5.1 INTRODUCTION

The pathophysiology of vitiligo is complex, although the role of various driving factors seems to become gradually elucidated. This may help to identify possible targets for treatment modalities (Speeckaert *et al*., 2017). Cytokines are low molecular weight glycoproteins produced by a myriad of cell types predominantly leukocytes. Cytokines regulate various physiological and pathological functions including innate immunity, adaptive immunity and a plethora of inflammatory responses. Redundancy, synergy and pleiotropism are the characteristics of cytokine action and account for the effectiveness of these proteins in regulating immune response (Kuby, Immunology, 6th Ed., 2007).

Cytokine research is one of the fastest growing areas of cell biology and it has been the delineation of receptor-linked signaling pathways (Hamilton, 1997). Cytokines exert their effect *via* interacting with their respective receptors and activating downstream signaling pathways. After binding to specific receptors on the cell surface of the target cells, cytokines produce multiple signals which regulate the expression of cytokine receptors. These target cells respond by regulation of mRNA and protein synthesis, resulting in specific biological phenomena (Khan, 2008). Different kinds of interactions exist between cytokines and their respective receptors as illustrated in figure 1. The fine and sensitive tuning of cytokines with their respective receptors is implicated by the presence of various regulatory mechanisms mentioned below:

- Short half life of cytokine and existence of high affinity receptors for quick biological response.
- Transcription, translation and post-translational modes of regulation in response to different environmental cues.
- Presence of natural antagonists, decoy receptors etc.

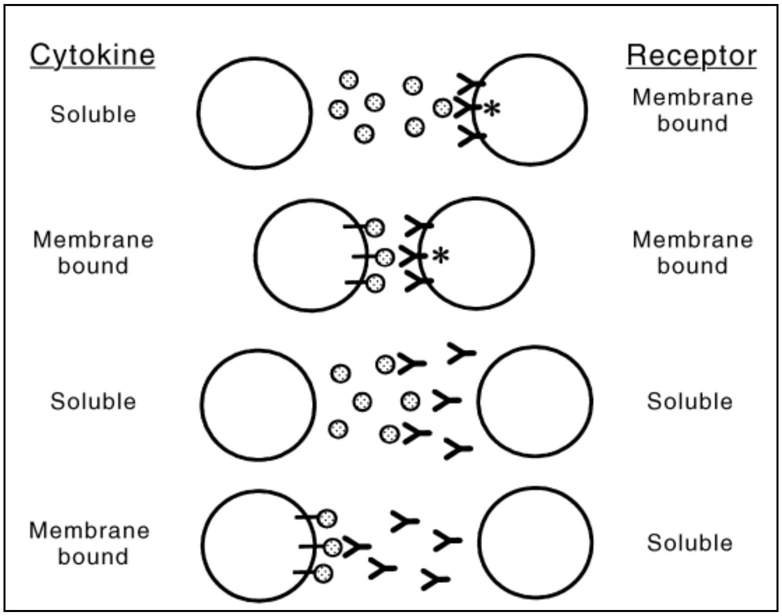

Figure 1: Cytokine-receptor interactions

Cytokines can exist in soluble and/or membrane bound form and their receptors are normally anchored to the cell membrane but a few of them are also secreted as soluble molecules. Signal transduction (*) can occur when membrane receptors interact with soluble or membrane bound cytokines, but this interaction can be competitively inhibited by soluble receptors (Kelso, 1998).

Keratinocytes obtain pigmentation from melanocytes, and in return provide the necessary microenvironment for melanocyte survival, proliferation, differentiation, and migration *via* production of ligands that interact with melanocyte receptors. Furthermore we are interested in exploring the following four cytokines receptors expression on melanocyte surface, upon stimulation with the respective cytokines i.e. TNF-α, IL-1α, IL-6 and IL-10. A brief introduction of the four cytokines and the respective receptors is as follows:

TNF-α and its receptors

TNF primarily occurs as a type II transmembrane protein of 26 kDa, which can be cleaved by the metalloprotease, TNF-α-converting enzyme to a 17 kDa TNF protein that is biologically active as a soluble homo-trimeric molecule of 51 kDa with a triangular pyramid shape (Wajant *et al*., 2003). The soluble 51 kDa trimeric sTNF tends to dissociate at concentrations below the nanomolar range, thereby losing its bioactivity. Both the secreted and the membrane bound forms are biologically active, although the specific function of each is controversial. But, both forms do have overlapping and distinct biological activities.

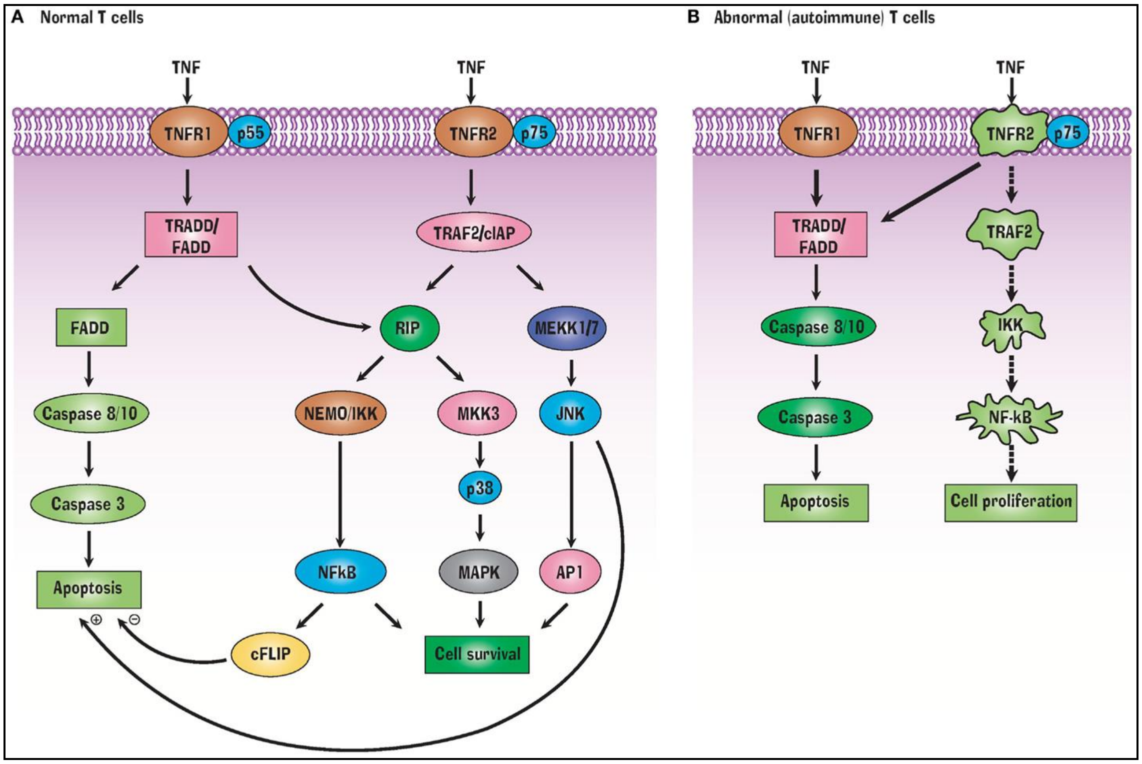

Figure 2: TNF signaling: TNF signals through TNFR1 and TNFR2 receptors (A) but abnormalities in this signaling pathway in autoimmunity (B) can favor a pathway of selective apoptosis of target cells due to excess of ligand or defects in downstream proteins involved in signaling pathway (Faustman & Davis, 2013).

TNF-α interacts with either a 55 kDa cell membrane receptor termed TNFR1 or a 75 kDa TNFR2. Both these receptors belong to the TNF receptor superfamily (Figure 1). Upon binding of TNF-α to TNFR1, the adapter molecule FADD recruits procaspase-8 to the activated receptor. The resulting death-inducing signaling complex (DISC) performs procaspase-8 proteolytic activation which initiates the subsequent cascade of caspases mediating apoptosis. Anti-apoptotic protein BCL2-associated athanogene 4 (BAG4/SODD) and adaptor proteins TRADD and TRAF2 have been shown to interact with this receptor, and thus play regulatory roles in the signal transduction. The fine regulation of these molecules explains different signaling upon sensing different environmental cues. A TNF-R1 selective apoptosis regulating process could be the TRAF2-mediated recruitment of the antiapoptotic cIAP1 and cIAP2 proteins to TNFR1. (Fotin-Mleczek *et al*., 2002; Shu *et al*., 1996) cIAP1 and the closely related cIAP2 protein have been originally identified as molecules present in the TNF-R2 signaling complex (Rothe *et al*., 1995) Both are typical members of the inhibitor of apoptosis protein family, which bind and inhibit caspase-3 and -7 via their amino-terminal BIR (baculovirus IAP repeat) domains, a structural feature common to all IAP family members. In agreement with an anti-apoptotic role of TRAF2-mediated recruitment of cIAP1 and cIAP2, it has been found that concerted overexpression of TRAF1, TRAF2, cIAP1 and cIAP2 efficiently interferes with TNF-R1- induced apoptosis and activation of caspase-8. So, it seems that in the TNF-R1 signaling complex TRAF2-bound cIAP1/2 molecules are able to block activation of caspase-8, which is independently recruited into the TNF-R1 signaling complex via the TRADD–FADD axis and which is otherwise no substrate for these IAP proteins (Wajant *et al*., 2003)

IL-1α Receptor

IL1R1 belongs to the interleukin 1 receptor family and is a chief mediator involved in many cytokine induced immune and inflammatory responses. This receptor binds to IL-1α, IL-1β and the natural antagonist IL1RN. Binding to the agonist leads to the activation of NF kappa B signaling pathway which involves formation of a ternary complex containing IL1RAP, TOLLIP, MYD88, and IRAK1 or IRAK2.

IL-6 Receptor

Human CD126 is also known as the alpha subunit of the human IL-6 Receptor (IL-6Rα). The human IL-6Rα is a 80 kDa type I transmembrane glycoprotein, also known as B cell stimulatory factor-2 (BSF-2) receptor and IL-6 receptor. The IL-6Rα subunit associates with the 130-160 kDa gp130 subunit (IL-6 receptor ß chain, CD130), that is shared with the receptors for Leukemia Inhibitory Factor (LIF), Ciliary Neurotropic Factor (CNTF), Oncostatin M (OSM), IL-11, Cardiotropin 1 (CT-1) and possibly Neurotrophin-1/B Cell-Stimulating Factor 3 (NNT-1/BSF-3). The IL-6Rα chain binds IL-6 with low affinity; however the association with CD130 stabilizes the IL-6/IL-6Rα complex resulting in the formation of a high affinity complex. The IL-6R ß chain mediates signal transduction. As IL-6 interacts with its receptor, it triggers the gp130 and IL-6R proteins to form a complex, thus activating the receptor. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through transcription factors, Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs). Dysregulated production of IL6 and its receptor are implicated in the pathogenesis of many diseases, such as multiple myeloma, other autoimmune diseases and prostate cancer.

IL-10 Receptor

Biologically functional IL-10 exists in the form of a 36 kD homodimer composed of two non-covalently bonded monomers each with 160 amino acids length (Zdanov, 2010). Two disulfide bridges exist between the monomers, which are required for their biological activity and maintaining structural integrity (Windsor *et al*. 1993). IL-10 homodimer binds to tetrameric IL-10 receptor complex, which consists of two IL-10R-α and two IL-10R-βsubunits. IL-10R-α is known to bind to the ligand and IL-10R-β is the accessory signaling subunit (Liu *et al*. 1994). CD210a, which is also known as, Interleukin-10 Receptor subunit alpha (IL-10R subunit alpha/IL-10Rα/IL10RA), or Interleukin-10 receptor subunit 1 (IL-10R1) is a 90-110 kDa type I transmembrane glycoprotein that belongs to the type II cytokine receptor family. IL-10 interacts with its tetrameric receptor complex consisting of two IL-10Rα and two IL-10Rβ chains resulting in the phosphorylation and activation of JAK1 and TYK2 kinases, which in turn phosphorylate two tyrosine residues in the intra cytoplasmic parts of the IL-10R chains that form docking sites for STAT3. IL-10 can suppress antigen presentation and the expression of pro-inflammatory type-1 immune responses while promoting type-2 immune responses. CD210a is expressed on T cells, B cells, NK cells, monocyte, macrophages and dendritic cells. The main activity for the IL-10 receptor is to bind IL-10 and initiate the transduction of a signaling cascade, which leads to the modification of biological responses.

In the epidermal melanin unit, surrounding keratinocytes secrete cytokines, such as TNF-α, IL-1α, IL-6, and transforming growth factor-β (TGF-β), which are paracrine inhibitors of melanocyte proliferation and melanogenesis (Cichorek *et al*., 2013, Swope *et al*., 1991; Moretti *et al.*, 2002). Our previous studies have shown increased levels of TNF-α, TNF-β, IL-1β in vitiligo patients (Laddha *et al*., 2012; Laddha *et al*., 2013; Laddha *et al*., 2014). However, there is paucity of data with regard to the regulation of cytokine receptors chiefly in melanocytes. It becomes an important aspect to monitor the expression levels of cytokine receptors upon stimulation with the respective cytokines. Henceforth, for better understating of the role of cytokine mediated melanocyte destruction in vitiligo or other pigmentary disorders, we aimed to assess transcript as well as protein expression levels of cytokine receptors: TNFR1, IL1R1, IL6R and IL10R on primary cultured normal human melanocytes upon stimulation with the respective cytokines i.e. TNF-α, IL-1α, IL-6 and IL-10.

5.2 MATERIALS AND METHODS

5.2.1 Ethics statement It is stated in Chapter II.

5.2.2 Culture establishment of primary normal human melanocytes (NHM) It is described in Chapter II.

5.2.3 RNA extraction, cDNA first and second strand synthesis As described in Chapter III. Primers and other details are given in Table 1.

Table 1: **Primer sequences used for gene expression analysis.**

Primer	**Sequence**	**Amplicon size (bp)**	**Annealing Temperature (^{0}C)**
IL1R1 FP	5’GGAGGCTGATAAATGCAAGG3’	256	62
IL1R1 RP	5’GTAAGATGAATTTACCACGC3’		
IL6R1 FP	5’GCGCAGGAGGTGGCGAGAGGCG3’	265	62
IL6R1 RP	5’CGGGGGGAACATCCACCAGC3’		
TNFR1 FP	5’GCTGCTGCCACTGGTGCTCC3’	175	62

TNFR1 RP	5’GTAGGTTCCTTTGTGGCAC3’		
TNFR2 FP	5’CCGCCCAGGTGGCATTTAC3’	118	62
TNFR2 RP	5’GCATGTTGGCCCGGCGAGC3’		
IL10R FP	5’GGGAAACTTCACGTTCACACAC3’	158	62
IL10R RP	5’ TGGTGAGGGAGATGCACTC3’		
GAPDH FP	5’CATCACCATCTTCCAGGAGCGAG3’	122	65
GAPDH RP	5’CCTGCAAATGAGCCCCAGCCT3’		

5.2.4 Receptor expression studies

TNFR1, IL1R1 and IL10R membrane expression on NHM was monitored by flow cytometry using the respective antibodies (Table 2). NHM were seeded ($0.4x10^6$ cells/well) in a 6 well plate and incubated overnight at 37^0C with 5% CO_2 in a humidified incubator. After overnight incubation, TNF-α, IL-1α and IL-10 treatments were given along with media replacement and the cells were further incubated for 48 hrs. Later the cells were trypsinised, washed with PBS and fixed with paraformaldehyde at 4^0C for 15 min, followed by blocking with 5% bovine serum albumin (Sisco Research Laboratories, Mumbai) for 1 hr at room temperature. Further, cells were incubated with primary antibodies overnight at 4^0C. Upon incubation, cells were washed with PBS +0.02% Tween 20 (PBST) and secondary antibody (Anti-Rabbit IgG - TRITC, Sigma-Aldrich, USA) was added and incubated for 1 hr at room temperature in dark conditions. Cells were washed with FACS buffer (BD FACS Flow sheath fluid) and IL1R1 membrane expression was monitored using flow cytomtery (BD Biosciences FACS-ARIA III).

5.2.5 Assessment of IL6R expression upon exogenous IL-6 stimulation on NHM

IL6 receptor expression on NHM was monitored by confocal microscopy using monoclonal antibody: APC-Mouse-Anti-Human CD126 (562090, BD Pharmingen). NHM were seeded on a coverslip ($0.4x10^6$ cells/ well) in a 6 well plate and incubated overnight at 37^0C with 5% CO_2 in a humidified incubator. After overnight incubation, IL-6 (0, 10 ng/ml) treatment was given along with media replacement and the cells were further incubated for 48 hrs. NHM were washed once in phosphate-buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 min at 4°C, then incubated in blocking solution (5% BSA) for one hr and incubated for 2 hrs in secondary conjugated IL6R antibody. After incubation, cells were washed 2–3 times in PBS and fluorescence was captured with confocal microscopy (60X magnification). Data were analyzed using Image Proplus software to calculate mean density of fluorescence and graphs were plotted using Graphpad prism software. This protocol was also followed for TNFR1 expression analysis.The IL10R and IL6R antibodies were secondary conjugated with PE and APC respectively (Table 2).

Table 2: **Details of Antibodies used for cytokine receptor analysis**

Antibody	**Commercial details**	**Dilution**	**MW** (kDa)
TNFR1	ab19139, Abcam, USA	1:100 Flow cytometry	55
IL1R1	ab40774, Abcam, USA	1:1000 W.B; 1:50 Flow cytometry	75
IL6 R	APC-Mouse-Anti-Human CD126 (562090, BD Pharmingen™).	5µl/assay	80
IL10 R	PE Rat Anti Human CDw210 (556013, BD Pharmingen™).	20µl/assay	90-110

Secondary antibody: Anti-Rabbit IgG (whole molecule) - TRITC	Sigma-Aldrich®,USA	1:200 Flow cytometry	-

5.2.6 Statistical analyses

All experiments were done independently at least three times in triplicate. Data are presented as the mean ± standard deviation (SD) and the statistical difference between two groups were analyzed by Student's t-test using Prism 4 software. *p* values less than 0.05 were considered statistically significant.

5.3 RESULTS

5.3.1 TNF receptors expression upon TNF-α treatment to NHM:

TNF-α mediates its action *via* binding and interacting with its two receptors *viz* TNFR1 and TNFR2. TNFR1 is present on most of the cells but the presence of TNFR2 is restricted to a few cell lineages. Melanocytes express both the receptors of TNF-α i.e. TNFR1 and TNFR2. We observed significant up regulation of *TNFR1* transcript levels (p=0.024; n=3) whereas there was no difference in the transcript levels of *TNFR2* upon TNF-α stimulation (10ng/ml; 48 hrs) (Figure 3). TNFR1 serves as the major mediator of TNF-α induced signaling pathways. In addition to transcript levels of TNFR1, we have found that the membrane expression of TNFR1 is increased upon TNF-α stimulation by confocal microscopy (p=0.004 & 17.2% increased expression as compared to untreated controls respectively; n=3) The results were further confirmed with flow cytometry using 10 ng/ml; 48 hrs TNF-α treatment (p = 0.047; n =5; Figure 4).

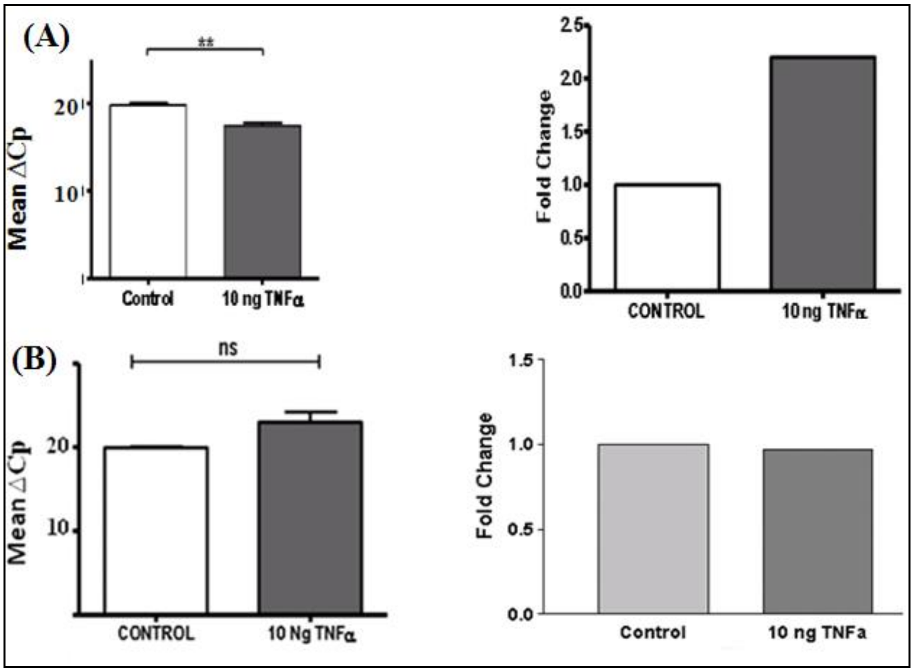

Figure 3: The NHM cells were treated with TNF-α (10ng/ml) and then *TNFR1* and *TNFR2* transcript levels were measured after 48 hrs of treatment.(A) Representation of mean ΔCp and fold change in expression of *TNFR1* at 10 ng/ml TNF-α at 48 hrs (n = 3; p = 0.0024). (B) Representation of Mean ΔCp and Fold Change in expression of *TNFR2* at 10 ng/ml TNF-α at 48 hrs (n = 3; p >0.05).

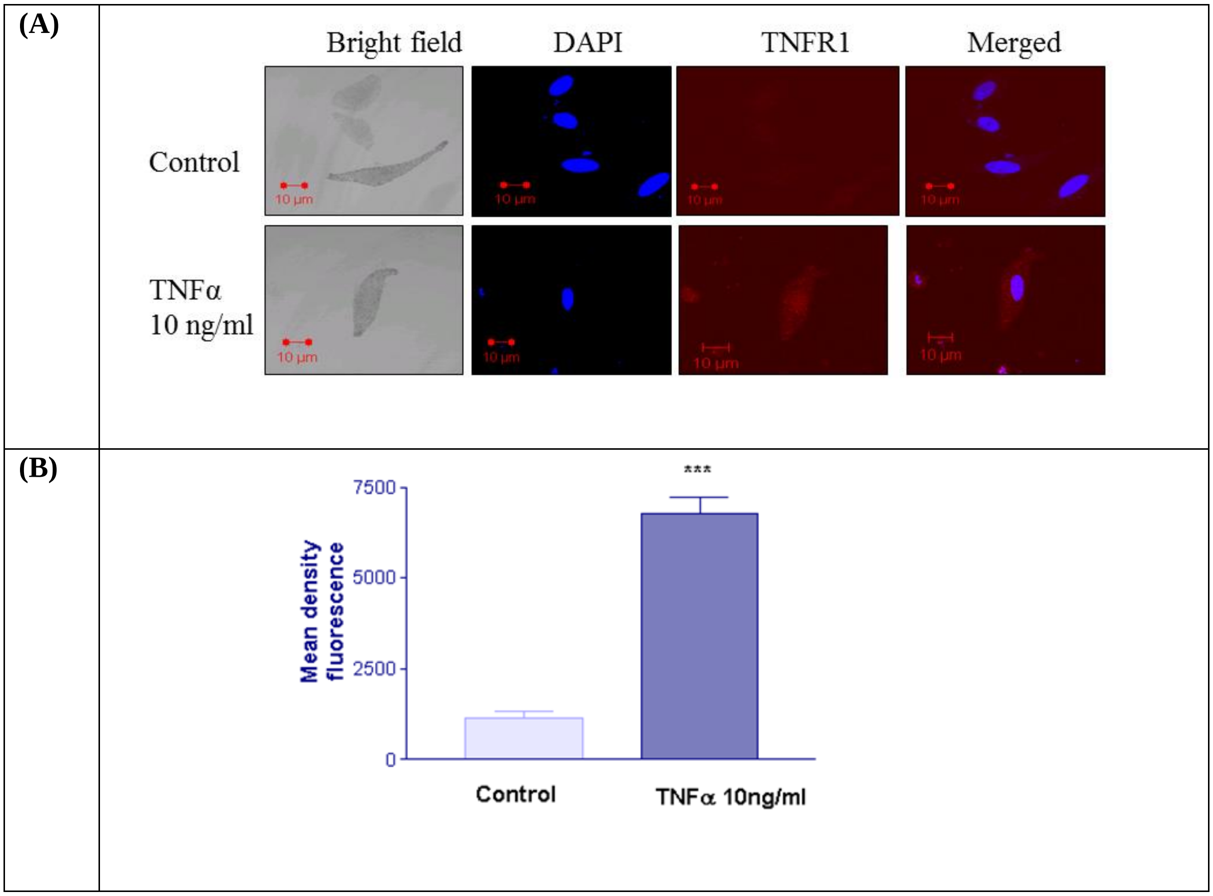

Figure 4: The NHM cells were treated with TNF-α (10ng/ml) and then TNFR1 expression levels were measured after 48 hrs of treatment. (A) Confocal microscopy analysis showed increased expression of TNFR1 on NHM. (B) Integrated mean density calculation showed significant increased expression of TNFR1 as compared to untreated control (p=0.0004; n=3).

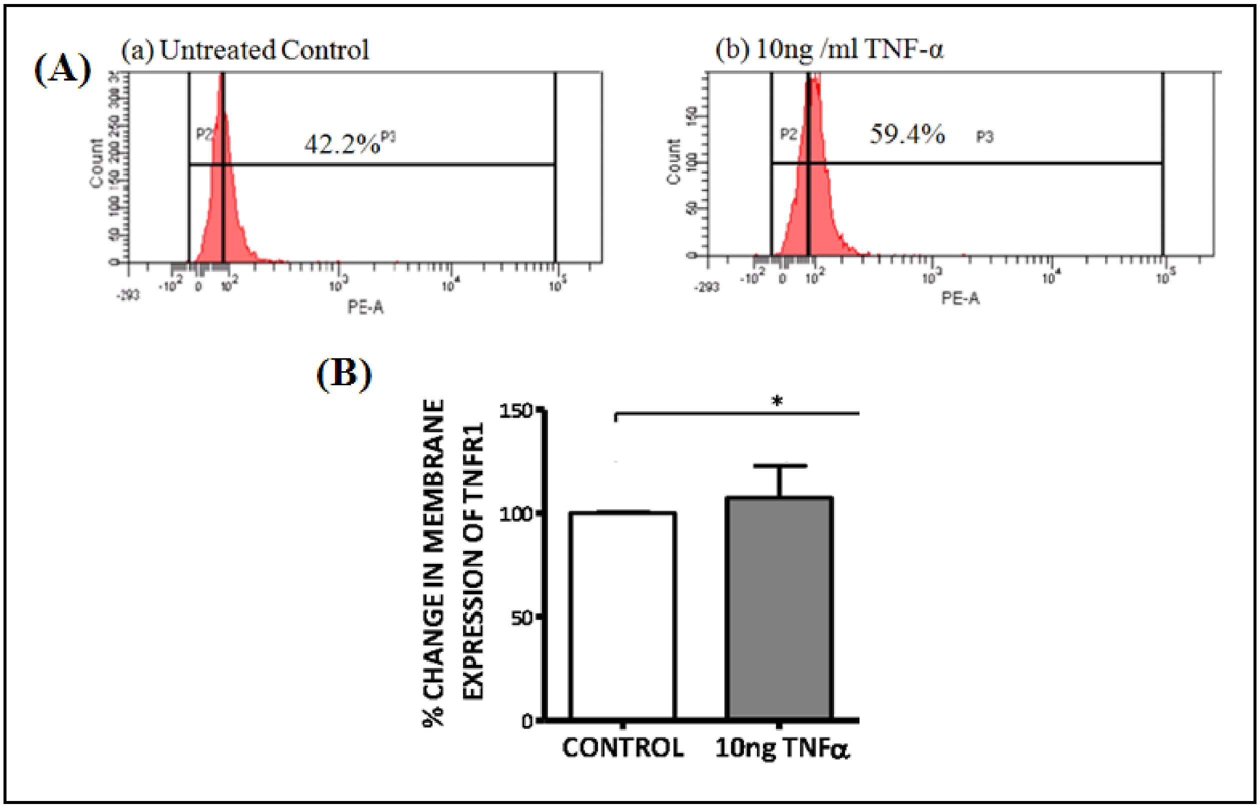

Figure 5: The NHM cells were treated with TNF-α (10ng/ml) and then TNFR1 expression levels were measured after 48 hrs of treatment. (A) Representative images showing the effect of TNF-α on expression of TNFR1 by Flow cytometry. (B) Mean % change in TNFR1 membrane expression at 10 ng/ml TNF-α at 48 hr (p = 0.047; n =5).

5.3.2 IL6R membrane expression upon IL-6 treatment to NHM:

Significant up regulation of *IL6R* transcript levels were observed upon exogenous stimulation with IL-6 and TNF-α to NHM (10ng/ml; 48 hrs) (n = 3; $p < 0.05$). However, at lower concentration (1ng/ml; 48 hrs) no difference was observed for *IL6R* transcript levels (Figure 5). Further, the membrane expression of IL6R upon exogenous IL-6 (10ng/ml) stimulation was validated with confocal microscopy (n =3; p=0.0262) (Figure 6).

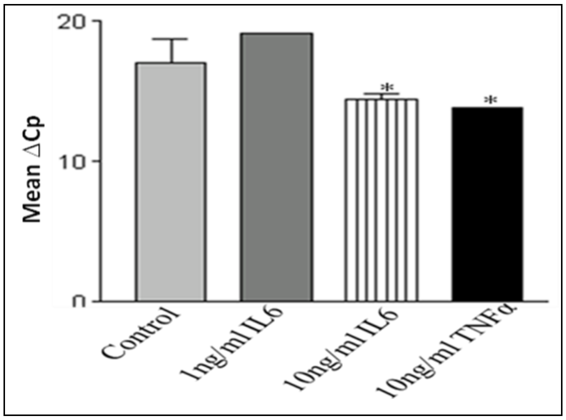

Figure 6: IL-6 and TNF-α induced *IL6R* gene expression in cultured melanocytes: The NHM cells were treated with IL-6 (1 and 10ng/ml; 48 hrs) and TNF-α (10ng/ml; 48 hrs) and analyzed for IL6R levels. Representation of Mean ΔCp showed significant increased expression of *IL6R* at 10 (ng/ml) treatment with IL-6 and TNF-α (n = 3; *$p < 0.05$).

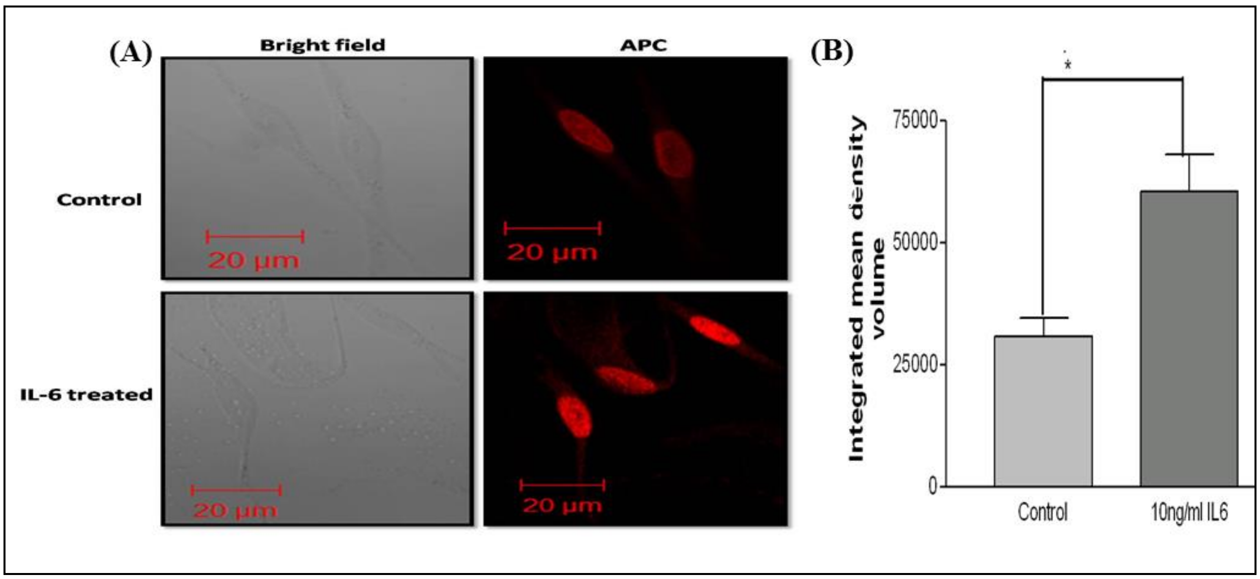

Figure 7 : IL-6 induced IL6 Receptor activation by confocal microscopy: The NHM cells were treated with IL-6 (10ng/ml) and then *IL6R* expression levels were measured after 48 hrs of treatment. (A) Representative images showing effect of IL-6 on IL6R expression by confocal microscopy. (B) Mean density fluorescence of IL6R upon exogenous stimulation with 10 ng/ml IL-6 for 48 hrs in NHM (n =3; p=0.0262).

5.3.3 IL1R1 membrane expression upon IL-1α treatment to NHM:

Cytokines mediate their action via interacting with their respective receptors and hence we monitored the effect of IL-1α on the membrane expression of IL1R1 (n=3). There was no significant difference in the transcript levels of *IL1R1* upon IL-1α exogenous stimulation (Figure 7). Interestingly, IL-1α stimulation showed significant increase in membrane expression of IL1R1 upon 10 ng/ml (~25%) and 100 ng/ ml (~22%) of IL-1α treated NHM as compared to untreated NHM (Figure 8), suggesting involvement of post translational modifications in regulation of membrane expression of IL1R1 on NHM.

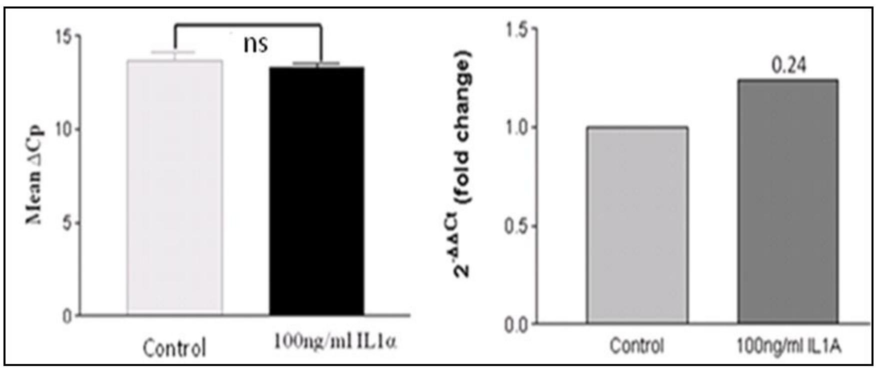

Figure 8 : Effect of IL-1αon IL1R1 expression in NHM: The NHM cells were treated with IL-1α (100ng/ml; 48 hrs). *IL1R1* transcript levels were measured after 48 hrs of treatment and the results showed no significant difference in the transcript levels of *IL1R1*. Representation of Mean ΔCp and Fold Change in expression of *IL1R1* at 100 ng/ml IL-1αat 48 hrs (n = 3; p = 0.5728).

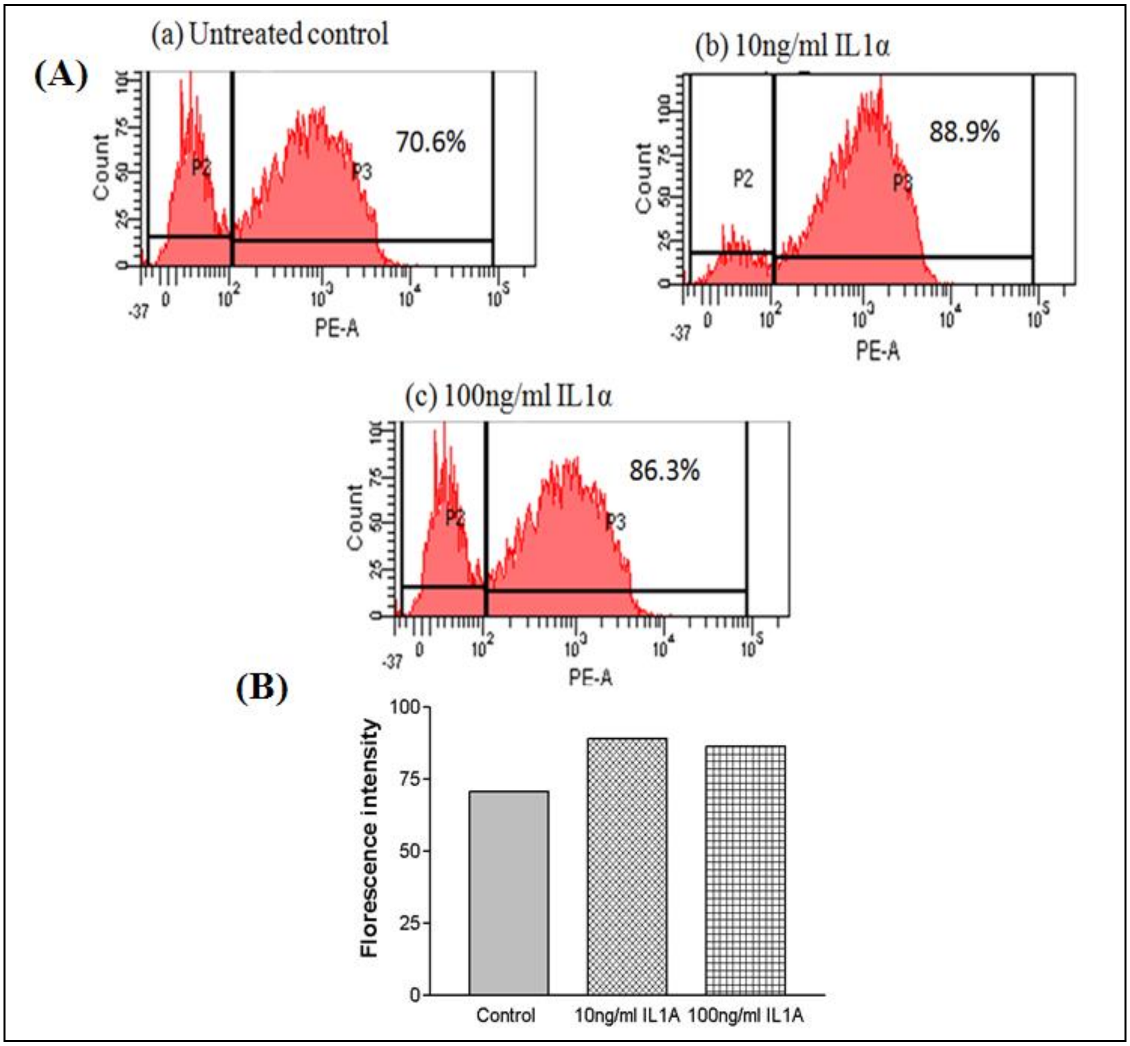

Figure 9: The NHM cells were treated with IL-1α (10 and 100ng/ml) and then IL1R1 expression levels were measured after 48 hrs of treatment. (A) Flow cytometry histogram shows increased expression of of IL1R1 upon IL-1α stimulation. (B) Graphical representation of fluorescence intensity of IL1R1 showed around 15% increased expression as compared to untreated control (n =3).

5.3.4 IL10R membrane expression upon IL-10 treatment to NHM:

The NHM cells were treated with IL-10 (10ng/ml) and *IL10R* transcript levels were measured after 48 hrs of treatment and the results showed no significant difference in the transcript levels of *IL10R* at 10ng/ml IL-10 (n = 3; p=0.6432) (Figure 9). Further, effect of IL-10 on expression of IL10R by Flow cytometry was monitored and non-significant difference i.e. ~0.9% increase in membrane expression was observed as compared to untreated controls (n =3) (Figure 10). Overall, our receptor expression studies on NHM cells indicate positive feedback regulation upon treatment with the respective cytokines.

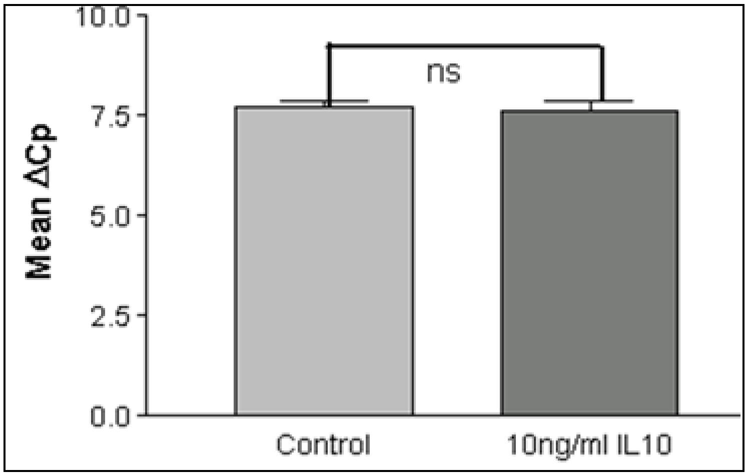

Figure 10: Effect of IL-10 on *IL10R* expression in NHM: The NHM cells were treated with IL-10 (10ng/ml) and *IL10R* transcript levels were measured after 48 hrs and the results showed no significant difference in the transcript levels of *IL10R*. Representation of Mean ΔCp of *IL10R* at 10 ng/ml IL-10 at 48 hrs (n = 3; p = 0.6432)

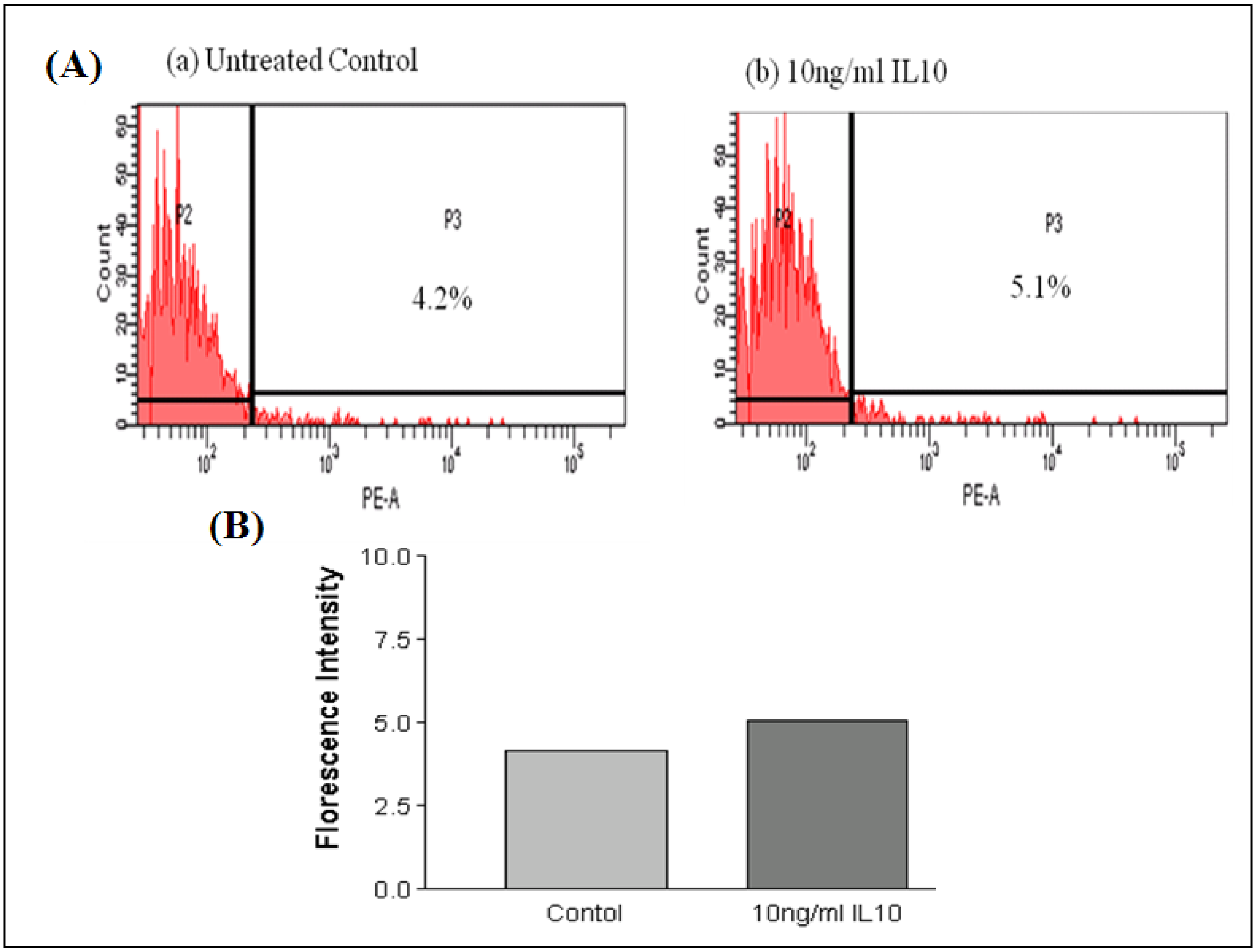

Figure 11: The NHM cells were treated with IL-10 (10ng/ml) and IL10R expression levels were measured after 48 hrs of treatment. (A) Effect of IL-10 on expression of IL10R by Flow cytometry. (B) Graphical representation of fluorescence intensity of IL10R upon exogenous stimulation of 10 ng/ml IL-10 for 48 hrs in NHM (n =3).

5.4 DISCUSSION

Cytokine receptors are key sensors and mediators of alterations in cytokine homeostasis under various pathological conditions, particularly associated with autoimmune and inflammatory responses. Various reports, including ours as discussed in chapter 1 advocates the existence of cytokine imbalance in vitiligo patients both in circulation as well as in the epidermis. Moreover, binding of cytokines with their respective receptors is essential for the activation of downstream signaling pathways. Therefore, it is interesting to investigate the receptor expression levels mimicking the vitiliginous melanocyte microenvironment condition.

TNF-α is a major mediator of inflammation and when over expressed, can give rise to chronic inflammatory and autoimmune diseases like vitiligo. Also, vitiligo patients exhibit higher TNF-α levels in skin and circulation. Melanocyte exhibits expression of both receptors for TNF i.e. TNFR1 and TNFR2. TNFR1 serves as the major mediator of TNF-induced signaling pathways. TNFR1 signaling has pleiotropic functions such as activation of nuclear factor kappaB (NF-κB) and induction of apoptosis, both of which depend on microenvironment of the cell (Naude *et al*., 2011). We were interested to investigate the effect of TNF-α on its receptor expression at transcript as well as protein levels. We observed a significant increase in *TNFR1* expression at the transcript and protein levels. But there was no difference in *TNFR2* expression levels. Crosstalk between TNFR1 and TNFR2 may take place which is controlled or influenced by many factors such as cell type, intracellular or extracellular environment, age, response to injury, inflammation and the occurrence of NF-κB activation (Faustman & Davis, 2013). TNFR1 is a type I transmembrane protein, which in resting cells is predominantly sequestered in the Golgi apparatus, from where it can be mobilized to the cell surface. The significance of the Golgi pool of TNFR1 molecules is unclear. One hypothesis is that it may act as a reservoir to increase surface receptor expression density, thereby sensitizing cells to the actions of TNF. There is precedence for this idea in smooth muscle cells, in which the TNF receptor family member, Fas localizes predominantly to the Golgi, from where it can be translocated to the cell surface, thereby sensitizing cells to Fas ligand-induced killing (Wang *et al*., 2003).

IL6 is a multifunctional pleiotropic cytokine which modulates expression of various genes. Our results showed significant decrease in NHM viability upon IL-6 treatment. IL-6 through its interaction with interleukin 6 receptor alpha (IL6αR) and the signal-transducing component gp130 induces transcription of inflammatory genes. It is thus implicated in chronic disease conditions associated with inflammation and is also suspected to cause increased susceptibility to various autoimmune disorders. Our results demonstrated increased transcript as well as protein expression of IL6R upon exogenous stimulation with IL-6 on NHM, suggesting the autocrine mode of IL6 regulation.

IL1 has two receptors, designated as IL1R1 and IL1R2. IL1R1 encodes a receptor for IL-1α, IL-1β and IL1RN. IL1β and IL1R1 are key regulators of the body's inflammatory responses which exert numerous biological effects and its defects result in various diseases (Schwanstecher, 2011). Our previous studies suggest that both miR-328 and miR-211 might target *IL1B* whereas, miR-1 and miR-211 might target *IL1R1* (Mansuri *et al*., 2016). Increased levels of *IL1R1* and *IL1B* are reported in vitiligo patients (Laddha *et al*., 2014; Mansuri *et al*., 2016) Unlike the IL-1β precursor, the IL-1α precursor is fully active and functions as an ''alarmin'' by rapidly initiating a cascade of inflammatory cytokines and chemokines, which accounts for sterile inflammation (Chen *et al*., 2007; Rider *et al*., 2011). IL1RN is a specific inhibitor of both IL-1α and IL-1β. IL-1RN binds tightly to IL-1RI and blocks the activity of either IL-1α or IL-1β. IL1 activation is believed to be mediated exclusively by IL1R1 and requires IL1 receptor accessory protein for cell signaling (Boraschi *et al*., 2006; Jacques *et al*., 2006). On the contrary, IL1R2 has no signaling properties, and appears to act as a 'decoy receptor' (Martin and Wesche, 2002; Dunne *et al*., 2003). Bellehumeur *et al*., (2009) have reported the regulation of IL1R1, IL1R2 and IL1RN by IL1-β in human endometrial cells. Our results showed increased IL1R1 membrane expression and *IL1RN* transcript levels upon IL-1α stimulation in NHM. However, *IL1R1* transcript levels remain unaltered in IL-1α treated NHM.

IL-10 activity is mediated by its specific cell surface receptor complex, which is expressed on a variety of cells including immune cells. Only a few copies of the IL-10R are expressed on the cell surface (Carson *et al*., 1995; Jurlander *et al*., 1997), and the expression is variable. Only a few regulating factors are known such as endotoxin increases the expression of IL-10R on fibroblasts (Weber-Nordt *et al*., 1994). Therefore, we were interested to monitor the effect of IL10 on its receptor expression. However, we did not find any significant difference in the expression of IL10R on melanocytes.

In conclusion, we can say that TNF-α, IL-1α, IL-6 mediate their action *via* receptor upregulation on NHM. This study will strengthen the understanding and correlation between increased levels of cytokines and their effect on melanocyte biology. Also, the receptor activation studies will be helpful in designing treatment regime for melanocyte cytokine imbalance related disorders like vitiligo, melanoma etc.

5.5 REFERENCES

- Bellehumeur, C., Blanchet, J., Fontaine, J.Y., Bourcier, N. and Akoum, A., 2009. Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. *Human reproduction*, *24*(9), pp.2193-2204.
- Boraschi, D. and Tagliabue, A., 2006. The interleukin-1 receptor family. *Vitamins & Hormones*, *74*, pp.229-254.
- Carson, W.E., Lindemann, M.J., Baiocchi, R., Linett, M., Tan, J.C., Chou, C.C., Narula, S. and Caligiuri, M.A., 1995. The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood*, *85*(12), pp.3577-3585.
- Chen, C.J., Kono, H., Golenbock, D., Reed, G., Akira, S. and Rock, K.L., 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nature medicine*, *13*(7), p.851.
- Cichorek, M., Wachulska, M., Stasiewicz, A. and Tymińska, A., 2013. Skin melanocytes: biology and development. *Advances in Dermatology and Allergology/Postępy Dermatologii I Alergologii*, *30*(1), p.30.
- Dunne, A. and O'Neill, L.A., 2003. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci. STKE*, *2003*(171), pp.re3-re3.
- Faustman, D.L. and Davis, M., 2013. TNF receptor 2 and disease: autoimmunity and regenerative medicine. *Frontiers in immunology*, *4*.
- Fotin-Mleczek, M., Henkler, F., Samel, D., Reichwein, M., Hausser, A., Parmryd, I., Scheurich, P., Schmid, J.A. and Wajant, H., 2002. Apoptotic crosstalk of TNF receptors: TNF-R2-induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. *Journal of Cell Science*, *115*(13),

pp.2757-2770.

- Hamilton, J.A., 1997. CSF-1 signal transduction. *Journal of leukocyte biology*, *62*(2), pp.145-155.
- Jacques, C., Gosset, M., Berenbaum, F. and Gabay, C., 2006. The role of IL-1 and IL-1Ra in joint inflammation and cartilage degradation. *Vitamins & Hormones*, *74*, pp.371-403.
- Jurlander, J., Lai, C.F., Tan, J., Chou, C.C., Geisler, C.H., Schriber, J., Blumenson, L.E., Narula, S.K., Baumann, H. and Caligiuri, M.A., 1997. Characterization of interleukin-10 receptor expression on B-cell chronic lymphocytic leukemia cells. *Blood*, *89*(11), pp.4146-4152.
- Kelso, A., 1998. Cytokines: principles and prospects. *Immunology & Cell Biology*, *76*(4).
- Khan, M.M., 2008. *Immunopharmacology*. Springer Science & Business Media.
- Laddha, N.C., Dwivedi, M. and Begum, R., 2012. Increased Tumor Necrosis Factor (TNF)-α and its promoter polymorphisms correlate with disease progression and higher susceptibility towards vitiligo. *PLoS One*, *7(12)*, p.e52298.
- Laddha, N.C., Dwivedi, M., Gani, A.R., Mansuri, M.S. and Begum, R., 2013. Tumor Necrosis Factor B (TNFB) genetic variants and its increased expression are associated with vitiligo susceptibility. *PloS one*, *8*(11), p.e81736.
- Laddha, N.C., Dwivedi, M., Mansuri, M.S., Singh, M., Patel, H.H., Agarwal, N., Shah, A.M. and Begum, R., 2014. Association of neuropeptide Y (NPY), interleukin-1B (IL1B) genetic variants and correlation of IL1B transcript levels with vitiligo susceptibility. PloS one, 9(9), p.e107020.
- Liu, Y., Wei, S.H., Ho, A.S., de Waal Malefyt, R. and Moore, K.W., 1994. Expression cloning and characterization of a human IL-10 receptor. *The Journal of Immunology*, *152*(4), pp.1821-1829.
- Mansuri, M.S., Singh, M. and Begum, R., 2016. miRNA signatures and transcriptional regulation of their target genes in vitiligo. *Journal of dermatological*

science, *84*(1), pp.50-58.

- Martin, M.U. and Wesche, H., 2002. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1592*(3), pp.265-280.
- Moretti, S., Spallanzani, A., Amato, L., Hautmann, G., Gallerani, I., Fabiani, M. and Fabbri, P., 2002. New insights into the pathogenesis of vitiligo: imbalance of epidermal cytokines at sites of lesions. *Pigment Cell & Melanoma Research*, *15*(2), pp.87-92.
- Naudé, P.J., den Boer, J.A., Luiten, P.G. and Eisel, U.L., 2011. Tumor necrosis factor receptor cross-talk. *The FEBS journal*, *278*(6), pp.888-898.
- Rider, P., Carmi, Y., Guttman, O., Braiman, A., Cohen, I., Voronov, E., White, M.R., Dinarello, C.A. and Apte, R.N., 2011. IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. *The Journal of Immunology*, *187*(9), pp.4835-4843.
- Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M. and Goeddel, D.V., 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell, 83(7)*, pp.1243-1252.
- Schwanstecher, M., 2011. *Diabetes-Perspectives in drug therapy*. Springer.
- Shu, H.B., Takeuchi, M. and Goeddel, D.V., 1996. The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proceedings of the National Academy of Sciences*, *93*(24), pp.13973-13978.
- Singh, M., Mansuri, M.S., Parasrampuria, M.A. and Begum, R., 2016. Interleukin 1-α: A modulator of melanocyte homeostasis in vitiligo. *Biochem Anal Biochem*, *5*(273), pp.2161-1009.
- Speeckaert, R., Speeckaert, M., De Schepper, S. and van Geel, N., 2017. Biomarkers of disease activity in vitiligo: A systematic review. *Autoimmunity Reviews*.
- Thomas J. Kindt.,2007. *Kuby Immunology (6th Edition).*

- Wajant, H., Pfizenmaier, K. and Scheurich, P., 2003. Tumor necrosis factor signaling. *Cell death and differentiation*, *10*(1), p.45.
- Wang, J., Al-Lamki, R.S., Zhang, H., Kirkiles-Smith, N., Gaeta, M.L., Thiru, S., Pober, J.S. and Bradley, J.R., 2003. Histamine antagonizes tumor necrosis factor (TNF) signaling by stimulating TNF receptor shedding from the cell surface and Golgi storage pool. *Journal of Biological Chemistry*, *278*(24), pp.21751-21760.
- Weber-Nordt, R.M., Meraz, M.A. and Schreiber, R.D., 1994. Lipopolysaccharide-dependent induction of IL-10 receptor expression on murine fibroblasts. *The Journal of Immunology*, *153*(8), pp.3734-3744.
- Windsor, W.T., Syto, R., Tsarbopoulos, A., Zhang, R., Durkin, J., Baldwin, S., Paliwal, S., Mui, P.W. and Pramanik, B., 1993. Disulfide bond assignments and secondary structure analysis of human and murine interleukin 10. *Biochemistry*, *32*(34), pp.8807-8815.
- Zdanov, A., 2010. Structural analysis of cytokines comprising the IL-10 family. *Cytokine & growth factor reviews*, *21*(5), pp.325-330.