40 versus 1–20 and 41–60 years (mean ± *SEM*: 2.64 ± 1.09 vs. 4.96 ± 0.94 and 1.08 ± 0.81; p = .122 and p = .203, respectively). (f) *TGFB* transcript levels in Tregs of the age of onset group 1–20 years versus 41–60 years (mean ± *SEM*: 4.31 ± 1.02 vs. 0.97 ± 0.63; p = .005). *TGFB* transcript levels in Tregs of the age of onset group 21–40 years versus 1–20 and 41–60 years (mean ± *SEM*: 2.56 ± 1.20 vs. 4.31 ± 1.02 and 0.97 ± 0.63; p = .141 and p = .098, respectively)

(Figure 4a,c,d,e,f). Similarly, there was no significant difference observed for *NFATs (1–5)*, *CD25*, *IL4*, *and IL2* transcripts in Tregs and plasma IL-10 protein level among any of the age of onset groups (i.e., 1–20 vs. 21–40; 1–20 vs. 41–60; 21–40 vs. 41–60) (Figure S10a–i). Moreover, there was no significant difference found in FOXP3 protein levels when the age of onset group 21–40 years was compared with other onset groups, that is, 1–20 and 41–60 years (p = .3326 and p = .2136, respectively) (Figure 4b). These results suggest that decreased *FOXP3*, *IL10*, *sCTLA4*, *flCTLA4*, *and TGFB* transcripts and FOXP3 protein may affect the onset of GV in these patients.

4 | DISCUSSION

The nuclear factors of activated T cells (NFATs) have not been addressed, so far in vitiligo. However, NFATs have been proven to be targets for immunomodulatory drugs (e.g., cyclosporine A, FK506) to control T-cell-mediated immunity in autoimmune diseases (Wells et al., 2000). The present study for the first time analyzed the expression of *NFATs* (1–5) in Tregs of GV patients and controls. Interestingly, we observed that transcript expressions of all five *NFATs* (1–5) were reduced drastically in Tregs of GV patients as compared to control Tregs (Figure 1). Earlier, the *NFAT1* and *NFAT*2 expressions were found to be essential for homing of Treg cells in preventing autoimmune diseases like systemic lupus erythematosus and experimental autoimmune encephalomyelitis (Ghosh et al., 2010; Vaeth et al., 2014). Further, the levels of *NFATC3*, *NFATC4*, and *NFAT5* transcripts were found to be significantly reduced in active vitiligo (AV) Tregs when compared to stable vitiligo (SV) Tregs (Figure 1), indicating their likely role in progression of GV as well. The decreased *NFAT* levels might lead to the altered Treg cells profile by further affecting the NFAT:FOXP3 complex formation (Figure 5). In induced Tregs (iTregs), NFATs regulate the expression of *FOXP3* by binding to the Cis-regulatory element of *FOXP3* CNS1 (Vaeth & Feske, 2018). Studies with double-deficient *NFAT* mouse models (*NFAT* 1, 2, and 4 knockout mice) also reported a diminished *FOXP3* expression by iTregs (Kwon et al., 2016; Vaeth et al., 2012). Moreover, Treg cell numbers were found to be reduced drastically in metastatic cancer patients treated with zoledronic acid, since it selectively inhibited Ca^{2+}/calcineurin/NFAT and IL-2

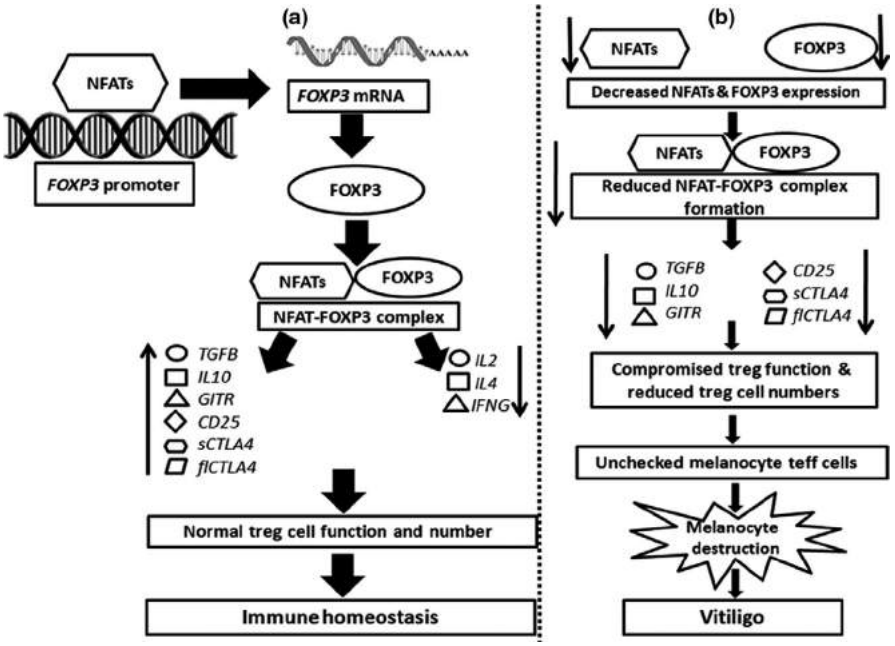

FIGURE 5 Mechanism of regulation of Treg cells by NFAT and FOXP3 in vitiligo pathogenesis. (a) NFATs are responsible to govern the expression of transcription factor FOXP3 by binding to the CNS1 promoter region of *FOXP3*. These NFATs and FOXP3 form a complex which has a crucial role in regulating expression 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

signaling pathways in Tregs resulting in reduced Treg cell capacity to suppress T and NK cell responses in vitro (Sarhan et al., 2017). This suggests that inhibition of NFAT signaling pathway represses Treg cells' suppressive function.

Forkhead box P3 (FOXP3) is indispensable molecule for Treg immunosuppressive function. Our results suggested that *FOXP3* transcript and FOXP3 protein expression in isolated Tregs of GV patients were significantly reduced (Figure 2a,b) and these are in concordance with the previous studies (Table S1). These results further indicate the crucial role of *FOXP3* in impairment of Treg cells in GV patients, which in turn might lead to $CD8^+$ T-cell-mediated pathogenesis of GV. In addition, a significantly reduced *FOXP3* transcript and FOXP3 protein were found in Tregs of AV patients when compared to SV patients (Figure 2a), suggesting a key role of *FOXP3* in disease progression as well. Moreover, the FOXP3 appeared to play an important role in onset of GV since early age of onset group exhibited reduced FOXP3 transcript and protein as compared to late age of onset group (Figure 4a,b). Earlier, both *FOXP3* transcript and protein levels were found to be reduced in vitiligo skin as compared to controls (Elela, Hegazy, Fawzy, Rashed, & Rasheed, 2013; Hegazy, Fawzy, Gawdat, Samir, & Rashed, 2014; Tembhre et al., 2015). Moreover, FOXP3 expression was significantly reduced in $CD4^+CD25^{high}$ Tregs of vitiligo patients compared to controls (Dwivedi et al., 2013). Similarly, significantly decreased FOXP3 in T cells was reported in non-segmental vitiligo patients in Egyptian patients (Hegab & Attia, 2015). Since FOXP3 is not just a suppressive marker of Treg, but it also plays a significant role in growth and development of Tregs; so, it could be one of the major factors leading to the reduced number of Tregs as observed earlier in vitiligo patients (Dwivedi et al., 2015, 2013).

To assess whether promoter polymorphisms are involved in the decreased expression of *FOXP3* in Tregs of GV patients, we further genotyped the three well-reported *FOXP3* promoter SNPs [single nucleotide polymorphisms; rs3761547 (A > G), rs3761548 (C > A), and rs2232365 (A > G)] and correlated them with the *FOXP3* transcript levels. We found a significant reduction in FOXP3 transcript and protein expression in GV Tregs with susceptible rs3761547 GG genotype as compared to controls (Figure S5b and Figure 2d), suggesting that rs3761547 GG genotype may be involved in decreased expression of *FOXP3*. Earlier, a meta-analysis showed significant association of *FOXP3* rs3761547 (A > G) SNP with vitiligo (Birlea et al., 2011). It is speculated that the presence of rs3761547 "G" allele at *FOXP3* promoter may prevent the binding of transcription factors leading to its decreased *FOXP3* expression. Furthermore, we found that GV patients with rs3761548 AA and CA genotypes exhibited reduced FOXP3 expression in Tregs as compared to controls (Figure 2c and Figure S5a), indicating the role of susceptible "A" allele in decreased *FOXP3* expression. Earlier, rs3761548 "A" allele was associated with Indian female vitiligo patients (Jahan et al., 2013). The Han Chinese vitiligo patients also showed increased frequency of AA genotype (Song et al., 2013) which has

been suggested to abrogates the binding of E-47 and c-Myb factors, leading to defective FOXP3 transcription factor (Shen et al., 2010). In addition, decreased expression of FOXP3 transcripts and protein in GV Tregs with susceptible rs2232365 GG genotype as compared to controls (Figure 2e and Figure S5c) suggested the crucial role of GG genotype. Previously, *FOXP3* rs2232365 (A > G) SNP was found to be associated with Han Chinese vitiligo patients (Song et al., 2013). It was suggested that due to the presence of rs2232365 SNP in DNA-binding site of GATA-3 transcription factor, the SNP regulates the expression of *FOXP3* and this could lead to impaired Treg cells.

The NFAT:FOXP3 complex regulates the Treg function by upregulating the expression of suppressive genes like *CTLA4*, *IL10*, *TGFB*, and downregulating the expression of *IL2* and *IL4*. However, the disruptive mutations and the altered levels of NFAT:FOXP3 complex lead to non-functional Tregs (Figure 5) (Vaeth & Feske, 2018). Since we found decreased expression of both *NFATs* and *FOXP3*, we further assessed their effect on downstream immune-suppressive genes in Tregs of GV patients. Interestingly, we found that the suppressive function of Tregs could be compromised in GV patients as the Tregs of GV patients showed significantly reduced expression of immune-suppressive genes: *CTLA4* (both *sCTLA4 and flCTLA4)* and *IL10* (both mRNA and protein) as compared to controls. Previous studies detected systemic levels of these genes in vitiligo patients; however, the present study has shown expression of these immune-suppressive genes in Tregs isolated from GV patients and controls. In particular, a significant decreased expression of *TGFB* and *flCTLA4* transcripts was observed in Tregs of AV patients compared to SV, indicating the likely role of *TGFB* and *flCTLA4* in disease progression. Further, the age of onset analyses revealed significantly reduced expression of *IL10*, *sCTLA-4*, *flCTLA4*, and *TGFB* transcripts in Tregs of early-onset groups (1–20 years) of GV patients compared to late-onset group (41–61 years) (Figure 4c–f), suggesting that these Treg suppressive genes may be involved in early onset of the disease. Our previous study reported a decreased systemic *flCTLA4* and *sCTLA4* transcript levels in vitiligo patients (Dwivedi et al., 2011). Recently, Zhang et al. (2018) have reported reduced serum levels of sCTLA4, IL10, and TGFB in vitiligo patients. The role of IL-10 in the pathogenesis of vitiligo has been evidenced by several studies demonstrating the reduced levels of IL-10 in serum (Ala, Pasha, Rao, Komaravalli, & Jahan, 2015; Tembhre et al., 2013), in lesional skin (Kidir, Karabulut, Ercin, & Atasoy, 2017), and in Tregs (Zhang et al., 2018) of vitiligo patients as compared to controls. The present study also found decreased *IL10* transcript in Tregs and decreased IL-10 protein level in plasma of GV patients as compared to controls (Figure 3a,b). The IL-10 is a pleiotropic, immunoregulatory cytokine which suppresses immune response by downregulating TNF-α, IL-1, and IFN-γ production (D'Andrea et al., 1993). Treg cells suppress immune response by cell-to-cell contact and by production of immune-suppressive cytokines such as IL-10 and TGF-β. Other than the immune-suppressive function, IL-10 in the presence of TGF-β also increases Treg cell expansion with enhanced CTLA-4 expression and suppressive capability via IL-10R-mediated STAT3 signaling pathway (Hsu et al., 2015). Additionally, increased IL10 expression in vitiligo lesions was shown after the topical tacrolimus treatment suggesting the inhibition of melanocyte destruction triggered by unchecked Th1 pathways in vitiligo (Taher, Lauzon, Maguiness, & Dytoc, 2009). Moreover, in a model of spontaneous melanoma, Treg depletion and IL10 neutralization led to increased occurrence of vitiligo that correlated with a decreased incidence of melanoma metastases (Pommier et al., 2013). Thus, these previous reports along with the present study in Tregs are suggestive of compromised function of Tregs in GV due to decreased expression of IL10 and *CTLA4*.

TGF-β is a pleiotropic immune-suppressive cytokine which maintains peripheral tolerance by regulating immune response. Treg-derived TGF-β exhibits suppressive function by inhibiting the proliferation of human B and T cells. It regulates the immune response by two means, firstly by inhibiting the function of inflammatory cells and secondly by promoting Treg cell proliferation (Wan & Flavell, 2007). TGF-β has been demonstrated to induce $CD4^+CD25^+$ Tregs from $CD4^+CD25^-$ cells in vitro (Fu et al., 2004). It also induces the expression of *FOXP3* in Tregs by activating the NFAT and SMAD3 binding to CNS1 region of *FOXP3* promoter (Wan & Flavell, 2007). In concordance with the earlier studies (Tembhre et al., 2015, 2013), the present study also reports a significant decreased *TGFB* transcript in Tregs and decreased plasma TGF-β protein in AV patients as compared to SV, indicating the likely role of *TGFB* in disease progression (Figure 3). Previous studies also showed reduced levels of TGF-β in serum, plasma, and tissue of vitiligo patients which could affect Treg cell function (Khan et al., 2012; Osman, Mukhtar, Bakheit, & Hamdan, 2015). Additionally, the decreased TGF-β levels of Tregs in vitiligo patients showed negative correlation with the percentage of depigmented body area (Tu, Jin, Lin, Wang, & Man, 2011), suggesting the crucial role of *TGFB* in immune suppression by Tregs.

Furthermore, the IL-4 which is a pleiotropic immunomodulatory cytokine mediates hypersensitivity reactions and studies have shown increased systemic IL-4 levels in vitiligo patients (Imran et al., 2012). The IL-2 is a potent T-cell growth factor required for activation and proliferation of T cells. Generally, upregulation of *IL4* and *IL2* is observed in activated T cells, whereas in Tregs they are downregulated (Wu et al., 2006). However, the present study did not find significant difference in *IL4* and *IL2* transcript levels between Tregs of GV patients and controls. Moreover, the transcript levels of *CD25* (interleukin-2 receptor alpha chain) did not differ between Tregs of GV patients and controls, suggesting that there was normal constitutive expression of CD25 in Tregs of GV patients and controls. Nevertheless, it has been reported that exogenous IL-2 consumption abrogates Treg's capacity to suppress conventional T cells (Tcon) (Schmidt, Oberle, & Krammer, 2012). Previously, vitiligo patients showed higher serum IL-2 levels (Singh, Singh, & Pandey, 2012) which might lead to higher consumption of IL-2 by Treg cells as they constitutively express high CD25 compared to Tcon cells, resulting in compromised Treg cell suppressive function.

In conclusion, the present study for the first time suggests that decreased transcript levels of *NFATs* and *FOXP3* and corresponding decreased protein levels of FOXP3 in Tregs of GV patients may

impair Treg cell function together with reduced IL10 (both mRNA and protein) and *CTLA4* (both *sCTLA4* and *flCTLA4*). However, protein levels of NFATs and NFAT:FOXP3 interaction studies in Tregs would be essential to draw unequivocal conclusion on their role in GV pathogenesis and progression.

ACKNOWLEDGEMENTS

We thank all vitiligo patients and control subjects for their participation in this study. This work was supported by grant to MD {ECR/2017/000858} Science & Engineering Research Board, Department of Science & Technology (SERB-DST), New Delhi, India. PSG thanks the Science & Engineering Research Board, Department of Science & Technology (SERB-DST), New Delhi, for awarding JRF. We thank Prof. B.K. Thelma, University of Delhi, South Campus, India, for helping in the revision of the article. We are thankful to Uka Tarsadia University, Gujarat, India, for providing necessary research facilities to conduct the study.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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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SUPPORTING INFORMATION

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

How to cite this article: Giri PS, Dwivedi M, Laddha NC, Begum R, Bharti AH. Altered expression of nuclear factor of activated T cells, forkhead box P3, and immune-suppressive genes in regulatory T cells of generalized vitiligo patients. *Pigment Cell Melanoma Res*. 2020;00:1–13. https://doi.org/10.1111/pcmr.12862