Chapter 3

To carry out immunophenotypic analysis of VTCN1 in vitiligo patients

3.1 Introduction

Generalized vitiligo (GV) is an autoimmune skin disfiguring disease typified by bilateral, symmetrical milky white patches all around the body. Early studies carried out for understanding vitiligo pathogenesis showed no significant difference in lymphocyte subpopulations in vitiligo patients and healthy individuals (Ortonne et al., 1978). Later on many experimental evidences highlighted breakdown in tolerance to melanocytes resulting in its destruction mediated by melanocyte specific cytotoxic T cells and autoantibodies (Dwivedi et al., 2013; Laddha et al., 2014; Le Poole et al., 1996). Increased number of cytotoxic $CD8^+$ T cells in the blood of vitiligo patients and its positive correlation with disease activity, *in vitro* experiments demonstrating of normal human melanocyte death by $CD8^+$ T cells isolated from vitiligo patient’s blood, increased infiltration of $CD8^+$ T cells into vitiligo skin lesions etc. suggest the involvement of immune cells in destruction of melanocyte during disease progression (Lang et al., 2001; Ogg et al., 1998; Palermo et al., 2001; Picardo et al., 2015).

Several regulatory mechanisms have been reported to induce tolerance and inhibit autoimmune response such as expression of costimulatory molecules, thymic cell selection and presence of regulatory T cells etc. Thymic negative selection is an important mechanism which renders the developing T cell tolerant to self-MHC/ peptide ligands. In contrast to that unusual large number of $CD8^+$ T cells specific to Melan-A/MART-1 was confirmed prenatally but the reason behind it remained mysterious (Pinto et al., 2014). Researchers found T cells expressing high levels of the skin homing receptors associated antigens in vitiligo patients which correlated with disease activity and depigmentation (Lang et al., 2001). Mystery behind presence of melanocyte specific autoreactive T cell generation is not well documented. Several reports showed that excessive co-stimulation and/ or insufficient co-inhibition results into abnormal T cell activation and breakdown in self-tolerance leading to autoimmunity (Yamada et al., 2002; Zhang et al., 2016). Previous reports mentioned these costimulatory &/ coinhibitory molecules on lymphocytes and APCs/Melanocytes were in complex cross talk (Alpdogan et al., 2012). Whether these molecules play any role in vitiligo development and progression? Are they the culprits for developing the autoimmune diseases? To get these answers we did the literature review and found that expression of these costimulatory molecules hampered in autoimmune and inflammatory diseases (Zhang et al., 2016). It is now well established that imbalance in costimulatory or coinhibitory signaling on T cells may have a role in triggering autoimmunity leading to autoimmune complications like vitiligo (Speeckaert et al., 2017a). Elevated expression of coinhibitory molecules such as PD-1, Tim-3 and galectin-9 was also reported in vitiligo

patients' blood and skin and correlated with disease activity (Rahimi et al. 2019). All members of B7 family members and their respective receptors are summarized in Figure 3.1 (Zhao et al., 2020).

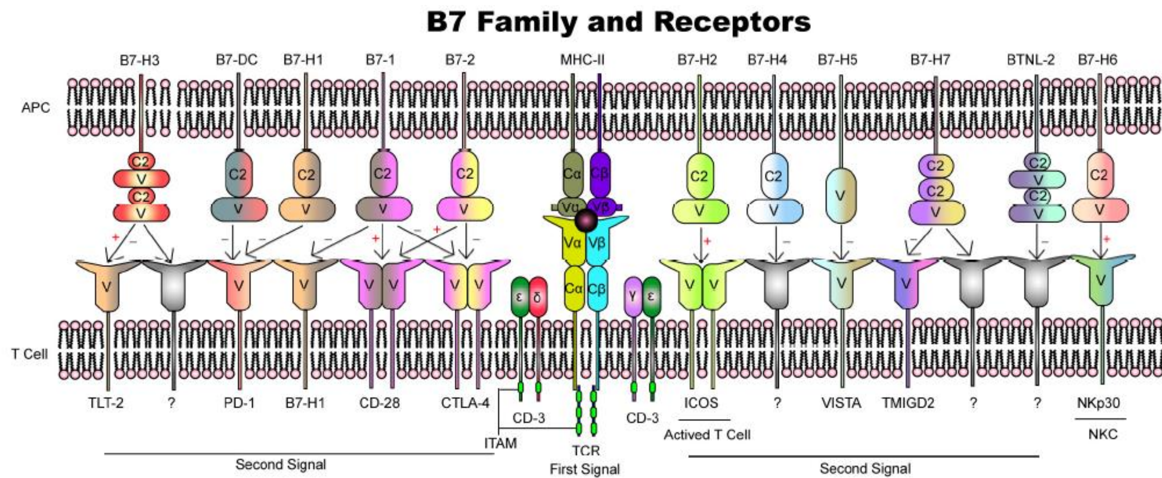

Figure 3.1: B7 family members and their receptors (Zhao et al., 2020).

Among the various costimulatory molecules, VTCN1 (V-set domain containing T cell activation inhibitor 1) also called B7-H4, B7S1, and B7x has attracted more attention due to its immunosuppressive role and involvement in regulation of innate and adaptive immune system (John

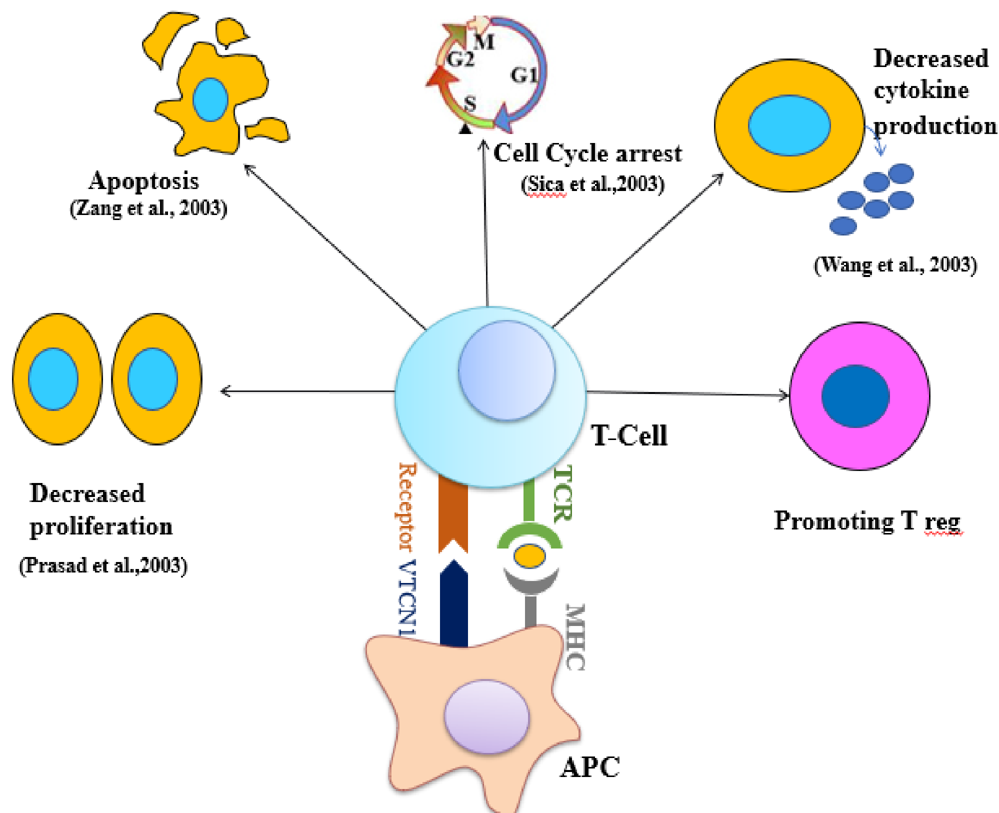

Figure 3.2: Effect of VTCN1 upon binding with its receptor

et al., 2019). It inhibits T cell proliferation via cell cycle arrest, reducing cytokine production and promoting activity of Treg cells by binding with its unknown receptor on T cells (Figure 3.2) (Podojil et al., 2018; Prasad et al., 2003; Sica et al., 2003; Zang et al., 2003).

Immunosuppressive role of VTCN1 was supported by experimental evidences such as aggressive diabetes developed in VTCN1 deficient mice, while complete abolition of diabetes was observed in mice with overexpression of VTCN1 (Wei et al., 2011). Similar to that in mouse model for kidney disease, VTCN1 knockout mice developed severe renal disease but VTCN1-Ig treatment showed decreased inflammation and kidney damage (Pawar et al., 2015). The role of VTCN1 has been evaluated and documented in various autoimmune diseases such as rheumatoid arthritis (Azuma et al., 2009), type-1 diabetes (Radichev et al., 2014), systemic lupus erythromatosus (SLE) (Xiao et al., 2017), multiple sclerosis (Wei et al., 2011). Involvement of VTCN1 and its expression is also well documented in ovarian cancer, breast cancer, renal cell carcinoma, lung cancer, melanoma, gastric cancer, colorectal cancer, pancreatic cancer and prostate cancer (Arigami et al., 2011; Chen et al., 2014; Krambeck et al., 2006; Kryczek et al., 2007; Mugler et al., 2007; Quandt et al., 2011; XU et al., 2014; Zang et al., 2007; Zhang et al., 2013; Zhang et al., 2013; Zhao et al., 2014). Though the extensive research on the role of VTCN1 in different cancers and autoimmune diseases has been carried out, its expression on immune cells is controversial. Early study done by Sica et al., reported low expression of VTCN1 on human T cell, B cells, monocytes and dendritic cells which is inducible upon *in vitro* stimulation, whereas another study by Wei et al., did not found VTCN1 expression on either human or murine immune cells with or without stimulation (Sica et al., 2003)(Wei et al., 2011). So, we performed immunophenotypic analysis of VTCN1 on different immune cell subpopulations from the blood and skin of healthy individuals and vitiligo patients.

3.2 Materials and methods:

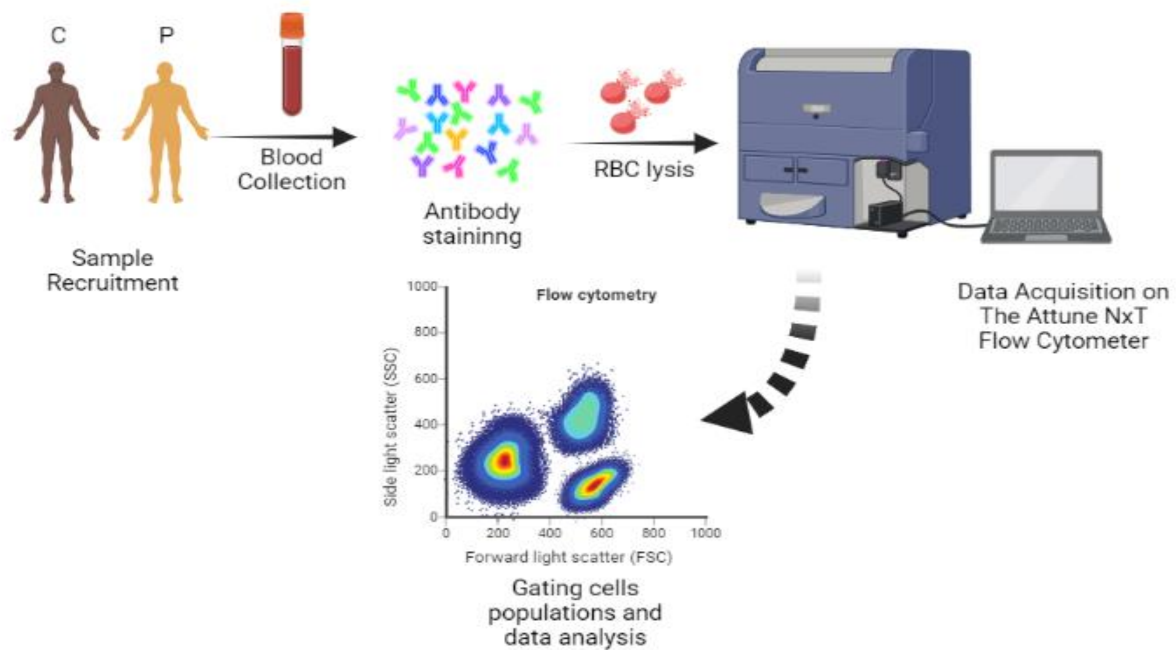

Figure 3.3: Overall strategy for immunophenotyping.

3.2.1 Ethical Committee Approval

The study plan and consent forms were approved by the Institutional ethical committee for human research (IECHR), Medical College Baroda, Faculty of Medicine, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (EC Reg No: ECR/85/Inst/GJ/2013/RR-16). Written consent was obtained from the next of kin, caretakers, or guardians on behalf of the minors/children enrolled in the study and significance of the study was explained.

3.2.2 Study population

The present study included 25 generalized active vitiligo patients and 30 healthy controls from S.S.G Hospital, Vadodara, Gujarat, India. The demographic details of patients and controls are given in Table 3.1. The inclusion criteria followed were: outpatients of age between 5 to 60 years and both the parents should be Gujarati by birth. Patients with other diseases and those unwilling to participate in the study were excluded. The diagnosis of vitiligo by dermatologists was clinically based on characteristic skin depigmentation with typical localization and white color lesions on the skin, under Woods lamp. Generalized or non-segmental vitiligo (NSV/GV) was characterized by depigmented patches varying in size from a few to several centimeters in diameter, involving one or both sides of the body with a tendency towards symmetrical distribution and active vitiligo (AV) was defined as

the appearance of new lesions and spreading of existing lesions observed during past two-years' duration (Ezzedine et al. 2012; Zhang et al. 2016).

Table 3.1: Demographic characteristics of vitiligo patients and unaffected controls for immunophenotyping from blood.

	Generalized Vitiligo Patients	**Controls**
	(n = 25)	(n = 30)
Average age (mean age ± SD)	37.83 ± 4.012 yr	35.45 ± 3.260 yr
Sex: Male	13 (52%)	16 (53.33%)
Female	12 (48%)	14 (46.66%)
Age of onset (mean age ± SD)	28.14±17.55 yr	NA
Duration of disease (mean ± SD)	24.6±10.60 yr	NA

3.2.3 Flow cytometric analysis of leucocytes from blood samples

Peripheral blood samples were obtained by venipuncture in K_3-EDTA tubes (BTL research labs) and stored at 4°C. Whole blood staining was performed on the same day using eleven directly conjugated monoclonal antibodies (mAbs) for T cells and APC cells from Thermo Fisher Scientific (Table 1). Briefly, 100 µl of blood was incubated with antibodies for 1 hour at room temperature in the dark, followed by RBCs lysis using 2 ml of RBC Lysing solution (BD FACS™Lysing solution, 349202) for 20 min. at room temperature. Cells were washed using FACS wash buffer, pelleted, and resuspended using 300 µl 1% paraformaldehyde. Samples were acquired on the Attune™ NxT Flow Cytometer (Thermo Fisher Scientific). Singlet cells were gated for leucocyte subpopulations and then VTCN1 expression was determined using different surface markers of T cells and APC cells using quadrant plots in Flow Jo software (Tree Star, Ashland, OR).

3.2.4 Flow cytometric analysis of leucocytes from skin samples

Skin samples were collected from 13 GV patients and 10 healthy controls with the help of expert dermatologist using 3 mm punch biopsies and transported to laboratory in transport media (Demographic details in Table 3.3). Single cell suspension was prepared in RPMI 1640 media and incubated with T cell panel as given in Table 1. After 1 hour incubation with primary tagged monoclonal antibodies for different T cell population, cells were washed using FACS wash buffer, pelleted, and resuspended using 300 µl 1% paraformaldehyde. Samples were acquired on the Attune™ NxT Flow Cytometer (Thermo Fisher Scientific). Singlet cells were gated for T cell subpopulations and then VTCN1 expression was determined using different surface markers of T cells using quadrant plots in Flow Jo software (Tree Star, Ashland, OR).

Table 3.2: List of antibodies used in immunophenotypic analysis.

Sr. No	Marker	Clone	Fluorochrome	Company	Catalogue
1	B7H4	H74	PE	Thermo Fisher Scientific	12-5949-42
2	CD3	SK7	APC-eFluor® 780	Thermo Fisher Scientific	47-0036-42
3	CD4	RM4-5	Super Bright 780	Thermo Fisher Scientific	78-0042-82
4	CD8a	SK1	Alexa Fluor® 700	Thermo Fisher Scientific	56-0087-42
5	CD11c	3.9	PE-Cyanine7	Thermo Fisher Scientific	25-0116-42
6	CD25	CD25-4E3	PE	Thermo Fisher Scientific	12-0257-42
7	CD69	FN50	PE-Cyanine7	Thermo Fisher Scientific	25-0699-42
8	CD80	MEM-233	APC	Thermo Fisher Scientific	MA1-19464
9	CD127	eBioRDR5	Alexa Fluor® 488	Thermo Fisher Scientific	53-1278-42
10	HLA-DR/DP/DQ	WR18	PE	Thermo Fisher Scientific	MA1-80680
11	CD19	SJ25-C1	PE-Cyanine7	Thermo Fisher Scientific	MHCD1912
12	CD14	Tuk4	FITC	Thermo Fisher Scientific	MHCD1401

Table 3.3: Demographic characteristics of vitiligo patients and unaffected controls for immunophenotyping from skin.

	Vitiligo Patients	**Controls**
	(n = 13)	(n = 10)
Average age (mean age ± SD)	35.67 ± 5.26 yr	27.80 ± 4.45 yr
Sex: Male	7 (60%)	5 (50%)
Female	6 (40%)	5 (50%)
Age of onset (mean age ± SD)	21.36 ± 3.51 yr	NA
Duration of disease (mean ± SD)	7.262 ± 8.60 yr	NA

3.2.5 Statistical Analysis:

Different subsets of T cells and APC cells were compared between controls and vitiligo patients using an unpaired Student's t-test. Protein expression analysis of pSTAT3, total STAT3, VTCN1 and β-Actin upon different cytokine treatment were compared using one-way ANOVA for multiple comparisons. Differences were considered as significant at $p \leq 0.05$. All the statistical analyses were done and graphs were plotted using Graph Pad Prism 8 software (Graph Pad Software Inc; 2003).

3.3 Results

3.3.1 Gating Strategy

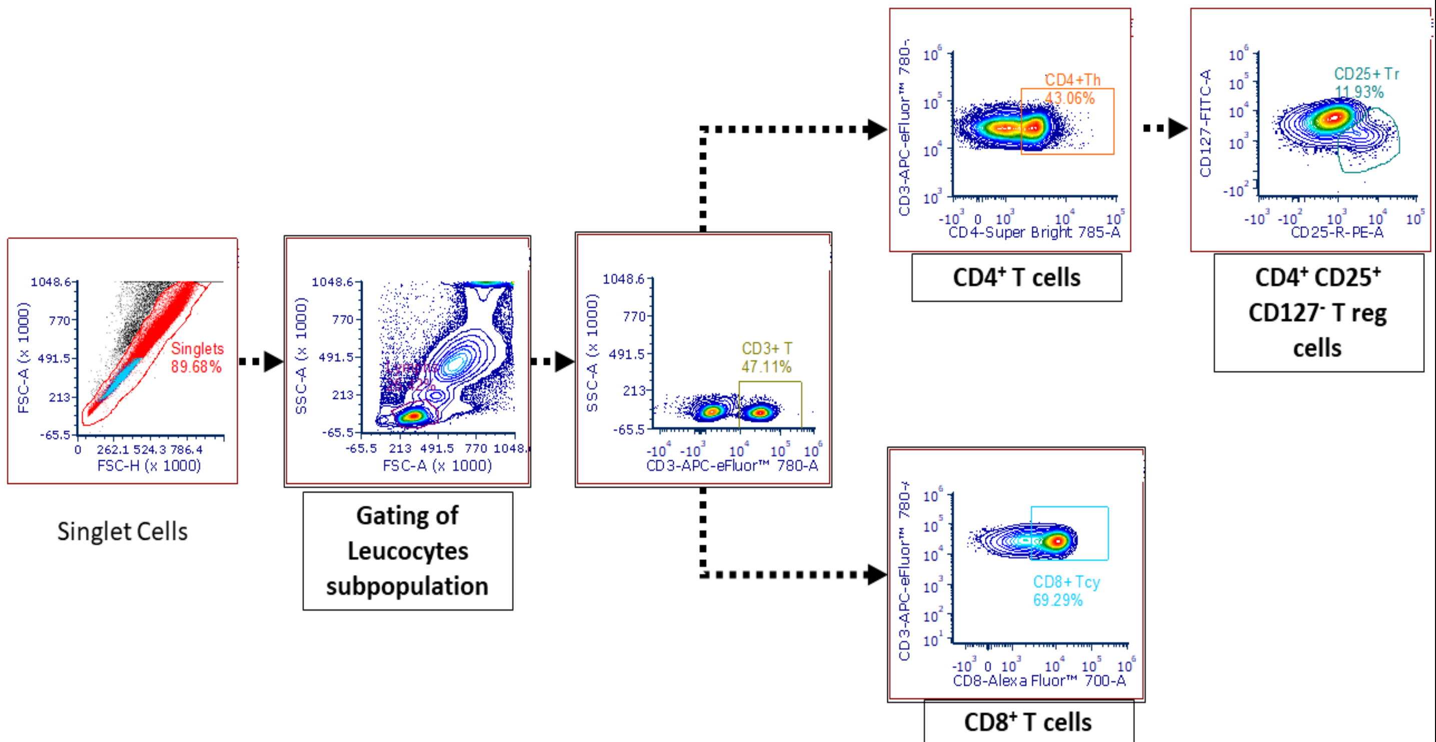

Figure 3.4: Gating strategy for subtyping of T cells into $CD4^+$, $CD8^+$ T cells and regulatory T cells.

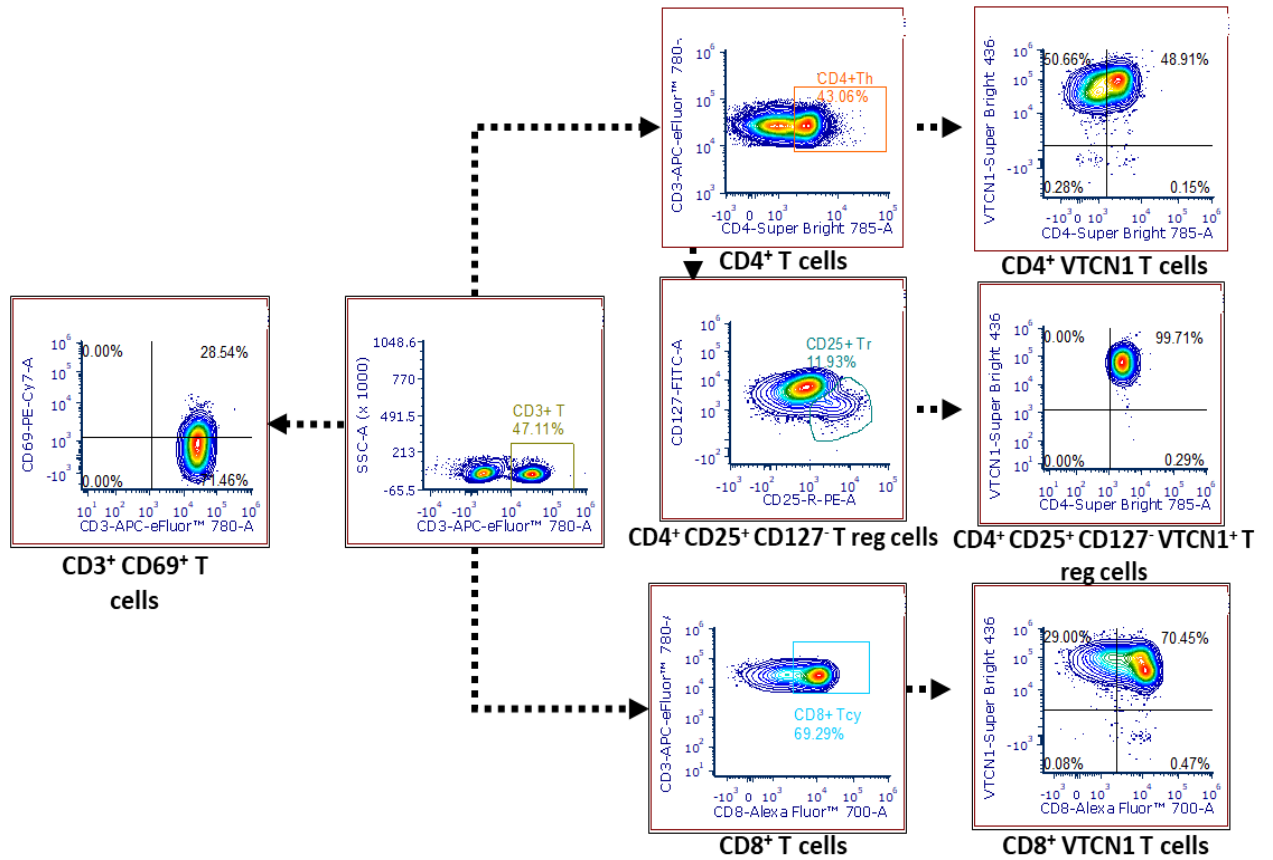

Figure 3.5: Gating strategy for VTCN1 positive CD4, CD8 and regulatory T cells.

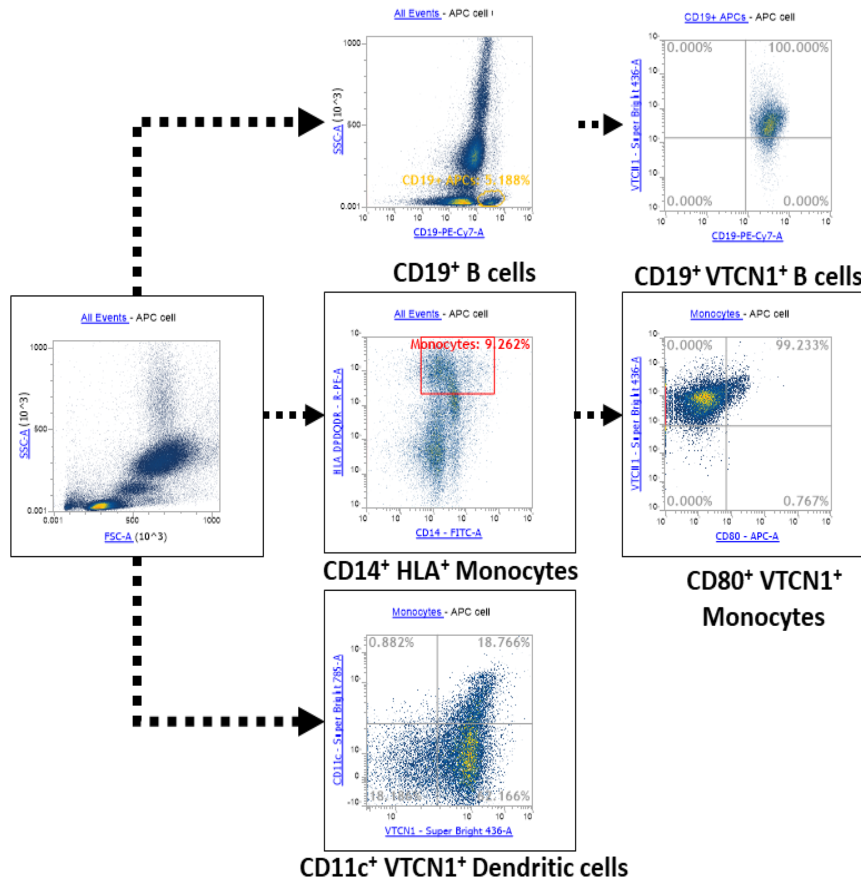

Figure 3.6. Gating strategy for VTCN1 positive APC cells (Dendritic cells, B cells, Monocytes).

3.3.2 Immunophenotyping of VTCN1 on different immune cells from Blood

Immunophenotypic analysis of VTCN1 on different immune cells from the blood of generalized active vitiligo patients and healthy controls revealed interesting findings. The percentage of lymphocytes in the peripheral blood of vitiligo patients was reduced compared to healthy individuals (*p*=0.026, Figure 1A). Further subtyping of $CD3^+$ T cells using CD4 and CD8 markers revealed no significant difference in $CD4^+$ cells in vitiligo patients as compared to controls (*p*=0.427, Figure 1B) however, $CD8^+$ cells were significantly increased in vitiligo patients (*p*=0.034, Figure 1C). As a result, significant decrease was observed in $CD4^+/CD8^+$ T cell ratio (*p*=0.038, Figure 1D). Upon analysis of $CD4^+$ and $CD8^+$ T cells for VTCN1 expression, we found a significant decrease in percentage of $CD4^+$ $VTCN1^+$ T cells (*p*=0.030, Figure 1E) and $CD8^+VTCN1^+$ T cells (*p*=0.004, Figure 1F) in vitiligo patients compared to controls. Next, we examined the activation of both T cells by using the early T cell activation marker CD69. Interestingly, we found significant increase in activated T cells of vitiligo patients (*p*=0.001, Figure 2A) along with significantly decreased $CD8^+$ $CD69^+$ $VTCN1^+$ positive cells (*p*=0.016, Figure 2C). As an important player in maintaining peripheral tolerance, exploring the status of VTCN1 expression on Treg cells in vitiligo patients is interesting. The analysis revealed significant decrease in the percentage of $CD4^+$ $CD25^+$ $CD127^-$ Treg cells (*p*=0.009, Figure 2D) along with a significant decrease in VTCN1 expression in vitiligo patients (*p*=0.012 Figure 2E).

Additionally, we used CD19 marker for the B cells and found significant increase in $CD19^+$ cells in vitiligo patients as compared to healthy controls (*p*=0.004 Figure 3A). We observed no significant difference in VTCN1 expression on B cells of vitiligo patients compared to controls (*p*= 0.99, Figure 3B). Moreover, we found significant increase in monocyte population in vitiligo patients as compared to controls (*p*=0.003, Figure 3C). CD11c was used as dendritic cell marker. Interestingly, we found significant decrease in VTCN1 expression on dendritic cells (*p*=0.028, Figure 3D). When we analyzed monocytes for the expression of positive costimulatory marker CD80 and negative costimulatory molecule VTCN1, we did not find any significant change in the percentage of CD80 and VTCN1 dual positive cell population in vitiligo patients compared to controls (*p*=0.290, Figure 3E).

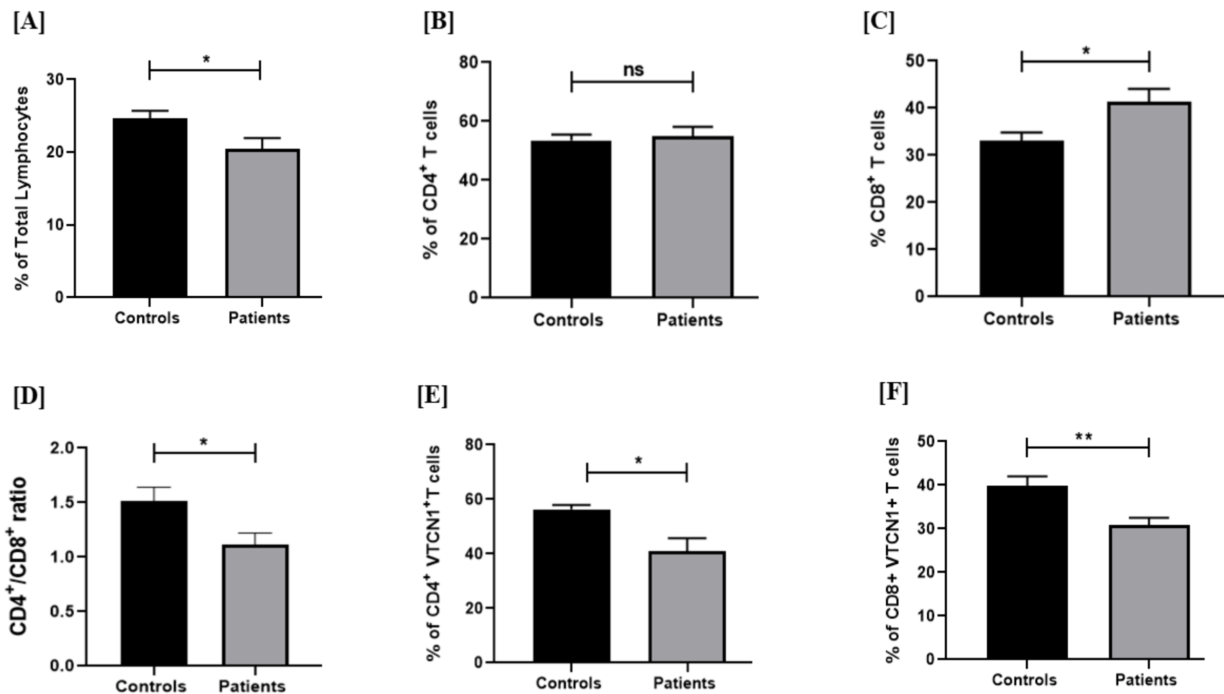

Figure 3.7: Immunophenotypic analysis of VTCN1 on different T cells from the blood of 25 generalized active vitiligo patients and 30 healthy controls. **[A]** The total percentage of lymphocyte count in the peripheral blood of vitiligo patients was reduced compared to healthy individuals (Mean±SEM: 20.67 ± 1.51 vs 24.63 ± 1.07, respectively; *p*=0.026). **[B]** No significant difference was observed in $CD4^+$ cells in vitiligo patients compared to controls (Mean±SEM: 55.04 ± 3.04 vs. 53.56 ± 2.03, respectively; *p*=0.427). **[C]** $CD8^+$ T cells found significantly increased compared to controls (Mean±SEM: 41.31 ± 2.81 vs. 33.10 ± 1.71, respectively; *p*=0.034). **[D]** Significant decrease was observed in $CD4^+/CD8^+$ T cell ratio in vitiligo patients compared to controls (Mean±SEM: 1.11 ± 0.12 vs. 1.51 ± 0.10, respectively; *p*=0.038). **[E]** Analysis of $CD4^+$ cells for VTCN1 expression revealed a significant decrease in percentage of $CD4^+$ $VTCN1^+$ T cells in vitiligo patients compared to controls (Mean±SEM: 40.98 ± 4.78 vs.56.36 ± 1.36 respectively; *p*=0.030). **[F]** Significant decrease in percentage of $CD8^+VTCN1^+$ T cells was observed in vitiligo patients compared to controls (Mean±SEM: 30.83 ± 1.70 vs. 39.91 ± 2.11, respectively; *p*=0.004).

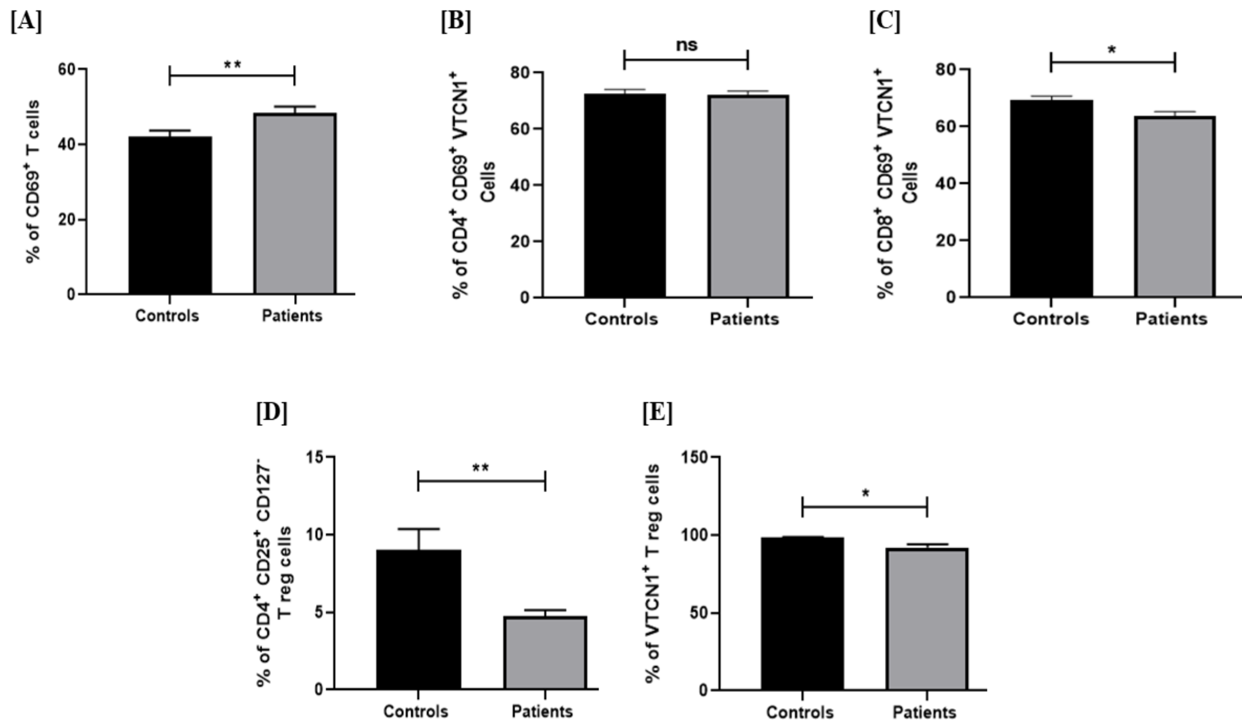

Figure 3.8: Immunophenotypic analysis of VTCN1 on activated T cells and Treg cells from the blood of 25 generalized active vitiligo patients and 30 healthy controls. **[A]** Significant increase in number of activated $CD69^+$ T cells of vitiligo patients (Mean±SEM: 48.49 ±1.64 vs. 42.14 ± 1.61 respectively; p=0.001) **[B]** No significant difference in $CD4^+$ $CD69^+$ $VTCN1^+$ cells in vitiligo patients compared to controls (Mean±SEM:72.03 ± 1.46 vs. 72.40 ± 1.76, respectively; p=0.450). **[C]** Significantly decreased percentage of $CD8^+$ $CD69^+$ $VTCN1^+$ positive cells in vitiligo patients compared to controls (Mean±SEM: 63.73 ±1.58 vs. 69.28 ± 1.51 respectively; p=0.016). **[D]** Significant decrease in the percentage of $CD4^+$ $CD25^+$ $CD127^-$ Treg cells in vitiligo patients compared to controls (Mean±SEM: 4.73 ±0.41 vs. 9.00 ± 1.35 respectively; p=0.009) was observed. **[E]** Significant decrease in VTCN1 expression on regulatory T cells in vitiligo patients (Mean±SEM: 92.13 ±2.00 vs. 98.45 ± 0.37 respectively; p=0.012).

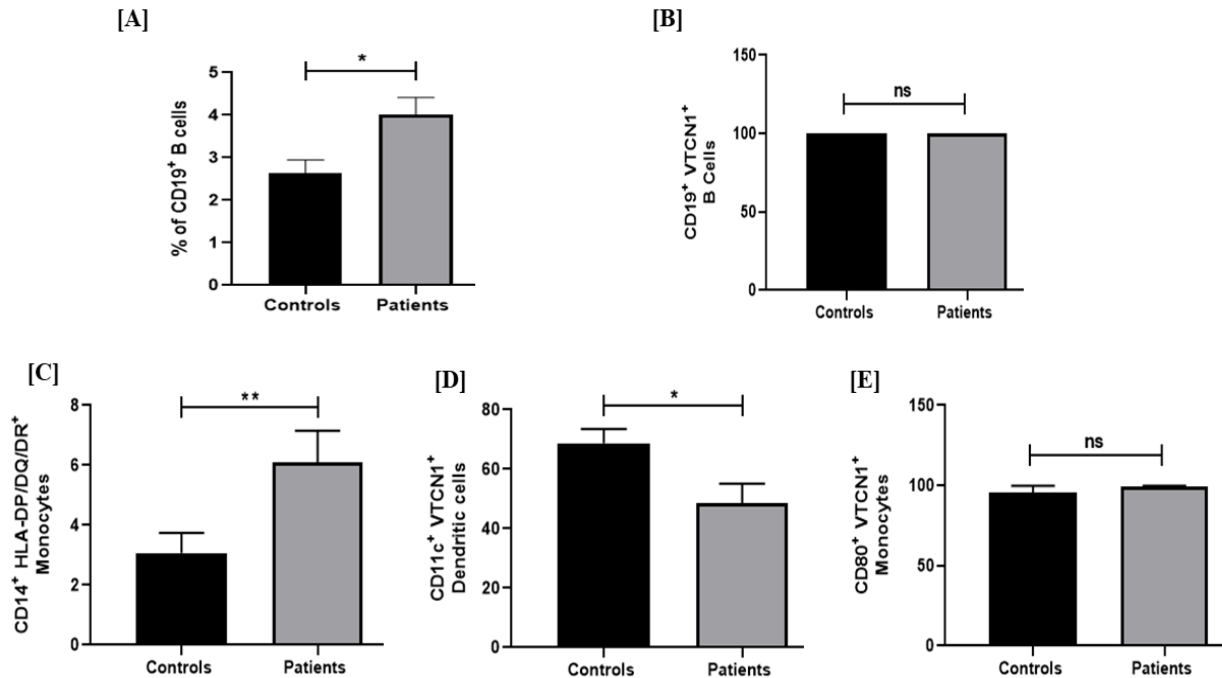

Figure 3.9: Immunophenotypic analysis of VTCN1 on different APC (Antigen presenting cells) from the blood of 25 generalized active vitiligo patients and 30 healthy controls. **[A]** Significant increase in $CD19^+$ cells of vitiligo patients as compared to healthy controls was observed (Mean±SEM: 4.41 ±0.44 vs. 2.49 ± 0.35 respectively; p=0.004). **[B]** No significant difference observed in VTCN1 expression on B cells of vitiligo patients compared to controls (p= 0.99). **[C]** Significant increase was observed in number of monocyte cells in vitiligo patients as compared to controls (Mean±SEM: 6.08 ±1.06 vs. 3.07 ± 0.66 respectively; p=0.003). **[D]** Significant decrease in VTCN1 expression on dendritic cells (Mean±SEM: 45.31 ±6.39 vs. 64.56 ± 5.49 respectively; p=0.028). **[E]** No significant change was observed in number of CD80 and VTCN1 dual positive cells (Mean±SEM: 99.71 ±0.07 vs. 99.77 ± 0.06 respectively; p=0.290).

3.3.3 Immunophenotyping of VTCN1 on different T cells isolated from Skin

The results from immunophenotypic analysis of VTCN1 on different immune cells from the skin of vitiligo patients and healthy controls unveiled few fascinating observations. After subtyping $CD3^+$ T cells using markers for CD4 and CD8 showed that there were no significant alterations in $CD4^+$ T cells in lesional and non-lesional skin of vitiligo patients as compared to healthy human skin (p=0.9580 and p=0.6998 respectively; Figure 4A) but there was a significant increase in $CD8^+$ T cells in lesional and non-lesional skin of vitiligo patients as compared to healthy human skin (p=0.0286 and p=0.0432 respectively; Figure 4C). Further analysis of $CD4^+$ and $CD8^+$T cells for VTCN1 expression showed a significant decrease in percentage of $CD4^+$ $VTCN1^+$T cells in lesional skin of vitiligo patients compared to controls (p=0.0052, Figure 4B) but no difference was observed in non-lesional skin compared to healthy skin (p=0.1499, Figure 4B). Also, a significant decrease was observed in the percentage of $CD8^+VTCN1^+$ T cells in lesional skin of vitiligo patients compared to controls (p=0.0016, Figure 4D), while no significant change was observed between non-lesional skin and healthy skin (p=0.071, Figure 4D). We also studied the alterations in the percentage of Treg cells and expression of expression in vitiligo. Analysis revealed significant decrease in the percentage of $CD4^+CD25^+CD127^-$ Treg cells in lesional skin of vitiligo patients as compared to controls (p=0.0329, Figure 4E) along with decrease in VTCN1 expression, in lesional skin and non-lesional skin of vitiligo patients as compared to controls (p=0.0010, Figure 4F). There was no significant difference in the percentage regulatory T cells between non-lesional skin and control skin as well as non-lesional skin and lesional skin (p=0.8654 and p=0.0904 respectively, Figure 4E)

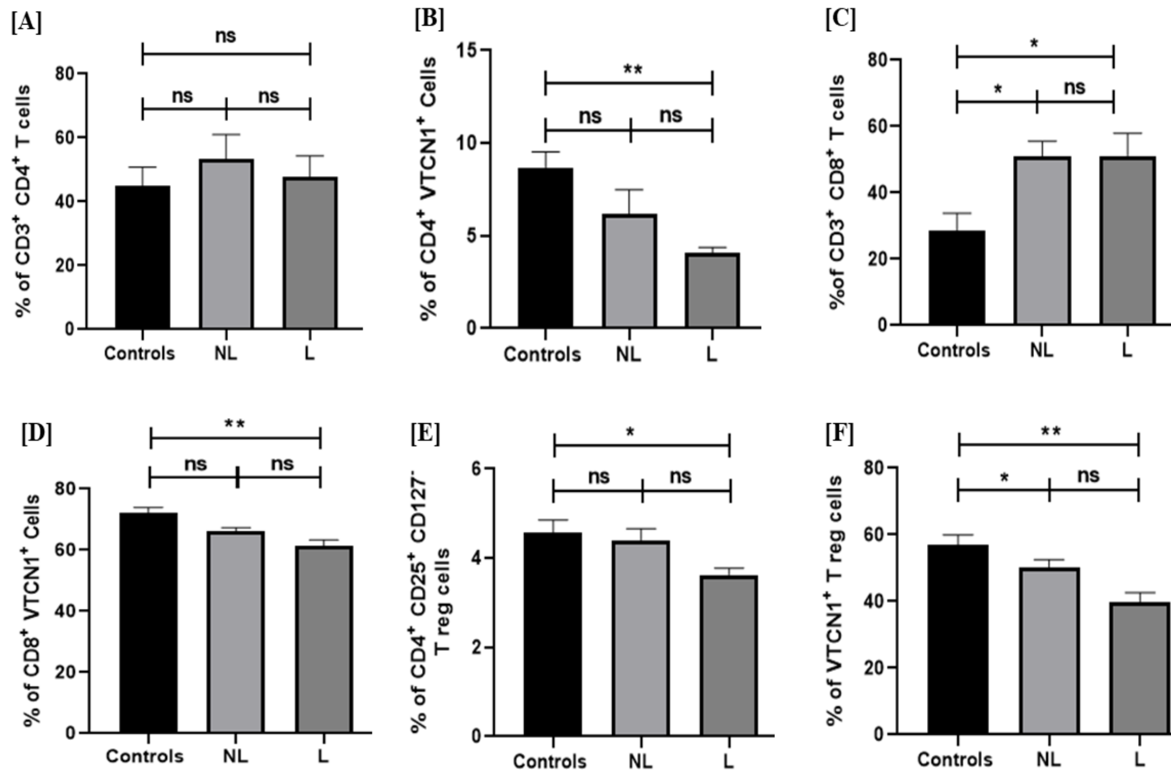

Figure 3.10: Immunophenotypic analysis of VTCN1 on different T cells from the non lesional and lesional skin of 13 generalized active vitiligo patients and 10 healthy controls. **[A]** No significant change was observed in $CD4^+$ cells in lesional and non lesional skin of vitiligo patients as compared to healthy human skin (Mean±SEM: 47.62 ±6.686 vs. 53.21 ± 7.815 vs. 44.94 ± 5.919 respectively; *p*=0.9580 and *p*=0.6998 respectively) **[B]** Significant decrease in percentage of $CD4^+$ $VTCN1^+$ T cells was observed in lesional skin of vitiligo patients compared to controls (Mean±SEM: 4.107 ± 0.2738 vs. 8.693 ± 0.8359 respectively; *p*=0.0052) **[C]** Significant increase in $CD8^+$ cells in lesional and nonlesional skin of vitiligo patients as compared to healthy skin (Mean±SEM: 51.18± 6.802 vs. 50.81 ± 4.750 vs. 28.42 ± 5.435 respectively; *p*=0.0286 and *p*=0.0447). **[D]** Significant decrease was observed in the percentage of $CD8^+VTCN1^+$ T cells in lesional skin of vitiligo patients compared to controls (Mean±SEM: 61.21 ± 2.114 vs. 72.03 ± 1.962 respectively; *p*=0.0016). **[E]** Significant decrease in the number of $CD4^+$ $CD25^+$ $CD127^-$ Treg cells in Lesional skin of vitiligo patients as compared to controls (Mean±SEM: 3.629 ±0.1472 vs. 4.751 ± 0.2865 respectively; *p*=0.0329). **[F]** Significant decrease in VTCN1 expression on Treg cells in lesional and nonlesional skin of vitiligo patients was observed as compared to controls (Mean±SEM: 39.87 ±2.774 vs. 50.24 ± 2.342 vs. 57.03 ± 2.913 respectively; *p*=0.0070 and *p*=0.0350, Figure 4F).

3.4 Discussion

Negative costimulatory molecules play a crucial role in both T cell activation phase and effector phase to advocate immune tolerance and prevent peripheral tissue damage by autoimmunity (June et al. 2017). Over the past several years, role of these molecules in immune regulation has been widely studied in different autoimmune diseases (Joller et al. 2012; Lu et al. 2019). Imbalance in expression of negative costimulatory molecules might be responsible for the exacerbated immune response resulting into development of autoimmune diseases like vitiligo (Speeckaert et al. 2017b). Significantly higher expression of immune checkpoint receptors Tim-3 and PD-1 was also reported on $CD8^+$ T cells from the blood of vitiligo patients by Ali Rahimi and his colleagues. Further analysis revealed positive correlation of it with the disease activity (Rahimi et al., 2019). All these reports invigorate us to explore the status of negative costimulatory molecule VTCN1 in vitiligo pathogenesis.

For the first time, we reported the role of a negative costimulatory molecule, VTCN1 in vitiligo pathogenesis. Increased soluble VTCN1 possibly due to Nardilysin mediated cleavage was observed in the blood of vitiligo patients that resulted in decreased VTCN1 protein expression in vitiliginous skin (Vaishnav et al., 2022). To take our previous lab study further and confirm VTCN1 expression on different immune cell population, we have carried out immunophenotypic analysis of VTCN1 on different immune cell subpopulations. In 2003, for the first time VTCN1 expression was found on human T cells, B cells, monocytes and dendritic cells upon *in vitro* stimulation by flow cytometric analysis (Sica et al., 2003). Further in 2010, one study was carried out to investigate expression of VTCN1 on immature myeloid and lymphoid dendritic cells from cord blood of neonates and peripheral blood of healthy adults. They also found higher expression of VTCN1 on dendritic cells from peripheral blood of adults (Serafin et al., 2010). Moreover, in 2011 a study reported VTCN1 expression on circulatory monocytes from peripheral blood of healthy individuals using flow cytometric analysis (Matsunaga et al., 2011). In addition to that we also found VTCN1 expression on different immune cells including T cells and its subpopulation, B cells, macrophages and DCs of healthy individuals and vitiligo patients.

Earlier reduced number of total peripheral blood lymphocytes were reported in vitiligo patients due to increased rate of activation induced cells death (AICD) of lymphocytes (Mahmoud et al. 2002), which supports our observation of decreased percentage of lymphocyte and increased activated T lymphocytes in vitiligo patients. In line with our previous lab study, we found decreased $CD4^+/CD8^+$

T cells ratio with increased number of $CD8^+$ T cells in blood of generalized active vitiligo patients (Dwivedi et al., 2013). These results were also similar with previous reports in vitiligo patients (Giri et al., 2020; Hegab et al., 2015; Willemsen et al., 2022). One of the previous report also showed importance of Treg cell mediated anergy in autoreactive T cell playing crucial role in tuning up self-tolerance (Maeda et al., 2014). So, we have investigated number of Treg cells from the blood of the vitiligo patients along with VTCN1 expression on them. Interestingly, we found decreased number of Treg cells in vitiligo patients which was supported by the previous studies (Dwivedi et al., 2013; Giri et al., 2021; Hegab et al., 2015; Raam et al., 2018). Depletion of Treg cells from blood of vitiligo patients pointing out increased risk for the development of autoimmunity and support unbridled response of autoreactive T cells to melanocytes in vitiligo (Klarquist et al., 2010). We also found decreased VTCN1 expression on Treg cells from blood and skin of vitiligo patients supporting breakdown in tolerance during the vitiligo development. Moreover, Wei et al., had reported decreased Treg cells in VTCN1 deficient mice during EAE and T1D (Wei et al., 2011). Our previous studies also showed reduced suppressive capacity of Treg cells in GV patients, suggesting that in addition to the reduced number of Treg cell, their function is also altered (Giri et al., 2020; Giri et al., 2022). All immunophenotyping studies from the blood of vitiligo patients along with markers are summarized in Table 3.4.

In inflammatory vitiligo, increase in T cell infiltration, increased $CD8^+/CD4^+$ T cells ratio and macrophage infiltration were observed in perilesional skin of vitiligo patients (Le Poole et al., 1996). These findings were supported by Wijngaard et al., 2000 who found increased influx of skin homing T cells and macrophages in perilesional skin of generalized active vitiligo patients using IHC (van den Wijngaard et al., 2000). Moreover, Van den boorn and his colleagues demonstrated destruction of melanocytes by perilesional cytotoxic T cell using skin explant model confirm T cell mediated destruction of melanocytes in vitiligo (van den Boorn et al., 2009). The similar finding was also reported in different vitiligo patient populations (Aslanian et al., 2008; Wu et al., 2013). In line with that above-mentioned finding, we also found increased $CD8^+$ T cells in non-lesional and lesional skin samples of vitiligo patients along with decreased VTCN1 expression on them. Earlier, drastic reduction in regulatory T cells in non-lesional, perilesional and lesional skin was also reported (Klarquist et al. 2010). In the present study, we also found decreased percentage of regulatory T cells and VTCN1 in the lesional skin of vitiligo.

Overall reduced percentage of a negative stimulatory molecule, VTCN1 on different immune cell populations pointing towards hampered immune cell activation in vitiligo patients leading to

exacerbated response of melanocyte specific autoreactive T cells resulting in development and progression depigmented patches in vitiligo.

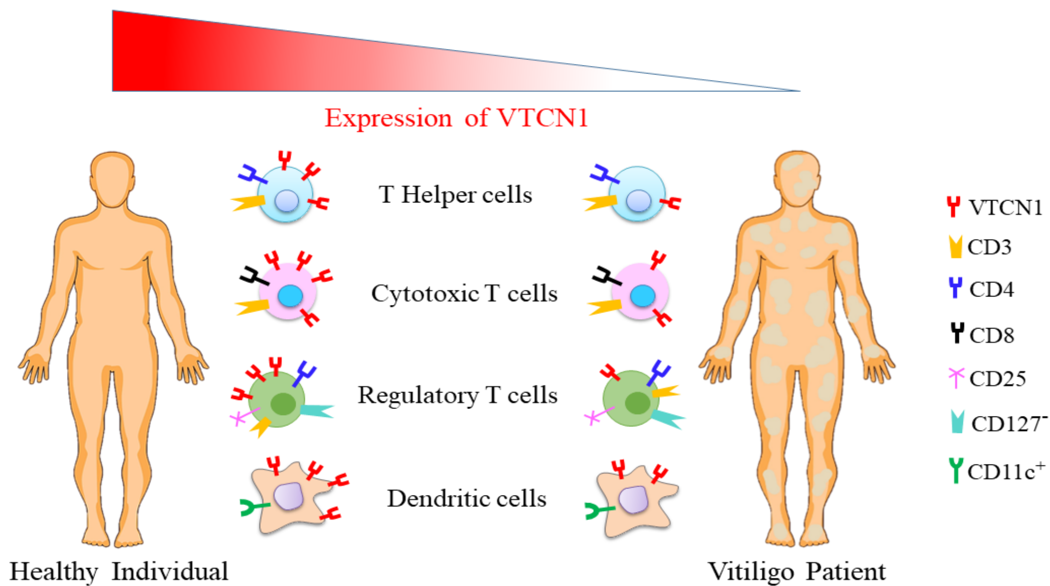

Figure 3.11: Summary figure: Overall reduced VTCN1 positive CD4, CD8, Treg cells, dendritic cells in blood and skin of vitiligo patients pointing out towards breakdown in tolerance which might result in unbridled response of autoreactive T cells leading to melanocyte destruction in vitiligo.

Table 3.4: Immunophenotyping studies in vitiligo.

Sr. no	Title of the study	No. of Patients	Cell Subtype	Markers used	Change as compared to healthy
1	Immunophenotypic Analysis Reveals Differences in Circulating Immune Cells in the Peripheral Blood of Patients with Segmental and Non segmental Vitiligo (Willemsen et al., 2022)	SV (n=12) Stable NSV (n=22) Control N=20 >18Y	Treg	$CD4^{+}CD25^{+}$ $FoxP3^{+}$	Decreased in NSV
			cTfh (Circulating T follicular helper cells)	$CXCR5^{+}CD4^{+}$	No change in NSV
			cTfh2 cells	CCR6– CXCR3–	Increased in SV
			cTfh17 cells	$CCR6^{+}$ CXCR3–	Increased in SV
			B cells	$CD19^{+}$ CD3–	No change
			Plasmablasts	CD24– $CD38^{+}$	Decreased in SV
			NK cells	CD3–$CD56^{+}$	No change in SV
2	Decreased suppression of $CD8^{+}$ and $CD4^{+}$ T cells by peripheral regulatory T cells in generalized vitiligo due to reduced NFATC1 and FOXP3 proteins (Giri et al., 2020)	GV: 55 Control: 45	Treg	FoxP3	Reduced
				NFATC1	Reduced
				CD25	Reduced
				CD44	No change
3	Immunophenotype of circulatory T-helper cells in patients with non-segmental vitiligo (Kalaiselvi et al., 2019)	NSV: 80 Control: 80	Th1	$CD4^{+}$ IFN-γ^{+}	Increased
			TH2	$CD4^{+}IL\text{-}4^{+}$	No change
			Th17	$CD4^{+}IL\text{-}17A^{+}$	Increased
			Treg	$CD4^{+}FoxP3^{+}$	No change
4	Lymphoid Stress Surveillance Response Contributes to Vitiligo Pathogenesis (Raam et al., 2018)	NSV: 19-60Y 6M, 15F (7A, 14S) Control: 8M, 20F	Treg cells	$CD4^{+}$ $Cd25^{+}$ CD127lo	Moderately Decreased
			Naïve B	IgD^{+} CD27-	Decreased in AV as compared to SV
			SM (Switched memory B cell)	IgD- $CD27^{+}$	Increased as compared to SV
			USM (Unswitched memory B cells)	IgD^{+} $CD27^{+}$	Increased
			Transitional B cells	$CD24^{hi}$ $CD38^{hi}$ naïve	No change
			NK cell	CD3- $CD16^{+}$ $CD56^{+}$	No change
5	Enhanced Th1 and Th17	NSV:	Treg	$CD4^{+}$ $CD25^{+}$	No change

	responses in peripheral blood in active non-segmental vitiligo (Zhen et al., 2016)	(18-45Y) 15M, 15F Control: 30		CD127-	
			Th1	IFN-γ $^+CD4^+$	Increased in progressive NSV
			Th2	$CD4^+$ IL-4^+	No change
			Th17	$CD4^+$ IL-$17A^+$	Increased in progressive NSV
				Th1/Tregs	Increased
				Th17/Tregs	Increased
6	Decreased Circulating T Regulatory Cells in Egyptian Patients with Nonsegmental Vitiligo: Correlation with Disease Activity (Hegab et al., 2015)	NSV: 36M, 44F Control: 36M, 24F	Peripheral T Cells	$CD4^+CD25^+$	Decreased
			Peripheral Treg Cells	$CD4^+FoxP3^+$	Decreased
7	Decreased regulatory T-cells and $CD4^+$ /$CD8^+$ ratio correlate with disease onset and progression in patients with generalized vitiligo (Dwivedi et al., 2013)	GV: 39M, 43F (56A, 26S) control: 24M, 26F	Treg cells	$CD4^+$ $CD25^+$ $FoxP3^+$	Decreased
			T helper	CD4	No change
			T cyt	CD8	Increased
			$CD4^+$/$CD8^+$ ratio		Decreased
8	Systemic analyses of immunophenotypes of peripheral T cells in non-segmental vitiligo: implication of defective natural killer T cells (Zhou et al., 2012)	Ac. NSV: 43 Control 43	T Helper Cells	$CD4^+$	No change
			T Cytotoxic Cells	$CD8^+$	No change
			Memory T helper cells	$CD45RO^+$	No change
			Naïve T helper cells	$CD45RA^+$	No change
			Memory T Cytotoxic Cells	$CD8^+CD45RO^+$	No change
			Naïve T Cytotoxic Cells	$CD8^+CD45RA^+$	No change
			Activated T cells	$CD4^+$ $CD69^+$	No change
				$CD8^+$ $CD69^+$	No change
			Treg cells	$CD4^+$ $CD25^+$ $Foxp3^+$	No change
				$CD4^+CD45RO^+$ cells/$CD4^+Foxp3^+CD25^+$ ratio	No change
			Peripheral blood iNKT cells	CD1d- aGalCer $tetramer^+$ $CD3^+$	Decreased
				$CD4^+$ iNKT subset	Decreased
9	Functional defects of peripheral regulatory T lymphocytes in patients with	Localized, Generalised: 15	Treg cells	$CD4^+CD25^+$	Increase
			T cells	$CD3^+$ $CD4^+$	No change

	progressive vitiligo (Ben Ahmed et al., 2012)	Control: 15			
10	Reduced skin homing by functional Treg in vitiligo (Klarquist et al., 2010)	GV, 30 ml blood	Circulating Treg Cells	$CD4^+$ $CD25^+$ CD127low $FoxP3^+$	No Change
				CD127	Decreased
				CD25	Increased
				Foxp3	Increased
11	Reduction of skin-homing cytotoxic T cells ($CD8^+CLA^+$) in patients with vitiligo (Antelo et al., 2011)	AGV: 18F, 4M Controls: 15	Skin homing T-Cytotoxic cells	$CD8^+CLA^+$	Decreased
				CD4/CD8 ratios	No change
			Skin homing T cells	CLA^+ Tcells	No change
			Skin homing T-helper cells	$CD4^+CLA^+$	No change
12	Decreased Total Numbers of Peripheral Blood Lymphocytes with Elevated Percentages of $CD4^+CD45RO^+$ and $CD4^+CD25^+$ of T-Helper Cells in Non-Segmental Vitiligo (Mahmoud et al., 2002)	NSV: 19M, 13F Control: 17M, 11F	Overall lymphocyte count		Decreased
			Memory T cell	$CD4^+CD45RO^+$	Increased
			Nk-T cells	$CD3^+CD16^+$ $CD56^+$	Decreased
			Naive T cells	$CD4^+CD45RA^+$	Decreased
			Treg cells	$CD4^+CD25^+$	Increased
			Activated T cell	$CD3^+HLADR^+$	No change
			Activated T-Cytotoxic Cells	$CD8^+HLADR^+$	No change
			Activated T-Helper Cells	$CD4^+HLADR^+$	No change
13	Vitiligo: peripheral T-cell subset imbalance as defined by monoclonal antibodies (SOUBIRAN et al., 1985)	Stable Vitiligo: 15 F, 17M Control: 9F 12M	B Cell	fluoresceine anti-IgM	Decreased
			T3	OKT3	No Change
			T4	OKT4	Increased
			T8	OKT8	Slightly increased
			T4/T8 ratio		Increased
			Monocytes	OKMi	No Change

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