Research Article

Decreased GZMB, NRP1, ITPR1, and SERPINB9 Transcripts Lead to Reduced Regulatory T Cells Suppressive Capacity in Generalized Vitiligo Patients

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Generalized vitiligo (GV) is an autoimmune skin disease characterized by bilateral white patches over the entire body. Regulatory T cells (Tregs) maintain peripheral tolerance; however, they are found to be reduced in numbers and function in vitiligo patients. The exact mechanism for reduced Treg suppressive capacity is unknown. Therefore, we aimed to assess transcript levels of Tregs-associated immunosuppressive genes (GZMB, NRP1, PDCD1, FASLG, and TNFRS18), regulatory molecules of Tregs suppressive function (SERPINB9, ITPR1, and UBASH3A), and Treg-associated transcription factors (GATA2, GATA3, RUNX1, STAT3, and STAT5) in 52 GV patients and 48 controls by real-time PCR (qPCR). We found significantly reduced GZMB, NRP1, SERPINB9, and ITPR1 transcripts in GV Tregs compared to controls (p = 0.03, p = 0.023, p = 0.0045, and p < 0.0001, respectively). There were 0.44-, 0.45-, 0.32-, and 0.54-fold decrease in GZMB, NRP1, SERPINB9, and ITPR1 transcripts in GV Tregs. Additionally, disease activity and severity-based analyses revealed significantly decreased GZMB (p = 0.019 and 0.034), SERPINB9 (p = 0.031 and p = 0.035), and ITPR1 (p = 0.0003 and p = 0.034) transcripts in active vitiligo and severe GV patients’ Tregs. Interestingly, we found a positive correlation for ITPR1 with GZMB (r = 0.45, p = 0.0009) and SERPINB9 (r = 0.52, p = 0.001) transcripts in GV Tregs. Moreover, we found positive correlation for percentage Treg mediated suppression of CD4+ and CD8+ T cells with ITPR1 (r = 0.54; r = 0.49), GZMB (r = 0.61; r = 0.58), NRP1 (r = 0.55; r = 0.52), and SERPINB9 (r = 0.56; r = 0.48) in GV Tregs. Further, calcium treatment of Tregs resulted into significantly increased ITPR1, SERPINB9, and GZMB transcripts in GV Tregs (p = 0.023, p = 0.0345, p = 0.02). Overall, our results for the first time revealed the crucial role of GZMB, NRP1, SERPINB9, and ITPR1 transcripts in decreased Treg suppressive capacity leading to GV pathogenesis, progression, and severity. In addition, our study highlighted that ITPR1 might be linked with decreased GZMB and NRP1 expression in GV Tregs. Moreover, our study for the first time suggest that increased SERPINB9 transcripts may lead to endogenous granzyme B-mediated Tregs apoptosis, and calcium treatment of Tregs may improve the Treg suppressive capacity. These findings may further aid in development of Treg-based therapeutics for GV.

1. Introduction

Generalized vitiligo (GV) is an autoimmune disease characterized by symmetrical white patches on the entire body [1]. Its prevalence is about 0.5 to 2% worldwide [2]. The key role of autoimmunity in GV has been suggested by the presence of autoantibodies and autoreactive CD4+ and CD8+ T cells in vitiligo skin lesions [3]. Furthermore, if they remain unchecked, these autoreactive T cells can lead to granzyme and FAS-FASL-mediated melanocyte destruction, leading to GV pathogenesis [4–8]. Additionally, our previous studies have found the role of inflammatory cytokines IFN-γ and TNF-α in melanocyte destruction [6, 9–11].

Regulatory T cells (Tregs) are crucial role in controlling such self-reactive T cells [12, 13]. However, previous studies have found altered Tregs number and function in vitiligo [6,
Moreover, our previous studies suggested impaired levels of the transcription factors of Tregs, such as nuclear factors of activated T cells (NFATs) and Forkhead box P3 (FOXP3) that led to reduced downstream immunosuppressive genes (IL-10, TGF-β, and CTLA-4), resulting in impaired Treg-mediated suppression of CD4⁺ and CD8⁺ T cells [6, 15, 16]. Thus, the impaired Treg suppressive capacity leads to widespread CD8⁺ and CD4⁺ T cell activation, proliferation, and IFN-γ production, which results in melanocyte destruction in GV patients [6, 15, 16].

In Tregs, the key immunosuppressive molecules such as granzyme B (GZMB), neuropilin-1 (NRPI), PDCD1 (programmed cell death protein 1 (PD-1) or CD279), FASLG (Fas ligand (FasL) or CD95L or CD178), and TNFRS18 (glucocorticoid-induced TNFR-related protein (GITR) or CD357) maintain Treg suppressive function [17–20]. Additionally, SERPINB9 (Serpin family B member 9) endogenous inhibitor of granzyme B protects Tregs from self-inflicted granzyme B-mediated apoptosis [21]. Moreover, inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) regulates calcium entry in T cells. Furthermore, UBASH3A (ubiquitin associated and SH3 domain-containing A) governs T cells’ function by regulating the TCR-CD3 complex [22]. Nevertheless, transcription factors such as GATA-binding factor 2 (GATA2), GATA3, runt-related transcription factor 1 (RUNX1), signal transducer and activator of transcription 3 (STAT3), and STAT5 also play a critical role in Treg cells’ function [23–27]. Although the role of NFATs and FOXP3 in Treg dysfunction has been suggested previously, the role of Tregs-associated immunosuppressive genes (GZMB, NRPI, PDCD1, FASLG, and TNFRS18), regulatory molecules of Tregs function (SERPINB9, ITPR1, and UBASH3A), and Treg-associated transcription factors (GATA2, GATA3, RUNX1, STAT3, and STAT5) is unknown in GV.

Therefore, to delineate the exact pathway of Treg cells dysfunction, the current study aimed to study the mRNA expression levels of (i) Tregs-associated immunosuppressive genes (GZMB, NRPI, PDCD1, FASLG, and TNFRS18), (ii) regulatory molecules of Tregs function (SERPINB9, ITPR1, and UBASH3A), and (iii) Treg-associated transcription factors (GATA2, GATA3, RUNX1, STAT3, and STAT5) in GV pathogenesis, progression, and severity. Additionally, we carried out the age of onset and gender-based analysis for these genes to study their effect on age of onset and gender biasness for GV pathogenesis.

2. Materials and Methods

2.1. Study Population. A total of 52 GV patients and 48 healthy controls were included in the study. Table 1 depicts the demographic details for the enrolled participants. Vitiligo was diagnosed by a dermatologist from Aura skin care clinic, Vyara, India, by observing symmetrical white color lesions on skin under woods lamps. Newborn babies, pregnant/lactating women, patients on treatment, and patients with other autoimmune diseases were excluded from the study. Controls were free from any signs of vitiligo and other autoimmune diseases. The study protocol was per the Institutional Human Research Ethics Committee (IHREC), Maliba Pharmacy College, UKA Tarsadia University, India. The study protocol followed the Helsinki Declaration of 1964 and its successful amendments. The GV patients were divided into active vitiligo (AV) patients and stable vitiligo (SV) patients as mentioned previously [15]. The patients were categorized as AV, if they developed any increase in lesions size or number within the past six months; otherwise, they were categorized as SV [28]. Moreover, patients were categorized based on the disease severity measured by the vitiligo area scoring index (VASI) as described by Bhor and Pande [29]. GV patients were divided into three groups: (i) 10%-25% VASI: mild GV; (ii) 25%-50% VASI: moderate GV; and (iii) 50%-75%: severe GV, as mentioned previously [6].

2.2. Isolation of CD4⁺CD25⁺ Treg Cells and CD4⁺ T Cells. CD4⁺CD25⁺ Treg cells and CD4⁺ T cells were isolated from three-milliliter peripheral blood of 52 GV patients and 48 controls using MACSxpress® whole blood Treg isolation kit (Miltenyi Biotec, Auburn, CA) as mentioned previously [15]. In the first step, all the non-CD4⁺ cells were removed from the whole blood using MACSxpress beads by negative selection. In the second step, through positive selection, the CD4⁺CD25⁺ Treg cells were enriched from CD4⁺ T cells using microbeads and LS columns under a strong magnetic field. Flow cytometry was carried out to confirm the purity of isolated Treg cells (Figure 1), the purity of isolated CD3⁺ CD4⁺CD25⁺FOXP3⁺ Treg cells was found to be 94.22%. The isolated Treg cells were immediately processed for the downstream experiments.

2.3. 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 and as mentioned previously [6]. 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.

2.4. Total RNA Isolation and cDNA Synthesis. The total RNA was extracted from CD4⁺CD25⁺ Treg cells using TRizol reagent (Invitrogen, Carlsbad, CA, USA) as mentioned previously [15]. RNA integrity, yield, and purity were determined by 1.5% gel electrophoresis and spectrophotometrically at 260/280 nm. The cDNA was synthesized from 1 μg of total RNA by iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA) as per the manufacturer’s instructions.

2.5. Quantitative Real-Time PCR. The mRNA expression levels of Treg-associated genes GZMB, NRPI, ITPR1, SERPINB9, PDCD1, FASLG, UBASH3A, IKZF4, GATA2, GATA3, TNFRS18, RUNX1, STAT3, and STAT5 were measured with qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression levels were used as a reference gene. Gene-specific primers for the expression study are mentioned in Table S1. mRNA expression
Table 1: Demographic characteristics of generalized vitiligo (GV) patients and controls.

|                          | GV patients (n = 52) | Controls (n = 48) |
|--------------------------|----------------------|-------------------|
| Average age (mean ± SD)  | 37.04 ± 11.22 years  | 32.18 ± 3.18 years|
| Gender                   |                      |                   |
| Male                     | 28 (53.84%)          | 26 (54.16%)       |
| Female                   | 24 (46.16%)          | 22 (45.83%)       |
| Age of onset (mean ± SD) | 18.23 ± 4.21 years   | NA                |
| Duration of disease (mean ± SD) | 7.22 ± 4.24 years | NA                |
| Extent of disease        |                      |                   |
| VASI score (mean ± SD)   | 58.12% ± 22.58%      | NA                |
| 10–25% VASI (mild GV)    | 12 (23.07%)          | NA                |
| 25–50% VASI (moderate GV)| 18 (34.61%)          | NA                |
| 50–75% VASI (severe GV)  | 22 (42.30%)          | NA                |
| Disease activity         |                      |                   |
| Active vitiligo          | 28 (54.00%)          | NA                |
| Stable vitiligo          | 24 (46.00%)          | NA                |
| Family history           | 20 (34.61%)          | NA                |

analysis was carried out using iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) as per the manufacturer’s instructions. The qPCR conditions for the gene expressions study are mentioned in Table S1. The specificity of the qPCR products was checked by dissociation curve analysis (Figures S1 and S2). The fluorescence data were collected during the extension step and the cycle at which the fluorescence intensity raised above the background was termed as cycle threshold (C_T). The difference between the target and reference genes C_T was considered as \(\Delta C_T\) value. The \(\Delta C_T\) value was determined as the difference between the \(\Delta C_T\) value of controls and patients. The fold change value in gene expression was calculated using 2^\(-\Delta\Delta C_T\) formula.

2.6. In Vitro Calcium Treatment of Treg Cells. Treg cells were subjected to calcium treatment by dissolving 750 \(\mu\)M calcium (Cayman, MI, USA) in RPMI media. The dissolved calcium level in the medium was confirmed using calcium assay kit (Cayman, MI, USA). Treg cells were seeded in 24-well plate at density of \(5 \times 10^4\) cells, in 1 ml RPMI medium supplemented with 5% FBS containing desired calcium concentration at 37°C at 5% CO_2 for 24 hours. The calcium-treated Treg cells were immediately processed for downstream in vitro functional assays. The standard curve for the estimation of calcium is presented in Figure S3.

2.7. In Vitro Treg Suppression Assay. CD4+CD25+ Treg cells (5000 cells) were co-cultured with CD8+ T cells and CD4+ T cells (10,000 cells) at a ratio of 1:2 individually, as mentioned previously [6]. The cells were activated with 200 IU rIL2 (PeproTech, NJ, USA) and anti-CD3/CD28 dynabeads Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a 1:1 (bead:cells) ratio in 200 \(\mu\)l RPMI supplemented with 10% fetal bovine serum for 5 days at 37°C and 5% CO_2 in 96 well U-bottom plate. On 4th day, the cells were labelled with 10 \(\mu\)M BrdU (Sigma-Aldrich, MO, USA) and incubated for 18 hrs and then further processed for BrdU cell proliferation assay as mentioned previously [6, 30, 31].

2.8. 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: \([(\text{proliferation of Tconv cells alone} – \text{proliferation of Tconv cells treated with Treg})/\text{proliferation of Tconv cells alone}] \times 100\) [6, 31].

2.9. Correlation of ITPR1, GZMB, NRPI, and SERPINB9 Transcripts with In Vitro Treg Suppressive Capacity. The isolated Tregs population was divided into two fractions. The inherent levels of ITPR1, GZMB, NRPI, and SERPINB9 transcripts were assessed from the first fraction, whereas in vitro Treg suppression assay was carried out from the second Treg fraction. Further, the inherent levels of ITPR1, GZMB, NRPI, and SERPINB9 transcripts in Tregs of GV were correlated with the Treg suppressive capacity by Spearman’s rank correlation analysis.

2.10. Statistical Analysis. The comparison of mean \(\Delta C_T\) values in GV patients and controls for relative mRNA expression analysis, disease activity analysis, disease severity analysis, age of onset analysis, and gender-based analysis was carried out using nonparametric Mann–Whitney U test. The 2^\(-\Delta\Delta C_T\) analysis was carried out to calculate the fold difference in gene expression. The statistical analysis was carried out using GraphPad prism software (Graphpad software
Additionally, we did not find any significant difference in the disease activity and disease severity-based analysis of SV and mild GV Tregs compared to AV and severe GV Tregs (p = 0.54). The disease activity and severity-based analysis suggested no significant difference in transcripts levels of PDCD1, FASLG, and TNFRS18 in AV and severe GV Tregs compared to SV and mild GV Tregs (p > 0.05; Figure S4).

3. Results

3.1. Transcript Levels of Tregs Associated Immunosuppressive Genes (GZMB, NRP1, PDCD1, FASLG, and TNFRS18) in GV Patients and Controls. The transcript levels of Tregs-associated immunosuppressive genes (GZMB, NRP1, PDCD1, FASLG, and TNFRS18) were assessed in 52 GV patients and 48 controls using nonparametric Mann–Whitney U test after normalization with GAPDH expression. We found significantly reduced transcript levels for GZMB and NRP1 expression in GV Tregs compared to control Tregs (p = 0.03 and p = 0.023; Figures 2(a) and 2(d)). The ΔΔCT analysis suggested a 0.44- and 0.45-fold difference in mRNA expression levels of GZMB and NRP1 in GV Tregs compared to control Tregs (Figures 2(c) and 2(e)). Further, the disease activity and disease severity-based analysis suggested significantly reduced mRNA expression levels of GZMB in AV and severe GV Tregs compared to SV and mild GV Tregs (p = 0.019 and p = 0.034, respectively; Figures 2(a) and 2(b)). However, there was no significant difference in mRNA expression levels of NRP1 in AV and severe GV Tregs compared to SV and mild GV Tregs (p = 0.453 and p = 0.2642; Figures 2(d) and 2(e)). Additionally, we did not find any significant difference in transcripts levels of PDCD1, FASLG, and TNFRS18 in GV Tregs compared to control Tregs (p > 0.05; Figure S4). Moreover, the disease activity and disease severity-based analysis suggested no significant difference in transcripts levels of PDCD1, FASLG, and TNFRS18 in AV and severe GV Tregs compared to SV and mild GV Tregs (p = 0.045 and p < 0.0001; Figures 3(a) and 3(d)). According to the 2^ΔΔCT analysis, there was a 0.32- and 0.54-fold difference in transcript levels of SERPINB9 and ITPRI in GV Tregs compared to control Tregs (Figures 3(c) and 3(f)). Moreover, the disease activity and severity-based analysis suggested a significantly decreased mRNA expression levels of SERPINB9 and ITPRI in AV Tregs (p = 0.031 and p = 0.0003; Figures 3(a) and 3(d)) and severe GV Tregs (p = 0.035 and p = 0.034; Figures 3(b) and 3(e)) as compared to SV and mild GV Tregs. However, we could not find any significant difference in transcript levels of UBASH3A for GV Tregs as compared to controls Tregs (p = 0.015; Figure S5). The disease activity and severity-based analysis also suggested no significant difference in mRNA expression levels of UBASH3A in AV and severe GV Tregs compared to SV and mild GV Tregs (p = 0.487; Figure S5).
Figure 2: Continued.
3.3. Transcripts Levels of Treg Associated Transcription Factors (GATA2, GATA3, RUNX1, STAT3, and STAT5) in GV Patients and Controls. The transcript levels for Treg-associated transcription factors (GATA2, GATA3, RUNX1, STAT3, and STAT5) were assessed in 52 GV patients and 48 controls using nonparametric Mann–Whitney U test after normalization with GAPDH expression. We did not find any significant difference for transcript levels of GATA2, GATA3, RUNX1, STAT3, and STAT5 in GV Tregs when compared to controls’ Tregs (p > 0.05; Figures S5 and S6). Moreover, the disease activity and severity-based analysis also suggested no significant difference in mRNA expression levels of GATA2, GATA3, RUNX1, STAT3, and STAT5 in AV and severe GV Tregs as compared to SV and mild GV Tregs (p > 0.05; Figures S5 and S6).

3.4. Effect of Calcium Treatment on ITPR1, GZMB, NRPI, and SERPINB9 Transcripts in GV Patients’ and Controls’ Tregs. Previously, we have found that calcium treatment enhances the calcium uptake in Tregs resulting in increased NFATc1 activity which leads to enhanced Treg suppressive capacity in GV [32]. As ITPR1 governs the release of calcium in Tregs and GZMB, NRPI, and SERPINB9 are crucial for Treg cells activity, we studied the effect of calcium treatment on ITPR1, GZMB, NRPI, and SERPINB9 transcripts in Tregs of GV patients and controls. Upon calcium treatment, we found significantly increased ITPR1 transcripts in Tregs of GV, SV, and AV patients compared to controls (p = 0.02, p = 0.0327, and p = 0.01; Figure 3(d)). Interestingly, the calcium treatment led to an increased SERPINB9 (p = 0.0345, p = 0.0432, and p = 0.02; Figure 3(a)) and GZMB (p = 0.023, p = 0.038, and p = 0.01; Figure 2(a)) transcripts in calcium-treated Tregs of GV, SV, and AV patients, respectively, compared to untreated Tregs of GV, SV, and AV patients, respectively. Moreover, the calcium treatment led to increased ITPR1 (p = 0.033, p = 0.021, and p = 0.01; Figure 3(e)), GZMB (p = 0.034, p = 0.022, and p = 0.01; Figure 2(b)), and SERPINB9 (p = 0.034, p = 0.02, and p = 0.001; Figure 3(b)) transcripts in mild GV, moderate GV, and severe GV Tregs. We did not find any significant differences in NRPI transcripts in GV, SV, and AV Tregs when compared to controls.
Figure 3: Continued.
difference in NRPI transcripts in calcium-treated Tregs of GV, SV, AV, mild GV, moderate GV, and severe GV patients \( p > 0.05 \); Figures 2(d) and 2(e)).

3.5. Correlation of ITPI Transcripts with GZMB, NRPI, and SERPINB9 Transcripts in Tregs and Correlation of In Vitro Treg Suppression Assay with of ITPI, GZMB, NRPI, and SERPINB9 Transcripts in Tregs of GV Patients. Interestingly, we found a positive correlation for ITPI transcripts with GZMB \( r = 0.45; p = 0.0009 \) and NRPI \( r = 0.52; p = 0.001 \) transcripts in Tregs of GV patients (Figures 4(a) and 4(b)). However, we could not find any correlation between ITPI and SERPINB9 transcripts in Tregs of GV patients \( r = 0.22; p = 0.2473 \); Figure 4(c)). Further, we correlated in vitro Treg suppression assay with GZMB, NRPI, SERPINB9, and ITPI transcripts in Tregs of GV patients. We found a positive correlation for percentage Treg-mediated suppression of CD4^+ T cells with GZMB \( r = 0.61; p = 0.0012 \), NRPI \( r = 0.55; p = 0.021 \), SERPINB9 \( r = 0.56; p = 0.002 \), and ITPI \( r = 0.54; p = 0.001 \) and percentage Treg-mediated suppression of CD8^+ T cells with GZMB \( r = 0.58; p = 0.004 \), NRPI \( r = 0.52; p = 0.022 \), SERPINB9 \( r = 0.48; p = 0.024 \), and ITPI \( r = 0.49; p = 0.032 \) in GV patients (Figures 5(a)–5(h)).

3.6. Age of Onset and Gender-Based Analyses for Transcripts Levels of Treg-Associated Genes (GZMB, NRPI, ITPI, SERPINB9, PDCD1, FASLG, UBASH3A, IKZF4, GATA2, GATA3, TNFRSF18, RUNX1, STAT3, and STAT5) in GV Patients. Further, the expression of GZMB, NRPI, ITPI, SERPINB9, PDCD1, FASLG, UBASH3A, IKZF4, GATA2, GATA3, TNFRSF18, RUNX1, STAT3, and STAT5 transcripts was monitored in different age at onset groups of GV patients. We did not find any significant difference in the
expression of \( \text{GZMB}, \text{NRPI}, \text{ITPR1}, \text{SERPINB9}, \text{PDCD1}, \text{FASLG}, \text{UBASH3A}, \text{IKZF4}, \text{GATA2}, \text{GATA3}, \text{TNFRSF18}, \text{RUNX1}, \text{STAT3}, \text{and STAT5} \) transcripts in Tregs between 1–20, 21–40, and 41–60 years of onset groups \((p > 0.05; \text{Figures S7 and S8}).\)

Next, we carried out gender-based analysis for expression of \( \text{GZMB}, \text{NRPI}, \text{ITPR1}, \text{SERPINB9}, \text{PDCD1}, \text{FASLG}, \text{UBASH3A}, \text{IKZF4}, \text{GATA2}, \text{GATA3}, \text{TNFRSF18}, \text{RUNX1}, \text{STAT3}, \text{and STAT5} \) transcripts. We did not find any significant difference in expression of \( \text{GZMB}, \text{NRPI}, \text{ITPR1}, \text{SERPINB9}, \text{PDCD1}, \text{FASLG}, \text{UBASH3A}, \text{IKZF4}, \text{GATA2}, \text{GATA3}, \text{TNFRSF18}, \text{RUNX1}, \text{STAT3}, \text{and STAT5} \) transcripts between male and female GV Tregs \((p > 0.05; \text{Figures S9 and S10}).\)

### 4. Discussion

Autoimmunity has been strongly implicated in GV pathogenesis by the presence of autoantibodies and autoreactive \( \text{CD4}^+ \text{ and CD8}^+ \) T cells in GV patients [3–8]. Additionally, studies have implicated the role of cytotoxic T cells in melanocyte death in vitiligo patients [7, 8, 15]. Tregs control such autoimmune responses against melanocytes by actively suppressing self-reactive T cells activation and expansion [13, 14]. However, studies have suggested impaired Tregs’ number and Tregs suppressive function in GV patients [6, 14, 15]. Moreover, our recent study has suggested the role of altered expression of NFATs and FOXP3 in impaired Treg suppressive function [6]. Thus, impaired Tregs fail to control the ongoing autoimmune response leading to widespread self-reactive T cells activation, resulting in GV pathogenesis [6]. Overall, these studies highlight that Tregs and their suppressive molecules may represent a potential therapeutic target for developing Treg-based therapeutics for GV.

Inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) governs the release of calcium from the endoplasmic reticulum [22]. The exact role of ITPR1 in Treg function in GV is unknown. However, upon TCR stimulations in T cells, ITPR1 controls the release of stored calcium from the endoplasmic reticulum [33]. Our previous study has suggested that optimal calcium levels in Tregs are a prerequisite for NFATc1 activation in Treg of GV patients [32]. In current study, we found significantly reduced ITPR1 transcripts in GV patients. Moreover, our study suggested the role of decreased ITPR1 transcripts in GV progression and disease severity. Thus, the reduced ITPR1 expression in Tregs may lead to reduced intracellular Treg calcium levels, resulting in impaired NFAT signalling pathway (Figure 6). Finally, the impaired NFAT signalling pathway may lead to decreased downstream immunosuppressive molecules leading to impaired Treg suppressive function.

As altered ITPR1 transcripts may lead to impaired Treg activation and suppressive function, we assessed the mRNA expression of Treg-associated suppressive molecules GZMB, \( \text{UBASH3A} \), \( \text{S10} \), \( \text{GZMB} \), and \( \text{NRP1} \). We did not find any correlation between ITPR1 transcripts and SERPINB9 transcripts in GV patients’ Tregs \((r = 0.22, p = 0.2473)).\)

![Figure 4](image-url) Correlation of ITPR1 transcripts with GZMB, NRPI, and SERPINB9 transcripts in GV patients. The correlation of ITPR1 transcripts with GZMB, NRPI, and SERPINB9 transcripts were analyzed by Spearman’s correlation analysis. (a) ITPR1 transcripts positively correlated with GZMB transcripts in GV patients’ Tregs \((r = 0.45, p = 0.0009)).\) (b) ITPR1 transcripts positively correlated with NRPI transcripts in GV patients’ Tregs \((r = 0.52, p = 0.001)).\) (c) No correlation was observed between ITPR1 transcripts and SERPINB9 transcripts in GV patients’ Tregs \((r = 0.22, p = 0.2473)).\)
Figure 5: Continued.
NRPI, PDCD1, FASLG, and TNFRS18 in GV patients. Studies have suggested that Tregs control the immune response by granyme B-dependent cytotoxicity [34]. Upon TCR stimulation and receptor activation, Treg cells produce granzyme B, and previous studies have found increased CD107a expression upon Treg activation, suggesting extracellular degranulation of granzyme B [21, 37]. Anti-NRP-1 antibodies have been shown to abrogate Treg immunosuppressive function [38]. Additionally, lack of NRP-1 on surface of Tregs has been linked with impaired Treg suppressive function and worsening of experimental autoimmune encephalomyelitis severity [38]. However, the role of NRPI in GV is unknown. Interestingly, we found significantly reduced NRPI transcripts in GV patients, suggesting impaired NFATc1 transcript expression [39]. Additionally, NRP-1 reduced transcripts which led to impaired Treg suppressive function in GV patients (Figure 6). However, future studies must explore the involvement of ITTPR1-calcium-NFAT signalling pathway, promoter SNPs, and epigenetic changes in NRPI promoter, as they might be responsible for decreased NRPI expression.

Next, we evaluated the expression levels of Treg suppressive molecule neuropolin 1 (NRP-1). NRP-1 is constitutively expressed on the surface of Tregs and mediates prolonged binding with immature dendritic cells [19, 37]. Anti-NRP-1 antibodies have been shown to abrogate Treg immunosuppressive function [38]. Additionally, lack of NRP-1 on surface of Tregs has been linked with impaired Treg suppressive function and worsening of experimental autoimmune encephalomyelitis severity [38]. However, the role of NRPI in GV is unknown. Interestingly, we found significantly reduced NRPI transcripts in GV patients, suggesting for the crucial role of NRPI in reduced Treg suppressive function leading to GV pathogenesis. Moreover, we found a positive correlation of NRPI transcripts positively correlated with in vitro Treg-mediated suppression of CD8+ T cells in GV patients’ Tregs (r = 0.49; p = 0.032).

Figure 5: Correlation of in vitro Treg suppression assay with ITTPR1, GZMB, NRPI, and SERPINB9 transcripts in Tregs of GV patients. The correlation of in vitro Treg suppression assay with ITTPR1, GZMB, NRPI, and SERPINB9 was analyzed by Spearman’s correlation analysis. (a) GZMB transcripts were positively correlated with in vitro Treg mediated suppression of CD4+ T cells in GV patients’ Tregs (r = 0.61; p = 0.0012). (b) NRPI transcripts were positively correlated with in vitro Treg-mediated suppression of CD4+ T cells in GV patients’ Tregs (r = 0.55; p = 0.021). (c) SERPINB9 transcripts were positively correlated with in vitro Treg-mediated suppression of CD4+ T cells in GV patients’ Tregs (r = 0.56; p = 0.002). (d) ITTPR1 transcripts were positively correlated with in vitro Treg-mediated suppression of CD4+ T cells in GV patients’ Tregs (r = 0.54; p = 0.001). (e) GZMB transcripts positively correlated with in vitro Treg-mediated suppression of CD8+ T cells in GV patients’ Tregs (r = 0.58; p = 0.004). (f) NRPI transcripts positively correlated with in vitro Treg-mediated suppression of CD8+ T cells in GV patients’ Tregs (r = 0.52; p = 0.0022). (g) SERPINB9 transcripts positively correlated with in vitro Treg-mediated suppression of CD8+ T cells in GV patients’ Tregs (r = 0.48; p = 0.024). (h) ITTPR1 transcripts positively correlated with in vitro Treg-mediated suppression of CD8+ T cells in GV patients’ Tregs (r = 0.49; p = 0.032).
endogenous inhibitor SERPINB9 [21]. Previous studies have shown an increase in granzyme B-mediated apoptosis in SERPINB9 knockout mice, suggesting that Tregs upon activation can undergo self-inflicted apoptosis mediated by granzyme B in absence of SERPINB9 [21]. Interestingly, our study suggested significantly reduced SERPINB9 transcripts in GV Tregs. Moreover, we found a significant association of SERPINB9 transcripts with GV disease severity and disease progression. Additionally, previous studies have suggested significantly decreased Treg cells in vitiligo patients [6, 14, 15], suggesting that the increased SERPINB9 transcripts can lead to Treg cells apoptosis, resulting into decreased Tregs number, thereby contributing to GV pathogenesis, progression, and severity.
However, future studies must confirm these findings by studying granzyme caspases-mediated apoptosis pathway in Tregs of GV patients.

Next, we aimed to study the key Treg-associated transcription factors (GATA2, GATA3, RUNX1, STAT3, and STAT5) in GV patients. The GATA transcription factors are zinc finger motif DNA-binding proteins, and they have a crucial role in Treg function [26, 27]. Genetic ablation in GATA3 has been associated with inflammatory disorder in mice [26]. Additionally, mutations in GATA2 have been associated with autoimmune hepatitis [27]. Additionally, RUNX1 is a runt-related transcription factor, and it plays a crucial role in generation and function of Treg cells [25]. Moreover, STAT transcription factors, i.e., signal transducers of activation of transcription, control Treg cells development [24, 41]. Genetic knockdown of STAT3 decreases the Tregs generation [24]. Moreover, STAT5 plays a crucial role in sustaining FOXP3 expression in Tregs [41]. However, we did not find an association for Treg-associated transcription factors (GATA2, GATA3, RUNX1, STAT3, and STAT5) with GV pathogenesis, progression, and severity.

Previously, we had studied the in vitro Treg suppression assay in GV patients [6]. Our study had revealed significantly decreased in vitro Treg-mediated suppression of CD4+ and CD8+ T cells in GV patients [6]. To study the role of ITPRI, GZMB, NRPI, and SERPINB9 on in vitro Treg suppressive capacity, we carried out correlation analysis. Interestingly, we found a positive correlation for Treg-mediated suppression of CD4+ and CD8+ T cells with ITPRI, GZMB, NRPI, and SERPINB9 transcripts in GV patients' Tregs. Therefore, our results suggest that decreased ITPRI, GZMB, NRPI, and SERPINB9 transcripts might impair Treg suppressive function, resulting in widespread activation of CD4+ and CD8+ T cells, which could lead to GV pathogenesis (Figure 6).

Our previous study suggested that calcium treatment of Tregs increased the intracellular calcium influx in Tregs of GV patients, due to increased expression of calcium ion channel gene ORAI1 after calcium treatment [42]. Moreover, the optimum calcium influx enhanced the calcineurin and NFATC1 activity in calcium treated Tregs, which led to increased Treg suppressive capacity [32]. As ITPRI is crucial for the release of calcium in Tregs, we studied the transcript levels of ITPRI in calcium-treated Tregs of GV patients and controls. Interestingly, our study suggested an increase in mRNA expression of ITPRI in Tregs after the calcium treatment (Figure 3(d)). As the increased expression of ITPRI after the calcium treatment may further lead to enhanced Treg suppressive activity, we accessed the transcript levels of GZMB and SERPINB9 in calcium-treated Tregs of GV patients. Our study suggested that the calcium treatment increased the expression levels of GZMB and SERPINB9 in GV Tregs (Figures 2(a) and 3(a)). Our results are in line with the previous findings which suggest that calcium signalling modulates cytolytic T lymphocyte function [42]. Overall, our study suggests that calcium treatment may improve Treg suppressive capacity, and targeting the Ca2+-calmodulin-calcineurin-NFAT signalling pathway may be a potent therapeutic target for GV.

5. Conclusions

Overall, our results for the first time suggest the crucial involvement of GZMB, NRPI, SERPINB9, and ITPRI transcripts in reduced Treg-mediated suppression of CD4+ and CD8+ T cells which lead to GV pathogenesis, progression, and severity. Moreover, our study highlighted that ITPRI might be responsible for decreased GZMB and NRPI transcripts in GV Tregs. Furthermore, our study revealed that the increased SERPINB9 transcripts might result in endogenous granzyme B-mediated Tregs apoptosis, and the Treg suppressive capacity can be enhanced after calcium treatment in Tregs. These findings may aid in development of Treg-based therapeutics for GV; however, in vivo studies must be carried out to validate the role of GZMB, NRPI, SERPINB9, and ITPRI in Treg-mediated GV pathogenesis.

Data Availability

Data available on request.

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 (IHREC), Malibya 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.

Consent

Informed consent was obtained from all patients and control participants of the study.

Conflicts of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

Authors’ Contributions

Prashant S. Giri has contributed to the investigation, methodology, validation, analysis, and writing-original draft preparation. Ankit H Bhart has contributed to the resources (patients' samples), clinical information, and analysis. Mitesh Dwivedi has contributed to the conceptualization, funding acquisition, investigation, methodology, resources, supervision, visualization, and writing-revewing and editing.
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Supplementary Materials

Supplementary 1. Table S1. Primer sequence for transcript analysis of GZMB, NRPI, ITPRI, SERPINB9, PDCD1, FASLG, UBASH3A, IKZF4, GATA2, GATA3, TNFRSF18, RUNX1, STAT3, and STAT5 genes by qPCR.

Supplementary 2. Figure S1. Melt curve analysis for GZMB, NRPI, ITPRI, SERPINB9, PDCD1, FASLG, UBASH3A, and IKZF4 transcripts. (a) Representative image for melt curve analysis for GZMB transcripts. (b) Representative image for melt curve analysis for NRPI transcripts. (c) Representative image for melt curve analysis for ITPRI transcripts. (d) Representative image for melt curve analysis for SERPINB9 transcripts. (e) Representative image for melt curve analysis for PDCD1 transcripts. (f) Representative image for melt curve analysis for FASLG transcripts. (g) Representative image for melt curve analysis for UBASH3A transcripts and (h) Representative image for melt curve analysis for IKZF4 transcripts. Figure S2: Melt curve analysis for GATA2, GATA3, TNFRSF18, RUNX1, STAT3, STAT5, and GAPDH transcripts. (a) Representative image for melt curve analysis for GATA2 transcripts. (b) Representative image for melt curve analysis for GATA3 transcripts. (c) Representative image for melt curve analysis for TNFRSF18 transcripts. (d) Representative image for melt curve analysis for RUNX1 transcripts. (e) Representative image for melt curve analysis for STAT3 transcripts. (f) Representative image for melt curve analysis for STAT5 transcripts. (g) Representative image for melt curve analysis for GAPDH transcripts. Figure S3. Standard curve for estimation of calcium levels: standard curve for calcium ELISA. Figure S4. PDCD1, FASLG, and TNFRSF18 transcripts in Tregs of GV patients and controls. PDCD1, FASLG, and TNFRSF18 transcripts in Tregs of 52 GV patients and 48 controls were analyzed by nonparametric Mann–Whitney U test. (a) PDCD1 transcripts in GV, SV, and AV vs controls’ Tregs (p = 0.4625, p = 0.2563, and p = 0.3627, respectively). (b) PDCD1 transcripts in GV vs SV Tregs (p = 0.1273). (b) PDCD1 transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.2427). PDCD1 transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.1225 and p = 0.1423, respectively). (c) There were 0.88-, 0.87-, and 0.98-fold changes in PDCD1 transcripts for GV, SV, and AV Tregs when compared to controls. (d) FASLG transcripts in GV, SV, and AV vs controls’ Tregs (p = 0.753, p = 0.999, and p = 0.213, respectively). FASLG transcripts in AV vs SV Tregs (p = 0.521). (e) FASLG transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.113). FASLG transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.7123 and p = 0.2321, respectively). (f) There were 0.72-, 0.65-, and 0.77-fold changes in FASLG transcripts for GV, SV, and AV Tregs as compared to controls. (g) TNFRS18 transcripts in GV, SV, and AV vs controls’ Tregs (p = 0.3362, p = 0.1272, and p = 0.3627, respectively). TNFRS18 transcripts in AV vs SV Tregs (p = 0.1265). (h) TNFRS18 transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.4653). TNFRS18 transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.1263 and p = 0.7625, respectively). (i) There were 0.86-, 0.87-, and 0.85-fold changes in TNFRS18 transcripts for GV, SV, and AV Tregs as compared to controls. Figure S5. UBASH3A, GATA2, and GATA3 transcripts in Tregs of GV patients and controls. UBASH3A, GATA2, and GATA3 transcripts in Tregs of 52 GV patients and 48 controls were analyzed by nonparametric Mann–Whitney U test. (a) UBASH3A transcripts in GV, SV, and AV vs controls’ Tregs (p = 0.145, p = 0.127, and p = 0.647, respectively). UBASH3A transcripts in AV vs SV Tregs (p = 0.487). (b) UBASH3A transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.2347). UBASH3A transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.1252 and p = 0.1625, respectively). (c) There was a 0.92-, 0.89-, and 1.1-fold changes in UBASH3A transcripts for GV, SV, and AV Tregs as compared to controls. (d) GATA2 transcripts in GV, SV, and AV vs controls’ Tregs (p = 0.1537, p = 0.1922, and p = 0.1189, respectively). GATA2 transcripts in AV vs SV Tregs (p = 0.7891). (e) GATA2 transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.1922). GATA2 transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.7654 and p = 0.1165, respectively). (f) There was a 0.89-, 0.86-, and 0.84-fold changes in GATA2 transcripts for GV, SV, and AV Tregs as compared to controls. (g) GATA3 transcripts in GV, SV, and AV vs controls’ Tregs (p = 0.2224, p = 0.86322, and p = 0.1152, respectively). GATA3 transcripts in AV vs SV Tregs (p = 0.3641). (h) GATA3 transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.8742). GATA3 transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.1645 and p = 0.8873, respectively). (i) There was a 0.89-, 0.92-, and 0.97-fold changes in GATA3 transcripts for GV, SV, and AV Tregs as compared to controls. Figure S6. RUNX1, STAT3, and STAT5 transcripts in Tregs of GV patients and controls. RUNX1, STAT3, and STAT5 transcripts in Tregs of 52 GV patients and 48 controls were analyzed by nonparametric Mann–Whitney U test. (a) RUNX1 transcripts in GV, SV,
and AV vs controls' Tregs (p = 0.1152, p = 0.1254, and p = 0.1455, respectively). (b) RUNX1 transcripts in AV vs SV Tregs (p = 0.2235). (b) RUNX1 transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.1455). RUNX1 transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.1152 and p = 0.1164, respectively). (c) There was a 0.11-, 1.2-, and 0.97-fold changes in RUNX1 transcripts for GV, SV, and AV Tregs when compared to controls. (d) STAT3 transcripts in GV, SV, and AV vs controls' Tregs (p = 0.2333, p = 0.2143, and p = 0.3541, respectively). STAT3 transcripts in AV vs SV Tregs (p = 0.3436). (e) STAT3 transcripts in 50-75% severe GV vs 10-25% mild GV Tregs (p = 0.4222). STAT3 transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.1455 and p = 0.8423, respectively). (f) There was a 1.01-, 1.3-, and 1.0-fold changes in STAT3 transcripts for GV, SV, and AV Tregs as compared to controls. (g) STAT5 transcripts in GV, SV, and AV vs controls' Tregs (p = 0.1454, p = 0.2241, and p = 0.1535, respectively). STAT5 transcripts in AV vs SV Tregs (p = 0.4353). (h) STAT5 transcripts in severe GV (50-75% VASI) vs mild GV (10-25% VASI) Tregs (p = 0.2532). STAT5 transcripts in moderate GV (25-50% VASI) vs mild GV (10-25% VASI) and severe GV (50-75% VASI) Tregs (p = 0.1432 and p = 0.1484, respectively). (i) There was a 0.99-, 1.1-, and 0.94-fold changes in STAT5 transcripts for GV, SV, and AV Tregs as compared to controls. Figure S7. Age of onset analysis for GZMB, NRPI, PDCDI, FASLG, TNFRS18, SERPINB9, and ITPR1 transcripts in GV Tregs and control Tregs. (a) GZMB transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1263; 1-20 vs 41-60 years: p = 0.6532; 21-40 vs 41-60 years: p = 0.9836). (b) NRPI transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1563; 1-20 vs 41-60 years: p = 0.8832; 21-40 vs 41-60 years: p = 0.6863). (c) PDCDI transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1348; 1-20 vs 41-60 years: p = 0.2842; 21-40 vs 41-60 years: p = 0.4923). (d) FASLG transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1323; 1-20 vs 41-60 years: p = 0.2932; 21-40 vs 41-60 years: p = 0.1937). (e) TNFRS18 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.4627; 1-20 vs 41-60 years: p = 0.2847; 21-40 vs 41-60 years: p = 0.2948). (f) SERPINB9 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.3533; 1-20 vs 41-60 years: p = 0.4242; 21-40 vs 41-60 years: p = 0.4363). (g) ITPR1 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.2648; 1-20 vs 41-60 years: p = 0.3762; 21-40 vs 41-60 years: p = 0.2635). Figure S8. Age of onset analysis for UBASH3A, GATA2, GATA3, RUNX1, STAT3, and STAT5 transcripts in GV Tregs and control Tregs. (a) UBASH3A transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.4836; 1-20 vs 41-60 years: p = 0.5247; 21-40 vs 41-60 years: p = 0.1938). (b) GATA2 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1321; 1-20 vs 41-60 years: p = 0.1380; 21-40 vs 41-60 years: p = 0.5221). (c) GATA3 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1241; 1-20 vs 41-60 years: p = 0.5384; 21-40 vs 41-60: p = 0.2411). (d) RUNX1 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1434; 1-20 vs 41-60 years: p = 0.3210; 21-40 vs 41-60 years: p = 0.1281). (e) STAT3 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.4826; 1-20 vs 41-60 years: p = 0.3292; 21-40 vs 41-60 years: p = 0.1196). (f) STAT5 transcript levels in Tregs of different age of onset groups (1-20 vs 21-40 years: p = 0.1284; 1-20 vs 41-60 years: p = 0.3421; 21-40 vs 41-60 years: p = 0.1248). Figure S9. Gender-based analysis for GZMB, NRPI, PDCDI, FASLG, TNFRS18, SERPINB9, and ITPR1 transcripts in GV Tregs and control Tregs. (a) GZMB transcript levels in Tregs of male and female GV patients (p = 0.2462). (b) NRPI transcript levels in Tregs of male and female GV patients (p = 0.1739). (c) PDCDI1 transcript levels in Tregs of male and female GV patients (p = 0.5642). (d) FASLG transcript levels in Tregs of male and female GV patients (p = 0.4852). (e) TNFRS18 transcript levels in Tregs of male and female GV patients (p = 0.6737). (f) SERPINB9 transcript levels in Tregs of male and female GV patients (p = 0.4513). Figure S10. Gender-based analysis for UBASH3A, GATA2, GATA3, RUNX1, STAT3, and STAT5 transcripts in GV Tregs and control Tregs. (a) UBASH3A transcript levels in Tregs of male and female GV patients (p = 0.4547). (b) GATA2 transcript levels in Tregs of male and female GV patients (p = 0.6780). (c) GATA3 transcript levels in Tregs of male and female GV patients (p = 0.6734). (d) RUNX1 transcript levels in Tregs of male and female GV patients (p = 0.6797). (e) STAT3 transcript levels in Tregs of male and female GV patients (p = 0.7621).

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