Chapter 4

To study the effect of pro inflammatory & anti-inflammatory cytokines on VTCN1 expression and STAT3 activation

4.1 Introduction

Generalized vitiligo (GV) is an autoimmune depigmentary skin disease typified by bilateral, symmetrical white macules around the body (Giri et al., 2021). Its worldwide prevalence is 0.5-2%, from which 85-90% of cases are of generalized vitiligo (Ezzedine et al., 2012; Picardo et al., 2015; Taïeb & Picardo, 2009). In India, prevalence of vitiligo was reported 0.25-4% (Mahajan et al., 2019). Immune-mediated selective destruction of melanocytes from the skin has been very well documented, which results in the white-colored patch on the body of vitiligo patients (Badri et al., 1993; Le Poole et al., 1996; Wańkowicz-Kalińska et al., 2003). Experimental evidence highlighted a 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). As a pivotal player in immune cell development, regulation, and effector function, cytokines have become a fascinating topic to be explored in autoimmune diseases (O'Shea et al., 2002). The balance between pro and anti-inflammatory cytokines is essential to produce a proper immune response and maintain the healthy state of human body. In vitiligo, previously excellent number of research articles reported an altered cytokine levels and these studies are described in the below section.

The role of proinflammatory cytokines is not yet fully defined in vitiligo, although interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) has been studied well (Dwivedi et al., 2013b; Harris et al., 2012; Laddha et al., 2013; Singh et al., 2019). INF-γ has numerous effects on melanocytes, including preventing melanogenesis, increasing the generation of ROS, and causing $CD8^+$ cells to cause senescence and death in them (Wang et al., 2014; Yang et al., 2015). The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway mediates the cellular response to INF-γ (Ivashkiv et al., 2009). TNF-α causes melanocyte toxicity and increases ROS production by acting on the microphthalmia-associated transcription factor (MITF), the melanocyte stimulating hormone receptor (MSH-R), and the expression of the melanocortin-1 receptor (MC1-R). These effects result in melanocyte dysfunction and cellular death (Salas-Alanis et al., 2013; Singh et al., 2021). Early reports showed that melanocyte-specific autoreactive cytotoxic T cells from lesional vitiligo skin can produce IFN-γ and TNF-α (van den Boorn et al., 2009). Further studies revealed higher IFN-γ and TNF-α levels in the blood and skin of vitiligo patients driving autoreactive T cell response and melanocyte destruction, pointing out their involvement in development and progression of the disease (Birol et al., 2006; Dwivedi, Laddha, Shah, et al., 2013a; Laddha et al., 2012; Moretti et al., 2002a). *In vitro* studies also demonstrated up-regulation of IFN-γ and TNF-α cytokines upon the polarization of T cells isolated from perilesional skin of vitiligo patients (Wańkowicz-Kalińska et al., 2003).

Interestingly, researchers also reported reduced expression of anti-inflammatory cytokine IL-10 from the PBMCs of vitiligo patients (Zhao et al., 2010). Further studies confirmed reduced serum levels of IL-10 in the blood of vitiligo patients, making it an attractive candidate to study in vitiligo (Ala et al., 2015). Additionally, one report showed an increase in IL-10 expression in vitiliginous skin after treatment of 0.1% tacrolimus, suggesting decreased IL-10 involvement in melanocyte destruction during the course of vitiligo (Taher et al., 2009).

In previous chapters, we have discussed the involvement of VTCN1 in vitiligo progression and development. However, the effect of above-mentioned pro and anti-inflammatory cytokines on VTCN1 expression has not been yet investigated. Moreover, regulatory mechanisms for VTCN1 expression in normal tissues are not well defined, but IL-6 and IL-10 have been reported to stimulate VTCN1 expression on macrophages in the tumor microenvironment (TME). In contrast, IL-4 and GM-CSF (Granulocyte/Macrophage colony-stimulating factor) have been reported to reduce VTCN1 expression (Kryczek, Zou, et al., 2006). Moreover, IL-6/STAT-3 mediated VTCN1 expression via enhancing *VTCN1* promoter was reported in BV2 microglia cells (Yao et al., 2016). Recently, decreased VTCN1 expression from the vitiliginous skin and higher sVTCN1 and Nardilysin in the blood of vitiligo patients were reported (Vaishnav et al., 2022). However, the status of VTCN1 expression upon treating with pro-and anti-inflammatory cytokines TNF-α, IFN-γ, and IL-10 are unchecked. To better understand the regulation of VTCN1 expression via STAT3 activation, we performed a western blot analysis of THP1 cells after treatment of TNF-α, IFN-γ, and IL-10 cytokines and assessed the expression of pSTAT3/STAT3 and VTCN1.

4.2 Materials and methods:

4.2.1 THP1 cell culture and cytokine treatment

Human monocytic cell line THP-1 was previously used to analyze specificity of novel developed antibody against VTCN1 and found to have good efficiency (Qian et al., 2011). So, we have selected THP-1 cell line for VTCN1 protein expression upon treatment of IFN-γ, TNF-α and IL-10 and performed the western blot analysis. Briefly, 1 x 10^6 THP1 cells were cultured in 10 cm cell culture plates in RPMI-1640 medium (Gibco, USA) containing 1% of antibiotics (Gibco, USA) and 10% heat inactivated serum (FBS) (Gibco, USA) at 37 °C in a CO_2 incubator (Thermo Fisher Scientific, USA). The cultured cells were then treated with 50-100 ng of IFN-γ (Cat. No I17001, Sigma-Aldrich Co. LLC), 10-20 ng of TNF-α (Cat. No C63721, PromoCell, USA) and 10-20 ng of IL-10 (Cat. No. Cyt-100-b, ProSpec-Tany Technologies, Israel) for 24 hours as per previous literature (Lim et al., 2014; Naiyer et al., 2013; Yoyen-Ermis et al., 2019).

4.2.2 Western Blot analysis of pSTAT3, t-STAT3 and VTCN1

Cells were harvested after the cytokines' treatment and total cell lysates were obtained by Laemmli buffer extraction. 20 µg of total protein was subjected to SDS-PAGE using 8-12% gel under reducing conditions and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Immuno-Blot®, Bio-Rad Laboratories Inc). The membranes were incubated with the following antibodies: p-STAT3 (Cat. No 9131), total STAT3 (Cat. No 4904), VTCN1 (Cat. No 14572) purchased from Cell signalling technologies, USA, and β-Actin (Cat. No AC0047) purchased form AB clonal, USA. β-actin was used as a loading control. Protein bands were detected by Clarity™ Western ECL substrate (Bio-Rad Laboratories Inc, USA) and blot images were captured using BIO-RAD ChemiDoc™ Imaging system.

4.2.3 Statistical Analysis:

Densitometric analysis of phospho-STAT3, total-STAT3 and VTCN1 expression upon different cytokine treatment was done using Fiji software (Schindelin et al., 2012) and each protein band was normalized with respective β-actin values. Statistical data was compared using one-way ANOVA for multiple comparisons. Differences were considered significant at $p \leq 0.05$. The statistical analysis was done and graphs were plotted using Graph Pad Prism 8 software (Graph Pad Software Inc; 2003).

4.3 Results

4.3.1 Assessment of VTCN1 expression upon IFN-γ, TNF-α and IL-10 treatment in THP1 cells

It has been reported that the STAT3 (p-STAT3) phosphorylated at 705 tyrosine residue could translocate into nucleus and acts as transcriptional factor to activate the expression of *VTCN1* gene (Yao et al. 2016). To assess the activation of STAT3 and VTCN1 expression upon the treatment of 50-100 ng of IFN-γ, 10-20 ng of TNF-α and 10-20 ng of IL-10 for 24 hours, we have treated THP1 cells with respective concentration of cytokines as per previous literature and performed western blot analysis. Interestingly, we found increased phosphorylation of STAT3 upon the treatment of 50ng and 100ng of IFN-γ for 24 hr (p=0.002, Figure 4.1 A) but there was no significant change observed in VTCN1 expression on THP1 cells upon 50ng IFN-γ treatment (p>0.05; Figure 4.1 A and B) while 100ng IFN-γ treatment showed significant increase in VTCN1 as compared to non-treated cells (p=0.03; Figure 4.1 A and B). When we treated the THP1 cells with 10ng and 20 ng TNF-α for 24 hrs, we found significant increase in phosphorylation of STAT3 along with VTCN1 expression (p=0.03; Figure 4.2 A and B). In addition, we also assessed the effect of 10 ng and 20 ng IL-10 treatment on phosphorylation of STAT3 and VTCN1 expression. We found significant increase in pSTAT3 levels in treated cells compared to non-treated cells (p=0.004 and 0.002 respectively; Figure 4.3 A and B). Significant increase in VTCN1 expression was observed in treated THP1 cells as compared to non-treated cells (p=0.003 and 0.004 respectively; Figure 4.3 B).

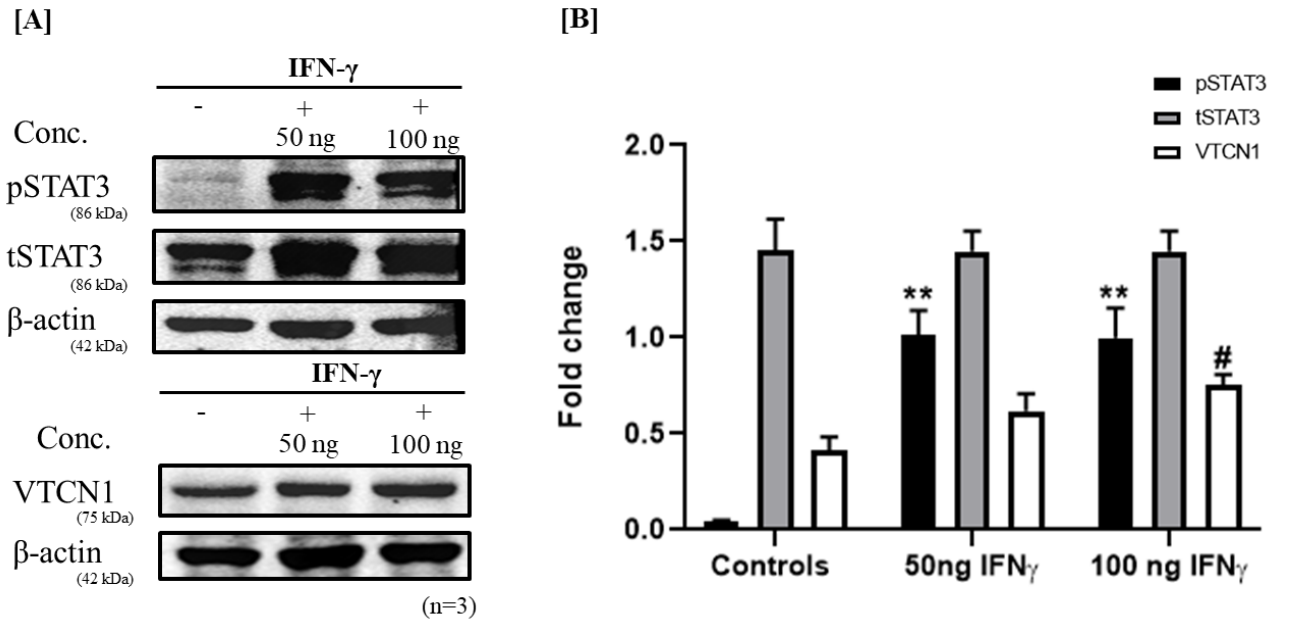

Figure 4.1: Western blot analysis of pSTAT3, total-STAT3 and VTCN1 upon 50ng and 100ng of IFNγ for 24 hours **[A]** Blot images **[B]** densitometric analysis [*: pSTAT3 compared to control, #: VTCN1 levels compared to controls]

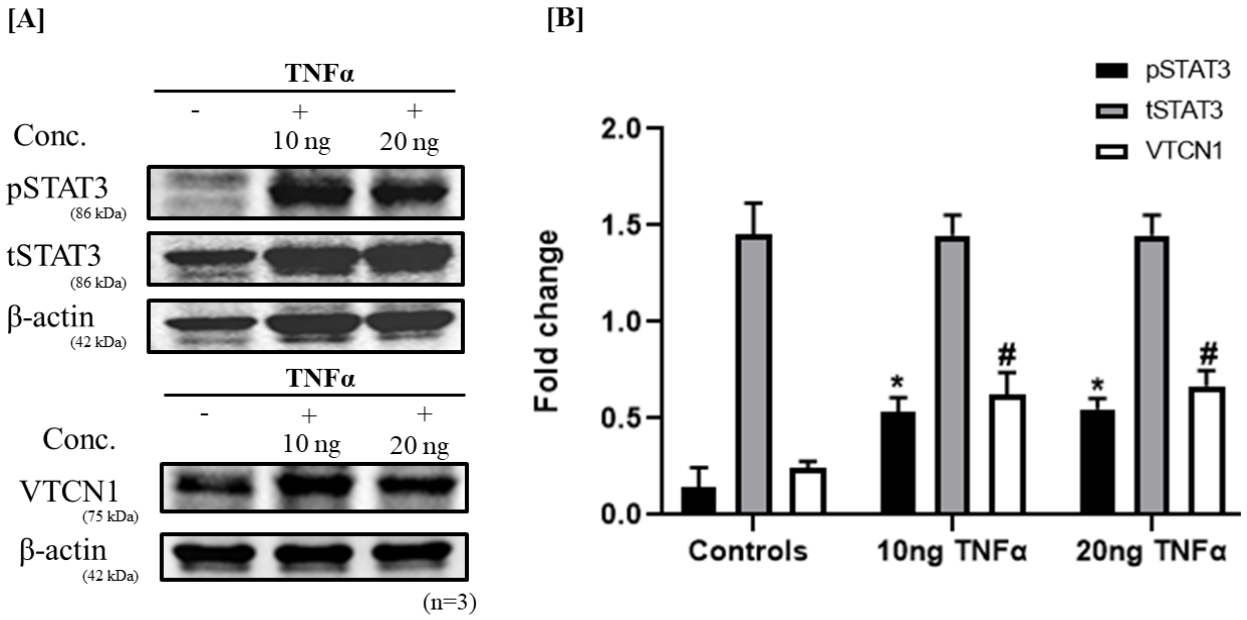

Figure 4.2: Western blot analysis of pSTAT3, total-STAT3 and VTCN1 upon 10ng and 20ng of TNF-α for 24 hours **[A]** Blot images **[B]** densitometric analysis [*: pSTAT3 compared to control, #: VTCN1 levels compared to controls]

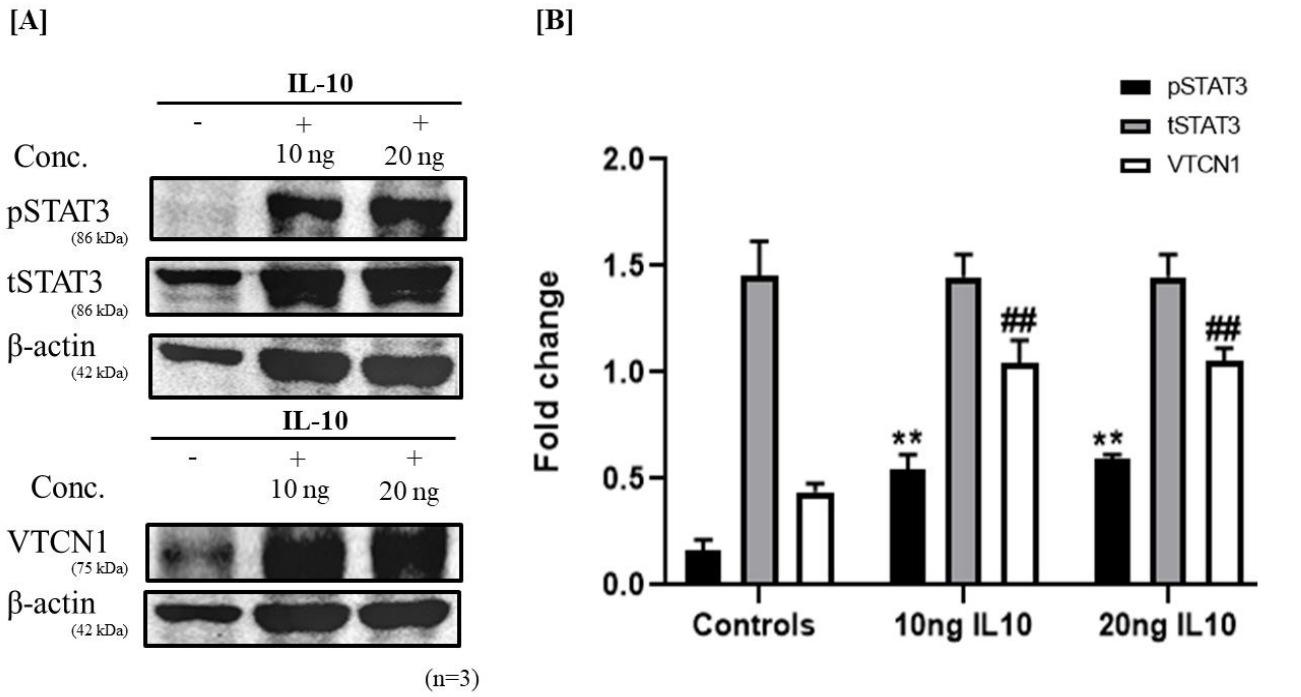

Figure 4.3: Western blot analysis of pSTAT3, total-STAT3 and VTCN1 upon 10ng and 20ng of IL-10 for 24 hours **[A]** Blot images **[B]** densitometric analysis [*: pSTAT3 compared to control, #: VTCN1 levels compared to controls]

4.4 Discussion

Previously we reported increased *VTCN1* transcripts from the vitiliginous skin compared to healthy skin, however, the reason is unknown (Vaishnav et al., 2022). Which factors are involved in the increase of VTCN1 transcripts in the patients? How VTCN1 expression is regulated? To answer these questions, we did a literature review. We found that regulatory mechanisms for VTCN1 expression are not very well documented in normal tissue. However, several reports showed transcriptional regulation and altered VTCN1 expression in cancerous tissue upon treatment of various stimuli (John et al., 2019). Regulatory mechanisms of VTCN1 expression are distinct from other B7 family members, such as PD-L1 (Ohaegbulam et al., 2017). In 2010, one report showed a higher concentration of IL-10 and IL-6 in the tumor microenvironment, which in turn stimulates VTCN1 expression on tumor macrophages, while GM-CSF and IL-4 inhibit VTCN1 expression (Kryczek, Zou, et al., 2006). In support of that, one report on glioblastoma showed that $CD133^+$ glioma cells were able to induce VTCN1 expression on tumor macrophages via IL-6 and IL-10. Upon further investigation, they found that IL-6 induces VTCN1 expression on microglia cells by activating STAT3 (Yao et al., 2016). STAT1 and STAT3 also found elevated in the vitiligo lesions (Samaka et al., 2019). Therefore, we carried out western blot analysis of activated pSTAT3, total STAT3, and VTCN1 upon treatment of with TNF-α, IFN-γ, and IL-10 cytokines to THP1 cells.

Interestingly, we found an increase in STAT3 activation upon IFN-γ treatment with both selected dosages, but no change was observed in VTCN1 expression upon 50 ng IFN-γ treatment. The phosphorylation of STAT3 upon IFN-γ treatment on THP1 cells was very well documented previously (Yoyen-Ermis et al., 2019). Moreover, increased STAT3 also reported in vitiliginous skin (Samaka et al., 2019). One of the previous reports of VTCN1 expression on different human and murine tumor cell lines such as MDA MB 468 [breast], SKBR3 [breast], U2OS [osteosarcoma], OVCAR4 [ovary] MC38 [colon], CT26 [colon], LLC [lung], B16F10 [melanoma] did not show any changes in VTCN1 expression upon IFN-γ treatment (Ohaegbulam et al., 2017). Our results align with this study. In contrast, few studies showed reduction of VTCN1 expression upon treatment of IFN-γ on NHL cells and ccRCC cells (Che et al., 2017; XU et al., 2014). However, in the present study, 100 ng of IFN-γ treatment showed significantly increased VTCN1 expression compared to the control. Hence, our results highlight the dose-dependent effect of IFN-γ on VTCN1 expression. However, the mechanism for dose-dependent effect of IFN-γ on VTCN1 expression still needs further investigation. In vitiligo patients, increased IFN-γ and TNF-α levels from blood and skin were reported previously (Dwivedi et al., 2013c; Harris, 2015; Laddha et al., 2012; Moretti et al., 2002b),

which might lead to increased VTCN1 transcripts in skin and protein in blood, as observed in our previous lab study (Vaishnav et al., 2022).

In 2006, Kryczek et al. reported for the first time that the suppressive activity of human APCs (Antigen Presenting Cells) relies on Treg cells triggered IL-10 release, which induces VTCN1 expression in them (Kryczek, Wei, et al., 2006). Increased IL-10 levels in the tumor microenvironment via tumor Treg cells resulting in high VTCN1 expression on tumor macrophages supports the earlier findings (Kryczek et al., 2007; Kryczek, Zou, et al., 2006). Therefore, we carried out a western blot analysis of THP1 cells to investigate the effect of IL-10 on VTCN1 expression and found increased pSTAT3 and VTCN1 expression. Thus in vitiligo patients, reduced Treg cells and IL-10 might be responsible for reduced VTCN1 in the lesional skin of the patients, as suggested by our previous findings (Vaishnav et al., 2022). There are report shown increased serum IFN-γ levels and decreased IL-10 levels in non-segmental vitiligo patients supporting our observation (Ala et al., 2015; Praharsini, 2018).

Overall, our data mechanistically link the increased IFN-γ and decreased IL-10 in the vitiligo skin microenvironment with decreased VTCN1 expression, which results in an unbridled response of autoreactive T cells leading to melanocyte destruction in vitiligo. Targeting this interconnected network may be therapeutically meaningful in treating patients with vitiligo.

4.5 References

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